JOURNAL OF LIQUID CHROMATOGRAPHY

VOLUME 5 NUMBER 10 1982

CODEN: JLCHD8 5(10) i-viii, 1809-2026 (1982)
ISSN: 0148-3919

ULTRASTYRAGEL™

Microparticulate GPC Columns

Highest Performance . . . more than doubles the efficiency of existing GPC columns.

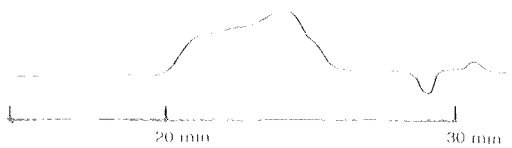
Waters announces a new break through in GPC column chemistry — ULTRASTYRAGEL.™ This innovation represents the latest technology in microparticulate gel permeation chromatography columns. These controlled pore size microparticulate columns are an evolutionary extension of Waters' proven STYRAGEL® line of GPC columns. ULTRASTYRAGEL columns provide the highest efficiency and resolution per column of any GPC column currently available. Major improvements in analytical results include:

- **Highest Resolution** — due to the extremely high efficiencies (plates) of ULTRASTYRAGEL columns, resolution of closely related molecular weight components is now possible.

- **Faster Separations** — ULTRASTYRAGEL will often provide the same or better resolution with fewer columns than you are currently using, thereby reducing the analysis time for each sample and improving productivity and throughput.

ULTRASTYRAGEL vs. μSTYRAGEL
SAMPLE = PREPOLYMER

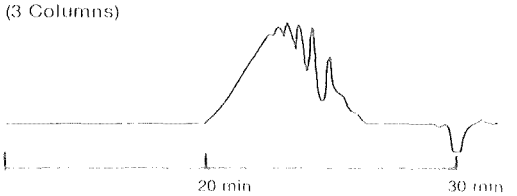
4,732 Plates/Foot
(3 Columns)



Column Bank: 10^3, 500, 100Å μSTYRAGEL
Total Length: 90 cm
Mobile Phase: THF
Flow Rate: 1.0 ml/min.

Improved Resolution

13,512 Plates/Foot
(3 Columns)



Column Bank: 10^3, 500, 100Å ULTRASTYRAGEL™
Total Length: 90 cm
Mobile Phase: THF
Flow Rate: 1.0 ml/min.

Increase resolution and speed in your GPC analyses with ULTRASTYRAGEL columns. ULTRASTYRAGEL columns are in stock and ready for shipment NOW!

MILLIPORE

Waters, The Liquid Chromatography People

For more information Circle No. 112 on the Readers Service Card

JOURNAL OF LIQUID CHROMATOGRAPHY

Editor: DR. JACK CAZES Editorial Secretary: ELEANOR CAZES
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

Aims and Scope. The journal publishes papers involving the application of liquid chromatography to the solution of problems in all areas of science and technology, both analytical and preparative, as well as papers that deal specifically with liquid chromatography as a science within itself. Included will be a thin-layer chromatography and all modes of liquid chromatography.

Indexing and Abstracting Services. Articles published in *Journal of Liquid Chromatography* are selectively indexed or abstracted in:

- Analytical Abstracts
- BioSciences Information Service of Biological Abstracts (BIOSIS)
- Chemical Abstracts
- Current Contents/Life Sciences
- Current Contents/Physical and Chemical Sciences
- Engineering Index
- Excerpta Medica
- Journal of Abstracts of the All-Union Institute of Scientific and Technical Information of the USSR
- Physikalische Berichte
- Science Citation Index

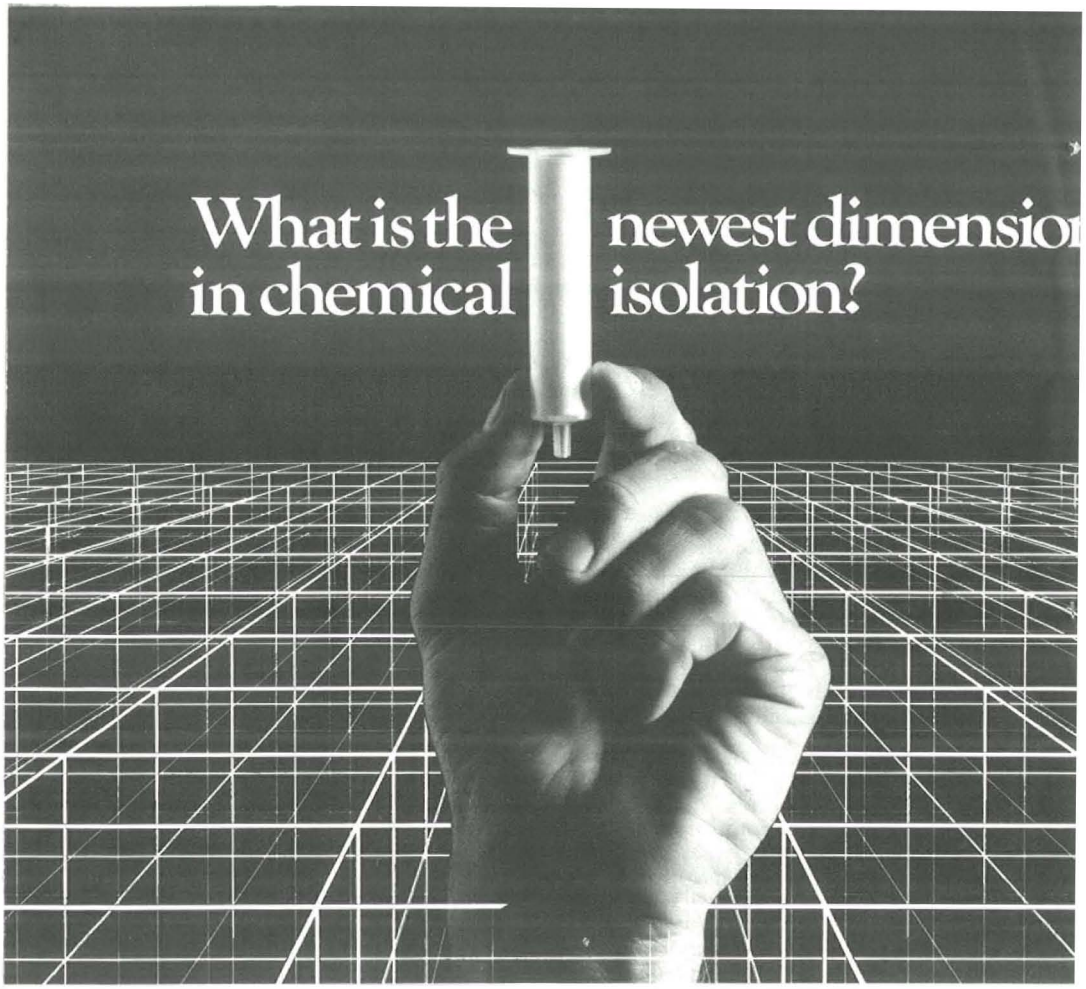
Manuscript Preparation and Submission. See the last page of this issue.

Subscription Information. *Journal of Liquid Chromatography* is published in twelve numbers and two supplements per volume by Marcel Dekker, Inc., 270 Madison Avenue, New York, New York 10016. The subscription rate for Volume 5 (1982), containing twelve numbers and two supplements, is $236.00 per volume (prepaid). The special discounted rate for individual professionals and students is $118.00* per volume. To secure this special rate, your order must be prepaid by personal check or may be charged to MasterCard or VISA. Add $29.40 for surface postage outside the United States. For airmail, add $49.70.

Mailing address. *Please mail payment with order to:* Marcel Dekker Journals, P. O. Box 11305, Church Street Station, New York, New York 10249.

Contributions to this journal are published free of charge.

*THIS REFLECTS A 50% DISCOUNT GIVEN TO INDIVIDUAL SUBSCRIBERS.



Analytichem introduces off-line Chromatographic Mode Sequencing

Now there is a simple new bench technique that provides more efficient chemical isolation than any technology available today. In a fraction of the time. And at a fraction of the cost. It's called Chromatographic Mode Sequencing. The technique has been developed by Analytichem International for use with Bond Elut solid phase extraction columns.

Chromatographic Mode Sequencing (CMS) is a simple off-line procedure that yields remarkably selective results. CMS allows the chemist to manipulate the interaction of various mobile and stationary phases to produce an almost limitless number of interactive modes. The result is that CMS, using Bond Elut, can solve virtually any chemical isolation problem. Yet it is disarmingly simple to perform.

The selectivity of this new technique is unsurpassed even by the most sophisticated column switching procedures. And the simplicity of CMS means you can process a far greater volume of samples than with any other method available. CMS makes a totally new dimension in chemical isolation possible. And Bond Elut makes CMS possible. To learn how CMS can simplify your chemical isolation problems, call Analytichem today.

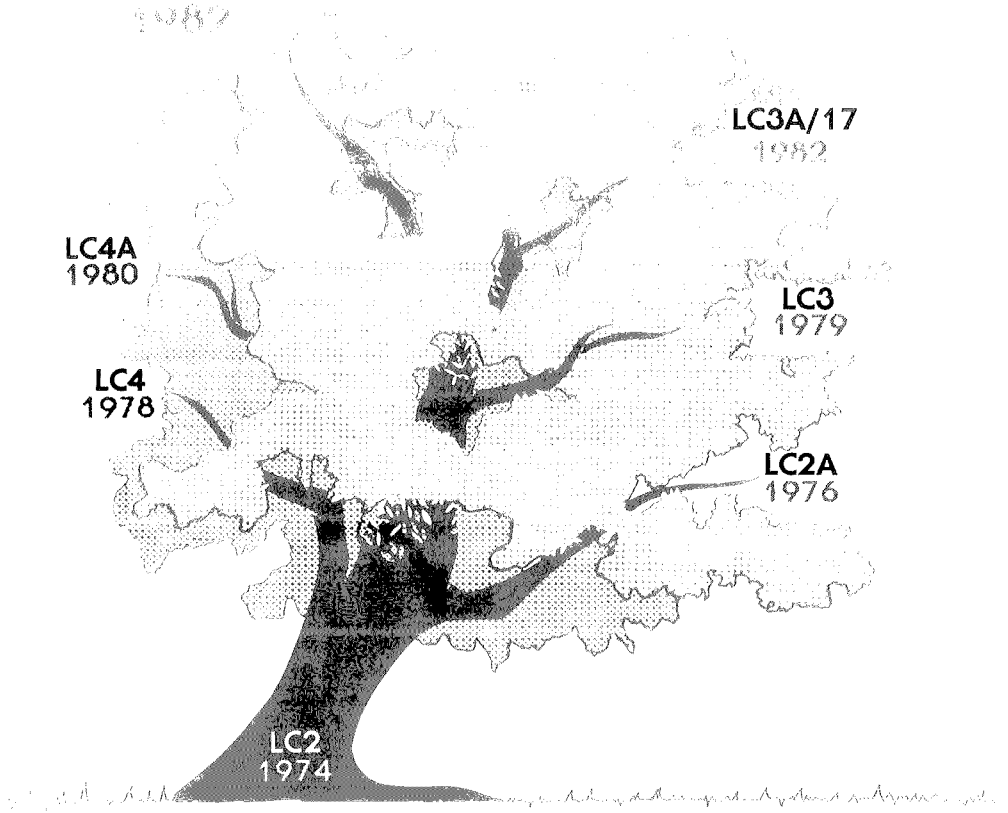
Analytichem International
24201 Frampton Ave., Harbor City
CA 90710. USA (800) 421-2825.
In California (213) 539-6490
Telex 664832 ANACHEM HRBO

Circle Reader Service Card No. 110

JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 5, Number 10, 1982

CONTENTS



LCEC is turning over a new leaf

Since 1974 when BAS developed the first commercial electrochemical detector we have been successful in introducing the LCEC technique to laboratories worldwide. Our first efforts took root and blossomed into a family of detectors. BAS is proud to announce the latest additions to our family tree: the LC3A/17 and the Dual LC4B/17.

With the dual electrode detector it is possible to enhance selectivity, detection limits, speed, and gradient compatibility in ways never before possible. BAS is the only company to offer both series and parallel amperometry at two potentials. Call or write today for details. We'll be pleased to show you how BAS can assist with your trace analysis problem.

bas bioanalytical systems

205 Kent Avenue, West Lafayette, IN 47906 (317) 463-2505 TLX 276141

Circle Reader Service Card No. 105

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1809-1823 (1982)

ELUTION BEHAVIOR OF LOW MOLECULAR WEIGHT COMPOUNDS IN GEL PERMEATION CHROMATOGRAPHY

Toshio Ogawa and Masakazu Sakai

Hirakata Plastics Laboratory
Ube Industries, Ltd.
3-10, Nakamiyakita-machi, Hirakata
Osaka 573, Japan

ABSTRACT

Elution behavior of organic compounds in gel permeation chromatography was investigated using chloroform as eluent. In aliphatic hydrocarbons, the elution counts decreased linearly with increasing the molecular volumes. In aromatic hydrocarbons, the relation between molecular volume and elution count slightly shifted toward lower counts. The elution counts in esters, ketones, amides, alcohols and carboxylic acids always fell in lower elution counts than expected by aliphatic hydrocarbons. This fact suggests that all these compounds are solvated by eluent molecules. Amines and chlorides exhibit an adsorption effect on cross-linked polystyrene gel. These compounds are eluted behind the corresponding hydrocarbons for given molecular volumes, which were obtained by dividing molecular weight by density.

INTRODUCTION

Gel permeation chromatography (GPC) is very useful, not only for polymer, but also for simple organic compounds such as hexane and benzene. Generally, organic compounds are expected to elute

according to molecular volume like high polymers. However, we can not conclude that elution counts of organic compounds depend only upon molecular volume. According to published papers,[1)-6)] so called universal calibration[7)] is not always observed for organic compounds. The order of elution may depend upon the kinds of solvents, i.e., eluent, polarity of compounds and experimental conditions, although the compounds are approximately eluted according to molecular volume.

As described above, it is very important to investigate the elution behavior of organic compounds as a function of molecular volume in GPC. In this study, experiments were carried out using chloroform as eluent. The logarithmic molecular volumes were plotted against elution counts. The elution behavior of esters, alcohols, etc. is discussed on the basis of elution behavior of aliphatic hydrocarbons.

EXPERIMENTAL

Instrument and Procedure

A Toyo Soda Model-807 high speed liquid chromatograph was used equiped with the two columns ($G2000H_8$) which were packed with cross-linked polystyrene gel. The columns have a nominal exclusion limit of 250Å. The instrument was operated using chloroform as eluent with a flow rate of 1.0ml/min. at ambient temperature. 100 µl of a 0.5 to 1.0% chloroform solution of samples was injected into the columns.

Samples

Aliphatic and aromatic hydrocarbons were supplied by various chemical producers. Esters and alcohols were supplied by Applied Science Laboratories, U.S.A., and ketones and carboxylic acids by Polyscience Corp., U.S.A. and P-L Biochemicals, Inc., U.S.A., respectively. Amides and amines were supplied by various Japanese and American chemical producers. The densities of these compounds were taken from the literature or estimated from those of homologous or similar compounds. The densities of solid compounds in

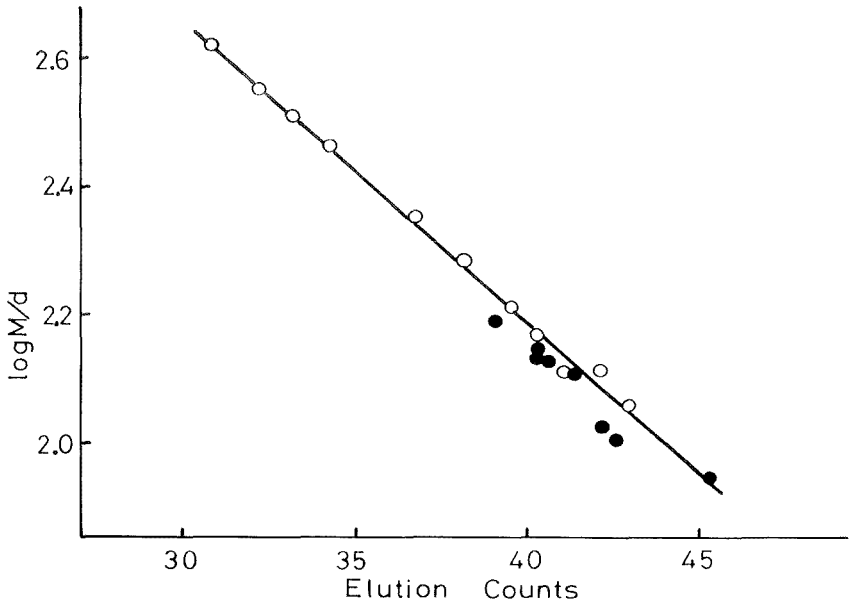


FIGURE 1. Logarithmic molecular volumes vs. elution counts of hydrocarbons.
○ : aliphatic, ● : aromatic.

solution were conventionally obtained by measuring the density of solution containing a given amount of the solute as follows:

$$\rho_2 = \frac{W_2}{\frac{V_1 + W_2}{\rho_3} - V_1} \qquad \text{.....(1)}$$

where ρ is the density, W is the weight, V is the volume, and subscripts 1, 2, 3 mean solvent, solute and solution, respectively.

RESULTS AND DISCUSSION

The relationship between molecular volume vs. elution count is expected to be linear, since the columns used in this study are

TABLE 1

Molecular Volumes and Elution Counts of Hydrocarbons

Compound	Molecular Volume	Elution Count
n-Tetracosane	423.9	30.8
n-Eicosane	359.4	32.1
n-Octadecane	328.4	33.1
n-Hexadecane	294.0	34.2
n-Dodecane	228.6	36.7
n-Decane	195.9	38.1
n-Octane	164.0	39.4
n-Heptane	147.5	40.2
n-Pentane	115.2	43.0
p-Cymene	157.4	39.0
Cumene	139.4	40.3
Tetralin	136.8	40.3
Diphenyl	134.1	40.6
Ethylbenzene	123.1	41.3
Styrene	114.8	42.5
Toluene	106.8	42.1
Benzene	89.4	45.3

commercially supplied. According to the usual application of GPC, the relationship will be hereafter called calibration curve. The molecular volumes of samples were obtained by dividing M by d (M: molecular weight, d: density). Strictly speaking, the molecular volume of organic compounds in solution may slightly deviate from M/d. However, we need not be concerned with such small differences in this study.

The calibration curve for aliphatic hydrocarbons is shown in FIGURE 1. and TABLE 1. As expected, elution counts increase linearly with decreasing molecular volumes. The elution counts of aromatic hydrocarbons shift slightly toward lower counts for a

TABLE 2

Molecular Volumes and Elution Counts of Esters

Compound	Molecular Volume	Elution Count
Methyl Behenate	410.9	30.2
Methyl Arachidate	378.0	30.8
Ethyl Stearate	359.0	31.3
Ethyl Oleate	356.9	30.9
Methyl Stearate	345.1	31.4
Methyl Myristate	279.3	33.2
Methy Laurate	246.3	33.7
Methyl Caprate	213.4	34.5
Methyl Caprylate	180.3	35.6
Phenyl Benzoate	160.5	37.8
Methyl Caproate	147.2	36.9
Phenyl Acetate	126.3	39.6
Methyl Benzoate	125.0	39.4
Diethyl Carbamate	121.2	37.6
Methyl Acetate	79.3	39.7

TABLE 3

Molecular Volumes and Elution Counts of Ketones

Compound	Molecular Volume	Elution Count
2-Nonanone	173.3	35.4
2-Octanone	156.3	36.1
2-Heptanone	140.8	36.6
2-Hexanone	123.5	37.4
2-Pentanone	106.5	38.0
2-Butanone	89.6	39.0
Acetophenone	117.4	39.1
Acetylacetone	102.5	38.5

TABLE 4

Molecular Volumes and Elution Counts of Amides

Compound	Molecular Volume	Elution Count
Erucamide	338.0	31.5
Stearamide	283.0	32.6
Oleamide	281.5	32.7
N,N-Dimethylformamide	77.4	37.9

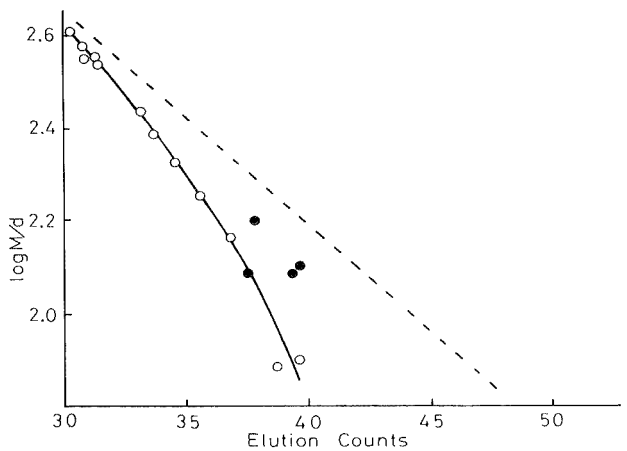


FIGURE 2. Logarithmic molecular volumes vs. elution counts of esters.
○ : alipahtic, ● : aromatic,
------- : hydrocarbons.

given molecular volume. This fact suggests that aromatic hydrocarbons exhibit interactions between solute and solvent molecules. Moreover, as will be discussed later, the effect of adsorption of solutes on the cross-linked polystyrene gel has to be also taken into account.[3),8)] At any rate, the calibration curve obtained for aliphatic hydrocarbons indicates the most reliable relationship between elution count and the molecular volume which is expressed by

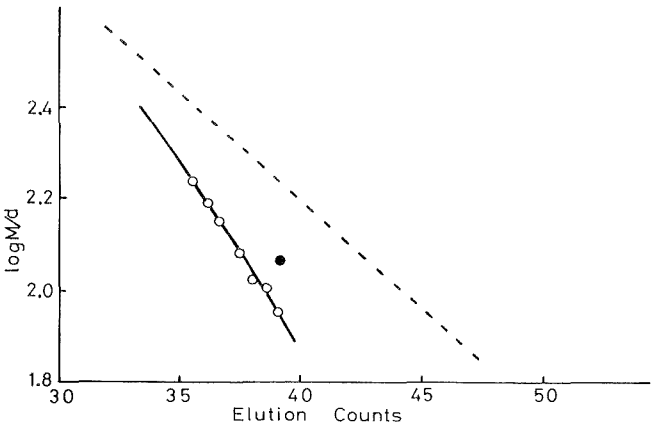


FIGURE 3. Logarithmic molecular volumes vs. elution counts of ketones.
○ : aliphatic, ● : aromatic,
-------- : hydrocarbons.

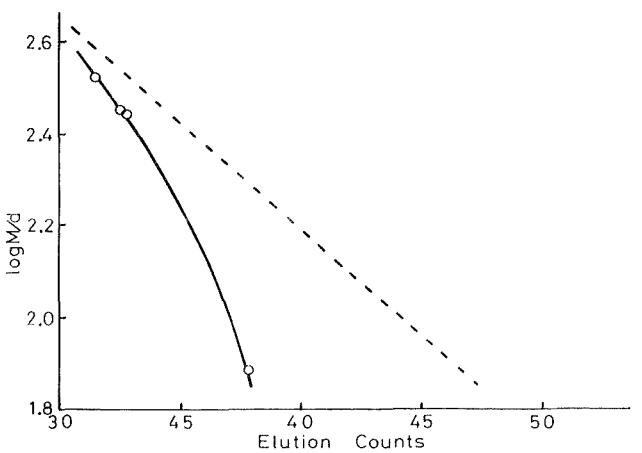


FIGURE 4. Logarithmic molecular volumes vs. elution counts of amides.
○ : aliphatic,
------- : hydrocarbons.

M/d. The elution behavior of other compounds will be always discussed on the basis of the curve for aliphatic hydrocarbons.

The elution behavior of esters, ketones and amides is shown in TABLES 2-4 and FIGURES 2-4. Like hydrocarbons, all these compounds are principally separated according to molecular volumes; the elution counts decrease with increasing molecular volumes. However, the compounds are eluted earlier than hydrocarbons, although experimental points in the lower molecular weight region are somewhat scattered. Similar tendency is observed for alcohols and carboxylic acids, as shown in FIGURES 5, 6 and TABLES 5, 6. Apparently, carboxylic acids are not so different from other polar compounds. However, the shapes of the peaks are skewed toward higher elution counts, as shown in FIGURE 7.

Two reasons are considered for these deviations: one is due to repulsion between solutes and cross-linked polystyrene gel,[1] the other is due to the association of solute molecules with solvent or other solute molecules. The latter is more probable. The effec-

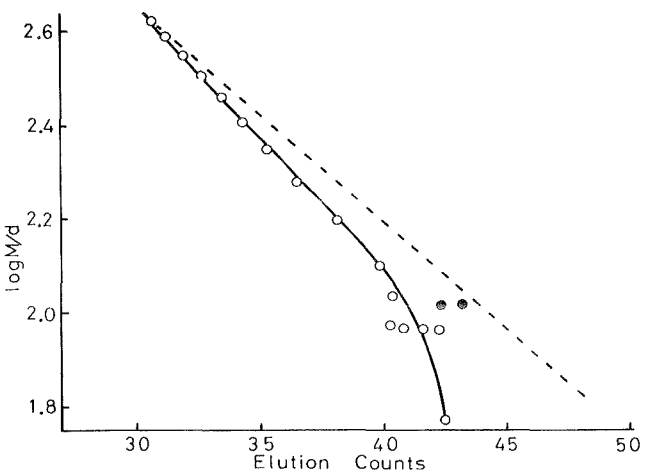


FIGURE 5. Logarithmic molecular volumes vs. elution counts of alcohols.
○ : alipahtic and alicyclic, ● : aromatic,
------- : hydrocarbons.

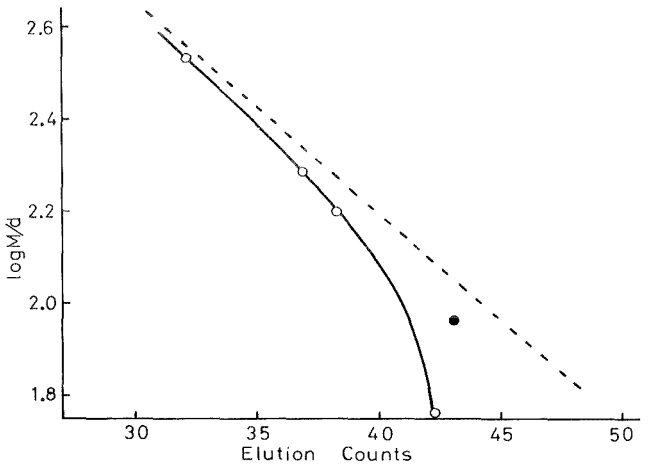


FIGURE 6. Logarithmic molecular volumes vs. elution counts of carboxylic acids.
○ : aliphatic, ● : aromatic,
------- : hydrocarbons.

TABLE 5

Molecular Volumes and Elution Counts of Alcohols

Compound	Molecular Volume	Elution Count
1-Tetracosanol	420.5	30.7
1-Docosanol	387.9	31.3
1-Eicosanol	355.2	31.9
1-Octadecanol	322.4	32.7
1-Hexadecanol	289.6	33.5
1-Tetradecanol	257.1	34.4
1-Dodecanol	224.3	35.4
1-Decanol	191.3	36.6
1-Octanol	158.4	38.2
1-Hexanol	125.3	39.8
n-Amyl Alcohol	108.7	40.4
t-Butyl Alcohol	94.9	40.2
s-Butyl Alcohol	92.3	40.8
n-Butyl Alcohol	92.0	41.6
Cyclopentanol	91.4	42.3
m-Cresol	105.0	43.2
Benzyl Alcohol	103.8	42.3

TABLE 6

Molecular Volumes and Elution Counts of Carboxylic Acids.

Compound	Molecular Volume	Elution Count
Stearic Acid	340.0	32.0
n-Capric Acid	192.9	36.8
n-Caprylic Acid	158.5	38.2
Acetic Acid	57.2	42.2
Benzoic Acid	92.0	43.0

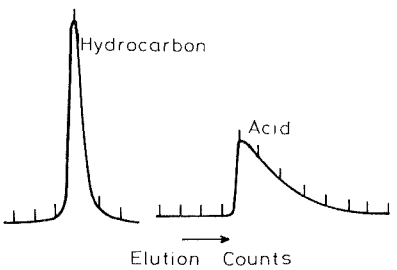


FIGURE 7. Elution peaks of hydrocarbon and carboxylic acid.

tive volumes of solutes should be enlarged if association takes place. Let us express the deviation of effective molecular volumes from M/d by ΔV_m,

$$\Delta V_m = V_o - V_c \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad (2)$$

where V_c is identical to M/d, V_o is the effective molecular volume. ΔV_m is schematically shown in FIGURE 8. If a polar group in a compound gives rise to association with eluent molecules, ΔV_m should be positive. FIGURE 9 clearly proves this idea. Generally, with decreasing molecular volume ΔV_m increases. Ketones, amides and esters have a similar tendency. ΔV_m for these compounds approaches

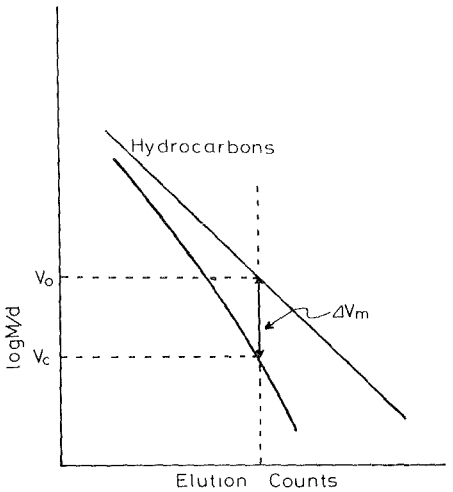


FIGURE 8. Deviation of molecular volumes of polar compounds from those of corresponding hydrocarbons.

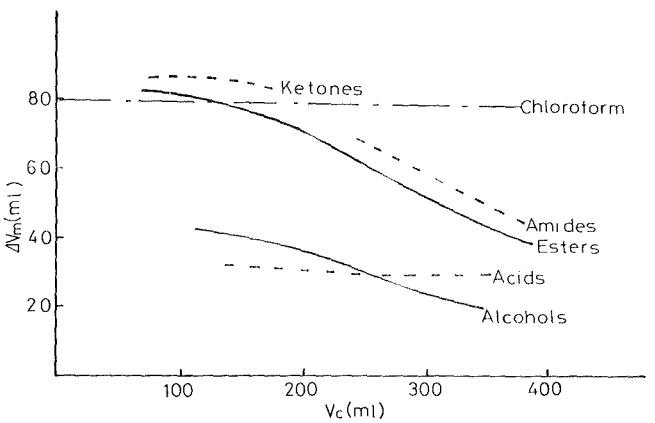


FIGURE 9. ΔV_m as a function of molecular volume in various solutes.

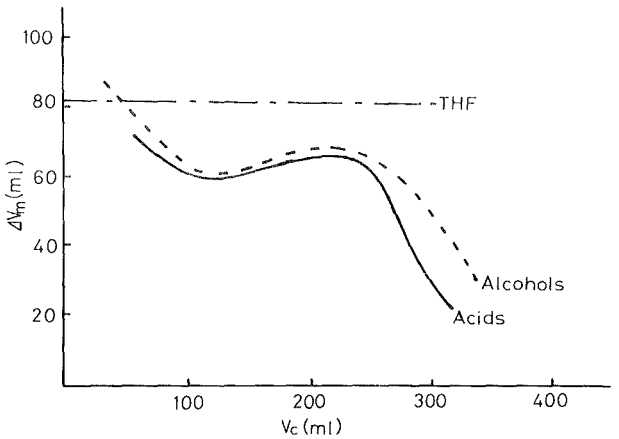


FIGURE 10. ΔV_m as a function of molecular volume in THF. These relationships were derived from data by Chang.

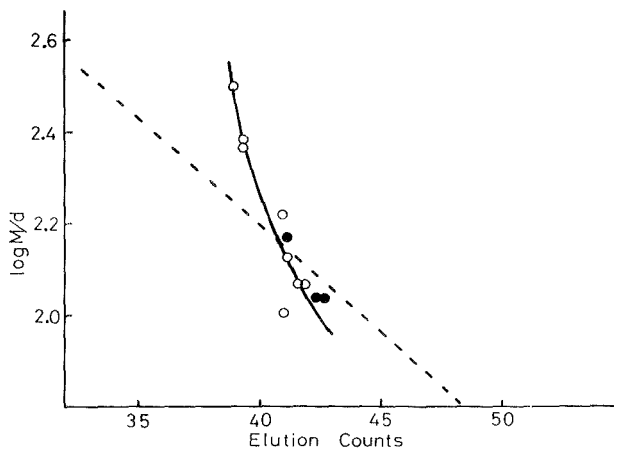


FIGURE 11. Logarithmic molecular volumes vs. elution counts of amines.
○ : aliphatic and alicyclic, ● : aromatic,
-------- : hydrocarbons.

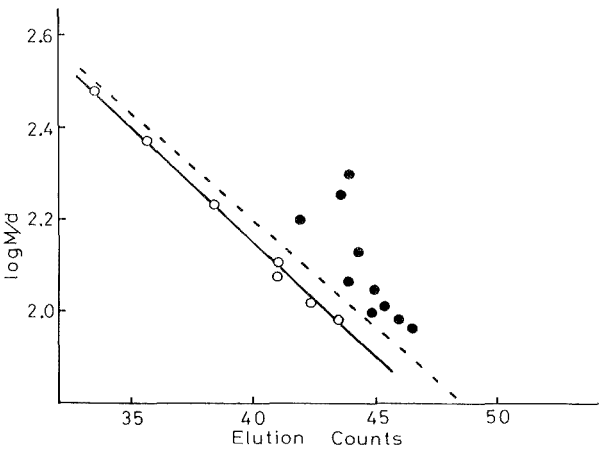


FIGURE 12. Logarithmic molecular volumes vs. elution counts of chlorides.
○ : aliphatic, mono- and di-chlorides,
● : other chlorides, ------- : hydrocarbons.

TABLE 7

Molecular Volumes and Elution Counts of Amines

Compound	Molecular Volume	Elution Count
Stearylamine	312.7	39.0
Tri-n-butylamine	238.2	39.4
Laurylamine	231.3	39.3
n-Octylamine	166.3	41.0
n-Hexylamine	133.1	41.2
n-Amylamine	115.6	41.7
Cyclohexylamine	115.0	41.8
s-Butylamine	100.4	41.0
N,N-Dimethylbenzyl-amine	147.8	41.2
Benzylamine	109.5	42.5
N-Methylaniline	108.9	42.7

TABLE 8

Molecular Volumes and Elution Counts of Chlorides.

Compound	Molecular Volume	Elution Count
Cetyl Chloride	301.5	33.5
Lauryl Chloride	235.9	35.6
n-Octyl Chloride	171.1	38.3
1,5-Dichloropentane	128.1	41.0
n-Amyl Chloride	120.9	40.9
n-Butyl Chloride	104.4	42.4
1,2-Dichloropropane	97.5	43.4
1,2,4,5-Tetrachlorobenzene	199.8	43.9
Hexachlorobenzene	181.5	43.5
Hexachlorocyclopentadiene	160.3	41.8
α-Chloronaphthalene	136.2	44.2
o-Chlorotoluene	117.1	43.8
Hexachloroethane	113.2	44.9
1,1,2,2-Tetrachloroethylene	104.5	45.3
1,1,1-Trichloroethane	99.6	44.7
Carbon Tetrachloride	97.1	45.8
1,1,2-Trichloroethane	92.5	46.4

the molecular volume of chloroform at M/d = 200. Carboxylic acids and alcohols exhibit a different pattern and their ΔV_m's are lower than those for the above compounds. This tendency is also similar to behavior in tetrahydrofuran (THF); the results were derived from the data of Chang (FIGURE 10).[2] However, the extent of solvation seems to be different from that in chloroform. At the present stage, it is very difficult to predict the magnitude of ΔV_m by an appropriate method. This problem will be fully discussed in the next paper with respect to infrared and NMR spectra.

Amines have specific behavior as shown in FIGURE 11 and TABLE 7. Elution counts are abnormally high for the compounds having a molecular volume greater than 150. In the low molecular weight region, elution behavior is similar to that of esters and ketones. Abnormal lag in high molecular volume region will come from adsorption on the cross-linked polystyrene gel. Chlorides are also complicated as shown in FIGURE 12 and TABLE 8. Mono- and dichlorides seem to follow the same rule as esters and ketones. On the other hand, polychlorides and chlorides containing aromatic rings show adsorption effects on the cross-linked polystyrene gel.

Other compounds containing aromatic rings, such as esters and alcohols, are eluted behind the corresponding aliphatic ones, as shown in FIGURES 1-3, 5 and 6. This behavior is also explained by the same idea. Consequently, these compounds exhibit both solvation and adsorption effects.

ACKNOWLEDGMENTS

The authors are indebted to Dr. J. Cazes, Waters Associates, Inc., and Mr. T. Inaba, Ube Industries, Ltd., for their helpful suggestions and critical readings of the manuscript.

REFERNCES

1. Cazes, J., and Gaskill, D.R., Reprints of Six International Seminar, "Gel Permeation Chromatography," p.147(1968)

2. Chang, T.L., Anal. Chim. Acta, 39, 519(1967)

3. Edwards, G.D., and NG, Q.Y., J. Polym. Sci., Part C, No.21, 105(1968)

4. Bergmann, J.G., Duffy, L.J., and Stevenson, R.B., Anal. Chem., 43, 131(1971)

5. Cazes, J., and Gaskill, D.R., Separ. Sci., 2, 421(1967)

6. Cazes, J., and Gaskill, D.R., Separ. Sci., 4, 15(1969)

7. Grubisic, Z., Rempp, P., and Benoit, H., Polymer Letters, 5, 753(1967)

8. Popl, M., Fähnrich, J., and Stejskal, M., J. Chromatogr. Sci., 14, 537(1976)

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1825-1845 (1982)

GEL PERMEATION CHROMATOGRAPHY OF POLYETHYLENE TEREPHALATE

Saleh A. Jabarin and Dennis C. Balduff

Owens-Illinois, Inc.
One SeaGate
Toledo, Ohio 43666

ABSTRACT

This paper describes the development of the gel permeation chromatography (GPC) technique for the measurements of cyclic trimer content, molecular weights, and molecular weight distribution of polyethylene terephthalate (PET), utilizing a solvent system of o-chlorophenol-chloroform. Mark-Houwink constants for this solvent system are also described.

The GPC technique was applied to the study of the cyclic trimer content, molecular weights and molecular weight distribution of a variety of commercial PET resins. The results indicate that the cyclic trimer content in PET is dependent on molecular weight, polycondensation process and catalyst system. Solid-state polymerized PET contains less cyclic trimer than PET made by the melt-phase process of the same molecular weight. The cyclic trimer content in solid-stated PET appears to be dependent on the conditions of solid-state polymerization.

The polydispersity index determine for a variety of PET samples is higher than the theoretically predicted value of 2.0; however, there is no systematic dependence on molecular weight or polycondensation process.

INTRODUCTION

Among the important characteristics of polyethylene terephthalate (PET), which determine the physical properties and uses

of this polymer are the molecular weight and the molecular weight distribution. The most frequently used technique in industry to evaluate the molecular weight of PET is solution viscosity. The use of solution viscosity in the determination of molecular weight for PET and other linear polymers is based on an empirical relationship between viscosity and molecular weight.

The solution viscosities may be expressed by several quantities. Their nomenclature and definitions are given elsewhere (1,2); however, the viscosity parameter of most importance to the impirical relation of viscosity to molecular weight is the intrinsic viscosity (I.V.) or limiting viscosity number, which is given by:

$$[\eta] = \left(\frac{\eta_{sp}}{c}\right)_{c=o} = \left(\frac{\ln \eta_{rel}}{c}\right)_{c=o}$$

where c = concentration (g./dl).

The intrinsic viscosity or the limiting viscosity number, $[\eta]$, is related to molecular weight, M, by the Mark-Houwink equation.

$$[\eta] = K\bar{M}^{a}$$

where K and "a" are empirical constants, which vary with the nature of the solvent.

The relationship between intrinsic viscosity and molecular weight for PET has been developed in a variety of solvents (3). Other correlations between the inherent viscosity and molecular weight are also known (4).

While the solution viscosity is a valuable tool for the molecular characterization of polymers, the procedure is laborious and time-consuming. In addition it is limited in providing direct and detailed information about the various molecular weight averages and the molecular weight distribution.

Gel permeation chromatography (4-10), GPC, is a well established technique for determining the molecular weight distribution (MWD), and in principle, can yield accurate measurements of molecular weight averages.

The application of GPC as a tool for determining molecular weight parameters of PET has been limited due to the availability of appropriate solvents. Previous work (11,12) involved the use of meta-cresol at 110-135°C as a solvent of polyethylene terephthalate for GPC; however, studies (13) have shown that meta-cresol can cause degradation of PET through acid catalyzed hydrolysis. Paschke, Bidlingmeyer and Bergmann (13) reported the development of a nitrobenzene-tetrachloroethane solvent system which can be used as a solvent for PET at room temperature.

In this paper, we describe the use of an o-chlorophenol-chloroform solvent system for the measurement of the molecular weight parameters of PET by GPC. This solvent system is also a solvent for polystyrene at room temperature; therefore, a calibration procedure, based upon polystyrene standards, can be developed to allow accurate and reproducible determination of molecular weight averages and molecular weight distribution of PET.

EXPERIMENTAL

Materials

Polymer Standards

Polystyrene standards of narrow molecular weight distribution were obtained from various sources. These standards, used in both viscometry and GPC experiments, cover the molecular weight range of 2,100 M_w to 7,100,000 M_w.

PET samples with a wide range of molecular weights were used in the viscometry experiments and in preliminary GPC trials (see Table 1).

TABLE 1

PET Samples

Sample #	I.V. (1)	Source
1	0.206	Owens-Illinois
2	0.492	Owens-Illinois
3	0.625	Owens-Illinois
4	0.64	Goodyear
5	0.67	Celanese
6	0.70	Eastman
7	0.72	Goodyear
8	0.83	Goodyear
9	0.95	Goodyear
10	1.00	Goodyear
11	1.08	Goodyear

(1) I.V. (inherent viscosity) measurements made in 60/40 - phenol/tetrachloroethane @ 25°C @ 0.25 g./dl.

Solvents

Ortho-chlorophenol, purified grade, and spectrophotometry grade chloroform, were obtained from Fisher. With the exception of filtration, no additional treatment of either solvent was performed.

Viscometry

All of the viscosity measurements were conducted in a Cannon-Ubbelohde suspended level capillary dilution viscometer. The viscometer was suspended in a constant temperature water bath with an accuracy of ± 0.01°C. Efflux times for the PET and polystyrene solutions were measured manually with an electric clock timer, accurate to 0.05 seconds.

The solution preparation for both PET and polystyrene standards was identical. The concentrations used in each viscosity measurement varied with the molecular weight of the sample being measured. In general, the concentration of the polymer solutions were made to yield an efflux time of 200 seconds or more for the highest solution concentration. Orthochlorophenol was added to a known weight of polymer and heated at 110°C, with agitation, until the polymer dissolved (∿10 to 30 minutes). After the polymer was dissolved, this solution was allowed to reach room temperature, at which time chloroform was added to make the final solution.

A given volume of the polymer-orthochlorophenol/chloroform solution was filtered into the viscometer through a Swinny-adapted sytinge, with a 0.45 μm filter. Efflux times of the

solutions were determined in triplicate, after allowing thermal equilibration of 10 minutes. The solutions were then diluted, equilibrated and remeasured for an additional three solution concentrations.

GPC

GPC experimentation was performed with a Waters Associates model ALC/GPC-201 gel permeation chromatograph equipped with a model U6K sample injector and a model R-401 differential refractometer. A circulating water bath, with an accuracy of ± 0.05°C, was attached to the differential refractometer and to temperature control blocks surrounding the column bank.

The solvents were vacuum filtered through a Millipore 0.45 μm filter. All sample solutions were also filtered through a Swinny-adapted syringe, fitted with a 0.45 μm Millipore filter.

The column bank for this system consisted of four μ-Styragel columns (Waters Associates), with porosities of 10^6–10^3 Å. The quoted column efficiency was 9000 plates/meter for each column.

PET solution concentrations were maintained at 0.25% (w/v) while the polystyrene standard solutions were made to be 0.10% (w/v). The normal operating conditions for this chromatographic system were:

Temperature	= 25°C
Flow rate	= 1.0 ml./min.
Eluent	= orthochlorophenol/chloroform (25/75)
System pressure	= 700-720 psig. @ 1 ml./min.
	= 1300-1500 psig. @ 2 ml./min.
Attenuation	= 8X
Injection	= 0.4 ml.

RESULTS AND DISCUSSION

Universal Calibration Curve

The hydrodynamic volume of a polymer chain, V_h, is usually measured as ($[\eta]M$), where $[\eta]$ is the intrinsic viscosity and M is the molecular weight. The parameter, $[\eta]M$, is the basis of the universal calibration curve.

The conversion from one polymer system to another involves the use of the Mark-Houwink equation

$$[\eta] = KM^a \tag{1}$$

The hydrodynamic volumes of polymers 1 and 2 can be written as

$$[\eta]M = K_1 M_1^{a_1+1} \tag{2}$$

$$[\eta]M = K_2 M_2^{a_2+1} \tag{3}$$

If the column combination, solvent, elution rate and temperature are constant when conducting the GPC experiment of the two polymers, then at a given elution volume, the relation

$$K_1 M_1^{a_1+1} = K_2 M_2^{a_2+1} \tag{4}$$

will hold. The molecular weight of polymer 2 may be obtained from polymer 1 by solving equation 4

$$\log M_2 = \frac{a_1+1}{a_2+1} \log M_1 + \frac{1}{a_2+1} \log \left(\frac{K_1}{K_2}\right) \tag{5}$$

The use of the universal calibration curve requires a knowledge of the Mark-Houwink constants which can be experimentally determined as described in the next section.

