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JOURNAL OF LIQUID CHROMATOGRAPHY

JOURNAL OF LIQUID CHROMATOGRAPHY

June 1983

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 thin-layer chromatography and all modes of liquid chromatography.

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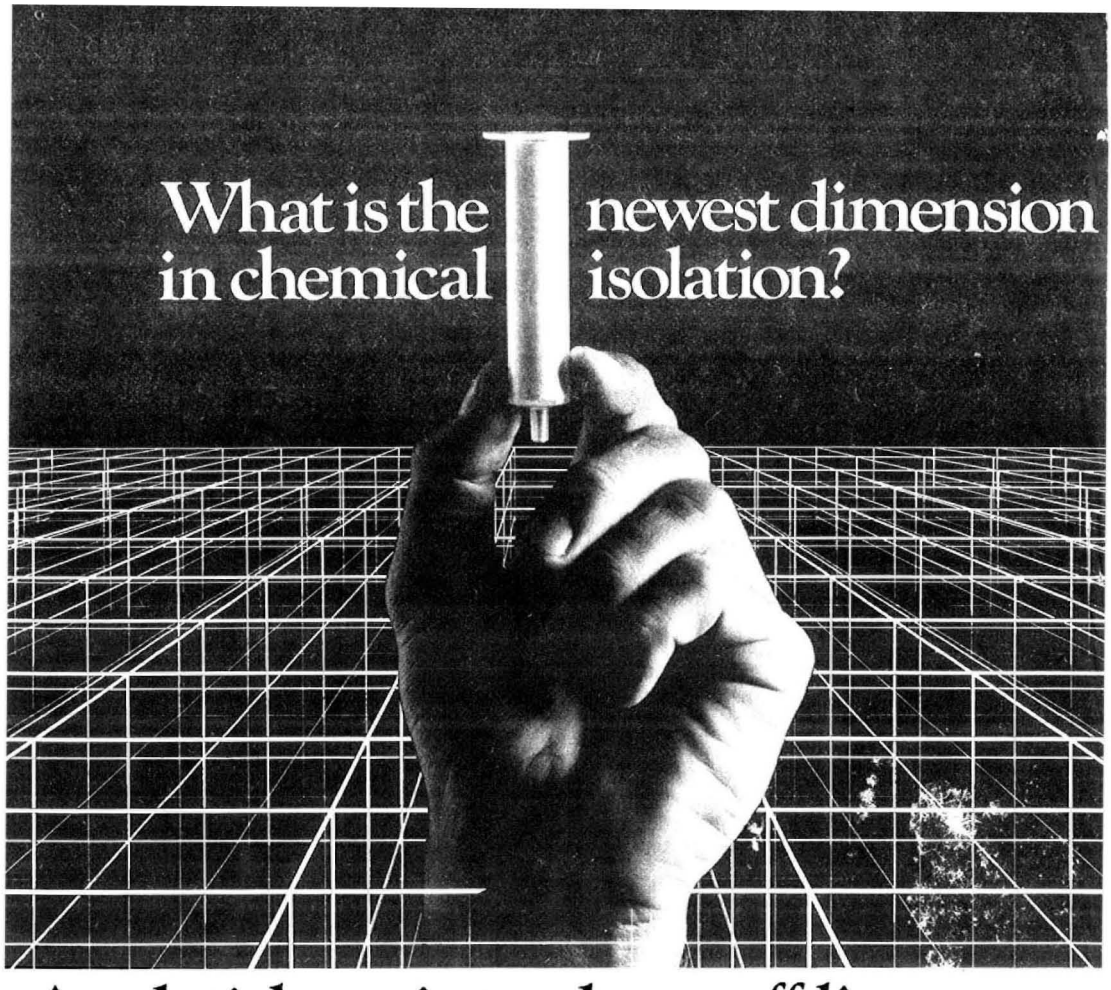

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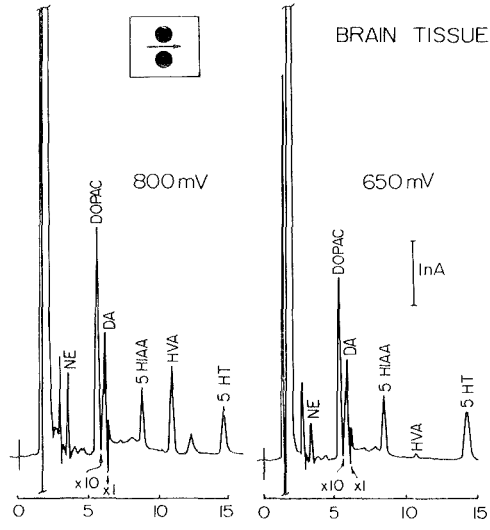

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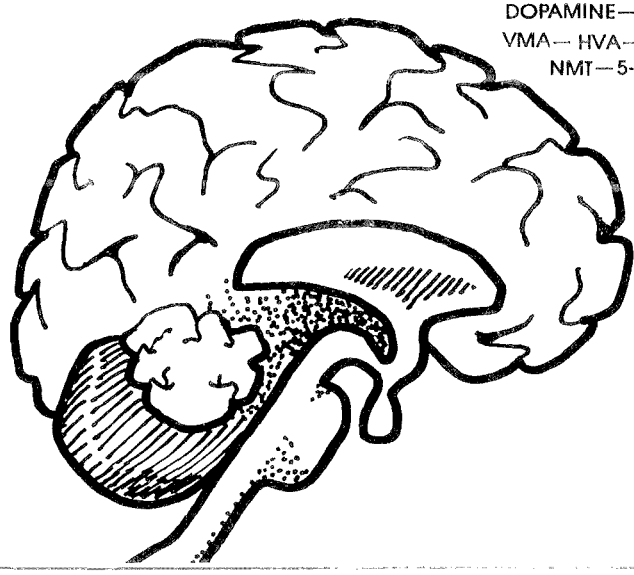

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(8), 1353-1357 (1983)

READER SURVEY
JOURNAL OF LIQUID CHROMATOGRAPHY

INTRODUCTION

A survey of Journal of Liquid Chromatography readers was conducted to gain insight into the

- nature of the readership
- its size
- their reading preferences
- their opinion of the journal's scientific stature and quality of the published papers, and
- the influence of advertisements in the Journal upon their buying decisions.

Two copies of a questionaire (reproduced at the end of this report) were inserted in 1300 copies of two issues of the Journal:

- Volume 5, Number 12: devoted to thin-layer chromatography, and
- Volume 6, Number 1: containing general papers

Respondents were asked to supply their names and addresses, but this was not a requirement for responding. It was noted that a significant number of those who did include this information were from countries outside the U.S., primarily Europe and Japan. Here's what we learned.

THE READERSHIP

Readers of The Journal of Liquid Chromatography are located in:

Industry	72.2%
Academia	12.4%
Government	8.2%
Private Research Center	3.1%
Hospital	2.1%
Other	2.0%

Their primary disciplines are:

Chemistry	82.1%
Biology	5.3%
Medicine	2.1%
Materials Science	1.1%
Other*	9.5%

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* includes biochem., polymer chem., toxicology, textile science, information science

Features that interest them:

Original Research	86.6%
LC News	55.7%
Reviews	52.6%
Topical Issues	26.8%
Book Reviews	26.8%
LC Calendar	26.8%

How thoroughly do they read the journal?

-8.8% read 90 - 100% of the papers
-61.5% read 25 - 75% of the papers
-28.6% read less than 25% of the journal

Almost all respondents (96.6%) read every issue of the Journal.

Each copy of the Journal is read by:

1-2 individuals	16.5%
3-5 individuals	27.8%
5-10 individuals	39.2%
>10 individuals	16.5%

Thus, more than half of the copies are read by five or more individuals. During 1982, when the total circulation was 2000 copies of each issue, an average of 12,500 individuals read the Journal.

In 1983, total circulation is 3000 copies of each issue. This translates to an average of 19,000 readers per issue, a substantial readership, indeed!

LC TOPICS OF INTEREST

Respondents to the Society indicated the following areas of primary interest:

Instrumentation	69.1%	Ion Chromatography	20.6%
Sample Prep'n	38.1%	Life Sciences	17.5%
Polymers/Plastics	36.1%	TLC	14.4%
Organic Analysis	34.0%	Food/Beverage	13.4%
LC Theory	29.9%	Agric. Chem.	12.4%
Derivatization	28.9%	Hydrodynamic Chrom.	11.3%
Aminoacids,Peptides,Proteins	26.8%	Forensic Analysis	9.3%
Pharmaceut.,Cosmetics	24.2%	Natural Products	8.2%
Data Handling	23.7%	Field-Flow Meth.	7.2%
Preparative LC	22.7%	Fossil Fuels	4.1%
Environmental	21.6%	Other*	5.2%

* Includes microbore technology, surtactants, multi-dimensional methods.

PERCEIVED STATURE AND QUALITY

	Agreed
"The Journal holds my interest"	92.9%
"Information in the Journal is accurate"	91.3%

"Papers in the Journal are well written"	89.9%
"The Journal is a prestigious place to publish"	73.2%
"The Journal provides professional leadership"	72.2%

CONCERNING ADVERTISEMENTS & PURCHASING

Although only 20.6% of the respondents said they approve purchases, a relatively large proportion told us they:

- determine need	79.4%
- select the supplier	66.0%
- evaluate products	59.8%
- establish specifications	46.4%

When questioned about the influence of advertisements in the Journal, they said:

- advertisements are informative	73.9%
- advertisements are useful	66.0%
- they contacted an advertiser as a result of seeing an ad in the Journal	32.6%
- made a purchase as a direct result of seeing an ad in the Journal	11.4%

Here's what they bought during the past year:

Solvents & Buffers	82.5%
Columns & Packings	81.4%
Chemicals	71.1%
LC instrumentation	63.9%
LC Books & Journals	57.7%
TLC Equipt/Materials	28.9%
Training Materials	6.2%

Dr. Jack Cazes
Editor

May we have your opinion?

Where do you primarily work? (one only)

_____ Industry _____ Academia _____ Private Testing Lab _____ Private Consultant
_____ Hospital _____ Government _____ Private Research Center _____ Other _____

What is your primary discipline? (one only)

_____ Chemistry _____ Physics _____ Biology _____ Other _____
_____ Medicine _____ Engineering _____ Materials Science

How many people read this copy of *Journal of Liqiud Chromatography?*

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_____ Book Reviews _____ LC News _____ LC Calendar

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_____ Hydrodynamic Chrom. _____ Life Sciences _____ Data Handling _____ Food/Beverage
_____ Polymers/Plastics _____ Forensic Anal. _____ Sample Prep'n. _____ Preparative LC
_____ Environmental _____ Theory _____ Natural Prod. _____ Fossil Fuels
_____ Derivatization _____ Ion Chrom. _____ TLC _____ Agric. Chem.
_____ Amino Acids, Peptides, Proteins _____ Other _____

Do you agree?	Agree	Disagree
Papers in *Journal of Liquid Chromatography* are well written	_____	_____
Journal of Liquid Chromatography provides professional leadership	_____	_____
Journal of Liquid Chromatography is a prestigious place to publish	_____	_____
Information in *Journal of Liquid Chromatography* is accurate	_____	_____
Advertisements in *Journal of Liquid Chromatography* are informative	_____	_____
Journal of Liquid Chromatography holds my interest	_____	_____

Are advertisements useful to you? _____ Yes _____ No

Have you used the reader service card? _____ Yes _____ No

Have you contacted an advertiser as a result of their ad in *Journal of Liquid Chromatography* _____ Yes _____ No

continued ▶

What have you purchased during the past year?

_____LC Instrumentation _____TLC/Equip't./Materials _____Training Materials

_____Solvents/Buffers _____Columns/Packings _____Other _____

_____LC Books/Journals _____Chemicals _____

Have you made a purchase because of a *Journal of Liquid Chromatography* ad? _____Yes _____No

Concerning purchasing, do you . . .

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_____Establish Specs? _____Select Supplier? _____Requisition Item?

May we have your name and address? (not required)

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SPECIFIC REFRACTIVE INDEX INCREMENT MEASUREMENTS ON MACROMOLECULES USING A WATERS R401 DIFFERENTIAL REFRACTOMETER

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Summit, New Jersey 07901

ABSTRACT

An accurate and simple method for determining the specific refractive index increment, dn/dc, of synthetic polymers during size-exclusion chromatography has been developed using a modified Waters R401 differential refractive index (DRI) detector. The only modification required on the R401 involved the replacement of the standard white light source with a monochromatic source. The use of this instrument offers a number of advantages over more classical instruments and procedures normally employed in evaluating this parameter. The only requirement which must be met in order to successfully measure this parameter is the conservation of mass (in terms of the amount of polymer injected on to the columm and which passes through the DRI detector). However, in some cases, even this requirement may be relaxed. The application of this procedure will be demonstrated for several synthetic polymers in mixed and single solvent systems.

INTRODUCTION

Intensity light scattering measurements represent a classical technique for obtaining molecular weight and shape information on macromolecules (1). Due to several experimental difficulties associated with this technique, it has in general not been suitable for routine analytical work. Hence its use has been limited. However, with the development by

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Kaye and co-workers (2,3,4) of a low-angle laser light scattering instrument with several novel features which eliminate or minimized most of these difficulties intensity light scattering measurement has become more popular, especially in its application to the area of size-exclusion chromotography (SEC) (5). With this renewed interest in light scattering (LS) has come a need for determining the refractive index increment (dn/dc), a key parameter used in light scattering theory and one which must be determined when new classes of polymers or old polymers in new solvents systems are investigated. Classically this parameter has been measured on a differential refractometer such as the Brice-Phoenix, and more recently, the Chromatix KMX-16, which are dedicated instruments for determining small differences in refractive index in a static mode. In conducting LS measurements on a new class of wholly aromatic polymers, which are only soluble at room temperature in a very strong mixed solvent and whose solutions were stable for only a short time ($\approx$ 1 day), a formidable problem in measuring the specific refractive index increment at constant chemical potential, $(\partial n/\partial c)_{\mu}$, was encountered. This parameter, however, was successfully evaluated by employing a modified Waters R401 differential refractive index (DRI) detector. Although this instrument finds wide use as a universal concentration detector in SEC and liquid chromatography, we have found no published use of this instrument for determining accurate dn/dc values of macromolecules. In using this instrument, it has become apparent that it offered a number of advantages over other differential refractometers. Hence the subject of this paper is concerned with the evaluation of this instrument in measuring dn/dc.

MATERIALS

A narrow distribution sample of polytetrahydrofuran, PTHF, having a molecular weight of 31,700 was obtained from Altex Scientific, Inc. Narrow distribution samples of polymethylmethacrylate, PMMA, and polystyrene, PS, having molecular weights of 92,000 and 200,000 respectively were obtained from Pressure Chemical Company. Broad distribution (MWD≈2) samples of poly (ethylene terephthalate), PET, having weight average molecular weight of 42,000 and 65,000 were obtained from Celanese Corporation. Tetrahydrofuran (THF), methyl alcohol (MeOH) and toluene were obtained from Burdick and Jackson Laboratories, Inc., and used as received. Hexafluoroisopropanol (HFIP) was obtained from DuPont Company and distilled once before use.

METHODS

Light scattering measurements were conducted with a Chromatix KMX-6 light scattering photometer. Static measurements of dn/dc and the refractive index increment at constant chemical composition, $(\partial n/\partial c)_c$, were made at room temperature at wavelengths of 546nm and 633nm using a Brice-Phoenix and/or a Chromatix KMX-16 differential refractometer.

SEC was conducted using a Waters M-45 pump to deliver solvent, a Waters U6K injector to apply samples on to the SEC-columns, and a Waters R401 DRI detector to monitor elutant. The light source on the R401 was replaced with a Hg-Vapor or quartz iodine lamp which was coupled to the detector using fiber optics. The emission line at 546nm and radiation centered at 633nm were isolated with bandpass

interference filters. Columns used in this work were either Waters 60Å μ-Porasil or 100Å μ-Styragel columns (which separate low molecular weight compounds). On some occasions, a Waters E-linear μ-Bondgel column was used with a 60Å μ-Porasil column. SEC traces were recorded on a Waters 730 data module.

The use of low molecular weight SEC-columns, either by themselves or in conjunction with other SEC-columns, causes the bulk solvent bathing the polymer sample and the low molecular weight additives and contaminates present in the sample to be well separated from the polymer peak (see Figure 1). This occurs as a result of the partitioning effect between the small molecules and the much larger polymeric material during the passage of the injected material through the porous packing material. Hence the area of the polymer peak in Figure 1 is directly proportional to the polymer mass injected on to the columns. Areas computed from DRI detector traces recorded during SEC experiments are related to the following parameters:

$$\text{Area} = (\beta)(\text{DRF})(dn/dc) \qquad \text{Eq. 1}$$

where β is a constant equal to the following collection of experimental known parameters:

$$\beta = \left[\begin{array}{c}\text{chart}\\ \text{speed}\end{array}\right]\left[\begin{array}{c}\text{DRI}\\ \text{detector}\\ \text{gain}\end{array}\right]\left[\begin{array}{c}\text{volume}\\ \text{injected}\end{array}\right]\left[\begin{array}{c}\text{concentration}\\ \text{of sample}\end{array}\right]\left[\begin{array}{c}\text{solvent}\\ \text{flow rate}\end{array}\right]^{-1}$$

Eq. 2

If the detector response factor, DRF, is known, one can calculate the dn/dc value for the polymer in the mobile solvent during

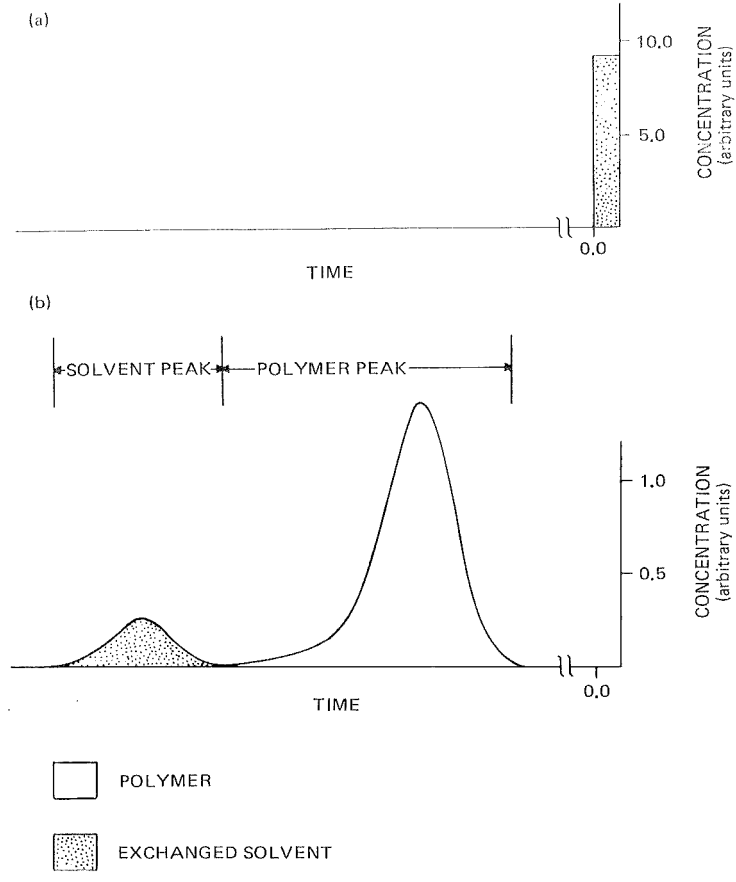

FIGURE 1. Hypothetical SEC-chromatograms of a polymer sample upon (a) injection and (b) elution.

SEC using Equation 1. Unfortunately, for a white light source, which is used in the standard Waters R401 DRI detector, DRF as well as dn/dc are both functions of wavelength, λ, (the former being a convolution problem of the intensity-wavelength spectrum of the light source and the response-wavelength spectrum of the detector). However, by using monochromatic light both DRF and

dn/dc become single value parameters permitting the use of Equation 1 to evaluate dn/dc by calibrating the DRI detector with a polymer having a known (k) dn/dc. Calibration is done by injecting an accurate volume and concentration of the polymer standard on a SEC-column bank. From several such injections, the average area is determined and combined with the known dn/dc and experimental terms contained within β. Similarly, a second equation can also be set up for the polymer sample having the unknown (unk) dn/dc. Since DRF is a constant for any given detector-light source combination and optical alignment, the combining of both equations eliminates the DRF term and on rearrangement yields the following equation for calculating the unknown dn/dc:

$$(dn/dc)_{unk} = \frac{(Area)_{unk}(\beta)_k}{(Area)_k(\beta)_{unk}} (dn/dc)_k \qquad \text{Eq. 3}$$

Since all the terms on the right side of Equation 3 are either measurable from data gathered or known, the unknown dn/dc can be evaluated. In performing the calibration, we have assumed conservation of mass (in terms of the amount of polymer injected on to the columns and which passes through the DRI detector), see Equation 2. It should be pointed out that the calibration procedure can be conducted in one solvent and dn/dc measurements conducted in another solvent (particular attention should be made in this situation to difference in flow rate between calibrating and experimental solvents).

RESULTS

The ability to accurately determine dn/dc values for macromolecules with the R401 detector during SEC was evaluated by com-

paring experimental values determined with this instrument to literature values. The polymers which we have chosen for this comparison work were PMMA and PTHF. The dn/dc value for both these polymers in THF at 546nm and 633nm were obtained from data listed by Huglin (6) and Chromatix (7). Actual experimental values determined with the R401 detector, using PS as our calibrating standard (the dn/dc values used for PS at 546nm and 633nm in THF were 0.194 (6) and 0.1845 (7) ml/g respectively), were found to be in good agreement with the literature values as shown in Table 1. A comparison of dn/dc values obtained for PET in HPIF using a Brice-Phoenix, KMX-16, and a R401 differential refractometers was also made. Date for this work is shown in Table 2. In this case, PMMA was used as the calibrating standard. The dn/dc value for PET in HFIP obtained with the R401 detector is in good agreement with the values determined using the other two instruments.

In situations where macromolecules are dissolved in a mixed solvent, preferential solution effects can seriously complicate dn/dc measurements (6,8,9). In this case, two important and different types of refractive index increments can be defined and measured. The first is measured at constant chemical composition, $(\partial n/\partial c)_C$. This simply involves measuring the refractive index difference between the mixed solvent and a solution made by the direct addition of the polymer sample to the mixed solvent. The second is measured at constant chemical potential, $(\partial n/\partial c)_\mu$. This requires the exhaustive dialysis of a polymer solution against the mixed solvent until thermodynamic equilibrium is attained. The refractive index difference between the dialyzed polymer solution and dialyzate is then determined. The difference

TABLE 1

Comparison of dn/dc Values Determined for PMMA and PTHF in THF with Literature Values.

Wavelength (nm)	dn/dc (ml/g)			
	PMMA		PTHF	
	Measured	Literature	Measured	Literature
633	0.084±0.002 *	0.083 **	———	———
546	0.087±0.005(4) §	0.0871±0.0001(2) †	0.063±0.004(4) †	0.063±0.001(2)

* Errors listed in this table are standard deviations.
** Data obtained from reference (7).
† The average value computed from data given in reference (6).
§ The number given within the parentheses represents the number of different samples used to calculate the mean.

TABLE 2

Comparison of dn/dc Determined on Several DRI Refractometers at 633nm for PMMA and PET in HFIP.

DRI Detectors	dn/dc (ml/g)	
	PMMA	PET
Brice-Phoenix	0.191	0.255
KMX-16	0.190	0.257
R401	———	0.259
Averages	0.191±0.001 *	0.257±0.004

* Errors listed in this table are standard deviation of the average value determined for each detector.

between these two refractive index increments for a binary solvent is given by the following relation:

$$(\partial n/\partial c)_{\mu} - (\partial n/\partial c)_{c} = \alpha \, (\partial n_{o}/\partial \phi) \qquad \text{Eq. 4}$$

Where α is the preferential absorption coefficient of solvent component 1 over the other solvent component, (this term is responsible for the difference in solvent composition near the macromolecule with respect to the bulk solvent) and $(\partial n_{o}/\partial \phi)$ is the change in the solvent refractive index with change in volume fraction of solvent component 1. Only in the case where either α is zero or all the solvent components have the same refractive index are these two refractive index increments the same. Hence, if the kinetics of exchange of the preferentially bound material on a polymer in a mixed solvent system is very rapid, in comparison to the length of time required for the polymer material to pass through the SEC column, the polymer sample which elutes from the column bank will be present in a solvent environment analogous to that which would be obtained by an exhaustive dialysis experiment. Hence the $\partial n/\partial c$ measured in this situation at any instant of time by the R401 detector would be equivalent to $(\partial n/\partial c)_{\mu}$ (assuming the pressure differentials between the column inlet and outlet are small or have little effect on $\partial n/\partial c$). This was experimentally verified by measuring both types of refractive index increments for a PS sample (NBS 705) of known molecular weight in a mixed solvent of 80% toluene and 20% methyl alcohol. The KMX-16 was used to determine $(\partial n/\partial c)_{c}$, and intensity light scattering measurements made during SEC experiments (see Figure 2) were used to determine $(\partial n/\partial c)_{\mu}$ by using the following equation:

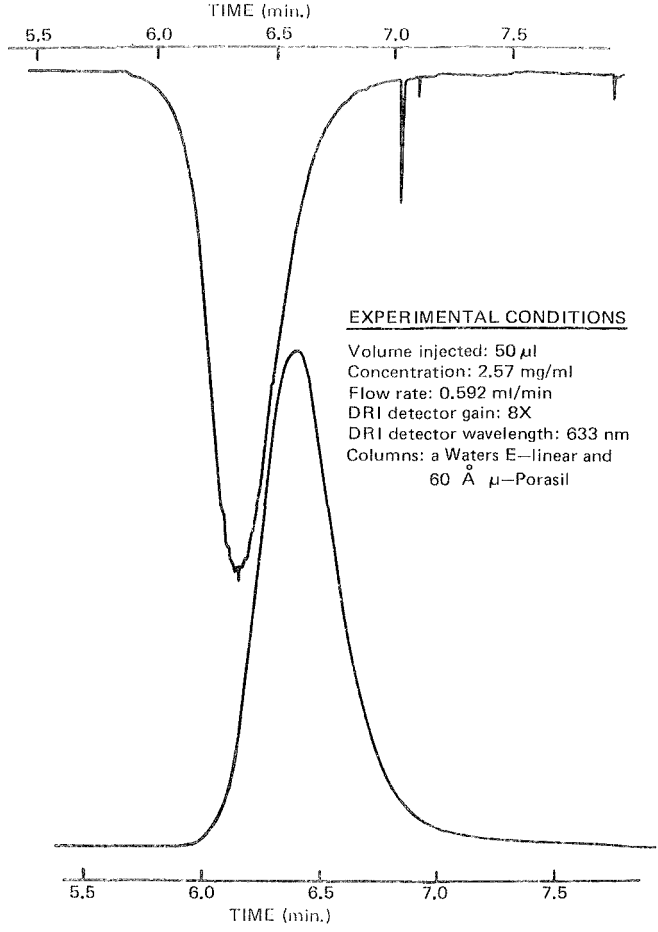

FIGURE 2. SEC-chromatograms of the NBS 705 polystyrene sample obtained from the modified R401 DRI detector (bottom) and the KMX-6 LS photometer (top) in toluene/methyl alcohol (80:20).

$$(\partial n/\partial c)_{\mu} = \left[\overline{R}_{\theta}/\overline{M}_{w}k'c\right]^{1/2} \qquad \text{Eq. 5}$$

where $\overline{R}_{\theta}$ is the excess Rayleigh ratio, $\overline{M}_{w}$ is the weight average molecular weight, K' is an optical constant, and c is concentration. The second and higher virial terms in equation 5

TABLE 3

$(\partial n/\partial c)_c$ and $(\partial n/\partial c)_\mu$ Values Determined for PS in Toluene: Methanol (80:20) at 633nm.

INSTRUMENT	KMX-16	LS	R401
Types of $(\partial n/\partial c)$	$(\partial n/\partial c)_c$	$(\partial n/\partial c)_\mu^*$	$(\partial n/\partial c)_\mu$
Experimental Value (ml/g)	0.150 ± 0.006 (3) † §	0.209 ± 0.009 (7)	0.207 ± 0.007 (3)

* The $\overline{M}_w$ determined at the National Bureau of Standards by LS was 179,300, and by sedimentation equilibrium was 189,800. Hence the average of 184,600 was used in Equation 4.

† Errors listed in this table are standard deviations.

§ The number given within the parentheses represents the number of different samples used to calculate the mean.

were neglected due to the dilute concentrations used and their low values. The refractive index increment determined with the R401 detector, using PS in THF as the calibrating standard, is shown in Table 3 to be in good agreement with the experimentally measured value for $(\partial n/\partial c)_\mu$.

DISCUSSION

In a review given by Huglin (6) on specific refractive index increment measurements, the Waters model R4 (which is now referred to as the model R401) DRI detector was mentioned as one of many commercially available differential refractometers capable of being used to evaluate dn/dc. However, the lack of monochromatic light and the mode in which the sample is introduced into the instrument were listed by this author as major drawbacks in using it in measuring dn/dc. In this paper, we have demonstrated the overlooked capability of this instrument to accurately deter-

mine dn/dc values of macromolecules during SEC. In so doing, we have realized that in comparison to other differential refractometers, this instrument offers the following advantages:

1. A very small amount of sample (microgram quantities) is required to conduct measurements; (this is especially important in dealing with biopolymers).

2. Baseline (or solvent) measurements are continuously monitored both before and after polymer material is eluted from the detector.

3. The amount of time required to perform measurements is very short, the procedure itself is very simple and readily automated using any automatic injector.

4. In performing LS measurements during SEC, both $\overline{M}_w$ and dn/dc values can be determined from the same experimental run.

5. The R401 detector is inexpensive in comparison to other differential refractometers. In addition it is not dedicated to static measurements, but can be used as a detector in liquid chromatography techniques.