Solution Properties

Intrinsic viscosities were determined according to the Huggins and Kraemer equations

$$\frac{\eta_{sp}}{C} = [\eta] + K' [\eta]^2 C \quad (6)$$

$$\ln \left(\frac{\eta_{rel}}{C}\right) = [\eta] - K'' [\eta]^2 C \quad (7)$$

A typical plot according to these equations is given in Figure 1, for low and high molecular weight PET. Similar treat-

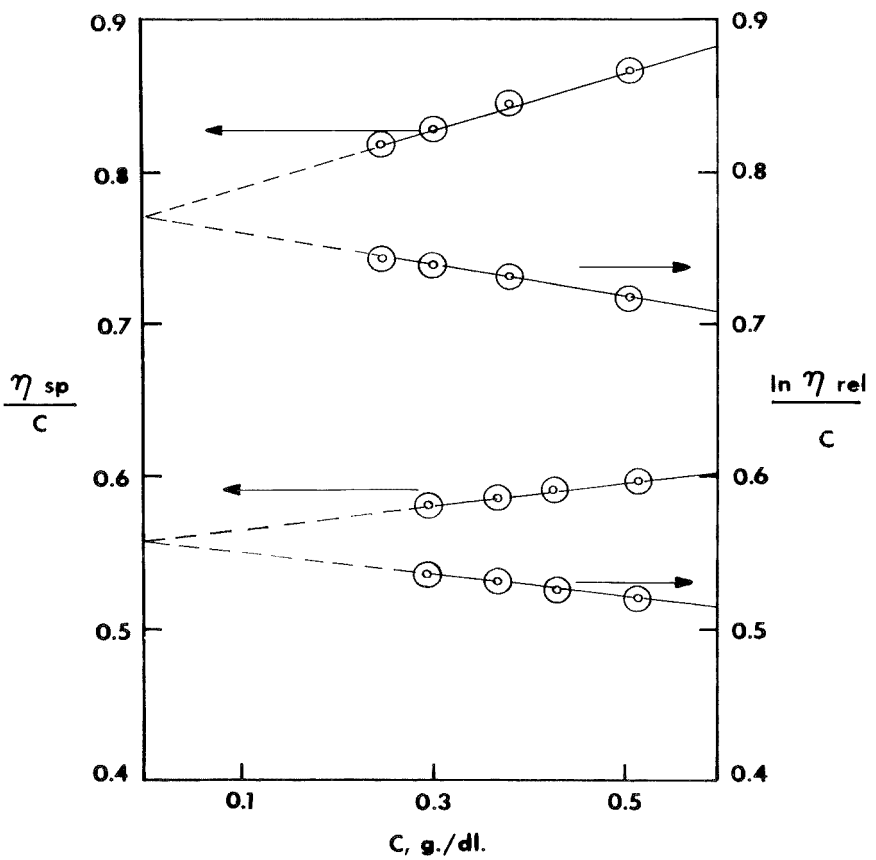


Figure 1 η_{sp}/C and $\ln\eta_{rel}/C$ versus concentration for a low and high molecular weight PET sample.

ment of viscosity data for the polystyrene standards is also required.

The intrinsic viscosities of PET are plotted as a function of M_W in Figure 2. Examination of the data in Figure 2 shows that the temperature effect on the $[\eta]$ -M relationship is negligible over the temperature range 20-30°C. The Mark-Houwink equations for PET and polystyrene were obtained by a least-squares fit. The results are

$$[\eta] = 1.49 \times 10^{-4} M_w^{0.56} \quad \text{(PET)} \tag{8}$$

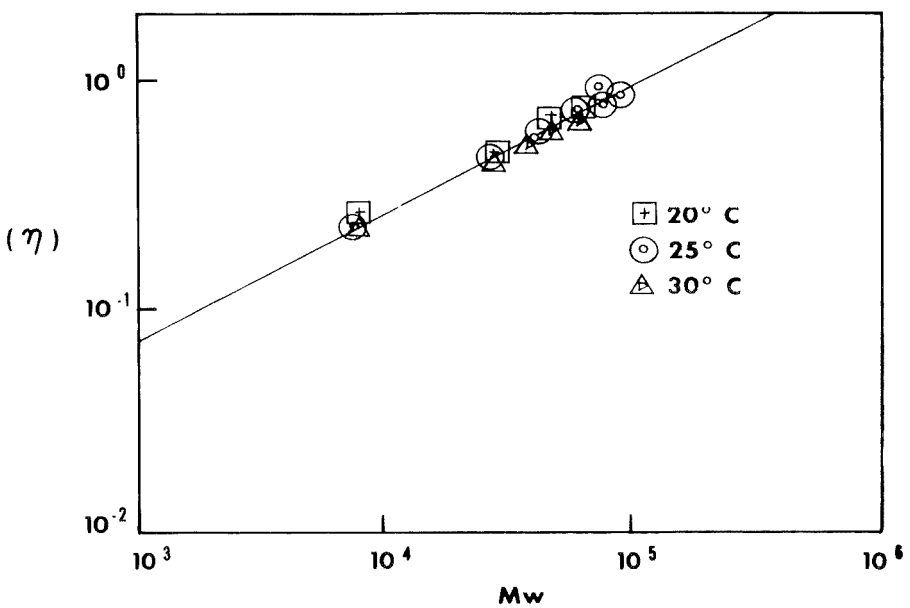


Figure 2 Intrinsic viscosity as a function of weight average molecular weight for PET in (25/75%) ortho-chlorophenol-chloroform.

$$[\eta] = 2.20 \times 10^{-4} M_w^{0.67} \quad \text{(Polystyrene)} \qquad (9)$$

The K (1.49×10^{-4}) and "a" (0.56) values for PET in orthochlorophenol-chloroform solvent system at 25°C determined in this study differ somewhat from those obtained for other solvent systems (3,4,14). The K value obtained here is in the same general range of K values obtained for other solvent systems. The value of the exponent "a" is low compared to other solvent systems; however, since the value of "a" is higher than 0.50, the solvent system can be considered as a good solvent for PET.

As indicated above, the effect of temperature on the viscosity - molecular weight relationship is negligible over the temperature range 20-30°C. This indicates that there is no association of the polymer in this solvent system.

The stability of PET in orthochlorophenol-chloroform solvent system was evaluated by solution viscosity. The results are shown in Table 2 for two PET samples having different molecular weights. It is seen that the efflux time is constant over

TABLE 2

Efflux Time (seconds) After Dissolving Samples in Orthochlorophenol-Chloroform Solvent System at 25°C

Period Min.	High M_w Sample C = 0.502 gm/dl	Period Min.	Low M_w Sample C = 0.613 gm/dl
31	223.40	25	211.67
160	223.37	139	211.73
1,731	223.38	1,420	221.68
2,881	223.30	3,039	221.34

extended periods, indicating that there is no breakdown in molecular weight.

Molecular Weight and Molecular Weight Distribution

Construction of Calibration Curves

Molecular weights for PET which have the same hydrodynamic volume ($[\eta]M$) and elute at the same elution volume as the polystyrene standards can be calculated from equation 5, using the Mark-Houwink constants given in equations 8 and 9. The results of these calculations are given in Table 3. The data are plotted

TABLE 3

Weight Average Molecular Weight, $\bar{M}_w$ for PET Calculated from Hydrodynamic Volume

Elution Volume V_e, ml	$\bar{M}_w$
15.9	6,330,120
16.0	3,516,620
16.4	2,168,470
17.5	505,770
17.8	368,150
19.6	138,640
20.8	73,820
23.3	32,100
25.7	12,290
27.2	5,790
29.1	2,100
29.9	1,060

in Figure 3, where M_w is plotted against elution volume. The results in Figure 3 constitute a calibration for PET in this solvent at 25°C.

Typical GPC Data

GPC Chromatograms

A typical GPC Chromatogram of PET in (25/75%) orthochlorophenol-chloroform at 25°C is given in Figure 4. The major portion of the chromatogram for this PET sample peaks at about 22 ml elution volume, with a minor peak at 31 ml. The identification and further characterization of this minor peak will be discussed in a later section.

Calculations

From the chromatogram a number of molecular weight parameters can be obtained. These include:

- Weight Average Molecular Weight (M_w)
- Number Average Molecular Weight (M_n)
- Polydispersity Index (D_n)

These molecular weight averages are calculated according to the general equation

$$M_a = \frac{\sum_{i=1}^{Q} N_i M_i^a}{\sum_{i=1}^{Q} N_i M_i^{a-1}} \qquad (10)$$

where a = 1,2,3 or 4. When a = 1, M_n is obtained. Higher weight-averages are obtained when a = 2,3 or 4. These are: a = 2 (M_w), a = 3 (M_z) and a = 4 (M_z + 1). The polydispersity index is expressed as the ratio of M_w/M_n and given the designation of D_n.

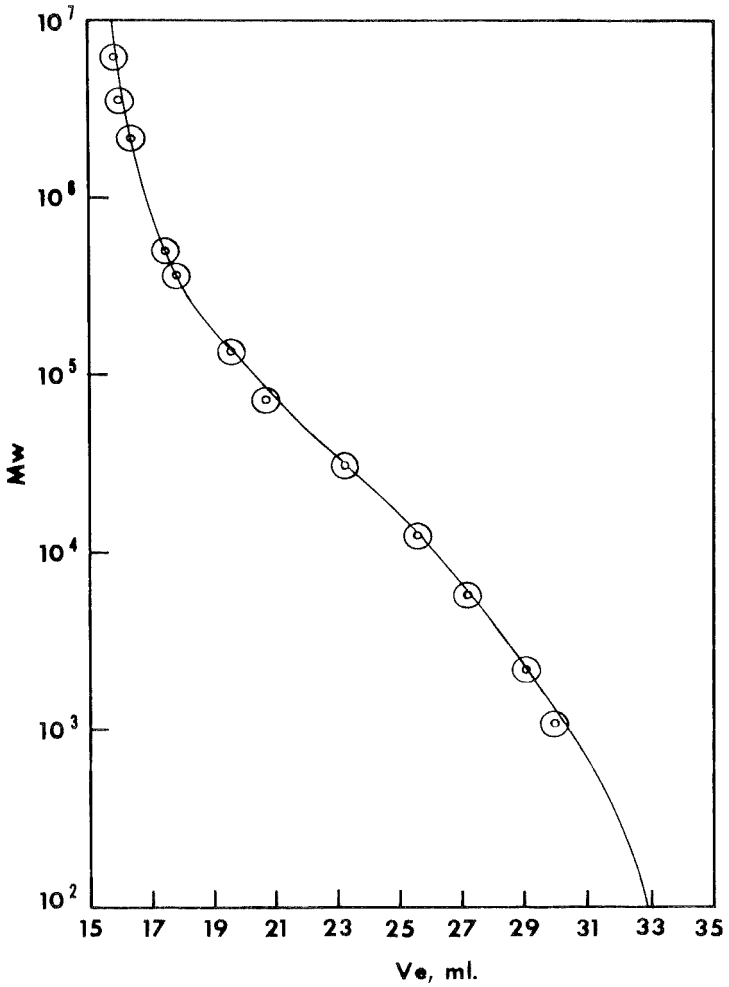


Figure 3 Weight average molecular weight of PET as a function of elution volume in (25/75%) ortho-chlorophenol-chloroform.

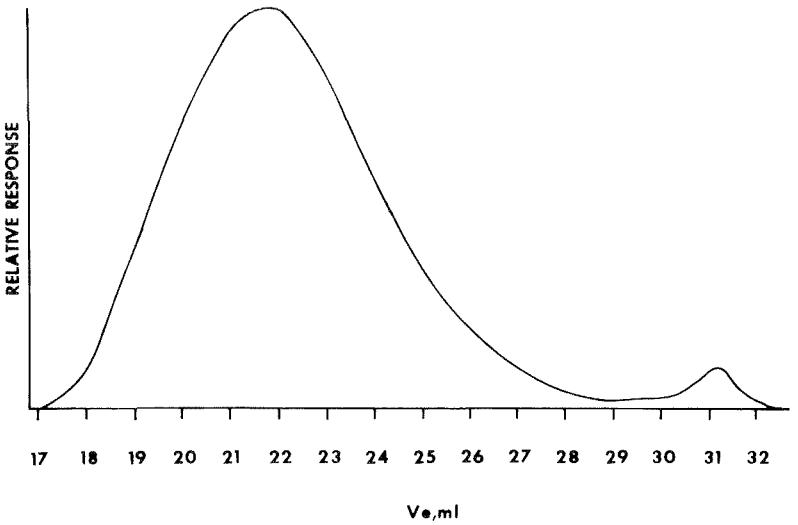


Figure 4 Typical GPC chromatogram of PET in (25/75%) ortho-chlorophenol-chloroform at 25°C.

Molecular Weights and Polydispersity Index

The molecular weight parameters for a variety of PET samples prepared by solid-state and melt-phase polymerization processes are given in Table 4. The polydispersity index M_w/M_n for all the samples studied is somewhat higher than the theoretically predicted value of approximately 2.0 (15) for polycondensation polymers; however, the polydispersity index values obtained here are in close agreement with other experimentally determined values (13). The results in Table 4 indicate that polydispersity indices for the melt-phase samples are, in general, lower than those of the solid-state samples.

TABLE 4

Molecular Weight Parameters Obtained by GPC of Various PET Samples

PET	I.V.*	Polymerization Process	M_w	M_n	$\bar{M}_w/\bar{M}_n$
Goodyear 5041	1.00	Solid-State	66,630	25,960	2.57
Goodyear 5737	0.72	Solid-State	52,870	18,640	2.84
Goodyear (O-I 11)	0.64	Solid-State	42,080	15,180	2.77
Celanese PT 101C	0.67	Solid-State	47,860	18,160	2.64
Eastman 44C	0.70	Melt-Phase	49,740	20,890	2.38
Owens-Illinois	0.86	Melt-Phase	59,180	25,450	2.33
Owens-Illinois	0.63	Melt-Phase	35,720	14,330	2.49
Owens-Illinois	0.49	Melt-Phase	29,540	12,990	2.27

*I.V. = inherent viscosity in (60/40) phenol-tetrachloroethane at 25°C and 0.25 gm./100 ml.

Correlations with Viscosity Data

From the GPC weight average molecular weights one can calculate the corresponding inherent viscosity, I.V., using the following relationship (4):

$$I.V. = 4.68 \times 10^{-4} (\bar{M}_w)^{0.68}$$

(60/40) phenol-tetrachloroethane at 25°C and 0.25 gm./100 ml.

Table 5 gives the calculated I.V. along with the experimentally determined I.V. A fair agreement is obtained over a wide range of I.V.

Low Molecular Weight Components

As was indicated above, all of the GPC chromatograms of PET exhibit a small peak at 31 ml elution volume. The corresponding

TABLE 5

Comparison Between Measured and Calculated I.V. of PET Samples

$\bar{M}_w$ GPC	Measured I.V. ± .01	Calculated I.V.
66,630	0.90	0.90
59,180	0.79	0.82
52,870	0.73	0.76
49,740	0.69	0.73
47,860	0.67	0.71
35,720	0.65	0.58
29,540	0.51	0.51

Measured I.V. was obtained in (60/40) phenol-teretrachloroethane @ 25°C and 0.25 gm/100 ml. Calculated I.V. was obtained using the GPC $\bar{M}_w$ in the relationship I.V. (calc.) = $4.68 \times 10^{-4}(M_w)^{0.68}$.

molecular weight is 550 to 610 according to the calibration curve in Figure 3. The fact that this peak encompasses the molecular weight of cyclic trimer and that cyclic trimers are often found in PET (16) leads one to conclude that the peak at 31 ml elution volume results from the presence of cyclic trimer. This conclusion was further confirmed by dissolving a sample of cyclic trimer and injecting into the GPC. The sample eluted with peak elution volume of 31 ml.

The cyclic trimer used in this study was obtained from Goodyear Company. It is a white, crystalline powder with a reported melting point of 319°C.

In order to establish a calibration for a semi-quantitative analysis of this oligomer, a "spiking" experiment was performed.

Six sample solutions with varying amounts of cyclic trimer were added to a PET sample and chromatographed.

The weight percentage of the oligomer in the solution was calculated from:

$$\text{weight \%} = \frac{\text{weight of cyclic trimer}}{\text{weight of PET}} \times 100$$

The weight percentage of the oligomer ranged from 0.206% to 3.55%. A solution containing only PET and no cyclic trimer was run as a control.

The peak height at 31 ml elution volume was measured for each solution containing the "spiked" samples. The peak height for the solution containing only PET was also measured and subtracted from the values obtained for the "spiked" samples.

The results of the "spiking" experiment are given in Figure 5. This is a calibration line which can be used to determine the amount of cyclic trimer in PET samples.

Table 6 gives the cyclic trimer content as determined by the above procedure for a variety of experimental and commercial PET resins. The quantitative content of the cyclic trimer in PET determined here is in good agreement with previous studies (16,17).

The quantitation of cyclic trimer in PET by this method demonstrates that the concentration is greater in melt-phase polymerized PET than in solid-state resins and that for melt-phase polymerized PET, the concentration of cyclic trimer decreases with increasing molecular weight. An additional correlation of molecular weight and cyclic trimer content may be found within solid-

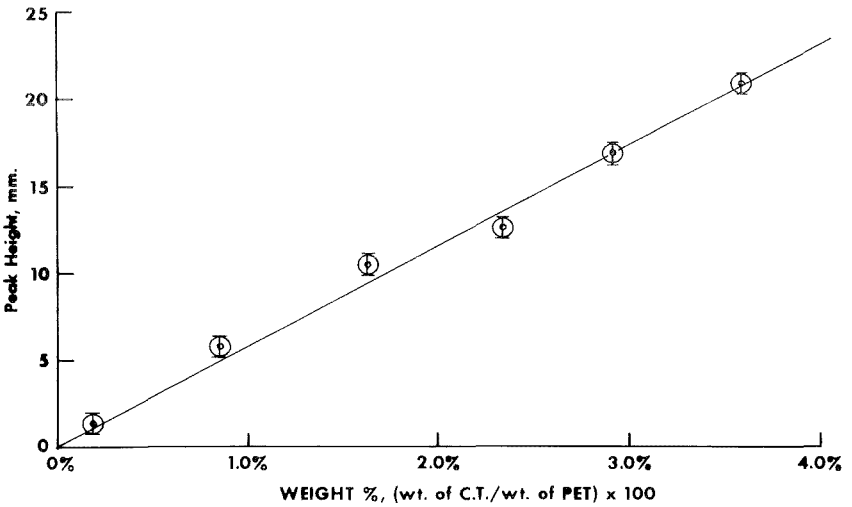


Figure 5 Peak height versus weight percent of cyclic trimer.

state polymerized PET utilizing the same catalyst system (i.e., Goodyear), with cyclic trimer content decreasing with increasing molecular weight. Furthermore, cyclic trimer content in PET prepared by solid-state polymerization, with PET samples of about the same molecular weight, appears to be dependent on the catalyst system used. Although these differences appear to be dependent on the catalyst system, a more likely cause is the solid-state polymerization conditions.

CONCLUSIONS

The gel permeation chromatography (GPC) technique has been developed for the measurements of cyclic trimer content, molecular weights and molecular weight distribution of PET, utilizing a solvent system of o-chlorophenol-chloroform.

TABLE 6

Cyclic Trimer Content in PET as Measured by the Peak Height Calibration in Figure 5

Resin	I.V.	Catalyst System	Polymerization Process	Weight % Cyclic Trimer
Eastman (experimental)	0.51	Sb-Ti-Co-Mn	Melt-phase	1.25
Zimmer (experimental)	0.62	Sb	Melt-phase	1.13
Eastman (experimental)	0.63	Sb-Ti-Co-Mn	Melt-phase	0.98
Eastman (experimental)	0.70	Sb-Ti-Co-Mn	Melt-phase	0.80
Goodyear (experimental)	0.64	Sb	Solid-state	0.60
Goodyear 5877	0.72	Sb	Solid-state	0.35
Celanese 2113 (Lot #202)	0.76	Sb	Solid-state	0.63
Eastman 7352	0.70	Sb-Ti-Co-Mn	Solid-state	0.62
Goodyear 5041	0.90	Sb	Solid-state	0.24
Goodyear 5041	1.0	Sb	Solid-state	less than 0.2

•The molecular weights determined by inherent viscosity (I.V.) measurement and those determined by GPC are in good agreement.

•The polydispersity index determined for a variety of PET samples is higher than the theoretically predicted value of 2.0; however, there is no systematic dependence on molecular weight or polycondensation process.

•The amount of cyclic trimer is greater in a melt-phase polymerized PET than that in a solid-state resin.

- For a melt-phase polymerized PET, the amount of cyclic trimer decreases with increasing molecular weight (or I.V.).

- For a solid-state polymerized PET having the same catalyst system, the cyclic trimer content decreases with increasing molecular weight.

- The cyclic trimer content in PET prepared by the solid-state polymerization process and having about the same molecular weight appear to be dependent on the catalyst system used and the solid-state polycondensation conditions.

REFERENCES

1. Van Krevelen, D. W., Properties of Polymers, Correlanous with Chemical Structure, Elsevier Publishing Company, Amsterdam-London-New York, 1972

2. Billmeyer, F. W., Jr., Textbook of Polymer Science, 2nd Ed., Wiley-Interscience, New York, 1971, 62-95.

3. Moore, W. R. and Sanderson, D., Viscosities of Dilute Solutions of Polyethylene Terephthalate, Polymer, 9, 153, 1968.

4. Moore, L. D., Jr., Cleveland ACS, Meeting 4, Vol. 1, 1960.

5. Cantow, M. J. R., Polymer Fractionation, Academic Press, New York, 1967.

6. Pebbles, L. H., Jr., Molecular Weight Distribution in Polymers, Interscience, New York, 1971, 190-232.

7. Moore, J. C., Chem. Eng. News, 40, No. 51, 43, 1962.

8. Moore, J. C., Gel Permeation Chromatography. I. A New Method for Molecular Weight Distribution of High Polymers, J. Poly. Sci., 2, 835, 1964.

9. Moore, J. C. Hendrickson, J. G., Gel Permeation Chromatography. II. The Nature of the Separation, J. Poly. Sci., 8, 233, 1965.

10. Johnson, J. F., Porter, R. S., Analytical Gel Permeation Chromatography, Cantow, M. J. R., ed., New York, 1967.

11. Shaw, G., Proceedings of the Seventh International GPC Seminar, Monte Carlo, Waters Associates, 1969, 309.

12. Overton, J. R., Rash, J., Moore, L. D., Jr., The Molecular Composition of Polyethylene Terephthalate as Determined by Gel Permeation Chromatography, Proceedings of Sixth International GPC Seminar, Miami Beach, Florida, Waters Associates, 1968, 422.

13. Paschke, E. E., Bidlingmeyer, B. A., Bergmann, J. G., A New Solvent System for Gel Permeation Chromatography of Polyethylene Terephthalate, J. Poly. Sci., 15, 983, 1977.

14. Brandup, I., Immergut, E. H., Polymer Handbook, Wiley Interscience, New York, 1965, VI-20.

15. Flory, P. J., Principles of Polymer Chemistry, Cornell University Press, Ithaca, New York, 1953.

16. Cooper, D. R., Semlyen, J. A., Equilibrium Ring Concentrations and the Statistical Conformations of Polymer Chains: Part II. Cyclics in Polyethylene Terephthalate, Polymer, 14, 185, 1973.

17. Pebbles, L. H., Jr., Huffman, M. W., Ableh, C. T., Isolation and Identification of the Linear and Cyclic Oligomers of Polyethylene Terephthalate and the Mechanism of Cyclic Oligomer Formation, J. Poly. Sci., 7, 479, 1969.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1847-1854 (1982)

SIMULTANEOUS DETERMINATION OF THE ANTIOXIDANT, THE CROSSLINKING-AGENT AND DECOMPOSITION PRODUCTS IN POLYETHYLENE BY REVERSE-PHASE HPLC

Michel Duval and Yves Giguère

Institut de recherche d'hydro-Québec,
Varennes, Québec, Canada J0L 2P0

ABSTRACT

A method has been developed for the extraction and analysis of the antioxidant, the peroxide crosslinking agent and some decomposition products in crosslinked polyethylene. The method involves the use of high-performance liquid chromatography (HPLC) in the reverse-phase mode, with a mixture of methanol and water as the mobile phase. Samples of polyethylene are ground at low temperature into a fine powder prior to extraction by methanol. The accuracy and sensitivity limits are given for each of the separated components.

INTRODUCTION

The initial formulation of chemically crosslinked polyethylene generally contains a peroxide crosslinking agent such as dicumylperoxide, and an antioxidant of the unhindered-phenol, sulfur-containing type. During the curing process, peroxide decomposition products are formed, e.g., acetophenone, cumylalcohol and α-methylstyrene (1).

The mechanical integrity of the crosslinked polyethylene (XLPE), as well as its resistance to oxidation, depend on the

remaining amounts of the above compounds, and these must therefore be carefully controlled.

The analytical procedures used in the determination of antioxidants in polyolefins, and the problems associated with them, have been discussed in several review paper (2-4). Most modern methods involve the use of high-performance liquid chromatography (HPLC) and differ mainly with regard to the extraction methods and phases used (4-9). HPLC has also been employed for the analysis of some organic peroxides (10-13). However, none of these methods can readily be applied to the case of XLPE.

A method has therefore been specially developed at IREQ for that purpose, allowing simultaneous determination of the antioxidant, the peroxide and decomposition products in XLPE, using reverse-phase HPLC. Applications to the more limited case of high voltage cables are described elsewhere (14).

EXPERIMENTAL

Materials

Commercial pellets of uncrosslinked polyethylene containing Di Cup(R)R dicumylperoxide crosslinking agent and Santonox(R)R antioxidant were obtained from Union Carbide Corp. (grade 4201B). Samples of partially and fully crosslinked polyethylene were prepared by heating pellets in a mold under pressure, at 160°C and 190°C respectively.

Standard samples of Di Cup(R)R dicumylperoxide and Santonox(R)R antioxidant were obtained from Hercules Inc. and Monsanto Chemical Co., respectively. Acetophenone, and 2-phenyl-2-propanol (cumylalcohol) were purchased from Aldrich Chemical Co., and α-methylstyrene from J.T. Baker Chemical Co.

HPLC and extraction solvents (methanol and water) were Omnisolv-grade from BDH (Canada), filtered on 0.4-μm Millipore filters prior to use.

Apparatus

A Waters Associates Model ALC-GPC 301 liquid chromatograph fitted with a Model 6000 high-pressure pump (0-40 MPa), a Model 7120 Rheodyne sample injector and a Lichrosorb RP 18 (10 μm) reversed-phase column was used with a 80 : 20 (v/v) methanol : water solution as the mobile phase. The flow rate was 1 mℓ/min and typical back pressure 17 MPa. The UV absorbance of the sample was monitored at 254 nm.

Cumylalcohol was analyzed by gas chromatography on Carbowax 20 M (1-m column, 160°C, FID detector).

Extraction Method

Additives cannot be recovered from XLPE by dissolution and selective precipitation of the polymer, because XLPE is cross-linked and consequently not soluble, and solvent extraction must be used.

XLPE samples were thus ground to 20 to 30 mesh powder in a blade grinder cooled with liquid nitrogen to avoid heat degradation, covered with methanol and stirred for 48 h at room temperature in amber glassware, then filtered and washed with methanol; the methanol solutions were subsequently concentrated to a known volume in a rotating evaporator at 30°C under vacuum, and injected directly into the liquid chromatograph.

Extractions performed directly on pellets or unpowdered specimens of XLPE were incomplete, and Sohxlet extraction with boiling methanol was not quantitative, even under a nitrogen atmosphere, possibly owing to the reaction of antioxidant and peroxide at that temperature.

RESULTS AND DISCUSSION

Qualitative Analysis

Typical chromatograms of XLPE samples are shown in Figure 1. In the reverse-phase mode, separation is according to polarity,

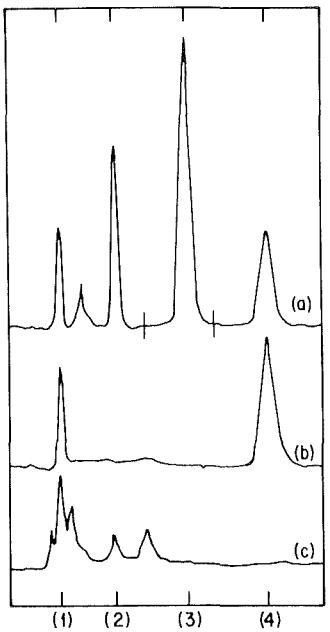


FIGURE 1 : Typical HPLC chromatograms of XLPE samples: (1) Acetophenone + cumylalcohol; (2) α-methylstyrene; (3) Santonox antioxidant; (4) Dicumylperoxide; (a) PE pellets, uncured, 0.08, 0.64 and 0.32 AUFS; (b) XLPE, partially cured, 0.08 AUFS; (c) XLPE fully cured, 0.08 AUFS.

i.e. the more polar compounds are eluted first: the peroxide decomposition products, mainly acetophenone, cumylalcohol and α-methylstyrene, then the antioxidant (Santonox(R)R) and the crosslinking agent (dicumylperoxide). Other decomposition products in a few cases were identified by mass spectrometric analysis of the eluting fraction, e.g., phtalates for the fully cured sample.

Quantitative Analysis

The antioxidant (Santonox(R)R), the crosslinking agent (dicumylperoxide) and α-methylstyrene are well separated. Acetophone and cumylalcohol elute together. However the UV absorption coef-

ficient of cumylalcoohol is very low, about 1000 times lower than that of acetophenone, while its concentration is only 1 to 5 times higher in all XLPE samples studied, as evaluated by gas chromatography. The contribution of cumylalcohol to the peak surface therefore is negligible (0.5%, i.e., less than the accuracy on the HPLC analysis) and this peak can be considered due to acetophenone only.

Quantitative HPLC results are obtained by calibrating with standard solutions of pure compounds. Accuracy on repeated injections is ±2%. The minimum amounts that can be determined by HPLC are 1 - 2 ng for acetophone and α-methylstyrene, 10 ng for the antioxidant and 400 ng for dicumylperoxide, at 0.04 AUFS.

HPLC values must be multiplied by a conversion factor, owing to the fact that extraction is not 100% complete after the 48 h extraction period chosen as a practical limit in the experimental procedure. A typical extraction curve relating the amount of additive extracted as a function of time is shown in Figure 2. Such curves are quite reproducible and a fairly reliable extraction value of 86% (±3%) is reached after 48 h.

The overall accuracy on XLPE analysis, combining the accuracy on extraction and on HPLC analysis, is ±5%. The sensitivity, i.e., the minimum concentration of additives and decomposition

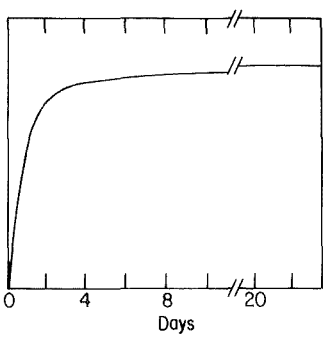


FIGURE 2 : Typical curve of extraction of the additives as a function of time.

TABLE 1
Sensitivity Limits on XLPE Analysis (in ppm)

Dicumyl-peroxide	Anti-oxidant	Aceto-phenone	α-methyl-styrene
100	2	0.5	0.5

TABLE 2
HPLC Quantitative Analysis of Various XLPE Samples

Ref Fig. 1	HPLC analysis / Samples	Dicumyl-peroxide %	Anti-oxidant %	Aceto-phenone ppm	α-methyl-styrene ppm
	Di-Cup R(R) Dicumylperoxide	100	-	3170	720
(a)	PE pellets uncured	1.2	0.14	25	3
(b)	XLPE sample partially cured	0.4	-	15	-
(c)	XLPE sample fully cured	-	-	5	22

products in XLPE that can be analyzed, is a function not only of the sensitivity of the HPLC analysis (values given above) but also of practical considerations such as the HPLC injection volume (20 µℓ max.), volume of concentrated extract (10 mℓ min.) and amount of XLPE which can be powdered (2 g min.). The sensitivity limits thus obtained are indicated in Table 1.

Application to the Curing Process

The quantitative values obtained for the XLPE samples shown in Figure 1 are listed in Table 2.

As expected, the peroxide content, starting from a measured value of 1.2% in uncured pellets, falls to 0.4% in the partially cured sample, then to 0% in the fully cured sample.

The amounts of the peroxide decomposition products vary slightly, with acetophenone present in all cases and α-methylstyrene being formed in the fully cured sample. Commercial dicumylperoxide itself contains relatively large amounts of acetophenone and α-methylstyrene.

The antioxidant, surprinsingly, starting from an initial value of 0.14%, seems to disappear in partially as well as in fully cured samples. Extended studies, however, have shown that it does not in fact disappear but is grafted to the polymer chain during the curing process and is not extracted by methanol (14).

ACKNOWLEDGMENTS

The authors would like to thank Dr. H. St-Onge for providing samples of XLPE, and Mrs. Lesley Regnier for reviewing the manuscript.

The cooperation of Union Carbide Corp., Hercules Inc. and the Monsanto Chemical Co., in providing materials and information, is also gratefully acknowledged.

REFERENCES

(1) Eichhorn, R. M., Organic Residues in Polyethylene Insulation Crosslinked with Dicumylperoxide, IEEE Underground Transm. Conf., Pittsburg, May 24, 1972.

(2) Wheeler, D. A., Determination of Antioxidants in Polymeric Materials, Talanta, 15, 1315, 1968.

(3) Crompton, T. R., Chemical Analysis of Additives in Plastics, Pergamon, New York, 1971.

(4) Walters, R. B., and Johnson, J. F., Analysis of Antioxidants in Polymers by Liquid Chromatography, J. Polym. Sci., Macromol. Rev., 15, 29, 1980.

(5) Wims, A. M. and Swarin, S. J., Determination of Antioxidants in Polypropylene by Liquid Chromatography, J. Appl. Polym. Sci., 19, 1243, 1975.

(6) Lehotay, J., Danecek, J., Liska, O., Lesko, J. and Brandsteterova, E., Analytical Study of the Additives System in Polyethylene, J. Appl. Polym. Sci., 25, 1943, 1980.

(7) Schabron, J. F. and Fenska, L. E., Determination of BHT, Irganox 1076, and Irganox 1010 Antioxidant Additives in Polyethylene by High performance Liquid Chromatography, Anal. Chem., 52, 1411, 1980.

(8) Haney, M. A. and Dark, W. A., A Reversed-Phase High Pressure Liquid Chromatographic Method for Analysis of Additives in Polyethylene, J. Chrom. Sci., 18, 655, 1980.

(9) Schabron, J. F. and Bradfield, D. Z., Determination of the Hindered Amine Additive CGL-144 in Polypropylene by High-Performance Liquid Chromatography, J. Appl. Polym. Sci., 26, 2479, 1981.

(10) Van Tilborg, W., Qualitative and Quantitative Determination of Hydroperoxides by High-Performance Liquid chromatography, J. Chromatogr., 115, 616, 1975.

(11) Hercules Technical Bulletin ORC-207, High-Performance Liquid Chromatographic Assay of Di-Cup(R) Dicumylperoxide, Available from Hercules Inc., Organics Department, Wilmington, Del. 19899, 1977.

(12) Cornish, L. A., Ferrie, R. and Paterson J. E., High Pressure Liquid Chromatography of Some Organic Peroxides, J. Chrom. Sci., 19, 85, 1981.

(13) Worstell, J. H., Daniel, S. R., Strand, G. O. and Frauenhoff, G. R., Comparative Separation of Tetralin Hydroperoxide from its Decomposition Products by High Performance Liquid Chromatography, J. Liquid Chrom., 4 (3), 539, 1981.

(14) Duval, M. and St-Onge, H., HPLC and DSC Analysis of Cross-linked PE from HV Cables, IEEE Trans. Elec. Insul., 14, 264, 1979.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1855-1867 (1982)

DONNAN EXCLUSION CHROMATOGRAPHY: THE ELUTION BEHAVIOR OF PHOSPHORUS OXOPOLYANIONS OF LARGE SIZE IN DEC

Yuji Tokunaga, Hirohiko Waki and Shigeru Ohashi

Department of Chemistry, Faculty of Science,
Kyushu University
Hakozaki, Higashiku, Fukuoka, 812 JAPAN

ABSTRACT

The elution behavior of ions of large size in Donnan exclusion chromatography has been elucieated by an assorted ionic and steric exclusion. An effective model for the swollen ion-exchanger phase such as a cross-linked dextran cation-exchanger and a structural size parameter for various oxoanions of phosphorus have been proposed. These studies may help the prediction for elution positions of ions excluded from an ion-exchanger column.

INTRODUCTION

Donnan exclusion chromatography (DEC) is a new type of separation method utilizing an electrostatic repulsion between sample ions and fixed ionic groups of an ion-exchanger. Accordingly, cation-exchangers should be used for the mutual separation of anions and anion-exchangers for cations(1-7). In the previous paper(1), a theoretical ground was given on the basis of the Donnan distribution equilibrium of sample ions between solution

and ion-exchanger phase; the logarithm of the distribution coefficient is found to be proportional to the practical ionic charge on sample ions. Further, it was pointed out that species of large size, such as phosphorus oxoanion polymers, undergo both ionic and steric exclusion effects and are eluted at a volume position before that which would be predicted purely from the ionic exclusion effect.

For a steric exclusion effect with ion-exchangers, Samuelson(8,9) performed many experiments with various organic substances. He generally correlated the elution behavior of oligomers such as sugar derivatives with their partial molar volumes, using the Gibbs-Donnan relationship. However, his treatment could not easily be applied to our case, where the sample components are highly charged and hydrated, and their conformations in solution or their effective sizes are not very clear.

It is very important to elucidate the elution behavior in terms of basic properties of sample ions, such as the molecular structure under an appropriate model on the interior structure of the ion-exchanger. For this purpose we employed oxopolyanions of phosphorus, which have a variety of sizes and structures.

EXPERIMENTAL

Chemicals Orthophosphate; $NaH_2PO_4 \cdot 2H_2O$ or Na_2HPO_4, diphosphate(pyrophosphate); $Na_4P_2O_7 \cdot 10H_2O$, phosphonate (phosphite); $Na_2PHO_3 \cdot 5H_2O$ and phosphinate(hypophosphite); $NaPH_2O_2 \cdot H_2O$ were of commercially available reagent grade. Triphosphate; $Na_5P_3O_{10} \cdot 6H_2O$ was prepared by recrystallization from commercial anhydrous triphosphate. Other linear polyphosphates, salts of lower oxoacid of phosphorus and metaphosphates were prepared in our

laboratory. Crotonaldehyde; *trans*-CH_3CH=CHCHO was of reagent grade.

Eluent The eluent was 1.00 M tetramethylammonium chloride solution prepared with a small amount of hydrochloric acid or tetramethylammonium hydroxide to desired pH. The solution of tetramethylammonium chloride was standardized titrimetrically with silver nitrate solution.

Elution procedure A pyrex tubing column of 15 mm I.D. x 95.8 cm was packed with SP-Sephadex C-25 (40–120 μm particle size, tetramethylammonium form). After the column was conditioned with the eluent, one ml of a sample solution, adjusted into the same pH as the eluent, was added onto the top of the column, then the eluent was passed through the column. The effluent was collected by a fraction collector (each one ml volume), and analyzed as described below.

Determination of sample ions Phosphorus for various oxoanions were determined colorimetrically with a molybdenum(V)-molybdenum(VI) reagent by a standard method(11). For lower oxoacid of phosphorus, sodium hydrogensulfite solution was added as oxidizing agent. Crotonaldehyde was determined UV-spectrophotometrically at wavelength 224 nm.

RESULTS AND DISCUSSION

It was found that the Donnan exclusion distribution coefficient of a certain ion X^{x-} eluted from a B^+-cation-exchanger column is expressed by the following equation (1);

$$K_D = R^x \qquad (1)$$

$$R = \frac{[B^+]}{[B^+]_r} \cdot \frac{y_X^{1/x} \; y_B}{\bar{y}_X^{1/x} \; \bar{y}_B} \qquad (2)$$

where y and $\bar{y}$ are activity coefficients of subscript species for solution and exchanger phase. Here R may be considered to be approximately constant under a given eluting condition. K_D is related to its elution position by an ordinary chromatographic relationship,

$$V_e = V_0 + K_D \cdot V_D \qquad (3)$$

where V_e is the elution volume, V_0 void volume and V_D the net internal volume of the exchanger phase. It has been experimentally supported by a linear relationship between log K_D and an anionic charge that R is constant if the size of sample anion is not very large. However, this does not fit ions which are of large size, exceeding a certain limit. In Fig. 1-(A), there was a considerable difference between elution volumes for octametaphosphate $P_8O_{24}^{8-}$ and long-chained linear polyphosphate P_nO_{3n+1} ($\bar{n}$= 55) ions, although these ions should be eluted at nearly the same volume position($K_D \approx 0$). Conventional theories could not explain such distribution of highly-charged ions which should be completely excluded by the ionic exclusion effect. On the other hand, as the ionic exclusion effect can be evaluated by Donnan exclusion chromatography, the steric effect can be discriminated from the ionic exclusion effect. Therefore, another model for the interior of an ion-exchanger of the present type must be proposed for quantitative explanation and a structural parameter for the steric effect is introduced.

The ion-exchanger interior can be divided, for convenience, into two phases where the fixed ionic groups are present in a concentrated state(Donnan exchanger phase) and in a diluted state(pseudo exchanger phase),

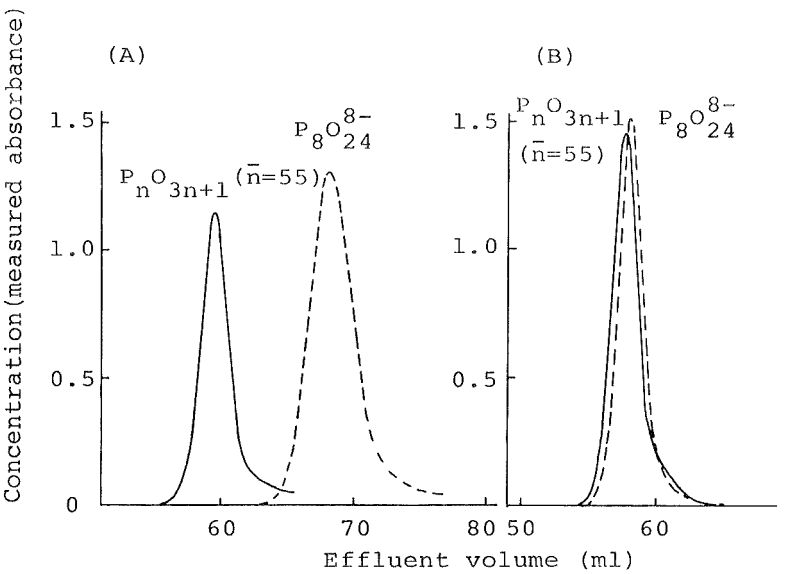


FIGURE 1

Elution behavior of Species sterically excluded. Column; SP-Sephadex C-25, $(CH_3)_4^+$-form, (A) 1.5 cm I.D. x 95.8 cm and (B) 1.5 cm I.D. x 95.5 cm. Eluent; (A) 1.00 M and (B) 0.10 M $(CH_3)_4NCl$ (pH 9.8). Flow rate; 0.75 ml/min. Sample amount; 0.7×10^{-6} mol $Na_8P_8O_{24}{\cdot}8H_2O$, 3.0×10^{-6} mol(as P) $Na_nP_nO_{3n+1}$ ($\bar{n}$ = 55).

as shown in Fig. 2. The phase of concentrated fixed ionic groups may maintain its ion-exchanger characteristics such as adsorption or ionic exclusion even after a considerable invasion of outside electrolytes takes place. On the other hand, the phase of dilute fixed ionic groups may be no longer considered as a real ion-exchanger phase, when the exchanger is immersed in a concentrated electrolyte solution, because the internal solution of this phase becomes nearly the same solution

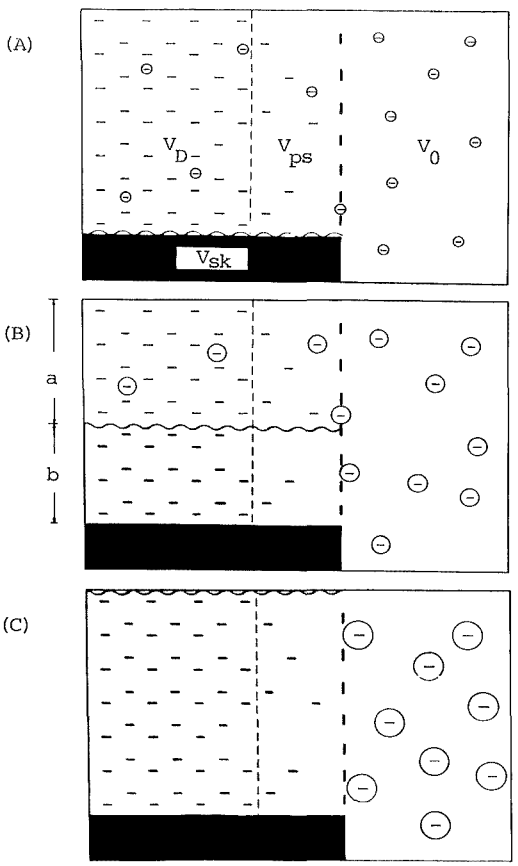


FIGURE 2

A proposed structure model for a swollen dextran cation-exchanger. (A) Anions of small size which cannot be excluded by steric exclusion effect. (B) Anions partially excluded by steric exclusion effect. (C) Anions of very large completely excluded by steric effect. V_0; external interstitial phase volume, V_{ps}; pseude exchanger phase volume, V_D; Donnan exchanger phase volume, V_{sk}; skeleton volume, -; fixed anion of cation-exchanger, $\ominus$; sample anion, α; ion-penetratable volume fraction in ion-exchanger (α = a/a+b).

as the external solution by remarkable electrolyte invasion. Even highly-charged anions could not be exluded from this pseudo exchanger phase as long as they are of small size while anions of very large size are completely excluded from both phases of cation-exchanger. However, the volume fraction of such pseudo exchanger phase varies with the electrolyte concentration of external solution(Fig. 1-(B)). When the exchanger is merely immersed in water, the difference between this phase and the external water becomes significant, and therefore, all movable anions should be excluded completely by the ionic exclusion effect irrespective of molecular size; this was confirmed by the fact that all anions are eluted together at the same effluent volume from a cation-exchanger column. Of course, it is not argued here that the distinct boundary between the inside phases is actually present. The concentration of fixed ionic groups may vary somewhat more continuously over the whole exchanger phase. Therefore, our treatment is just an approximation for theoretical convenience.