6. This procedure allows $(\partial n/\partial c)_\mu$ and preferential solution effects to be evaluated in mixed solvent systems without the need to conduct lengthy dialysis experiments. This is particularly important in cases where chemically resistant membranes are not available and where polymer-solvent systems are stable for only a

short period of time. Although the concept of studying preferential solvation during SEC with a DRI detector has already been demonstrated by Berek et al (10) and Campos et al (11), the procedure outlined in this paper, which makes use of the polymer peak, differs in approach to that used by Berek et al, and Campos et al, which uses the solvent peak (see Figure 1). By using the polymer peak, we have eliminated potential problems discussed by Berek et al, and problems which might arise from the presence of contaminants in the sample solution (such as air, water, etc.) which would elute in the solvent peak region and lead to erroneous results.

Although the procedure in this paper offers the above advantages in determining dn/dc values, several important assumptions were necessary. This includes the following:

1. Conservation of mass is valid for the polymer-solvent-column resin system used.

2. Pressure effects on dn/dc are negligible.

3. Thermodynamic equilibrium between polymer and solvent components is reached before polymer is eluted from the column.

In the case where assumption "1" is in question or is known not to be valid the use of a second detector, such as a UV-Vis or IR spectrophotometer (if the macromolecule contains a chromophore and if the solvent is transparent at the appropriate spectral

regions), which is capable of giving concentration values from known extinction coefficients should permit the correct concentration to be determined. In fact, point by point dn/dc values across the chromatogram can be calculated. In the case where assumptions "2" and "3" are suspected, measurements conducted over a wide range of flow rates should allow the correct dn/dc value to be determined by extrapolation to conditions corresponding to zero flow rate. The extrapolated value would be equivalent to conditions of infinite time and zero pressure differential between the column inlet and outlet.

In conclusion, we have clearly demonstrated the ability of the Waters R401 detector to correctly determine dn/dc values for macromolecules. Although the accuracy of this procedure is 1-2% less than that capable from static measurements using standard differential refractometers, which is due mainly to the greater accuracy in the calibrating salt solution standards used for static type measurements in comparison to the calibrating polymer solutions used in this procedure, the advantages gained more than outweight this small loss in accuracy.

ACKNOWLEDGEMENTS

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATIONS USING SHORT COLUMNS PACKED WITH SPHERICAL AND IRREGULAR SHAPED ODS PARTICLES*

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ABSTRACT

In a comparative study of the separation of a mixture of polycyclic aromatic hydrocarbrons on 5 cm and 10 cm columns packed with 3μ and 5μ ODS spherical particles, the separations achieved on the 10 cm column were not significantly better than those on the 5 cm column. Although, columns packed with 3μ ODS spherical particles gave slightly better resolution than those packed with 5μ ODS spherical particles having the same physical properties, differences were observed when columns packed with ODS spherical particles were compared with columns packed with ODS irregular shaped particles of the same size.

The results show that separations on a 5μ or 3μ packed columns takes place at the first few centimeters.

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INTRODUCTION

The last decade witnessed the emergence of high performance liquid chromatography (HPLC) as a powerful and indispensable separation technique.

Initially, long and narrow columns were used (50 cm x 2 mm). As time passed the dimensions of the column changed. The length shrinked to 3-5 cm and the internal diameter to 4-6 mm. This is due to the change in particle size of the packing material (stationary phase) from 35μ to 3μ which resulted in high back pressure and greater efficiency. The higher efficiency of the small particles permitted the use of shorter columns.

The advantage of the shorter columns over 25-30 cm columns packed with 5μ or 10μ particles is the speed of analysis which results in savings of materials and time. Katz and Scott (1) described an HPLC system in which the analysis was completed in less than 1 minute. However, to achieve such rapid analyses, special equipment was required.

The objectives of this study are (a) to compare the separation of a five component mixture on 5 and 10 cm columns packed with 3 and 5μ spherical particles using standard HPLC equipment without modification, (b) to compare a 5 and 10 cm columns packed with 5μ spherical ODS particles and 10 and 25 cm columns packed with irregular ODS particles, and finally (c) to see where separation takes place on the column.

It must be noted that to compare columns, especially from different manufacturers, is not without pitfalls. However, care has been taken to pack the columns under the same conditions, and using materials, (reversed phase silica) from the same manufacturer with the same physical properties, (Table 1). The 3 and 5μ Spherisorb packings used in each of the 5 and 10 cm columns were from the same batch.

EXPERIMENTAL

Materials: Perylene, benzo(a)pyrene, benz(a)anthracene, methyl cholanthrene and coronene were received from the chemical carcinogens reference standard

TABLE I

Physical Properties of 3 and 5μ Spherisorb ODS Packings*

Mean particle size (μ)	5	3
Size distribution (±1μ)	70%	95%
Pore size (nm)	8	8
Pore diameter (nm)	5.5-11	5.5-11
Surface area (m^2/g)	220	220
ODS capacity (m mol/g)	~ 0.3	~ 0.5

*Data supplied by Phase Sep., 255 Oser Avenue, Hauppauge, NY

repository, function of the Division of Cancer Cause and Prevention, NCI/NIH, Bethesda, MD 20205. Acetonitrile was glass distilled (Burdick & Jackson).

Apparatus: A modular HPLC system consisting of Laboratory Data Control (LDC) constametric I and II pumps attached to an LDC Gradient Master, a Chromatronix dual-channel UV absorbance detector (254 and 280 nm), a Rheodyne injector, and a strip-chart recorder operated at 0.2 in/min was used.

Three sets of columns were used of which two sets (50 mm x 4 mm and 100 mm x 4 mm) were each packed with 3 and 5μ Spherisorb ODS packings obtained from Phase Sep., (see Table 1 for packing physical properties). The third set (100 mm x 4 mm and 250 mm x 4 mm) of columns were packed with irregular shaped ODS materials having a mean diameter of 5μ, a surface area of $400m^2/g$, a pore diameter of 80 A°, 10% total carbon and 95% silanization, obtained from Whatman, Inc.

The experiments were run at room temperature using a mobile phase of 75% acetonitrile/water at a flow rate of 1 ml/min. unless specified. Ten μl

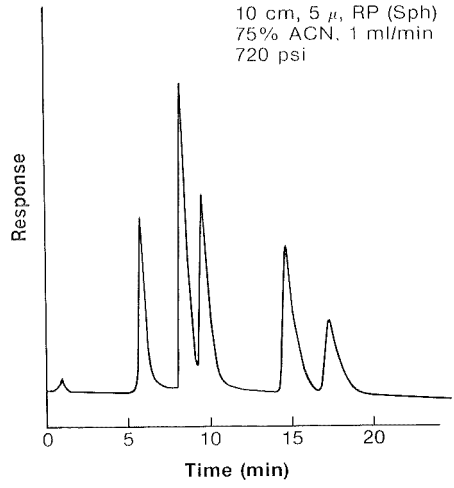

Figure 1. Separation of perylene, benzo(a)pyrene, benz(a)anthracene, methyl cholanthrene and coronene of a 10 cm long column packed with 5μ Spherisorb ODS. 75% acetonitrile/water was used as the mobile phase.

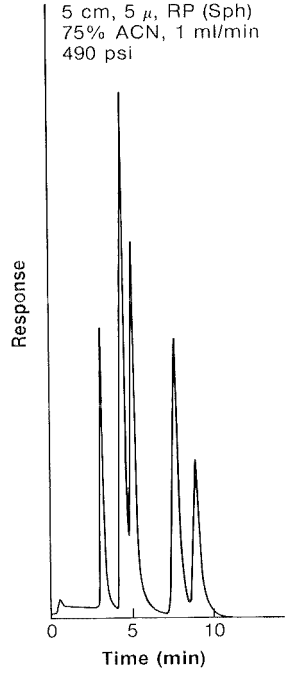

Figure 2. Same as Figure 1, but 5 cm column.

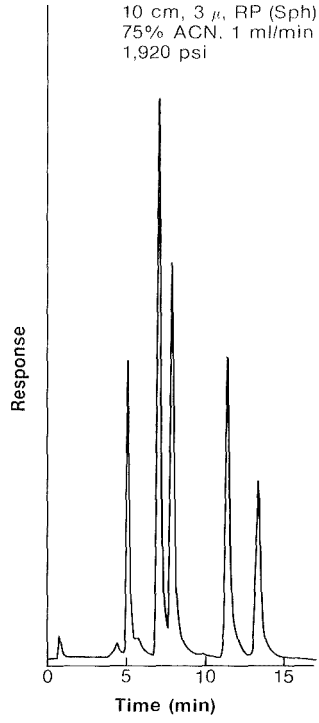

Figure 3. Same as Figure 1, but 10 cm column packed with 3μ Spherisorb ODS.

sample solutions was injected. The mobile phase was degassed before use. No modifications of any kind were made on the instrument to accommodate the requirements of the short columns.

Column Packing: Supports were slurry packed into columns with acetone: acetonitrile (1:1), at 8000 psi by the upward technique using a Haskel pneumatic pump.

RESULTS AND DISCUSSION

The objective of this study was to examine the effect and possibility of using short columns with standard HPLC equipment. The results (Figures

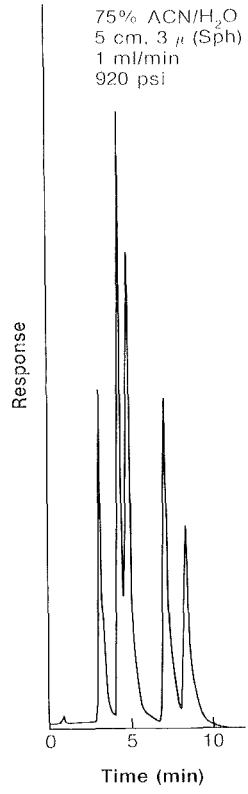

Figure 4. Same as figure 3, but 5 cm cloumn.

1-4) indicate that, other than peak broadening, due to the 100 ul injector loop and the long tube connections to the column and the detector, the short columns can be adapted to standard instruments with a small loss of resolution which may not affect the results drastically.

Figures 1 and 2 show the chromatograms from 10 and 5 cm columns packed with 5μ ODS Spherisorb material. It is clear, from the figures, that in both columns the mixture was separated into its five components. However, the peaks were broader with the 10 cm column. When back pressure, peak heights and retention times are compared, the result favor the shorter

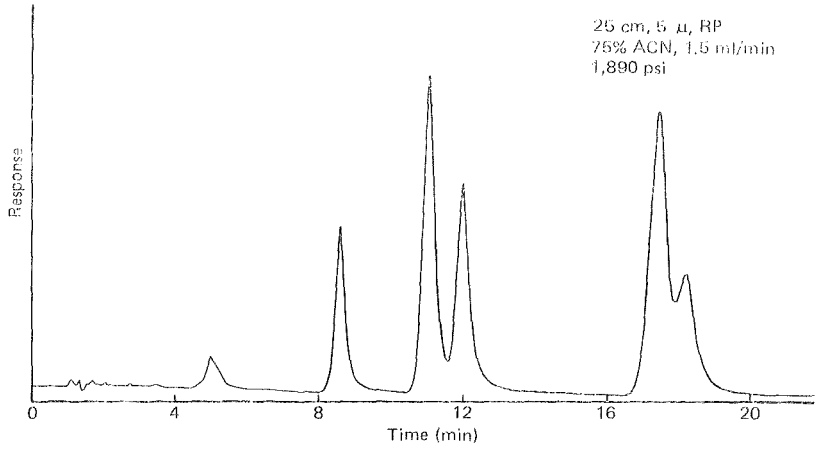

Figure 5. Same as Figure 1, but 25 cm column packed with 5μ irregular shape ODS.

column by approximately 2 to 1. It was quite surprising that the 5 cm column performed as well as the 10 cm column. This was also true when the same test mixture was injected into the 10 cm and 5 cm columns packed with 3μ ODS Spherisorb material (Figures 3 and 4). However, the peak heights differential, i.e., sensitivity, observed with 5μ packed columns (Figures 1 and 2) was absent here.

When the results obtained using the 3μ and 5μ packed columns were compared, no significant differences in separation was observed. The differences observed were in retention times and back pressures. Otherwise each set of columns separated the five components mixture. The chromatographer can probably double the flow rate, using the 10 cm column packed with 5μ particles, without any loss of resolution, but shortening the retention times.

The results with the 10 and 25 cm columns packed with irregular 5μ ODS materials (Figures 5 and 6) were different from those using 3 and 5μ Spherisorb packings (Figures 1-4). The 25 cm column (Figure 5) did not give significantly improved resolution when the results are compared with those with the 10 cm column (Figure 6).

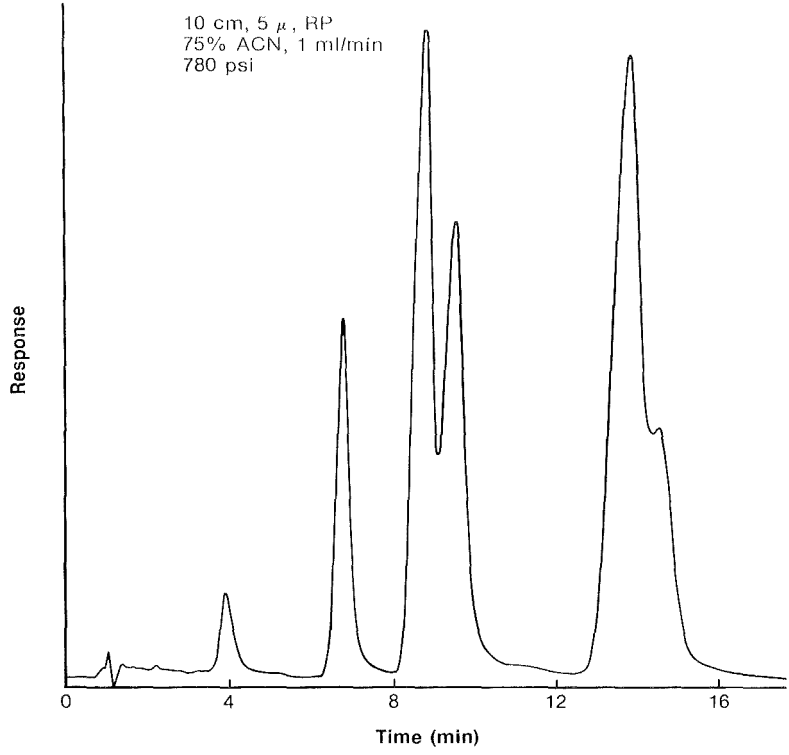

Figure 6. Same as Figure 5, but 10 cm column.

CONCLUSION

The results showed that when the physical properties of the particles are closely related, the differences in the results with 3 and 5μ particles or 5 and 10 cm columns is minimal. The results also showed that there was a difference, under our experimental conditions, between columns packed with spherical and irregular particles. This is because both particles were manufactured under different conditions by different manufacturers. This may be due to carbon loading,particle diameter and particle size distribution and end capping.

It is also clear that separations take place in the first few centimeters of a column, and increasing the length of the column does not improve the resolution significantly, although this will increase the retention time and back pressure. This phenomenon has been reported by Regnier (2) for large molecules.

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SOLVATION AND ADSORPTION EFFECTS IN GEL PERMEATION CHROMATOGRAPHY

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ABSTRACT

Saturated hydrocarbons are generally eluted according to molecular volumes in gel permeation chromatography(GPC). Other compounds, containing polar groups such as hydroxyl and carbonyl groups, deviate from the molecular volume/elution count relationship which is prepared using saturated hydrocarbons. The deviation is more or less observed regardless of the kinds of eluents. In this paper, this problem is investigated in detail with respect to infrared(IR) and proton magnetic resonance(NMR) spectra, and concentration dependences of peak heights and elution volumes in GPC. IR and NMR spectra reveal that alcohols, ketones and esters are strongly solvated by eluent molecules such as chloroform and tetrahydrofuran(THF). The solvation effect leads to faster elution for these compounds than expected for aliphatic hydrocarbons. On the other hand, the concentration dependences of elution counts and peak heights prove the adsorption of amines on polystyrene gel in chloroform. In fact, the elution rates of amines and polychlorides are retarded. In the case of aliphatic carboxylic acids, the elution mechanism is more complicated: association and adsorption effects would be overlapped.

INTRODUCTION

Many organic compounds such as ketones and alcohols are eluted at lower elution counts than expected from data for aliphatic hydrocarbons, as reported previously.[1)] The phenomenon is assumed to be due to solvation by eluent molecules. On the other hand, some other organic compounds such as amines and polychlorides are eluted at higher elution counts. This case is assumed to be due to adsorp-

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tion of solute molecules on polystyrene gel. However, no evidence other than deviation of elution volume has been presented yet. The solvation phenomenon is detectable by observing the wavenumbers of absorption bands due to polar groups in IR spectra, or by the δ values of peaks in NMR spectra. For example, the stretching vibration band of hydroxyl groups should appear from 3200 to 3600 $cm.^{-1}$ If solvation occurs, the band will appear at a lower wavenumber. If adsorption of solute molecules occurs, the peak height or the area under the peak will not be proportional to the concentration, because some amount of solute molecules will stay longer in columns than remaining molecules. Some other anomalous behavior would be expected.

EXPERIMENTAL

GPC

A Shimadzu Model 1-A gel permeation chromatograph was used, equipped with the two columns which were packed with cross-linked polystyrene gel. One column had a nominal exclusion limit of 10^{8}Å and the other 10^{2}Å. Each column was 4ft in length and 0.305in. in inner diameter. The instrument was operated using o-dichlorobenzene(ODCB) as eluent with a flow rate of 1.0ml/min at ambient temperature and 80°C. One milliliter of a 1.0% ODCB solution of sample was injected into the columns. One count corresponded to 5ml of eluent, which was measured by a siphon.

Another instrument was used for investigating concentration dependences of peak heights and elution counts in chloroform. A Toyosoda HLC-807 high speed liquid chromatograph was used euipped with the two columns($G2000H_8$) which were packed with cross-linked polystyrene gel. The columns had a nominal exclusion limit of 250Å. Each column was 2ft in length and 0.305in. in inner diameter. The instrument was operated with a flow rate of 1.0ml/min at ambient temperature. One hundred microliter of a 0.5% chloroform solution of sample was injected into the columns.[1] In this case, one count corresponded to 128 drops of eluent coming out from a special nozzle which forms a part of an eluent volume detector. This count also corresponded to nearly 0.74ml of eluent.

IR spectroscopy

A Hitachi Model EPI-G3 infrared spectrophotometer was used with liquid NaCl cells of 0.5mm thickness. Measurements were done at a concentration 1 to 2g/dl in chloroform, or in Nujol for solid compounds. Infrared spectra of neat samples were measured by spreading on NaCl plates or as a Nujol mull. Calibration was accomplished by referring the spectra to that of a thin film of polystyrene.

NMR spectroscopy

A Nihondenshi Model JNM4H-100 high resolution NMR spectrometer was used to determine the chemical shifts in ^{1}H-NMR. NMR spectra should be measured at 1% concentration, that is, the same concentration as in GPC. However, at such low concentrations, NMR spectra are not readily obtainable. A concentration of 5% was usually provided for the measurement. Tetramethylsilane($Si(CH_3)_4$) was used as an internal standard.

Samples and solvents

Aliphatic and aromatic hydrocarbons were supplied by various chemical producers. Esters and alcohols were supplied by Applied Science Laboratories Inc.,(State College, Pa., U.S.A.), and ketones by Polyscience Corp.,(Niles, IL, U.S.A.). ODCB is a special grade purchased from Kishida Chemical Co.,(Higashi-ku, Osaka, Japan).

RESULTS AND DISCUSSION

Elution behavior in ODCB

The elution behavior in chloroform was shown in the previous paper.[1)] To understand the elution behavior it is very valuable that measurements are done in different eluents. Logarithmic molecular volumes log V_M were plotted against elution counts by the same way as in the previous paper,[1)] where V_M equals M/d (M: molecular weight, d: density) in this discussion. The result at ambient temperature is shown in Figure 1. The extent of deviation of the elution volumes is much smaller in ODCB than was previously reported in chloroform.[1)] The elution behavior at 80°C is shown in Fig-

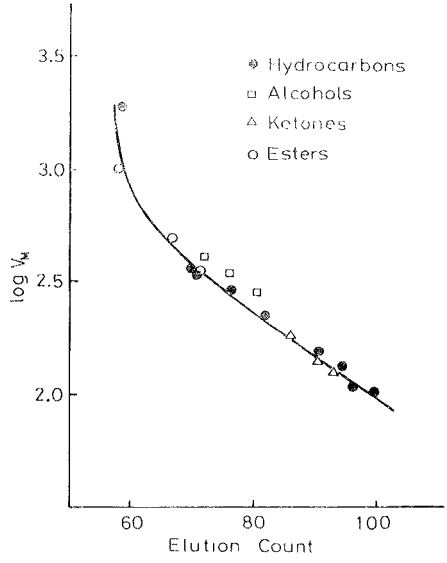

FIGURE 1. Logarithmic molecular volumes vs. elution counts in ODCB at ambient temperature. Two columns having nominal exclusion limits of 10^3 and 10^2 Å were used.

ure 2. Experimental points seem to fall on the same curve as obtained for aliphatic hydrocarbons. However, this apparent agreement comes from the decrease of resolution, because only one column having a nominal exclusion limit of 10Å was used at 80°C. It is difficult to compare exactly the result shown in Figure 1 with that in Figure 2. In that sense, these results do not involve so detailed information on the temperature dependence of elution counts as presented by Cantow et al.[2] At any rate, it is sure that there is no substantial change of experimental results at high temperature. If solvation was the major factor, the deviation should disappear or decrease at high temperature. We conclude that the deviation is caused by solvation, but by adsorption of solute molecules.

Elution behavior depends upon the kind of eluent. Moreover, we cannot expect to prepare a universal calibration for low molecular weight compounds even if any eluents were carefully selected.

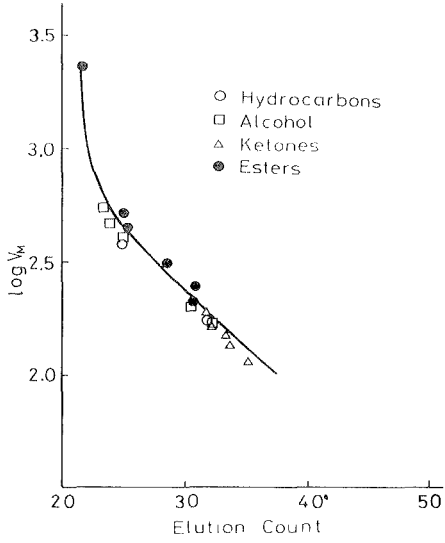

FIGURE 2. Logarithmic molecular volumes vs. elution counts in ODCB at 80°C. One column having a nominal exclusion limit of 10^4 Å was used. This column was different in history from that used for experiment of Figure 1.

Solvation

In the previous paper, we suggested that solvation and adsorption are the major factors by which the deviation occurs in GPC elution behavior. Concerning solvation, NMR and IR spectra reveal directly the presence of hydrogen bonding between solutes and eluent molecules. Various cases are demonstrated in Table 1. For example, stearyl alcohol has an absorption band at 3323.5cm^{-1} due to hydroxyl group, when the spectrum is measured for neat sample. This low frequency is the result of intermolecular hydrogen bonding. The same compound in a 1% solution shows the absorption band at 3445.9cm^{-1} in chloroform, at 3465.2cm^{-1} in THF and 3607.0cm^{-1} in ODCB. These wavenumbers reveal that stearyl alcohol is solvated strongly by chloroform and THF. NMR spectra also clearly show this tendency. The signals of hydroxyl group lie at lower magnetic field in chloroform and THF than in ODCB. Solute/solute interac-

tion may be taken into account. However, the fraction of eluent in the solution is almost 100%. Solute/eluent interaction is more predominant than solute/solute interaction in their probabilities if we assume that interaction forces are similar.

In Table 1, the estimated results from IR and NMR spectra are shown in the column, "solvation." The extent of the deviation observed in GPC is shown in the column, "deviation." In most cases, the agreement between the estimated deviation, i.e., "solvation," and the observed deviation, i.e., "deviation," is good. This fact clearly proves that elution volumes are largely influenced by solvation between solute and eluent molecules. Large discrepancies between the estimated and observed deviation are, however, present for amines and carboxylic acids, especially when chloroform is used as eluent. In the former, the elution volumes are smaller than in hydrocarbons. This behavior is completely different from that expected from IR and NMR spectra. This fact suggests that the adsorption effect is more predominant than solvation in this system.

Free carboxylic acids generally do not exist in solution. Even in fairly diluted solution, carboxylic acids exist as dimers.[3] IR and NMR spectra do not simply reflect the phenomenon of solvation by eluent molecules in this case. Carboxylic acids should be eluted at considerably lower counts than expected from hydrocarbons, since the dimer is the most probable structure in solution. However, these compounds are eluted at almost the same elution counts as corresponding hydrocarbons, as compared with amines and esters.[1] This abnormal phenomenon would be due to the adsorption of solute molecules on the gel surface.

Adsorption

To confirm the adsorption effect, the following experiments were carried out in amine/chloroform system. If adsorption of solute molecules takes place on the gel surface, the peak heights in GPC will not be proportional to the concentration of solutes because of retardation of some amount of solute molecules. The concentration dependence of peak heights was measured for various compounds. Figures 3 and 4 show these results. In n-octadecane and toluene,

TABLE 1 Comparison of NMR and IR Data with Elution Behavior

Compound		Solvent			Neat sample
		$CHCl_3$	C_4H_8O**	C_6H_4Cl	
Stearyl Alcohol	$\nu_{OH}(cm^{-1})$	3445.9	3465.2	3607.0	3323.5
	δ(ppm)	3.72	...	3.64	
	Solvation*	M	M	S	
	Deviation*	M	L	M	
Stearic Acid	$\nu_{C=O}(cm^{-1})$	1706.7	1736.3	1710.2	1702.3
	δ(ppm)	2.36	2.10	2.28	
	Solvation*	L	S	M	
	Deviation*	S	L	M	
Stearyl Amine	$\nu_{NH}(cm^{-1})$	3375.0	3561.0	3375.0	3323.5
	δ(ppm)	1.58	1.46	1.64	
	Solvation*	M	S	M	
	Deviation*	-L	S	S	
2-Octanone	$\nu_{C=O}(cm^{-1})$	1709.4	1718.4	1714.5	1718.9
	δ(ppm)	2.16	2.04	2.04	
	Solvation*	L	S	M	
	Deviation*	L	S	M	
Methyl Arachidate	$\nu_{C=O}(cm^{-1})$	1727.6	1740.0	1730.5	1742.8
	δ(ppm)	2.34	2.55	2.26	
	Solvation*	L	S	M	
	Deviation*	L	S	M	

*: L > M > S, **: the data on "deviation" is quoted from reference (4).

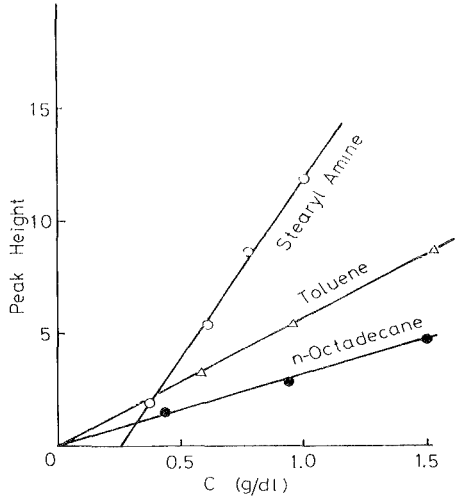

FIGURE 3. Concentration dpendence of peak heights in chloroform.

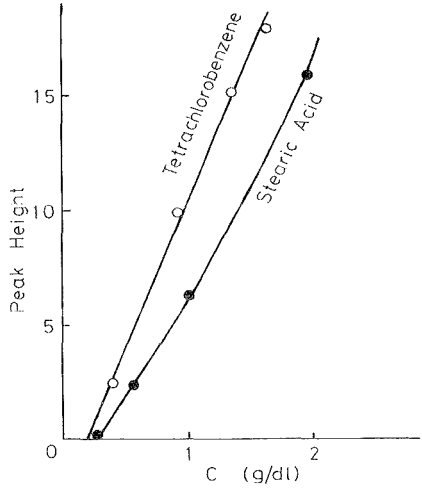

FIGURE 4. Concentration dependence of peak heights in chloroform.

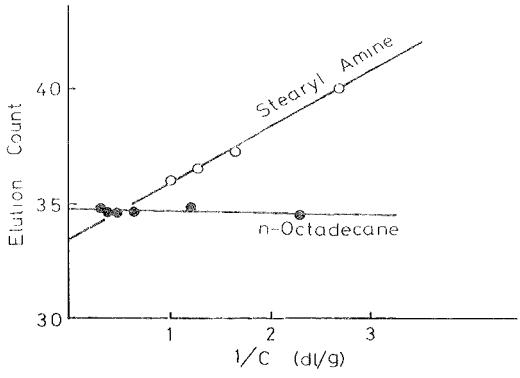

FIGURE 5. Concentration dependence of elution counts in chloroform.

the peak heights are completely proportional to the concentration. These straight lines pass through the origin. In stearyl amine, 1,2,4,5-tetrachlorobenzene and stearic acid, the peak heights vs. concentration curves, however, do not pass through the origin. These figures demonstrate that the peak of the compounds disappears at a certain concentration. The solúte molecules are completely adsorbed on the gel surface at the concentration where the peak disappears.

As shown in Figure 5, the elution counts in stearyl amine decreased with increasing concentration: the elution volume is considerably increased by adsorption at low concentration. On the other hand, the behavior in octadecane is contrary to that of stearyl amine and is rather normal for GPC.[5] The concentration dependence of elution counts also proves the occurence of adsorption in stearyl amine.

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RAPID AMINO ACID ANALYSIS IN BIOTECHNOLOGY: DETERMINATION OF ALANINE AND ASPARTIC ACID

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ABSTRACT

A fast and sensitive chromatographic method was developed to monitor the enzymatic conversion of aspartic acid into alanine in a membrane reactor. The amino acids were converted into dansyl derivatives which were separated by ion-pair chromatography on a reversed-phase column and detected by fluorimetry using only conventional HPLC equipment.

INTRODUCTION

The use of biotechnological processes to produce amino acids has recently been gaining in importance (1). Their main advantage in comparison with most chemical syntheses is that pure enantiomers are formed. L-amino acids are produced not only using cultures of microorganisms, but also with isolated enzymes as reaction catalysts (2). For example, L-amino acids are produced very successfully by using soluble enzymes in an enzyme-membrane reactor where the substrate solution is pumped continuously into a stirred tank reactor. Ultrafiltration is used to retain the enzymes in the reaction vessel and to allow the amino acids formed to be collected in the effluent (3,4). L-α-alanine is produced from L-aspartic acid by the soluble enzyme L-aspartate-ß-dicarboxylase. Measuring the optical rotation of the products is one way to record the conversion (5). This method, however, is problematic in low

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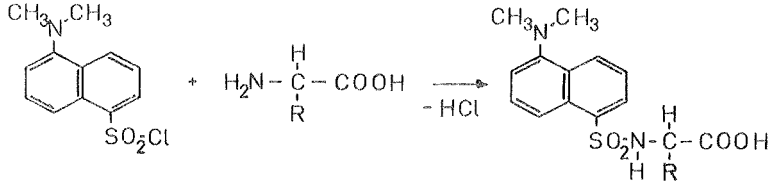
concentration ranges (c $\ll$ 100 mM) and is hampered by problems like bubble formation (CO_2) and protein precipitation.

Chromatographic methods can be used for measuring process kinetics if run in a repetitive way. A chromatographic method suitable for amino acid analysis of the effluent of a membrane reactor has to be fast to allow analysis of a large number of samples and sensitive to allow measurements of the beginning and end phase of the reaction with low concentrations of the end product and of the precursor. In spite of its routine use for amino acid analysis, ion-exchange chromatography is too slow to be used in the monitoring of the conversion in a membrane reactor and, furthermore, accumulation of contaminants in the column necessitates frequent regenerations. Systems with column-switching could overcome some of these problems.

Reversed-phase columns are, by far, most frequently used in HPLC. However, compounds like polar amino acids are hardly retained on these columns. Therefore, pre-column derivatization has to be performed to convert amino acids into more unpolar derivatives which also can be more easily and sensitively detected. The main disadvantage of pre-column derivatization is the time and labor necessary to treat the samples. However, preparation time per sample reduces with increasing numbers of samples.

Conversion of amino acids into fluorescent dansyl derivatives (see equation below) has been used to improve chromatographic separations and to enhance sensitivity (6, 7).



dansyl chloride + amino acid ⟶ dansylated amino acid

There are several publications on the separation of complex mixtures of dansyl amino acids (8 - 12). The task of this investigation, however, was to find out conditions which allowed the analysis of just two amino acids in the shortest possible time. We now describe the use of the dansylation technique in combination with high-pressure liquid chromatography for the rapid and sensitive monitoring of amino acid conversion in a membrane reactor.

MATERIALS AND METHODS

Apparatus

A Waters (GmbH) M 6000A pump was connected with a U6K injector and a μ-Bondapak C18 column (30 x 0.4 cm, 10 μm particles, octadecylsilyl phase). Detection was achieved with a fluorescence detector (Du Pont 836) equipped with a 16 μl flow cell. The excitation wavelength was λ = 250 - 390 nm while emitted light passed through a cut-off filter of λ = 451 nm. Signals were recorded by a Laumanr (Selb, Bavaria) recorder and processed by a Spectraphysics System I Computing Integrator.

Chemicals

Acetonitrile was p.a. grade (Merck). It was distilled prior to use. L-amino acids were purest available grade (Sigma). Other chemicals were p.a. reagents (Merck) with the exception of tetrabutylammonium bromide (Fluka).

Sample Derivatization

A modification of the method described by Tapuhi et al. (13) was used. In a glass vial with a teflon-lined screw cap, 10 μl of a sample containing up to 0.2 M amino acid were mixed with 1 ml dansyl chloride solution (3 mg/ml in acetonitrile) and 2.0 ml buffer containing the internal standard (40 mM Li_2CO_3, adjusted to pH 10 with HCl, 1.0 mM glycine). The solution was

kept 15 min at 60 °C. Thereafter, 100 µl of proline solution (5 % in water) was added to bind the excess of dansyl chloride, and the reaction was completed at 60 °C (5 min). After cooling to room temperature, 25 µl of this mixture was injected for analysis. The derivatized samples are sufficiently stable at room temperature and in the dark.

Eluent Preparation and Chromatographic Conditions

The eluent consisted of acetonitrile (32 %, 34 %, 36 % or 39 %) and aqueous buffer. To prepare the aqueous buffer, 100 ml of a stock solution (aqueous solution of 40.8 g sodium acetate x 3 H_2O and 16.1 g of tetrabutylammonium bromide in 1000 ml) were mixed with 0.9000 to 5.400 g acetic acid and brought to 1000 ml with water. These concentrations correspond to 30 mM sodium acetate, 5 mM tetrabutylammonium bromide, and 15 - 90 mM acetic acid. The eluent selected for amino acid analysis consisted of 36 % acetonitrile (v/v) and 64 % aqueous buffer (30 mM sodium acetate, 45 mM acetic acid, and 5 mM tetrabutylammonium bromide).

HPLC was performed at room temperature (21 °C) and with a flow of 2.0 ml/min.

Determination of Amino Acid Concentrations

A calibration curve was obtained with standard solutions of 0 - 0.2 M amino acid, derivatization as described above and by measuring the peak areas. Amino acid concentrations of samples were calculated using the program of the integrator (method 2: Internal Standard).

RESULTS AND DISCUSSION

For the reliable analysis of aspartic acid and alanine by HPLC of the dansyl derivatives, the derivatization procedure as well as the chromatographic separation had to be optimized.

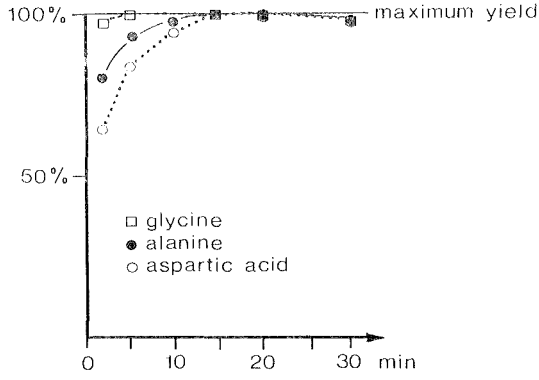


FIGURE 1. Time course of the reaction of aspartic acid, alanine and glycine with dansyl chloride.

Previous problems with varying yields depending on the excess of dansyl chloride in relation to the amino acid concentration have been mostly overcome by the use of acetonitrile as solvent and lithium carbonate as buffer component in the derivatization procedure (13). In order to establish a stable baseline and to increase the stability of the reaction products, it was found that binding of the excess of dansyl chloride is important. The use of an internal standard increased the accuracy of the method considerably. A suitable internal standard had to show similar reactivity with all compounds analyzed. Glycine has a similar reaction rate with dansyl chloride when compared with alanine and aspartic acid (14) and was, therefore, chosen as internal standard. As can be seen from figure 1, the yield of the dansylation reaction of the amino acids reached the maximum after 5 - 15 minutes. A reaction time of 5 minutes was found sufficient for analysis. Proline, which was used to bind the excess of dansyl chloride, reacted within seconds under the reaction conditions applied.

The separation of dansyl derivatives of amino acids on reversed-phase columns has been achieved mainly using phosphate or acetate buffers with acetonitrile or methanol as organic

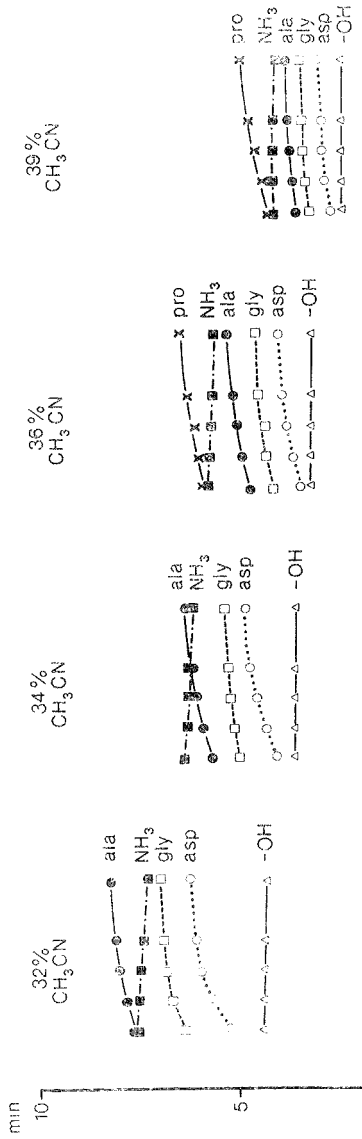


FIGURE 2. Retention of dansyl derivatives with varying eluent composition.

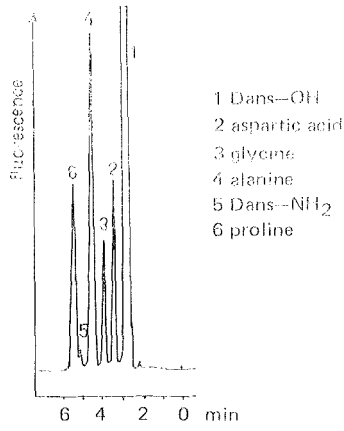


FIGURE 3. HPLC chromatogram of a dansylated sample containing alanine, aspartic acid, glycine (internal standard) and proline (dansyl chloride scavanger).

modifiers (8 - 12). In order to resolve all 20 protein amino acids, gradient programs had to be applied. For the complete resolution of the amino acids investigated in this study in the shortest possible time more suitable separation conditions had to be found. In addition to dansyl aspartic acid, alanine and glycine (internal standard), dansyl proline (bound excess of dansyl chloride), Dans-OH (hydrolyzed dansyl chloride) and dansyl amide (side product) had to be separated. It was found that the cationic ion-pair reagent tetrabutylammonium bromide improved the peak shape of the eluted dansyl amino acids considerably. Optimum separation conditions were obtained by keeping concentrations of sodium acetate and ion-pair reagent constant and by varying acetic acid and acetonitrile concentrations.

From figure 2 it can be seen that a relatively small increase in acetonitrile concentration caused strong reductions in retention time of all compounds analyzed. Ionization of the carboxylic group of the dansyl amino acids is reduced by the addition of acetic acid, and this loss in polarity is responsible for the increased retention with increasing acid concentrations.

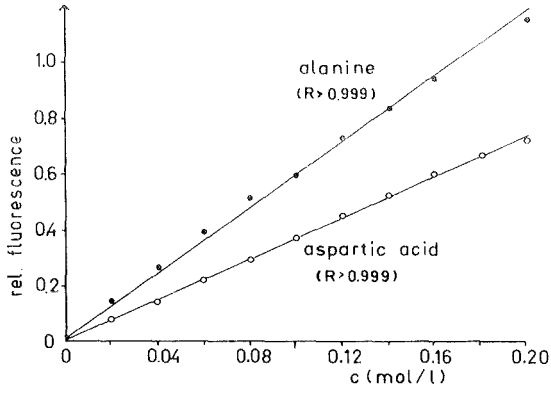


FIGURE 4. Calibration curve for the determination of aspartic acid and alanine.

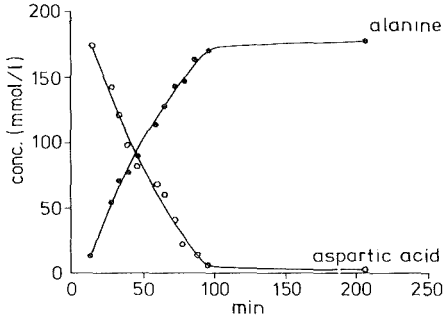


FIGURE 5. Time course of alanine production from aspartic acid in a membrane reactor.

Separation conditions were found to be optimal with 36 % acetonitrile in the solvent under the HPLC conditions used. Under these conditions all dansylated amino acids involved in the analytical system and the dansyl derivatives of ammonia and water were separated well enough to allow the analytical procedure to be established (see fig. 3). Higher acetonitrile concentrations would give even smaller retention times, but

aspartic acid would then be eluted too near to Dans-OH resulting in loss of accuracy.

With concentrations between 2.0 and 200 mM amino acid, typical for the reactor, an excellent calibration curve was obtained ($R > 0.999$; see fig. 4). The use of this calibration curve allowed the measurement of aspartic acid conversion into alanine (see fig. 5).

CONCLUSION

Using the chromatographic separation described, one run was completed within six minutes. The dansylation procedure (about 15 minutes for 10 samples) lengthened the process time per sample only slightly. Regeneration of the column after several runs yielded hardly any fluorescent material. Therefore, time-consuming regeneration procedures are not necessary adding to the convenience and speed of this method. In comparison, the analysis using a routine amino acid analyzer would require at least one hour and even with an abbreviated program not more than one injection in 30 minutes would be possible.

Although the presented method describes the determination of alanine and aspartic acid, the principles of optimization can be applied to any dansylated amino acid to be analyzed. This way, amino acids produced by fermentation or by any other process can be analyzed conveniently using standard HPLC equipment.

For semi-continuous reactor analysis automatic sample treatment would be required. Investigations are now under way to convert the method described into such a semi-continuous method for membrane reactor monitoring.

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PURIFICATION OF HUMAN PLATELET MONOAMINE OXIDASE B BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Human platelet monoamine oxidase (MAO B), a membrane bound enzyme was purified to homogeneity by DEAE-Sephacel column chromatography, chromatofocusing, and high performance liquid chromatography (HPLC). The crucial purification step was HPLC on a anion exchange column (SynChropak AX 300). The HPLC column was eluted initially with potassium phosphate buffer (100 mM, pH 7.4) for 10 min at a flow rate of 1.0 ml/min, followed by a gradient (0-1%) of octyl-β-D-glucopyranoside (octylglucoside) in the same buffer for 10 min, and finally with buffered octylglucoside (1%) for 40 min. The elution of pargyline-bound or active MAO was established by determining either radioactivity in each fraction when MAO B had previously been covalently labeled with [^{3}H]-pargyline [^{3}H(G)] or catalytic activity using [^{14}C-methylene]-benzylamine as substrate. [^{3}H]-pargyline-bound and active MAO B eluted from the column at approximately 34 min. The extent of homogeneity and the subunit M_r (approximately 59,000) of MAO B were determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis followed by silver staining for proteins.

INTRODUCTION

Monoamine oxidase (amine: oxygen oxidoreductase, E.C. 1.4.3.4.) (MAO) is an intrinsic membrane flavoprotein localized in the outer mitochondrial membrane (1). This enzyme has an approxi-

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mate molecular weight of 120,000 and consists of two subunits, one of which contains covalently bound flavin adenine dinucleotide. MAO has been classified into two types, A and B, depending upon their differences in sensitivity to specific inhibitors and their preference for different amines as substrates. MAO A preferentially deaminates 5-hydroxytryptamine and norepinephrine and is sensitive to inhibition by clorgyline (2, 3), while MAO B deaminates benzylamine and β-phenylethylamine (4) and is preferentially inhibited by pargyline (5) and deprenyl (2). Both types of MAO are present in most tissues, each being expressed in varying proportions in different tissues (6-10). In human tissues, however, platelets contain mostly MAO B, whereas placenta has a high proportion of MAO A.

In this article, we describe the purification of MAO B from outdated human platelets, employing a three-step procedure which includes DEAE-Sephacel column chromatography, chromatofocusing, and high performance liquid chromatography.

MATERIALS AND METHODS

Chemicals:

Octylglucoside (octyl-β-D-glucopyranoside) was obtained from Calbiochem-Behring Corp. [^{3}H]-pargyline (Pargyline-HCl, specific activity, 15 Ci/mmol) was purchased from New England Nuclear and [^{14}C]-benzylamine (Benzylamine·HCl [methylene-^{14}C], specific activity, 14 mCi/mmol) was obtained from ICN Pharmaceuticals, Inc.

Purification of MAO from Human Platelets:

Platelet rich plasma (PRP) was obtained from The University of Texas Medical Branch Blood Bank immediately after they became out-dated (72 hours after blood drawing). The PRP was stored in a cold-room overnight. In each MAO preparation, a batch of 25 units (65 ml/unit) was used.

Pooled PRP was centrifuged at 600 x g for 3 min to remove contaminating red blood cells and lymphocytes. The supernatant was

centrifuged at 2,500 x g for 20 min and the platelets collected. The platelets were washed by dispersing in 0.9% saline-5 mM EDTA (saline-EDTA), pH 7.4, and then centrifuged at 2,500 x g for 20 min. After two more washings with saline-EDTA solution, the washed platelets were suspended in cold distilled water to a final protein concentration of 5 mg/ml. The suspension was frozen at -20°C overnight, thawed, and centrifuged at 35,000 x g for 60 min. The pellet was suspended in 50 mM potassium phosphate buffer, pH 8.0, containing 0.1% Triton X-100 (freshly prepared). After the suspension was stirred for 60 min at 4°C, it was centrifuged at 35,000 x g for 30 min. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 8.0, containing 0.5% Triton X-100, stirred for 60 min at 4°C and centrifuged at 150,000 x g for 60 min. The supernatant containing solubilized MAO was dialyzed against 3 x 6000 ml of 10 mM potassium phosphate buffer, pH 8.0, for 36-40 h.

Then the dialyzed-extracted MAO was fractionated on a DEAE-Sephacel (Pharmacia) column (2.6 x 40 cm) which had been previously equilibrated with 10 mM potassium phosphate buffer, pH 8.0. The column was developed by stepwise elution with 10 mM and 100 mM potassium phosphate buffer, pH 8.0, and MAO was eluted with 100 mM potassium phosphate buffer, pH 8.0, containing 0.25% Triton X-100. The fractions containing high MAO activity were pooled together and the active protein was precipitated by adding solid $(NH_4)_2SO_4$ to 50% saturation. The mixture was centrifuged at 30,000 x g for 60 min and the precipitate which floated on the surface was collected. The precipitate was dissolved in 50 mM potassium phosphate, pH 8.0, containing 1% octylglucoside and then dialyzed against 3 x 6000 ml of 0.025 M Tris-acetate buffer, pH 7.4, for 36-40 h. Solid octylglucoside was added to the dialyzed solution to a final concentration of 1%. This preparation was further fractionated on a Polybuffer Exchanger 94 (Pharmacia) chromatofocusing column (0.9 x 27cm) according to the instructions from Pharmacia Fine Chemicals. The pH gradient was developed by elution with 200 ml of eight times-diluted Polybuffer 74 (Pharmacia), pH 4.0, (adjusted with 1 M HCl) containing 1% octylglucoside. The 2.8

ml fractions were collected and were assayed for absorption at 280 nm, pH, and MAO activity. The fractions showing high MAO activity near pH 5.3 were pooled together and the active protein fraction was precipitated by adding solid $(NH_4)_2SO_4$ to 80% saturation. The floating precipitate collected after centrifugation at 30,000 x g for 20 min was washed once with 80% saturated solution of $(NH_4)_2SO_4$ in 50 mM potassium phosphate buffer, pH 7.4. The washed precipitate was dissolved in 50 mM potassium phosphate buffer, pH 8.0, containing 1% octylglucoside and dialyzed against 6000 ml of 10 mM potassium phosphate buffer, pH 7.4, for 24 h. This dialyzed, chromatofocused fraction was further fractionated by HPLC.

HPLC was performed on a Beckman model 334 Gradient Liquid Chromatograph using a SynChropak AX 300 column (4 x 300 mm), a Beckman model 153 analytical UV detector containing 280 nm filter, and an Altex C-R1A processor. One ml of the chromatofocused fraction, (0.5-0.7 mg protein) in 10 mM potassium phosphate buffer, pH 7.4, was injected and eluted with potassium phosphate buffer, (100 mM, pH 7.4) for 10 min, followed by a gradient (0-1%) of octylglucoside in the same buffer for an additional 10 min. Catalytically active MAO was eluted with phosphate buffer containing 1% octylglucoside for 40 min. A flow rate of 1.0 ml/min was maintained throughout the elution profile.

Fractions containing catalytically active MAO were pooled together and dialyzed against 10 mM potassium phosphate buffer, pH 7.4, for 24 h. The dialyzed fraction (2.0 ml) was rechromatographed on the HPLC column as described above. The active fractions were pooled, dialyzed, and lyophilized before electrophoresis.

MAO Activity Determination:

MAO activity was assayed by the basic procedure of Wurtman and Axelrod (11). The assay mixture contained 2 mM [^{14}C]-benzylamine (specific activity, 2 mCi/mmole), 50 mM potassium phosphate buffer, pH 7.4, and the enzyme in a total volume of 15 µl. The mixture was incubated at 37°C for 30 min. The reaction was terminated by transferring the mixture to an ice bath and adding 3 µl of 6 M HCl.

TABLE 1

Purification of Human Platelet MAO

FRACTION		TOTAL PROTEIN (mg)	PROTEIN RECOVERY (%)	ENZYME SPECIFIC ACTIVITY (nmol/h/mg prot)	TOTAL ENZYME ACTIVITY (nmol/h)	ENZYME ACTIVITY RECOVERY (%)	PURIFICATION
A.	Crude	1349	100	90.8	123,000	100	-
B	Dialyzed 0.5% TX-100 Extract	121	8.97	526	60,900	49.4	5.8
C	DEAE-Sephacel Fraction 0-50% $(NH_4)_2SO_4$ precipitate	27	2.00	1330	35,700	28.9	14.7
D	Chromatofocused Fraction	1.8	0.13	1290	2,320	1.9	14.2
E	HPLC-Fraction	0.08	0.006	3920	314	0.25	43.2

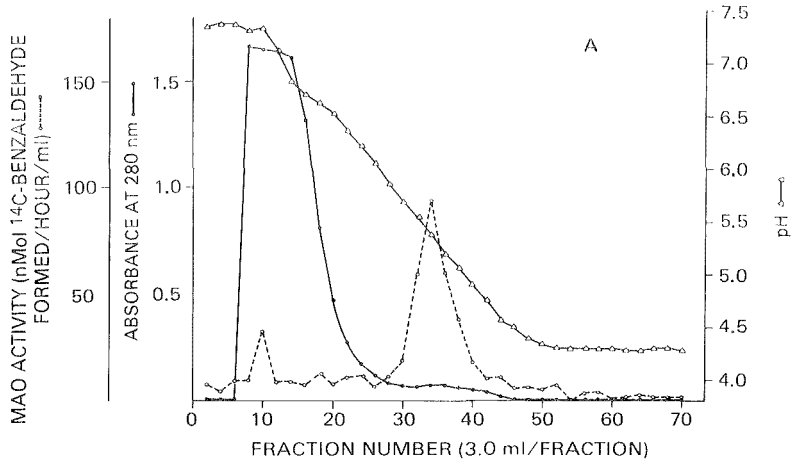

FIGURE 1

Chromatofocusing of DEAE-Sephacel fraction proteins on Polybuffer Exchanger 94 column. Column size: 0.9 x 30 cm. Bed height: 27 cm. Elution conditions: Starting buffer -- 0.025 M Tris-acetate, pH 7.4, containing 1% octylglucoside; Elution buffer -- Polybuffer 74 (8 times diluted), pH 4.0, containing 1% octylglucoside; Flow rate: 32 mL/h.

A. Sample: 48 mg of DEAE-Sephacel fraction protein in 0.025 M Tris-acetate, pH 7.4, containing 1% octylglucoside applied on the column. Each individual fraction was assayed for pH, absorbance at 280 nm, and catalytic activity.

Two types of blanks were used: a tissue blank, in which 3 µl of 6 M HCl was added to the complete reaction mixture before incubation; and a water blank, in which the enzyme was replaced by an equivalent volume of water. The [^{14}C]-benzylaldehyde formed was extracted with 120 µl of toluene and centrifuged. Sixty µl of the toluene layer was mixed with 3.0 ml of ScintiVerse I (Fisher Scientific Co.) cocktail and counted. The specific activity of the

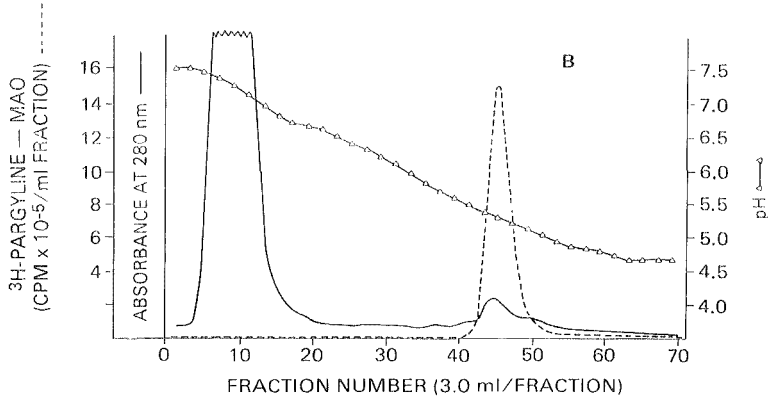

FIGURE 1 (continued)

B. Sample: 35 mg of [^{3}H]-pargyline treated DEAE-Sephacel fraction proteins in 0.025 M Tris-acetate, pH 7.4, containing 1% octyl-glucoside applied on the column. Absorbance profile at 280 nm was obtained by coupling LKB Uvicord II to the column. Each individual fraction was assayed for pH and [^{3}H] activity (10 µl aliquot from each fraction was mixed with 4.0 ml PCS counting fluid from Amersham Corporation and counted in Beckman LS 8000 scintillation counter).

enzyme was expressed as nmoles of [^{14}C]-benzylaldehyde formed/h/mg protein.

[^{3}H]-Pargyline Binding to MAO:

The DEAE-Sephacel fraction (22.6 mg protein/8.0 ml) was mixed with 0.96 ml of 0.5 M potassium phosphate buffer, pH 7.4, and 0.097 ml of [^{3}H]-pargyline solution (specific activity, 15.3 Ci/mMole) in a total volume of 9.6 ml. The mixture was incubated at 37°C for 60 min, cooled on ice, and then dialyzed against 3 x 6000 ml of 25 mM Tris-acetate buffer (pH 7.4) for 60 h. SDS-polyacrylamide gel electrophoresis showed that the [^{3}H] activity was concentrated in the protein band having a M_r of about 59,000, demonstrating specific labeling of MAO.

Analytical Methods:

The protein concentration was determined by the method of Lowry et al. (12), using bovine plasma gamma globulin as standard. SDS-polyacrylamide gel electrophoresis analyses of the fractions were carried out according to Fairbanks (13), with 2.5% stacking gel and 7.5% running gel. Proteins were stained with silver as described by Merrill et al. (14). Commercial Triton X-100 was purified before use by the procedure described by Chang and Bock (15).

RESULTS AND DISCUSSION

A three step method including DEAE-Sephacel column chromatography, chromatofocusing on Polybuffer Exchanger 94 (Pharmacia) column, and HPLC employing an anion exchange column has been used to purify human platelet MAO to a single band on SDS-polyacrylamide gels. Treatment of the membrane fraction obtained after centrifugation of a freeze-thawed platelet suspension with buffered detergent (0.1% Triton X-100) did not release MAO activity into the wash. Further treatment with buffered 0.5% Triton X-100 solubilized 45-55% (4 preparations) of the platelet MAO activity. This extraction step increased the specific activity 5.8 times (Table 1, Fraction B). On further fractionation of the dialyzed Triton X-100 solubilized extract on a DEAE Sephacel column, MAO activity was eluted with 100 mM potassium phosphate buffer, pH 8.0, containing 0.25% Triton X-100. The enzyme from the pooled active fractions after precipitation with 50% ammonium sulfate saturation showed a 2.5 fold increase in specific activity (total purification: 14.7 fold; Table 1, Fraction C). Upon chromatofocusing, which separates proteins on the basis of differences in isoelectric pHs, MAO activity was eluted as a sharp peak at pH 5.3 (Figure 1A and 1B). Proteins are eluted from the column by pH gradients generated by interaction of polybuffer exchanger equilibrated at one pH with polybuffer (eluent) which has been adjusted to a second, lower pH value. This step of purification did not result in an increase in

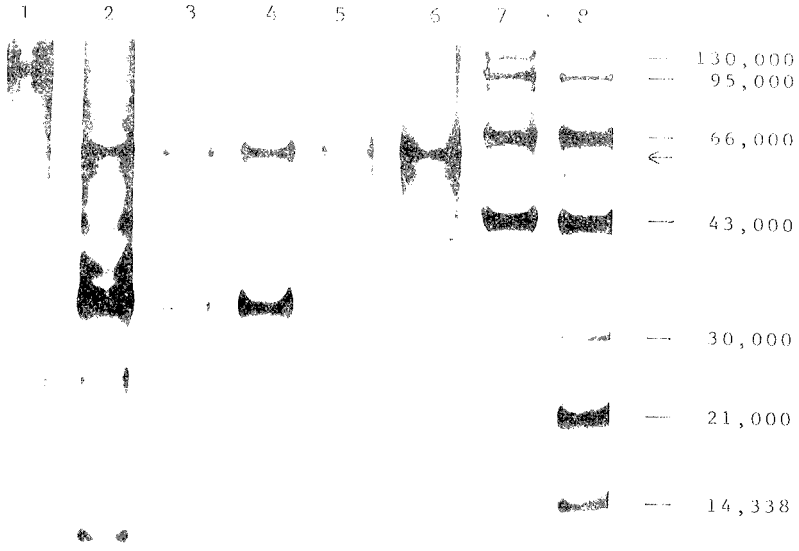

Figure 2

SDS-polyacrylamide gel profile of various fractions. Electrophoresis was run and stained for proteins with silver as described in Materials and Methods. Lane 1: DEAE-Sephacel fraction (10 µg protein); Lane 2. Chromatofocused fraction (10 µg protein); Lane 3 and 4: HPLC fraction after first run (2.6 and 5.2 µg protein); Lane 5 and 6: HPLC fraction after second run (1.0 and 2.0 µg protein); Lane 7: BioRad high molecular weight markers (0.3 µg protein per band--from top to bottom, β-galactosidase, 130,000; phosphorylase B, 95,000; bovine serum albumin, 66,000; ovalbumin, 43,000); Lane 8: BioRad low molecular weight markers (0.3 µg protein per band--from top to bottom, phosphorylase B, 95,000; bovine serum albumin, 66,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; lysozyme, 14,338). The arrow indicates the position of MAO, corresponding to M_r 59,000.

specific activity of the MAO (Table 1, Fraction D), but SDS-polyacrylamide gel electrophoretic analysis showed increased concentration of the MAO protein (M_r around 59,000) with elimination of several other proteins present in the DEAE-Sephacel fraction (Figure 2, lanes 1 and 2). The loss of activity at this stage could be attributed either to removal of a protein or lipid component required for MAO activity, or exposure of the MAO protein to its isoelectric pH, with partial denaturation of the enzyme.