Under these assumptions, the elution volume V_e for large anions which undergo both ionic and steric exclusions, can be given by the following equation

$$V_e = V_0 + \alpha (V_{ps} + K_D \cdot V_D) \qquad (4)$$

instead of Eqn (3). Here V_0 is the volume of the external interstitial solution, V_{ps} that of pseudo exchanger phase and V_D that of the Donnan exchanger phase. The α is the volume fraction of the ion-penetratable part in an ion-exchanger(a/a+b in Fig, 2-(B)), which depends upon the ion structure and becomes unity in case of small ions (corresponding to the distribution coefficient K_d in gel-permeation chromatography). The K_D in Eqn (4) is not an apparent distribution ratio, but an authentic distribution coefficient between the Donnan exchanger

phase and the solution phase. Among these quantities, V_0 can be determined from the elution volume for extremely large ions such as long-chained polyphosphate ions ($\bar{n}$= 55), and evaluated to be 59.5 ml for a column of 169.3 ml. On the other hand, V_{ps} can not directly be measured since the small ions of extremely high charge are not available. This was determined by a trial and error treatment with the consideration that the assumption of an appropriate V_{ps} value should bring about the linear relationship between log K_D and ionic charge(1). The log K_D—ionic charge plot with small ions at V_{ps} = 18.2 ml for the same column gave a straight line, as shown in Fig. 3.

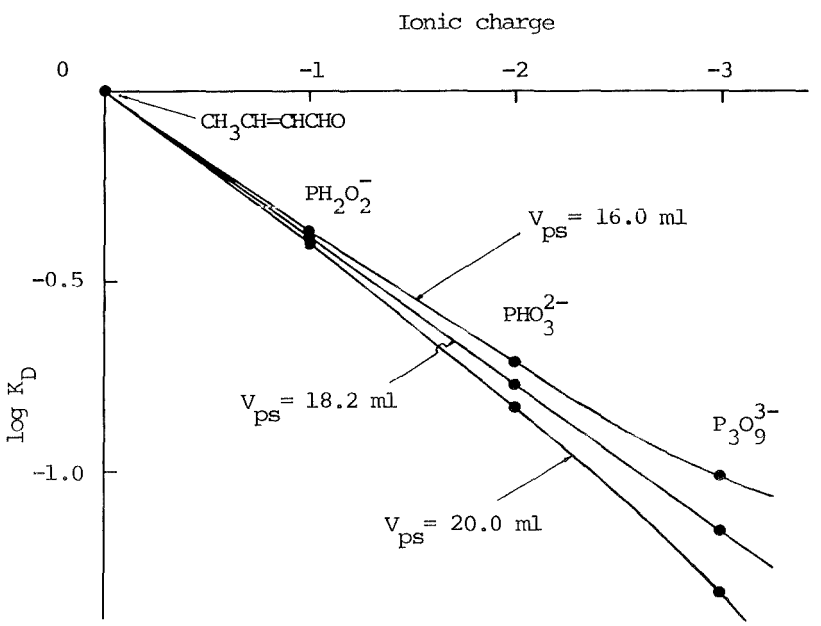


FIGURE 3
Estimation of V_{ps}.

Next, V_D was estimated to be 74. 8 ml by substracting $V_0 + V_{ps}$ from the elution volume of neutral species of 152.5 ml (crotonaldehyde; $K_D = 1$, $\alpha = 1$). The skeleton volume of an ion-exchanger can also be calculated from these date and total column volume. The volume allocation of the cation-exchanger, SP-Sephadex C-25 equilibrated with 1.00 M tetramethylammonium chloride solution is given in TABLE 1. Although these relative volumes are, of course, dependent upon types of ion-exchangers and compositions of external solutions, the understanding of the phase composition of a swollen ion-exchanger may serve to interpret the elution phenomena of various ions from such an ion-exchanger.

The α value indicates to what extent, in terms of volume fraction, the exchanger permits the penetration of a particular ion and directly depends upon the dimension of the ion. To know the dependence of α on the ion size, we measured α on series of linear polyphosphates, cyclic metaphosphates and lower oxoanions of phosphorus. The K_D of a highly charged anion was estimated from the theoretical straight line in Fig. 3, since it can not be determined experimentally. Then K_D and V_e values of individual sample anions were

TABLE 1

Composition of SP-Sephadex C-25 Column Equilibrated with 1.00 M $(CH_3)_4NCl$ Solution

	Volume allocation(%)
V_0	35.1
V_{ps}	10.8
V_D	44.2
V_{sk}	9.9

TABLE 2

Structural Size Parameters for Oxoanions of Phosphorus

Number	Species	l	i_e	p	α
Linear phosphorus oxoanions					
1	$PH_2O_2^-$	3	1	4	1.00
2	PHO_3^{2-}	3	2	5	1.00
3	$H_2PO_4^-$	3	1	4	1.00
4	$H_2P_2O_7^{2-}$	5	2	7	0.95
5	$HP_2O_7^{3-}$	5	3	8	0.93
6	$P_2O_7^{4-}$	5	4	9	0.86
7	$P_2H_2O_5^{2-}$	5	2	7	1.00
8	$P_3O_{10}^{5-}$	5	4	9	0.83
9	$P_3O_9^{5-}$	6	4	10	0.68
10	$P_4O_{13}^{6-}$	7	4	11	0.65
Cyclic phosphorus oxoanions					
11	$P_3O_9^{3-}$	5	2	7	1.00
12	$P_4O_{12}^{4-}$	7	2	9	0.86
13	$P_6O_{18}^{6-}$	9	2	11	0.61
14	$P_8O_{24}^{8-}$	11	2	13	0.47
15	$P_6O_{12}^{6-}$	6	2	8	0.86

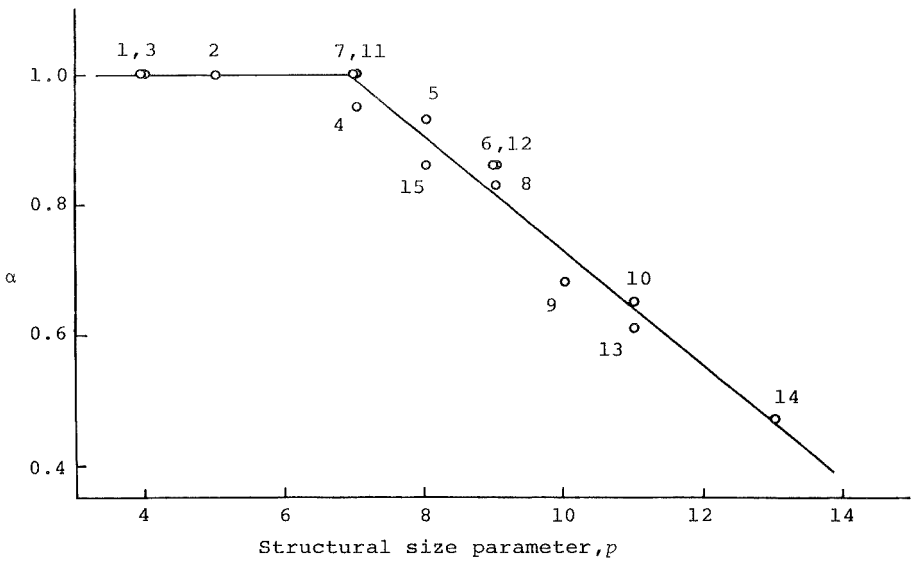


FIGURE 4

Relationship between α and the structural size parameter. Numbers in the figure are the same as in TABLE 2.

substituted in Eqn (4) to calculate α. The α values obtained are tabulated in TABLE 2; this order of α is essentially the same as that of the distribution coefficient obtained by gel chromatography in which influences of eluting agents are taken into account(10).

In order to correlate α with the ion structure, a structural size parameter p was introduced. The parameter was, in principle, taken as the molecular length of hydrated phosphorus oxoanion. Since the conformation of various phosphorus polymers are, in general, not exactly known, we first assumed the net molecular chain length l as the total number of P and O atoms along the molecular chain as a first approximation; for example, l = 5 and 7 for trimeta- and tetrametaphosphate ions,

respectively. For triphosphate and longer linear phosphates, the l value was assumed to have the same value as that in the corresponding metaphosphate of the same phosphorus numbers, for instance, l for triphosphate is not seven but five, because a linear chained anion is considered to take a spiral conformation. Furthermore, we added to l the total anionic charge of the two end groups i_e to make a contribution by bound water molecules which affect the actual length of species. The p thus obtained should reflect, at least qualitatively, the hydrated length of an polymer anion in the simplest way. These parameters are given in TABLE 2 together with α's; the correlation between α and p is shown in Fig. 4. For large phosphorus oxoanions which undergo a steric exclusion from an ion-exchanger, a fairly good relationship can be seen between α and the structural size parameter. This relationship may serve in elucidating the exclusion or elution behavior of a large ionic species and, in some cases, in deducing the molecular conformation in solution state.

ACKNOWLEDGEMENTS

This work was partially supported by a Grant-in-Aid for Scientific Research Nos. 411706 and 510804 from the Ministry of Education, Science and Culture.

REFERENCES

1. Waki, H. and Tokunaga, Y., J. Chromatogr., 201, 259 (1980).

2. Crone, H. D., J. Chromatogr., 92, 127(1974).

3. Crone, H. D., Dawson, R. M. and Smith, E. M., J. Chromatogr., 103, 71(1975).

4. Crone, H. D., J. Chromatogr., 107, 25(1975).

5. Tanaka, K. and Sunahara, H., Bunseki Kagaku (Jap. Anal.), 27, 95(1978).

6. Tanaka, K., Ishizuka, T. and Sunahara, H., J. Chromatogr., 174, 153(1979).

7. Tanaka, K. and Ishizuka, T., J. Chromatogr., 190, 77(1980).

8. Martinsson, E. and Samuelson, O., J. Chromatogr., 50, 429(1970).

9. Samuelson, O., Adv. Chromatogr., 16, 113(1978).

10. Miyajima, T., Yoshikawa, Y., Yoza, N. and Ohashi, S., J. Liq. Chromatogr., 5, 73(1982).

11. Conde, F. L. and Prat, L., Anal. Chim. Acta, 16, 473(1957).

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1869-1897 (1982)

COMPARISON OF COMMERCIAL COLUMN TYPES IN LIQUID CHROMATOGRAPHY

R. W. McCoy and R. E. Pauls

Standard Oil Company (Indiana)
P. O. Box 400
Naperville, IL 60566

ABSTRACT

Several commercially available liquid chromatographic column types have been experimentally evaluated. A conventionally-sized column containing 5 μm packing, a microbore column containing 10 μm packing, a column containing 3 μm packing, and two short, wide-bore columns containing 5 μm packing were compared at optimum velocity (van Deemter minimum) and at twice the optimum velocity where possible. All columns contained reversed-phase media of the C-18 type. Attention was focused on establishing advantages and limitations of each column with regard to maximum available plate count, minimum separation time, and required pressure drop. A van Deemter plot was constructed for each column type and the number of plates generated per unit length, time, and pressure was determined. In addition reduced parameters, separation impedance, peak capacities, and analysis times at a given k′ were calculated. Calculations indicated the highest possible plate counts should be obtained with coupled microbore columns. Small particle (3 μm) columns provided the best performance for high speed, moderate plate count separations. Conventional-sized columns containing 5 μm packing material appeared to be a good compromise between high speed and high total plate count.

INTRODUCTION

For the past several years most commercial manufacturers have employed a common design for analytical-scale liquid

chromatographic columns. These columns typically are stainless steel tubing of 4-5 mm internal diameter and 150-250 mm length packed with porous particles 5-10 μm in diameter. Recently several columns which deviate from this basic design have become available. These changes reflect a growing interest in high speed and high resolution separations. In striving toward these goals, the compromises in terms of column efficiency, analysis time, and available pressure dictated by each column design must be understood. With this knowledge, the practicing analytical chemist can wisely select the column design best suited for a particular separation problem.

Design, construction, and operation of packed, microbore columns have been the subject of several publications by Scott and co-workers (1-3). Their work demonstrates the extremely high efficiencies, on the order of 500,000 theoretical plates (1), obtained with 1 mm internal diameter columns packed with 10-20 μm particles. These columns provide maximum efficiency at volumetric flow rates in the range of 2-100 μl/min. These low flow rates provide improved solvent economy and mass sensitivity when utilizing concentration sensitive detectors such as the uv absorbance detector. However, these advantages are realized only with an extreme sacrifice in separation speed. At optimum conditions, separation times with microbore columns can be several hours (1).

Developments in the field of small-bore columns have not been limited to packed columns. The use of open tubular capillary columns of borosilicate glass (4,5) and fused silica (6) have been reported. Chemically-bonded, octadecyl groups provide separation surfaces similar to those of conventional reversed-phase packings. Packed capillary columns (7-9) containing either bare or surface modified alumina or silica gel have also been applied to the liquid chromatographic separation of complex mixtures. Recent theoretical comparisons (10,11) of microbore packed columns (PC), open tubular columns (OTC) and packed capillary columns (PCC) indicate no advantages for OTC and PCC type columns given the current operational limitations imposed by available instrumentation, principally detector cell volumes. However, if open tubular columns can be prepared with 5-10 μm internal diameter and detector cells of 1 nl or less volume are available, the inherent permeability advantages of these columns should provide performance beyond that realizable from packed columns (10).

The minimum height equivalent to a theoretical plate (H) for a well packed column is approximately equal to 2 particle diameters (3). Therefore, two equally well packed columns of the same length containing different size packing materials will provide total plates inversely proportional to the packing diameter. Conversely, if total plates are held constant, shorter columns containing smaller particles will provide faster analysis

times. Recently, several column manufacturers have begun offering columns packed with 3 μm particles to take advantage of potential savings in analysis times. Ettre, et. al. (12) have discussed high speed separations utilizing conventional sized columns containing these 3 μm packing materials.

According to Darcy's law (13), the pressure drop across a column is determined by both the packing particle diameter and the column length. Several commercial columns have recently become available utilizing conventional 5-10 μm packing materials in 100 mm lengths and 8-10 mm internal diameters. The stated advantages (14,15) of these columns is reduced back pressures allowing operation at higher volumetric flow rates resulting in reduced analysis times.

In this paper, various column designs will be compared at the optimum flow (minimum of the van Deemter plot) for each and at twice the optimum flow where possible. Based on this optimized evaluation, relative advantages and disadvantages of each design will be presented.

EXPERIMENTAL

Apparatus

The pumping system utilized in this work consisted of a Waters Associates (Milford, MA.) 6000A pump controlled by a Waters

660 controller. The single pump was connected to the "B-Pump" output of the controller. With this arrangement, the volumetric flow rate delivered by the pump was determined by the product of "% B" and "total flow" settings of the controller. Although it was conceptually possible to deliver flows from 1 µl/min to 10 ml/min with this arrangement, delivered flow rates below 50 µl/min differed significantly from the set values. Actual volumetric flow rates in these cases were determined by collecting mobile phase in a tared vessel for a fixed period of time (typically 30 minutes).

A Valco (Houston, TX) Model CFSV-6-HPAX sample injection valve with 0.2 µl internal loop volume was employed for all studies on microbore columns. For all other columns, a Rheodyne (Cotati, CA) Model 7125 sample injection valve with 0.5 µl internal loop was utilized. The maximum pressure ratings of the Valco and Rheodyne valves are 3000 and 6000 psi, respectively. Both valves were manually controlled.

The five reversed-phase columns employed in this study are listed in Table 1. Column dimensions, packings, particle sizes, and suppliers are provided. All columns were of fixed wall stainless steel construction except the RCSS column, which utilized flexible wall construction. During use, the RCSS column cartridge was placed in a Waters Model RCM 100 compression module.

TABLE 1

Columns Evaluated

Column Type	Column Dimensions	Packing Type and Size	Manufacturer
Conventional	250x4.6mm	Ultrasphere, ODS-5 μm	Altex/Beckman (Berkeley, CA)
Microbore	500x1.0mm	C-18 - 10 μm	Peterson Assoc. (Nutley, NJ)
Small Particle (3 μm)	100x4.6mm	C-18 - 3 μm	Perkin Elmer (Norwalk, CN)
RCSS[a]	100x8.0mm	C-18 - 5 μm	Waters Assoc. (Milford, MA)
RAC[b]	100x9.4mm	Partisil, ODS-3 - 5 μm	Whatman (Clifton, NJ)

(a) Radial Compression Separation System.
(b) Rapid Analysis Column.

The microbore column was connected directly to the injection valve and the detector flow cell. For all other columns, a minimum length (~6 cm) of 0.010 in internal diameter tubing was used for these connections. These connections accounted for approximately 3 μl of added dead volume.

A Schoeffel (Westwood, NJ) Model 770 variable wavelength detector equipped with Model SFA 234 0.5 μl flow cells was employed. Detector wavelength was adjusted to 254 nm for all studies and the detector time constant was set to the minimum value (fastest response). The detector analog output was

monitored with a Linear Instruments (Irvine, CA) Model 232 10 mv strip chart recorder. The analog output was also connected to a Modcomp II computer (Modular Computer Systems, Ft. Lauderdale, FL). Graphical display of chromatograms was possible on either a Tektronix (Beaverton, OR) Model 4010 video terminal or Model 4611 printer. All measurements of retention time and peak width were made from the output of the computer graphics system. Digitization rates of the chromatographic data were varied from 7.5 to 1 points per second to suit individual experiments.

Reagents

A mobile phase of 60% (v/v) acetonitrile (Burdick and Jackson, Muskegon, MI) in water was employed in all studies. The water was distilled and purified through a Millipore (Bedford, MA) Milli-Q treatment system. Mobile phase was prepared by mixing exactly 400 ml water with exactly 600 ml acetonitrile and degassing the mixture under vacuum while stirring. Preparation in this manner negated volume changes due to mixing phenomena encountered with these two solvents.

The test solution for all column studies contained acetone (50% v/v), acetophenone (0.50% v/v), anisole (16.5% v/v), benzene (16.5% v/v), and toluene (16.5% v/v) of analytical reagent grade or better. This mixture was injected directly without further dilution.

Calculations

The number of theoretical plates, N, was calculated by

$$N = 5.54\ (t_r/w_{\frac{1}{2}})^2 \tag{1}$$

where t_r is the solute retention time and $w_{\frac{1}{2}}$ is the width of the solute peak at half height. The plate height, H, is given by

$$H = L/N \tag{2}$$

where L is the column length. The reduced plate height, h, and reduced velocity, v, both dimensionless quantities, are defined by

$$h = H/d_p \tag{3}$$

$$v = ud_p/D_m \tag{4}$$

where d_p is the mean particle diameter, u is the linear mobile phase velocity, and D_m is the solute diffusion coefficient in the mobile phase.

The capacity factor, k´, is defined as

$$k' = (t_r - t_m)/t_m \tag{5}$$

where t_r and t_m are solute and nonretained peak retention times,

respectively. The separation impedance as defined by Bristow and Knox (16) was calculated by

$$E = (t_m/N)(\Delta P/N)(1/\eta) \quad (6)$$

where ΔP is the pressure drop across the column, η is mobile phase viscosity, and other terms are as previously defined. A value of 0.76 centipoise was assumed for η, the mobile phase viscosity, and values of 1.3×10^{-5} cm^2/sec and 1.6×10^{-5} cm^2/sec for toluene and acetone, respectively, were assumed for D_m, the solute diffusion coefficient (17).

RESULTS AND DISCUSSION

Plate height (H) values for all solutes were determined over linear mobile phase velocities of 0.1 to 5.0 mm/sec. These data for acetone ($k' \sim 0$), anisole ($k' \sim 1.2$), and toluene ($k' \sim 2$) are presented in Figures 1-5 in the form of van Deemter plots for each of the five columns under study. Since all columns were compared with the same mobile phase composition, k' varied for each component from column to column.

The van Deemter plot for a Altex/Beckman Ultrasphere-ODS column, representative of conventional liquid chromatographic columns, is provided in Figure 1. The optimum linear velocity was 1.5 mm/sec corresponding to a volumetric flow rate of 1.0 ml/min.

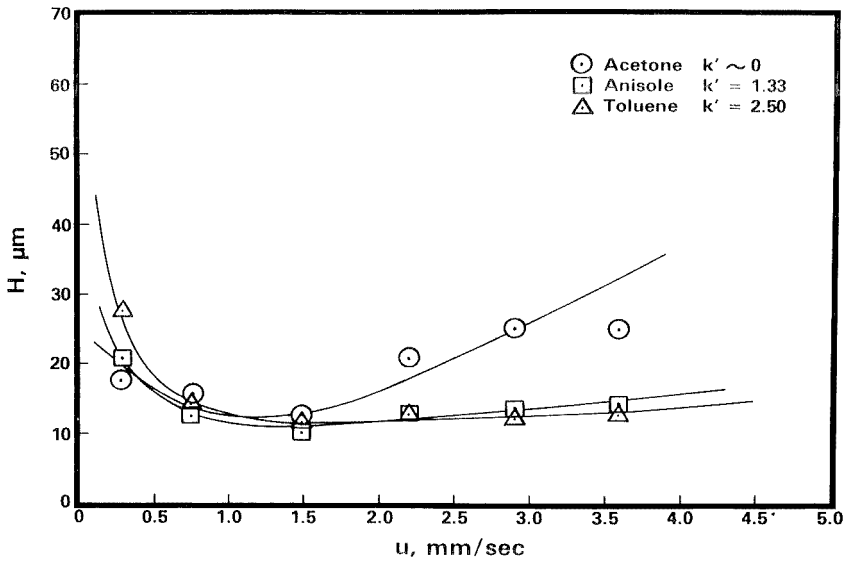


Figure 1. The van Deemter plot for test solutes on a 250 x 4.6 mm column containing 5 µm Ultrasphere ODS packing.

The plate height at optimum flow was roughly 11 µm for anisole and toluene and was slightly higher for acetone. The minimum of the van Deemter plot for this column was broad for anisole and toluene while the nonretained peak, acetone, displayed a rapid loss in efficiency at increased velocities.

Figure 2 contains the van Deemter plot for a 50 cm microbore column. The minimum plate height occurs at a velocity of approximately 0.5 mm/sec corresponding to a volumetric flow rate of 28 µl/min. At this flow H values ranged from 26 µm for toluene to 33 µm for acetone. For all solutes, the minimum of the van

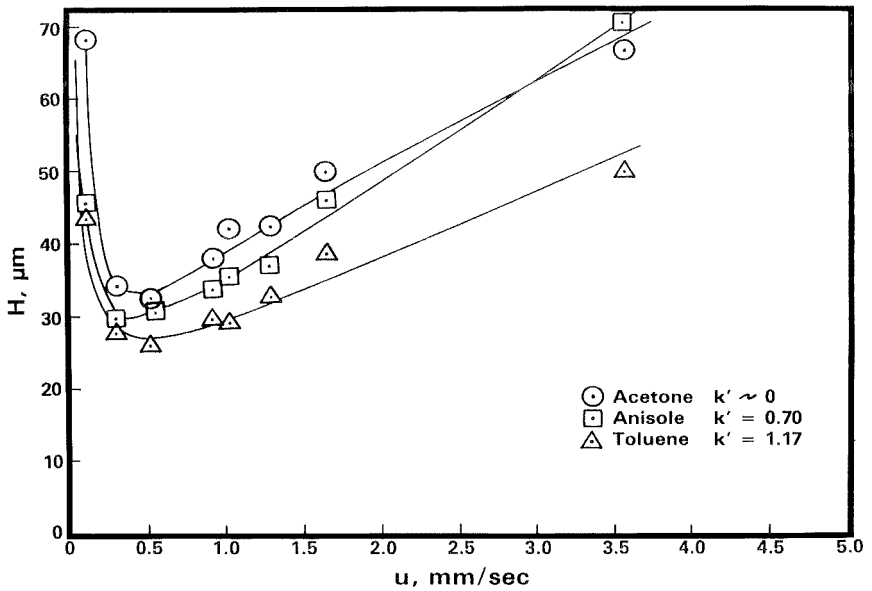


Figure 2. The van Deemter plot for test solutes on a 500 x 1.0 mm microbore column containing 10 µm packing.

Deemter plot was sharp and column efficiency degraded quickly with increased flow. Plate height values for the microbore were significantly larger than those found for the Ultrasphere column reflecting the increased particle diameter (10 µm vs. 5 µm).

Figure 3 shows the van Deemter plot for a Perkin-Elmer column packed with small particles (3 µm). This plot is flat throughout the entire linear velocity range examined. The minimum plate height for toluene of roughly 8 µm occurred at a velocity of 2.5-3.0 mm/sec. This velocity corresponded to a volumetric flow rate of 2.0 ml/min.

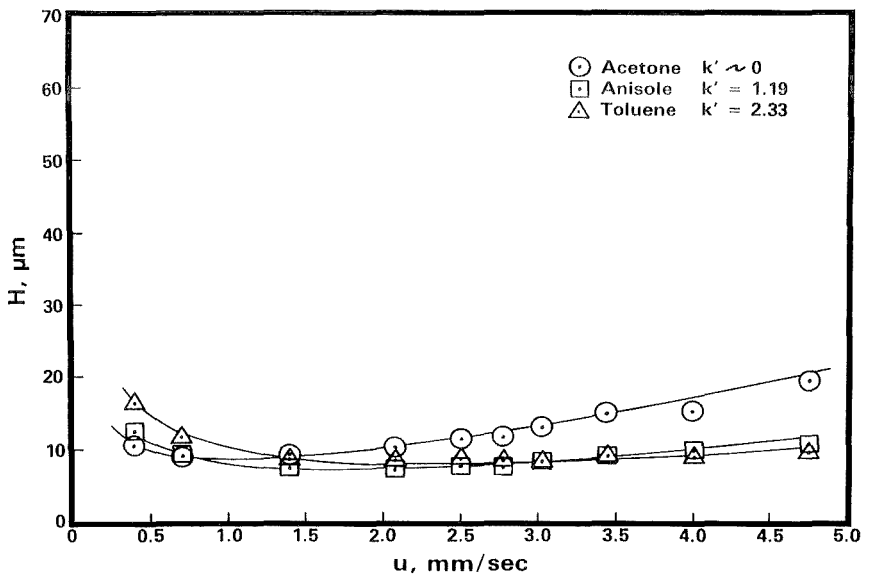


Figure 3. The van Deemter plot for test solutes on a 100 x 4.6 mm column containing 3 µm packing.

The van Deemter plot for a radial compression column (RCSS) packed with 5 µm particles is given in Figure 4. Acetone exhibits a minimum at a lower velocity than found for anisole or toluene. The minimum H for acetone occurred at a velocity of approximately 0.7 mm/sec while for toluene the minimum occurred at 1.0 mm/sec corresponding to a volumetric flow of 2.1 ml/min. The minimum H for toluene was approximately 10 µm.

Plate height data for the Whatman RAC column is given in Figure 5. This plot is similar to that obtained with the RCSS column since acetone exhibited a minimum at a lower flow rate than

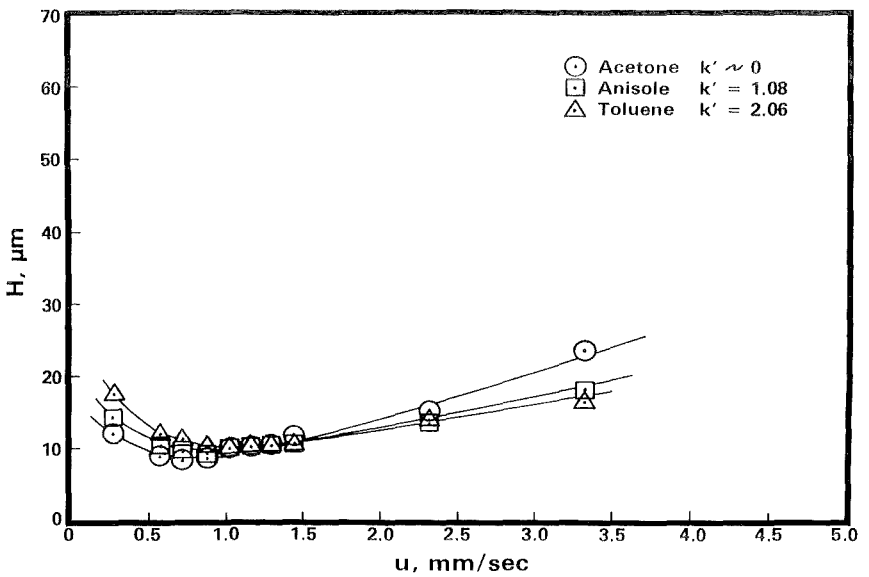


Figure 4. The van Deemter plot for test solutes on a 100 x 8.0 mm RCSS column containing 5 µm packing.

the other solutes. The minimum H for toluene was 14 µm. The linear velocity at the minimum was 2.1 mm/sec corresponding to a volumetric flow rate of 7.5 ml/min.

Figure 6 summarizes the plate height data obtained with toluene as solute for all five columns. As expected columns containing smaller diameter packing materials gave lower minimum H values. The smallest plate height was obtained with the Perkin-Elmer 3 µm column followed by the RCSS column and the Ultrasphere column. The minimum H value for the microbore column packed with 10 µm particles was significantly larger than those of the other columns.

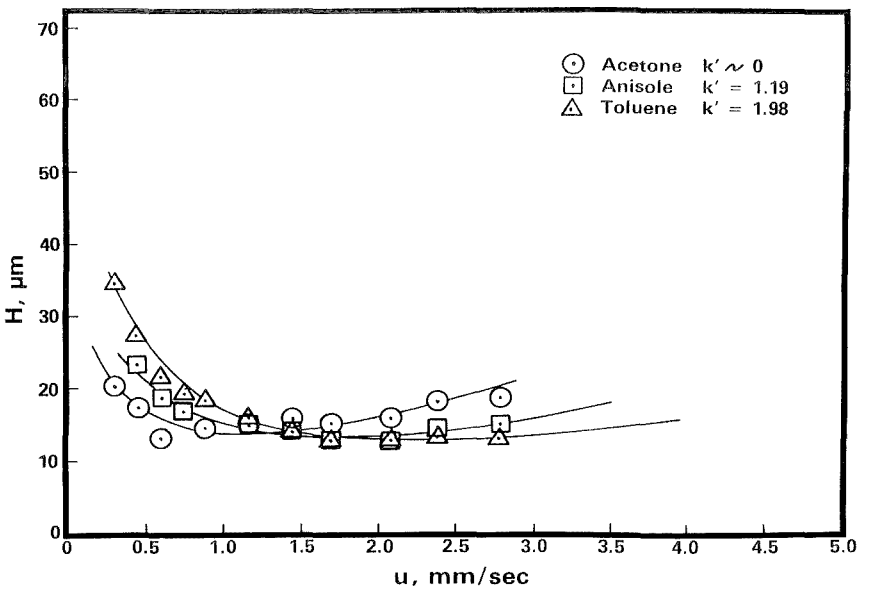


Figure 5. The van Deemter plot for test solutes on a 100 x 9.4 mm RAC column containing Partisil ODS-3 5 µm packing.

The optimum linear velocity for the microbore column was 0.5 mm/sec, smaller by a factor of 2-5 compared to the other columns. This, combined with the small internal diameter of the column, requires operation at low volumetric flow rates to achieve maximum column efficiency. The optimum linear velocities for the other four columns ranged from 1.0 to 2.5 mm/sec.

All columns except the microbore exhibited broad, flat van Deemter plots indicating only small losses in column efficiency need be sacrificed with operation at higher flow rates. However, with the microbore column severe losses in column efficiencies

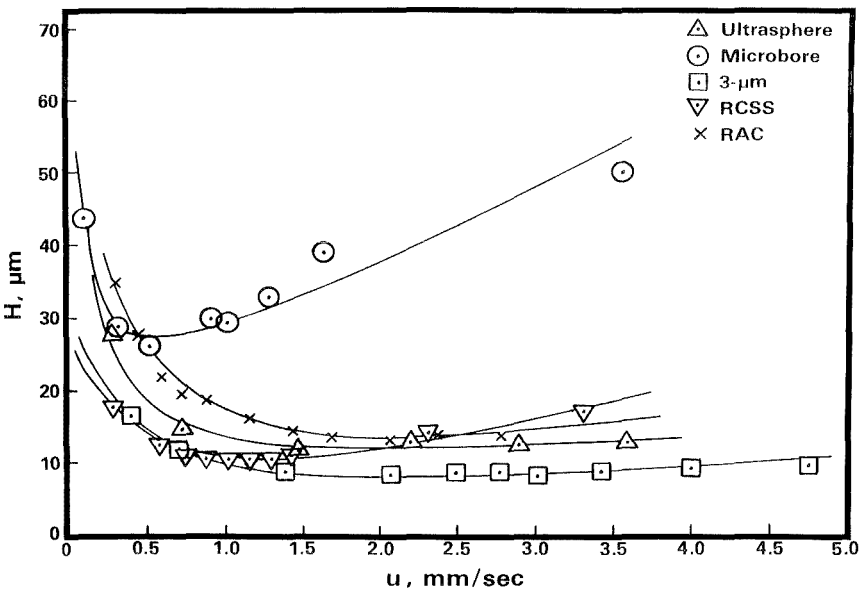


Figure 6. The van Deemter plot for toluene on all five columns evaluated.

would result with operation at velocities in excess of twice the optimum.

Well packed liquid chromatographic columns will normally have minimum reduced plate heights (h) of approximately 2 at reduced velocities (v) of approximately 3-20 (16). Values for h and v are presented in Table 2 for acetone and toluene. These values were calculated at the minimum of the van Deemter plot for each column. Reduced plate height values for toluene range from 2.04 to 2.80 with the RCSS column having the lowest value and the 3 µm column having the highest. This larger h value obtained with the 3 µm

TABLE 2

Reduced Plate Height and Velocity at Optimum Velocity

Column Type	Acetone, k´∼0 h	v	Toluene, k´∼2 h	v
Conventional	2.52	4.6	2.30	5.7
Microbore	3.24	3.2	2.62	3.9
Small Particle	2.93	1.3	2.80	6.4
RCSS	1.68	2.2	2.04	3.9
RAC	2.80	1.9	2.62	8.0

column might indicate some difficulties in packing columns with these small particles. The H value for this column, however, is still better than that obtained with any other column evaluated. All columns exhibit h values close to the expected 2 in a reduced velocity range of 1.3 to 8, consistent with the description of a well packed column. Therefore, subsequent comparisons are based on columns representative of state of the art commercially available.

A typical chromatogram is presented in Figure 7. The elution order was identical for all columns examined. Linear velocities, volumetric flow rates, total plate counts, column pressure drop, and retention characteristics for acetone and toluene at optimum conditions are summarized in Table 3. For the acetone peak (k´∼0), the greatest number of theoretical plates, 19500, was

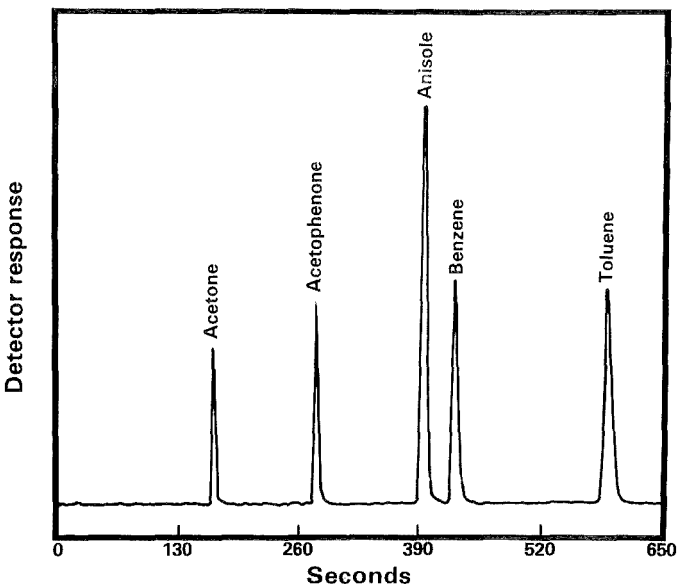


Figure 7. Chromatogram produced by test mixture on a 250 x 4.6 mm column containing 5 µm Ultrasphere ODS packing operated at optimum linear velocity, 1.48 mm/sec.

generated by the conventional Ultrasphere column followed by the microbore column with 15400 plates. The retention time for toluene was much greater at the low optimum flow rate for the microbore column than with any other column evaluated. The shorter columns, as expected, produced the fastest analyses. The column pressure drop varied significantly from 300 psi for the microbore to approximately 4000 psi for the RAC column.

The data in Table 3 indicate the raw number of plates available per column and the relative sacrifices in time and

TABLE 3

Column Characteristics at Optimum Linear Velocity

Column Type	u,mm/sec	Flow,ml/min	Pressure Drop,psi	Acetone Retention Time,sec	Acetone N	Acetone k´	Toluene Retention Time,sec	Toluene N	Toluene k´
Conventional	1.48	1.0	1750	169	19500	~0	592	21800	2.50
Microbore	0.513	0.028	300	975	15400	~0	2111	19100	1.17
Small Particle	2.78	2.0	3120	36	8330	~0	120	11900	2.33
RCSS	1.02	2.1	590	98	10000	~0	300	9820	2.06
RAC	2.08	7.5	3760	48	6250	~0	143	7650	1.98

pressure required to achieve these plate counts. To better assess the relative efficiencies of these columns, plates per column length, plates generated per second, and plates per unit pressure are given in Table 4. This table also contains values for the separation impedance, E. Separation impedance is a dimensionless quantity which measures the expense in time and pressure required to achieve a given plate count. Reports in the literature (11) demonstrate values of less than 1000 indicate excellant performance while values greater than 20000 indicate poor performance.

The data in Table 4 reveal the largest number of plates per meter were obtained on the 3 μm column (119000 plates per meter). This column also generated the largest number of plates per unit time (99 plates per second). At the same time, this column was next to last in plates generated per unit pressure drop

TABLE 4

Quantitative Column Comparison at Optimum Velocity

Column Type	N/column	N/meter	N/sec	N/psi	E
Conventional	21800	87200	36.8	12.5	5650
Microbore	19100	38200	9.05	63.7	7220
Small Particle	11900	119000	99.2	3.81	7200
RCSS	9820	98200	32.7	16.6	5440
RAC	7650	76500	53.5	2.03	27970

demonstrating the continual sacrifices that must be made between resolution, time, and pressure. The microbore column demonstrated the lowest number of plates per meter and plates per unit time. These plates however were achieved with a minimal sacrifice in pressure drop as indicated by the high value of 64 plates per psi. The remaining three columns were intermediate in plates per unit length and plates per unit time. Separation impedance values indicate that four of these columns had roughly equivalent performance. The RAC column demonstrated inferior performance as measured by this parameter.

For high speed analyses, the mobile phase velocity of choice is not necessarily the optimum linear velocity. By operating the column at twice the optimum velocity (optimum practical velocity) a reduction in analysis times of approximately 2 can be realized with a minimal sacrifice of 10-20% in plate count (3). The exact value of this reduction will be determined by the steepness of the van Deemter plot or the relative importance of the van Deemter C term for each column. The Ultrasphere, microbore, and RCSS columns were compared at the optimum practical velocity. Data for the three columns are presented in Table 5. The 3 μm and RAC columns could not be included in this comparison due to pressure (6000 psi) and flow (10 ml/min) restraints, respectively, imposed by the equipment.

Comparison of Tables 4 and 5 indicates analysis times were reduced as expected by a factor of 2 by operation at optimum

TABLE 5

Quantitative Column Comparison at Optimum Practical Velocity[a]

Column Type	N/column	N/meter	N/sec	N/psi	E
Conventional	19960	79800	66.5	5.75	6770
Microbore	17060	34120	16.3	28.9	8880
RCSS	8330	83300	53.7	6.77	8150

(a) Optimum practical velocity equals twice optimum linear velocity.

practical velocity with a loss in total plates of 8%, 11%, and 15% for Ultrasphere, microbore, and RCSS columns, respectively. At the same time, however, the number of plates generated per unit pressure decreased by approximately a factor of 2. Table 5 shows the Ultrasphere column provided the largest number of plates per unit time and highest column performance as measured by the impedance parameter.

Guiochon (18) has shown that optimum analytical performance of a liquid chromatographic column is obtained when the last component of a mixture is eluted at $k'=6.4$ (analysis time of 7.4 times the dead time) and that the peak capacity under these conditions is given by $N^{\frac{1}{2}}/2$. Peak capacity is defined as the number of components which can be reasonably well resolved in a given analysis time provided a column/mobile phase combination can be found which spread these components uniformly throughout the

chromatogram. Analysis times and peak capacities have been calculated at optimum linear velocities for the five columns and are presented in Table 6. The plate count of toluene for each column was employed in this calculation. The 3 μm column gave the shortest analysis time followed by the RAC column. This data again reflects the excellent potential for high speed separations with the 3 μm column. The other columns had considerably longer analysis times with the microbore column giving the longest. The conventional Ultrasphere column had the greatest peak capacity in this evaluation followed closely by the microbore column.

It has been shown (1) that several lengths of individually packed microbore columns can be connected to produce a column with plate counts equivalent to the sum of the parts. Since these columns

TABLE 6

Calculated Analysis Time ($k'=6.4$) and Peak Capacity[a]

Column Type	Analysis Time[b], sec	Peak Capacity
Conventional	1251	74
Microbore	7215	69
Small Particle	266	54
RCSS	725	50
RAC	355	44

(a) Values calculated at optimum linear velocity.

(b) Analysis time equals retention time of component at $k'=6.4$.

operate at low volumetric flows and low pressure drops, several meters of microbore column can produce several hundred thousand plates at moderate pressures thus allowing separation of extremely complex mixtures. Assuming linear additivity of both total plates and pressure drops, the allowable column length consistent with a given maximum pressure drop was calculated for each of the five columns under study. For these calculations, operation at optimum linear velocity was selected and a maximum operating pressure of 5000 psi was imposed for all except the RCSS column.

The pressure limit of the compression module is 2000 psi and this was the value utilized for this column. The results from these calculations as well as other column characteristics are given in Table 7. The data show the microbore is clearly the column of

TABLE 7

Calculated Column Characteristics at Maximum Pressure, 5000 PSI[a]

Column Type	Maximum Column Length,m	N at Maximum Length	Analysis Time ($k'=6.4$) at Maximum Length,sec	Peak Capacity at Maximum Length	Elution Volume ($k'=6.4$) at Maximum Length,ml
Conventional	0.714	62300	3570	125	59.5
Microbore	8.33	318000	120000	282	56.0
Small Particle	0.160	19000	425	69	14.2
RCSS[b]	0.339	33300	2460	91	86.1
RAC	0.133	10180	472	50	59.0

(a) Values calculated at optimum linear velocity.
(b) This column is limited to 2000 psi maximum pressure due to the design of the radial compression system.

choice for high efficiency separations with a peak capacity more than twice its closest rival. This column could separate 282 components evenly spaced over a 33 hour analysis at an expense of 56 ml of mobile phase. A total of 318000 theoretical plates could be generated. For separations requiring moderate efficiency (30-60000 plates) either the Ultrasphere or RCSS columns produce reasonable analysis times (40-60 min). The maximum lengths for the RAC and 3 μm columns are limited due to high operating pressures per unit length. This in turn limits these columns to 10-20000 total plates. However, this limited efficiency is obtained in a short time period.

The length of column necessary to generate a fixed number of theoretical plates is another useful comparison of various chromatographic columns. Table 8 summarizes the length of each

TABLE 8

Calculated Column Characteristics at 10,000 Theoretical Plates[a]

Column Type	Column Length,mm	Pressure Drop,psi	Analysis Time (k'=6.4),sec	Elution Volume (k'=6.4),ml
Conventional	115	805	575	9.59
Microbore	262	157	3780	1.76
Small Particle	84	2621	224	7.46
RCSS	102	602	738	25.8
RAC	131	4926	466	58.2

(a) Values calculated at optimum linear velocity.

column necessary to deliver 10000 theoretical plates and the resulting pressure drop, analysis time, and elution volume when operating at optimum velocity with $k'=6.4$. As expected, the column length and analysis time are shortest for the highly efficient 3 μm column. An analysis time of 224 seconds would be required for a component with $k'=6.4$. This is faster by a factor of at least 2 than any other column. The microbore column required the greatest length and the longest analysis time but achieved the separation at the expense of only 1.76 ml of mobile phase.

SUMMARY

This experimental study has compared the relative merits of various designs of commercial, analytical-scale liquid chromatographic columns. These comparisons were based on one column of each type and no effort was made to account for column-to-column variability. In addition all columns were evaluated with a single mobile phase composition resulting in solute k' values of 2 or less. Hence, no effort was made at evaluations at higher k' values or under conditions where all columns exhibited constant k' values.

The Ultrasphere ODS column, typical of most commercially available columns, exhibited intermediate performance in most aspects of the evaluation. This column produced more theoretical plates, 21800 for toluene, on a per column basis than any of the

other columns examined. Under the experimental conditions, assuming linear additivity of plates, approximately three 250 mm lengths placed in series would produce 62000 plates in 1 hour with a k´ of 6.4.