The chromatofocusing step gave 0.13% recovery of protein and 1.9% recovery of MAO activity. We also labeled MAO B protein in the DEAE-Sephacel fraction with [^{3}H]-pargyline, a selective irreversible MAO B inhibitor, and fractionated the labeled material by chromatofocusing. The [^{3}H]-pargyline labeled MAO eluted at pH 5.3 (Fig. 1B), with 70% recovery of radioactivity. SDS-polyacrylamide gel electrophoresis of either the [^{3}H]-pargyline labeled DEAE-Sephacel fraction or the chromatofocused fraction resulted in recovery of the [^{3}H] in a single protein band corresponding to M_r 59,000 (results not shown). Thus, the [^{3}H]-pargyline labeling technique provided a convenient way to follow the purification of MAO.

In the next step of purification, an HPLC with an anion exchange column (SynChropak AX 300) was employed using a gradient of buffered octylglucoside (Fig 3, A and B). The catalytically active MAO or [^{3}H]-pargyline labeled MAO was consistently eluted from the HPLC column in fraction 34. The electrophoretic patterns of this fraction on SDS-electrophoretic gels showed two major bands with M_r 59,000 and 28,000 (Figure 2; lanes 3 and 4). The 28,000 M_r protein was easily removed by repeating the HPLC step (Figure 2; lanes 5 and 6). This step of purification resulted in a further 3 fold increase in specific activity (total purification: 43.2 fold; Table 1, Fraction E). The final preparation of purified MAO B from human platelets is suitable for production of antibodies and protein characterization.

The extensive purification of MAO B using this procedure demonstrates that this purification scheme can be successfully applied to the purification of membrane bound proteins, although we

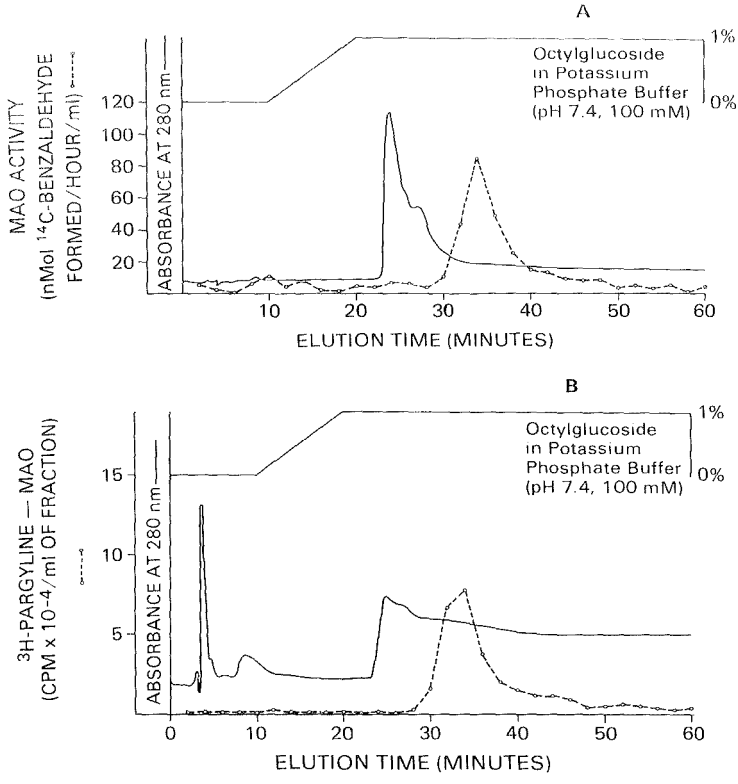

Figure 3

Separation of MAO protein from chromatofocused fraction by HPLC. The sample (0.5 - 0.7 mg protein) in 10 mM potassium phosphate buffer, pH 7.4, was injected on SynChropak AX 300 column (4 x 300 mm).

A. Catalytically active chromatofocused fraction: Alternate fractions were assayed for MAO activity.

B. [^{3}H]-pargyline treated chromatofocused fraction: 10 µl aliquots from the alternate fractions were mixed with 4.0 ml of PCS counting fluid (Amersham Corporation) and [^{3}H] activity determined in Beckman LS 8000 scintillation counter.

lost considerable enzyme activity during the chromatofocusing step. In such purification schemes, octylglucoside represents the detergent of choice because it does not interfere with UV detection, it is a relatively mild nonionic detergent permitting retention of catalytic activity, and it is easily removed by dialysis.

ACKNOWLEDGMENTS

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^{125}I-LABELED PEPTIDE MAPPING AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ^{125}I-PEPTIDE SEPARATION OF PROTEIN I OF FOUR STRAINS OF NEISSERIA GONORRHOEAE

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ABSTRACT

^{125}I-labeled α-chymotryptic peptides of the principal outer membrane proteins (P.Is) of four strains of Neisseria gonorrhoeae were separated and visualized by two-dimensional (2-D) ^{125}I-peptide mapping and by high-performance liquid chromatography (HPLC) coupled with a Beckman Biogamma counter. In addition, ^{125}I-peptides were recovered from the HPLC separation and re-separated by the 2-D ^{125}I-peptide mapping system. The results indicated that the 2-D ^{125}I-peptide mapping procedure was best suited for comparative analyses of α-chymotryptic digests whereas the HPLC system, which is able to detect many more peptides than the 2-D system, is ideally suited for preparative separation of the ^{125}I-peptides. ^{125}I-peptides separated by HPLC could be recovered, rerun on the 2-D system, and the location of each peptide ascertained. The coupling of these two procedures allows for the isolation of specific ^{125}I-labeled peptides for further immunological and structural analyses of these outer membrane proteins.

INTRODUCTION

The technique of ^{125}I-labeled peptide mapping has proved to be a useful tool for evaluating the primary structural relationships of proteins. The coupling of this procedure with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7), which provides excellent protein separation, and in-gel radiolabeling of single protein bands (3, 12) has extended the applicability of

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^{125}I labeled peptide mapping to the outer membrane proteins of several Neisseria species and strains (4, 5, 6, 12, 13, 16).

The separation of ^{125}I-peptide residues is accomplished by high voltage thin-layer electrophoresis (TLE) in the first dimension, followed by thin-layer chromatography (TLC) in the second dimension. Autoradiography then yields characteristic ^{125}I-peptide patterns (i.e., two-dimensional [2-D] ^{125}I-peptide maps). By comparing these patterns, structural relationships can be ascertained.

Until recently, the resolution and reproducibility of 2-D ^{125}I-peptide mapping have been restricted, due to batch variations in TLE sheets and the difficulty of maintaining precise temperature control during TLE (unpublished observations). The use of identical sheets and TLE chambers which maintain precise temperature control by immersing the thin-layer sheet in a cooling menstrum such as Varsol has greatly improved both the resolution and reproducibility of 2-D ^{125}I-peptide mapping (5, 6, 17). One drawback of this system is the inability to recover usable quantities of the separated ^{125}I-peptides for further experimentation.

In this study, I describe a system which compares an improved 2-D ^{125}I-peptide mapping procedure with a high-performance liquid chromatography (HPLC) ^{125}I-peptide separation using the major outer membrane proteins (P.Is) of four strains of Neisseria gonorrhoeae (GC). The results show both procedures yield similar information about the proteins' primary structure. In addition, ^{125}I-peptides separated by HPLC can be recovered, rerun using the 2-D ^{125}I-peptide mapping procedure, and identified by their migration characteristics, making it possible to isolate useful quantities of ^{125}I-peptides of outer membrane proteins for further investigations.

MATERIALS AND METHODS

Bacteria

Nonpiliated, transparent (11) GC strains JS1, JS2, JS3, and JS4 (5) were grown on clear typing medium and harvested as previously described (11).

SDS-PAGE

Unreduced lysates of whole GC were separated by SDS-PAGE using the discontinuous system of Laemmli (7) as previously described (5).

Radioiodination and enzyme digestion

The appropriate Coomassie-stained P.I bands were excised and radioiodinated by the chloramine-T procedures of Elder (3) and Swanson (12). Radioiodinated protein bands were digested with α-chymotrypsin in 0.05 M NH_4HCO_3 buffer, pH 8.5, and the ^{125}I-labeled peptides washed eight times by dissolving the peptides in 250 μl in distilled water (5, 12) and drying in a Savant (Savant Insts., Hicksville, New York) Speed-vac.

2-D ^{125}I-peptide mapping.

The washed ^{125}I-peptides from each P.I were rehydrated at 1 x 10^5 counts per minute (CPM) per μl in distilled water containing 1 mg/ml each of L-leucine, L-aspartate, and L-tyrosine as markers. ^{125}I-peptides recovered from the HPLC separation of the JS1 P.I (see below) were dried in a Speed-Vac and washed eight times in distilled water. The dried residues were resuspended in 10 μl of distilled water containing the above amino acid markers.

Two microliters of the P.I ^{125}I-peptide preparations (2 x 10^5 CPM) were spotted onto a Polygram Cel 300, 20 x 20 cm thin-layer sheet (Brinkmann Instruments, Westbury, N.Y.). Three preparations, spotted 8 cm from the anodal edge of the sheet and 1 cm, 7.5 cm, and 14.5 cm from

the side of the sheet, were run on each sheet. All 10 µl of each ^{125}I-peptide preparation recovered from the HPLC separation of the JS1 PI were spotted either 4.5 cm or 11.5 cm from the anodal edge of the sheet and 1 cm, 7.5 cm, and 14.5 cm from the side of the sheet. Six preparations could thus be run on each sheet (a pattern similar to that seen on a playing card having six spots, viewed side-on [Hoyle1).

Each thin-layer sheet was electrophoresed at a constant 1200 V for 30.5 min in a Savant TLE 20 (Savant Instruments, Inc.) apparatus, which immerses the sheet in Varsol during electrophoresis. A Forma Scientific 2095 bath and circulator (Forma Scientific, Marietta, Ohio) maintained a constant 8.5°C circulating coolant which held the Varsol menstrum at a constant 13.5°C throughout the run. Auxiliary cooling coils, fashioned by the Rocky Mountain Laboratories staff, were inserted horizontally, about 1 cm below the surface of the Varsol. These coils were mandatory for precise temperature control. The electrophoresis buffer was a pH 3.7 solution of water-acetic acid-pyridine (200:10:1, v:v:v). Following electrophoresis, the plate was removed, air dried, and cut in thirds longitudinally, so that each piece contained either one preparation, if three were added to the sheet, or two preparations, if six ^{125}I-peptides recovered from the HPLC separation were added to each sheet. Each piece was turned 90°C and subjected to ascending TLC in a solution of n-butanol-pyridine-water-acetic acid (13:10:8:2, v:v:v:v). A moat was scored 2 mm from the top of each piece of thin-layer sheet prior to TLC separation. When the solvent front reached the moat, the sheets were removed, air dried, and sprayed with 0.25% ninhydrin in acetone to locate the amino acid markers. ^{125}I-peptides were visualized by placing the thin-layer sheets on Kodak XAR-5 film and exposing for 18 h at -76°C using Cronex Par-Speed (E. I. dupont de Nemours and Co., Wilmington,

Delaware) intensifying screens. Origins on the 2-D maps of ^{125}I-peptides recovered from the HPLC were marked with 1-5 x 10^3 CPM of ^{125}I for reference.

HPLC.

All HPLC separations were performed on a Waters System (Waters Associates, Inc., Milford, Massachusetts), equipped with dual M 6000A pumps, system controller, data module, 440 UV detector, and a WISP 710 sample injector. A Waters μ Bondapak C_{18} reverse-phase column (3.9 mm ID x 30 cm) was used to separate the ^{125}I-peptides.

Approximately 2.1 x 10^5 CPM of each of the P.Is ^{125}I-peptide preparations were dissolved in 210 μl of distilled water-0.05 trifluoroacetic acid (TFA) Aldrich Chemical Co., Inc., Milwaukee, Wisconsin) containing 0.3 mg/ml each of L-tyrosine, L-tryptophan, and L-phenylalanine (Sigma Chemical Co., St. Louis, Missouri) as markers. Two hundred microliters of this solution was injected by the WISP 710 injector at time 0. The dual pump system, with pump A delivering filtered, distilled water-0.05% TFA and pump B delivering acetonitrile (reagent grade, Sigma)-0.05% TFA, then delivered 1 ml/min of a 0 to 8% acetonitrile-0.05% TFA linear gradient (06 gradient program) in the first 10 min. From 10 to 55 min, an 8 to 72% acetonitrile-0.05% TFA elution gradient, using the 09 gradient program, was generated followed by a 5-min period of 72% acetonitrile-0.05% TFA (60 min total separation time). Many different gradient profiles were tested; this profile was chosen as being representative of type of separation which can be obtained using these procedures.

At the time of injection, an LKB 7000 Ultrorac (LKB Instruments, Sweden) was started. Three hundred 2/10-min fractions were collected in Biovials (Beckman Instruments Inc., Palo Alto, California). Each

fraction was transferred to a Beckman Biogamma Counter and counted. Twice the background (100 CPM) was subtracted from the CPM of each fraction. The CPM of each fraction was then plotted versus retention time (RT) as derived from the fraction number. The elution of the amino acid markers was monitored at 254 nm by the model 440 UV detector. No ^{125}I-peptides could be detected by the 440 UV detector. A model 450 detector was unable to detect ^{125}I-peptidic peaks at 210 nm but was able to very weakly respond to autodigestion products of α-chymotrypsin at beginning concentrations of 200 µg.

RESULTS

The P.Is of the four strains of GC used in this study are seen in the Coomassie-stained SDS-PAGE gel shown in Fig. 1. These bands were excised, radioiodinated, and digested with α-chymotrypsin. The results of 2-D ^{125}I-peptide mapping of these preparations are displayed in Fig. 2. Previous studies using this technique (5, 6, 12) have indicated that the JS1 and JS2 P.Is are members of one "homology" group, whereas the JS3 and JS4 P.Is are in a second group. These relationships are seen in the 2-D ^{125}I-peptide maps (Fig. 2). However, the improved resolution provided by the technique described here shows a greater heterogeneity among these proteins than has been previously demonstrated by 2-D mapping.

The same preparations and the same number of CPM used to generate the 2-D ^{125}I-peptide maps seen in Fig. 2 were subjected to HPLC separation. The results of this procedure are seen in Fig. 3, which shows the plots of the CPM versus RT (fraction number) of the ^{125}I-peptides of the four P.I molecules under study. The gradient profile is an approximate representation of the 09 gradient program used to elute the peptides from the µ Bondapak C_{18} column. The RTs of the amino acid markers, as calculated by the data module from UV adsorbence

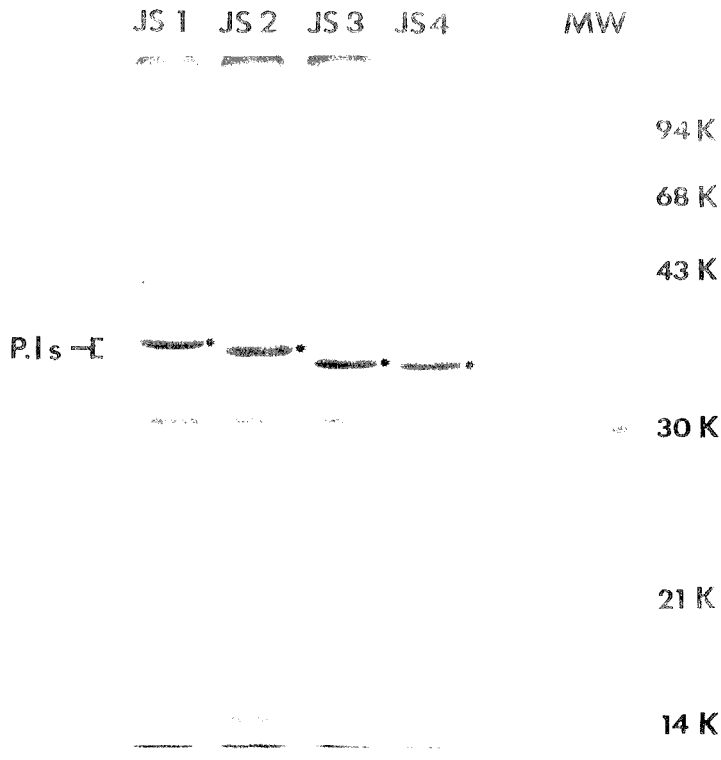


FIG. 1. Coomassie-stained SDS-PAGE gel of unreduced lysates of GC strains JS1, JS2, JS3, and JS4. P.I bands (asterisks) were excised and used in ^{125}I-labeled peptide mapping studies.

peaks, were 8.50 min (tyrosine), 9.65 (phenylalanine), and 13.90 (tryptophan) with no more than 5/100 min variation in all four runs for any marker.

The most striking feature of these separations is the large number of radioemitting fractions. Comparative interpretation is difficult due to the numerous small peaks which can be resolved by this very sensitive method. The JS1 and JS2 P.Is appear to be slightly more similar to one another than they are to the JS3 or JS4 P.Is, and vice versa,

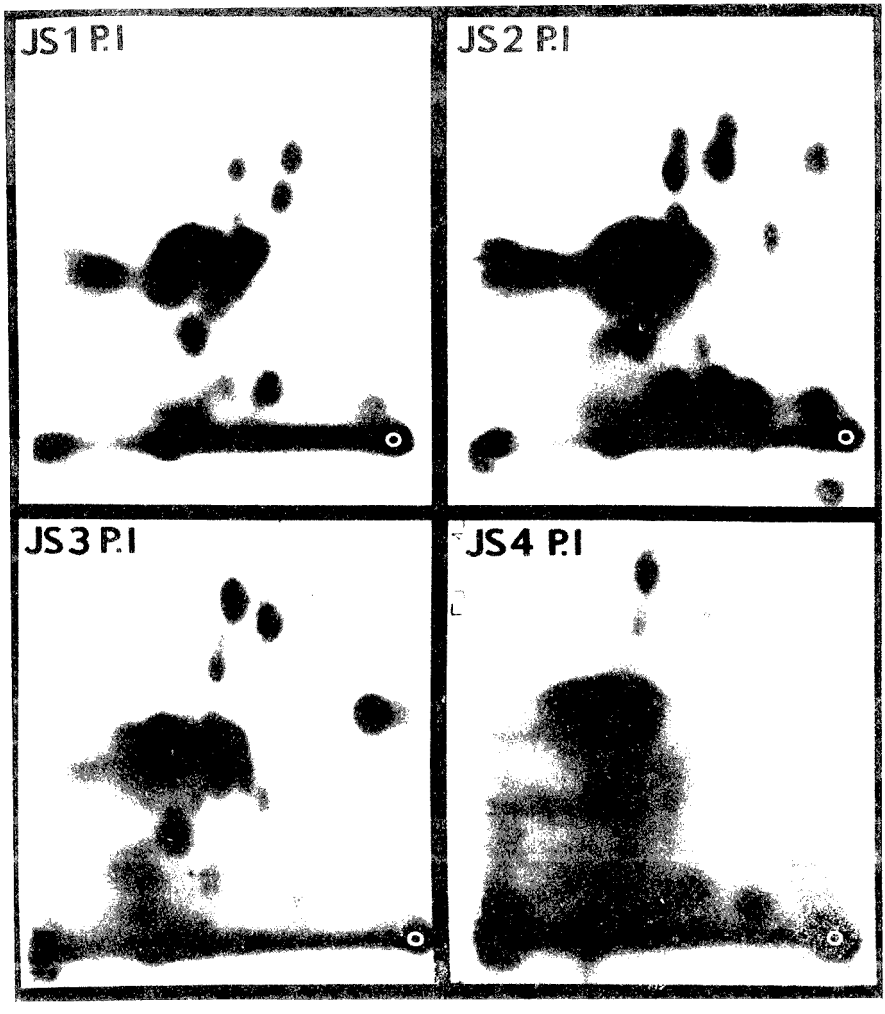


FIG. 2. 2-D ^{125}I-labeled peptide maps of α-chymotrypsin digested P.Is from GC strains JS1, JS2, JS3, and JS4. White circle marks the origin. Direction of TLE and TLC is as shown in Fig. 5.

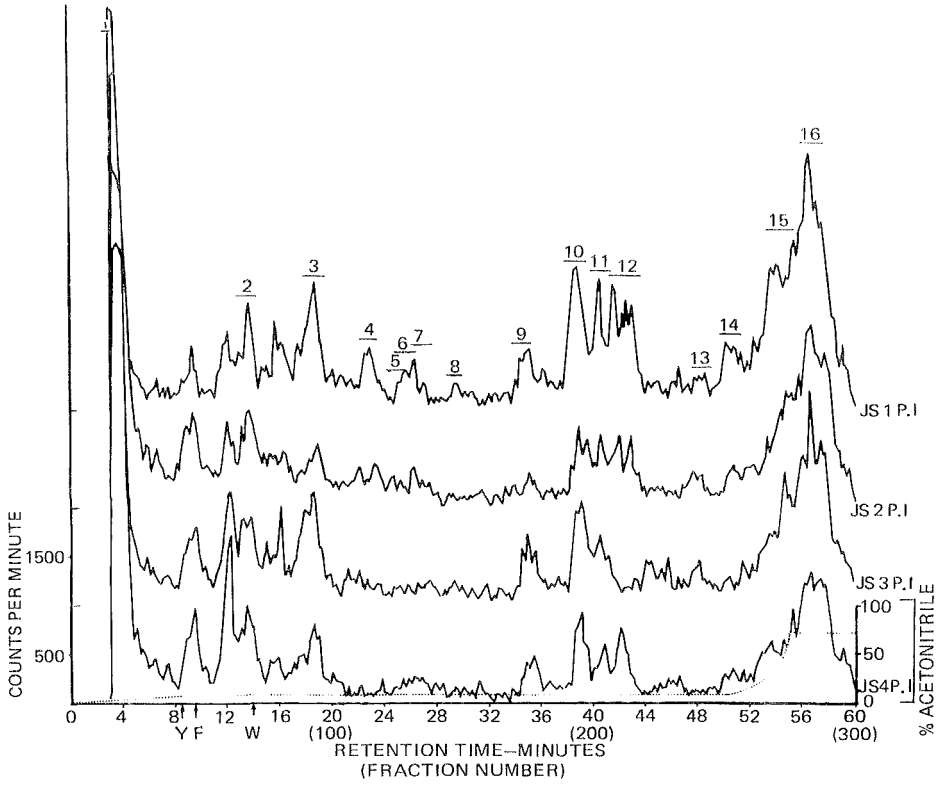


FIG. 3. HPLC profiles of α-chymotryptic ^{125}I-labeled peptides from P.Is of strains JS1, JS2, JS3, and JS4. Fractions of the JS1 P.I, designated by numbered bars over the JS1 P.I profile, were pooled for reseparation by 2-D ^{125}I-labeled peptide mapping. Y (tyrosine), F (phenylalanine), and W (tryptophan) designate amino acid markers RTs.

particularly in the 38- to 44-min region. Clearly, all the P.Is share several heavily emitting peaks having the same RT. Each also has a few strongly emitting and many weakly emitting peaks which are unique.

Complete separation of the peptides has not been accomplished using these elution parameters. Longer separation times and/or rechromatography of portions of these gradients using different

parameters can easily separate any peptide of interest. By altering the gradient profiles, the separation of peptides in any given region can be expanded; unfortunately, other regions are concomitantly compressed (data not shown and 10). The profiles shown here are presented as representative separations of ^{125}I-peptides of these proteins.

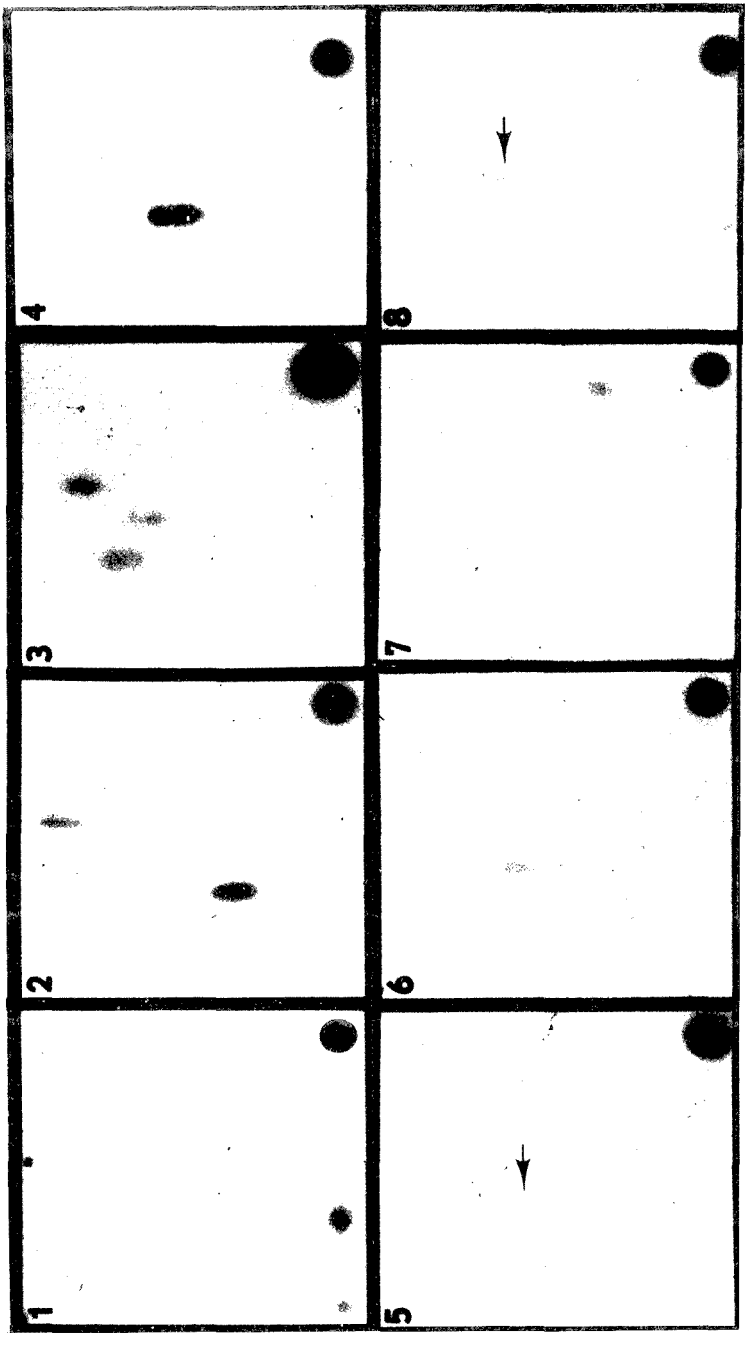
Fractions of the JS1 P.I HPLC separation, designated by numbered bars, were pooled, dried, washed, and subjected to 2-D ^{125}I-peptide mapping. [Note: all the fractions of one HPLC separation of the JS1 P.I were pooled, dried, washed, and subjected to 2-D mapping. All the peptides seen in the JS1 P.I 2-D map (Fig. 2 & 5) were recovered (data not shown)]. The results of these experiments are shown in Fig. 4. The number in the upper left of each ^{125}I-peptide map in this figure corresponds to the pooled fractions designated in Fig. 3. Every effort was made to quantitatively recover these peptides (only 10 to 15% of the original radioactivity was lost during handling). The relative intensities of the various peptides agree well with the height of the HPLC peaks from which they were recovered. The exception occurs with Peak 1, where a very large HPLC peak yielded two moderately emitting peptides (#1, Fig. 4). It appears that much of the material eluting with the HPLC solvent front migrates off the thin-layer sheet towards the anode since the anodal buffer became radioactive following the TLE separation. This suggests that this peak represents unbound ^{125}I.

It is clear from this figure that the HPLC separated ^{125}I-peptides can be recovered and returned to the 2-D ^{125}I-peptide mapping system for identification. The number of peptides recovered from each HPLC peak is consistent with the profile of the peak, e.g., Peak #10 is very broad, suggesting several peptides might be present, and this is confirmed in Fig. 4, #10, where four peptides are seen, etc. Also, all the P.Is have

an HPLC peak which has the same RT and approximate radioactivity as the dominant peak in the #2 pooled fractions (Fig. 3). The 2-D map of this peak (#2, Fig. 4 and 5) shows that this is a peptide which is present in about the same location and same approximate relative intensity in the 2-D maps of all the P.Is. This suggests that peaks having the same RT and radioactivity in the various PI separations contain the same peptides.

The isolated peptides in Fig. 4 recovered from the peaks in Fig. 3 are identified by number on a 2-D ^{125}I-peptide map of the JS1 P.I in Fig. 5. Note: due to differences in the positions that each preparation was spotted onto the thin-layer sheets and differences in the batches of sheets used in the 2-D separation, the peptides seen in Fig. 4 cannot be directly related to the 2-D ^{125}I-peptide map of the JS1 P.I in Fig. 2 and 5). The locations of these peptides were established by comparing the peptides with 2-D ^{125}I-peptide maps of the JS1 P.I which were run on the same batch of thin-layer sheets in the same position on the sheet and whose amino acid markers migrated to the same location (data not shown). It is characteristic of the TLE separation that samples placed near the anode migrate considerably further than identical samples run near the center of the sheet, as the JS1 P.I ^{125}I-peptides were in Fig. 2 and 5. This is evident in #7 of Fig. 4, where the two peptides have moved quite far from the origin but are identified as peptides closer to the origin in Fig. 5. Preparations spotted near the center of sheets from the same batch are highly reproducible.

A remarkable number of peptides from all regions of the HPLC separation fall in the central region of the 2-D ^{125}I-peptide map, suggesting many peptides are present in this area but are not easily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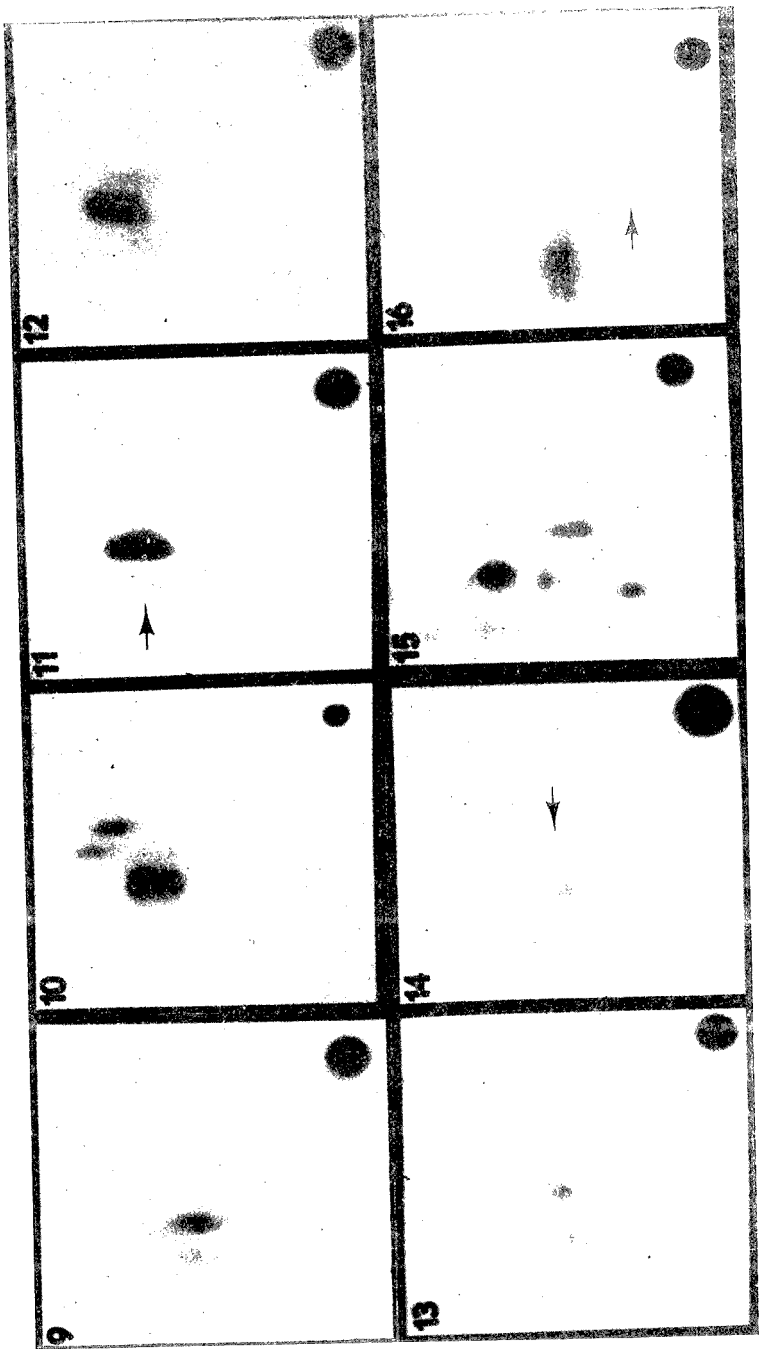

FIG. 4. 2-D ^{125}I-labeled peptide maps of pooled fractions from the HPLC separation of the α-chymotryptic ^{125}I-peptides of the JS1 P.I. Numbers correspond to the numbered bars over the JS1 P.I profile in Fig. 3. Arrows point to peptides which are very faint. Dark circles in the lower right corner of each 2-D map mark the origin. Direction of TLE and TLC is as shown in Fig. 5.

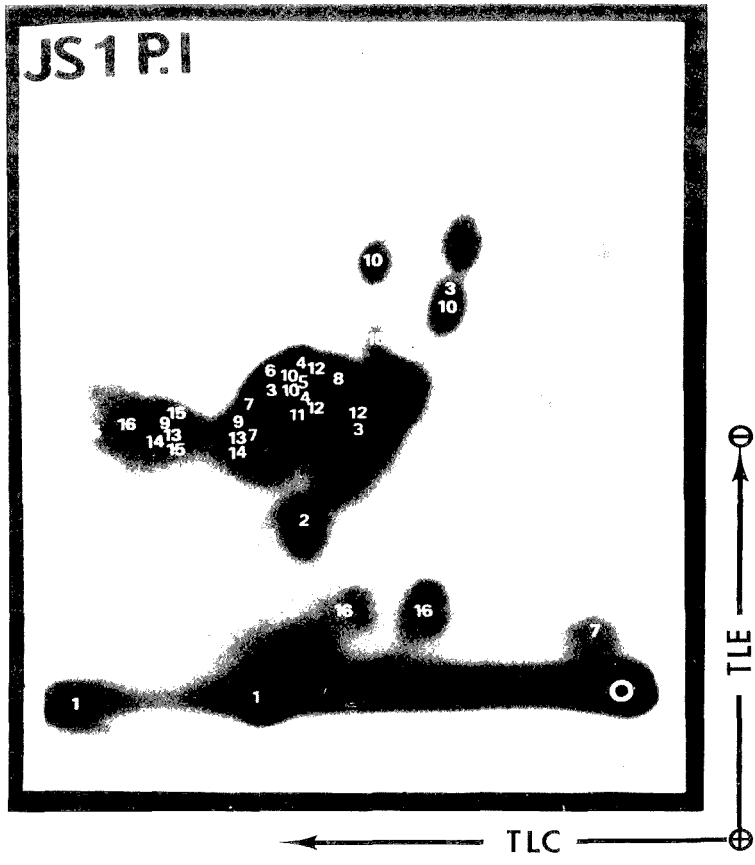

FIG. 5. 2-D ^{125}I-labeled peptide map of α-chymotrypsin digested JS1 P.I. Numbers identify location of isolated peptides (Fig. 4) recovered from HPLC separation (Fig. 3). See text for explanation of how locations were identified. White circle marks the origin. TLE, thin-layer electrophoresis; and TLC, thin-layer chromatography.

visualized. Several other 2-D ^{125}I-peptides also appear to have multiple components that are not seen due to overlapping. This, and the demonstration that ^{125}I-peptides from adjacent peaks (e.g., #2, #3, Fig. 3 and 4) can behave very differently in 2-D mapping, verifies that the HPLC and 2-D ^{125}I-peptide mapping procedures separate on different

physical parameters (8). It is not possible to accurately predict where a peptide will elute in the HPLC profile based on its migration in a 2-D map and vice versa.

DISCUSSION

In this study, two techniques of ^{125}I-peptide separation were compared. The P.Is of GC strains JS1, JS2, JS3, and JS4 were chosen for investigation because they have been well characterized by 2-D ^{125}I-peptide mapping (5, 6, 12). Both the 2-D ^{125}I-peptide mapping system and the HPLC separations gave similar information about the structural relationships of these proteins. However, due to the limitations and advantages of each technique, it appears that the two systems find their best use when used in tandem.

The 2-D ^{125}I-peptide mapping procedure for separation and visualization of α-chymotryptic digests has several advantages over the HPLC system that make it the preferable method for comparative analyses. Forty or more individual ^{125}I-peptide maps can be generated daily, allowing for multiple repeat runs of each preparation. With the improved cooling system described here and if strict attention is paid to precision, the 2-D system becomes highly reproducible and provides good resolution of the ^{125}I-labeled peptides.

Comparison of the 2-D system with the HPLC separations shows that the HPLC-Biogamma counter system is much more sensitive, detecting many very weakly emitting peptides, perhaps representing incomplete cleavage products and/or mono- versus diiodinated individual peptides (9). Whatever the reason for so many small peaks, this extensive "noise" makes comparative analysis of α-chymotryptic digests, on a fine scale, difficult. We are in the process of comparing 2-D maps with HPLC profiles of both gonococcal and chlamydial proteins digested with

Staphylococcal V-8 protease. Preliminary results indicate that V-8 protease yields many fewer peptides that are more easily resolved and comparable by HPLC. However, the HPLC is exquisitely sensitive to minor differences in the amount of dissolved gases, evaporation, oxidation, etc., in the elution buffers (especially the acetonitrile) which can result in very different elution profiles, even over a period of a few hours (unpublished observation and 10). This makes it difficult to run more than a few comparative separations at one time.

The demonstration that ^{125}I-peptides can be recovered from the HPLC separation, subjected to the 2-D ^{125}I-peptide mapping system, and their locations ascertained, indicates the HPLC is ideally suited for the preparative recovery of ^{125}I-labeled peptides for use in further analyses. In addition, the HPLC separation indicates several of the ^{125}I-peptides visualized by 2-D mapping may actually be multiple peptidic residues which, due to their proximity, are difficult to resolve in the 2-D system, showing that the HPLC separation coupled with the 2-D analysis can be used to dissect the heavily emitting regions seen in the 2-D ^{125}I-peptide maps.

In previous studies I demonstrated that surface-exposed portions of gonococcal outer membrane proteins I and III (6) could be identified and compared by 2-D mapping. Recent work by Tam, et al. (15), and Sandstrom (personal communication and MS submitted) has demonstrated that the differences observed by surface-mapping of P.Is correlate exactly with their P.I serotyping scheme (i.e. JS1 and JS2 being W11 and JS3 and JS4 being W1 - see references 6 and 15) using monoclonal reagents. In addition, Swanson, et al. (14) has confirmed that P.IIIs from all strains studied react identically with monoclonal antibody, confirming predictions based on the identical surface-peptide maps of P.IIIs. The

serological correlation with surface-peptide mapping of chlamyelial major outer membrane proteins (1) using polyclonal antisera and borrelial outer membrane proteins (Judd and Barbour, MS submitted) confirms that surface-peptides represent immunoreactive sites of native outer membrane proteins. With the technologies described in this paper, it will now be possible to not only identify and compare surface-peptides, but to physically isolate these peptides for further immunogenic-antigenic studies.

The coupling these techniques, then, provides a tool for acquiring defined peptides for use in antigenic, sequencing, or topographical studies of membrane proteins (15). The HPLC may also be helpful in comparing (and identifying) surface-labeled ^{125}I-peptides with chloramine-T iodinated peptides. There has been several reports of difficulty in relating surface peptides with chloramine-T peptides (1, 4, 6), possibly due to the greatly differing relative intensities of labeling by these procedures (1, 6). The sensitivity of the HPLC system described here may help resolve this problem. The HPLC may also be of value in comparing the α-chymotryptic peptides of ^{125}I-labeled proteins with peptides of intrinsically ^{14}C-labeled proteins, which are not efficiently visualized by 2-D peptide mapping (unpublished observation), in order to establish if the ^{125}I-labeling truly reflects the total primary structure of these proteins.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF T-RNA

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ABSTRACT

Fractionation of amino acid specific tRNAs by descending salt gradient on a reversed-phase high-performance liquid chromatography column is demonstrated. Stationary phase variables such as n-alkyl chain length and silica type are discussed in terms of stability and selectivity. Mobile phase parameters including salt concentration, organic modifier, pH, and temperature are investigated. The concentration of organic modifier to adjust elution profiles can be critical in terms of tRNA retention.

INTRODUCTION

Fractionation of tRNAs has been investigated by many laboratories. The earliest method devised was liquid-liquid countercurrent distribution (1). Later, chromatography materials such as hydroxyapatite (2), DEAE-Sephadex (3,4), methylated albumin on kieselguhr (MAK) (5), and benzoylated DEAE-cellulose (BD-cellulose) (6) were used with some success. However, much higher resolution of oligo- and polynucleotides has been obtained with i)

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reversed-phase chromatography (RPC-5) adsorbants (7), based on polychlorotrifluoro-ethylene beads (Plascon 2300) coated with a trialkylquaternary amine (Adogen 464), and 2) Sepharose 4B beads (2,8,9), which are carbohydrate polymers. Elution from both columns utilizes salt gradients, ascending for RPC-5 and descending for Sepharose 4B. Most interestingly, the common denominator of selectivity in both systems involves an ionically facilitated hydrophobic interaction. Narihara et al (10) have adapted a trialkylamine coating onto silica instead of Plascon beads via a silicone oil interface. The system allowed increased flow rates while retaining the selectivity of RPC-5 type supports.

This paper reports an increased flow rate system utilizing high-performance liquid chromatography (HPLC)[1] columns. The concept used for fractionation is a hybrid based on siliceous support materials with reversed-phase character (7) and a mobile phase similar to that used for Sepharose 4B chromatography (2).

Reversed phase liquid chromatography (RPLC) has been extensivley used in the life sciences because many biological molecules are at least slightly hydrophobic. To accomplish elution of hydrophobic species from RPLC columns it is common practice to use increasing concentrations of organic solvent. This technique works well with small molecules but has a disadvantage with proteins and polynucleotides: organic solvents in greater than 3-10% concentration may alter or destroy secondary, tertiary and quaternary structure of biopolymers (11). The general structure of tRNA species is maintained through base pairing and a variety of stacking interactions that stabilize its tertiary structure (12).

The literature cited above would suggest that a very weakly hydrophobic high-performance column should allow the control of tRNA retention with descending salt gradients and little or no organic solvent. It was the objective of this research to: 1) identify HPLC bonded phases that allow retention of tRNA when high salt concentration is used in the aqueous mobile

phase, then release tRNA when the salt concentration is reduced; 2) optimize the mobile phase in terms of resolution and selectivity; and 3) select the best silica matrix for the optimal mobile phase/bonded phase system. The method developed consists of a descending salt gradient on a reversed-phase column (DSG-RP). Fractionations of various tRNA isoacceptor species have been achieved in an hour or less.

DSG-RP was used to fractionate both yeast and E. coli tRNA mixtures. Alkylsilane bonded phases of the C_1, C_2, and C_4 type all worked well on macroporous, microparticulate silica to effect tRNA separations. A very low concentration of organic modifier such as propanol, ethanol, or methanol was found to enhance the fractionation process. Other variables such as initial salt concentration, temperature, and pH also affected DSG-RP selectivity.

MATERIALS AND METHODS

Hypersil (5μm) was purchased from Shandon Southern Instruments (Sewickley, PA). Vydac TP (5μm) was obtained from The Separations Group (Hesperia, CA). LiChrospher Si 500 (10μm) was purchased from E. Merck (Darmstadt, West Germany). n-Alkylchlorosilanes were purchased from Petrarch Systems (Levittown, PA). 1-Propanol was obtained from Burdick and Jackson Laboratories (Muskegon, MI). 2-Propanol was purchased from Fisher Scientific Company (Fair Lawn, NJ). Methanol was obtained from J. T. Baker Chemical Company (Phillipsburg, NJ). All solvents were HPLC grade. Absolute ethyl alcohol was purchased from U. S. Industrial Chemicals Company (New York, NY). Transfer ribonucleic acid (tRNA) No. R-9001 Type X from baker's yeast with amino acid acceptor activity for glutamic acid, phenylalanine, valine, and alanine was purchased from Sigma Chemical Company (St. Louis, MO). tRNA from Escherichia coli MRE 600 (RNase negative) specific for phenylalanine (CAT. No. 109 673), valine (CAT. NO. 109 720) and a mixture (CAT. No. 109 517) of lysine, phenylalanine, serine, and valine activity was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN).

Preparation of Stationary Phases

n-Alkylation of silicas via silylation was achieved by methods analogous to those previously reported (13,14).

Column Packing

Supports were slurry packed (2% w/v) into columns with 2-propanol at 8000 psi by means of a pneumatic pump, (Haskel, Burbank, CA). LiChrospher Si 500 was packed at 4000 psi (10). All columns were 0.41 x 5.0 cm LiChroma stainless-steel precision tubing (Anspec Co., Inc., Warrenville, Ill) with 2.0 µm frits. A precolumn (0.41 x 4.5 cm) was fitted to the slurry vessel and connected to the analytical column to channel microparticulate silica into the column during the packing process. Allowed packing time was ca. 10 min.

High-Performance Liquid Chromatography

Analyses by HPLC were done using a Varian Vista System (Varian Associates, Walnut Creek, CA). The system was fitted with a Valco model 9080 sample injector (Anspec Company, Inc., Warrenville, IL) with a 100µl injection loop.

Mobile Phase Conditions

A binary gradient system was used for all tRNA separations. Buffer A contained ammonium sulfate and 0.2M potassium phosphate. Buffer B was 0.2M potassium phosphate. Initial ammonium sulfate concentrations, amount and type of organic modifier, temperature, and pH varied and are described in the text. Gradients were from 0 to 100% buffer B in 40 or 80 min. after which 100%B was left running isocratically until solute elution was complete.

Sample Preparation

Fresh tRNA samples of about 2µg/µl concentration were prepared every 2-3 weeks using buffer A as diluent. It was found that slightly better resolution

was achieved when tRNAs were prepared, stored, and injected in solutions not containing organic modifiers.

Recovery Determination

The tRNA profile in Fig. 6a was collected. A blank gradient was then collected for a background absorbance zero. The amount of tRNA injected onto the column in Fig. 5a was directly added to the collected blank gradient and a comparison was made. Recovery is reported as relative absorbance.

RESULTS AND DISCUSSION

Spencer (2,8) has shown that Sepharose 4B can be used for descending ammonium sulphate gradient elutions of tRNAs. The mechanism of retention is thought to be two-fold. First, the high ionic strength of the initial buffer induced "interfacial precipitation" of tRNAs, which could then be released as the salt concentration was reduced. Second, solubilized tRNAs experienced an "adsorptive retardation" through hydrophobic interactions with the stationary phase as they were eluted from columns. Manipulation of the latter mechanism has been shown to be more important for tRNA fractionation (8). The present study investigates this retention and elution protocal with regard to the design of a silica-based HPLC column.

Stationary Phase

Since Spencer found that relatively hydrophilic Sepharose 4B (a polymer of alternating D-galactose and anhydrogalactose residues) was sufficiently lipophilic for descending salt gradient chromatography of tRNAs, it was concluded that a short alkyl ligand attachment to silica would provide an optimal HPLC material. Short chain length supports have already seen some utility in the separation of biopolymers (15,16). The C_8 and C_{18} (ODS) ligands are used most often, but recent reports (16) have indicated low recoveries for some large solutes (>40,000 daltons) such as proteins. It has

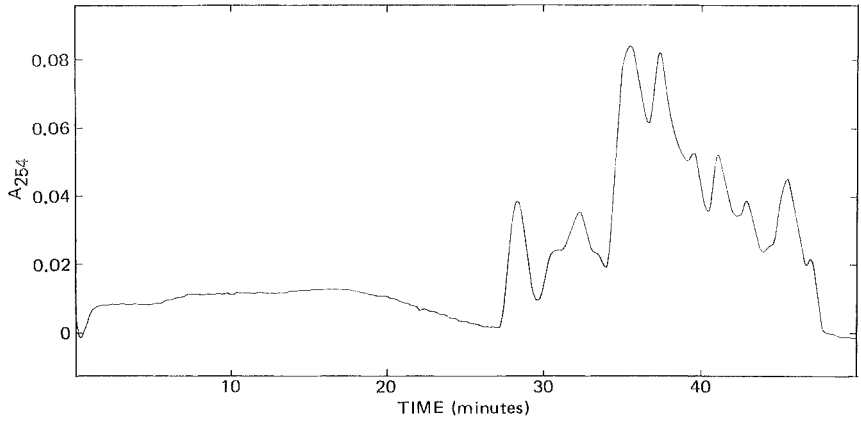

FIGURE 1 Fractionation of yeast tRNA on a reversed-phase column (0.41 x 5cm) packed with TMCS coated Vydac (5μ) silica. Buffer A, 2.0M ammonium sulfate and 0.2M potassium phosphate pH 7, Buffer B, 0.2M potassium phosphate pH 7. Gradient was 0-100% buffer B in 40 min. then 100% B for an additional 15 mins. Flow, 0.7 ml/min; sample, 50μg in 100μl buffer A; temperature, ambient.

been demonstrated that protein loadability (17), recovery (16), and resolution (17) could be increased by employing shorter n-alkyl ligands. Another advantage, relative to longer chains, is higher efficiency due to better mass transfer (18). Therefore, a trimethylsilylated silica support was prepared for these studies. Fig. 1 shows an elution profile of a yeast tRNA mixture high in glutamic acid, phenylalanine, valine, and alanine acceptor activity. tRNAs did not elute until near the end of the 40 min. gradient, which indicated strong retardation forces were still apparent. In an effort to negate the interfacial precipitation contribution to the retention mechanism (2) the column temperature was increased to 55°C- just below the 50-90°C transition range of tRNA melting (19). tRNAs eluted during the 40 min. descending gradient but the useful lifetime of trimethylchlorosilane (TMCS) bonded phase was approximately 2 hrs. at this temperature. A polymeric C_1

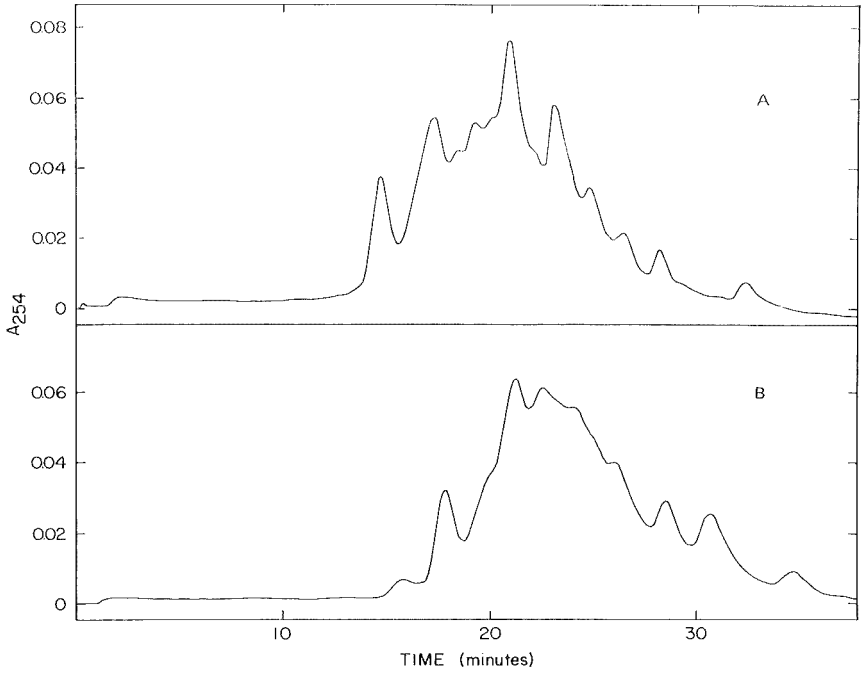

FIGURE 2 Effect of temperature on retention. Same conditions in Fig. 1 except packing was polymeric C_1 coated and loading was 200µg tRNA. A, gradient ran at 55°C; B, 24°C.

coating proved to be more temperature stable. As shown in Fig. 2 the yeast tRNA profile eluted about 3 min. earlier at 55°C as opposed to room temperature. This polymeric C_1 column reproduced four 55°C gradient elution profiles prior to the 24°C run in Fig. 2b. Polymeric C_1 columns could be run for about ten gradients under conditions of Fig. 2a before thermal exposure erroded the bonded phase and diminished resolution. Polymeric C_2 and C_4 coatings were much more stable at 55°C and were employed for the remainder of the study. The C_4 column was used for approximately 45 gradients, 20 of which were at 55°C. At room temperature, short n-alkyl chain columns have reportedly lasted over 200 hrs. (16).

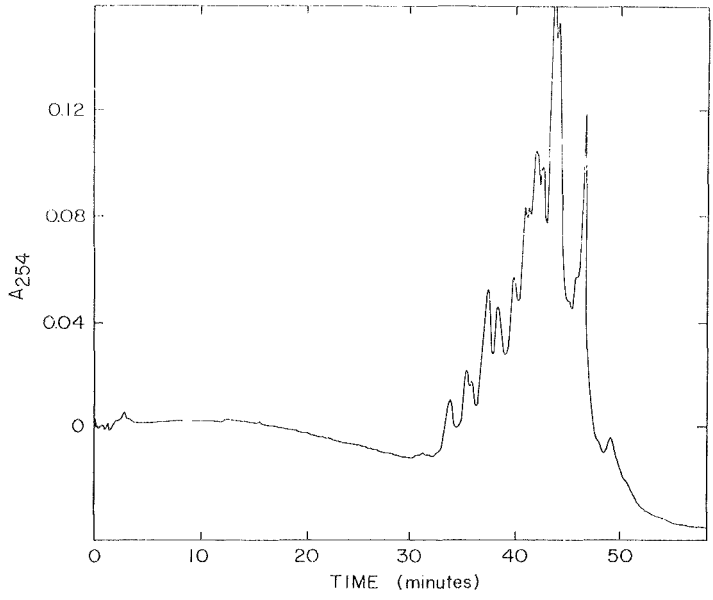

FIGURE 3 Fraction of yeast tRNA on a polymeric C_2 coated packing. Elution conditions as in Fig. 1 except temperature was 55°C.

Mobile Phase

Identification and manipulation of adsorptive retardation contributions on agarose media have been done by pH, temperature, column dimension and gradient slope variations (2,8). We have found that another parameter, addition of small amounts of alcohol to the mobile phase, can alter selectivity in tRNA fractionations (compare Fig. 3 with Figs. 4a and 4b). Interestingly, the less hydrophobic tRNAs have a marked difference in fractionation pattern depending upon whether 1% 1- or 2-propanol is used, although there seems to be no selective difference for the more hydrophobic tRNAs eluted in the last third of the gradient profiles (Figs. 4a and 4b). In general, peaks eluted faster using 1-propanol as opposed to 2-propanol. This same effect has been noted in reversed-phase peptide separations (20). In that type of system protein adsorption-desorption is thought to be a result of

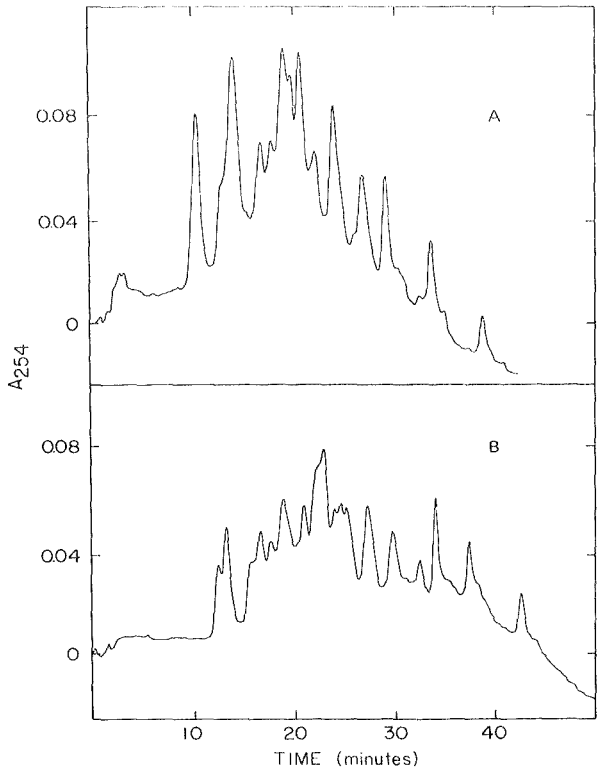

FIGURE 4 Beneficial effect of organic modifier on tRNA retention and resolution. Conditions as in Fig. 3 except 1% propanol was added to both buffers. A, 1-propanol; B, 2-propanol.

multisite binding and release mediated by a critical concentration of organic modifier (14,21). The effect an organic modifier has on DSG-RP chromatography may be somewhat analogous in that only a small amount of propanol in the mobile phase can cause drastic reductions in tRNA retention times. Under conditions of higher initial salt concentration, addition of 1% (v/v) 2-propanol to the mobile phase for fractionation of tRNAs gave excellent results, whereas addition of 2.5% resulted in non-retention of all species (Table 1). The same type of phenomenon was observed with ethanol and

TABLE 1

Effect of Organic Modifier on Retention

Organic Modifier	Percent Modifier in Mobile Phase[a]	
	Optimal tRNA Profile	No tRNA Retention
2-Propanol	1%	2.5%
Ethanol	2	4
Methanol	5	8

[a]All elution profiles are E. coli tRNA on a C_2 column at 55°C and salt gradient conditions as in Fig. 4a.

methanol. The data in Table 1 imply there is a narrow concentration range where organic modifiers may enhance resolution, with an upper threshold which, when surpassed, results in a sharp reduction of tRNA adsorptive retardation. It is apparent from the above examples that increased initial salt concentration in DSG-RP raises the critical concentration of organic modifier.

An organic modifier gradient was investigated next. An ascending 2-propanol gradient (0-2%) run concommitantly with the 2.0M $(NH_4)_2SO_4$ descending salt gradient in Fig. 5a could fractionate E. coli tRNA into 12 peaks (chromatogram not shown), but with a constant 0.5% 2-propanol concentration during the descending salt gradient, about 20 peaks were present (Fig. 5a). It was apparent that the use of organic modifiers worked better in an equilibrium rather than dynamic process. Therefore the investigation continued utilizing constant organic modifier concentrations during gradients.