The microbore column when operated at optimum linear velocity exhibited minimal pressure drop, however, analysis times were greatest for this column compared to all others in this evaluation. This column demonstrated the poorest performance in terms of plates per meter and plates per unit time while exhibiting best performance in terms of plates per unit pressure drop. Due to the low pressure drop, it is conceptually possible to link 8.3 meters of column to produce 318000 plates within a 5000 psi pressure limitation. However, the price for this high plate count is long retention time, 33 hours for a component eluting at a k´=6.4.

The small particle (3 μm) column exhibited the greatest number of plates per unit length (lowest H value) and greatest plates per unit time of the five columns examined. Due to the small packing diameter, the pressure drop was high resulting in low plates per unit pressure. The combination of short column length (100 mm) and high plates per unit time results in the fastest analysis time of the five columns evaluated. Due to the large pressure drop generated by 3 μm particles, only 160 mm of column length could be utilized at a 5000 psi pressure limit with

the above experimental conditions. This column length would generate 19000 plates with a component of $k'=6.4$ eluting in approximately 6 minutes.

The radial compression column offered moderate plate count at low operating pressures, however, the potential advantage of this lower pressure drop is counteracted by the 2000 psi pressure limitation imposed by the compression module. This column gave the smallest reduced plate height of any column evaluated.

For the RAC column, the optimum linear velocity occurred at the high volumetric flow rate of 7.5 ml/min resulting in short retention times with a corresponding high back pressure. The separation impedance for this column was significantly higher than for any other column evaluated which might indicate an atypical column.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the contributions of R. D. Snyder in performing some of the experiments reported here.

REFERENCES

1. Scott, R.P.W. and Kucera, P., Mode of Operation and Performance Characteristics of Microbore Columns for use in Liquid Chromatography. J. Chromatogr., 169, 51 (1979).

2. Scott, R.P.W., Microbore Columns in Liquid Chromatography. J. Chromatogr. Sci., 18, 49 (1980).

3. Reese, C.E. and Scott, R.P.W., Microbore Columns - Design, Construction, and Operation. J. Chromatogr. Sci., 18, 479 (1980).

4. Tsuda, T., Hibi, K., Nakanishi, T., Takenchi, T., and Ishii, D., Studies of Open-Tubular Micro-Capillary Liquid Chromatography. II. Chemically Bonded Octadecylsilane Stationary Phase. J. Chromatogr., 158, 227 (1978).

5. Ishii, D. and Takenchi, T., Open Tubular Capillary LC. J.Chromatogr. Sci., 18, 462 (1980).

6. Yang, F. J., Fused Silica Open Tubular Column for Liquid Chromatography. J. of HRC and CC, 3, 589 (1980).

7. Hirata, Y. and Novotný, M., Techniques of Capillary Liquid Chromatography. J. Chromatogr., 186, 521 (1979).

8. Novotný, M., Capillary HPLC: Columns and Related Instrumentation. J. Chromatogr. Sci., 18, 473 (1980).

9. Hirata, Y., Novotný, M., Peaden, P., and Lee, M., A Comparison of Capillary Chromatographic Techniques for the Separation of Very Large Polycyclic Aromatic Molecules. Anal. Chimica Acta, 127, 55 (1981).

10. Guiochon, G., Conventional Packed Columns vs. Packed or Open Tubular Microcolumns in Liquid Chromatography. Anal. Chem., 53, 1318 (1981).

11. Knox, J.H., Theoretical Aspects of LC with Packed and Open Small-Bore Columns. J. Chromatogr. Sci., 18, 453 (1980).

12. DiCesare, J.L., Deng, M.W., and Ettre, L.S., Very-High-Speed Liquid Chromatography: The System and Selected Applications. Chromatographia, 14, 257 (1981).

13. Giddings, J.C., Dynamics of Chromatography, Part I, Principles and Theory. Dekker, New York, 1965, Chapter 5.

14. Waters Associates Technical Bulletin, Radial Compression Separation System (RCSS)., 1979.

15. Whatman, Chem-Sep News, 1, (1981).

16. Bristow, P.A. and Knox, J.H., Standardization of Test Conditions for High Performance Liquid Chromatography Columns. Chromatographia, 10, 279 (1977).

17. Snyder, L.N. and Kirkland, J.J., Introduction to Modern Liquid Chromatography, Second Edition. Wiley, New York, 1979, p.838.

18. Guiochon, G., Preparation and Operation of Liquid Chromatographic Columns of Very High Efficiency. J. Chromatogr., 185, 3 (1979).

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1899-1920 (1982)

GPC ANALYSIS OF ETHYLENE-PROPYLENE COPOLYMERS*

Ke-Qiang Wang, Shi-Yu Zhang,
Jia Xu, Yang Li
Research Institute of Chemical Industry
He Ping Li, Beijing,
People's Republic of China

ABSTRACT

Determination of the values of K and alpha of the Mark-Houwink equation by applying the universal calibration curve and using a trial and error method was performed with samples of ethylene - propylene copolymers with broad MW distributions and different intrinsic viscosities. The following equation was obtained, correlating K with composition of ethylene-propylene copolymer and alpha:

$$\log K = \log(5.755 - 4.65C3) - 5.75 \text{ alpha}$$

where C3 is the mole percent of propylene in the copolymer, alpha is a value within the range of 0.73 and 0.755 (i.e., K and alpha are dependent upon each other). Values of intrinsic viscosity or molecular weight determined by means of the universal calibration curve agree well with those determined by solution viscosity, light scattering, and osmometry.

INTRODUCTION

GPC experiments have shown that the universal calibration method proposed by Benoit (1) is valid for

*Presented at the GPC Symposium, Chinese Chemical Society, Guilin, People's Republic of China, June 2-6, 1981.

widely different species. One, therefore, can calculate the weight- and number-average molecular weights and molecular weight distribution (MWD) from the universal calibration curve if the Mark-Houwink relationship for the particular polymer-solvent pair is known. However, in the case of ethylene-propylene copolymer, the monomer contents have an effect on the values of K and alpha, especially the former (2,3) . Thus, it is difficult to determine, by GPC, the molecular weight and MWD of the copolymer. So far, a satisfactory method for solving this problem is yet to be found.

In our experiments, a number of ethylene - propylene copolymers, having broad MWD's and different intrinsic viscosities, were used as calibration samples. The intrinsic viscosity and GPC chromatogram for each sample were measured in o-dichlorobenzene (ODCB) at 135 deg.C. and the values of K and alpha were determined by the universal calibration and trial and error method. However, the column peak spreading effect was ignored.

The experimental data were treated by applying regression analysis and the following relatiuonship was set up:

$$\log K = \log(5.755 - 4.65\ C3) - 5.75\alpha$$

where C3 is the mole percent of propylene in the copolymer; alpha can take any value between 0.73 and 0.755.

EXPERIMENTAL

Materials:

Ethylene and propylene copolymerization products, prepared by vanadium catalysis, were used as samples; they were purified by extraction with methanol.

The propylene contents of the samples (C3-mole%) were 50.8 +/- 1% (six samples), 45.3 +/- 1.5% (six samples), 40.1 +/- 1% four samples), and 36 +/- 1% (six samples), respectively.

The solvent employed for all experiments described in this paper was distilled ODCB.

Intrinsic Viscosity:

Intrinsic viscosities were measured in ODCB at 135 deg.C. using Ubbelohde viscometers with flow times greater than 100 sec. For some of the data, the required extrapolation to zero concentration was made from measurements at five concentrations; for others, however, only a single measurement was made and Cheng's equation was employed (4) for calculating the intrinsic

viscosity. The results have shown that, for the same sample, there was not much difference between the two methods.

Gel Permeation Chromatography:

The instrument employed for measuring the GPC data was a Waters Model 200 GPC, with Styragel-packed columns. Most of the measurements were made with a five-column assembly (10E7, 10E6, 10E5, 10E4, and 10E3 Angstroms, respectively). However, some measurements were done with a four-column assembly (10E7, 10E6, 10E4, and 10E3). ODCB was used as solvent; the flow rate was 1 ml/min.

In order to minimize error, the copolymer solution, after determination of intrinsic viscosity, was divided into four parts, which were then separately injected into the GPC instrument. Thus, four GPC chromatograms were obtained from each sample. These were then individually normalized, and the average value

$$1/4 \sum_{j=1}^{4} (H_i / \sum_{i=1}^{n} H_i)_j = W_i$$

of the normalized height of the i-th count was taken.

$$(H_i / \Sigma H_i)$$

Methods:

Weiss (5) has proposed that the K and alpha of the Mark-Houwink relationship may be determined by using two polymers having relatively broad MWD with different intrinsic viscosities. The procedure is as follows:

$$J_i = [\eta]_i M_i \tag{1}$$

$$[\eta]_i = KM_i^{\alpha} = K^{1/1+\alpha} J_i^{\alpha/1+\alpha} \tag{2}$$

$$[\eta] = K^{1/1+\alpha} \Sigma W_i \, J_i^{\alpha/1+\alpha} \tag{3}$$

$$\frac{[\eta]_1}{[\eta]_2} = \frac{\sum_i W_{1i} \, J_i^{\alpha/1+\alpha}}{\sum_i W_{2i} \, J_i^{\alpha/1+\alpha}} \tag{4}$$

where wi and $[\eta]_i$ are, respectively, the weight-fraction and the intrinsic viscosity of the i-th species; $[\eta]$ is the intrinsic viscosity of the polymer. Therefore, using wi's from GPC, Ji's from the universal calibration curve, and $[\eta]$ determined by viscometry, one can solve for alpha and K from equations (4) and (3), respectively, and ultimately calculate weight- and number-average molecular weights according to the definition for molecular weight of polymer.

In applying Weiss's method to ethylene - propylene copolymers, besides errors inherent in the determination of intrinsic viscosity and the GPC chromatogram, compositional distribution might also

introduce errors. Our experiments have shown that the calculated alpha and K from data based on samples 1 and 2 might not be appropriate for other samples. Therefore, we employ samples having the same compositions but different intrinsic viscosities for determinig alpha and K by means of a trial and error method. There are two steps in this procedure. In the first step, let

$$\left| \left(\frac{[\eta]_1}{[\eta]_2} - \frac{\sum W_{1i} J_i^{\alpha/1+\alpha}}{\sum W_{2i} J_i^{\alpha/1+\alpha}} \right) \cdot \frac{[\eta]_2}{[\eta]_1} \times 100 \right| \leqslant u (0<u\leqslant 7) \quad (5)$$

After determination of the intrinsic viscosity, the GPC chromatogram and the universal calibration curve, using polystyrene standards (the intrinsic viscosity - molecular weight relationship of polystyrene in ODCB at 135 deg.C. is $[\eta] = 1.38 \times 10^{-4} M^{0.70}$), the α values (denoted as α_0) may be calculated from equation (5) with given values of u by an iteration method with a computer. Since molecular chains of ethylene - propylene copolymer are flexible, and ODCB is a good solvent, the range of iteration for alpha values may be set as 0.59 - 0.9. By substituting α_0 and the intrinsic viscosity as well as the GPC data of sample 1 into equation (3), K_1 can be calculated. Similarly, K_2 for sample 2 may be obtained. Since α_0

is obtained from the inequality (5), K_1 and K_2 are not equal. Take K_3 as the average of K_1 and K_2. Apply α_0 and the experimental data of sample 3 to determine K_4 from equation (3). Take the average of K_1, K_2, and K_4 as K_5. Calculate, by means of the iteration method, the value of alpha (denoted as α_1) from equation (6):

$$\left| ([\eta]_3 - K_5^{1/1+\alpha} \Sigma W_{3i} J_i^{\alpha/1+\alpha} \Big/ [\eta]_3 \times 100 \right| \leqslant z \qquad (6)$$

$$(0 < z \leqslant 7)$$

Substitute α_1 and experimental data of samples 1, 2, and 3, respectively, into equation (3) to calculate K_6, K_7, and K_8. Take K_9 as the average of these three values. Take the average of α_0 and α_1 to obtain α_2 and, subsequently, K_{10}, K_{11}, K_{12} and their average value K_{13}. Thus, 13 groups of K and alpha

(α_0, K_1, K_2, K_3, K_4, and K_5; α_1, K_6, K_7, K_8, and K_9; α_2, K_{10}, K_{11}, K_{12}, and K_{13})

were obtained. Finally, using the corresponding values for K and alpha of each group, and applying equation (3), the intrinsic viscosity $[\eta]$ of each sample having the same composition may be calculated. Then, the relative error between $[\eta]$ and the measured value of the intrinsic viscosity $[\eta]$ is calculated and is used as a criterion for the preliminary selection of K and alpha.

Assume new values for u and z, repeat the foregoing procedure until the accumulation of sufficient data.

Exchange the experimental data of sample 3 in equation (6) with that of sample 1 or sample 2 in equation (5), or, alternatively, substitute the experimental data of another sample of the same composition for the corresponding ones in (5) or (6). Calculate as before using a computer. The flow diagram for the iteration calculation is presented in Figure 1.

The result of the above treatment has shown that, within the range of allowance error of intrinsic viscosity, most of the ethylene - propylene copolymers of different composition have the same value of alpha. It has been reported in the literature that alpha is independent of composition (2,3). Therefore, we treated the experimental data with respect to alpha and thereby simplified the situation. Thus, with alpha within the range of 0.73 - 0.78, we obtained 11 groups of alpha's and the corresponding K's, K being dependent upon composition. The group of experimental data with alpha value of 0.745 are tabulated in Table I.

After having obtained 11 groups of values of K and alpha, we proceeded to apply a trial and error approach to determine the optimal value of K corresponding to each value of alpha. Again, take alpha = 0.745 as an

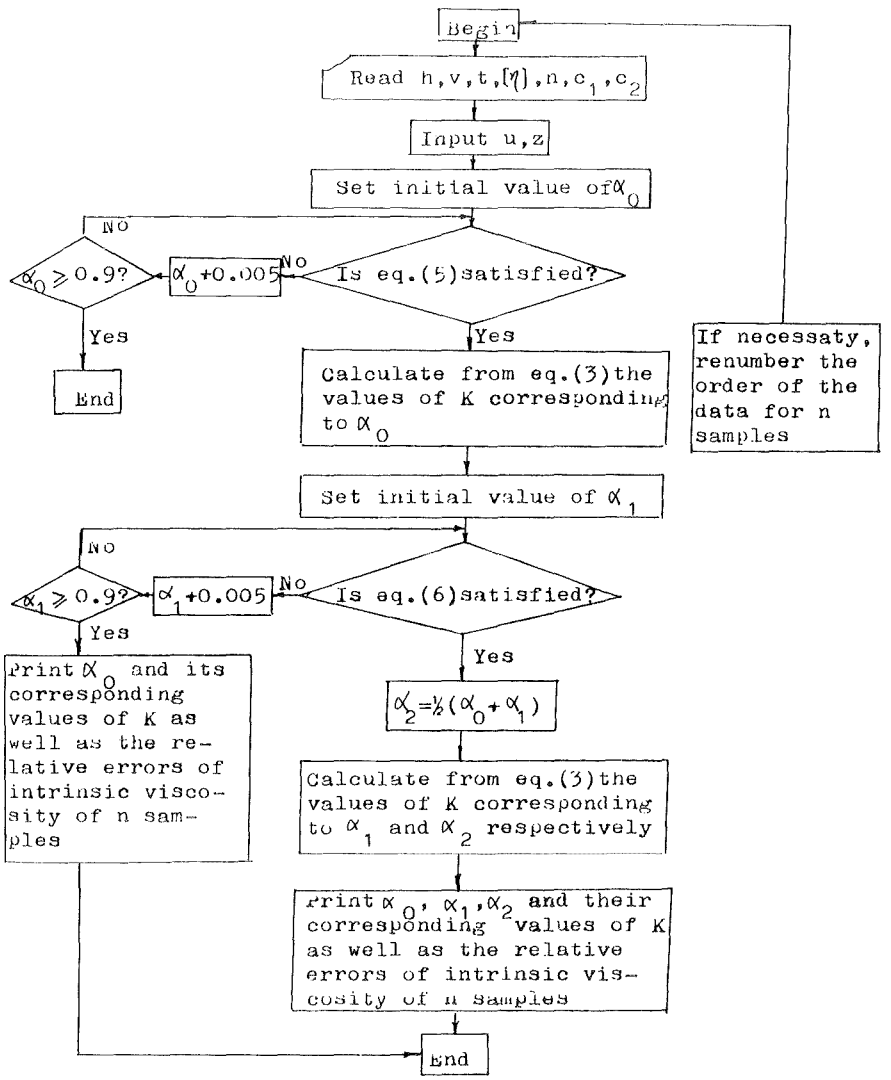


FIGURE 1

Flow Diagram of Iterative Calculation. h = height of interval from Baseline; v = elution volume (count number; t = total count intervals; [η] = intrinsic viscosity of sample; n = number of samples; c_1 and c_2 are the intercept and slope of the universal calibration curve.

TABLE I

Composition of Ethylene-Propylene Coploymers and Values of K Corresponding to α= 0.745

Composition of C_3, mol%	K x 10^4
50.8	1.72 - 1.90
45.3	1.76 - 1.97
40.1	1.97 - 2.07
36.0	2.03 - 2.15

example. When the C3 content of the copolymer is 50.8%, the corresponding value of K falls between 1.72 - 1.90. If alpha remains constant, the value of K increases by constant steps. Calculate the relative error of the intrinsic viscosity of each sample and the standard deviation

$$\sigma = \sqrt{\sum_i ([\eta]_c - [\eta]_d)_i^2 \; / \; n}$$

where n is the number of samples. The K value is then determined on the basis of the minimum value of σ ; in the case of successive appearance of two equal values of minimum σ , the K-value should be determined by reference to all the corresponding relative errors of the intrinsic viscosities of the various samples of that particular composition. The

same procedure was employed in the treatment of experimental data with the C3 contents of the polymer samples equal to 45.3%, 40.1%, and 36%, respectively.

The remaining 10 groups of K and alpha values were treated with the same procedure; the results are tabulated in Table II.

RESULTS AND DISCUSSION

It can be seen from Table II that, when alpha is greater than 0.75, the standard deviation of the intrinsic viscosities of the ethylene - propylene copolymers having different compositions tend to increase with alpha. For samples having compositions of 50.8% and 36%, minimum values of σ, corresponding to alpha values between 0.73 - 0.755, have lower values than the rest. The relative errors of intrinsic viscosities calculated from different groups of values of K and alpha show little deviation. It may be inferred from Table III that most of the intrinsic viscosity deviations for the group of data with alpha = 0.745 as an example are below 7% and that the determined values of K and alpha are applicable to all ethylene - propylene copolymers having molecular weights of 10E5 to 10E6, MWD'S (Mw/Mn) of 3.2 to 12.9, and GPC chromatograms of either single peak or double peaks.

TABLE II

Optimal Value of K and Stad. Deviation Corresponding to Composition of Ethylene-Propylene Coploymer and Alpha.

Comp. C_3-mol%	K,σ	Alpha 0.73	0.735	0.74	0.745	0.75	0.755	0.76	0.765	0.77	0.775	0.78
50.8	$Kx10^4$	2.20	2.02	1.94	1.78-1.80	1.66-1.68	1.56	1.46	1.34-1.36	1.28	1.18	1.08-1.10
	σ	0.196	0.194	0.197	0.197	0.198	0.199	0.199	0.200	0.201	0.202	0.204
45.3	$Kx10^4$	2.26-2.28	2.12	1.98	1.86	1.74	1.62	1.52	1.42	1.32	1.24	1.14
	σ	0.090	0.092	0.095	0.099	0.103	0.110	0.110	0.114	0.116	0.121	0.123
40.1	$Kx10^4$	2.48-2.50	2.32-2.34	2.16	2.04	1.90	1.78	1.66	1.56	1.46	1.36	1.26-1.28
	σ	0.058	0.058	0.059	0.058	0.059	0.059	0.059	0.060	0.060	0.060	0.062
36.0	$Kx10^4$	2.56-2.58	2.38-2.40	2.22	2.10	1.96-1.98	1.84	1.72	1.60	1.50	1.40	1.32
	σ	0.122	0.122	0.120	0.122	0.122	0.123	0.123	0.123	0.124	0.124	0.125

TABLE III

Examples of Viscosity Deviation and Molecular Weight Calculated from the Determined Values of K and alpha (alpha = 0.745)

Comp. C_3-mol%	Sample No.	Type Peak	$[\eta]_d$	Kx10^4	$(M_w)_c$ x10^{-4}	$(M_w/M_n)_c$	$\frac{[\eta]_c - [\eta]_d}{[\eta]_d}$ %
50.8	1	doub	4.22	1.80	99.7	12.9	2.67
	2	doub	3.62	1.80	85.3	6.98	8.92
	3	sing	1.35	1.80	22.1	4.81	4.41
	4	doub	4.52	1.80	93.2	9.30	-5.68
	5	sing	2.48	1.80	42.1	7.39	-5.89
	6	doub	0.90	1.80	10.3	5.84	-6.65
45.3	7	doub	2.17	1.86	33.8	4.96	-6.60
	8	doub	1.69	1.86	23.9	3.82	-4.79
	9	sing	1.29	1.86	17.8	2.58	4.76
	10	doub	4.33	1.86	97.1	9.72	3.32
	11	sing	1.83	1.86	25.8	5.28	-4.79
	12	sing	1.68	1.86	24.9	3.19	0.62
40.1	13	doub	3.07	2.04	50.1	4.55	-0.30
	14	doub	2.24	2.04	32.1	5.78	-1.64
	15	doub	2.95	2.04	48.4	5.69	-0.86
	16	doub	2.11	2.04	37.1	9.47	6.29
36.0	17	sing	3.87	2.10	64.4	4.51	0.17
	18	sing	2.83	2.10	43.9	5.72	4.23
	19	doub	1.77	2.10	28.6	6.81	7.84
	20	doub	3.07	2.10	45.8	5.03	-1.22
	21	sing	2.65	2.10	34.2	4.79	-8.72
	22	doub	1.43	2.10	16.6	4.25	1.84

Using data in Table II, if the values of K are plotted against copolymer composition (C3-mole%), a linear relationship is obtained.

With a view to testing the validity of the linear relationship between K and copolymer composition, we determined the intrinsic viscosity and GPC chromatogram of an additional five samples and, by interpolation and extrapolation of the straight line of Figure 2, the corresponding values of K and alpha were obtained. Intrinsic viscosity errors of the various samples were then calculated and the results are tabulated in Table IV. Moreover, we determined the number-average molecular weight, by osmometry, of samples 24 and 25 using a Knauer membrane osmometer and the weight-average molecular weight of sample 24 using a low-angle laser light-scattering photometer (Type KMX-6, Chromatix, Inc., USA). These are compared with data obtained by GPC (after corrections for peak spreading effects) as shown in Table V.

From Tables IV and V, it is apparent that deviations in intrinsic viscosities and molecular weights of the different samples all fall within the permissible range, and the data calculated from different groups of K and alpha show little variation. It is thus inferred that the linear relationship, as shown in Figure 2 is reliable and is applicable to

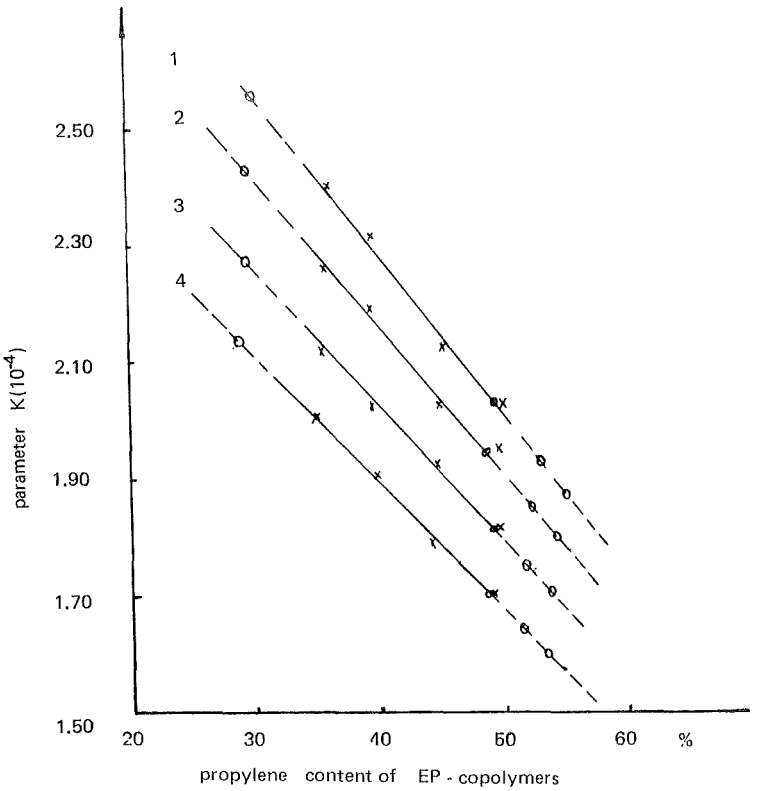


FIGURE 2

Relationship Between K and Propylene Content of EP-copolymers. -x-: Experimental values; -o-: Values of linear interpolation or extrapolation; 1 :α = 0.735; 2: α = 0.740; 3: α = 0.745; 4: α = 0.750.

samples with a narrow MWD, i.e., equal to 1.69 (see Table V).

In order to further investigate the relationship between k, alpha, and copolymer composition, we treated the data by means of regression analysis, i.e., to plot log K against alpha; this resulted in a very good

TABLE IV

Comparison of Measured Values of Intrinsic Viscosity With Those Calculated Form K & Alpha as Indicated in Figure 2.

No.	Comp. C_3-mol%	α=0.75 Kx10^4	$\frac{[\eta]_c-[\eta]_d}{[\eta]_d}$%	α=0.745 Kx10^4	$\frac{[\eta]_c-[\eta]_d}{[\eta]_d}$%	α=0.735 Kx10^4	$\frac{[\eta]_c-[\eta]_d}{[\eta]_d}$%
23	30.0	2.12	-5.49	2.26	-5.45	2.56	-5.54
24	49.5	1.68	-5.43	1.80	-5.40	2.04	-6.56
25	53.9	1.58	1.68	1.68	1.58	1.90	1.39
26	55.4	1.54	6.30	1.64	6.30	1.86	6.63
27	55.4	1.54	-0.43	1.64	-0.43	1.86	-0.12

TABLE V

Comparison of Data From GPC and Absolute Methods.

No.	Kx10^4	Alpha	$(M_n)_{GPC}$ x10^{-4}	$(M_n)_{OSM}$ x10^{-4}	Rel. Error %	$(M_w)_{GPC}$ x10^{-4}	$(M_w)_{LS}$ x10^{-4}	Rel. Error%	$(M_w/M_n)_{GPC}$
24	2.04	0.735	29.9	29.8	0.33	63.3	62.3	1.60	2.12
	1.92	0.74	30.1	29.8	1.00	63.3	62.3	1.60	2.10
	1.80	0.745	30.0	29.8	0.67	62.8	62.3	0.82	2.09
	1.68	0.75	30.1	29.8	1.00	62.9	62.3	0.90	2.09
25	1.90	0.735	13.9	13.0	7.0	23.8	-----	-----	1.71
	1.80	0.74	14.0	13.0	7.9	23.7	-----	-----	1.69
	1.68	0.745	14.0	13.0	7.9	23.8	----	----	1.70
	1.58	0.75	14.0	13.0	7.7	23.7	-----	-----	1.69

linear fit. The lines were parallel to each other, as shown in Figures 3 and 4. The following general equation was established from the plots:

$$\log K = A - 5.75\alpha = \log B - 5.75\alpha \qquad (7)$$

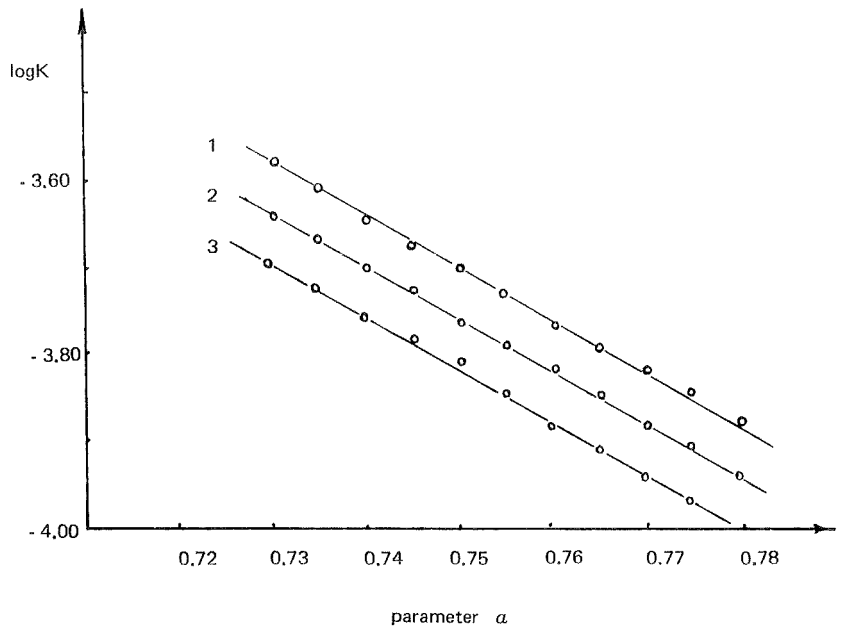


FIGURE 3

Relationship Between K and Alpha. 1: C_3=36%; 2: C_3=45.3% 3: C_3=55%.

The relationship between A, B, and composition is shown in Table VI. By plotting B against composition, straight lines as shown in Figure 5 were obtained and, by solving the straight lines graphically, we obtained

$$B = 5.755 - 4.65\ C_3 \tag{8}$$

Substituting (8) into (7), we obtain

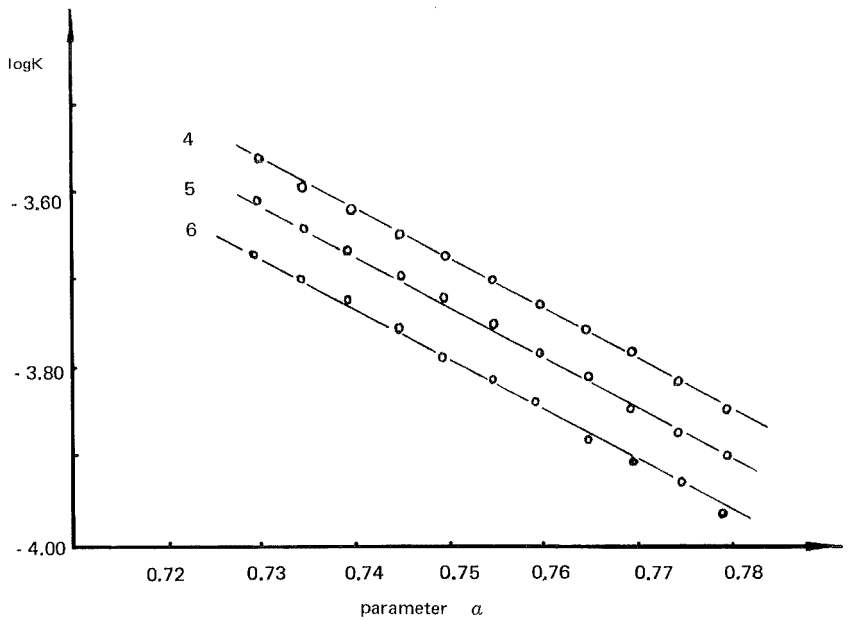


FIGURE 4

Relationship Between K and Alpha. 4: C_3=30%; 5: C_3=40.1% 6: C_3=50.8%.

TABLE VI

Values of A and B in Equation (7)

	C_3-mol %					
	30.0	36.0	40.1	45.3	50.8	55.0
A:	0.635	0.610	0.590	0.555	0.533	0.500
B:	4.32	4.07	3.89	3.59	3.40	3.16

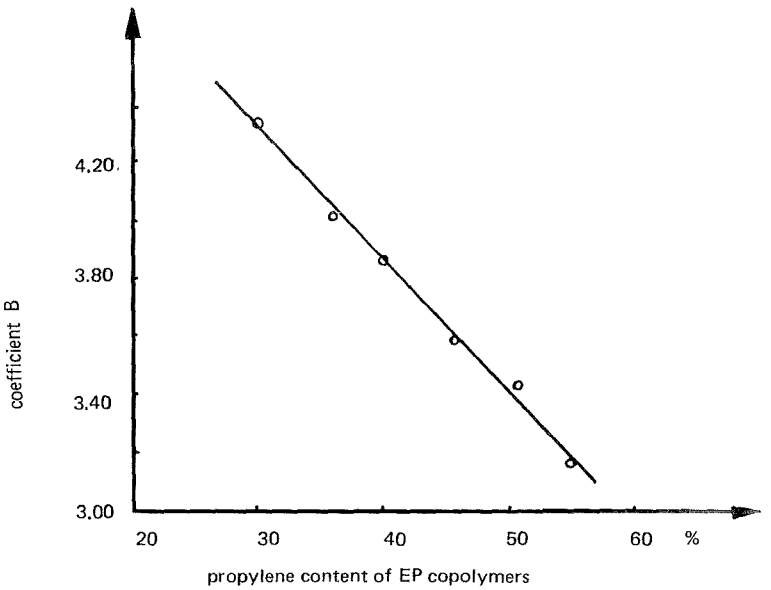


FIGURE 5

Relationship Between B of Equation (7) and Propylene Content of EP-Copolymers.

$$\log K = \log(5.755 - 4.65\ C_3) - 5.75\ \alpha \qquad (9)$$
(ODCB, 135°C)

Substituting (9) into the Mark - Houwink equation and rearranging,

$$[\eta] = (5.755 - 4.65C_3)\ (M/5.623\times10^5)^{\alpha} \qquad (10)$$
(ODCB, 135°C)

Table VII shows that the K_c value calculated from (9) agrees well with values of K previously determined. This proves that equation (9) is valid, as is equation

TABLE VII

Comparison of Calculated and Determined Values of K (10^{-4})

Compos'n, C_3-mol %	K_d, K_c	Alpha 0.73	0.735	0.745	0.75	0.755	0.76	0.775	0.78
30.0	K_d	2.72	2.57	2.27	2.13	2.01	1.88	1.55	1.44
	K_c	2.77	2.58	2.27	2.12	1.98	1.86	1.53	1.43
50.8	K_d	2.20	2.02	1.80	1.68	1.56	1.46	1.18	1.08-1.10
	K_c	2.16	2.02	1.77	1.66	1.55	1.45	1.19	1.11

(10). In fact, intrinsic viscosity calculated from equation (19) agrees well with measured values, with errors generally below 7%. Moreover, equation (9) shows that K and alpha are mutually dependent; the logarithm of K is a function of alpha.

Since equations (9) and (10) are obtained from experimental data through regression analysis, they are primarily applicable to ethylene - propylene copolymers prepared with a vanadium catalyst system, with propylene contents in the range of 30 - 55%, and alpha in the range of 0.73 - 0.755. The intrinsic viscosity or molecular weight calculated from different values of alpha approximate each other.

With the establishment of equation (9), it is possible to calculate the molecular weight and MWD from GPC chromatogram using the universal calibration curve.

Also, by using equation (10), the viscosity - average molecular weight may be calculated from intrinsic viscosity data of the samples.

CONCLUSION

Using ethylene - propylene copolymers of broad molecular weight distribution and different intrinsic viscosities as samples, and by employing the trial and error method reported in this paper, we have been able to determine the parameters K and alpha of the Mark - Houwink equation and, by further treating these by regression analysis, the following relationship is ultimately established:

$$\log K = \log(5.755 - 4.65\ C_3) - 5.75\ \alpha$$

$$[\eta] = (5.755 - 4.65\ C_3)\ (M/5.623 \times 10^5)^{\alpha}$$

(ODCB, 135°C)

The above relationship shows that K and alpha are mutually dependent, the logarithm of K is a function of alpha, and alpha may be any value between 0.73 and 0.755. The above relationship is applicable to ethylene - propylene copolymers having a weight - average molecular weight of 10E5 to 10E6, a Mw/Mn of 1.7 - 12.9, a propylene content of 30 - 50 mole% and a GPC curve with either a single peak or two peaks. It

is thus possible to apply universal calibration to calculate molecular weight and molecular weight distribution from the GPC chromatogram.

ACKNOWLEDGEMENTS

The authors wish to express their thanks to Shi Lianghe for helpful comments; to Ma Shubin for supplying the copolymer samples, and Gao Guoli for helping simplify the computer program.

REFERENCES

1. Grubisic, Z., et al., J. Polymer Sci., Part B, 5, 753 (1967).

2. Moraglio, G., Chim. Ind., (Milan), 10, 984 (1959).

3. Ogawa, T., Inaba, T., J. Appl. Polymer Sci., 21, 2979 (1977).

4. Cheng Rongshi, Gaofenzi Tongxun (Polymer Communications), 4, 159 (1969).

5. Weiss, A. R., Cohn-Ginsberg, E., J. Polymer Sci., Part B, 7, 379 (1969).

6. Evans, J. M., Polymer Eng. Sci., 13, 401 (1971).

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1921-1931 (1982)

THE PURIFICATION OF PROSTATIC ACID PHOSPHATASE FROM SEMINAL PLASMA BY REVERSE PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY

M. Patricia Strickler, John Kintzios, and M. Judith Gemski

Division of Biochemistry
Walter Reed Army Institute of Research
Washington, D.C. 20012

ABSTRACT

We report here the rapid isolation of PAP directly from dialyzed human seminal plasma by reverse phase high performance liquid chromatography (RP-HPLC). The recovery of PAP of high specific activity was found to be dependent on the treatment of the fractions after separation. The collection of fractions into a stabilizing medium was essential to the preservation of the specific activity of the enzyme. This finding extends the use of RP-HPLC to the purification of active enzymes from complex biological matrixes.

INTRODUCTION

Prostatic acid phosphatase (PAP) is a clinically important marker used for the detection of prostatic cancer. This enzyme is a glycoprotein with a molecular weight of approximately 102,000, and has been purified to near homogeneity from seminal plasma by numerous protein purification techniques. Proteins having high specific enzymatic activity and immunologic re-activity have seldom been

separated using reverse phase high performance liquid chromatography (RPHPLC). The critical role of stabilizing agents in the recovery of enzymatic activity and the chromatographic methodology for the isolation and purification of PAP is hereby described.

MATERIALS

Prostatic acid phosphatase was obtained from human seminal plasma as described by Mahan and Doctor (1). Acetonitrile and trifluoroacetic acid of HPLC grade were obtained from Fisher Scientific, (Silver Spring, MD). The o-carboxyphenyl phosphate was obtained from Sigma Chemical Co., (St. Louis, MO) and the RIA-Quant PAP Test Kit from Mallinckrodt, (St. Louis, MO). All other chemicals and reagents were of the highest purity available.

INSTRUMENTATION

A Waters Associates, (Milford, MA) liquid chromatograph consisting of two M6000A solvent delivery systems, a M660 solvent flow programmer, a U6K universal injector, an M440 absorbance detector set at 280nm, an M450 variable wavelength detector set at 214nm and an M730 data module was utilized. Enzymatic activity was surveyed using a Beckman UV 5230 Spectrophotometer (Fullerton, CA) equipped with an automatic sample changer. An LKB 1270 Rackgamma II gamma counter (Rockville, MD) was used for the RIA analysis. An LKB Multiphor unit, Model 2117 was used for the isoelectric focusing.

HPLC CHROMATOGRAPHIC PROCEDURE

A 30 minute linear gradient was run at a flow rate of 1.5ml/min on a μBondapak C_{18} column (3.9mm x 30cm, Waters Associates). The

mobile phase consisted of solution A, 0.1% aqueous trifluoroacetic acid (TFA) and solution B, 0.1% TFA in acetonitrile, run from 12% to 70% B. Both solvent systems were filtered and degassed prior to use. The column effluent was monitored at 214nm and 280nm. All separations were carried out at ambient temperatures.

PREPARATION OF HUMAN SEMINAL PLASMA

Pooled ejaculates from persons undergoing routine fertility examinations were centrifuged at low speed to remove cells. A 150ml sample of the seminal plasma was dialyzed against 4 liters of 0.1M Tris buffer at pH 7.2 for 48 hours, with one change. The dialyzed sample was centrifuged at 100,000 x g for 30 minutes, and supernatant stored at -70°C.

ENZYMATIC ACTIVITY PROCEDURE

A stock of prostatic acid phosphatase (a gift of Dr. D. Mahan) at a concentration of 1mg/ml was prepared in distilled water. From this stock 200µl was injected into the liquid chromatograph and 30 - 1 minute fractions were collected. Aliquots (0.1ml) of each fraction were added to 2.4ml of a 0.15M sodium acetate, solution pH 5.0, and 0.5ml of 3.65mM o-carboxyphenyl phosphate substrate. The change in absorbance at 300nm over the initial period of linearity was used to calculate the activity. The units of activity were calculated by the following equation:

$$\text{U of Activity/mg} = \frac{A_{300}/\text{min} \times 1000}{3500 \times \text{mg enzyme/ml reaction vol.}} \quad (2)$$

Aliquots of the dialyzed human seminal plasma and fractions from the

resulting chromatographic separation of seminal plasma were assayed similarly. Protein concentrations were estimated by the Lowry method (3) using bovine serum albumin as the standard, and the PAP concentration was determined by RIA.

ELECTROPHOSESIS AND ISOELECTRIC FOCUSING

SDS polyacrylamide tube gels (10% acrylamide, 0.3% bis-acrylamide, and 1.0% sodium dodecyl sulfate) were prepared as a modification of the Maizel method (4). Electrophoresis was carried out in 0.01M Tris-glycine buffer at pH 8.2 containing 1% glacial acetic acid and 10% isopropyl alcohol. The electrophoretic separation of acid phosphatase isoenzymes was based on the buffer system of Reisfeld, et al. (5). Visualization was accomplished by immersing the gels in 50mM acetic acid-sodium acetate buffer at pH 5.0 which contained sodium α-napthyl acid phosphate as a substrate (1mg/ml) and fast garnet salt as a coupler. Isoelectric focusing was performed at 5°C on acrylamide tube gels, pH range 4-6 according to Mahan, et al. (1).

RESULTS AND DISCUSSION

Prostatic acid phosphatase (PAP) is a glycoprotein with a molecular weight of approximately 102,000. It has been purified to near homogeneity from human seminal plasma by a combination of techniques such as gel filtration, affinity chromatography, and preparative gel electrophoresis (1, 6, 7). A stock solution of PAP, was chromatographed on a reverse phase HPLC column under the conditions described in materials and methods. This separation technique revealed only minor impurities, (Figure 1), which corresponded by retention times

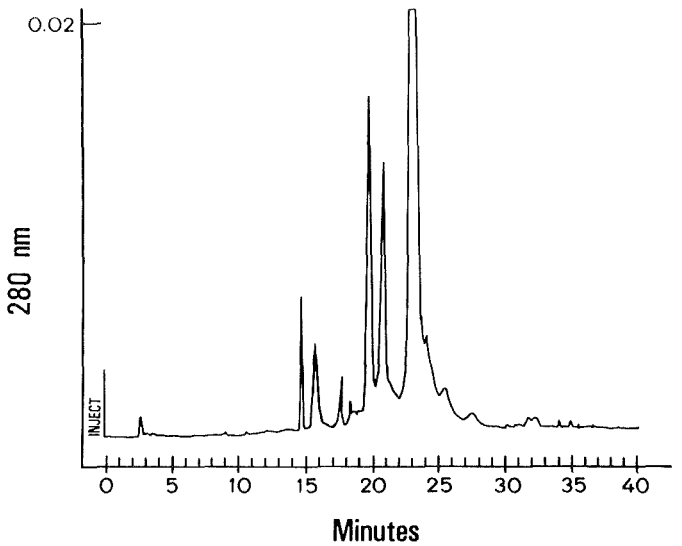


Figure 1: RP-HPLC chromatography of prostatic acid phosphatase purified by gel filtration. Column μBondapak C_{18} (3.9 mm x 30cm), thirty minute linear gradient. Flow rate, 1.5ml/min., mobile phase, 0.1% TFA in water, 12% to 70% 0.1% TFA in acetonitrile, column temperature, ambient. Activity occurs at 22-24 minutes.

to peaks present in the RP-HPLC profile of dialyzed human seminal plasma, (Figure 2). Based on this data, the purification of PAP directly from dialyzed human seminal plasma was attempted.