Descending salt gradient chromatography of tRNAs on Sepharose 4B requires a high ammonium sulfate concentration (> 2.0M) to initiate interfacial

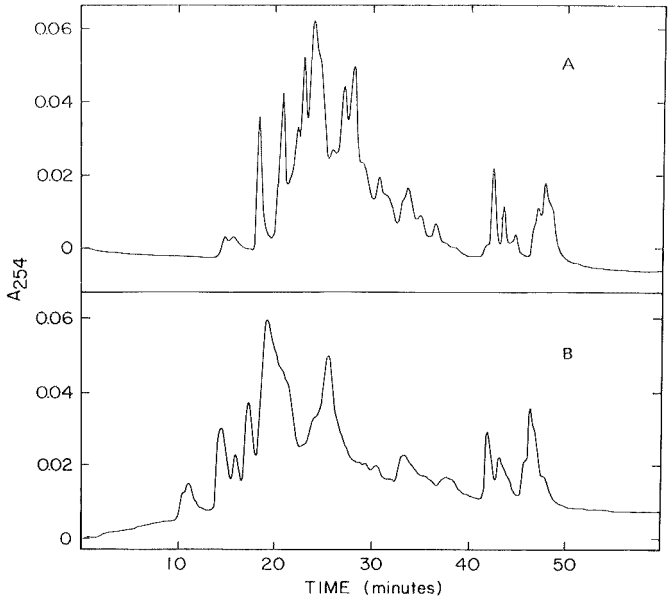

FIGURE 5 Fractionation of E. coli tRNA on a polymeric C_4 coated column. Same elution conditions as in Fig. 1, loads were 186µg, and pH varied. A: pH 7.05. B: pH 5.1.

precipitation and a long column bed (> 15cm) to facilitate absorptive retardation (2,8). 150mg tRNA loads typically require 50-100 hrs for elution. Comparable resolution of tRNAs by DSG-RP can be achieved in about 1 hr. at lower salt concentration (<2.0M) on a 5cm analytical column.

The elution profiles of E. coli tRNA in Figs. 6a-e illustrate that after a descending salt gradient has been run, organic modifier additions can then be used to adjust elution times. It was found for both yeast and E. coli tRNA that resolution at neutral pH was superior to slightly acidic conditions (Fig. 5). Retention times for relatively hydrophobic tRNAs did not vary with pH whereas less hydrophobic tRNAs eluted faster at pH 5.1.

The E. coli tRNA sample used in this study was a heterogeneous mixture of isoacceptors specific for lysine, phenylalanine, serine and valine (Fig. 7a).

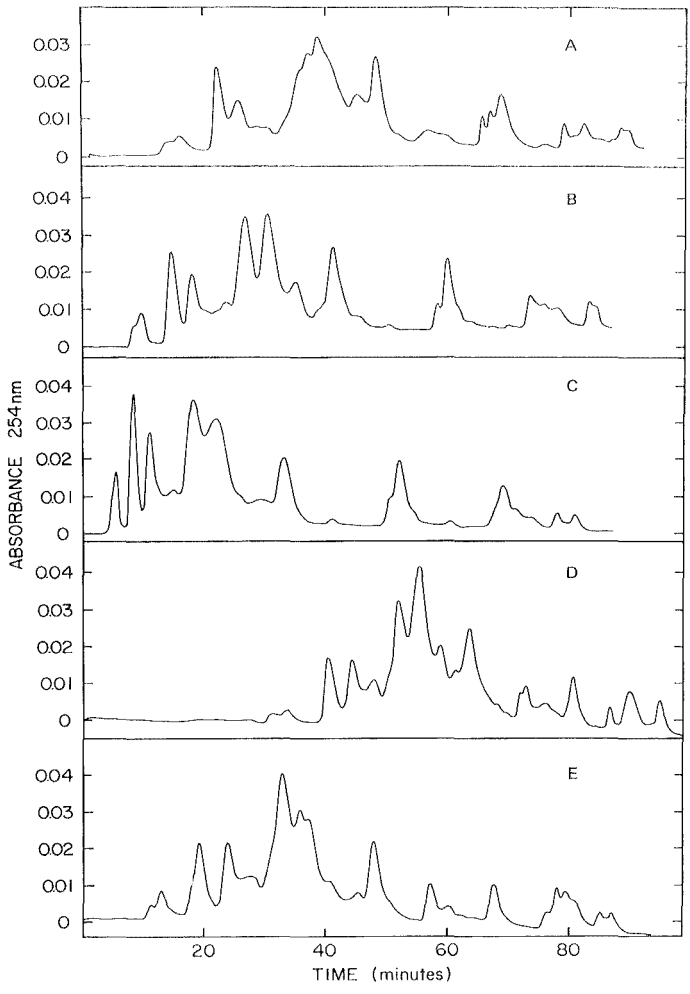

FIGURE 6 Effects of initial salt concentration and % organic modifier on tRNA retentions. 0.41 x 5.0cm polymeric C_2 column. Buffer A, ammonium sulfate and 0.2M potassium phosphate pH 7: Buffer B, 0.2M potassium phosphate pH 7. Gradient was 0-100% buffer B in 80 min. then 100% for an additional 20 min. Flow, 0.7ml/min; temperature, 55°C; injections, 100µl of 180-230µg E. coli tRNA in buffer A. Initial concentration of ammonium sulfate in buffer A and % 2-propanol maintained during the salt gradient: A, 1.5M, no propanol; B, 1.5M, 0.25%; C, 1.5M, 0.5%; D, 2.0M, no propanol; E, 2.0M, 1%.

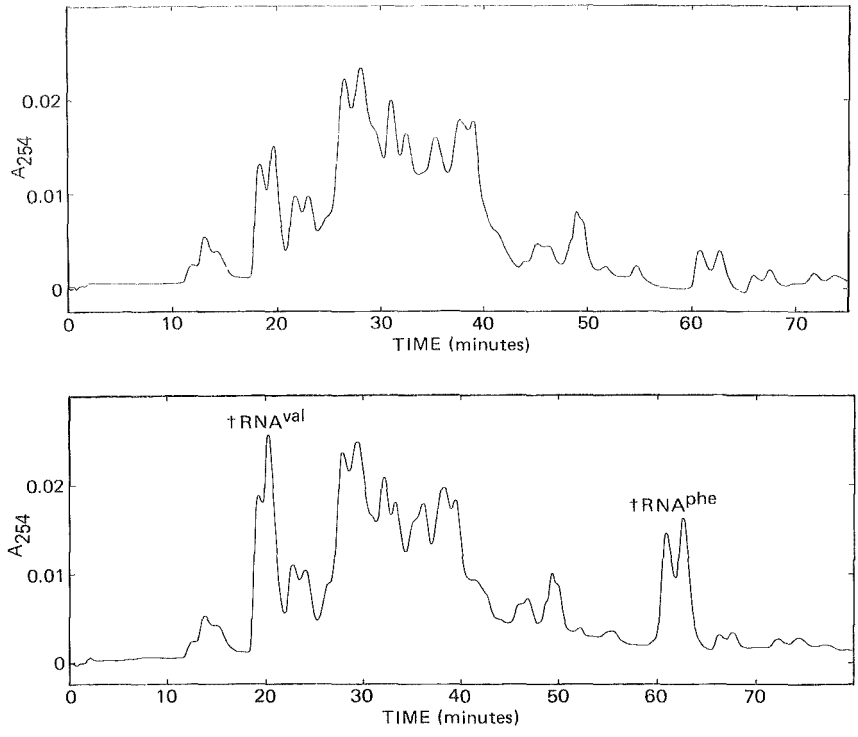

FIGURE 7 Separation of $tRNA^{val}$ from $tRNA^{phe}$ isoacceptors. Conditions as in Fig. 6D but with 0.75% 2-propanol added to buffers. A: 180µg tRNA. B: 180µg tRNA plus addition of commercially purified $tRNA_{val}$ and $tRNA_{phe}$.

Phenylalanine specific isoacceptors would be predicted to elute late in the profile because of their known hydrophobic character on classical reversed-phase supports (22,23), while the relatively less hydrophobic $tRNA^{val}$ should elute early (22,23). Chromatography of commercially purified $tRNA^{phe}$ isoacceptors was shown to elute two peaks (60 and 62 min.) late in the gradient, characteristic of relatively hydrophobic species, while commercially purified $tRNA^{val}$ isoacceptors were identified as

eluting much earlier (19 and 20 min.). A double addition of both species to the E. coli sample is illustrated in Fig. 7b. The recovery determined by absorbance at 254nm for a 180µg analytical load of E. coli tRNA using elution conditions specified in Fig. 5a was 80%.

Silica

An important consideration was the optimal type of microparticulate silica that could serve as the support for the stationary phase. Recent studies have shown that pore-diameter and other inherent silica matrix properties are important for optimizing HPLC fractionation of macromolecules such as peptides (13,14), proteins (14,24-26), and oligonucleotides (27). For example, pore sizes from 100Å for oligonucleotides (27) to 1000Å for large proteins (26) have been espoused as optimal. A qualitative study of resolution using C_2 and C_4 n-alkylsilyated silicas with pore-diameters of 120, 330, and 500Å indicated that tRNAs fractionated best on the 330Å silica (data not shown). From a purely geometrical standpoint, the dimensions of a monoclinic, crystalline form of yeast $tRNA^{phe}$ are 56 x 33.4 x 63.0Å (12), which suggests that utilization of a high-surface area, mesopore (100Å) silica would suffice. Actually, three lines of physical evidence dictate the desirability of macroporosity:

1) In solution tRNA (27,000 daltons) has considerable linear structure (28) which causes the hydrodynamic volume to be comparable to a 60,000 dalton protein (29);
2) A 60,000 dalton sphere or solute that kinetically behaves like one has diffusivity problems in mesoporous silica, resulting in poor efficiency (30);
3) The DSG-RP separation mechanism is partly based on interfacial precipitation where possible multilayer stacking (2) must be considered.

In addition to better selectivity, the 330Å pore-diameter silica was preferred over the 500Å silica on the basis of pressure stability (14) and greater surface area (100 vs. 50 m^2/g).

CONCLUSION

Fractionation of tRNA isoacceptors was achieved by a descending salt gradient on a reversed-phase high-performance liquid chromatography column. This system (DSG-RP) was demonstrated on C_1, C_2, and C_4 type coatings. Taking into consideration the physical nature of tRNA molecules, it was found that macroporous, as opposed to mesoporous, microparticulate silica was desirable. Retention time could be manipulated by 1) initial salt concentration or 2) amount organic modifier added to the mobile phase. The type and amount of organic modifier used were important variables. Resolution was increased at elevated temperature, but at the expense of a concomitant decrease in column lifetime. At room temperature the columns were very stable. Hydrophobic interactions are normally enhanced at a high temperature (2). In this study, elution at 55° as opposed to 24° decreased retention times. Apparently the thermal retardation enhancement was more than offset by the effect increasing temperature had on solubilizing tRNA to negate interfacial precipitation. The same affect has been noted by Spencer for Sepharose 4B (2). The effects of initial salt concentration, type and amount of organic modifier, temperature and pH had, in general, a greater effect on the less hydrophobic tRNAs. The efficacy of this new HPLC method has yet to be explored for separation of larger polynucleotides.

[1]Abbreviations used: HPLC, high-performance liquid chromatography; RPLC, reversed-phase high-performance liquid chromatography; DSG-RP, descending salt gradient reversed-phase.

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REMOVAL OF THE NONIONIC DETERGENT EMULGEN 911 FROM SOLUBILIZED MICROSOMES BY HPLC

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ABSTRACT

The removal of a nonionic detergent, Emulgen 911, by HPLC from solubilized microsomes is described. Microsomal samples were dissolved in phosphate buffer containing sodium cholate and tritiated Emulgen 911. The HPLC column was eluted with phosphate buffer containing 20% (v/v) glycerol, sodium cholate and, for one set of conditions with Lubrol WX. The detergent, Emulgen 911, when eluted with sodium cholate, binds tightly to the column eluting after the inclusion volume. Elution in the presence of Lubrol WX resulted in decreased retention, with Emulgen 911 eluting within the inclusion volume. Microsomal samples chromatographed with sodium cholate are resolved into two peaks. The first eluting peak is associated with the cytochrome P-450 fraction. Effective detergent removal under these conditions was 88% of the eluted radioactivity. For purified cytochrome $P\text{-}450_1$ detergent removal was 99% efficient and provided a sample with a ratio of 2.5 microgram detergent per nanomole of P-450. Elution with Lubrol WX did not provide a full quantitative picture because of overlapping protein and detergent peaks but it provided evidence for strong hydrophobic interactions between the bonded phase and Emulgen 911, and between the two nonionic detergents.

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INTRODUCTION

Proteins which are an integral parts of membranes require solubilization by use of detergents prior to any purification step (1). Protein constituents of the endoplasmic reticulum are solubilized by means of nonionic/or ionic detergents. A combination of non-ionic and ionic detergents has been successfully used for the solubilization of microsomal preparations prior to purification (2). One of the most frequently used combinations in the purification of cytochrome P-450 is sodium cholate and Emulgen 911. The latter is a nonionic surfactant consisting of a mixture of polyoxyethylene nonylphenyl ethers (3). The presence of detergents in the purified protein samples represents a serious problem due to potential inhibition of enzymatic activity and slow denaturation of the enzyme (2,4). These effects are associated with both types of detergents but removal of nonionic detergents from solubilized proteins is comparatively more difficult than removal of ionic detergents (1,2). Consequently, it is worthwhile to devise efficient methods for removal of nonionic detergents from protein samples. A frequently used method for removal of Emulgen 911 involves the use of XAD-2 resin; by sequential batch treatments the concentration of detergent is lowered to acceptable levels. The use of Sephadex LH-20 has been reported for removal of Lubrol WX (5). Application of this latter procedure to Emulgen 911 is not as efficient, with an optimal 60% effective removal in a slow process (2). A recently

published procedure involving chromatography in hydroxylapatite (6) has become the most widely used method for removal of nonionic detergents. In the present communication we explore the removal of Emulgen 911 from rat liver microsomes by HPLC. This technique has been recently used to fractionate cytochrome P-450 samples either on ion exchange (7) or size exclusion columns (8).

MATERIALS AND METHODS

Charles River CD strain male rats were used in the present study. A sample of [^{3}H]-Emulgen 911 was generously provided by Mr. Wayne Levin, Hoffman LaRoche, Nutley, NJ. No specific activity was given for the tritiated detergent, a neat aliquot of 1 µl represented ca. 10^6 cpm and appropriate dilutions were made from this stock. A sample of highly purified cytochrome P-450$_1$ from rabbit lung was provided by Dr. Richard M. Philpot, Laboratory of Pharmacology, NIEHS, Research Triangle Park, N.C.

SOLUBILIZATION OF MICROSOMES

Rat liver microsomes were solubilized at room temperature in 100 mM potassium phosphate buffer, pH 7.5, containing 0.5% sodium cholate and 0.1% Emulgen 911 according to literature procedures (9). For the experiments with [^{3}H]-Emulgen 911, 2 ml of protein solution (10 mg protein/ml) was diluted with 2 ml of radiolabeled buffer and equilibrated at room temperature for 1 hr. The final activity of the solubilized microsomes was 102 408 cpm/10 µl at a protein concentration of 5 mg/ml. From this solu-

tion, aliquots of 250 µl (250 µg Emulgen, 1.25 mg protein) were injected into the HPLC column.

CHROMATOGRAPHIC CONDITIONS

The eluents used were: buffer A, 100 mM potassium phosphate (pH 7.5) buffer solution containing 0.5% sodium cholate, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 20% (v/v) glycerol; buffer B, as above with the addition of 0.2% Lubrol WX. A control (blank) sample of [^{3}H]-Emulgen 911 buffer (20 µl, ca. 95 547 dpm) was run under each set conditions. The equipment consisted of a M6000A pump, U6K injector, model 440 dual-channel UV absorbance detector, and a model 730 data module (all from Waters Associates, Inc., Milford, MA 01757). Peak absorbance was monitored simultaneously at 280 nm and 405 nm. The column used was a Spherogel TSK-3000SW (0.75 x 60 cm, Altex-Beckman). Samples were eluted isocratically at a flow rate of 1 ml/min. In all cases fractions were collected between 8 and 60 minutes. Instagel (6 ml) was mixed with the fractions and counting was done in a Packard Tricarb 460 CD liquid scintillation counter.

HPLC of [^{3}H]-Emulgen 911. a) Elution with 0.5% sodium cholate/Lubrol WX. An aliquot of a 0.1% solution of tritiated Emulgen 911 buffer (20 µl, 123 261 dpm) was chromatographed as described above. Fractions (1 ml) were collected from 8 to 60 min. The column was subsequently eluted with water (2 x 30 ml) and methanol (2 x 30 ml); aliquots (1 ml) of these fractions were taken for scintillation counting. Total recovery of radioac-

tivity from the column was 94%. The distribution of label among the fractions was: sodium cholate/Lubrol WX buffer (91.2%), water (6.4%), and methanol (2.3%). b) Elution with 0.5% sodium cholate. As described above, radiolabeled Emulgen 911 (20 µl, 95 547 dpm) was chromatographed using sodium cholate as the only detergent component in the buffer. Total recovery of radioactivity was 94%. The distribution of label was: sodium cholate buffer (51%), water (23%), and methanol (26%).

HPLC of cytochrome $P\text{-}450_1$ Solubilized cytochrome $P\text{-}450_1$ (0.8 nmol heme) in labeled 0.05% Emulgen 911 buffer (220 µl, 120 µg Emulgen 911, 71 142 dpm) was chromatographed as above with 0.5% sodium cholate buffer. Total recovery of radioactivity was 96%. The distribution of label was: sodium cholate buffer (73%), water (13%), and methanol (14%). Fractions 5-22 (10 to 19 min) were associated with cytochrome $P\text{-}450_1$ (determined by absorbance at 405 nm) and contained 0.96% of total eluted radiolabeled detergent.

RESULTS AND DISCUSSION

A preliminary indication of the potential application of HPLC for removal of nonionic detergents was obtained during the analysis of cytochrome P-450 fractions from a DE-52 column under conditions previously described (8). A late eluting peak with strong absorbance at 280 nm, too intense in relation to the amount of protein injected, was observed. (Fig. 1). This peak was identified as Emulgen 911 by comparing retention characteristics and

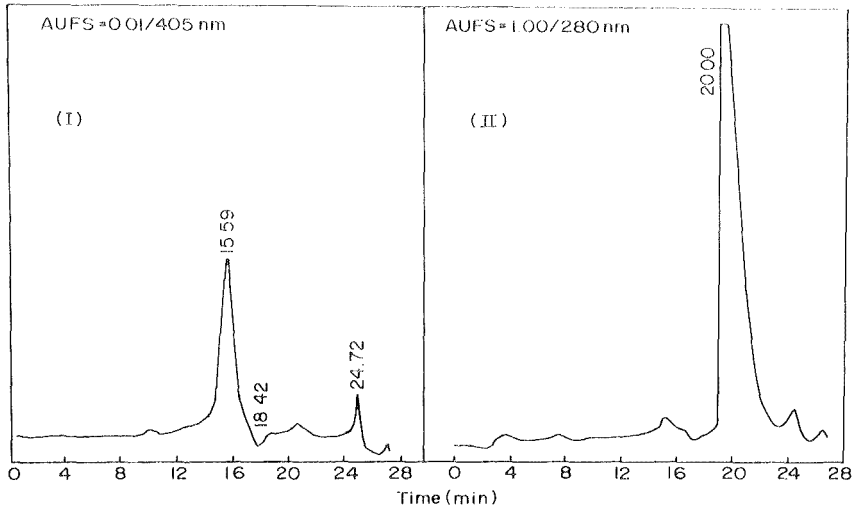

Figure 1. Partially purified cytochrome P-450 from a DE-52 column chromatographed on a Spherogel TSK-3000SW column eluted at 1 ml/min with 0.1M phosphate (pH 7.5) containing 0.2% Lubrol WX and 0.5% sodium cholate. Trace I, absorbance at 405 nm, Trace II, absorbance at 280 nm.

coelution on HPLC with an authentic standard. This particular sample of cytochrome P-450 from the ion-exchange column (DE-52) had been treated with Amberlite XAD-2 for a period of one hour following literature procedures (2). Elution of the HPLC column with a Lubrol WX-sodium cholate combination (8) allowed monitoring of the separation at 280 nm. At this point it became clear that the HPLC column was efficient in removing tightly bound detergent and that this approach could have a useful complementary role in the final stages of protein purification.

For the removal of Emulgen 911, two sets of conditions were explored. First, elution with a buffer containing 0.5% sodium

cholate and, second, elution with a buffer containing 0.2% Lubrol WX and 0.5% sodium cholate. The removal of detergent from the protein fractions was quantitated by incorporation of [^{3}H]-Emulgen 911 in the detergent mixture used to solubilize the microsomes. Because of our particular interest in the behavior of cytochrome P-450 under HPLC conditions, the profiles of the Emulgen 911 solubilized microsomal samples used in the experiments is shown in Fig. 2. The number 1 identifies the peak associated with the major cytochrome P-450 fraction. It is interesting to observe that in the absence of a nonionic detergent in the buffer eluent the cytochrome P-450 proteins aggregate readily and elute near the void volume (10-13 min, Fig. 2). The cytochrome P-450 is not denatured under these conditions, and, as in the case of elution with Lubrol WX, the isolated fraction from HPLC provided a carbon monoxide binding difference spectrum after reduction with sodium dithionite. The elution profile with Lubrol WX was as previously described (8), the cytochrome P-420 elutes near the exclusion volume (10-13 min), and the major cytochrome P-450 fraction elutes between 12-18 min peaking at 16 min (Fig. 2).

The elution profiles, radioactive and 280 nm absorbance, obtained by chromatography of the [^{3}H]-Emulgen 911 buffer are shown in Fig. 3A. Three radioactive peaks designated as A, B, and C are distinguishable. The distribution of radioactivity among these peaks is shown in Table 1. Peak A eluted at the

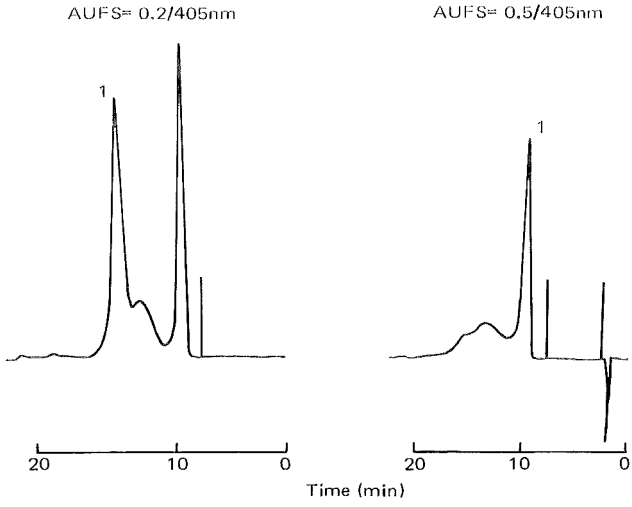

Figure 2. HPLC profile for the microsomal sample in the presence (right-hand trace) and absence (left-hand trace) of the nonionic detergent Lubrol WX. The number 1 identifies the fraction corresponding to cytochrome P-450.

inclusion volume (25 ml) as determined with tritiated water. Peak B is a broad, disperse band, and peak C is highly retained and eluted well beyond the inclusion volume. Peak C contained 44% of the eluted radioactivity and was associated with the major absorbance peak at 280 nm. Obviously, the mechanism of retention of Emulgen 911 in this column is not based exclusively on size, perhaps involving hydrophobic interactions with the bonded phase. It was also clear that the labeled detergent was not strictly pure; however, since other studies have been conducted with [^{3}H]-Emulgen 911 from the same source, a comparison would be possible only by using the original detergent sample (*2*,10).

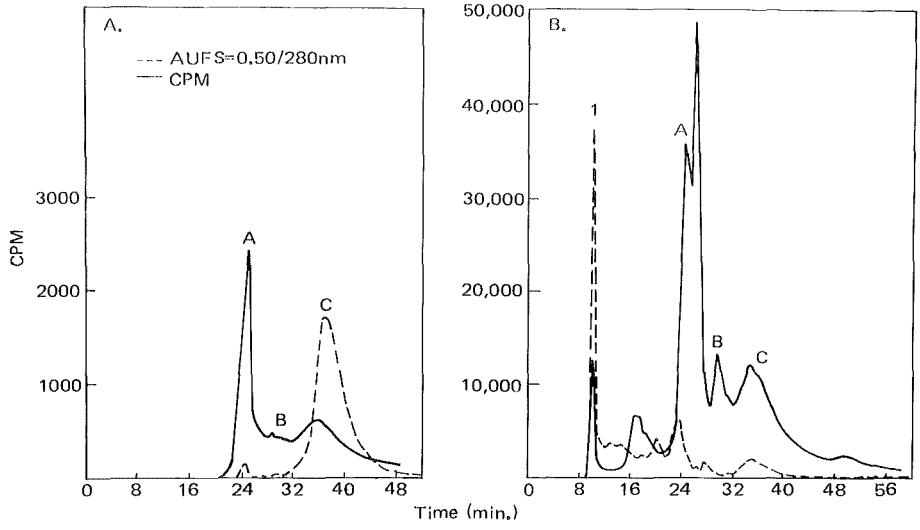

Figure 3. HPLC profile for samples eluted with buffer/0.5% sodium cholate. Trace A, [^{3}H]-Emulgen 911 buffer solution. Trace B, microsomal sample solubilized in [^{3}H]-Emulgen 911 buffer. The number 1 designates the cytochrome P-450 fraction.

The elution profile obtained from a sample of rat liver microsomes solubilized in [^{3}H]-Emulgen 911 and eluted with 0.5% cholate buffer is shown in Fig. 4B. Two distinct areas were established for the protein sample. Peak 1, from 10 to 13 min., corresponds to the major cytochrome P-450 peak (labeled 1, in Fig. 3B) as determined by absorbance at 405 nm (Fig. 2); peak 2, 13-16 min, contains other proteins with lesser amounts of heme proteins, including cytochrome b_5. As shown in Table 1, the total amount of radioactivity emerging in the protein fractions was 12% of the recovered radioactivity. By examining the amounts

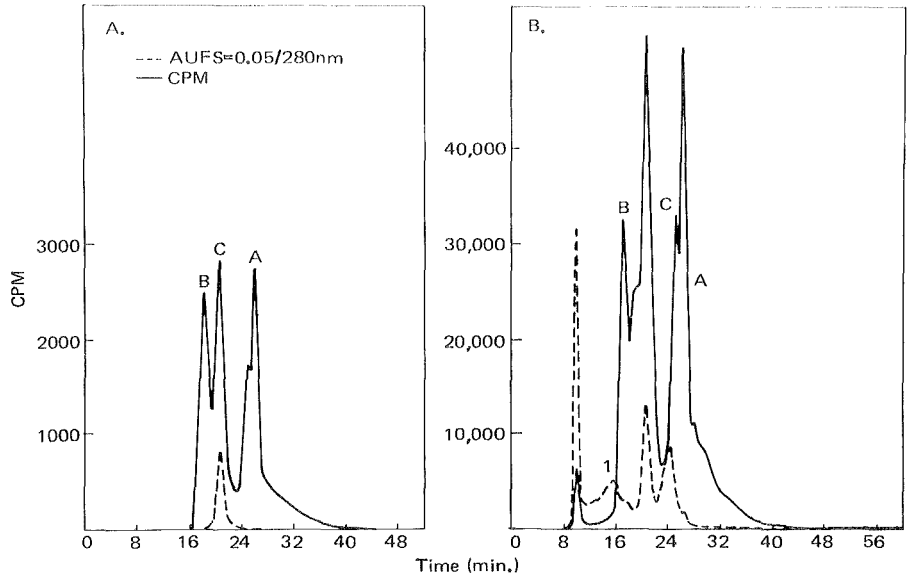

Figure 4. HPLC profile for samples eluted with buffer/0.5% sodium cholate 0.2% Lubrol WX. Trace A, [^{3}H]-Emulgen 911 buffer solution; Trace B, microsomal sample solubilized in [^{3}H]-Emulgen 911 buffer. The number 1 designates the cytochrome P-450 fraction.

recovered for the detergent peaks (Table 1), peak C shows a significant decrease, relative to blank, in cpm content. Peak C is the major detergent component observable by UV and it is tempting to speculate that the detergent eluting in the protein fractions is derived largely from this particular pool. As a whole, 88% of the [^{3}H]-Emulgen 911 is removed in one pass through the column; if we consider only the cytochrome P-450 fraction (peak 1), close to 96% removal is achieved. The efficiency of this approach to the removal of large amounts of nonionic detergents may be best visualized by considering the effective

Table 1

Distribution of Radioactivity Obtained by Chromatography of [^{3}H]-Emulgen 911 and Microsomes Solubilized in [^{3}H]-Emulgen 911

Sample	Eluent Buffer	Relative Distribution (%) Of Recovered Radioactivity				
	Peak:	1	2	A	B	C
Blank	A	-	-	41.4	14.4	44.2
Microsomes	A	4.2[a]	8.0[b]	40.2	12.0	35.7
Blank	B	-	-	43.4	25.3	31.3
Microsomes	B	2.1	-	47.0	13.7	37.3

[a]Fraction collected between 10-13 min, Figures 2 and 3B.

[b]Fraction collected between 13-16 min, Figures 2 and 3B.

[c]Fraction collected between 10-13 min, Figures 2 and 4B.

load of detergent and the ratio of detergent to protein before and after HPLC. For the microsomal sample, the initial ratio was 1 μg detergent/5μg protein; the recovered fraction contained 12% (30 μg) of the original detergent load and, assuming 70% as an average protein recovery (7,8), a final ratio of 1 μg detergent/30 μg protein.

This remaining 4% [^{3}H]-Emulgen 911 cannot be considered as representative of the extent of binding of this detergent to cytochrome P-450 since there are numerous other proteins eluting in the same fraction. This aspect was investigated with purified cytochrome P-450$_1$ from rabbit lung. The cytochrome P-450 (0.8 nmol) in 0.05% [^{3}H]-Emulgen 911 (220 μl, 120 μg detergent) gave

after HPLC, using cholate buffer, a solution containing heme protein (0.48 nmol by UV analysis (6)) and [^{3}H]-Emulgen 911 (1% of recovered label, 1.2 μg) for a final ratio of 2.5 μg Emulgen 911/nmol P-450. For Emulgen 911 the average molecular weight is ca. 900 (3) which indicates that after HPLC 2.7 nmol of detergent remain bound to one nmol of cytochrome P-450.