The chromatographic profile of seminal plasma was reproducible. Injection on column of 100 to 500 microliters of seminal plasma gave a linear response in terms of the area of integration of selected peaks, including the peak at 22-23 minutes which corresponded to PAP. Recovery of active enzyme, though, was negligible. In contrast to our previous experience with trypsin (8) removal of the mobile phase by lyophilization was only marginally effective in restoring enzymatic

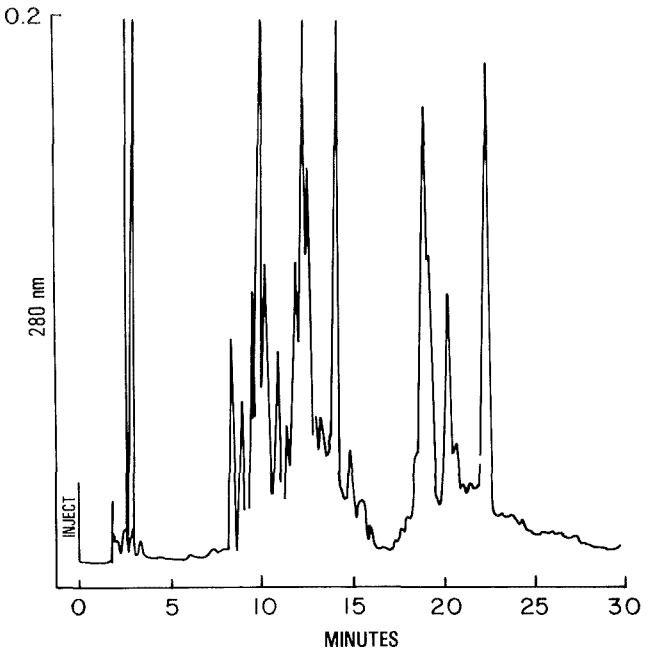


Figure 2: RP-HPLC chromatograph of dialyzed human seminal plasma. 200 μl injected on column. PAP activity at peak elution time 22-24 minutes.

activity. An investigation was therefore undertaken to determine whether the poor recovery was attributable to loss of enzyme on the column or denaturation by the mobile phase after fractionation. The chromatographic fractions were collected into a series of buffers and solvents known to stabilize activity of a variety of enzymes. These substances and their effectiveness in protecting PAP activity are summarized in Table 1. Glycerol was found to be the best protector of PAP activity; Hanks' Balanced Salt Solution and Dulbecco's Phosphate Buffered Saline, though not nearly as good as glycerol,

TABLE 1

Protection of PAP Activity

	STABILIZING AGENT	RELATIVE ACTIVITY
1.	No protective agent (d)	marginal
2.	Hanks' Balanced Salt Solution (a,c)	moderate
3.	Delbecco's Phosphate Buffered Saline (a,c)	moderate
4.	Potassium phosphate, 0.1M, pH 7 (a,c)	none
5.	Sodium Acetate, 0.05M, pH 5 (a,c)	none
6.	Ammonium Bicarbonate, 0.01M pH 8 (a,c)	none
7.	DMSO (b,c)	none
8.	PEG (b,c)	none
9.	Glycerol (45% v/v) (c)	very active

a) Aliquots of 0.5ml of the buffered solutions were added to each 1.5ml fractions.
b) Aliquots of 0.1ml of solvents (7 and 8) were added to each 1.5ml fractions.
c) None of the fractions were lyophilized prior to testing the activity. The acetonitrile, however, was evaporated under N_2.
d) Lyophilization of any of these fractions gave ambigious results, due to the instability of PAP under freezing and thawing conditions. The glycerol was found to be the most effective protector of prostatic acid phosphatase activity.

did significantly increase the recovery of enzymatic activity. The dramatic change in recovery of PAP activity seen in this study suggests that the previous difficulty with reverse phase HPLC may be due to the collection and processing after fractionation and not to denaturation or loss of material in the chromatographic separation.

The homogeneity of the fractions containing PAP was assessed by rechromatography on RP-HPLC, (Figure 3) and sodium dodecyl sulfate

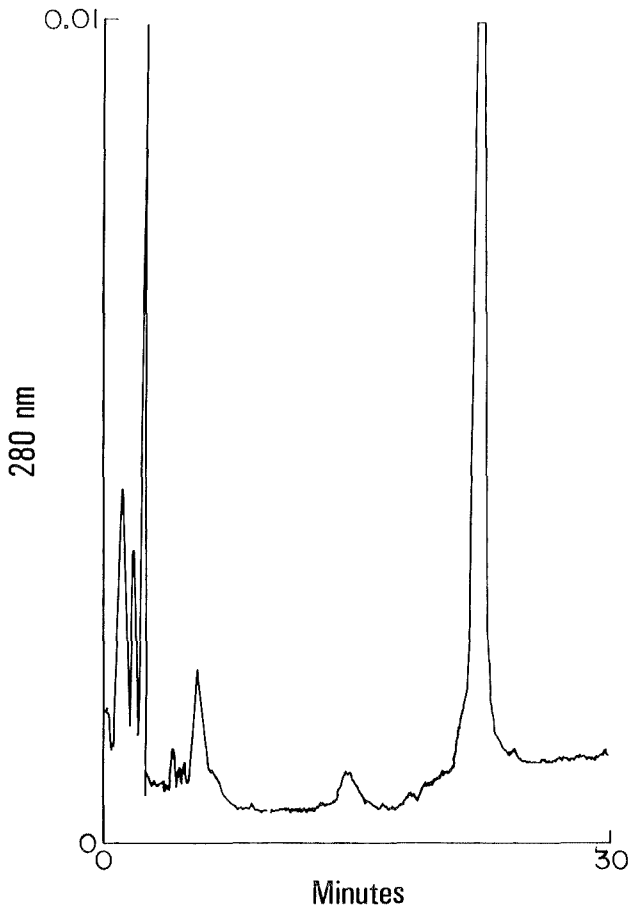


Figure 3: Re-chromatograph of the active fraction obtained from the separation of the human seminal plasma.

gels (Figure 4). The electrophoretic pattern of SDS gels clearly shows one major band corresponding in molecular weight to the purified PAP and to the active enzyme in seminal plasma. Assay of the fractions for isoenzyme activity revealed a single band. This band,

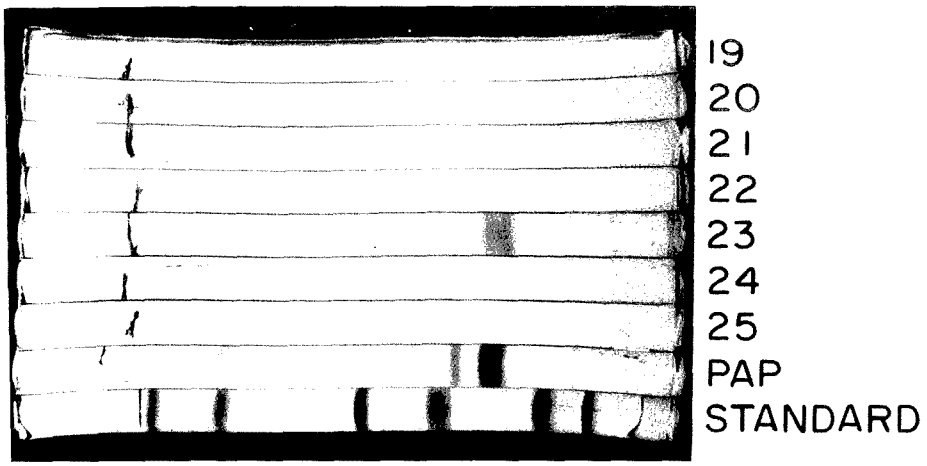


Figure 4: SDS polyacrylamide gel electrophoresis analysis of RP-HPLC fractions. PAP, purified by gel filtration, was included for identification of the HPLC fraction containing the enzyme; protein standards were obtained from Pharmacia (HMW).

compared with the PAP purified by affinity chromatography, showed a similar electrophoretic mobility. To test the validity of the previous assay, aliquots resulting from the fractionation were subjected to isoelectric focusing as per the methods section. The profile showed that all samples contained the same number of bands focused at the same area.

The enzymatic activity of the fractionated PAP was surveyed by the reaction to the substrate o-carboxyphenyl phosphate and with a RIA. The latter also confirms the immunological integrity of the purified PAP. The recovery of immunologically re-active protein from 100µl of human seminal plasma, injected on column, was 57.5%. The PAP activity and protein concentration of each fraction from the chromatographic separation was determined and is graphically shown in Figure 5.

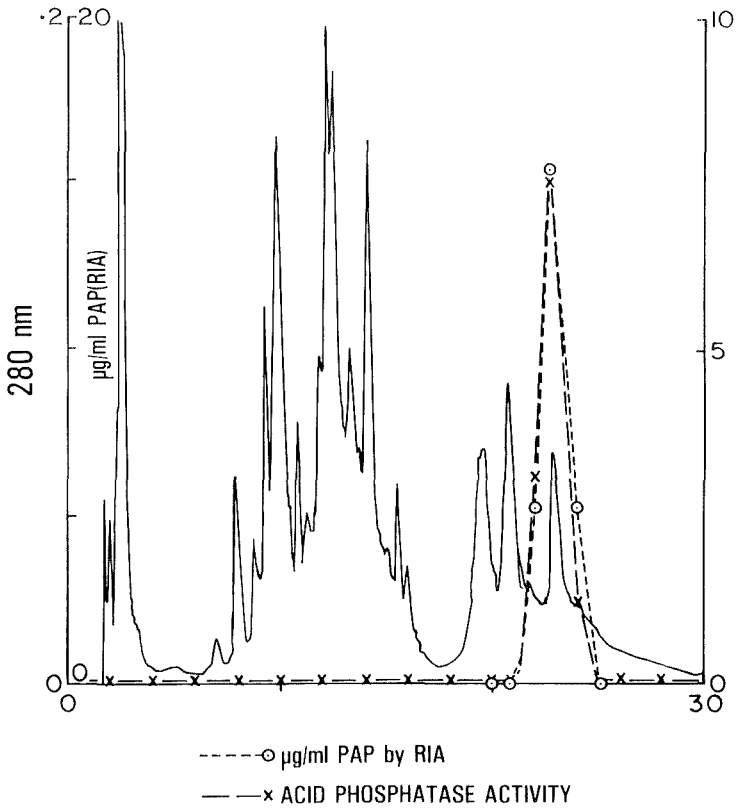


Figure 5: PAP activity and protein concentrations of each fraction from the chromatographic separation of human seminal plasma.

Purification of PAP by RP-HPLC yields highly active, immunologically and chromatographically homogenous enzyme, directly from dialyzed human seminal plasma in thirty minutes. With the proper selection of post-fractionation conditions, RP-HPLC can be demonstrated to be a highly effecient and simple technique for the isolation of enzymes.

ACKNOWLEDGEMENTS

We wish to thank Dr. S. Leto, Washington Fertility Clinic, Washington, D.C., for supplying us with the seminal plasma and Mrs. B. Libys for her secretarial support.

REFERENCES

1. Mahan, Donald E. and Doctor, B. P., A Radioimmune Assay for Human Prostatic Acid Phosphatase-Levels in Prostatic Disease, Clin. Biochem., 12, 10-17, 1979.

2. Decker, L. A., ed., Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, N.J., 1977, p. 145.

3. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R. J., Protein Measurement with the Folin Phenol Reagent. J. Biol Chem 193, 265-275, 1951.

4. Maizel, J. Jr., Polyacrilamide Gel Electrophoresis of Viral Proteins, Methods in Virology, ed. Maramorosch, K. and Koprowski, H., Vol 5, Academic Press, N.Y. 1971, p. 179-247.

5. Reisfeld, R.A., Lewis, U. J., Williams, D.E., Disk Electrophoresis of Basic Proteins and Peptides on Polyacrylamide Gels., Nature 195, 281-283, 1962.

6. Vihka, P., Kontturi, M., and Korhonew, L.K., Purification of Human Prostatic Acid Phosphatase by Affinity Chromatography and Isoelectric Focusing, Part I, Clin. Chem. 24, 466-470, 1978.

7. Lam, W.K.W., Yam, L.T., Wilbur, H.J., Taft, E. and Li, C.Y., Comparison of Acid Phosphatase Isoenzymes of Human Seminal Fluid, Prostate and Leukocytes. Clin. Chem. 25(F), 1285-1289, 1979.

8. Strickler, M.P., Gemski, M.J., and Doctor, B.P., Purification of Commercially Prepared Bovine Trypsin by Reverse Phase High Performance Liquid Chromatography, Journal of Liquid Chromatography, 4, 1765-1775, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1933-1940 (1982)

PEPTIDE MAPPING OF VARIANT GLYCOPROTEINS FROM TRYPANOSOMA RHODESIENSE BY REVERSE PHASE LIQUID CHROMATOGRAPHY

M. PATRICIA STRICKLER, RICHARD W. TRAVIS, AND JOHN G. OLENICK

DEPARTMENT OF APPLIED BIOCHEMISTRY
DIVISION OF BIOCHEMISTRY
WALTER REED ARMY INSTITUTE OF RESEARCH
WALTER REED ARMY MEDICAL CENTER
WASHINGTON, D.C. 20012

ABSTRACT

Peptides of trypsin-digested surface coat glycoproteins isolated and purified from 4 cloned variants of Trypanosoma rhodesiense (Wellcome strain) were mapped by reverse phase high performance liquid chromatography. The peptide maps provide definitive chemical data demonstrating a lack of structural homology among the variant glycoproteins.

INTRODUCTION

The African trypanosomes are parasitic protozoa that cause sleeping sickness in humans and pose a serious threat to the health of 35 million people (1). The disease in animals is known as nagana, the prevalence of which prevents the use of 4 million square miles of Africa for raising livestock (2-4). Trypanosome infections may persist for several months and are often characterized by recurrent waves of parasitemia, in which the parasite population of each successive wave differs antigenically from that of preceding waves. Antigenic variation appears to be the primary mechanism enabling the African trypanosome

to evade the host's immune response (5-7). This phenomenon is generally regarded as a serious obstacle to the development of successful immunoprophylactic measures.

Antigenic variation is mediated through sequential elaboration of variant-specific cell surface glycoproteins (6,7). As each variant-specific glycoprotein (VSG) is expressed, induced host antibodies apparently destroy most of the parasite population. Subsequently, another population arises that carries a new VSG immunologically distinct from the previous VSG. VSG's have been isolated and purified from several species and variants of African trypanosomes and consist of a single polypeptide chain having an apparent molecular weight of 55-65,000 (8-13).

Structural studies suggest that the immunological uniqueness of VSG's results from considerable variation in the sequence of amino acids comprising the polypeptide chain. Partial tryptic hydrolysis of native VSG's has not revealed any common features associated with the cleavage sites (14). Isoelectric points and amino acid compositions have been shown to differ greatly (8,13,15). In addition, analyses of N-terminal amino acid sequences have demonstrated a complete lack of homology (14,16). These findings indicate extensive structural diversity and imply an absence of any significant sequence and conformational homology.

Attempts to determine total sequence variation of VSG's by means of conventional peptide mapping techniques are regarded as inconclusive (7,14). High performance liquid chromatography (HPLC) is a versatile technique for the rapid and effective separation of various biological compounds (17). The application of reverse phase HPLC to the separation of small underivatized peptides was recently described (18,19). Although this procedure was shown to provide a high degree of resolution and extreme sensitivity, only a few investigators have applied the technique to peptide mapping. Hancock and co-workers (20), for example, employed reverse phase HPLC to demonstrate similarities and differences in the sequences of several related proteins. The technique was also used by Fullmer and Wasserman (21) to map and subsequently purify tryptic fragments for amino acid sequence determinations. In the present study, a reverse phase HPLC separation system was developed and employed to map tryptic digests of 4 cloned variant antigenic types (VAT's) of the Wellcome strain (22) of Trypanosoma rhodesiense.

MATERIALS AND METHODS

Surface coat glycoproteins were purified from VAT's CP3B4, 6, 10 and 13 as previously described (13). One mg (dry wt) of each VAT preparation was

denatured by successive treatments including reduction and alkylation (23) and finally suspended in 1 ml of 0.05 M ammonium bicarbonate. TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)-treated trypsin (Millipore) was further purified by HPLC (24) just prior to use. Ten µg quantities of the purified trypsin were added to the 1 ml suspension of denatured glycoprotein at zero time and at each succeeding 24-hr interval of incubation at 25°C. Immediately after the addition of trypsin, 25 µl aliquots were removed from the digestion mixture, mixed with 5 µl of glacial acetic acid to stop trypsinization, and injected directly into the liquid chromatograph.

A Waters Associates (Milfore, MA) liquid chromatograph equipped with two M6000A solvent delivery systems, an M660 solvent flow programmer, a U6K universal injector, an M440 absorbance detector set at 280 nm, an M450 variable wavelength detector set at 215 nm, and an M730 data module were utilized. All separations were performed in a µ Bondapak C_{18} reverse phase column (3.9 mm x 30 mm), also from Waters Associates.

RESULTS AND DISCUSSION

Figure 1 shows the progressive tryptic digestion of VAT 13 glycoprotein. Glacial acetic acid appeared as a large peak at 3-4 min. In the zero time chromatogram, undigested glycoprotein emerged as a peak at 34 min. Following incubation for 24 hr, numerous peaks representing different peptide fragment peaks were clearly evident, although some intact glycoprotein still remained. At 72 hr, digestion was complete as indicated by the absence of the glycoprotein peak. The appearance of several additional peptide fragment peaks was also observed. The height and shape of peptide peaks were generally altered with increasing incubation time. Since further changes were not apparent at 96 hr, subsequent digestions were stopped at 72 hr with glacial acetic acid and the digest lyophilized.

The lyophilized digest of each VAT glycoprotein was dissolved in 400 µl of aqueous 0.1% trifluoroacetic acid, and 100 µl samples used for chromatography. As shown in Figure 2, the number of peaks resolved for the different VAT digests ranged from 30 to 40. Based on the hydrolytic peptide specificity ascribed to trypsin, this number of fragment peaks or cleavage sites is consistent with the quantities of lysine and arginine previously found in each of these VAT glycoproteins (13). To demonstrate reproducibility, three different preparations of each VAT glycoprotein were digested and each digest was chromatographed in triplicate. The within-day peptide fragment retention times of these runs varied by no more than $\pm$ 0.04 min, while day-to-day variation was within a range of $\pm$ 0.14 min. All digests were run under

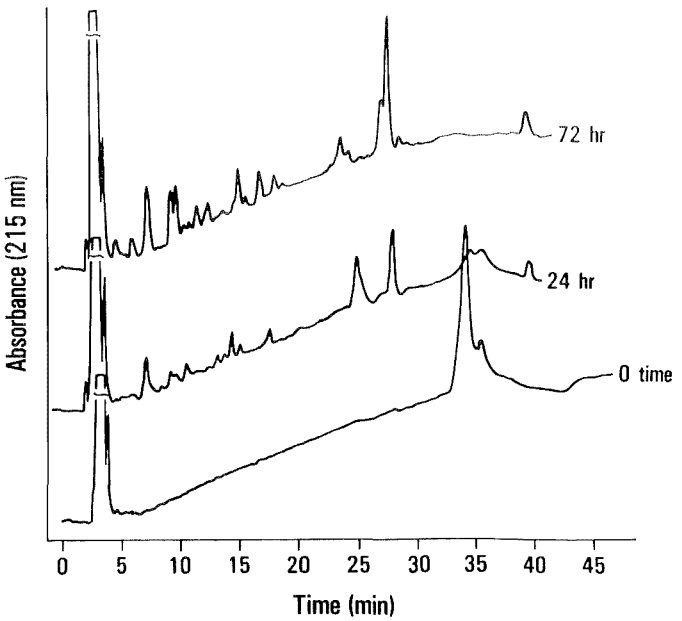


FIGURE 1. High performance liquid chromatographic analysis of the progress in trypsin digestion of VAT 13 glycoprotein. Elution of peptides was achieved by use of a 30 min linear gradient of 12% to 45% acetonitrile-water mixtures containing 0.1% trifluoroacetic acid at a flow rate of 2 ml/min. All runs were conducted at room temperature. Note position of undigested glycoprotein peak at 34 min.

comparable conditions to permit comparative observations. To facilitate comparison of peptide retention times among the four VAT's, a schematic representation consisting of vertical bars is presented in Figure 3. None of the vertical bars were shared by all four VAT's. Moreover, the patterns of retention times were totally different. We conclude from these findings that the purified VAT glycoproteins under present investigation do not contain significant stretches of amino acid homologies and that variation in the sequence of amino acids occurs throughout the polypeptide chains.

Richards et al. (25) found homology to the extent of 27% in the amino terminal region of two sequentially expressed VSG's in T. congolense, but not in non-sequential T. brucei variants nor between T. brucei and T. congolense VSG's. C-terminal amino acid sequences, as deduced from nucleotide sequencing of T. brucei VSG cDNA's, reveal regions of homology in the final 120-130

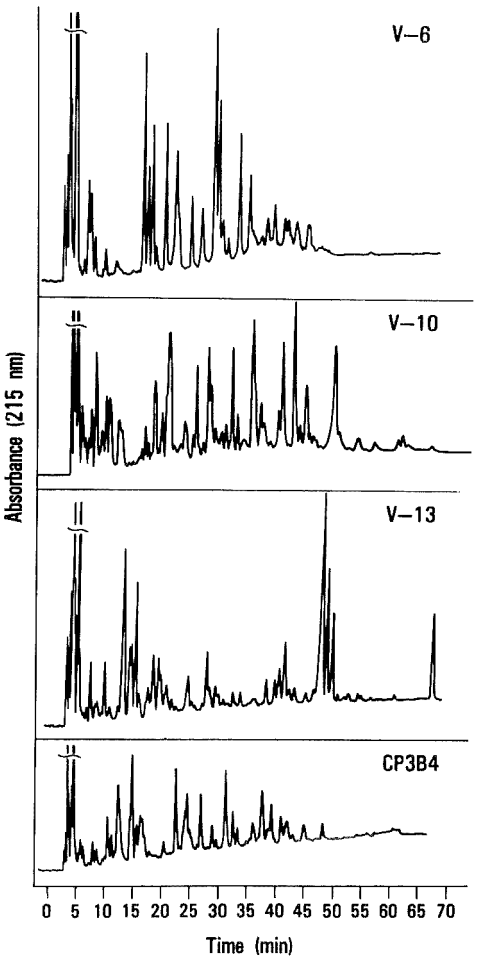


FIGURE 2. Peptide map chromatograms of variant glycoproteins 6, 10, 13 and CP3B4. Separations of peptide fragments of 72-hr trypsin digestion mixtures were obtained by a 60 min gradient as described in Figure 1 at a flow rate of 1.5 ml/min.

amino acids (26,27). A hydrophobic terminus of 17-23 amino acids and an uncharged polar region of 17-18 residues are the longest conserved sequences. However, the hydrophobic terminal sequence is not found on purified mature VSG's (28,29). Since the variants employed in this study, with the exception of CP3B4, were obtained at designated intervals (13) from a single rabbit in

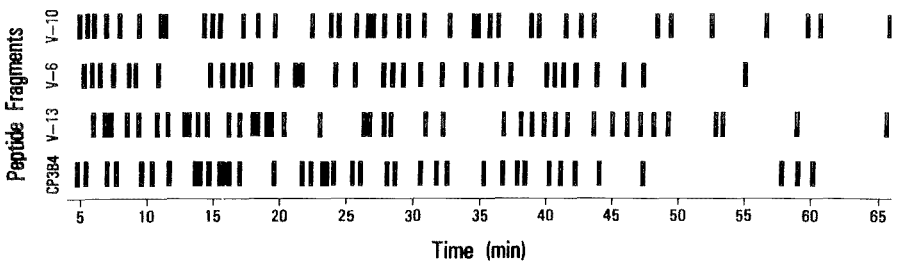


FIGURE 3. Schematic representation of the peptide map chromatograms of variant glycoproteins 6, 10, 13 and CP3B4. The vertical bars represent the average retention times of peptide peaks of triplicate runs ($\pm$ 0.15 min) for each trypsin-digested glycoprotein.

which the course of infection was characteristically mild and generally uniform throughout the period of our observations, it is not clear whether such variants represent sequential (or non-sequential) populations of trypanosomes. Nonetheless, it is reasonably certain that significant stretches of conserved amino acid homologies would be revealed by the present investigative approach employing reverse phase HPLC peptide mapping.

The present findings suggest that this technique may have significant application in the immunochemical characterization of VSG's. Recently, hybridoma-derived monoclonal antibodies have been prepared against VSG's of T. rhodesiense (30) and T. brucei (31). Coupled with the use of monoclonal antibodies directed against VSG's or any glycoprotein antigens, the technique should permit the recovery of peptides that can be employed as possible inhibitory haptens, thus facilitating the location or definition of immunogenic determinant sites. In addition, immunochemical mapping studies may be designed to probe the relationship of these sites to protective immunity.

REFERENCES

(1) de Raadt, P., World Health Organisation Documentation for Special Programme for Research and Training in Tropical Diseases (TDR/WP/76.12), Geneva, 1976.

(2) Jordan, A.M., Principles of the eradication or control of tsetse flies, Nature, 273, 607, 1978.

(3) Mulligan, H.W., and Potts, W.H., The African Trypanosomiases, Wiley Interscience, New York, 1970.

(4) WHO, World Health Statistics Report 30, 1977.

(5) van Meirvenne, N., Janssens, P.G., and Magnus, E., Antigenic variation in syringe-passaged populations of Trypanosoma (Trypanozoon) brucei. I. Rationalization of the experimental approach, Ann. Soc. Belge Med. Trop., 55, 1, 1975.

(6) Vickerman, K., Antigenic variation in trypanosomes, Nature, 273, 613, 1978.

(7) Cross, G.A.M., Antigenic variation in trypanosomes, Proc. R. Soc. Lond. B, 202, 55, 1978.

(8) Cross, G.A.M., Identification, purification and properties of clone-specific glycoprotein antigens constituting the surface coat of Trypanosoma brucei, Parasitology, 71, 393, 1975.

(9) Baltz, T., Baltz, D., and Pautrizel, R., Affinite de la concanavaline A pour Trypanosoma equiperdum: Applications a l'isolement de la fraction glycoproteique specifique du type antigenique, Ann. Immunol. (Inst. Pasteur), 127, C, 761, 1976.

(10) Reinwald, E., Risse, H-J., and Salker, R., Diazoniobenzenesulfonate as marker for cell surface proteins: Study of the surface coat of Trypanosoma congolense, Hoppe-Seyler's Z. Physiol. Chem., 359, 939, 1978.

(11) Rovis, L., Barbet, A.F., and Williams, R.O., Characterisation of the surface coat of Trypanosoma congolense, Nature, 271, 654, 1978.

(12) Strickler, J.E., Mancini, P.E., and Patton, C.L., Trypanosoma brucei brucei: Isolation of the major surface coat glycoprotein by lectin affinity chromatography, Exp. Parasitol., 46, 262, 1978.

(13) Olenick, J.G., Travis, R.W., and Garson, S., Trypanosoma rhodesiense: Chemical and immunological characterization of variant-specific surface coat glycoproteins, Mol. Biochem. Parasitol., 3, 227, 1981.

(14) Johnson, J.G., and Cross, G.A.M., Selective cleavage of variant surface glycoproteins from Trypanosoma brucei, Biochem. J., 178, 689, 1979.

(15) Baltz, T., Baltz, D., Pautrizel, R., Richet, C., Lamblin, G., and Degand, P., Chemical and immunological characterization of specific glycoproteins from Trypanosoma equiperdum variants, FEBS Lett., 82, 93, 1977.

(16) Bridgen, P.J., Cross, G.A.M., and Bridgen, J., N-terminal amino acid sequences of variant-specific surface antigens from Trypanosoma brucei, Nature, 263, 613, 1976.

(17) Biological/Biomedical Applications of Liquid Chromatography, Chromatographic Science Series, vol. 10, Hawk, G.L., ed., Marcel Dekker, New York, 1978.

(18) Molnar, I., and Horvath, C., Separation of amino acids and peptides on non-polar stationary phases by high-performance liquid chromatography, J. Chromatography, 142, 623, 1977.

(19) Hollaway, W.L., Bhown, A.S., Mole, J.E., and Bennett, J.C., Biological/Biomedical Applications of Liquid Chromatography, Hawk, G.L., ed., Marcel Dekker, New York, 1979, p. 163.

(20) Hancock, W.S., Bishop, C.A., Prestidge, R.L., and Hearn, M.T.W., The use of high pressure liquid chromatography (hplc) for peptide mapping of proteins IV, Anal. Biochem., 89, 203, 1978.

(21) Fullmer, C.S., and Wasserman, R.H., Analytical peptide mapping by high performance liquid chromatography: Application to intestinal calcium-binding proteins, J. Biol. Chem., 254, 7208, 1979.

(22) Rane, L., Rane, D.S., and Kinnamon, K.E., Screening large numbers of compounds in a model based on mortality of Trypanosoma rhodesiense-infected mice, Am. J. Trop. Med. Hyg., 25, 395, 1976.

(23) Weintraub, S.B., and Frankel, F.R., Identification of the T4rIIB gene product as a membrane protein, J. Mol. Biol., 70, 589, 1972.

(24) Strickler, M.P., Gemski, M.J., and Doctor, B.P., Purification of commercially prepared bovine trypsin by reverse phase high performance liquid chromatography, J. Liquid Chromatog., 4, 1765, 1981.

(25) Richards, F.F., Rosen, N.L., Onodera, M., Bogucki, M.S., Neve, R.L., Hotez, P., Armstrong, M.Y.K., and Konigsberg, W.H., Antigenic variation and the surface glycoproteins of Trypanosoma congolense, Federation Proc., 40, 1434, 1981.

(26) Matthyssens, G., Michiels, F., Hamers, R., Pays, E., and Steinert, M., Two variant surface glycoproteins of Trypanosoma brucei have a conserved C-terminus, Nature, 293, 230, 1981.

(27) Rice-Ficht, A.C., Chen, K.K., and Donelson, J.E., Sequence homologies near the C-termini of the variable surface glycoproteins of Trypanosoma brucei, Nature, 294, 53, 1981.

(28) Boothroyd, J.C., Cross, G.A.M., Hoeijmakers, J.H.J., and Borst, P., A variant surface glycoprotein of Trypanosoma brucei synthesized with a C-terminal hydrophobic "tail" absent from purified glycoprotein, Nature, 288, 624, 1980.

(29) Boothroyd, J.C., Paynter, C.A., Cross, G.A.M., Bernards, A., and Borst, P., Variant surface glycoproteins of Trypanosoma brucei are synthesized with cleavable hydrophobic sequences at the carboxy and amino termini, Nucleic Acids Res., 9, 4735, 1981.

(30) Lyon, J.A., Pratt, J.M., Travis, R.W., Doctor, B.P., and Olenick, J.G., Use of monoclonal antibody to immunochemically characterize variant-specific surface coat glycoprotein from Trypanosoma rhodesiense, J. Immunol., 126, 134, 1981.

(31) Pearson, T.W., Kar, S.K., McGuire, T.C., and Lundin, L.B., Trypanosome variable surface antigens: Studies using two-dimensional gel electrophoresis and monoclonal antibodies, J. Immunol., 126, 823, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1941-1946 (1982)

IMPROVED SEPARATION OF POLYOLS AND CARBOHYDRATES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

C. Vidal-Valverde, C. Martin-Villa and B. Olmedilla

Centro Nacional de Investigaciones Médico-Quirúrgicas
de la Seguridad Social
Faculty of Medicine. Universidad Autónoma
S. Martin de Porres 4. Madrid-35 (Spain).

ABSTRACT

A reliable High Performance Liquid Chromatographic analysis of glucose-fructose-sucrose-sorbitol and mannitol is developed. A Sugar Pak I column at 85ºC is used employing water as the mobile phase. The separation is completed within 20 minutes, and the resolution is very acceptable.

INTRODUCTION

The application of high performance liquid chromatography (HPLC) to the analysis of carbohydrates has signified a great improvement in their qualitative and quantitative determination (1-4).

Polyhydric alcohols, such as mannitol and sorbitol, usually employed as artificial sweeteners in food products, have been determined by different authors (5).

Nonetheless, the simultaneous presence of sugars and polyols in fruits and dietetic foodstuffs

poses a difficult analytical problem due to difficulty of resolution of these compounds by HPLC.

Various attempts have been carried out in this regard. Brandao et al. (6) and Richmond et al.(7) achieved the separation of polyols and carbohydrates by HPLC, using two columns joined in tandem, which increased the cost of the analyses. Gordy et al. (8) and Dokladalova et al. (9) give some chromatographic constants for carbohydrates and polyols.

EXPERIMENTAL

High performance liquid chromatography was carried out in an ALC/GPC (Model 201) equipped with model 6000 A pump dual reciprocating piston heads, model U 6K septumless injector, Sugar Pak I column 30cm x 6.5 mm i.d., water jacketed at 85ºC, pre-column filter, and model R-401 differential refractometer detector optical deflection type, maintained at 30 C (Waters Associates,Milford,Mass, USA). The detector signal was recorded on a Houston Instrument Omniscribe recorder.

Mobile phase: Bidistilled water was degassed by immersion in an ultrasonic bath and filtered through a Millipore HA (0.45µm) membrane (Millipore Corp., Bedford, Mass., USA).

Standard solution: Various amounts of sucrose, glucose, fructose, sorbitol and mannitol (Merck) were dissolved in bidistilled water and filtered through a Millipore HA (0.45µm) membrane.

RESULTS AND DISCUSSION

The performance of the Sugar Pak I column for a mixture of sucrose, glucose, fructose, mannitol

TABLE 1

Standardization of Waters Sugar Pak I High Performance Liquid Chromatographic Column

	RT_S	Sucrose	Glucose	Fructose	Mannitol	Sorbitol	k'	N
		Separation factor (α)						
Sucrose	0.472	\	1.923	2.846	3.615	4.692	0.433	1183
Glucose	0.604	2.182	\	1.480	1.880	2.440	0.833	1344
Fructose	0.736	4.000	1.846	\	1.270	1.649	1.233	1466
Mannitol	0.846	6.182	3.666	1.539	\	1.298	1.567	2635
Sorbitol	1.000	8.727	6.000	3.692	2.545	\	2.033	3680
		Resolution (R)						

RT_S=relative retention time to sorbitol; Separation factor $(\alpha)=k'_2/k'_1$;
W = peak width; Resolution $(R)=V_2-V_1/\frac{1}{2}(W_2+W_1)$; V=retention volume;
V_0=void volume; Capacity factor $(k')=V-V_0/V_0$;
Number of theoretical plates $(N)= 16\ (V/W)^2$

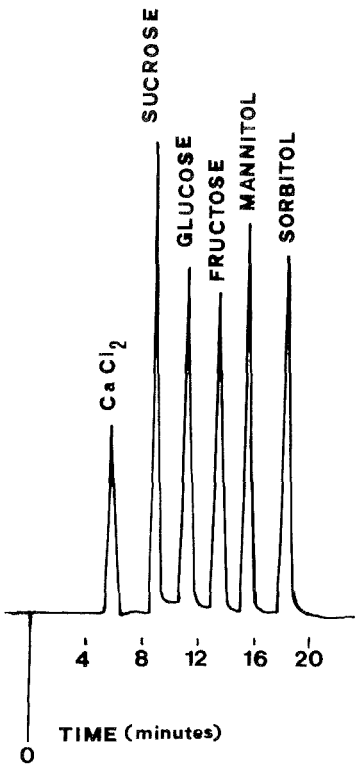


Fig.1: Flow rate: 0.4ml/min.; Attenuation 32x

and sorbitol, was characterized by the following numerical values (see Table 1): retention time to sorbitol (RT_S), separation factor (α), resolution (R), capacity factor (k'), and theoretical plates (N). The void volume used to calculate (k') was determined using the retention time of $CaCl_2$.

A typical chromatogram of the standard solution is shown in Fig.1.

The response of the detector was a rectilinear response between peak height (cm) and weight,(25,

50,75 and 100 µg in 50 µl) of the carbohydrates and polyols. The equations y=a+bx of the 5 lines are:

for sucrose	a=0.050	b=0.082	r=0.999
for glucose	a=0.063	b=0.066	r=0.999
for fructose	a=0.063	b=0.061	r=0.999
for mannitol	a=0.025	b=0.071	r=0.999
for sorbitol	a=0.062	b=0.064	r=0.999

The number of theoretical plates and the capacity factor for the polyhidric alcohols, as well as for the mono- and disaccharides analyzed were substantially greater than those obtained previously (8).

Dokladalova et al. (9) indicate only the retention times of the products separated, which are similar to those reported here, though ours, especially those corresponding to sucrose and glucose, are more adequate for the achievement of complete resolution of a mixture of them.

In general, it can be concluded that the chromatographic conditions established here are appropiate for the separation and quantitation of a mixture of sucrose, glucose, fructose, mannitol and sorbitol.

ACKNOWLEDGEMENT

The authors are indebted to Dr. E. FERNANDEZ (Waters Associates Española) for his constant encouragement during the realization of this work.

REFERENCES

1. De Vries, J.W., Heroff, J.C., and Egberg, D.C., High pressure liquid chromatographic determination of carbohydrates in food products: Evaluation of method, J. Assoc. Off. Anal. Chem., 62, 1292, 1979.

2. Hurst, W.J. and Martin, R.A., High performance liquid chromatographic determination of carbohydrates in chocolate: Collaborative study, J. Assoc. Anal. Chem., 63, 595, 1980.

3. Vidal-Valverde, C., Martin-Villa, C., Herranz, J., and Rojas-Hidalgo, E., High performance liquid chromatographic determination of carbohydrates in soft drinks , Z. Lebensm. Unters. Forsch., 172, 93, 1981

4. Martin-Villa, C., Vidal-Valverde, C., and Rojas-Hidalgo, E., High performance liquid chromatographic determination of carbohydrates in raw and cooked vegetables , J. Food Sci., (in press).

5. Samarco, E.C., High pressure liquid chromatographic determination of mannitol and sorbitol in sugarless chewing gums, J. Assoc. Off. Anal. Chem. 60, 1318, 1977.

6. Brandao, S.C.C., Richmond, M.L., Gray, J.I., Morton, I.D. and Stine, C.M., Separation of mono- and di-saccharides and sorbitol by high performance liquid chromatography, J. Food Sci. 45, 1492, 1980.

7. Richmond, M.L., Brandao, S.C.C., Gray, J.I., Markakis, P., Stine, C.M., Analysis of simple sugars and sorbitol in fruit by high performance liquid chromatography, J. Agric. Food Chem., 29, 4, 1981.

8. Gordy, V., Baust, J.G., Hendrix, D.L., A high pressure liquid chromatographic method for analysis of carbohydrates and polyols from lichens, The Bryologist, 81, 532, 1978.

9. Dokladalova, J., Barton, A.Y., Mackenzie, E.A., High pressure liquid chromatographic determination of sorbitol in bulk sorbitol, J. Assoc. Off. Anal. Chem., 63, 664, 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1947-1965 (1982)

DETERMINATION OF PLASMA NOREPINEPHRINE AND EPINEPHRINE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY USING A TWO-COLUMN SYSTEM AND AN ELECTROCHEMICAL DETECTOR

G. Lachâtre*, G. Nicot*, C. Magnet**, J.L. Rocca**, L. Merle* and J.P. Valette* (with the technical assistance of P. Pignolet*)

* Département de Pharmacologie Clinique, Centre Hospitalier Régional Universitaire, Hôpital Dupuytren, 87031 Limoges, France.

** Laboratoire de Chimie Analytique III (E.R.A. n° 474, M. Porthault), Université Claude Bernard - Lyon I, 43 Bd du 11 novembre 1918, 69622 Villeurbanne, France.

ABSTRACT

A method for the simultaneous plasma norepinephrine (NE) and epinephrine (E) determination by reversed-phase ion-pair liquid chromatography with electrochemical detection has been developed. Catecholamines were extracted from a 4 ml plasma sample using an alumina adsorption procedure. A two-pump, two-injection valve, two-column system allowed both to detect plasma NE and E with a good sensitivity due to large injected volumes of extract without any electrochemical detector disturbance and to eliminate uric acid and dopa the low k' of which would prevent the NE detection. Using this method, NE and E would be detected in respective injected amounts down to 30 and 50 picograms. Plasma NE and E determinations were found to be linear in the range of 288 to 788 pg/ml and 24 to 274 pg/ml respectively. The reproducibility, expressed as the coefficients of variation, varied from 2.1 % for NE to 10.8 % for E.

INTRODUCTION

The determination of the plasma catecholamines norepinephrine (NE) and epinephrine (E) can be useful both in clinical studies

when a pheochromocytoma is suspected (1, 2) and in physiopathological and pharmacological studies (3, 4, 5, 6, 7, 8, 9, 10).

The detection of low plasma levels of NE (less than 500 picograms per milliliter) and E (less than 100 pg/ml) in healthy individuals requires the use of techniques involving extremely sensitive procedures, such as gas chromatography/mass spectrometry (11, 12) or radioenzymology (13, 14).

High performance liquid chromatography (HPLC), appeared in recent years as being a new efficient method for the separation of catecholamines and their derivatives (15, 16). If numerous techniques dealing with the assay of catecholamines in tissues or urines have been published, few authors have described the assay of plasma catecholamines by HPLC. Two detection methods have been used in plasma : fluorimetric and electrochemical. Fluorimetric detection has been employed after products had been derivatized with orthophthalaldehyde (17) or transformed into trihydroxyindole derivatives (18). Electrochemical detection which seemed to be an accurate method in order to measure catecholamines concentrations has been used by Hallman et al (19), Hjendahl et al (20) and Allenmark et al (21) together with ion exchange HPLC.

Like Davis et al (22), we have chosen to use a reversed-phase ion-pairing chromatography technique. This method allows us to modify many parameters : pH of the mobile phase, ionic strength, type and number of carbon atoms in the n-alkyl chains, concentration of the counter-ion, in order to improve the selectivity of the system.

A major problem which remains unanswered is the close elution of uric acid and NE, the capacity factor (k') of which are similar. The method we report allows us to overcome these problems using two injection valves and two columns one of which is placed in a loop position.

EXPERIMENTAL

Instrumentation

The chromatographic system (scheme 1) consisted of - two pumps : Waters 6000 A (Waters Assoc. Inc., Milford, MA., U.S.A.) - two injection valves : Rheodyne 7125 and 7110 (Rheodyne, Santa Rosa, U.S.A.) equipped, the first (I) with a 250 µl loop, the other (II) with a short column placed in loop position - a thin layer electrochemical detector : Eldec 102 (Chromatofield, Chateauneuf-les-Martigues, France) provided with a glassy carbon electrode maintained at a + 0.75 V potential vs a Ag/AgCl reference electrode. The detector output was connected to a recorder : Kontron W + W 610 (W.W. Electronic Inc., Basel, Switzerland).

The two stainless steel columns we used (a short column : 4.6 × 35 mm and an analytical column : 4.6 × 150 mm) were packed under pressure by a slurry packing technique with Nucleosil C18, 5 µm (Macherey-Nagel, Düren, F.R.G.) according to the method described by Coq (23).

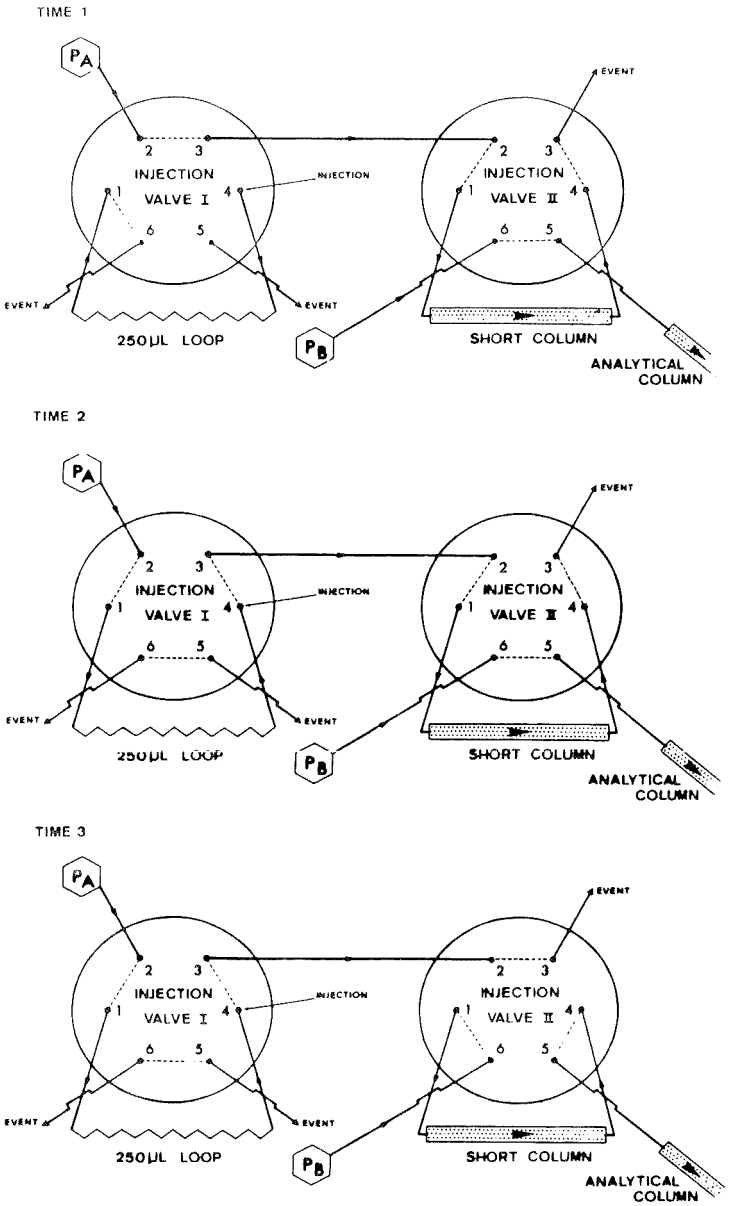
A pulse damper (Touzart-Matignon, Vitry-sur-Seine, France) was inserted between the pump and the injection valve in order to decrease the background.

The 250 µl syringe we used was fitted with a teflon piston nozzle (Hamilton, Bonaduz, Switzerland).

The detector was mounted within its proper Faraday cage. The detector, the column and the metal capillaries were grounded.

Chemicals and solvents

Catecholamines (NE, E, dopamine (DA), epinine), their precursors (phenylalanine, tyrosine, phenylethylamine, tyramine, dopa), their derivatives (3,4,5,trihydroxyphenylethylamine, metanephrine, normetanephrine, vanilmandelic acid, homovanillic acid) as well as the internal standard (dihydroxybenzylamine (DHBA)) were provided by Sigma (Sigma Chemical Co., Saint-Louis, MO., U.S.A.).



Scheme 1. - Diagram of the HPLC system showing the three steps of the chromatography.

1 mg/ml standard solutions were prepared in 0.1 M $HClO_4$ and stored at + 4°C for a month, in brown glass flasks. 10 ng/ml working solutions (8 ng/ml for DHBA) were obtained daily by dilution of the standard solutions with 0.1 M $HClO_4$. The heptane sulfonic acid sodium salt was provided by Eastman Kodak (Eastman Kodak, Rochester, NY, U.S.A.), the disodium ethylenediaminetetraacetate (EDAT Na_2) and the acid type alumina were provided by Fluka (Fluka, Buchs, Switzerland). Alumina was reactivated before use according to the Anton and Sayre technique (24). 1 M tris buffer (pH = 8.6) and sodium metabisulfite were from Merck (Merck, Darmstadt, F.R.G.).

Chromatographic conditions

The mobile phase we used consisted of 15 g/l phosphate buffer ($NaH_2PO_4, 2H_2O$), 300 mg/l of heptane sulphonate and 200 mg/l of EDTA. The pH was ajusted to 4.2 with 3 M phosphoric acid. 5% (Vol%) acetonitrile was used as an organic modifier of this mobile phase. The mixture was filtered through a 0.45 µm filter then degassed in an ultrasonic bath.

The elution was carried out at ambient temperature. They were cleaned every day using 60 ml of methanol. The electronic system was left continuously under tension, the cell put to counterbalance for at least 90 minutes before each manipulation.

Two pumps (P_A and P_B) delivered independently the same mobile phase which was thus divided into two parts (MP_A and MP_B). The flow rates of MP_A and MP_B were identical (1 ml/min). The usual positions "load" and "inject" on valve II (Rheodyne 7110) were inverted and so were the entry numbers (2←→6, 3←→5) thus allowing the flow in the short column to be always in the same way (whatever the position of the valve might be). Valve I (Rheodyne 7125) was used according to the operating instructions.

The chromatography was carried out over three periods (scheme 1) :

1. Valve I and valve II were placed in the "load" position. The loop was filled with 200 µl of eluate containing catecholamines. During this period, MP_A flowed through the short column and MP_B through the analytical column.
2. Valve I was placed in the "inject" position while valve II remained in the "load" position. Thus, catecholamines in the loop eluate were sent through the short column, while MP_B was flowing through the analytical column.
3. After a 60 second elution through the short column, valve II was placed in the "inject" position. So catecholamines were eluted by MP_B from the short column into the analytical column. MP_A was discarded.

Sample preparation

Polystyrene tubes containing 100 µl of the following mixture : heparine (50 mg/ml) and metabisulfite (25 mg/ml) were kept in an ice bath.

In fasting volunteers asked to remain in the supine position, an arm vein was catheterized. 20 min after the veinipuncture a 10 ml blood sample was drawn using a heparinized syringe. Blood was immediately transfered into the polystyrene tubes.

The tubes were centrifuged at + 4°C ; the plasma was separated from blood cells and stored in polystyrene tubes at + 4°C if the assay was to be done within the following 12 hours. If the assay was done later, plasma was stored at - 20°C for a maximum of 7 days.

Extraction was done at room temperature by adsorption on alumina according to the classic method without deproteinization. In 10 ml polystyrene tubes, to 4 ml of plasma were added 500 µl of 1 M HCl tris buffer and 200 µl of DHBA (8 ng/ml) used as an internal standard. The mixture was homogenized for 15 sec (final pH = 8.6) and 75 mg of alumina was added. The tubes were capped and placed on a reciprocal shaker for 15 min and then spun down at 1000g for 5 min. The supernatant was discarded and alumina was

washed three times with bidistilled water. The washings were removed by aspiration. Following the last wash, alumina was aspirated to near dryness. Catecholamines were eluted from the alumina by 250 µl of 0.1 M $HClO_4$. All the eluate was transfered to a small Eppendorf conic tube, spun for 1 min (8000g) and stored in the dark at + 4°C until the assay. A 200 µl eluate sample was injected in the chromatographic system.

As the samples contained an internal standard, catecholamines concentrations were calculated by measuring the following ratio : height of catecholamine peak/height of internal standard peak, and comparing that to the mean of equivalent ratios obtained from two aqueous extracts containing known amounts of catecholamines (250 pg of NE and E) assayed following the same procedure.

RESULTS

As most of the perchloric acid was eliminated before it entered the analytical column, the k' we measured were rough estimates : 0.9 (NE), 1.7 (E), 2.3 (DHBA), 4.1 (DA). (Fig. 1a, 1b).

Linearity

Figure 2 shows the linearity plots when injecting increasing amounts of each catecholamine as pure solutions (from 50 pg to 1 ng). The plots indicate that linearity was good up to 1 ng.

Figures 3 and 4 show linearity plots obtained after extraction from aqueous solutions of catecholamines and plasmas charged with NE and E. No linearity discrepancy appeared in plasma concentrations ranging from 288 to 788 pg/ml for NE and from 24 to 274 pg/ml for E.

Reproducibility (table 1)

It was tested by repeated measurements of NE and E in two different plasmas at a 15 day-interval. Five assays were done in the first plasma and seven in the second.

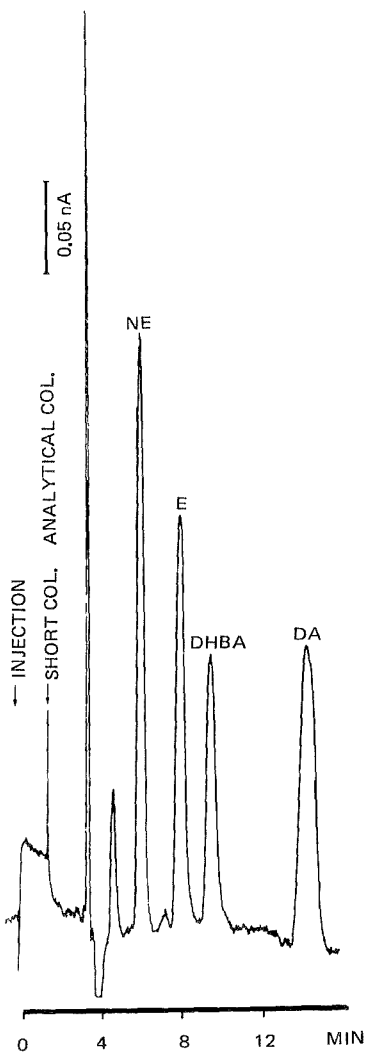

0,05 nA
NE
E
DHBA
DA
INJECTION
SHORT COL.
ANALYTICAL COL.
0
4
8
12
MIN

FIG. 1A

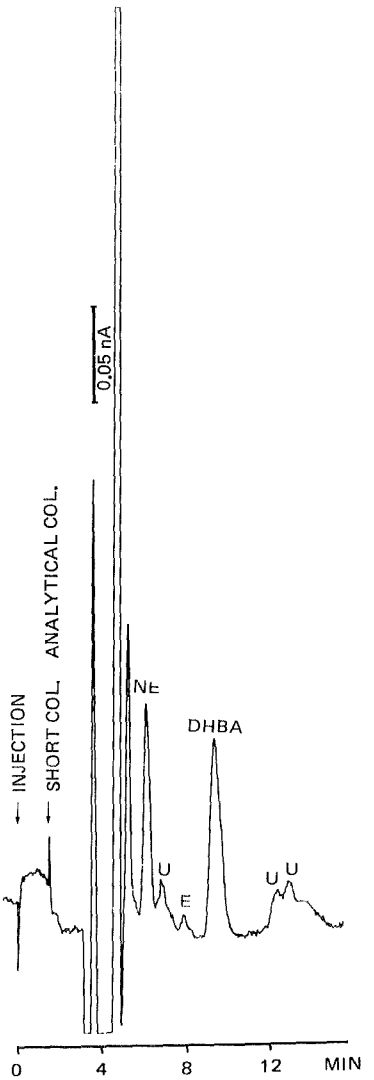

0,05 nA
NE
DHBA
U
E
U
U
INJECTION
SHORT COL.
ANALYTICAL COL.
0
4
8
12
MIN

FIG. 1B

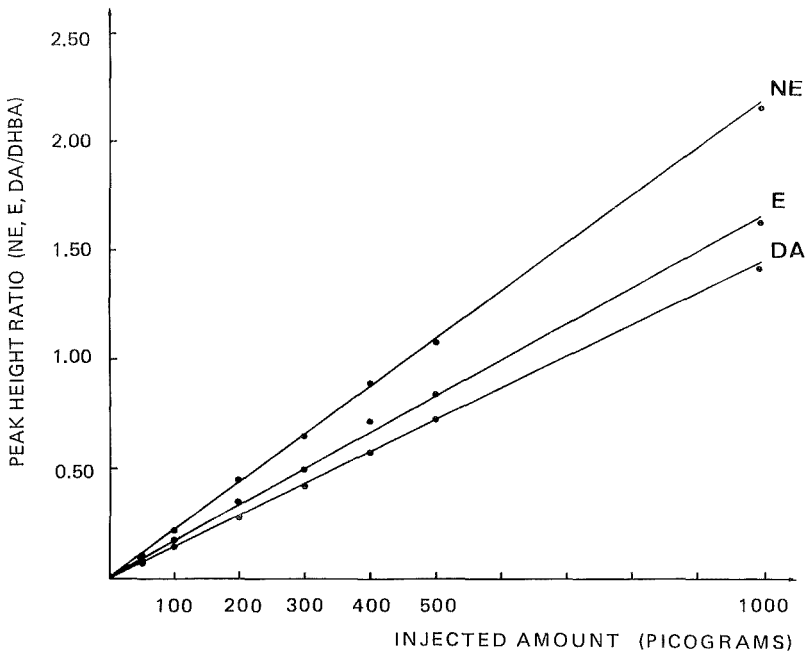


Figure 2. - Standard curves obtained after chromatography of increasing amounts of catecholamines (NE, E, DA) and 800 picograms of internal standard (DHBA) in 0.1 M $HClO_4$.

Figure 1. - a. Injection of 1 ng of each catecholamine and 0.8 ng of internal standard, in 200 ul of 0.1 M $HClO_4$.

b. Chromatogram of catecholamines obtained from 4 ml of human plasma to which 200 ul of a 8 ng/ml DHBA solution were added. This sample was extracted with alumina eluated by 250 ul of 0.1 M $HClO_4$ and a 200 ul eluate was injected. The NE and E peaks are respectively equivalent to 230 and 33 pg/ml of plasma. (U = unknown peak).

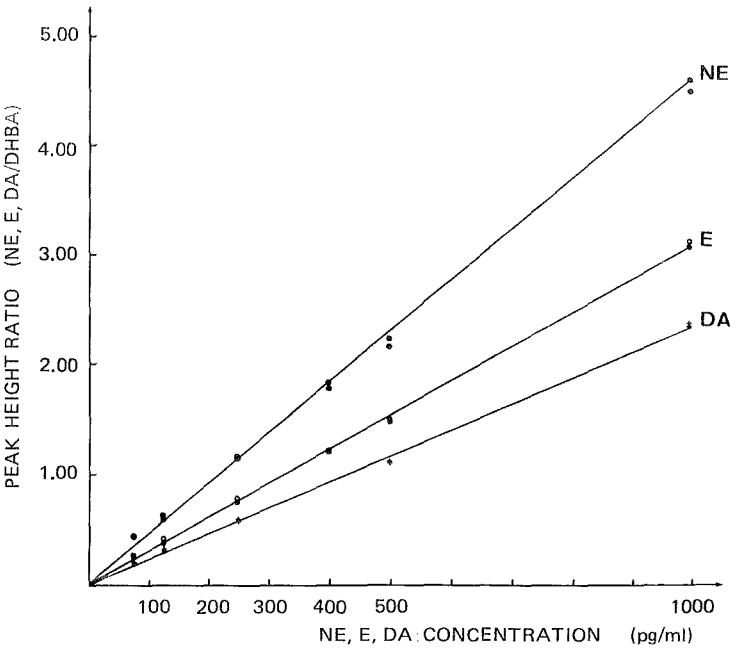


Figure 3. - Standard curves obtained after extraction and chromatography of NE, E, DA at different concentrations in aqueous solutions.

Detection limit

A study of the applied voltage between 0.5 and 0.8 volts has shown that the best signal-to-noise compromise (S/N) was reached at 0.75 volts. Under these conditions, the detection limit defined as the amount of catecholamines leading to a S/N ratio of 5 was 30 pg of NE and 50 pg of E.

Recovery (table 2)

This study was done after extraction of each catecholamine (250 pg/ml) and of the internal standard (400 pg/ml) from a 4 ml aqueous solution. Four extracts were recovered. In order to compare the peak heights of the extracts, we injected an amount

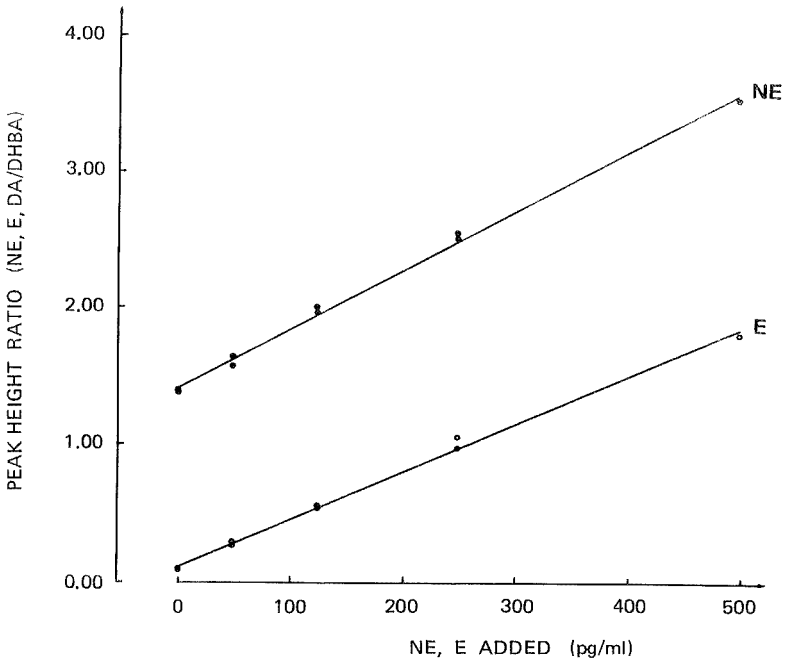


Figure 4. - Standard curves obtained after extraction and chromatography of a human plasma charge with NE and E. The plasma sample contained 288 and 24 pg/ml of NE and E respectively before addition.

TABLE 1

Reproducibility of the Method (Extraction, Chromatography)

	Extracts							Mean	Coefficient of variation (percentage)
	1	2	3	4	5	6	7		
Plasma I[a]									
NE (pg/ml)	258	260	256	256	271			260	2.4
E (pg/ml)	128	109	100	122	103			112	10.8
Plasma II[a]									
NE (pg/ml)	274	261	276	270	272	279	268	272	2.1
E (pg/ml)	47	43	43	50	37	43	43	43	9.2

a - Blood was collected without taking special precaution (subjects were not resting or lying before sampling, blood was drawn by veinipunture and not through an indwelling catheter).

TABLE 2

Recovery Percentage of Catecholamines and Internal Standard after Extraction and Chromatography

Compounds	Peak height after extraction (mm)				Mean of peak height without extraction (mm) (mean of 3 injections)	Recovery % (±2 SD)
	1	2	3	4		
NE (800 pg)	62	67	62	67	113	57.3 ± 5.1
E (800 pg)	41	43	38	45	74	56.1 ± 8.6
DA (800 pg)	33	33	28	37	60	54.8 ±12.3
DHBA (1280 pg)	50	54	49	53	97	53.1 ± 4.9

of catecholamines and internal standard equivalent to a 100 % yield. Taking into account the fact that only a 200 µl volume out of the 250 µl eluate was injected, an amount of 800 pg of each catecholamine and 120 pg of internal standard was injected. The recovery percentage was calculated by measuring the peak height ratio (53.1 - 57.3 %).

Conservation of catecholamines and interference (tables 3, 4)

The samples of catecholamines in 0.1 M $HClO_4$ were divided into four portions. Two samples were processed after 4.5 hours of storage at + 4°C either in the dark or exposed to daylight ; two other samples were processed after 2.5 hours of storage at room temperature either in the dark or exposed to daylight. No significant difference was noted (table 3) between the results of stored samples.

Table 4 illustrates that in no case was a detectable interference between catecholamine or internal standard and tested endogenous compounds.

DISCUSSION

During our first chromatographic studies of catecholamines, we used two detection methods : flucrimetric (direct or after post

TABLE 3

Conditions of Conservation of Catecholamines

Conditions of conservation[a]	Peak height of each compound (mm)[b]		
	NE	E	DA
4.5 hours (+ 4°C, darkness)[c]	49	31	25
4.5 hours (+ 4°C, light)	50	31.5	25
2.5 hours (room temperature, darkness)	48	28	25
2.5 hours (room temperature, light)	50	30	25

a - Concentrations are close to those in the eluate (2,5 ng/ml).
b - Injection of 200 µl of a 2,5 ng/ml solution of each catecholamine in 0.1 M $HClO_4$.
c - Considered as ideal conditions of conservation.

column derivatization with orthophthalaldehyde) or electrochemical. Then, we chose electrochemical detection which appeared to be one of the most effective method, thus confirming results obtained by Yui (25).

Using this method, the most effective stationary phase was Nucleosil C18, 5 µm (26).

At the beginning of our work, the mobile phase pH was set at 5.0 in order to facilitate the oxydation of catecholamines so as to enhance the detector response (16). Later as we used a different batch of the same stationary phase, we observed a band spreading and so had to set the pH at 4.2. This pH induces a loss of ionization of OH and above all facilitates the protonation of the amine, thus improving the efficiency of the counter-ion. Consequently, we had to slightly increase the amount of acetonitrile (5 %).

During these tests, we noticed that when using an aliquot of 4 ml and an eluate of 250 µl, the best extraction yield needed 75 mg of alumina.

Among the electro active compounds extracted together with catecholamines were uric acid and dopa. This lack of selectivity

TABLE 4

Interference from Endogenous compounds

Compounds	Capacity factors	Extraction with alumina[a]	Detection in our HPLC system
Norepinephrine	0.9	+	+
Epinephrine	1.7	+	+
Dopamine	4.1	+	+
Dihydroxybenzylamine	2.3	+	+
Dopa	no peak		-
Epinine	7.1	-	-
Homovanillic acid	no peak		-
Metanephrine	6.4	-	-
Normetanephrine	4.7	-	-
Phenylalanine	no peak		-
Phenylethylamine	no peak		-
Tyramine	no peak		-
Tyrosine	no peak		-
3,4,5 trihydroxy-phenylethylamine	2.3	-	-
Vanilmandelic acid	no peak		-

a - Concentration of each compound = 2 ng/ml

due to the alumina extraction is a major problem which could induce interferences during chromatography. Effectively, the close k' of uric acid and NE as well as the destabilization of the detector due to the injection of 200 µl of 0.1 M $HClO_4$ impede the detection of NE and E. As the retention time of uric acid did not seem to be modified by the use of various counter-ions and as a double extraction by ion exchange and alumina adsorption (22) could lead to more inaccuracy and increase the length of the assay we used the chromatographic system previously described. This

system uses a preset optimal elution time of 60 seconds in the short column and a 1 ml/min flow. Thus uric acid and 0.1 M $HClO_4$ are eliminated and the analytical column is protected. The mixture which is injected in the analytical column is very similar to the mobile phase, so the detector is less disturbed. All this procedure permits to inject a large volume.

Of course this large volume leads to a slight band spreading and to a loss of resolution. Yet chromatograms are quite satisfactory as peaks are perfectly resolved.

One of the drawbacks of this method using ion-pairing is the loss of efficiency which could limit the detection of E.

We applied this method to the detection of catecholamines in ten volunteers. We measured NE concentrations ranging from 137 to 553 pg/ml and E concentrations ranging from 23 to 100 pg/ml. These results are similar to those previously published (9, 22, 27). We were not able to detect plasma DA which is conjugated as sulfates or glucuronides in a considerable proportion (28). Though a recent study has shown a good stability of catecholamines in plasma (29) we prefered not to store plasma samples more than a week at - 20°C.

We have presented a reliable method permitting the simultaneous assay of plasma norepinephrine and epinephrine. We think this method is useful, first as a chemical tool using reversed-phase ion-pair liquid chromatography with electrochemical detection, second as a clinical tool in order to establish some difficult diagnosis and third as a research tool in physiology and pharmacology.

REFERENCES

1. Devynck, M.A., Elghozi, J.L., Le Quan Bui, K.H., Meyer, P. Dosage rapide des catécholamines plasmatiques pour le diagnostic d'urgence des phéochromocytomes. Chromatographie à haute pression en phase liquide et détection électrochmique. Nouv. Presse méd., 9, 2061, 1980.

2. Plouin, P.F., Duclos, J.M., Menard, J., Comoy, E., Bohuon, C., Alexandre, J.M.
Biochemical tests for diagnosis of phaeochromocytoma : urinary versus plasma determinations.
Br. Med. J., 282, 853, 1981.

3. Sever, P.S., Osikowska, B., Birch, M., Tunbridge, R.D.G.
Plasma-noradrenaline in essential hypertension.
Lancet, 1, 1078, 1977.

4. Ziegler, M.G., Lake, C.R., Kopin, I.J.
Plasma noradrenaline increases with age.
Nature, 261, 333, 1976.

5. Cryer, P.E., Silverberg, A.B., Santiago, J.V., Shah, S.D.
Plasma catecholamines in diabetes. The syndrome of hypo-adrenergic and hyperadrenergic postural hypotension.
Am. J. Med., 64, 407, 1978.

6. Dechamplain, J., Farley, L., Cousineau, D., Van Ameringen, M.R.
Circulating catecholamine levels in human and experimental hypertension.
Circul. Res., 38, 109, 1976.

7. Vlachakis, N.D.
Blood pressure variability and plasma catecholamines in man. Effect of propranolol therapy.
Biochem. Med., 21, 253, 1979.

8. Christensen, N.J., Mathias, C.J., Frankel, H.L.
Plasma and urinary dopamine : studies during fasting and exercise and in tetraplegic man.
Eur. J. Clin. Invest., 6, 403, 1976.

9. Johnson, G.A., Peuler, J.D., Baker, C.A.
Plasma catecholamines in normotensive subjects.
Cur. Ther. Res., 21, 898, 1977.

10. Plouin, P.F., Comoy, E., Bohuon, C., Corvol, P., Menard, J.
Activité rénine et noradrénaline plasmatiques au cours du traitement antihypertenseur par l'acébutolol.
Nouv. Presse méd., 8, 1905, 1979.

11. Ehrhardt, J.D., Schwartz J.
Advances in the biosciences - Peripheral dopaminergic receptors. Imbs, J.L., Schwartz, J. eds., Oxford, 1978, p. 41.

12. Yoshida, J.I., Yoshino, K., Matsunaga, T., Higa, S., Suzuki, T., Hayashi, A., Yamamura, Y.
An improved method for determination of plasma norepinephrine : isolation by boric acid gel and assay by selected ion monitoring.
Biomed. Mass Spectrometry, 7, 396, 1980.

13. Da-Prada, M., Zürcher, G.
Simultaneous radioenzymatic determination of plasma and tissue adrenaline, noradrenaline and dopamine within the femtomole range.
Life Sci., 19, 1161, 1976.

14. Peuler, J.D., Johnson, G.A.
Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine.
Life Sci., 21, 625, 1977.

15. Crombeen, J.P., Kraak, J.C., Poppe, H.
Reversed-phase systems for the analysis of catecholamines and related compounds by high-performance liquid chromatography.
J. Chromatogr., 167, 219, 1978.

16. Moyer, T.P., Jiang, N.S.
Optimized isocratic conditions for analysis of catecholamines by high performance reversed-phase paired-ion chromatography with amperometric detection.
J. Chromatogr., 153, 365, 1978.

17. Davis, T.P., Gehrke, C.W., Gehrke, C.W.Jr, Cunningham, T.D., Kuo, K.C., Gerhardt, K.O., Johnson, H.D., Williams, C.H.
High-performance liquid-chromatographic separation and fluorescence measurement of biogenic amines in plasma, urine and tissue.
Clin. Chem., 24, 1317, 1978.

18. Yui, Y., Fujita, T., Yamamoto, T., Itokawa, Y., Kawai, C.
Liquid-chromatographic determination of norepinephrine and epinephrine in human plasma.
Clin. Chem., 26, 194, 1980.

19. Hallman, H., Farnebo, L., Hamberger, B., Jonsson, G.
A sensitive method for the determination of plasma catecholamines using liquid chromatography with electrochemical detection.
Life Sci., 23, 1049, 1978.

20. Hjemdahl, P., Daleskog, M., Kahan, T.
Determination of plasma catecholamines by high performance liquid chromatography with electrochemical detection : comparison with a radioenzymatic method.
Life Sci., 25, 131, 1979.

21. Allenmark, S., Hedman, L.
Cation exchange liquid chromatography with amperometric detection as a method for the analysis of endogenous catecholamine concentrations in plasma or serum.
J. Liquid Chromatogr., 2, 277, 1979.

22. Davis, G., Kissinger, P.T.
Strategies for determination of serum or plasma norepinephrine by reversed-phase liquid chromatography.
Anal. Chem., 53, 156, 1981.

23. Coq, B., Gonnet, C., Rocca, J.L.
Techniques de remplissage de colonnes de chromatographie liquide à haute performance avec des microparticules poreuses.
J. Chromatogr., 106, 249, 1975.

24. Anton, A.H., Sayre, D.F.
A study of the factors affecting the aluminium oxide-trihydroxyindole procedure for the analysis of catecholamines.
J. Pharmacol. Exp. Ther., 138, 360, 1962.

25. Yui, Y., Kawai, C.
Comparison of the sensitivity of various post column methods for catecholamine analysis by high-performance liquid chromatography.
J. Chromatogr., 206, 586, 1981.

26. Magnusson, O., Nilsson, L.B., Westerlund, D.
Simultaneous determination of dopamine, dopac and homovanillic acid. Direct injection of supernatants from brain tissue homogenates in a liquid chromatography-electrochemical detection system.
J. Chromatogr., 221, 237, 1980.

27. Franco-Morselli, R., Elghozi, J.L., Joly, E., Digiuilio, P., Meyer, P.
Increased plasma adrenaline concentration in benign essential hypertension.
Br. J. Med., 2, 1251, 1977.

28. Kuchel, O., Thanh Buu, N., Unger, T., Lis, M., Genest, J.
Free and conjugated plasma and urinary dopamine in human hypertension.
J. Clin. Endocrin. Metab., 48, 425, 1979.

29. Pettersson, J., Hussi, E., Jänne, J.
Stability of human plasma catecholamines.
Scand. J. Clin. Lab. Invest., 40, 297, 1980.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1967-1972 (1982)

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF NEW SEMISYNTHETIC DAUNOMYCINONE DERIVATIVES

M. Beran, J. Jizba, V. Přikrylová, H. Lipavská, V. Schön and M. Podojil

Department of Biogenesis of Natural Substances
Institute of Microbiology
Czechoslovak Academy of Sciences
CS-142 20 Praha 4, Czechoslovakia

ABSTRACT

Reversed-phase HPLC utilizing LiChrosorb RP-8 was used to separate reaction mixtures of new semisynthetic daunomycinone derivatives and determine their relative occurrence.

Chromatographic behaviour of the following compounds was studied: daunomycinone (I), 7(S) and 7(R)-O-(2-hydroxyethyl)-13-ethyleneacetal daunomycinone (II and III), 13-ethyleneacetal daunomycinone (IV), 13-ethyleneacetal bisanhydrodaunomycinone (V), 7(S) and 7(R)-O-(3-hydroxypropyl)-13-propyleneacetal daunomycinone (VI and VII), 13-propyleneacetal daunomycinone (VIII), 13-propyleneacetal bisanhydrodaunomycinone (IX), 7(S) and 7(R)-O-(4-hydroxybutyl) daunomycinone (X and XI), 4-toluenesulfonylhydrazone daunomycinone (XII).

INTRODUCTION

Different stationary phases and different detection methods are used in the HPLC of known anthracyclines and anthracyclinones. The above compounds are separated on

silica gels (1, 2), normal bonded-phases (3, 4) and reversed-phases (5, 6, 7). The use of the HPLC method in the analysis of new anthracyclines and anthracyclinones requires modifications of the experimental conditions with respect to the optimum separation of mixtures of these compounds.

In the present paper HPLC was used for the separation of new semisynthetic derivatives of daunomycinone (Figure 1) and determination of relative representation of components of the reactions yielding these new derivatives. Solutes were identified by comparing their capacity ratios k' with those of standards.

MATERIAL AND METHODS

Chemicals. Daunomycinone (I) was obtained from Medexport (U.S.S.R), derivatives (II-XI) were prepared according to Jizba et al. (8), compound (XII) according to Smith et al. (9). UV-Grade solvents (methanol, acetone, acetonitrile and redistilled water) were used.

Apparatus. The high pressure liquid chromatograph SP-8000 (Spectra Physics Corp., Santa Clara, CA, U.S.A) equipped with a 250 x 4.6 mm column (LiChrosorb RP-8, 10 μm) and a detector SP-770 was used. Experimental conditions were as follows: flow 1.5 mL/min, pressure 9.1 - 11.2 MPa, temperature 25 ^{o}C, detection at 480 nm. Dead time for calculation of the capacity ratio k' was determined by injecting redistilled water.

Procedure. The following mixtures of reaction components (after a reaction time of 8 - 12 h), and, in parallel, corresponding mixtures of standards were subjected to the chromatographic analysis (see Table 1):

A. Daunomycinone (I), 7(S) and 7(R)-O-(2-hydroxyethyl)-13-ethyleneacetal daunomycinone (II and III), 13-ethyleneacetal daunomycinone (IV), 13-ethyleneacetal bisanhydrodaunomycinone (V).

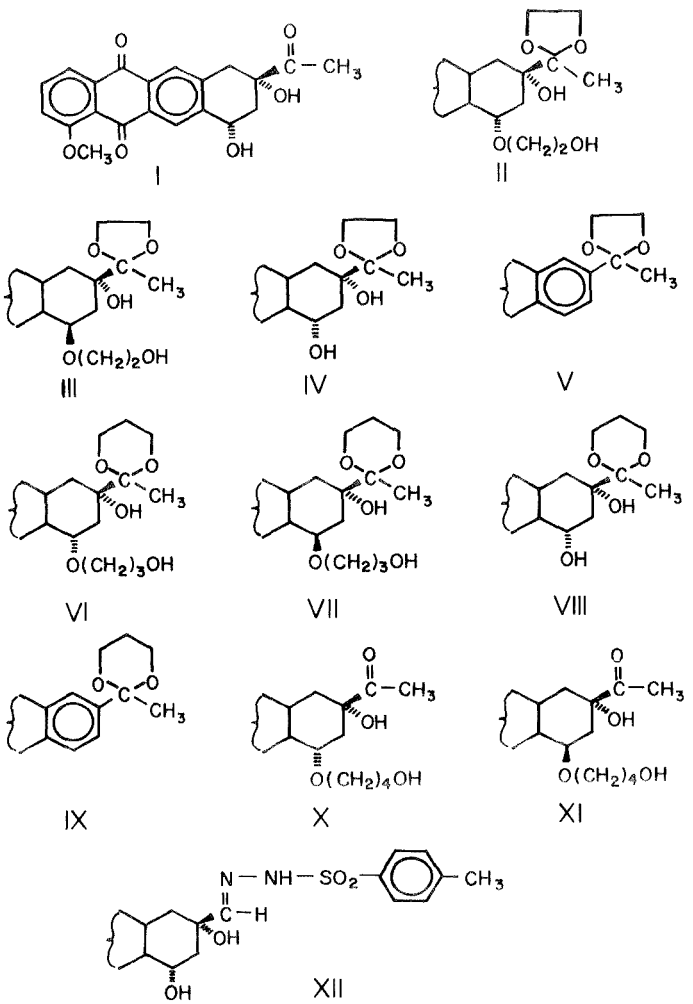


FIGURE 1

Chemical structures of daunomycinone derivatives

TABLE 1

Capacity ratios k´ of the solutes and their relative occurence in reaction mixtures

Compound	k´		%	Conditions
	A	B		
III	3.2	3.2	16	methanol-water (55:45) (20 min) linear gradient to acetone (30 min)
I	5.1	5.1	5	
IV	7.5	7.5	9	
II	10.8	11.0	60	
V	20.2	20.0	10	
	C	D		
VII	1.1	1.1	31	methanol-water (70:30) (10 min) linear gradient to acetone (30 min)
I	1.1	1.1		
VIII	1.9	2.0	48	
VI	3.1	3.2	18	
IX	16.5	16.7	3	
	E	F		
XI	1.5	1.5	7	acetonitrile-water (40:60) (12 min)
I	2.1	2.1	48	
X	4.0	4.1	45	
	G	H		
I	4.0	4.0	10	methanol-water (60:40) (15 min)
XII	5.5	5.5	90	

A, C, E, G - mixture of standards

B, D, F, H - reaction mixtures

C. Daunomycinone (I), 7(S) and 7(R)-O-(3-hydroxypropyl)-13-propyleneacetal daunomycinone (VI and VII), 13-propylene daunomycinone (VIII), 13-propyleneacetal bisanhydrodaunomycinone (IX).

E. Daunomycinone (I), 7(S) and 7(R)-O-(4-hydroxybutyl) daunomycinone (X and XI).

G. Daunomycinone (I), 4-toluenesulfonylhydrazone daunomycinone (XII).

Evaporates of reaction mixtures B and D and corresponding standard mixtures A and C were dissolved in acetone. Evaporates of the reaction mixtures F and H and corresponding standard mixtures E and G were dissolved in methanol. The samples (0.8 - 1.5 μg) were injected to a 10 μL loop. Concentrations of individual reaction components were determined according to calibration relationships and their relative occurrence in the reaction mixtures was calculated.

RESULTS AND DISCUSSION

Table 1 summarizes capacity ratios k' of solutes of individual reactions and corresponding standards. The relative representation of individual components after the reaction is also presented. Under the experimental conditions used the baseline separation of the solutes was reached. Only in the reaction mixture D it was not possible to separate daunomycinone (I), the starting compound of the reaction, from the reaction product (VII), even when using various modifications of the mobile phase.

The separation of configuration isomers of individual pairs (II and III, VI and VII, X and XI) was remarkably good. The different retention in individual pairs might be due to formation of the intramolecular hydrogen bridge between the hydroxyl group on carbon C-9 and hydroxyl group on 7(S)-O-(n-alkyl)-13-alkyleneacetal of

daunomycinone. The formation of the hydrogen bond results in a decreased polarity of the compound and, thus, in prolongation of the retention time of these compounds on the reversed-phase. This phenomenon is not observed in the case of 7(R)-O-(n-alkyl)-13-alkyleneacetal of daunomycinone. Compounds (V) and (IX) differing chemically from other solutes were also present in the cromatographic mixtures. An acetone gradient had to be used for their elution, and, hence, the time required for their analysis was extended by 30 min. Other compounds were eluted with a retention time of up to 20 min.

REFERENCES

1. Hulhoven, R., Desager, J.P., and Harvengt, C., Cancer Chemother. Pharmacol., 3, 133, 1979.
2. Ogasawara, T., Goto, S., Mori, S., and Oki, T., J. Antibiot., 34, 47, 1981.
3. Baurain, R., Deprez-De Campeneere, D., and Trouet, A., Cancer Chemother. Pharmacol., 2, 37, 1979.
4. Averbuch, S.D., Finkelstein, T.T., Fandrich, S.E., and Reich, S.D., Pharm. Sci., 70, 265, 1981.
5. Pierce, R.N., and Jatlow, P.I., J. Chromatogr., 164, 471, 1979.
6. Pandey, R.C., Toussaint, M.W., J. Chromatogr. 198, 407, 1980.
7. Matějů, J., Beran, M., Jizba, J., and Podojil, M., J. Liquid Chromatogr. 4, 977, 1981.
8. Jizba, J.V., Sedmera, P., Vokoun, J., Lipavská, H., Podojil, M., and Vaněk, Z., Collect. Czech. Chem. Commun., 1982 (in press).
9. Smith, T.K., Fujiwara, A.N., and Henry, D.W., J. Med. Chem., 21, 280, 1978.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1973-1990 (1982)

DETERMINATION OF TETRACYCLINE AND RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J.Y.C. Hon and L.R. Murray

Antibiotics Section, National Biological Standards Laboratory
P.O. Box 462, Canberra City, 2601, Australia
and
B. Walker, Waters Associates Pty. Ltd.,
82-96 Myrtle Street, Chippendale, 2008, Australia

ABSTRACT

An isocratic high-performance liquid chromatography method for the determination of tetracycline and its related compounds is described. The method uses a reverse phase (C_{18}) column, a modified acetonitrile/water mobile phase, and benzoic acid as the internal standard. Elution of all compounds of interest is complete within seven minutes. Results are presented for thirteen commercial capsule formulations and are compared with results by microbiological assay and thin-layer chromatographic methods.

INTRODUCTION

The most widely accepted method for the analysis of tetracycline (TC) and its formulations is the microbiological assay; it is the method of choice of both the British Pharmacopoeia 1980 (1) and the Code of Federal Regulations (2). The British Pharmacopoeia (BP) monograph specifies the large plate agar diffusion method, while that of the Code of Federal

Regulations (CFR) is the turbidimetric assay method. Non-chromatographic chemical methods generally encounter interference from one or more of the related compounds which may be present in TC. The more important of these compounds are 4-epi-tetracycline (ET), anhydrotetracycline (AT), 4-epianhydrotetra-cycline (EAT) and chlortetracycline (CT). (The latter may be found in TC which has been made by dehalogenation of CT.) Of these, EAT is the most significant, since it is nephrotoxic. The CFR specifies a spectrophotometric screening test for total anhydrotetracyclines in TC bulk substances and formulations; where the results exceed specified limits, determination of EAT by column chromatography is required. The BP determines all related compounds in bulk substances by thin-layer chromatography (TLC), but simply limits the concentration of the anhydrotetracyclines in capsules by a spectrophotometric test.

The microbiological assay and the chromatographic tests for related compounds are time consuming and their replacement by a single quantitative method for all compounds is desirable.

Tsuji and Robertson (3) introduced a gas-liquid chromatography method, but it necessitates a difficult derivatisation, and TC degradation may occur in the process.

In recent years a number of methods using high-performance liquid chromatography (HPLC) have been proposed; some of them employ ion exchangers, but the majority use reverse phase bonded materials. Complete resolution of TC and its related compounds has not been achieved in some of the proposed methods (4,5) while others do not present evidence for quantitative application to commercial formulations (6,7). While gradient elution systems are able to achieve good resolution in a short time, they may give rise to sloping baselines, e.g. the systems proposed by Mack and Ashworth (8). Tsuji and Robertson (9) developed a gradient elution system with a flat baseline, which they successfully applied to the analysis of pharma-ceutical preparations. A single step gradient system was employed by Muhammad and Bodnar (10) in a method for the related compounds in bulk substances and formulations.

The objective of this work was to develop an isocratic HPLC method suitable for the rapid determination of TC and its related compounds in bulk materials and in commercial capsule formulations.

MATERIALS

(a) Instrumentation

The instrument used was a Waters Associates liquid chromatograph, consisting of a 6000A pump, a U6K injector, a 440 dual channel detector, and an OmniScribe (Houston Instrument) dual pen recorder. A microBONDAPAK C_{18} (reverse phase) column, 3.9 mm X 30 cm (Waters Associates) was used for all separations.

(b) Solvents and Reagents

Acetonitrile (ACN) was supplied by Waters Associates; anhydrous methanol, diammonioum hydrogen phosphate and ortho-phosphoric acid were Analytical Reagent grade; ethanolamine and dimethylformamide (DMF) were Laboratory Reagent grade, and glass distilled water was freshly prepared before use. Benzoic acid (Merck, GR volumetric standard) was used as the internal standard.

The strong internal standard solution (SIS) was 0.5% W/v benzoic acid in 50% methanol, and the weak internal standard (WIS) was 0.05% benzoic acid in 50% methanol.

The mobile phase was prepared as follows:
In 760 mL water were dissolved 5.2 g diammonium hydrogen phosphate and 5.0 mL ethanolamine. To the solution were added 240 mL ACN and 60 mL DMF. Finally the pH was adjusted, using orthophosphoric acid, to 2.5. After filtration through a 0.5 μm pore size membrane filter (Millipore Corporation Type FH), the mobile phase was ultra-sonically degassed. The work involved in the development of this mobile phase is summarised below under METHODS.

(c) Tetracycline and Related Compounds Standards

In this work, the form of TC and its related compounds was the hydrochloride, unless otherwise specified.

The TC standard was a commercial sample of BP quality, supplied by Cyanamid Pty. Ltd. It was microbiologically standardised against the World Health Organisation's Second International Standard for Tetracycline (1970), giving a potency of 968 units/mg, "as is". An HPLC analysis by the method described here gave an ET content of 2.5%, an AT content of 0.2% and a negligible EAT content. The loss on drying (60°C, 5 mm pressure) was 0.7%. The microbiological and HPLC analyses were therefore consistent within experimental error, and the standard was assumed to be of 96.8% purity for this work. The ammonium salt of ET was prepared according to McCormick et al (11); AT was prepared by the method described by Schlecht and Frank (12), and EAT, by an adaptation of the latter method, from the ammonium salt of ET.

(d) Formulation Samples

All of the TC capsule formulations, for human use, on the Australian market, were sampled. There were thirteen different formulations (seven of which were buffered), representing nine manufacturers.

METHODS

(a) Mobile Phase Development

The evaluation and optimisation of the mobile phase were based on an analysis time-constrained concept. The retention time of the last eluting peak (AT) was adopted as a measure of analysis time. Optimisation of the system then became a matter of achieving the best resolution, within that time, for the least resolved pair, TC and ET.

The column suppliers recommend using ethanolamine in all mobile phases to reduce the adsorptive effect of active silanyl groups on the column; it was therefore routinely included at 0.5% v/v.

Preliminary work showed that acidified aqueous ACN was the most promising of the range tested, but that the system would

require modification in order to produce acceptable chromatograms. The column suppliers recommend working above pH 2 in order to avoid hydrolytic loss of the C_{18} phase. The pH range 2.5 to 3.3 was investigated by comparing the chromatograms given by different ACT/water mixtures acidified to pH 2.5, 2.9 and 3.3 with ortho-phosphoric acid. A system consisting of 24 ACN/76 water at pH 2.5 gave the best ET-TC peak separation; it was also effective in reducing the tailing of the AT peak which was observed at higher pH. Below pH 2.5, the ET-TC peak separation was further improved, but as continuous work at such acidities may have shortened the effective column life, it was decided to standardise all phases to pH 2.5.

Investigation of a number of solvents and modifiers showed that DMF and ammonium phosphate both markedly improved the chromatogram. The latter, when present at 1% w/v in the mobile phase (19ACN, 81 water, 6DMF) reduced the analysis time from 23 minutes (for mobile phase without the salt) to 9 minutes. However, the ET-TC peak separation was significantly better in the salt-free system, and it was necessary to compromise at an intermediate concentration. The salt, at 0.52 g diammonium phosphate per 100 mL ACN/water mixture, became a standard addition to all experimental phases.

It was found that additional marked improvement in the chromatogram could be affected by the addition of 5-10% DMF. This modifier was used by Knox and Jurand (13), who developed mobile phases based on simple mixtures of it with water. However, in the conditions used in this work, ACN could not be completely replaced by DMF to give satisfactory chromatograms. In particular, with DMF/water mixtures, AT and EAT were poorly resolved. When used at 5-10%, DMF brought about only small reductions in retention times, but had a striking effect in reducing tailing and in sharpening all peaks.

At this point, after evaluation of a number of chromatograms, the desired analysis time was fixed at seven minutes. Within that constraint, optimisation of the system with respect to ET-TC separation was undertaken by varying the ACN/water ratio and the DMF concentration.

Three different concentrations of DMF (6, 8 and 10 volumes per 100 volumes ACN/water mixture) were examined. At each DMF concentration, the ACN/water ratio was varied and the ET-TC peak separations were measured according to the method proposed by Morgan and Deming (14). The results are shown graphically in Figs. 1, 2 and 3. It is evident that the analysis times increase,

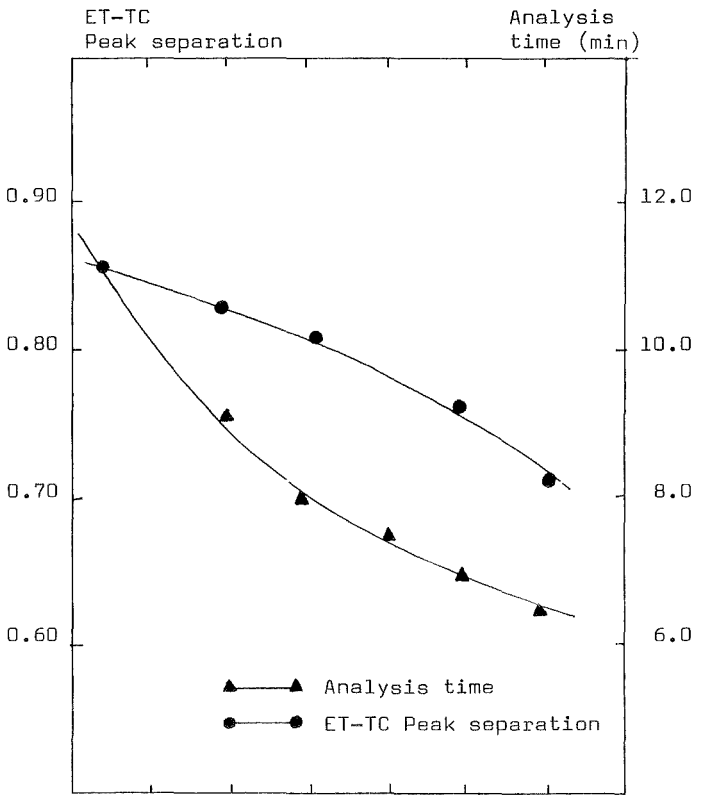


Figure 1

The dependence of ET-TC peak separation and analysis time on the ACN to water ratio of the mobile phase; DMF concentration constant at 6 volumes per 100 volumes ACN/water mixture.