The elution profile of [^{3}H]-Emulgen 911 when Lubrol WX is incorporated in the buffer (buffer B) is shown in Fig. 4A. As with buffer A (Fig. 3A), three radioactive peaks are discernible, except that now all components are eluted within the inclusion volume of the column. Peak A still designates the peak eluting at the inclusion volume; for consistency we designate C the peak associated with strong absorbance at 280 nm, and in this case it elutes before peak A. The distribution of radioactivity in Table 1 shows an increase in the cpm in peak B with a concomitant decrease in peak C. The elution experiment with Lubrol WX was carried out largely in order to gain information on the nature of the interaction between Emulgen 911 and the HPLC bonded phase, and about the possible exchange between nonionic detergents under the chromatographic conditions used. Chromatography of the microsomal sample with buffer B is shown in Fig. 4B. Under these conditions only peak 1 (Table 1) could be quantitated because of the strong tailing of peak B. As shown in Fig. 4B the peak corresponding to cytochrome P-450, identified as 1 on the trace, overlaps partially with a detergent peak. As shown in Table 1, only 2% of the

eluted radioactivity remains associated with this protein fraction. There are changes in the relative amounts of peak B and peak C but our inability to quantitate protein peak 2 makes analysis rather uncertain. The elution experiment with Lubrol WX does provide a clue as to the mechanism of action of the HPLC column towards nonionic detergents. In the absence of Lubrol WX, i.e., buffer A, Emulgen 911 interacts strongly with the column and elutes beyond the inclusion volume (Fig. 3A). In the presence of Lubrol WX, i.e., buffer B, Emulgen 911 is unable to compete for hydrophobic sites on the column and elutes considerably faster (Fig. 4A). Supporting evidence for this mechanism was found in the distribution of [^{3}H]-Emulgen 911 among buffer, water, and methanol fractions (see experimental). With Lubrol WX, 91.2% of the radiolabel was eluted in the buffer fraction compared to 51% with sodium cholate; in the latter water (23%) and methanol (26%) accounted for a significant portion. Differences in the distribution of [^{3}H]-Emulgen 911 among peaks A, B, and C (Table 1) are also suggestive of the formation of mixed aggregates with Lubrol WX. In all cases the formation of multiple high molecular aggregates observed in the LH-20 procedure does not materialize (2).

Under both sets of conditions, the removal of [^{3}H]-Emulgen 911 from the microsomal preparation compares favorably with optimal conditions reported for LH-20 and XAD-2 treatments which range from 50-60%. For the purified cytochrome P-450 examined, the

ratio obtained (2.5 µg detergent/nmol P-450) closely approaches the optimal values reported for hydroxylapatite (6). The recoveries of heme protein from HPLC columns have been demonstrated to be high (6,7), and since methods are available for the efficient removal of sodium cholate and Lubrol WX (5), the approach described here has practical potential in the removal of tightly bound nonionic detergents from proteins. Experimental factors to bear in mind are the load of detergent and concentration of protein solution, both of which may affect the efficiency of the procedure.

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HPLC SEPARATION OF THE DIASTEREOMERIC GLUTATHIONE ADDUCTS OF STYRENE OXIDE

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ABSTRACT

The four diastereomeric thioether adducts resulting from the addition of glutathione to racemic styrene oxide were separated on a Radial Pak C_{18} column using pH 7 Tris-phosphate buffer solution containing methanol as eluent. The benzylic thioether (1) eluted earlier than the benzylic alcohol (2) regioisomers. A complete stereochemical profile was established with the first eluting stereoisomer assigned as (S,R)-1, followed by (R,R)-1, (S,R)-2, and (R,R)-2,. The diastereomers with S configuration at the benzylic carbon emerged first for each set of regioisomers. The use of glutathione as a chiral probe for the analysis of enantiomerically enriched epoxides was illustrated with β-methylstyrene oxide formed from (1R,2S)-N,N-dimethylephedrium bromide during the course of a chiral phase-transfer synthesis of oxiranes.

INTRODUCTION

The reaction of glutathione (GSH) with electrophilic compounds is an important component in the defense mechanisms available to many organisms. This reaction is catalyzed by a group of enzymes known as the glutathione transferases (1). Among the substrates for these enzymes, epoxides constitute a

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major group of environmental interest. Epoxides are products of the oxidative metabolism of unsaturated hydrocarbons catalyzed by the cytochrome P450 dependent monoxygenase system (2). Styrene oxide has been found a useful substrate for the in vitro determination of glutathione transferase activities (3). In addition, this epoxide has provided a model for examining the stereochemical aspects of the reaction of GSH in the presence and absence of enzyme (4). The product profile for GSH and styrene oxide indicated various degrees of stereoselectivity for the chemical and enzymatic reactions. Further work was hampered by the lack of a suitable analytical procedure. Optimal analytical conditions provided only partial separation of the stereomeric GSH conjugates of styrene oxide (5).

In the present work we describe a modified reversed-phase HPLC (RP-HPLC) procedure for the separation of all the diastereomeric glutathione adducts of this epoxide. The practical value of this procedure is illustrated by examining the stereoisomer distribution resulting from reaction of GSH with a styrene oxide sample obtained by a phase- transfer procedure utilizing a chiral catalyst reported to produce epoxide with high enantiomeric enrichment (6).

MATERIALS AND METHODS

Enantiomerically pure styrene oxides were prepared from the corresponding mandelic acids. Reduction of (+)-(S)-mandelic acid with borane-dimethyl sulfide gave (+)-(S)-phenylethane diol

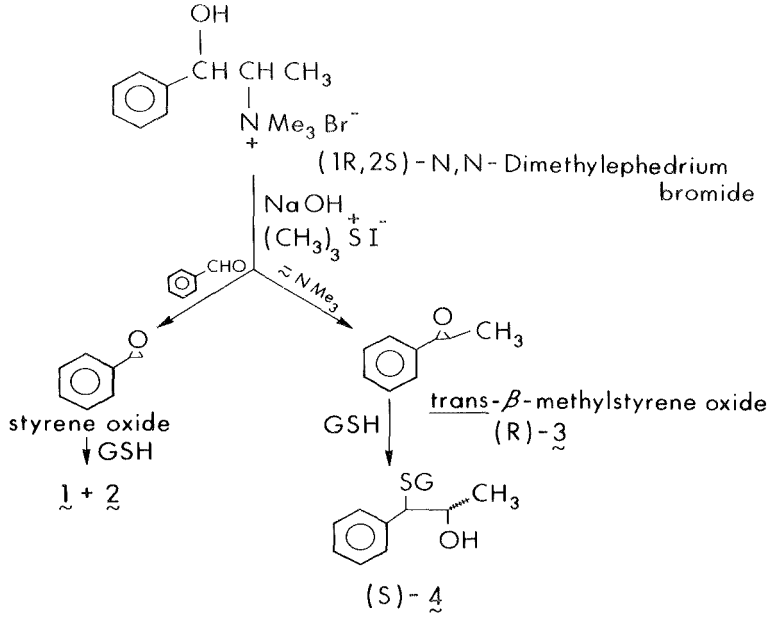
which by the orthoester procedure (7) gave (-)-(S)-styrene oxide ($[\alpha]_D^{25}$ -42.5° (c2.0, benzene), lit.(8)-41.5° (c5.0). Similarly, (-)-(R)-mandelic acid afforded (+)-(R)-styrene oxide $[\alpha]_D^{25}$ +35.5°(c2.27, benzene), lit.(9) + 42.2° (c3.09).

Chiral phase-transfer procedure

By following the published procedure (6), benzaldehyde (10.7 mmol), (-)-(1R,2S)-N N-dimethylephedrium bromide (2.13 mmol), trimethylsulfonium iodide (11 mmol), and sodium hydroxide (20 mmol) were refluxed for 60 hr under argon in a mixture of water (18 ml) and dichloromethane (8 ml). The crude product was purified by filtration through deactivated neutral alumina, followed by elution with dichloromethane, to provide 0.63 g of an oil, $[\alpha]_D^{25}$+4.2° (c1.7, acetone), lit. +4.4° (c5.5, acetone). No significant product formation ocurred at room temperature. A control experiment was conducted as above except that benzaldehyde was omitted from the reaction mixture. The product, 0.17 g (57% yield), $[\alpha]_D^{25}$ + 79.75° (c1.6, acetone), was identified as trans-β-methylstyrene oxide by proton nmr and its reaction products with GSH (Scheme 1).

Glutathione Conjugates

The GSH conjugates were prepared by reaction of GSH (5 eq) with the corresponding epoxide (1 eq) in 0.5 M potassium bicarbonate for 12 hr at room temperature under argon. Isolation and purification methods for these compounds have been described (4,5).



SCHEME 1

HPLC

The equipment used consisted of a M6000A pump, model 440 UV absorbance detector (254nm), and a model U6K injector all from Waters Associates. The column used was a 8 mmID 5 micron C18 Radial-PAK (Waters Associates). A precolumn (5 micron C18-Spherisorb, Rainin Instruments Co.) and an in-line 2 micron filter were also used. A stock solution of Tris-phosphate buffer (pH 7) was prepared as follows: 5 ml of 85% phosphoric acid was added to 1000 ml of water and neutralized to pH 7 (Corning 125 pH meter with combination glass electrode) with tris-(hydroxylmethyl)aminomethane (Tris-base). Buffer A

consisted of 500 ml of stock solution plus 500 ml of 50 mM sodium sulfate solution. Buffer B contained 475 ml of stock, 25 ml methanol, and 500 ml 50 mM sodium sulfate. The column was conditioned by pumping 10 column volumes of B followed by 15 column volumes of A. For the separations, a step gradient procedure at a flow rate of either 3 ml/min or 2 ml/min was used. After sample injection, buffer A was pumped for 10 min followed by a step switch to buffer B until elution was complete. For reequilibration, 15 column volumes of buffer A were pumped through the column.

RESULTS AND DISCUSSION

The reaction of styrene oxide with glutathione produces two positional isomers, 1 and 2 (Fig. 1). Because of the chiral benzylic center in styrene oxide each positional isomer may consist of one or two diastereomers depending on the optical purity of the starting epoxide. As shown in Fig. 1 the benzylic thioether isomers are designated as (R,R) - 1 and (S,R) - 1, and the benzylic alcohols as (R,R) - 2 and (S,R) - 2. The first letter designates the absolute configuration at the benzylic carbon and the second letter the configuration of the asymmetric carbon of the cysteine residue in GSH. For simplicity, the configuration of the γ-glutamyl residue is not included in this notation. This nomenclature is preferred to rotation signs since, for styrene oxide, the magnitude and sign of rotation are solvent and

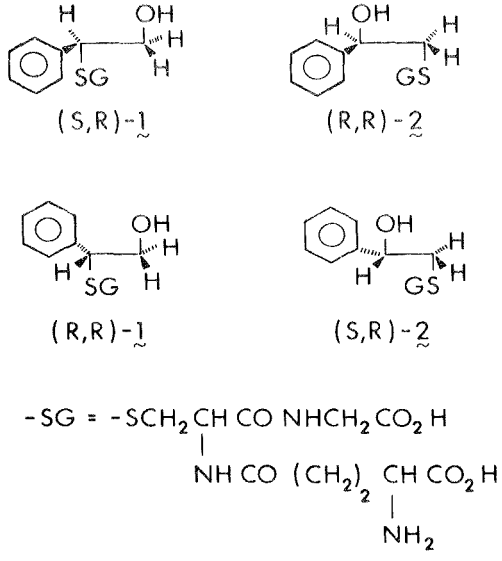


Figure 1. Relative stereochemistry of the GSH adducts of styrene oxide. The first letter designates the stereochemistry at the benzylic carbon center and the second letter the configuration of the cysteinyl residue in GSH.

concentration dependent (10). Reaction of GSH with optically pure (R)-styrene oxide would give rise to (S,R) - 1 and (R,R) - 2; (S)-styrene oxide would produce (R,R) - 1 and (S,R) - 2. Racemic styrene oxide would produce a mixture of all four diastereomers (Fig. 1).

RESULTS AND DISCUSSION

The separation of the glutathione conjugates of styrene oxide as originally developed (4) is illustrated in Fig. 2a.

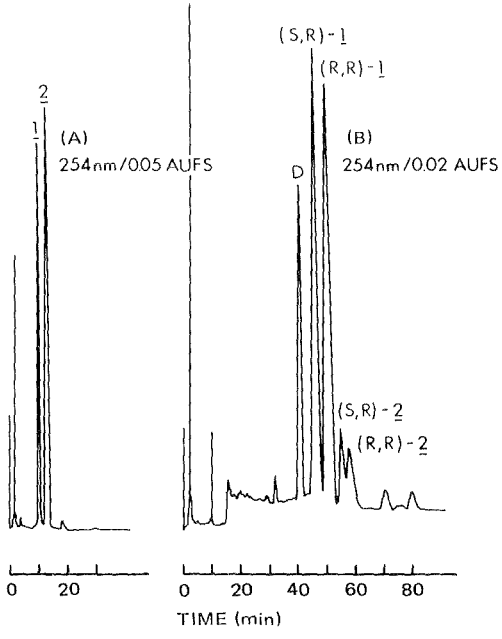


Figure 2. HPLC profiles of the diastereomeric GSH adducts of (±)-styrene oxide. Trace 2a, Tris-phosphate pH 3 buffer/15% MeOH; trace 2b, step gradient from buffer A (Tris-phosphate pH 7, 25mM sodium sulfate) to buffer B (Tris-phosphate pH 7, 2.5% MeOH, 25mM sodium sulfate). Flow rate was 2ml/min in both cases. For stereochemical N rotation see Fig. 1. The peak labeled D in Fig. 2b is 1-phenylethane 1,2-diol.

As described earlier, the first eluting peak contained a single benzylic thioether diastereomer, now identified as (S,R) - 1, the remaining three diastereomers coeluted in the second peak. These conditions were found useful in the analysis of thioether metabolites of styrene oxide but were not adequate for more detailed analysis of the stereochemical aspects of the alkylation reaction. In order to study the regio- and stereoselectivity of the enzymatic reaction we explored conditions that would allow separation of all four diastereomers (Fig. 1) and still be compatible with enzymatic samples. The separation of the four diastereomers from the reaction of racemic styrene oxide and GSH is shown in Fig. 2b. By operating at pH 7 with a minimal volume (2.5%) of methanol resolution is achieved in a reasonable time. The order of elution in this case is (S,R) - 1, (R,R) - 1,(S,R) - 2 and (R,R) - 2. Stereochemical assignments were based on the analysis of reaction products of GSH with optically pure epoxides the traces for which are shown in Fig. 3. The GSH adducts of (+)-(R)-styrene oxide (Fig. 3a) correspond to the first and last eluting peaks on the racemic sample (Fig. 2b). The (-)-(S)-sytrene oxide forms the GSH conjugates corresponding to the inside peaks (Fig. 3b) in the racemic sample (Fig. 2b). For both sets, the benzylic thioether (1) eluted ahead of the benzylic alcohol (2) regioisomer. These assignments were based on: 1) isolation and structural character-

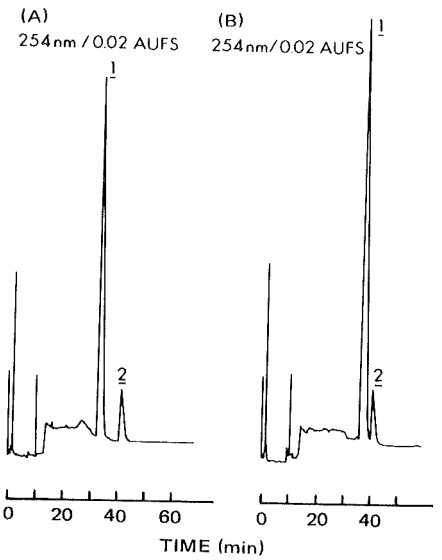


Figure 3. HPLC profiles of the diastereomeric GSH adducts of (+)-(R)-styrene oxide (trace 3a) and (-)-(S)-styrene oxide (trace 3b). Mobile phase and flow rate as on Fig. 2b. The numbers designate regioisomers (Fig. 1).

ization of the GSH conjugates of (+)-(R)-styrene oxide (14); 2) coinjection with authentic benzylic thioether samples (5). A step gradient was introduced as a cleaning step for samples from incubation mixtures. After pumping buffer A (0% methanol) for 10 min the solvent was manually switched at the pump head (M6000A pump) to buffer B (2.5% methanol). This procedure helps in the removal of large amounts of salts and other polar components and it provided for more reproducible separations. The early

eluting peak shown in Fig. 2b and identified as D corresponded to phenylethane 1,2-diol, demonstrated by coelution with authentic diol. This diol is formed by hydrolysis of styrene oxide and it is a potential contaminant in samples from enzymatic experiments. An organic amine in the buffer seemed to be critical for the separation of these compounds. We have used Tris-base because it is readily available in high purity although it is likely that other organic bases may produce similar results (11). As noted by Horvath (12), the role of organic bases in HPLC separations goes well beyond a simple buffering action and it is likely that ion pairing, and masking of silanols on the column bonded phase play an important role in the separation mechanism.

The ability to separate all four diastereomers from the reaction of styrene oxide with GSH provides an excellent tool to study the stereochemical aspects of the enzymatic and chemical reactions of GSH with styrene oxide. In addition to its obvious application to enzymatic studies, it was clear that we could use this approach to determine the optical purity of styrene oxide samples. The ring opening reaction of styrene oxide with GSH, under basic conditions, has been shown to occur without racemization (4,5) and this is verified by the traces shown in Fig. 3. As noted earlier the optical purity of styrene oxide is difficult to ascertain from the optical rotation of the sample since its value varies with

concentration and solvent. However, if a sample of styrene oxide of unknown optical purity is allowed to react with GSH as described here, the ratio of the first two eluting peaks will reflect the optical purity of the sample.

An attractive test for this approach was found in a recently published method (6) for the preparation of enantiomerically pure styrene oxides. Since these epoxides are now widely used in enzymatic reactions the operational simplicity and high optical yields obtained made this the synthetic method of choice. The reaction was conducted as described in the literature with (-)-(1R,2S)-N,N-dimethylephedrium bromide as the chiral phase-transfer catalyst. Under these conditions the (S)-styrene oxide was reported to be formed with enantiomeric purity up to 97% (6). The product obtained from this reaction was quenched with GSH (0.5 M $KHCO_3$ solution) and analyzed as described above. The trace for the product mixture (Fig. 4a) shows that all four diastereomers were formed. At the levels of enantiomeric enrichment reported (6) for this reaction a trace similar to Fig. 3b would have been anticipated. It follows that the level of optical induction by the chiral catalyst under the conditions reported is negligible. In a later note (13) the authors lamented contamination of their samples by β-methylstyrene oxide (3, Scheme 1) which could be formed from the ephedrium salt chiral catalyst. We have verified that this is indeed the case by:

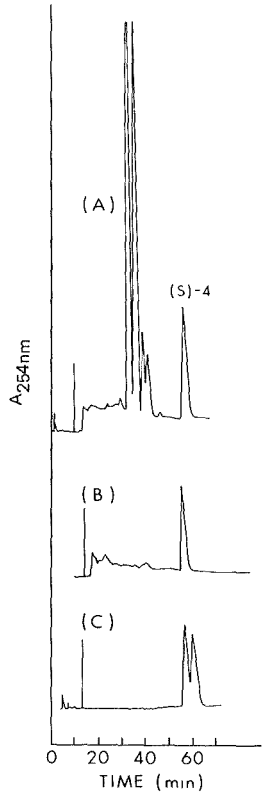


Figure 4. HPLC profiles of GSH adducts of (a) styrene oxide obtained in the presence of an ephedrium salt chiral phase-transfer catalyst; (b) optically pure β-methylstyrene oxide from fragmentation of the ephedrium salt catalyst; (c) racemic β-methylstyrene oxide. Flow rate was 3ml/min with mobile phase as on Fig. 2b.

1) a control sample without benzaldehyde and quenched with GHS; 2) coelution of authentic β-methylstyrene oxide glutathione adduct (4) with the product obtained above. The late eluting peak designated as (S)-4 on Fig. 4a corresponded to the glutathione conjugate of β-methylstyrene oxide (3) formed when benzaldehyde was ommitted. Fig. 4c shows the diastereomers derived from racemic β-methylstyrene oxide. It is clear that the extent of optical induction, if any, is very remote from the original claim. The optical activity detected in the product(s) is largely, if not exclusively, derived from optically pure β-methylstyrene oxide formed by the elimination of trimethylamine from the chiral phase-transfer catalyst. These conclusions are summarized in Scheme 1. The elimination of trimethylamine proceeds with retention of configuration at the benzylic carbon and the configuration at this site of the β-methylstyrene oxide (3) is R. Reaction of 3 with GSH has been shown to be regioselective with the sulfur nucleophile adding exclusively to the benzylic carbon (4). Thus, the configuration of the GSH adduct of 3 becomes S at this center, and in relation to the adducts from racemic 3, it corresponds to the first eluting peak (Fig. 4b and 4c). Similarly, by examining the traces of the adducts of (R)-and (S)-styrene oxide (Fig.2 and 3) one finds that for each set of regioisomers (i.e. 1 and 2) the diastereomer with S configuration at the benzylic center elutes

earlier than the corresponding R diastereomer ((S)-1 vs (R)-1, and (S)-2 vs (R)-2). This undoubtedly reflects a strong stereochemical influence in the separation mechanism involving these compounds. Further studies are necessary in order to ascertain whether this observation may be of use in elucidating the stereochemistry of GSH adducts of epoxides. The present analytical procedure significantly expands the range of experiments possible, with styrene oxide as substrate, in the study of the glutathione transferase enzymes. In addition, an interesting observation, the use of GSH as a chiral trap may develop into a practical approach to the determination of the optical purity of epoxides, particularly those from metabolic processes.

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DETERMINATION OF NALOXONE HYDROCHLORIDE IN DOSAGE FORM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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KEY WORDS

High-performance liquid chromatography. Naloxone, determination of.

ABSTRACT

A new, sensitive and rapid method for the determination of naloxone hydrochloride as drug in dosage entity and form using HPLC has been developed. Authentic naloxone hydrochloride was used to establish a calibration curve. A linear relationship was obtained for concentrations ranging from 10 µg/ml to 50 µg/ml. The column used was C_{18}, Micropak MCH-10 (monomeric) and the mobile phase was acetonitrile : 0.01 M KH_2PO_4 (70 : 30) at a flow rate of 2 ml/min. Retention time for naloxone hydrochloride was 3.3 minutes. The proposed method has been proved accurate and precise compared to other pharmacopoeia methods of assay for naloxone hydrochloride.

INTRODUCTION

Naloxone (17-Allyl-4,5-expoxy-3, 14-dihydroxy-morphinan-6-one) is a specific antagonist of pentazocine having a similar action to that of nalorphine but with greater potency.

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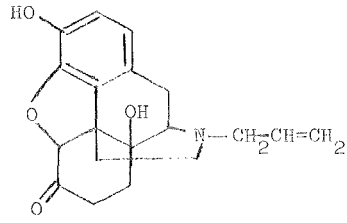


Naloxone hydrochloride is official in the USP XIX[1] where it is assayed by a non-aqueous titrimetric method using standard $HClO_4$ after the addition of mercuric acetate. An injectable solution is also official in the same pharmacopoeia but it is assayed by gas liquid chromatography.

A radioimmunoassay for naloxone in either serum or brain[2] has been developed and several procedures for the gas liquid chromatographic determination of naloxone hydrochloride and naloxone in biological fluids have been reported (3-7).

Thin layer chromatographic techniques, for the identification of the drug in studies of drug abuse using a urine screening program has been developed by Kaistha[8].

Baker[9] and co-workers have characterised several drugs of forensic interest, including naloxone, using hplc reversed phase and normal phase systems whereby the drugs, were identified by their relative retention times and by the ratios of their absorbances at 254 and 280 nm.

The present work describes a rapid, accurate and precise method for the assay of naloxone hydrochloride in dosage form using high-performance liquid chromatography.

EXPERIMENTAL

Apparatus

The HPLC unit is a Varian 8500 LC. Accessory units attached to the instrument are computer model CDS 111, autosampler model

TABLE (1)

The Optimum Values for the HPLC Parameters

Volume injected	:	10 μl
Detector	:	VAR, CHROM, 254 nm, 0.10 AUFS
Flow rate	:	2 ml/min.
Column	:	Commercially available stainless steel (4.0 mm id X 30 cm) packed with C_{18}, micropak MCH - 10 (monomeric).
Eluent	:	70/30 (acetonitrile/0.01 M K H_2PO_4)

8000 and a recorder model 9176. The optimum values for the HPLC parameters established are presented in Table (1).

Non-aqueous potentiometric titrations were performed using a combined glass-calomel electrode assembly and an automatic potentiograph model E576, Metrohm, Herisau, Switzerland.

MATERIALS

Authentic naloxone hydochloride USP[1] was used as obtained without further purification. Water used was doubly distilled in an all-glass still. Acetonitrile was spectral grade Fluka AG.; and all other reagents were analytical grade.

Glacial acetic acid and potassium hydrogen phthalate used for the non-aqueous potentiometric experiments were products of BDH and analytical grade. Mercuric acetate and perchloric acid employed were products of Riedel-De Haen AG., Seezle, Hannover.

METHODS

(1) Determination of Naloxone Hydrochloride by Non-Aqueous Potentiometry:

About 150 mg of authentic naloxone hydrochloride was accurately weighed and dissolved in a mixture of 20 ml glacial acetic acid and 5 ml of mercuric acetate (5% w/v). The potentiometric titration curve was recorded using the potentiograph and the end point of the titration was estimated by the method of parallel tangents (10). The percentage of naloxone hydrochloride was computed from the following expression:

$$\text{Percentage of Naloxone Hydrochloride} = \frac{V \times F \times 36.38}{\text{weight of naloxone hydrochloride sample}} \times 100$$

where V and F are the volume (in millilitres) and factor for the standard (0.1 N) acetous perchloric acid respectively, and 36.38 is the number of milligrams of naloxone hydrochloride chemically equivalent to 0.1 N - perchloric acid.

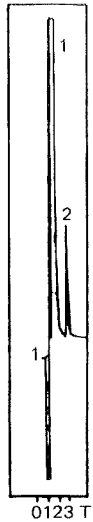
The calculated percentage of naloxone hydrochloride was used for correction of concentrations employed in the establishment of the calibration curve.

(2) Preparation of Standard Curve:

A stock solution containing 50 mg of naloxone hydrochloride per 100 ml of the eluent solution was prepared, and serial dilutions were made in the same vehicle to give solutions containing 0.1, 0.15, 0.2, 0.25, 0.3, 0.4 and 0.5 mg per 10 ml. Triplicate injections of 10 µl each were made onto the column. The integrator count was plotted versus concentration of naloxone hydrochloride.

(3) Determination of Naloxone Hydrochloride in Dosage Form:

Naloxone hydrochloride is available commercially as an injectable



Peak No. 1 – Solvent peak

Peak No. 2 – Naloxone Hydrochloride (Retention time–3.3 min)

Fig. 1

(Narcan(R), neonatal) labelled to contain 0.02 mg per ml of naloxone hydrochloride in addition to 0.86% sodium chloride and 0.2% of 9:1 mixture of methyl to propylparaben.

Six ampoules were randomly sampled. Triplicate injections of 10 µl each were withdrawn from each ampoule and made onto the column. Using the average integrator count, the concentration of naloxone hydrochloride was determined from the standard curve. Similarly added recovery experiments were carried out by spiking the ampoule contents with known amounts of authentic naloxone hydrochloride.

RESULTS AND DISCUSSION

Fig. 1 shows a tracing of a typical chromatogram obtained by HPLC. Under the conditions described above, the retention time was 3.3 min.

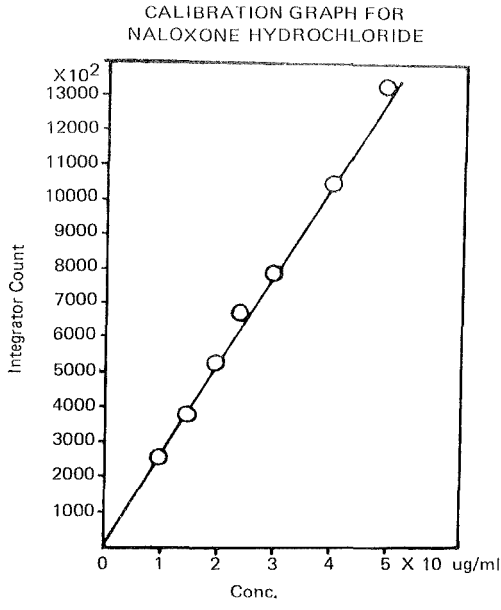


Fig. 2

Fig. 2 shows the relationship between the concentration of naloxone hydrochloride and the integrator count. A straight line is obtained demonstrating the applicability of this procedure for assaying naloxone hydrochloride in concentration range 10μg/ml to 50μg/ml. Furthermore the results of applying the new HPLC method to the determination of naloxone hydrochloride in its dosage form (Narcan(R), Neonatal) of injections are shown in Table 2. The results of spiking shown in Table (2) indicate a percentage recovery of 99.0 ± 2.6. It can be concluded that the HPLC method described here for the determination of

(R) Endo Laboratories, Inc.
Subsidiary of EI. du Pont de Nemours & Co
Garden City N.Y. 11530.

TABLE (2)

High Performance Liquid Chromatographic Determination of Naloxone Hydrochloride and in Dosage Form.

Sample	Stated amount of Naloxone hydrochloride µg/ml	Added amount µg/ml	Percentage recovery*	Standard deviations
Authentic Naloxone hydrochloride.	15	-	98.0	± 2.4
Narcan Neonatal injectable (R).	20	-	97.1	± 2.8
	20	19.98	99.0	± 2.6

*Mean of six runs.

naloxone hydrochloride in bulk and in its dosage form is rapid, accurate and simple to perform.