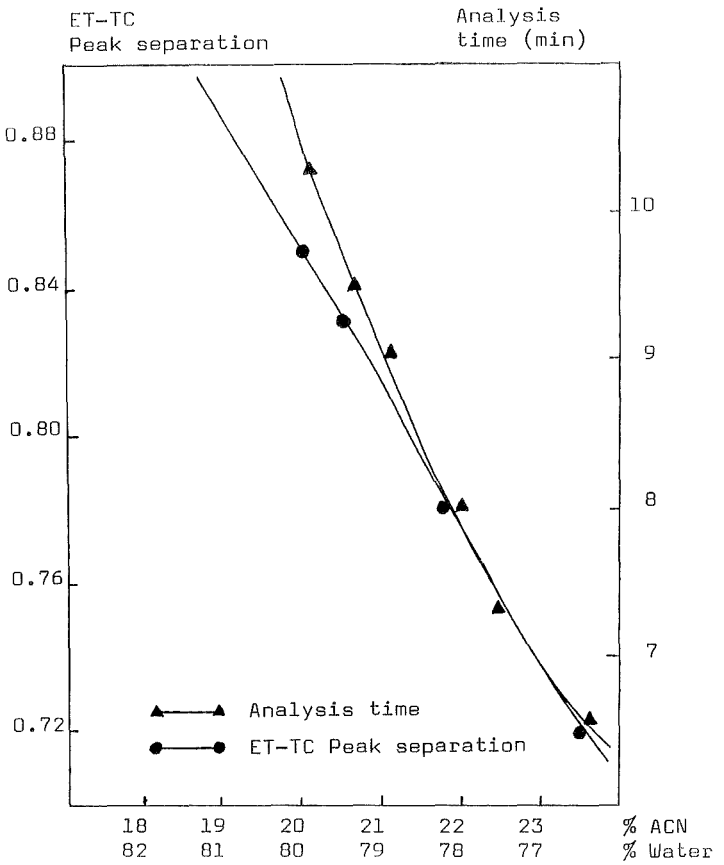


Figure 2

The dependence of ET-TC peak separation and analysis time on the ACN to water ratio of the mobile phase; DMF concentration constant at 8 volumes per 100 volumes ACN/water mixture.

and the ET-TC separations improve, with increasing water content. The relationship between analysis time and peak separation for the three DMF concentrations is shown in Fig. 4. For the desired analysis time of seven minutes, a mobile phase containing 6 volumes DMF per 100 volumes ACN/water mixture provides better peak separa-

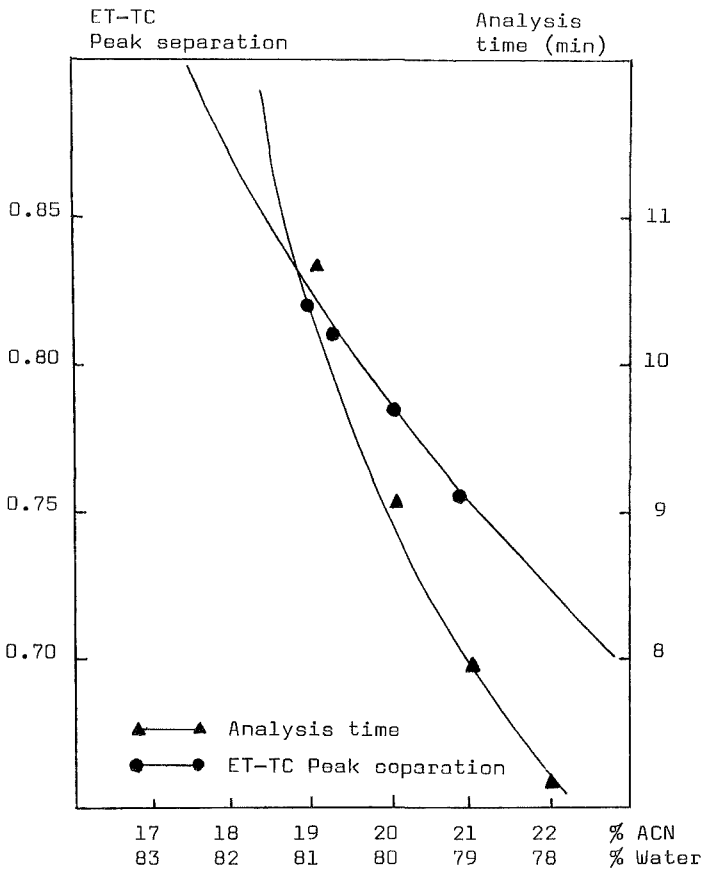


Figure 3.

The dependence of ET-TC peak separation and analysis time on the ACN to water ratio of the mobile phase; DMF concentration constant at 10 volumes per 100 volumes ACN/water mixture.

tion than the higher DMF concentrations. Reference to Fig. 1 indicates that the ACN to water ratio should be approximately 24 to 76 to achieve the required analysis time.

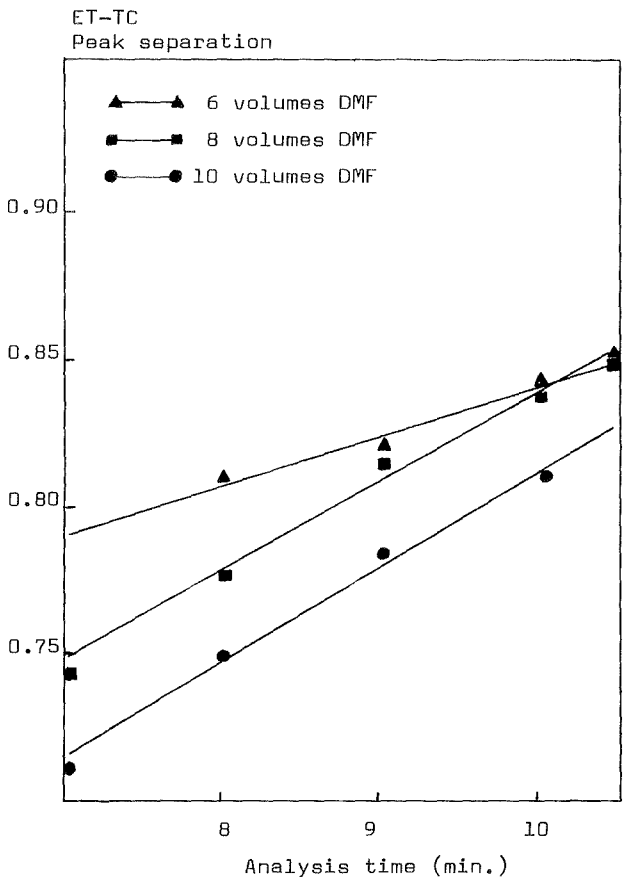


Figure 4.

The relationship between ET-TC peak separation, DMF concentration in the mobile phase and the analysis time.

(b) Analysis of Capsule Samples by HPLC

A weighed quantity of the mixed contents of 20 capsules equivalent to about 250 mg TC was shaken with 60 mL anhydrous methanol for 20 minutes. For capsules containing TC base,

sufficient 1M hydrochloric acid should be added to the suspension to convert the base to hydrochloride, as solutions of the base are relatively unstable. Some samples required brief untrasonication in order to complete the solution of the tetracycline. After making the volume to 100 mL, the solution was filtered to remove insoluble excipients (Filtrate A).

For the determination of tetracycline, filtrate A was diluted 1 to 5 with anhydrous methanol, and the diluted solution mixed 1 to 1 with SIS just before injection.

For the determination of related compounds, filtrate A was mixed 1 to 1 with WIS just before injection.

The standard solution contained in 100 mL anhydrous methanol: 50 mg TC, 20 mg ET (ammonium salt), 10 mg CT, 2.5 mg AT and 2.5 mg EAT. For the determination of tetracycline, the standard solution was mixed 1 to 1 with SIS, and for the determination of related compounds, it was mixed 1 to 1 with WIS. As with the sample solutions, the mixing with the internal standards was carried out just prior to injection, since there was a slow increase in the ET content (about 10% in 2 hours) of the mixed solutions. Three successive injections of each mixed solution were made (standard and sample), in order to determine the mean ratio of TC (or related compound) to internal standard, before discarding the solution. The unmixed standard solution in methanol was stable for at least six hours and therefore one preparation was used for a number of samples.

(c) Analysis of Bulk Substances by HPLC

Solutions of TC bulk substances for HPLC were prepared to contain 500 mg TC in 100 mL anhydrous methanol. In the case of TC base, sufficient 1M hydrochloric acid was added to the solution to effect conversion to the hydrochloride. For determination of TC, the solution was diluted 1 to 10 with methanol before mixing 1 to 1 with SIS. For determination of related compounds, the solution was mixed 1 to 1 with WIS. The standard solution and its dilutions were those specified above for the analysis of capsules.

(d) Chromatographic Conditions

The chromatographic conditions were as follows:

Temperature	:	Ambient (20^{o} - 22^{o}C)
Flow rate	:	2.5 mL/min
Chart speed	:	1 cm/min
Detector	:	280 nm 0.5 AUFS for tetracycline 0.1 AUFS for related compounds
Injection volume	:	10 µL

Quantitation was effected via peak heights, these being measured directly from the recorder chart graduations.

(e) Microbiological Assays of Capsules

Microbiological assays for TC were carried out on the capsules using the large plate agar diffusion system, in which the standard and unknown preparations were contrasted at two corresponding dose levels (3.0 and 0.75 units/mL).

The TC standard and the capsule contents were dissolved in 0.01 M hydrochloric acid, and diluted to the assay working doses with phosphate buffer pH 5.8.

A seed layer of Medium C, pH 6.6 (BP A 67) was inoculated with a spore suspension of Bacillus cereus ATCC 11778 and the antibiotic solutions applied to the surface of the medium by the use of Schleicher and Schuell 740-E, 12.7 mm diameter paper discs.

The statistical validity of the test results, the potency of the unknown and the fiducial limits of error of the potency estimate were calculated from the 64 responses (zones of inhibition) obtained from each plate assay.

(f) TLC Examination of Capsules

The capsule samples were examined for the presence of related compounds by TLC on kieselguhr, using the method of Murray (15). The sample solutions examined were those prepared for the HPLC

analyses (Filtrate A), and the standard solutions contained 2.5 mg AT, 2.5 mg EAT, 10 mg CT and 20 mg ET (ammonium salt), each separately dissolved in 100 mL anhydrous methanol. These standard solutions correspond to proposed limits of 1% for AT and EAT, 4% for CT and 8% for ET. The intensities of the spots from the samples were compared with those from the standards, and were arbitrarily rated as follows:

0 - no spot detected
1 - faint compared with standard
2 - moderate compared with standard
3 - intense, but less than standard
4 - more intense than standard

(g) Precision and Statistical Evaluation

In order to establish the precision of the HPLC assay method for TC, ten determinations were carried out on Sample 1 over a period of three days.

A statistical comparison of the two assay methods was made, using the paired t-test and by simple regression.

RESULTS & DISCUSSION

A chromatogram of a mixture of TC, all the related compounds, and the internal standard is shown in Fig. 5.

Calibration studies showed that the detector responses were linear for all five compounds of interest, up to the following concentrations, in mg/mL:

TC 0.65, ET 0.60, AT 0.038, EAT 0.038, CT 0.15

These concentrations represent 130% of the expected (label strength) for TC in capsules, 300% of the proposed upper limit for ET, and 150% of the proposed upper limits for AT, EAT and CT in

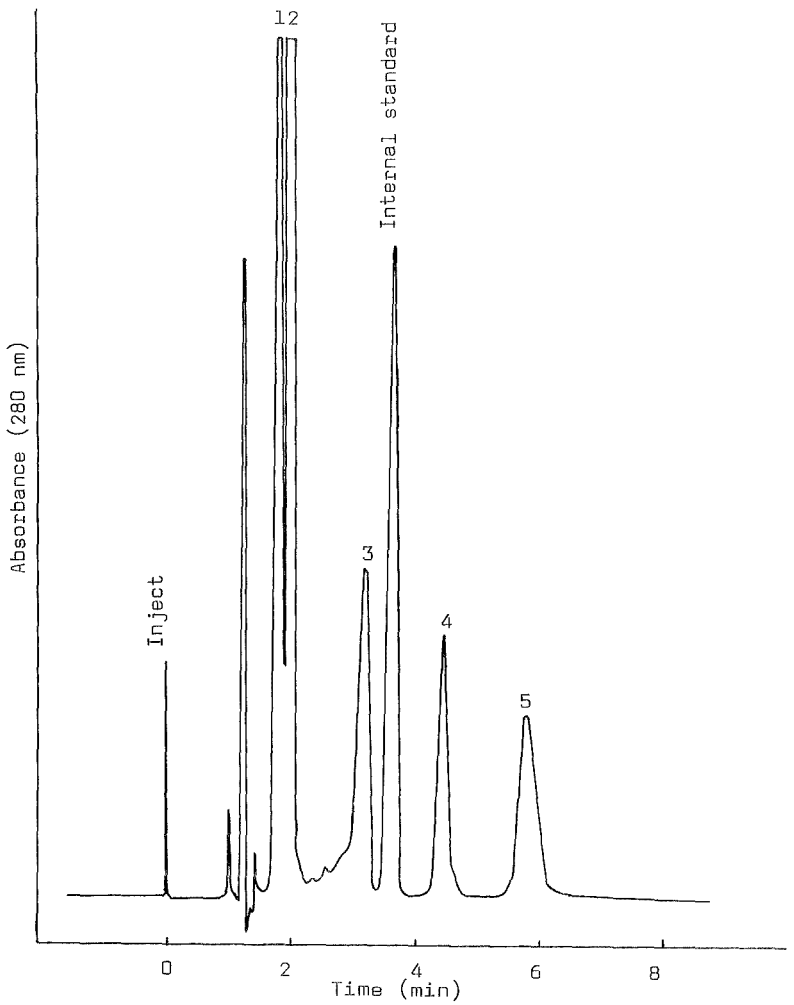


Figure 5

HPLC separation of tetracycline (peak 2) and its related compounds (4-epitetracycline, peak 1; chlortetracycline, peak 3; 4-epianhydrotetracycline, peak 4; anhydrotetracycline, peak 5).

Chromatographic conditions are described in the text

capsules. The linearity of response indicates that the method is valid for the determination of all compounds in a formulation, providing there is no interference from excipients. Confirmatory evidence for the validity of the method as applied to capsules is afforded by the results of the microbiological assay and the TLC examinations. These are presented, together with the HPLC results, in Table 1.

The chromatograms of samples 11 and 12 included peaks which were initially interpreted as being from CT, and the peak heights indicated contents of 1.00% and 1.50% respectively in the samples. However, no spots for CT were detectable in the TLC, and a closer examination of the HPLC chromatogram showed that the retention time of the compound in question was 0.1 minute longer than that for CT. The identity of the compound was not established.

The results of the ten HPLC assays on capsule Sample 1 ranged from 97.3% to 98.8% label strength, with a mean of 98.18% and a coefficient of variation of 0.48%.

In the statistical comparison of the HPLC and the microbiological assay data, the paired t-test gave a value for t of 0.508. A linear regression line, constrained to pass through the origin, when fitted to the data, resulted in the equation $Y = 1.0033\ x$, where x is the potency estimated by the microbiological assay and y is the HPLC assay. These results suggest that there is no significant difference in the two methods.

Comparison of the TLC and HPLC results for related compounds indicates excellent agreement in the visual appraisal of TLC spots and the quantitation of peak heights in HPLC. The latter procedure is clearly superior with respect to speed and precision, but TLC may be valuable in certain circumstances for identification purposes, since observations may be made on both R_f value and the colour of the fluorescent spot; in HPLC only the retention time is useable for identification. It is suggested that HPLC is the method of choice for determining

TABLE 1

HPLC Analyses, Microbiological Assays and TLC Examination of 13 TC Capsule Samples

Sample	H.P.L.C. Analysis, percent					Microbiological Assay Percent	T.L.C. Examination Spot Intensity Relative to Standard			
	TC[b]	AT[c]	EAT[c]	CT[c]	ET[c]		AT	EAT	CT	ET
1	98.6	0.40	0.05	ND	4.52	96	2	1	0	3
2	95.8	0.54	0.15	ND	6.90	96	2	1	0	3
3[a]	106.1	0.33	0.06	ND	6.29	103	2	1	0	3
4	102.8	0.46	0.03	ND	4.97	101	2	1	0	3
5	105.3	0.40	0.07	ND	3.62	102	2	1	0	3
6[a]	102.7	0.45	0.11	ND	6.11	101	1	1	0	3
7[a]	101.8	0.32	0.06	ND	5.42	102	2	1	0	3
8[a]	96.2	0.38	0.06	ND	5.34	98	2	1	0	3
9[a]	100.2	0.70	0.23	ND	4.55	101	3	1	0	3
10	95.2	0.98	0.25	ND	6.00	96	3	1	0	3
11[a]	96.2	1.03	0.23	ND	8.42	96	3	1	0	4
12[a]	88.8	1.52	0.34	ND	9.46	92	4	2	0	4
13	98.9	2.25	1.42	0.53	4.63	101	4	3	2	2

a Buffered Sample
b Mean of 3 determinations, expressed as % of label
c Expressed as % of assayed TC
d For ratings, see text
ND Not detected

related compounds, and that the TLC examination should be used for confirmation of identity where doubt exists.

The proposed method determines TC and the related compounds by means of two chromatograms obtained at different detector sensitivities on one channel at 280 nm. Where a dual channel detector is used, it is possible to simultaneously determine TC and the related compounds, by employing different sensitivities for the channels, both set to 280 nm. Under such conditions it would be valid to use the internal standard peak heights on the first channel (TC) for the quantitation of the related compounds on the second channel. For the quantitation of the related compounds it is desirable for the sample and standard solutions to be of similar composition. Thus an appropriate standard solution for the simultaneous determination of TC and related compounds in a bulk substance would contain 50 mg TC, 0.25 mg AT, 0.25 mg EAT, 1.00 mg CT and 2.00 mg ET (ammonium salt) in 100 mL anhydrous methanol. These quantities of the related compounds are only 10% of those used in the standard solution recommended for the two chromatogram system. The TC standard would contribute significant amounts of the related compounds to the standard solution; these amounts should therefore be determined with suitable precision so that accurate determination of the related compounds is assured. Where samples are subject only to a limit test for the related compounds, the single chromatogram system could be used for rapid screening purposes.

With regard to the reverse phase column life, there was no detectable drop in plate count in the column used for much of this work, after more than 400 injections. The column was dedicated to tetracycline work, and was well washed with water and then 70% methanol after each day's use. These factors, together with the restriction of the mobile phase to pH 2.5, may be responsible for the excellent stability of the column.

It is planned to investigate the application of this method to other TC formulations (ointments, oral suspensions, injections), to tetracycline - nystatin formulations and to other tetracycline antibiotics and their formulations.

ACKNOWLEDGEMENTS

The authors thank Mrs. J. Andres for carrying out the microbiological assays, and Mr. R. Barge for the statistical evaluations.

REFERENCES

1. British Pharmacopoeia 1980, Her Majesty's Stationery Office, London, 1980.

2. Code of Federal Regulations. Title 21, April, 1981, Office of the Federal Register, Washington, 1981.

3. Tsuji, K. and Robertson, J.H , Formation of trimethylsilyl derivatives of tetracyclines for separation and quantitation by gas liquid chromatography. Anal. Chem., 45, 2136, 1973.

4. Tsuji, K., Robertson, J.H. and Beyer, W.F., High pressure liquid chromatographic determination of tetracyclines. Anal. Chem., 46, 539, 1974.

5. Lindauer, R.F., Cohen, D.M and Munnelly, K.P., Determination of anhydrotetracyclines in tetracycline by high-pressure liquid chromatography. Anal. Chem., 48, 1731, 1976.

6. Butterfield, A.G., Hughes, D.W., Pound, N.J. and Wilson, W.L., Separation and detection of tetracyclines by high-speed liquid chromatography. Antimicrob. Agents Chemother., 4, 11, 1973.

7. Gstrein, K. and Nachtmann, F., Bestimmung von Abbauprodukten in Tetracyclin-Präparaten mittels HPLC. Acta. Pharm. Technol., 26, 247, 1980.

8. Mack, G.D. and Ashworth, R.B., A high performance liquid chromatographic system for the analysis of tetracycline drug standards, analogs, degradation products and other impurities. J. Chromatogr. Sci., 16, 93, 1978.

9. Tsuji, K. and Robertson, J.H., Analysis of tetracycline in pharmaceutical preparations by improved high performance liquid chromatographic method. J. Pharm. Sci., 65, 400, 1976.

10. Muhammad, N. and Bodnar, J.A., Separation and quantitation of chlortetracycline, 4-epitetracycline, 4-epianhydrotetracycline, and anhydrotetracycline in tetracycline by high-performance liquid chromatography. J. Pharm. Sci., 69, 928, 1980.

11. McCormick, J.R.D., Fox, S.M., Smith, L.L., Bitler, B.A., Reichenthal, J., Origoni, V.E., Muller, W.H., Winterbottom, R. and Doerschuk, A.P., Studies of the reversible epimerization occuring in the tetracycline family. The preparation, properties and proof of structure of some 4-epi-tetracyclines. J. Am. Chem. Soc., 79, 2849, 1957.

12. Schlecht, K.D. and Frank, C.W., Dehydration of tetracycline. J. Pharm. Sci., 64, 352, 1975.

13. Knox, J.H. and Jurand, J., Mechanism of reversed-phase separation of tetracyclines by high-performance liquid chromatography. J. Chromatogr., 186, 763, 1979.

14. Morgan, S.L. and Deming, S.N., Optimization strategies for the development of gas-liquid chromatographic methods. J. Chromatogr., 112, 267, 1975.

15. Murray, L.R., Thin layer chromatography of the tetracyclines. Aust. J. Pharm. Sci., 10, 82, 1981

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 1991-2003 (1982)

SIMULTANEOUS DETERMINATION OF FLUNIXIN, PHENYLBUTAZONE, OXYPHENBUTAZONE AND γ-HYDROXYPHENYLBUTAZONE IN EQUINE PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: WITH APPLICATION TO PHARMACOKINETICS

Gregory E. Hardee and Jin-Wang Lai
Department of Pharmaceutics
School of Pharmacy

and

James N. Moore
Department of Large Animal Medicine
College of Veterinary Medicine

University of Georgia
Athens, GA 30602

ABSTRACT

A high performance liquid chromatographic method was developed for the simultaneous determination of flunixin, phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone in equine plasma. Samples of plasma or sera were deproteinated by addition of acetonitrile containing the internal standard naproxen. The concentration step consisted of taking an aliquot of deproteinated plasma, evaporating under nitrogen to dryness and redissolving in mobile phase. The extracts were chromatographed on a Spherisorb 5 μm ODS column using an isocratic mobile phase of methanol (30% v/v), acetonitrile (20% v/v) and pH 3.0 1% acetate buffer (50% v/v) at a flow rate of 1.2 ml/min using naproxen as the internal standard. The detection limit for flunixin, phenylbutazone, oxyphenbutazone and γ-hydroxyphenylbutazone was 50 ng/ml.

The developed chromatographic method was applied to the determination of equine nonsteroidal anti-inflammatory treatment. Plasma samples from clinically treated horses administered flunixin and phenylbutazone simultaneously are reported. Effect of different anticoagulants used in sampling is reported.

1991

INTRODUCTION

Flunixin Meglumine (3-Pyridine carboxylic acid, 2 [[2-methyl-3 trifluromethyl] phenyl] amine) is an analgesic agent with anti-inflammatory and antiprostaglandin synthetase activities which has been approved for use in horses. Recently, flunixin has been used in the management of endotoxin-induced cardiovascular derangements associated with equine intestinal ischemia (1-3). Although physical improvement is seen immediately after and for the duration of flunixin therapy, little is done to correct the underlying cause of the condition. Consequently, the clinical signs upon which the veterinarian must rely to make the diagnosis are masked and referal of patients requiring emergency surgery is delayed. More recently, we have noticed the simultaneous use of phenylbutazone and flunixin further exacerbating the problems. A rapid, reliable method for determining extent of prior-to-clinic treatment with nonsteroidal anti-inflammatory agents may help in further cases or therapeutic management.

Recently, reports have been published on the simultaneous analysis of some anti-inflammatory agents in human serum and plasma (4, 5). These papers present a general method for the analysis of many agents but point out the necessity of developing a separation scheme which addresses the particular need of each assay. The concurrent use of phenylbutazone and flunixin appears to be increasing in veterinary practice and to determine pretreatment and proper therapy, an assay which determines therapeutic and subtherapeutic concentrations of phenylbutazone, oxyphenbutazone, and flunixin in icteric horse plasma is required. In this paper we describe the extraction, concentration and chromatographic analysis of these drugs. Results obtained in clinical cases of intestinal ischemia treated with nonsteroidial anti-inflammatories are also presented.

MATERIALS AND METHODS

Reagents

Acetonitrile and methanol were HPLC grade (Baker Chemical Co., Phillipsburg, N. J.). Oxyphenbutazone, γ-hydroxyphenylbutazone (γ-OH) and phenylbutazone reference standards were gifts from Ciba-Geigy (Ciba-Geigy Corp., Summit N. J.). Flunixin Meglumine reference standard was a gift from Schering Corporation (Schering Corp., Bloomfield, N. J.). Naproxen was a gift from Syntex Corporation (Syntex Corp., Palo Alto, CA.).

Instrumentation and Quantitation

An Altex model 110A HPLC pump with a 254 nm fixed wavelength absorbance detector (Altex model 153) was used with a strip chart recorder. Signals were read as peak heights above the base line. A pellicular ODS 30 μm, 5 cm guard column coupled with an analytical column of Spherisorb 5 μm, ODS I 25 cm (Universal Scientific Inc., Atlanta, GA) was eluted with a degassed mobile phase of 30% v/v acetonitrile, 20% v/v methanol and pH 3.0 acetate (1%) buffer at a flow rate of 1.2 ml/min.

Peak heights were measured by a recorder-integrator (Hewlett-Packard 3390A, Hewlett-Packard, Atlanta, GA.). Peaks were identified by comparison of retention times to samples spiked with authentic standards. Under the conditions described the elution times for γ-OH, oxyphenbutazone, naproxen, flunixin and phenylbutazone were 3.5, 5.8, 7.7, 9.0 and 14.2 min. respectively. Quantitation of each compound was achieved using the peak ratio method with respect to the internal standard, naproxen.

Sampling and Extraction Procedure

Blood was drawn into heparinized syringes and centrifuged at 1000 xg at 4C for 10 minutes. Plasma was pooled from healthy horse samples for preparation of the standard curves. Clinical samples were collected in the same way except that selected samples were

collected with sodium fluoride (NaF), citrate-phosphate-dextrose (CPD), ethylenediaminetetraacetic acid (Na_2EDTA) or no anti-coagulants. Plasma or serum samples were stored at -10C as 2.5 ml aliquots until use. All samples were thawed and two 1 ml aliquots taken for extraction.

To 1 ml of plasma (or serum) 4 ml of acetonitrile containing naproxen (250 ng/ml) was added to precipitate the plasma proteins (6). Samples were vortexed for 30 seconds and then centrifuged for 15 minutes at 1000 x g. From the supernatant a 4 ml aliquot was taken and evaporated to dryness under a stream of nitrogen at 37C. The residue was redissolved in 500 µl of the HPLC mobile phase and a portion was withdrawn to rinse and load the 50 µl loop for injection.

One normal, thoroughbred mare (454 kg) was fitted with a 12 gauge cannula inserted into the right jugular vein. Phenylbutazone (4.4 mg/kg) and flunixin (1.1 mg/kg) were mixed in a syringe and administered. Blood samples (10 ml) were withdrawn at 0 min. and 5, 10, 15, 30, 45, 60, min., 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, 8, 12, 16, 20 and 24 hr post administration. Blood samples were immediately transferred to the heparinized tubes, refrigerated, centrifuged, separated and frozen. Other samples were drawn from clinical cases at the time of admission.

RESULTS

Sensitivity, Linearity, Reproducibility and Recovery

Standard solutions containing 1 µg/ml, 10 µg/ml and 100 µg/ml of flunixin, phenylbutazone, γ-OH and oxyphenbutazone each in acetonitrile/methanol 50% were prepared. These three solutions were used to spike known amounts of drug into empty test tubes. To tubes containing .05, .10, .20, .25, .40, .50, 1.0, 2.0, 2.5, 5.0, 10.0 and 25.0 µg of each drug 1 ml of pooled normal plasma or 1 ml of mobile phase was added. Figure 1 shows the chromatograms obtained from injections of: 1) spiked mobile phase, 2) spiked

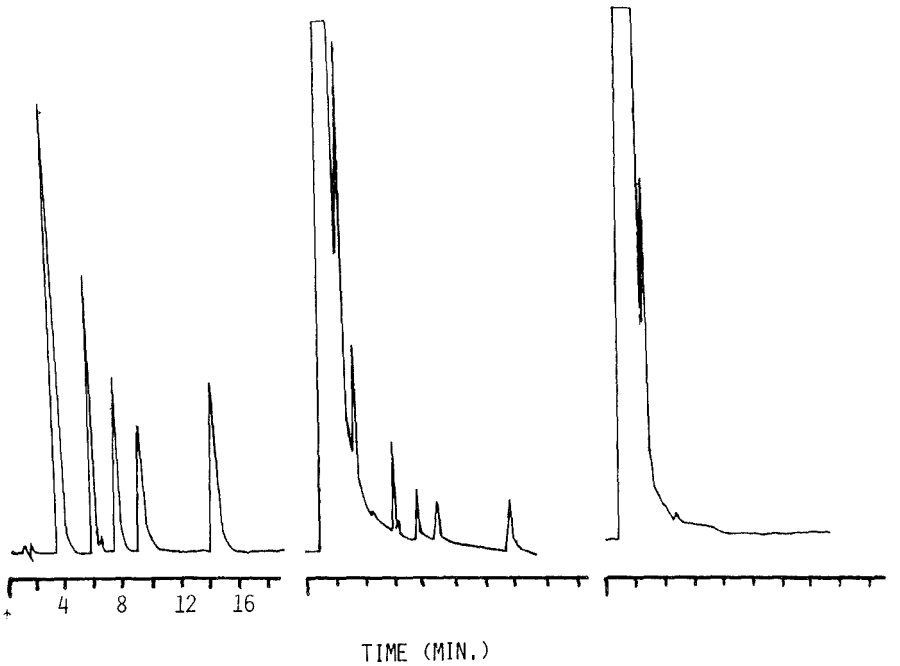


Figure 1. Chromatograph of: a) mobile phase spiked with 10 µg/ml each of γ-hydroxyphenylbutazone, oxyphenbutazone, naproxen, flunixin and phenylbutazone (.08 AUFS), b) extracted normal plasma spiked with 1 µg/ml of each drug (.02 AUFS), c) extracted normal plasma blank (.02 AUFS).

plasma carried through the extraction procedure, and 3) blank plasma carried through the extraction procedure. The peak eluting at 6.4 minutes was identified as the lactone form of γ-hydroxyphenylbutazone which exists in equilibrium with the γ-OH form. This lactone form, which accounts for approximately 4% of the total γ-OH present, appears in both spiked and experimental samples but was not quantitated in this study. The chromatograph of the blank plasma shows the absence of interferring peaks for γ-OH, naproxen, flunixin and phenylbutazone but shows a component of horse plasma which elutes before oxyphenbutazone. This peak fused with the

oxyphenbutazone peak at lower concentrations but the peak of interest was still able to be quantitated.

It was necessary to calculate recoveries by comparing spiked plasma to spiked mobile phase because: 1) recoveries from distilled-deionized water or 0.1 M phosphate buffer (pH 7.4) gave lower absolute and relative (to naproxen) recoveries than that obtained from plasma, 2) injection of the extract containing 4 volumes acetonitrile gave excessive broadening of the peaks and subsequent decreases in peak heights and 3) solubilization of the drugs by pure methanol, water or acetonitrile was incomplete. Absolute recoveries calculated by comparing spiked plasma to spiked mobile phase yielded: 99±2% for oxyphenbutazone, 96±3% for naproxen, 97±4% for flunixin, 96±3% for phenylbutazone and 98±2% for the γ-OH. Relative to the internal standard, naproxen, the recoveries were: 100±2% for oxyphenbutazone, 99±2% for flunixin, 97±2% for phenylbutazone and 100±2% for γ-OH.

Figure 2 shows the standard curves prepared from spiked plasma samples. Each point represents the average of three to six separation determinations. Table 1 lists the linear regression parameters for this peak height versus added drug concentration data.

The overall precision and accuracy of the method for each compound are represented in Table 2. Presented are the mean concentrations calculated, standard deviations and number of samples. The precision and accuracy were tested in three ways: 1) six different plasma samples were spiked with 5 µg of each compound followd by extraction and analysis the same day, 2) eight different aliquots of an actual sample from a dosed horse were analyzed the same day and 3) a spiked sample (5 µg each compound) was analyzed once a week for 5 weeks.

For this extraction, concentration and chromatographic procedure the minimum detectable limit, twice the signal to noise ratio, was 50 ng/ml for each compound.

The amounts of extraneous components carried through the extraction procedure are very important when concentrating the sample

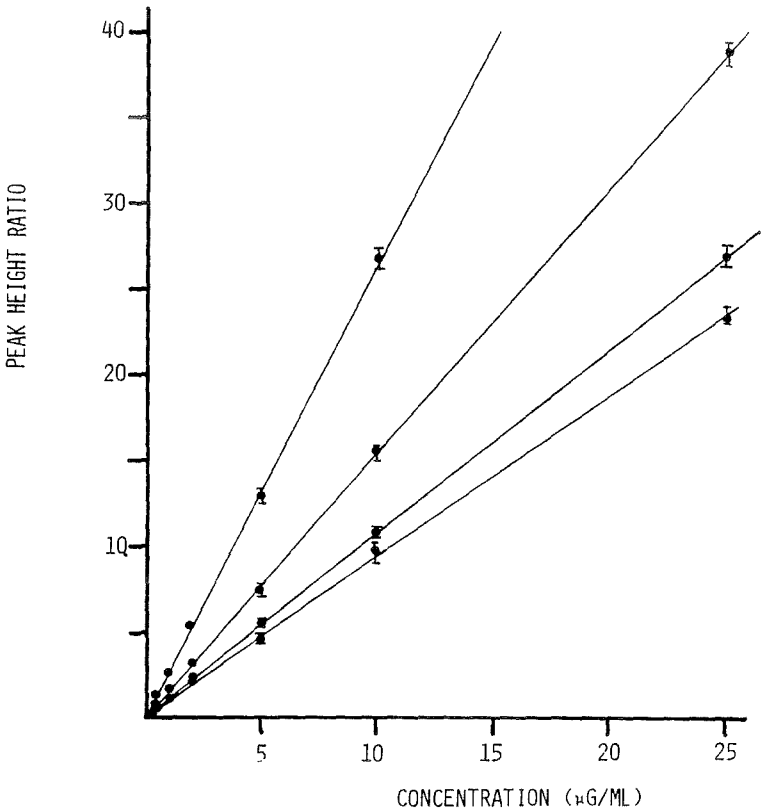


Figure 2. Calibration curves constructed for each compound in plasma. Lower concentrations not shown but included in regression functions included in Table 1.

TABLE 1

Linear Regression Parameters for the Drug Calibration Curves Presented in Figure 2.

Compound	Slope	Intercept	Correlation Coefficient
γ-OH	2.631	-.041	.9995
Oxyphenbutazone	1.541	.024	.9994
Flunixin	1.162	.035	.9992
Phenylbutazone	.9359	-.030	.9997

TABLE 2

Precision and Accuracy of Method for Different Samples

Sample		γ-OH	Oxyphen-butazone	Fluni-xin	Phenyl-butazone
1) Spiked Plasma (5 μg)	mean concentration	4.94	4.96	4.84	4.85
	std. dev.	.13	.10	.26	.16
	n	6	6	6	6
2) Plasma Sample (60 minute)	mean concentration	5.25	.42	5.31	12.23
	std. dev.	.10	.01	.14	.57
	n	8	8	8	8
3) Spiked Plasma (5 μg repeated)	mean concentration	4.99	4.84	4.81	4.90
	std. dev.	.11	.23	.17	.13
	n	10	10	10	10

to obtain maximum sensitivity. In the dosed horse, 30 and 60 minutes post administration, samples were collected in different anticoagulants. Standard blood collection tubes containing: 1) no anticoagulants, 2) NaHeparin, 3) Na_2EDTA, 4) NaF and 5) CPD were used for collection of samples. All samples were separated and stored in the same way except the samples in the tubes containing no anticoagulants were allowed to clot at room temperature before separation and storage.

All anticoagulants produced the same absolute and relative extraction ratios. However, the sample which was allowed to clot produced higher relative extractions due to lower absolute amounts of naproxen recovered. Each of the samples gave acceptable, peak free, blanks for the analysis of naproxen, flunixin and phenylbutazone except CPD which produced excessive solvent front tailing. Samples taken in the EDTA tubes proved to be the cleanest and this became especially important when measuring low levels of γ-OH and

oxyphenbutazone where heparin samples gave peaks which fused with the compounds of interest.

The volume of acetonitrile used for deproteinization was found to be critical; volume ratios of less than 4:1 gave much dirtier extractions which made quantitation of lower concentrations difficult. Also, methanol and acetone were used to precipitate the proteins but failed to give samples as clean as the acetonitrile treatment.

Blood Levels and Pharmacokinetic Data

The log plasma concentration versus time course of flunixin and phenylbutazone obtained in a single horse after simultaneous IV administration of flunixin (1.1 mg/kg) and phenylbutazone (4.4 mg/kg) is shown in Figure 3. These doses are recommended by the manufacturer and commonly used in clinical practice. The plasma concentration versus time curves for γ-OH and oxyphenbutazone are shown in Figure 4.

Clearly, this data suggests that a two compartment model best fits the data derived from this horse for both flunixin and phenylbutazone. While the distribution component of the phenylbutazone disposition is shallow, frequent sampling at the early times allows for its delineation. Flunixin shows a more pronounced distribution phase which requires six to eight hours for equilibration. Applying two compartment model fitting to this data the pharmacokinetic parameters, presented in Table 3, are calculated. Data for the first 15 minutes are omitted from these calculations as this appears to be the time required for complete mixing of the drug and blood after I. V. administration (see Figure 3).

DISCUSSION

The procedures described allow for the rapid and sensitive assay of flunixin, phenylbutazone, oxyphenbutazone, γ-hydroxyphenylbutazone and naproxen in plasma samples. In developing the assay it became apparent that not only the choice of mobile phase

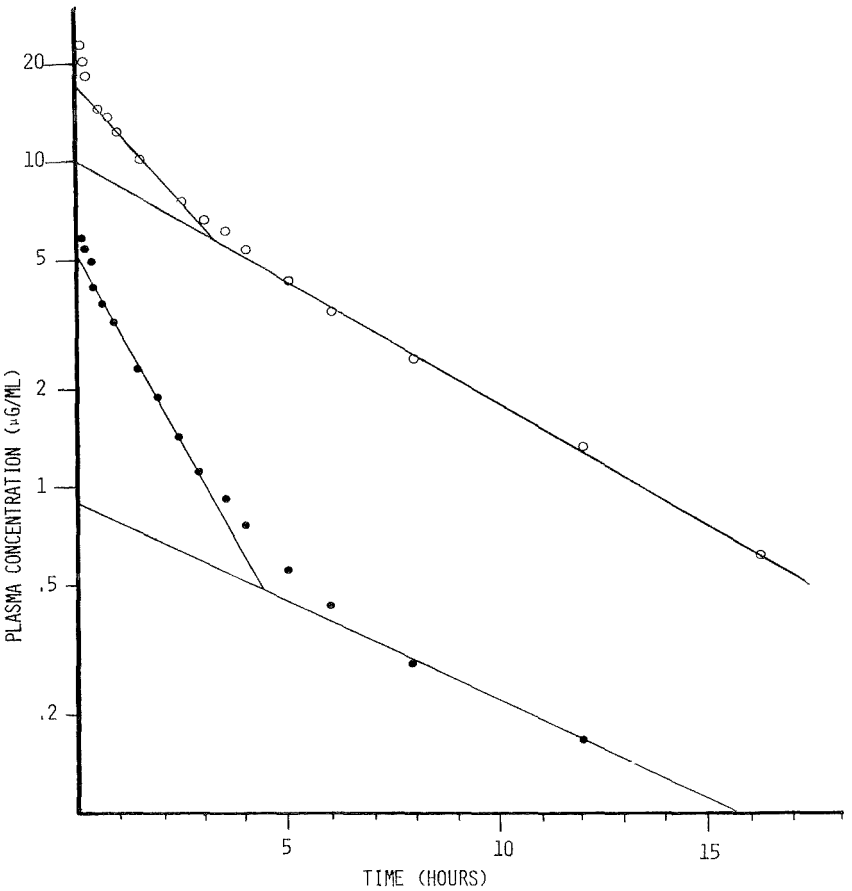


Figure 3. Semilog plot of plasma concentrations; (○) - phenylbutazone, (●) - flunixin. Lines drawn represent best fit of data to a two compartment pharmacokinetic model, 10 and 20 hr samples used for computations not shown.

composition but the sampling procedure and protein precipitation procedure are all extremely important when seeking the maximum sensitivity. The sensitivity obtained with this assay provides a useful tool in studying the pharmacokinetics of flunixin.

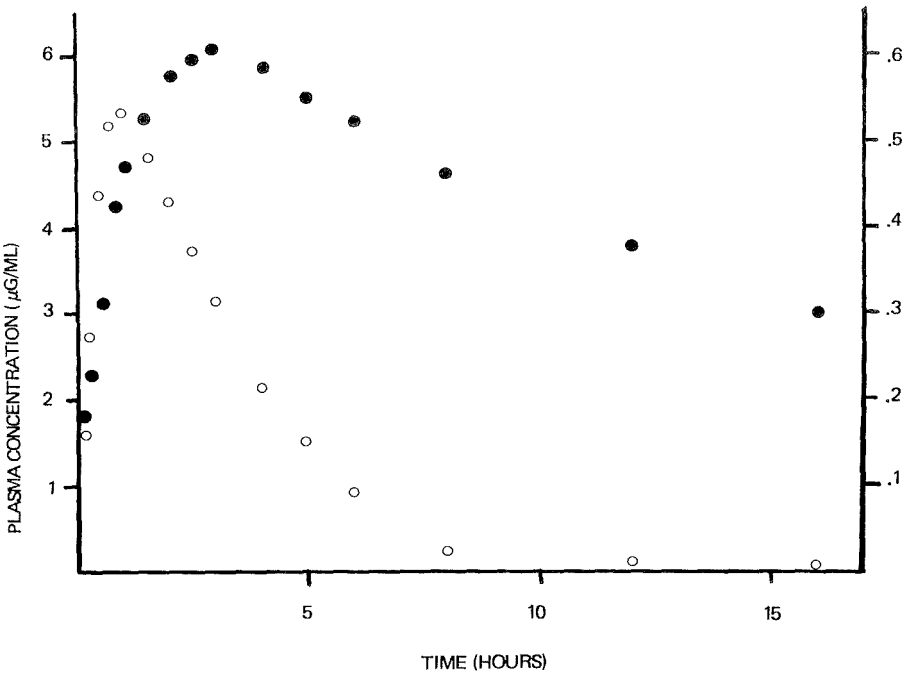


Figure 4. Linear plot of the major metabolites appearing in plasma from phenylbutazone; (○) - γ-hydroxyphenylbutazone (scale on right hand axis 0-6 μg/ml), (●) - oxyphenbutazone (scale on left 0-.6 μg/ml).

Previous studies on the pharmacokinetics of flunixin in horses report a single compartment model with an elimination half-life of 1.6 hours (2). These data were derived using an assay with a sensitivity of 2 μg/ml (7). Although the data derived here is from a single horse it agrees quite well with previous studies if data greater than 1 μg/ml are considered, i.e., one compartment model with an elimination half-life of 1.4 hours. The increased assay sensitivity allows delineation of the second compartment for flunixin. The importance of this second compartment in light of multiple dosing and minimum effective concentration is currently being studied in our laboratories.

TABLE 3

Pharmacokinetic Parameters Derived from the Two Compartment Treatment of the Experimental Data

Parameter	Flunixin	Phenylbutazone
Dose, mg/kg	1.1	4.4
β , hr^{-1}	.145	.172
$t_{\frac{1}{2}}$ elim., hr	4.8	4.0
α, hr^{-1}	.636	.624
A, µg/ml	4.61	6.05
B, µg/ml	.94	10.16
Vd, ℓ/kg	.552	.372
Vc, ℓ/kg	.198	.271
k_{21}, hr^{-1}	.221	.455
k_{10}, hr^{-1}	.417	.236
k_{12}, hr^{-1}	.143	.105

In a random sampling of clinic cases admitted to the University Veterinary Teaching Hospital for colic treatment, two of eight horses showed signs of pretreatment with flunixin. Plasma concentrations were measured to be 0.10 µg/ml and 0.50 µg/ml at the time of admission. Still another horse admitted for observation and subsequently diagnosed as a case of phenylbutazone toxicity presented no measureable levels of phenylbutazone upon admission. Oxyphenbutazone (650 ng/ml) and γ-hydroxyphenylbutazone (50 ng/ml) were measured in this sample; both decreased to undetectable levels (<50 ng/ml) within twenty four hours. The clinical consequences of prior-to-clinic treatment with nonsteroidal anti-inflammatory agents will be discussed elsewhere.

ACKNOWLEDGMENTS

This research was supported in part by funds from the Bolshoi Gastrointestinal Research Program. The authors wish to thank Ms. Nancy Reynolds for her skillful preparation of this manuscript and Mr. Rick Rentz for his technical assistance during the project.

REFERENCES

1) Moore, J. N., Garner, H. E., Shapland, J. E.: Prevention of Endotoxin-Induced Aterial Hypoxemia and Lactic Acidosis with Flunixin Meglumine in Conscious Ponies. Equine Vet J., 13 (2): 95-98, 1981.

2) Bottoms, G. D., Fessler, J. F., Roesel, O. F., Moore, A. B., Frauenfelder, H. C.: Endotoxin-Induced Hemodynamic Changes In Ponies: Effects of Flunixin Meglumine. Am. J. Vet. Res., 42 (9) 1514-1518, 1981.

3) Fessler, J. F., Bottoms, G. D., Roesel, O. F., Moore, A. B., Frauenfelder, H. C., Boon, G. D.: Endotoxin-Induced Change in Hemograms, Plasma Enzymes and Blood Chemical Values in Anesthetized Ponies: Effects of Flunixin Meglumine. Am. J. Vet. Res., 43 (1) 140-144, 1982.

4) Dusci, L. J., Hackett, L. P.: Determination of Some Anti-inflammatory Drugs in Serum by High-performance Liquid Chromatography. J. Chrom. 172, 516-519, 1979.

5) Nielsen-Kudsk, F.: HPLC-Determination of Some Anti-inflammatory Weak Analgesic, and Uricosuric Drugs in Human Blood Plasma and its Application to Pharmacokinetics. Acta Pharmacol et Toxicol. 47, 267-273, 1980.

6) Chiou, W. L., Gadalla, M. A. F., Peng, G. W.: Simple, Rapid, and Micro High-pressure Liquid Chromatographic Determination of Endogenous "True" Creatinine in Plasma, Serum and Urine. J. Pharm. Sci. 67, 182-187, 1978.

7) Palmer, K., Schering Research Corp., Personal Communication.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 2005-2016 (1982)

SEP-PAK PREPARATIVE CHROMATOGRAPHY: USE IN RADIOPHARMACEUTICAL SYNTHESIS

Michael R. Kilbourn, Douglas D. Dischino,
Carmen S. Dence, and Michael J. Welch

Mallinckrodt Institute of Radiology
Washington University School of Medicine
510 South Kingshighway
St. Louis, Missouri 63110

ABSTRACT

The use of SEP-PAK^R C_{18} cartridges for the isolation and purification of radiopharmaceuticals, labeled with the 20.4 minute half-life radionuclide carbon-11, is reported. Synthesis and SEP-PAK preparative chromatographic purification of [1-^{11}C]palmitic acid, [^{11}C-methyl]benzyl methyl ether, [1-^{11}C]butan-1-ol, and [1-^{11}C]pyruvic acid are described. The use of SEP-PAK C_{18} cartridges has allowed development of rapid and remote methods for handling of high amounts (>100 mCi) of radioactive products.

INTRODUCTION

In the synthesis of organic radiopharmaceuticals labeled with radionuclides of short half-life (for example carbon-11, $t_{1/2}$ = 20.4 minutes, or nitrogen-13, $t_{1/2}$ = 9.98 minutes) severe restrictions are placed on the methods of isolation and purification of the labeled product. Such procedures must necessarily be very rapid, yet they must be reproducible and

yield products of high radiochemical purity. Furthermore, the high radiation levels associated with handling of large amounts (up to 2 Ci) of these positron-emitting radionuclides require procedures amenable to incorporation into remotely controlled or automated apparatus, which will minimize radiation exposure of personnel involved.[1,2] Finally, procedures must be useful for the isolation of extremely small amounts of radioactive material; in no-carrier-added synthesis with these radionuclides, the actual amount of radiochemical produced is typically less than 20 μmol.

Various methods of chromatography (open column[1], flash column[3] and high pressure liquid chromatography[2,4]) are widely used in radiopharmaceutical syntheses. In many cases, however, a prior isolation and partial purification of reaction products is necessary, especially in conjunction with purification by HPLC, where resolution and column life expectancy can be severely compromised by certain impurities. We would like to describe here the application of SEP-PAK^R C_{18} preparative chromatography to the isolation and purification of several carbon-11 labeled organic radiopharmaceuticals. The use of SEP-PAK chromatography allows for the development of rapid, remotely controlled procedures for isolation of radiolabeled products.

EXPERIMENTAL

Materials and Methods

The SEP-PAK^R C_{18} cartridges were obtained from Waters Associates (Milford, MA, USA) and pre-equilibrated prior to use by

washing with 2 ml of ethanol and 5 ml of water. High pressure liquid chromatograpphy was done using a Waters Model 210 liquid chromatograph equipped with two 6000A pumps and a Waters Model 660 solvent programmer. Gas chromatographic analysis was done using a Varian Model 3700 gas chromatograph equipped with either a 5' x 1/4" stainless steel column packed with 10% FFAP on Chrom 60/80, or a 5' x 1/4" nickel column packed with Porapak Q (80/100 mesh).

The $^{11}CO_2$ was produced as previously described[7]. Syntheses of [1-^{11}C]palmitic acid, [^{11}C-methyl]benzyl methyl ether and [1-^{11}C]pyruvic acid have been previously reported (5,6,7): the modified procedures using SEP-PAK chromatography are detailed below.

Radiochemical Synthesis

[1-^{11}C]Palmitic Acid. A stream of nitrogen carrying the $^{11}CO_2$ was bubbled through 1 mL of a 0.1 M solution of pentadecylmagnesium bromide in diethyl ether. After complete transferral of the $^{11}CO_2$, 1 mL of 1 N hydrochloric acid was added, followed by 10 mL of water. The mixture was then passed through an activated SEP-PAK. The SEP-PAK was sequentially washed with 10 mL of water and 2 mL of 50% ethanol, then the [1-^{11}C]palmitic acid eluted from the SEP-PAK with 2 mL of 95% ethanol. The ethanol solution was analyzed for diethyl ether content using GC (FFAP column, 60° C, 20 cc/min helium flow, FI detector: R_T diethyl ether = 1.5 min, R_T ethanol = 2.4 min).

[^{11}C-Methyl]Benzyl Methyl Ether. A stream of $^{11}CO_2$ in nitrogen was bubbled through 0.15 mL of 1 M lithium aluminum hydride in tetrahydrofuran. After the $^{11}CO_2$ was transferred, the nitrogen flow was stopped, and 0.2 mL of water added. To the aqueous mixture was added 1.0 mL of dimethylsulfoxide, 40 μL of benzyl bromide (57 mg, 0.34 mmol), and 0.4 g of potassium hydroxide, and the mixture stirred for seven minutes. To this was added 8 mL of water and 1 mL of 1N hydrochloric acid, and the aqueous solution passed through a SEP-PAK. The SEP-PAK was rinsed with 10 mL of water, then the labeled methyl ether eluted off the SEP-PAK using 6 mL of 25% ethanol. This solution could be directly injected onto a HPLC column for purification (Waters Partisil 5 ODS-3 column, 45% ethanol, 3.0 mL/min, R_T = 3.9 min).

[1-^{11}C]Butan-1-ol. In a conical vessel were placed 0.5 mL of dry diethyl ether and 0.5 mL of a 2.2 M propyl magnesium chloride solution. Through this was bubbled helium containing $^{11}CO_2$. When the activity was transferred, the helium flow was increased and most of the ether evaporated. The helium was shut off and 0.5 mL of 1 M lithium aluminum hydride in THF added. The vessel was shaken briefly, let set 2 min, then the solution poured into 2.5 mL of cooled (ice-water bath) 1.0 M hydrochloric acid. The solution was swirled to dissolve the salts, then passed through the two SEP-PAKS. The SEP-PAKS were washed with 1 mL of water, and finally the [1-^{11}C]butan-1-ol eluted using 1.5 mL of 95% ethanol. Analysis of the ethanol solution was by HPLC (Waters Partisil 5 ODS-3 column, 30% ethanol, 3 ml/min, R_T = 5.8 min).

[1-^{11}C]Pyruvic Acid. Methyllithium (1.0 mL of a 1.6 M solution in diethyl ether) was added to 1 mL of dry tetrahydrofuran, the solution cooled (ice-water bath), and 250 μL (1.8 mmol) of 1,1,3,3-tetramethylbutylisocyanide added. After 30 min. a stream of nitrogen carrying $^{11}CO_2$ was bubbled through the solution. When transfer of the $^{11}CO_2$ was complete the nitrogen flow was stopped, 250 μL of 95% ethanol added, and the solvents evaporated. To the residue were added 3 mL of 5% hydrochloric acid, and the oily brown mixture boiled vigorously for 10 min. The solution was cooled, neutralized by dropwise addition of saturated sodium bicarbonate solution, then passed through two SEP-PAK cartridges into an empty sterile vial. The clear, colorless solution of sodium [1-^{11}C]pyruvate was analyzed by GC by withdrawal of an aliquot, acidifiction (pH 2), and injection onto a Porapak Q column. Analysis showed 95% [1-^{11}C]pyruvic acid and 5% radiochemical impurities ([1-^{11}C]acetic acid and [2-^{11}C]acetone), and no chemical impurities.

RESULTS AND DISCUSSION

The SEP-PAK C_{18} cartridges have been previously used to absorb lipophilic compounds present in low concentrations in large volumes of water (8,9). We have utilized the SEP-PAK C_{18} cartridges in a strictly analogous fashion in the isolation of [1-^{11}C]palmitic acid and [^{11}C-methyl]benzyl methyl ether. We have also used these cartridges to isolate a not-so-hydrophobic molecule, [1-^{11}C]-butanol, and to purify (but not isolate) a

hydrophilic product, [1-^{11}C]pyruvic acid. The results of these applications of SEP-PAK C_{18} chromatography are detailed below.

[1-^{11}C]Palmitic Acid

This labeled fatty acid is prepared by a classical Grignard reaction. The separation of the labeled palmitic acid from diethyl ether and excess hydrochloric acid is crucial; at the end of the synthesis the [1-^{11}C]palmitic acid is prepared for in vivo injection by addition of a saline solution of human serum albumin, and traces of diethyl ether or hydrochloric acid can cause denaturation of the albumin and render the preparation useless[5]. Previous methods for ^{11}C-palmitic acid purification have involved extraction of the product into diethyl ether, repeated washings of the ether with saline to remove HCl, then evaporation of the ether. The use of a SEP-PAK C_{18} cartridge, as shown in Table 1, can substitute for these isolation and purification steps. The ethanol solution of [1-^{11}C]palmitic acid obtained via SEP-PAK isolation is at neutral pH and contains less than 0.5% diethyl ether impurity.

TABLE 1
Isolation and Purification of [1-^{11}C]Palmitic Acid

	Solution through C-18 SEP-PAK	Activity, mCi SEP-PAK	Eluant
(1)	Crude reaction mixture	32	2
(2)	10 ml H_2O	25	7
(3)	2 ml 50% ethanol	23	2
(4)	2 ml 95% ethanol	5	18

[^{11}C-Methyl]Benzyl Methyl Ether

This labeled ether is prepared in two steps, the reduction of $^{11}CO_2$ to $^{11}CH_3OH$ using lithium aluminum hydride, and the reaction of this labeled methanol with benzyl bromide using potassium hydroxide in water-dimethylsulfoxide solution. Although a reverse-phase HPLC separation of methanol, benzyl bromide, and benzyl methyl ether is easily achieved, the presence of dimethylsulfoxide and potassium hydroxide in the crude reaction mixture (both deleterious to HPLC columns) makes a preliminary isolation of the product absolutely necessary. In this regard the SEP-PAK works exceptionally well; the lipophilic benzyl methyl ether and benzyl bromide are quantitatively retained on the SEP-PAK, and washing with water removes the KOH, DMSO, and 90% of the ^{11}C-methanol (see Table 2). Washing of the SEP-PAK with 25% ethanol is effective in eluting off 84% of the ^{11}C-benzyl methyl ether, along with only a small amount of the benzyl bromide. Using higher concentration of ethanol (up to 50%) gives more complete elution of the labeled benzyl methyl ether, but also elution of proportionately more benzyl bromide. Finally, a reverse-phase HPLC

TABLE 2
Isolation and Partial Purification of [^{11}C]Benzyl methyl ether

	Solution through C-18 SEP-PAK	Activity, mCi SEP-PAK	Eluant
(1)	Crude reaction mixture + 10 ml H_2O	125	62
(2)	6 ml 25% ethanol	19	105

separation affords [^{11}C-methyl]benzyl methyl ether with a radiochemical purity of 99% and free of chemical impurities.

[1-^{11}C]Butan-1-ol

This low molecular weight alcohol is obtained by a two step synthesis, a Grignard synthesis of [1-^{11}C]butanoic acid followed by lithium aluminum hydride reduction to the alcohol. The ^{11}C-butanol is separated from chemical impurities (diethyl ether, tetrahydrofuran, and hydrochloric acid) using two SEP-PAK C_{18} cartridges in series; typical results are shown in Table 3. As butanol is somewhat water soluble, a procedure using a minimum of water and a slower flow rate is necessary: however, 20% of the ^{11}C-butanol is lost in the water wash step. HPLC analysis of the final product solution shows [1-^{11}C]butanol in 100% radiochemical purity with small amounts of diethyl ether and methanol as chemical impurities. Attempts to entirely remove these chemical impurities by selective washings of the SEP-PAK with various ethanol:water mixtures proved unsuccessful. At present, these impurities do not interfere with the use of [1-^{11}C]butan-1-ol for in vivo animal studies. In those instances where a product of

TABLE 3
Isolation and Purification of [1-^{11}C]butan-1-ol

	Solution through C-18 SEP-PAK	Activity, mCi SEP-PAK	Eluant
(1)	Crude reaction mixture	117	12
(2)	1.5 ml H_2O	94	23
(3)	2.0 ml 95% ethanol	85	9

higher chemical purity is desired, the solution of ^{11}C-butanol in ethanol isolated using SEP-PAKS in this manner can be diluted with water and safely injected onto a reversed-phase HPLC column for preparative chromatographic purification. The use of the SEP-PAKS has been particularly rewarding in this synthesis: it should be noted that the alternative method of isolation by liquid-liquid extraction and evaporation cannot be utilized, due to the volatility of the radiolabeled product.

[1-^{11}C]Pyruvic acid

This α-keto acid is prepared in two steps: the addition of $^{11}CO_2$ to a lithium aldimine followed by acid hydrolysis of the intermediate α-imino acid[7]. The crude product solution contains two major impurities, 1,1,3,3-tetramethylbutylamine and [1-^{11}C]-2-(1,1,3,3-tetramethylbutyl)-iminopropionic acid. By using two SEP-PAK C_{18} cartridges in series, 100% of these impurities can be easily and quickly removed. As a typical example, the passage of a solution of 50 mCi of crude products through two SEP-PAKS gave 17 mCi of [1-^{11}C]pyruvic acid, with 11 mCi retained on the SEP-PAK cartridges (93% of which could be eluted with 2 mL of 95% ethanol). The eluant from the SEP-PAK chromatography then contains the desired [1-^{11}C] pyruvic acid in greater than 95% radiochemical purity (by GC analysis: remainder is [1-^{11}C]acetic acid and [2-^{11}C]acetone). The absence of chemical impurities was confirmed by both GC (FI detector) and thin layer chromatography. In this example, the SEP-PAKS are used to purify (but not isolate) the desired radiolabeled product by selective absorption of the

hydrophobic side products, and is thus in a sense an application "opposite" to the three previous examples of isolation of hydrophobic products.

The use of SEP-PAK C_{18} cartridges in the syntheses of carbon-11 labeled palmitic acid, butanol, benzyl methyl ether, and pyruvic acid are only four examples of our many applications of SEP-PAK C_{18} chromatography to radiochemical syntheses. The syntheses of these compounds were chosen to illustrate the versatility of this isolation and purification technique. We have also used SEP-PAK C_{18} chromatography in syntheses of carbon-11 labeled ethers[6] and long-chain alcohols[10], in the syntheses of several other carbon-11 labeled α-keto acids, and in the synthesis of a carbon-11 labeled α-amino acid, [1-^{11}C]norvaline. In general, incorporation of a SEP-PAK chromatography step has allowed for shorter preparation times and safe handling of larger amounts of radioactive products. Finally, in the synthesis of fluorine-18 labeled spiroperidol[11] (fluorine-18 is a positron-emitting radionuclide with $t_{1/2}$ = 110 minutes), we have recently used SEP-PAK C_{18} chromatography to concentrate the organic products prior to HPLC separation. For fluorine-18 and other longer-lived radionuclides the speed of synthesis is not as crucial as with carbon-11, but methods amenable to incorporation in remotely controlled apparatus are still needed.

We feel that SEP-PAK chromatography, and related methods of C_{18}-silica gel bonded phase chromatography[12], will be a valuable

addition to the chromatographic options of the radiopharmaceutical chemist. The SEP-PAK cartridges have proven very versatile; they can be used for product isolation, for isolation and purification, or product purification alone. Finally, use of a SEP-PAK C_{18} cartridge should allow for concentration of a radiolabeled product obtained in an eluant from an HPLC column; this is important in situations where the product is obtained in a solvent or volume incompatible with in vivo animal or human studies.

SEP-PAK chromatography has thus taken its place alongside column and high-pressure liquid chromatography as a purification technique used daily in our laboratories.

ACKNOWLEDGEMENTS

The authors would like to thank Mr. William Margenau for operation of the Washington University Medical School cyclotron. This work was supported by the National Institutes of Health (Grants HL13851, HL17646 and NS06833).

REFERENCES

1. Fowler, J.S., MacGregor, R.R., Wolf, A.P., Farrell A.A., Karlstrom, K.I., and Ruth, T.J., A Shielded Synthesis System for Production of 2-Deoxy-2-[^{18}F]Fluoro-D-Glucose, J. Nucl. Med., 22, 376, 1981.

2. Berger, G., Maziere, M., Knipper, R., Prenant, C., and Comar, D., Automated Synthesis of ^{11}C-Labelled Radiopharmaceuticals: Imipramine, Chloropromazine, Nicotine, and Methionine, Int. J. Appl. Radiat. Isot., 30, 393, 1979.

3. Stille, N.C., Kahn, M., and Mitra, A., Rapid Chromatographic Technique for Preparative Separations with Moderate Resoution, J. Org. Chem., 43, 2923, 1978.

4. Berger, G., Maziere, M., Godot, J.M., Prenant, C., and Comar, D., HPLC Purification of ^{11}C-Labelled Radiopharmaceuticals, Int. J. Appl. Radiat. Isot., 31, 641, 1980.

5. Welch, M.J., Wittmer, S.L., Dence, C.S., and Tewson, T.J., Radiopharmaceuticals Labeled with ^{11}C and ^{18}F: Considerations related to the Prepartion of ^{11}C-Palmitate, in Short-Lived Radionuclides in Chemistry and Biology, J.W. Root and K.A. Krohn, eds., American Chemical Society, Washington, D.C., 1981, p. 407.

6. Dischino, D.D., Wittmer, S.L., Raichle, M.E., and Welch, M.J., Synthesis and Cerebral Extraction of Carbon-11 Labeled Ethers, J. Lab. Compd. Radiopharm., 18, 239, 1981.

7. Kilbourn, M.R., and Welch, M.J., No-Carrier-Added Synthesis of [1-^{11}C]Pyruvic Acid, Int. J. Appl. Radiat. Isot., 33, 359 (1982).

8. Whitney, J.O., and Thaler, M.M., A Simple Liquid Chromatographic Method for Quantitative Extraction of Hydrophobic Compounds from Aqueous Solutions, J. Liquid Chromat., 3, 545, 1980.

9. Wolkoff, A.W., and Creed, C., Use of SEP-PAK C_{18} Cartridges for the Collection and Concentration of Environmental Samples, J. Liquid Chromat., 4, 1459, 1981.

10. Fowler, J.S., Gallagher, B.M., MacGregor, R.R., Wolf, A.P., Carbon-11 Labeled Aliphatic Amines in Lung Uptake and Metabolism Studies: Potential for Dynamic Measurements in vivo, J. Pharmacol. Exp. Ther. 198, 133, 1976.

11. Maeda, M., Tewson, T.J., and Welch, M.J., Synthesis of High Specific Activity ^{18}F-Spiroperidol for Dopamine Receptor Studies, J. Lab. Compd. Radiopharm. 18, 183, 1981.

12. Good, T.J., and Andrews, J.S., The Use of Bonded-Phase Extraction Columns for Rapid Sample Preparation of Benzodiazepines and Metabolites from Serum for HPLC analysis, J. Chromat. Science, 19, 562, 1981.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 2017 (1982)

BOOK REVIEW

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY - Theory, Practice, and Biomedical Applications, A. M. Krstulovic and P. R. Brown, John Wiley & Sons, Inc., 605 Third Avenue, New York, NY, 10158, 1982, 312 pp., $35.00 (US).

HPLC has become the method of choice for the solution of problems related to the analysis of substances of biomedical origin. Reverse phase techniques have all but replaced the classically employed approaches of ion exchange, adsorption, and gel filtration, no doubt due to the ultra-resolution and relatively mild experimental conditions they afford.

There is a need for a unified treatment of reverse phase chromatography including instrumentation, theory, and applications. This volume, well organized and expertly written by two eminent authorities, meets this need in a way that is easy to understand and to use. It is sprinkled throughout with tidbits of useful and practical information.

Most of the applications are taken from the life sciences and the book will be most useful to scientists in this area. However, the first 217 pages (more than 70% of the book) deal with a general treatment of chromatographic theory and reverse phase technology, including theory, instrumentation, mechanism, methods development strategy, peak characterization, and quantitation; thus, it will be of interest to anyone who plans to work with reverse phase liquid chromatography.

As with any volume, there are some weaknesses here. Derivatization, a very important subject, is barely mentioned in a two-page chapter. Also, a much more thorough treatment of ion-pairing would have been a welcome enhancement.

It is the reviewer's opinion that this volume is required reading for all chromatographers and for other scientists who will use reverse phase liquid chromatographic techniques for the solution of separations problems in their respective fields.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 2019-2022 (1982)

LC NEWS

LIQUID CHROMATOGRAPH/MASS SPECTROMETER INTERFACE continuously concentrates the effluent from a conventional liquid chromatograph and delivers the concentrated solution into the mass spectrometer. Combination with a mass spectrometer/data system provides capability for analysis of complex and intractable biological, environmental, and petrochemical samples. Extranuclear Laboratories, Inc., JLC/82/10, P. O. Box 11512, Pittsburgh, PA, 15238, USA.

NEW COLUMNS FOR OLD column replacement service permits chromatographers to send in their old, obsolete columns to be emptied, cleaned, and repacked with the packing material of their choice. Columns are returned with a full evaluation report, as for new HPLC columns. HPLC Technology, JLC/82/10, P.O.Box 7000-196, Palos Verdes Peninsula, CA, 90274, USA.

EXPANDABLE COMMUNICATION SYSTEM for the chromatography lab uses a simple 3-wire connection to integrate chromatographs and data systems into an integrated network. This approach eliminates the need for a system controller or host computer to control communications. It can be interfaced with CRT terminals, printers, modems, computers via a communications interface board. Spectra-Physics, JLC/82/10, 3333 North First Street, San Jose, CA, 95134, USA.

SHORT HPLC COLUMNS perform many analyses faster and with reduced solvent consumption. Three-micron diameter packing materials yield efficiencies comparable to longer conventional columns. Advanced bonding and packing technology combine to assure long column lifetimes and consistent performance. Rainin Instrument Co., JLC/82/10, Mack Rd., Woburn, MA, 01801, USA.

PROTEIN SEQUENCING SOLVENTS eliminate solvent impurities that contribute to losses in PTH amino acid derivatives during protein sequencing even at the picomole level. Each solvent undergoes recovery testing using a representative mixture of 16 different PTH amino acid derivatives. This assures higher yields for Edman Degradation procedures. Burdick & Jackson Laboratories, JLC/82/10, 1953 S. Harvey St., Muskegon, MI, 49442, USA.

GUIDE TO MEMBRANE SEPARATION TECHNOLOGY is applications oriented. A unique "Applications Guide" leads one to the proper product for his needs, plus available literature. Millipore Corp., JLC/82/10, Bedford, MA, 01730, USA.

LC COLUMN SELECTOR switches five columns. It enables the user to exchange columns rapidly, without wrenches, and without subjecting fittings to repeated wear. A column that is switched off-line is sealed at both ends. One can be sure that no column is exposed to a solvent that is intended for another. Rheodyne, Inc., JLC/82/10, P. O. Box 996, Cotati, CA, 94928, USA.

PROGRAMMABLE WAVELENGTH DETECTOR is microprocessor controlled. It permits selection of any number of wavelengths in one-nanometer increments from 190 to 370 nm for optimal detection of all components. Utilizes keyboard entry that may be changed at any point and as often as desired. Varian Instrument Group, JLC/82/10, 2700 Mitchell Drive, Walnut Creek, CA, 94598, USA.

SEPARATE OPTICAL ISOMERS by HPLC with chiral columns. Preparative chiral sorbents are also

available for scale-up of separations. J.T.Baker Research Products, JLC/82/10, 222 Red School Lane, Phillipsburgh, NJ, 08865, USA.

AUTOMATED LC SYSTEM combines chromatograph with an advanced computer with high resolution graphics. It features a pulse-free pump, ternary gradient capability and a variety of sensitive detectors. Up to four chromatographs can be controlled with user-written software. A wide range of data calculations can be performed, including area pct., normalization, internal and external standards, single or multilevel and linear or non-linear calibrations. IBM Instruments, Inc., JLC/82/10, Orchard Park, P. O. BOX 332, Danbury, CT, 06810, USA.

HIGH PRESSURE FLUID CELLS for UV monitors are rated at 1000 psi back pressure and incorporate enhanced chromatographic flow characteristics to optimize the plate count in microparticulate columns without sacrificing detector sensitivity. The cells also improve bubble clearing and allow additional detectors to be added downstream without excessive band spreading. LDC/Milton Roy Co., JLC/82/10, P. O. Box 10235, Riviera Beach, CA, 33404, USA.

APPLICATIONS DEVELOPMENT KIT permits application of solid phase technology in the development of new sample preparation methods. One can experiment with new methods or solve existing problems with the phases included in the kit. Included are octadecyl, phenyl, cyanopropyl, aminopropyl, benzenesulfonic acid, quaternary amine, diol, and unbonded silica. Analytichem Internat'l, Inc., JLC/82/10, 24201 Frampton Avenue, Harbor City, CA, 90710, USA.

HPLC GRADIENT PROGRAMMER/SYSTEM CONTROLLER uses a microcomputer based on the Zenith/Heath Z-89 and controls up to 3 pumps to produce virtually any type of gradient or flow profile. Control functions include sample injection, solvent selection, integration, fraction collection, and recorder speed. The Anspec Co., JLC/82/10, P. O. Box 7044, Ann Arbor, MI, 48107, USA.

QUALITY ANALYZER, an LC system designed specifically for quality control applications will operate unattended 24 hours/day. It requires half the bench space of a conventional HPLC system and one can learn to operate it in 20 minutes. Waters Associates, Inc., JLC/82/10, 34 Maple street, Milford, MA, 01757, USA.

TLC TECHNICAL SERIES VOLUME, by Dr. Joseph Sherma at Lafayette College, deals with practice and applications of TLC. This volume is part of a Technical Series consisting of individual comprehensive volumes on current techniques of TLC. Each is the work of an outstanding scientist, and each is confined to a single area. Whatman Chemical Separation, Inc., JLC/82/10, 9 Bridewell Place, Clifton, NJ, 07014, USA.

SEPARATION OF BIOPOLYMERS such as proteins, peptides, oligonucleotides, are performed with the FPLC system. Separation times as low as one minute have been achieved with high yields of protein mass and activity. Pharmacia Fine Chemicals, Inc., JLC/82/10, 800 centennial Avenue, Piscataway, NJ, 08854, USA.

POSITIVE DISPLACEMENT MICROPIPETTORS are available in 3 models ranging from 5 to 1000 microliters. Featured are snap-on, snap-off, precision plastic tips. They are precalibrated, but can be recalibrated with a calibration rod stored in the handle. Labindustries, Inc., JLC/82/10, 620 Hearst Avenue, Berkeley, CA, 94710, USA.

ABSORBANCE DETECTOR FOR HPLC offers extremely high sensitivity, is a continuously variable wavelength system suited for microbore LC, analytical scale HPLC, and semi-preparative LC applications. Kratos Analytical Instruments, JLC/82/10, 24 Booker Street, Westwood, NJ, 07675, USA.

POST COLUMN REACTOR can be usewd to determine metals. It features a pneumatic pump, mixing tee, and a packed bed reactor in a self-contained unit. Dionex, JLC/82/10, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(10), 2023-2026 (1982)

LC CALENDAR

1982

OCTOBER 4-6: Capillary Chromatography '82: International Symposium, Tarrytown, NY. Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX, 77004, USA.

OCTOBER 10 - 13: 21st Annual Mtg. of ASTM Committee E-19 on the Practice of Chromatography, Marriott Hotel, New Orleans. Contact Mr K. Riley, ASTM Headquarters, 1916 Race Street, Philadelphia, PA, 19103, USA.

OCTOBER 12 - 14: 3rd International Symposium On Chlorinated Dioxins and Related Compounds, International Congress Center, Salzburg, Austria. Contact: Prof. O. Hutzinger, University of Amsterdam, Nieuwe Achtergracht 166, 1018WV Amsterdam, The Netherlands.

OCTOBER 14: Ninth ANACHEM Symposium, sponsored by The Association of Analytical Chemists, Dearborn Inn, Dearborn, MI, USA. Contact: J. W. Auld, Detroit Edison Co., Detroit, MI, 48226, USA.

OCTOBER 14 - 15: "New Perspectives in Racemic Coumpound Separation" sponsored by CNR-PF Chimica Fine e Secondaria, Societa Chimica Italiana, and Universita degli Studi di Roma, in Rome, Italy. Contact: Prof. Domenico Misiti, Inst. di Chimica Organica, Via del Castro Laurenziano 9, 00161 Roma, Italy.

OCTOBER 19 - 20: short Course on LC/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postal 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

OCTOBER 21 - 22: 2nd Workshop On LC/MS and MS/MS, Maison des Congres, Montreux, Switzerland. Sponsored by Int'l. Assoc. of Environmental Anal. Chem. Contact: Dr. Alain Donzel, Workshop Office, Case Postale 5, CH-1052 Le Mont-sur Lausanne, Switzerland.

NOVEMBER 2 - 5: 1st Inter-American Congress in Forensic Medicine and Sciences, Pan-American Assoc. of Forensic Sci., Sacramento, CA. Contact: John D. DeHaan, Calif. Department of Justice Lab. Box 13337, Sacramento, CA 95813,USA.

NOVEMBER 11 - 14: Applied Seminar for the Association of Clincial Scientists, Chicago, IL. Contact: Dr. F. M. Sunderman, Jr., Dept. of Lab. Medicine, Univ. of Connecticut School of Medicine, 263 Farmington Avenue, Farmington, CT 06032.

NOVEMBER 16 - 18: Medical and Laboratory Instrumentation Soc. Annual Int'l. Congress and Exhibition, Sheraton-Washington Hotel, Washington, DC. Contact: John Wolf, MLIS, 11310 Palisades Court, Kensington, MD, 20895, USA.

NOVEMBER 17 - 19: Eastern Analytical Symposium, Statler-Hilton Hotel, New York. Contact: Dr. H. Issag, Frederick Cancer Res. Facility, P.O. Box B, Frederick, MD, 21701, USA, or Dr. D. Strumeyer, Rutgers University, Chem. Dept, New Brunswick, NJ 08903.

DECEMBER 6 - 8: 3rd Biennial TLC Symposium-Advances in TLC, Hilton Hotel, Parsippany, NJ. Contact: J.C. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, 19104, USA.

1983

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

MAY 2 - 6: VIIth International Symposium On Column Liquid Chromatography, Baden-Baden, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

MAY 30 - JUNE 3: International Conference on Chromatographic Detectors, Melbourne University. Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria, Australia 3052.

JULY: 3rd Int'l. Flavor Conf., Amer. Chem. Soc., The Corfu Hilton, Corfu, Greece. Contact: Dr. S. S. Kazeniac, Campbell Inst. for Food Research, Campbell Place, Camden, NJ, 08101, USA.

JULY 17 - 23: SAC 1983 International Conference and Exhibition on Analytical Chemistry, The University of Edinburgh, United Kingdom. Contact: The Secretary, Analytical Division, Royal Society of Chemistry, Burlington House, London W1V 0BV, United Kingdon.

1984

OCTOBER 1 - 5: 15th International Symposium on Chromatography, Nurenberg, West Germany. Contact: K. Begitt, Ges. Deutscher Chemiker, Postfach 90 04 40, Varrentrappstrasse 40-42, D-6000 Frankfurt (Main), West Germany.

The Journal of Liquid Chromatography will publish announcements of LC meetings and symposia in each issue of The Journal. To be listed in the LC

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

an instructive laboratory guide to . . .

STEROID ANALYSIS BY HPLC
Recent Applications

(Chromatographic Science Series, Volume 16)

edited by
MARIE P. KAUTSKY
University of Colorado
School of Medicine
Denver

July, 1981
424 pages, illustrated

Steroid Analysis by HPLC: Recent Applications is a laboratory guide for clinical researchers, pharmaceutical manufacturers, food and drug analysts, and steroid chemists who need to be able to perform successful separations and analyses of steroids and related compounds. This manual is written by leading chromatographers in the field who describe in precise detail the methodologies used in their laboratories. Scientists involved in the separation, isolation, or quantitation of steroids should take immediate advantage of the instructive material contained in this authoritative workbook.

Steroids are difficult compounds to isolate and analyze. The widespread use of HPLC for purifying and analyzing steroids is a recent development. *Steroid Analysis by HPLC: Recent Applications* covers the two major uses of HPLC in steroid separation and analysis: the analysis of specific steroid groups; and the utilization of HPLC in solving analytical problems common to all steroid-like compounds. Concise methods for the separation and analysis of bile acids, cardiac glycosides, progestins, adrenal and testicular steroids estrogens, D vitamins, steroid epimers, and hormones found in feed and food are presented. Complete procedures for the HPLC of sterol intermediates in cholesterol biosynthesis and the study of enzymatic steroid reactions are also included. Flow sheets and typical chromatogram scans accompany the articles. An appendix identifying various types of column packing will be especially helpful to scientists unfamiliar with the new column nomenclature.

The use of HPLC has developed rapidly over the past few years. This collection of papers by practicing chromatographers presents tested approaches to problems in steroid separation and quantitation. With *Steroid Analysis by HPLC: Recent Applications*, analytical chemists, clinical analysts, endocrinologists, organic chemists, and biochemists have at their disposal a practical, concise guide for successful isolation and analysis of steroids.

CONTENTS

ISBN: 0-8247-1324-9

MARCEL DEKKER, INC.
270 MADISON AVENUE
NEW YORK, N.Y. 10016
(212) 696-9000

the most valuable guide to . . .

Applications of Glass Capillary Gas Chromatography

(Chromatographic Science Series, Volume 15)

edited by
WALTER G. JENNINGS
University of California
Davis

August, 1981
648 pages, illustrated

HERE is your fully illustrated, authoritative reference and working laboratory manual on one of the most exacting forms of analysis – glass capillary gas chromatography . As an analytical chemist, whether in industry or academia, you should have this book in order to fully understand and stay abreast of developments in this field.

Gas chromatography remains the world's most widely used analytical technique. However, glass capillary gas chromatography is a more precise form that offers advantages the packed column cannot provide: more complete separation; faster analysis time; higher sensitivity; and a more inert system.

This book contains contributions from 20 leading experts in this field from around the world. They explore the special requirements for the various areas of application and give detailed examples of the uses of this technique to a wide variety of specific analytical fields.

Analytical chemists, particularly those in food, flavor, and beverage analysis; clinical analysis; forensic analysis; and environmental analysis, should have this book on their shelf. No other work can provide the "how-to" information to begin work in the field or help experimental analysts upgrade and refine their analytical techniques.

CONTENTS

ISBN: 0-8247-1223-4

a valuable source of problem-solving information . . .
for analytical chemists, forensic scientists, pharmaceutical scientists, biologists, microbiologists, and clinical chemists . . .

THIN-LAYER CHROMATOGRAPHY

TECHNIQUES AND APPLICATIONS

(Chromatographic Science Series, Volume 17)

BERNARD FRIED and JOSEPH SHERMA
Lafayette College
Easton, Pennsylvania

January, 1982
328 pages, illustrated

THIN-LAYER CHROMATOGRAPHY (TLC) is experiencing a rebirth as a result of the recent development of higher-efficiency technology. This versatile separation technique is simpler to use than other chromatographic methods, offering separation scientists faster results in greater detail and at lower cost. The authors of *Thin-Layer Chromatography: Techniques and Applications* masterfully present the principles, practices, and applications of this method. Designed specifically for those without extensive training in analytical chemistry, this volume

- presents the first modern, self-teaching treatment of this inexpensive technique
- offers a valuable source of practical, problem-solving information for the scientist using TLC
- introduces TLC for use as an aid in solving basic and applied problems for scientists considering this technique
- serves as an aid for the introduction of TLC into the classroom, presenting various detailed experiments which utilize inexpensive, readily available materials

Thin-Layer Chromatography: Techniques and Applications is the primary source of information for analytical chemists, biochemists, clinical chemists, and laboratory and medical technicians requiring a greater knowledge of this technique. Zoologists and botanists interested in the separation sciences will find it a valuable reference; and it will serve college teachers of biology and chemistry equally well.

CONTENTS

ISBN: 0-8247-1288-9

MARCEL DEKKER, INC. 270 MADISON AVENUE, NEW YORK, N.Y. 10016 •

INSTRUCTIONS FOR PREPARATION OF MANUSCRIPTS FOR DIRECT REPRODUCTION

Journal of Liquid Chromatography is a bimonthly publication in the English language for the rapid communication of liquid chromatographic research.

Directions for Submission

One typewritten manuscript suitable for direct reproduction, carefully inserted in a folder, and two (2) copies of the manuscript must be submitted. Since all contributions are reproduced by direct photography of the manuscripts, the typing and format instructions must be strictly adhered to. Noncompliance will result in return of the manuscript to the authors and delay its publication. To avoid creasing, manuscripts should be placed between heavy cardboards and securely bound before mailing.

Manuscripts should be mailed to the Editor:

Dr. Jack Cazes
Journal of Liquid Chromatography
P. O. Box 1440-SMS
Fairfield, Connecticut 06430

Reprints

Owing to the short production time for articles in this journal, it is essential to indicate the number of reprints required upon notification of acceptance of the manuscript. Reprints are available in quantities of 100 and multiples thereof. For orders of 100 or more reprints, twenty (20) free copies are provided. A reprint order form and price list will be sent to the author with the notification of acceptance of the manuscript.

Format of Manuscript

1. The general format of the manuscript should be as follows: title of article; names and addresses of authors; abstract; and text discussion.

2. Title and Authors: The entire title should be in capital letters and centered on the width of the typing area at least 2 inches (5.1 cm) from the top of the page. This should be followed by three lines of space and then by the names and addresses of the authors in the following way (also centered):

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN SURFACE WATERS BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakra Way
Salt Lake City, Utah 84108

3. Abstract: Three lines below the addresses, the title ABSTRACT should be typed (capitalized and centered on the page). This should be followed by a single-spaced, concise, abstract comprising less than 10% of the length of the text of the article. Allow three lines of space below the abstract before beginning the article itself.

4. Text Discussion: Whenever possible, the text discussion should be divided into such major sections as INTRODUCTION, MATERIALS, METHODS, RESULTS, DISCUSSION, ACKNOWLEDGMENTS, and REFERENCES. These major headings should be separated from the text by two lines of space above and one line of space below. Each heading should be in capital letters, centered, and underlined. Secondary headings, if any, should be flush with the left margin, underscored, and have the first letter of all main words capitalized. Leave two lines of space above and one line of space below secondary headings.

5. Paragraphs should be indented five (5) typewriter spaces.

6. Acknowledgment of collaboration, sources of research funds, and address changes for an author should be listed in a separate section at the end of the paper.

7. References (including footnotes) in the text will be numbered consecutively by numbers in parentheses. All references (and footnotes) should then be aggregated in sequence at the end of the communication. No footnotes should be shown at the bottom of pages. The reference list follows immediately after the text. The word REFERENCES should be capitalized and centered above the reference list. It should be noted that all reference lists should contain initials and names of all authors; *et al.* will not be used in reference lists. Abbreviations of journal titles and styles of reference lists will follow the American Chemical Society's Chemical Abstracts List of Periodicals. References should be typed single-spaced with one line space between each reference.

8. Each page of manuscript should be numbered lightly at the bottom of the sheet with a light blue pencil.

9. Only standard symbols and nomenclature approved by the International Union of Pure and Applied Chemistry should be used.

10. Any material that cannot be typed, such as Greek letters, script letters, and structural formulae, should be drawn carefully in black India ink (do not use blue ink).

Typing Instructions

1. The manuscript must be typewritten on good quality white bond paper measuring approximately 8½ x 11 inches (21.6 cm x 27.9 cm). Do not use Corrasible bond or its equivalent. The typing area of the article opening page, including the title, should be 5½ inches wide by 7 inches deep (14 cm x 18 cm). The typing area of all other pages should be no more than 5½ inches wide by 8½ inches deep (14 cm x 21.6 cm).

2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

3. It is essential to use black typewriter ribbon (carbon film is preferred) in good condition so that a clean, clear impression of the letters is obtained. Erasure marks, smudges, creases, etc., may result in return of the manuscript to the authors for retyping.

4. Tables should be typed as part of the text but in such a way as to separate them from the text by a three-line space at both top and bottom of each table. Tables should be inserted in the text as close to the point of reference as possible, but authors must make sure that one table does not run over to the next page, that is, no table may exceed one page. The word TABLE (capitalized and followed by an Arabic number) should precede the table and be centered on the page. The table title should have the first letters of all main words in capitals. Titles should be typed single-spaced. Use the full width of the type page for the table title.

5. Drawings, graphs, and other numbered figures should be professionally drawn in black India ink (do not use blue ink) on separate sheets of white paper and placed at the end of text. Figures should not be placed within the body of the text. They should be sized to fit within the width and/or height of the type page, including any legend, label, or number associated with them. Photographs should be glossy prints. A typewriter or lettering set should be used for all labels on the figures or photographs; they may not be hand drawn. Captions for the pictures should be typed single-spaced on a separate sheet, along the full width of the type page, and preceded by the word FIGURE and a number in arabic numerals. All figures and lettering must be of a size to remain legible after a 20% reduction from original size. Figure numbers, name of senior author, and arrow indicating "top" should be written in light blue pencil on the back or typed on a gummed label, which should be attached to the back of the illustration. Indicate approximate placement of the illustrations in the text by a marginal note in light blue pencil.

6. The reference list should be typed single-spaced although separated from one another by an extra line of space. Use Chemical Abstract abbreviations for journal titles. References to journal articles should include (1) the last name of all author(s) to any one paper, followed by their initials, (2) article title, (3) journal, (4) volume number (underlined), (5) first page, and (6) year, in that order. Books should be cited similarly and include (1) author, surname, first and middle initials, (2) title of book, (3) editor of book (if applicable), (4) edition of book (if any), (5) publisher, (6) city of publication, (7) year of publication, and (8) page reference (if applicable). E.g., Journals: Craig, L. C. and Konigsber, W., Use of Catechol Oxygenase and Determination of Catechol, Chromatogr., 10, 421, 1963. Books: Albertsson, P. A., Partition of Cell Particles and Macromolecules, Wiley, New York, 1960. Article in a Book: Walter, H., Proceedings of the Protides of Biological Fluids, XVth Colloquim, Pteeters., H., eds., Elsevier, Amsterdam, 1968, p. 367.

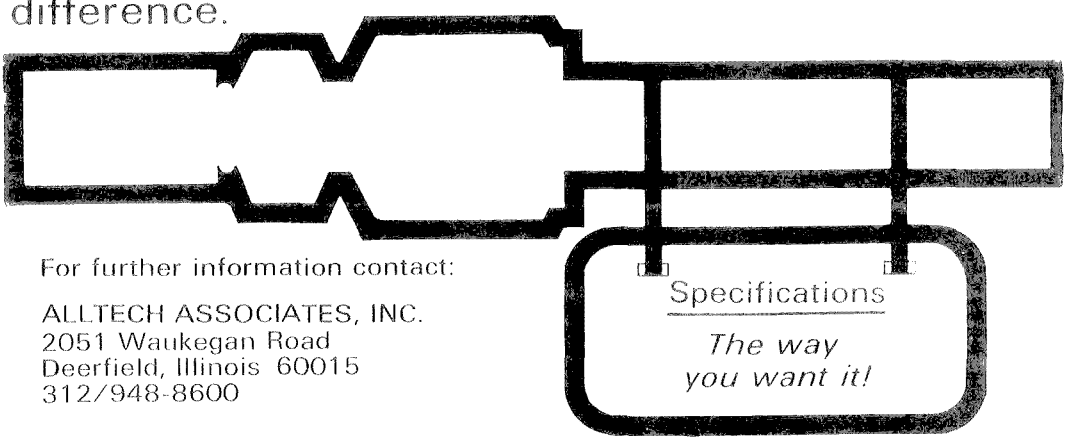
Custom packing HPLC columns has become our specialty. Any length, several ID's (including 3.2mm) and almost any commercially available packing material may be specified. We'll supply the column others won't.

With each column, you will receive the original test chromatogram plus a vial of the test mixture. Our advanced technology and computer testing is your assurance of a quality product.

When custom packing and testing is your special concern, we make the difference.

Each one is our special concern

CUSTOM PACKED HPLC COLUMNS



For further information contact:

ALLTECH ASSOCIATES, INC.
2051 Waukegan Road
Deerfield, Illinois 60015
312/948-8600

ALLTECH ASSOCIATES

Circle Reader Service Card No. 102