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LIQUID CHROMATOGRAPHIC ASSAY OF ARBAPROSTIL

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ABSTRACT

Arbaprostil was extracted from the solution formulations with ethyl ether-chloroform. The extraction efficiency was 94%. After evaporation of the extraction solvent, the samples were derivatized with p-nitrophenacyl bromide at 40°C for 30 minutes in the presence of N,N-diisopropylethyl amine. The prepared samples were dissolved in mobile phase and chromatographed on a silica gel column with acetonitrile, methylene chloride and water (150/350/2.5, v/v) as mobile phase. The chromatography separated the p-nitrophenacyl esters of arbaprostil, its 15-s-epimer and the degradation products derived from arbaprostil. When monitored by UV absorption, the degradation products could not be detected as they eluted near the solvent front under the peak of the derivatization reagent. The chromatographic responses were linear with the concentrations of arbaprostil. Assay results with good precision and accuracy were obtained.

INTRODUCTION

Most prostaglandins have small UV-extinction coefficients and derivatization with suitable chromophores to enhance UV absorbance is usually necessary for their quantitative determination. For prostaglandin free acids, various functionally substituted phenacyl bromide (1,2) and α-bromoacetonaphthone (3) are conveniently employed as derivatizing reagents to form the corresponding esters

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which can be separated by high pressure liquid chromatography (HPLC) and quantitated by monitoring the UV absorbance at 254 nm.

The derivatization of prostaglandin with p-nitrophenacyl bromide and the subsequent separation of the resulting esters by HPLC have been investigated (1). This procedure was utilized for the determination of the 15-epimer of dinoprost in bulk drug (4). The same reagent was also used to study the epimerization kinetics of arbaprostil (5). The present paper describes the application of p-nitrophenacyl bromide derivatization on a micro-scale for the quantitative determination of arbaprostil in aqueous formulations containing dextrose, alcohol, triacetin and polyoxyethylated vegetable oil.

Arbaprostil, like other C-15 alkyl substituted prostaglandins, is more resistant to enzymatic oxidation than the C-15 unsubstituted analog by 15-prostaglandin dehydrogenase (6,7). It is readily converted under acidic conditions into its biologically more active 15-s-epimer (5) and therefore serves as a prodrug of the s-epimer which inhibits gastric acid secretion (8) and promotes healing of duodenal and gastric ulcers (9,10). Like other E series prostaglandins, arbaprostil also dehydrates under acidic and basic conditions to form degradation products (5,11). These chemical and biological properties potentially make arbaprostil a highly potent but unstable drug. The facile epimerization and degradation also render the quantitative determination of arbaprostil difficult.

EXPERIMENTAL

Chemical

Arbaprostil, 15-s-epimer of arbaprostil and 17β-hydroxy 17-methyl-4-androstene-3,11-dione (internal standard) were supplied by Pharmaceutical Research and Development Laboratories of The Upjohn Co. (Kalamazoo, MI). p-Nitrophenacyl bromide and N,N-diisopropylethyl amine were obtained from Pfaltz and Bauer, Inc.

(Stamford, CT) and Aldrich Chemical Co. (Milwaukee, WI), respectively, and used as received. Solvents for HPLC were from Burdick and Jackson Laboratory (Muskegon, MI). All other chemicals were reagent grade.

The aqueous formulations of arbaprostil contained 5% dextrose solution U.S.P., alcohol, triacetin and polyoxyethylated vegetable oil (Emulphor® EL-620, GAF Corp., N.Y.).

Extraction

Aliquots of 1 mL of the solution of arbaprostil in the formulations in 15-mL culture tubes fitted with teflon-lined screw caps were supplemented with internal standard, acidified with 0.5 mL 2% phosphoric acid and immediately extracted with 10 mL of ethyl ether-chloroform (4/1, v/v). After centrifugation, 8 mL of the organic extract was transferred to a new tube and the solvent was evaporated at 40°C under a stream of nitrogen. Aliquots of blank formulation vehicle were supplemented with arbaprostil and internal standard to prepare calibration standards for the quantitative determination of arbaprostil.

Derivatization

p-nitrophenacyl bromide, 2.5 mg in 1 mL acetonitrile, and N,N-diisopropylethyl amine, 6.25 µL in 0.5 mL acetonitrile, were added to the residues of the ether-chloroform extract of the samples. The mixtures were tightly capped with teflon-lined screw caps, vortexed briefly and placed in a water bath at 40°C. After 30 minutes, the solvent in the derivatization mixture was evaporated at 40°C under nitrogen. A 2 mL aliquot of HPLC mobile phase was added to each tube containing the derivatization residues. The samples were vortexed prior to chromatography.

Chromatography

The chromatographic analysis of arbaprostil was carried out using a model 6000 A solvent delivery pump (Waters Associates,

Milford, MA), a model 440 detector (Waters Associates) or a model LC 55-B detector (Perkin-Elmer Corp., Norwalk, CT) at 254 nm, a model 7120 sample valve injector (Rheodyne, Inc., Cotati, CA) and a variable span strip chart recorder.

The chromatographic separation was accomplished using a commercially prepared silica gel column (μ-Porasil, Waters Associates) with a mobile phase of acetonitrile, methylene chloride and water (150/350/2.5, v/v). The mobile phase flow rate was 1.5 mL/min. which generated a back pressure of about 1,000 psig.

The peak height ratios of arbaprostil p-nitrophenacyl ester/internal standard were calculated from the peak height measurements. The concentrations of arbaprostil in the samples were calculated from the peak height ratios of the samples and the slope and intercept obtained by linear regression analysis of the calibration curve data.

RESULTS AND DISCUSSION

Extraction

Good recovery of arbaprostil from the formulations was obtained using ethyl ether-chloroform (4/1, v/v) as the extraction solvent. The extraction efficiency was found to be 94.2 ± 0.67% based on the total radioactivity recovery of tritium-labelled arbaprostil from the formulation.

The extraction of arbaprostil from the formulations was reproducible and simple with no emulsion formation and almost complete phase separation between the aqueous and the organic phases even though the formulation vehicle contained alcohol, triacetin and polyoxyethylated vegetable oil. However, some difficulties were encountered in our experience with the extraction of arbaprostil from its solutions in water. At low concentrations (e.g. 24 μg/mL), neither p-nitrophenacyl esters of arbaprostil nor its s-epimer was detected in the chromatograms of the prepared samples. At higher concentrations, the peak

height derived from arbaprostil was not reproducible among the samples and the peak derived from the s-epimer was often observed in the chromatograms.

The difficulties encountered in the extraction of arbaprostil from water were not associated with poor extraction recovery, since the recovery of total radioactivity of tritium-labelled arbaprostil from water was essentially complete. The chromatography of samples prepared from tritium-labelled arbaprostil solution in water showed that at least 50% of the radioactivity was eluted from the column at early retention times and, when monitored by UV absorption, the radioactive peaks were near the solvent front and masked by the peak from the reagent. Most of the remainder of the radioactivity was eluted at the same retention times as the p-nitrophenacyl esters of arbaprostil and its s-epimer. Figure 1 shows the chromatograms of a sample prepared from labelled arbaprostil solution in water monitored by both UV absorption and radioactivity. The radioactivity chromatogram was obtained by counting the collected fractions of the column effluent. Similar experiments with a solution of tritium-labelled arbaprostil in the formulation vehicle showed that only very small quantities of the radioactivity were eluted at retention times different from that of arbaprostil ester. When the glassware was siliconized (Surfasil® Pierce Chemical Co., Rockford, IL) before use for the sample preparation, most of the difficulties encountered in the extraction of arbaprostil from water were eliminated and the extraction recovery became reproducible. Therefore, the extraction recovery of arbaprostil from water was complicated by rapid epimerization and degradation of arbaprostil in water and these processes were probably catalyzed by the surface of the glassware. The siliconization of the glassware had no obvious effect on the extraction recovery of arbaprostil from the formulation solutions.

Derivatization

The formation of p-nitrophenacyl esters of various prostaglandins for chromatography was reported previously (1). For the

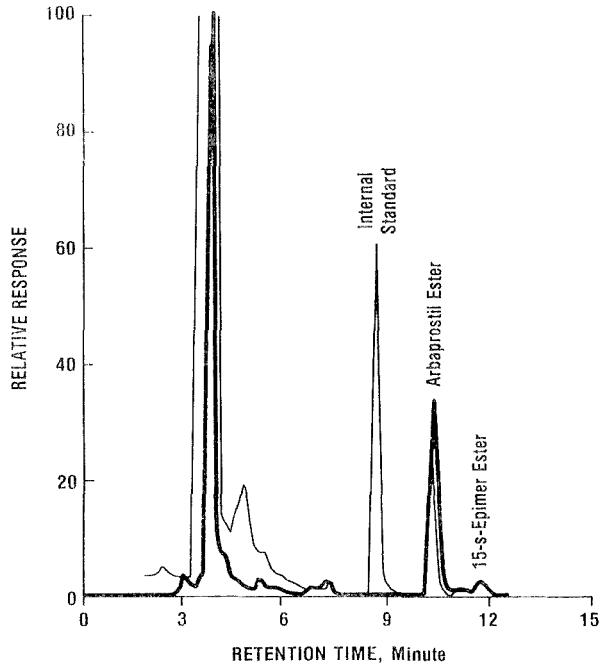


FIGURE 1. Chromatograms of a sample of ^{3}H-arbaprostil extracted from water. Mobile phase flow rate: 1 mL/min., UV absorption: ——, Radioactivity: ▬▬.

present application, we found that the reaction of the extract of 1 mL aliquots of the formulations containing about 20-800 µg (0.05-2.19 µmole) arbaprostil with 2.5 mg (10.25 µmole) of p-nitrophenacyl bromide and 6.25 µL (0.04 µmole) of N,N-diisopropylethyl amine at 40°C for 30 minutes produced the best results. These conditions resulted in minimal interferences in the chromatographic separation and a rapid and quantitative esterification of arbaprostil. Derivatization at lower temperatures (e.g., 22 and 30°C) required longer reaction times and derivatization at higher temperatures (e.g., 50°C) caused a decrease in arbaprostil peak heights after 15 minutes, indicating a potential stability problem. The conditions of p-nitrophenacyl esterification of arbaprostil at room tempera-

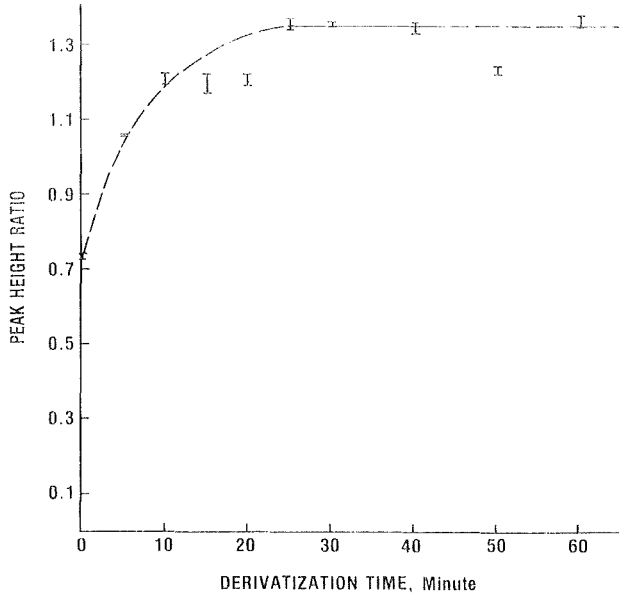


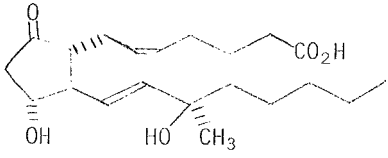
FIGURE 2. Time course of p-nitrophenacyl esterification of arbaprostil. Derivatization time vs. peak height ratio.

ture overnight were employed previously (5). Figure 2 shows the time course of p-nitrophenacyl esterification of arbaprostil under the optimal conditions. The arbaprostil ester/internal standard peak height ratios were measured to monitor the progress of the derivatization. The results shown in Figure 2 indicate that the reaction reached completion at about 25 minutes and the arbaprostil ester was stable in the reaction mixture at 40°C for at least 40 minutes. The internal standard was stable under the conditions of the derivatization.

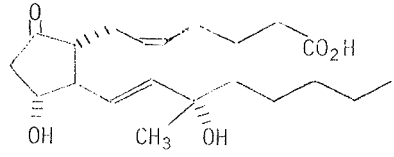
Chromatographic Separation and Linearity of Detection

Representative chromatograms of the p-nitrophenacyl ester of arbaprostil preparation from the solutions in formulation and in water as well as a sample of a blank formulation vehicle are

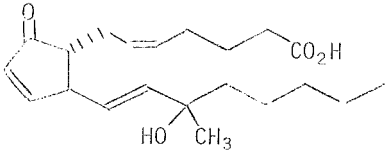
shown in Figure 3. The background interferences from the derivatization reagents and the extract of the formulation vehicle (Figure 3-a) were negligible and did not hinder the analysis of the formulation samples having arbaprostil concentrations of 20 µg/mL or higher. The internal standard and the p-nitrophenacyl esters of arbaprostil and s-epimer were eluted from the column with mobile phase flow of 1.5 mL/min. at the retention times of about 5.8, 6.8 and 7.7 minutes, respectively. The s-epimer was often present in arbarprostil solutions in water (Figure 3-d) but was very seldom observed in the formulations (Figures 3-b and -c).



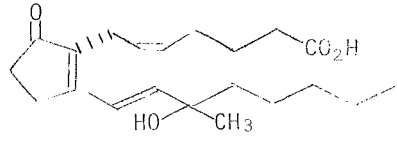
arbaprostil



15-s-epimer



15-methyl prostaglandin A_2



15-methyl prostaglandin B_2

As revealed by radioactivity, the early elution peaks near the solvent front derived from arbaprostil and masked by the peaks of reagent were probably from the A_2 and/or B_2 type prostaglandins (5,11) and other degradation products (5). Under the chromatographic conditions, the esters of 15-methyl prostaglandins A_2 and B_2 were eluted early. Under the conditions of gradient elution, using mobile

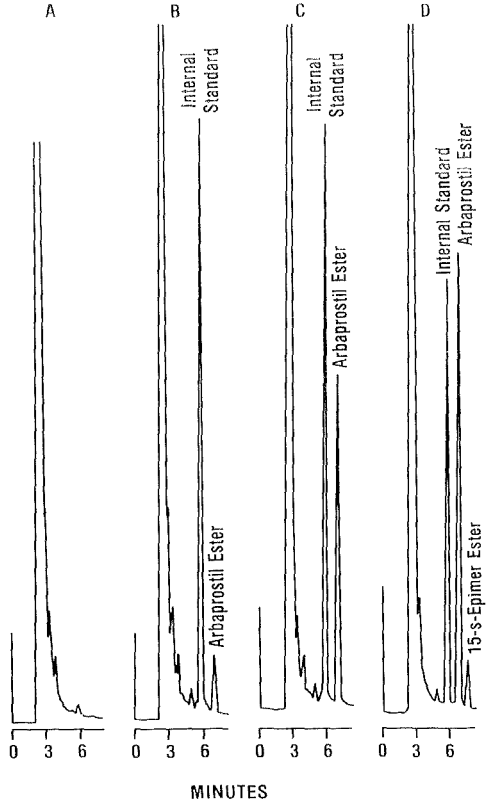


FIGURE 3. Chromatograms of samples of (A) blank formulation, (B) formulation with 24 μg/mL arbaprostil, (C) formulation with 150 μg/mL arbaprostil, and (D) solution in water with 301 μg/ml arbaprostil. Samples B, C, and D contained internal standard.

phase of methylene chloride, acetonitrile and 2-propanol from 350/50/5 (v/v) to 350/150/5 (v/v) and a silica gel column (μ-Porasil), these degradation products were separated into 4 peaks. Two of these peaks had retention times similar to those of the epimers of 15-methyl prostaglandins A_2 and B_2. The other two peaks had different retention times. From these preliminary results, the identity of these components could not be established.

The esterified arbaprostil and the internal standard were stable in the mobile phase solution at ambient temperature (22°C) for at least 72 hours. The peak heights of arbaprostil ester and the internal standard showed no change when the sample was chromatographed during this period of time. The chromatography of the calibration standards immediately after preparation and 72 hours thereafter also showed no significant change in the slopes of the resulting calibration curves. For example, the calibration curves with slopes of 0.00396 and 0.00401 and the intercepts of 0.00890 and -0.00653 were obtained by repeated chromatography of a set of calibration standards immediately and 72 hours, respectively, after their preparation.

When the derivatized samples were evaporated, redissolved in mobile phase and chromatographed, the arbaprostil ester/internal standard peak height ratios were proportional to the arbaprostil concentration in the range from about 20 to at least 600 μg/mL in formulation solution. This proportionality was indicated by the constant peak height ratios obtained by the chromatography of a 600 μg/mL sample and its dilutions and by the linearity of the calibration curves. The correlation coefficients of the calibration curves were usually better than 0.999 with negligible intercepts. The slopes did not vary significantly from day to day. For example, 21 calibration curves prepared over a span of 7 weeks had an average slope of 0.003994 ± 0.000141 (mean ± S.D., RSD = 3.5%, range = 0.003756 to 0.004284).

When the derivatization mixture in acetonitrile was directly chromatographed, the peak height ratios were found to vary with the injection volume. Replacing acetonitrile (by evaporation) with the mobile phase as the solvent for injection of the derivatization mixture, identical peak height ratios were obtained independent of the injection volume (5-20 μL). These observations were reproducible simply by switching the solvent. Table 1 shows some typical peak height ratios obtained by injection of the acetonitrile solution of a sample. The peak height ratios

TABLE 1

Effect of Injection Volume on Peak Height Ratio of Arbaprostil (200 µg) p-Nitrophenacyl Ester and Internal Standard (75 µg) in Acetonitrile (2 mL).

Injection Volume, µL	2.5	5	10	15
Peak Height Ratio	2.1987	2.5370	3.4528	4.3243

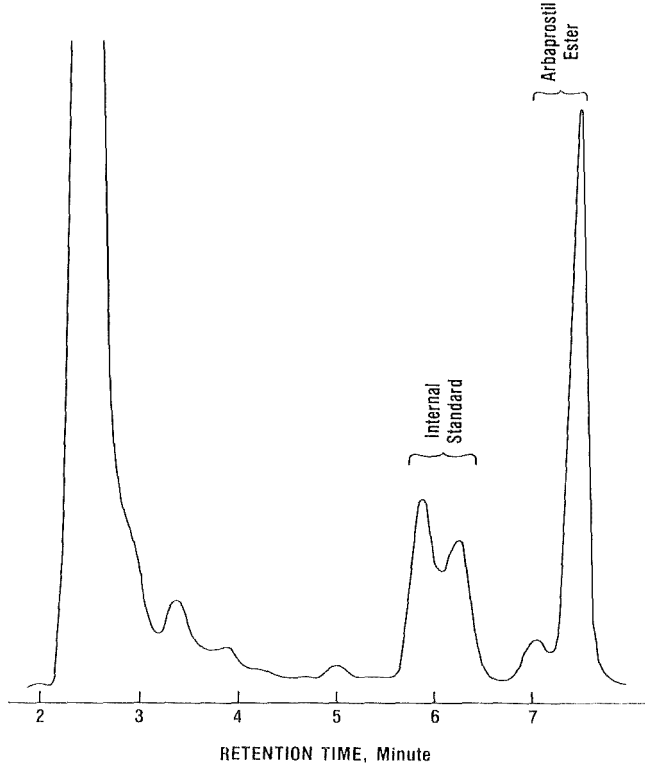


FIGURE 4. Chromatograms of acetonitrile solution of the derivatization mixture of arbaprostil and internal standard. Injection volume 25 µL.

increased from 2.1987 for a 2.5 µL injection to 4.3243 for a 15 µL. For injection volumes of 20 and 25 µL, the formation of double peaks was obvious for both the internal standard and the arbaprostil ester as shown in Figure 4.

The above phenomena of changing peak height ratios with injection volume were related to the peak broadening which led to the double peak formation (Figure 4). Injection of acetonitrile probably disturbed the equilibrium between the stationary and the mobile phases with respect to water present in the system. This disturbance influenced the adsorption interaction between the solutes and the silica stationary phase and changed the chromatographic separation. When small quantities of water (5-15 µL) were added to the acetonitrile solution (2 mL) of the derivatization mixture, the chromatographic peak broadening and splitting were effectively suppressed. Methylene chloride and ethyl ether did not completely dissolve the derivatization mixtures. However, the supernates resulting from centrifugation of the mixtures in methylene chloride and in ethyl ether behaved like the mobile phase and the water supplemented acetonitrile solutions in that there was no peak broadening and that the chromatographic peak height ratios did not vary with the injection volume. The proportionality between peak area and concentration was not investigated.

TABLE 2

Assay Accuracy and Precision

Label Conc. µg/mL	No. of Sample	Concentration found Mean ± S.D., µg/mL	% RSD	% of Label
24.1	4	23.95 ± 0.25	1.05	99.4
301.6	4	295.10 ± 1.58	0.54	97.9
626.8	4	615.60 ± 1.69	0.28	98.2

Assay Accuracy and Precision

Table 2 shows some data indicating the accuracy and precision of this analytical method. At arbaprostil levels of 24.1, 301.6 and 626.8 µg/mL in the formulations, the concentrations found were within 3% of the corresponding theoretical values and the relative standard deviations were less than 2%. These represented the excellent assay accuracy and the intra-assay reproducibility. The reproducible slopes of the 21 calibration curves with a relative standard deviations of 3.5% as mentioned earlier were an indication of the excellent inter-assay reproducibility.

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SIMULTANEOUS DETERMINATION OF CHLOROQUINE AND DESETHYLCHLOROQUINE IN BLOOD, PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high-performance liquid chromatographic method is described for the determination of chloroquine and its major metabolite desethylchloroquine in blood, plasma and urine. The procedure employs reversed-phase chromatography, with ultraviolet detection, and chlorpheniramine as an internal standard. One milliliter samples of biologic fluid are extracted in a single step with ether. The method has a sensitivity limit of 5 ng/ml for chloroquine and its metabolite. The applicability of the method is demonstrated by the analysis of blood and plasma samples obtained from rabbits following intravenous administration of chloroquine.

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INTRODUCTION

Chloroquine is the most effective drug employed in the treatment of malaria, a disease that affects approximately twenty percent of the world population. Chloroquine is also used in the treatment of systemic lupus, intestinal amebiasis and rheumatoid arthritis. Recently, several high performance liquid chromatographic methods have been reported (1-3) for the determination of chloroquine in biologic fluids. Two of these methods required fluorescence detection (1,2). The other method (3) employed ultraviolet detection, but did not separate the parent drug from its major metabolite, desethylchloroquine (4). Further, two of the assays (1,3) utilized internal standards which had to be synthesized.

The present study concerns the development of a simple one-step extraction method, employing a readily available drug, chlorpheniramine, as an internal standard. The reversed-phase HPLC procedure utilizes ultraviolet detection, and permits the simultaneous assay of chloroquine and desethylchloroquine in plasma, whole blood and urine.

EXPERIMENTAL

Reagents and Materials

Chloroquine diphosphate was obtained from Sigma Chemical Co. (St. Louis, MO); desethylchloroquine was provided by Sterling-Winthrop Research Institute (Rensselaer, NY); and chlorpheniramine

maleate was obtained from Berlex Laboratories, Inc. (Cedar Knolls, NJ). Methanol and chloroform were HPLC-grade, Fisher Scientific (Fair Lawn, NJ). Deionized water was used throughout the experiment and all other chemicals were of reagent grade.

Glass tubes employed in the extraction and evaporation procedures were placed in a 500° kiln for 1 hr to eliminate any contaminants remaining after washing with detergent, rinsing with deionized water and drying at 100° overnight. The 15 ml conical tubes used for evaporation were silanized with 4% trimethylchlorosilane from Alfa Products (Danvers, MA) in dry toluene. After silanization, the tubes were rinsed with methanol and dried at 100° overnight.

Chromatography Equipment and Conditions

The HPLC system (Waters Assoc., Milford, MA) consisted of a Model M6000 solvent delivery system, a U6K loop injector and a prepacked 30 cm x 3.9 mm i.d. stainless steel column containing 10 µm C_{18} µ-Bondapak, along with a 23mm x 3.9mm guard column, hand packed with C_{18}/Corasil. The variable wavelength detector, Kratos Analytical Instruments (Westwood, NJ), was set at 225 nm, 0.02 aufs.

The mobile phase, consisting of 0.18% ammonium hydroxide (30%) in methanol, was deaerated by filtering through a type HA filter, pore size 0.45 µm, using a solvent clarification kit (Millipore, Bedford, MA). The flow rate was 2 ml/min, and the system was operated at ambient temperature.

Standard Solutions

Aqueous solutions were prepared containing 5 µg/ml of chloroquine diphosphate and desethylchloroquine. Aliquots of this solution were diluted with deionized water to provide standard solutions for the calibration curves ranging from 25 ng/ml to 2.0 µg/ml of drug and metabolite. The aqueous internal standard solution contained 0.5 µg/ml of chlorpheniramine maleate.

Standard Curves and Biologic Samples

Standard curves were prepared with pooled human or rabbit plasma and blood, and human urine. One milliliter of blood, plasma or urine was combined with 1 ml of chloroquine/desethylchloroquine standard solution, 1 ml of 0.5 µg/ml internal standard solution, and 1 ml of 1.0 N sodium hydroxide in a 20-ml polytef-lined screw-cap centrifuge tube. The mixture was extracted with 8 ml of chloroform for 20 min, with gentle mixing on a platform shaker. After centrifugation at -10° for 15 min at 3000 rpm, the aqueous layer was aspirated off and the chloroform layer was transferred to 15 ml conical tubes and evaporated under a stream of nitrogen at 40°. The residue was reconstituted with 100 µl of methanol and vortexed for 20 sec. A 10 µl aliquot was then injected into the HPLC. An unweighted least-squares regression was employed to fit plots of peak height ratio (drug or metabolite/internal standard) versus drug or metabolite concentration.

Studies in Rabbits

Chloroquine diphosphate was administered intravenously at a dose of 10 mg/kg (equivalent to 6.2 mg/kg base) through the marginal ear vein of New Zealand White rabbits. Five milliliter blood samples were withdrawn over an 8 hr period through an arterial catheter, and were placed into tubes containing 50 U of heparin. A 2 ml aliquot of whole blood was frozen, and a 3 ml aliquot was centrifuged to obtain the plasma, which was also frozen until the time of assay.

RESULTS AND DISCUSSION

Figure 1 illustrates typical chromatograms obtained for the assay of human urine, blood and plasma containing 0.5 µg/ml of chloroquine diphosphate and desethylchloroquine, as well as 0.5 µg/ml of internal standard. The retention times for the internal standard, drug and metabolite were 2, 4 and 8 min, respectively. Blank samples of each biologic fluid did not exhibit any interfering peaks in the chromatograms. The recovery of drug and metabolite from urine, plasma and blood samples containing 0.5 µg/ml of each component was consistently in the range of 85-87 percent. The recovery of internal standard averaged 90-95 percent from urine, plasma and blood. The precision of the assay was evaluated with ten replicate urine, plasma and blood samples containing 0.05 µg/ml of drug and metabolite, and ten samples of each biologic fluid containing 0.15 µg/ml of drug and metabolite.

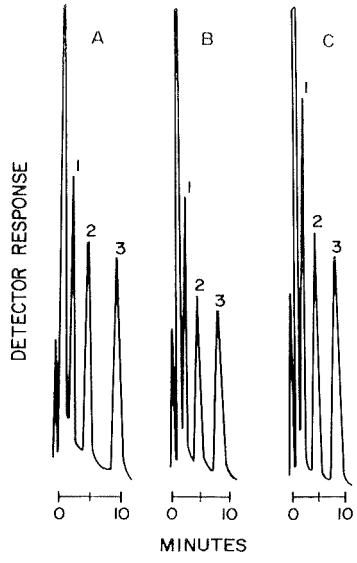

Figure 1 - Chromatograms from the assay of human urine (A), blood (B) and plasma (C) containing 0.5 µg/ml of internal standard (1), chloroquine (2) and metabolite (3).

The relative standard deviations (SD x 100/mean) were 8.7 percent and 4.9 percent for the lower and higher concentrations, respectively. Standard curves for drug and metabolite in each biologic fluid exhibited excellent linearity over a concentration range of 25 ng/ml to 2.0 µg/ml, with coefficients of determinations (r^2) which were consistently at least 0.998. The intercepts did not differ significantly from zero. The limit of detection of both chloroquine and its metabolite is 5 ng/ml of biologic fluid.

Figure 2 is representative of chromatograms obtained by assaying blood and plasma samples obtained from rabbits receiving

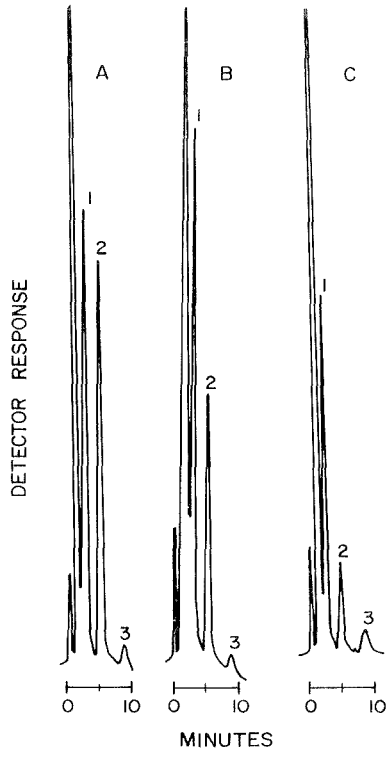

Figure 2 - Chromatograms for the assay of rabbit blood and plasma following administration of 6.2 mg/kg of chloroquine base intravenously. Key: (A), 15 min blood; (B), 30 min blood; (C), 30 min plasma; 1, internal standard; 2, chloroquine; 3, metabolite.

an intravenous dose of chloroquine. Two features of these chromatograms are of particular interest. First, the metabolite is well separated from the chloroquine, and is present in blood and plasma obtained within 15 min after an intravenous dose. In subsequent studies to be reported elsewhere, desethylchloroquine was detected in blood samples obtained as early as 5 min after dosing,

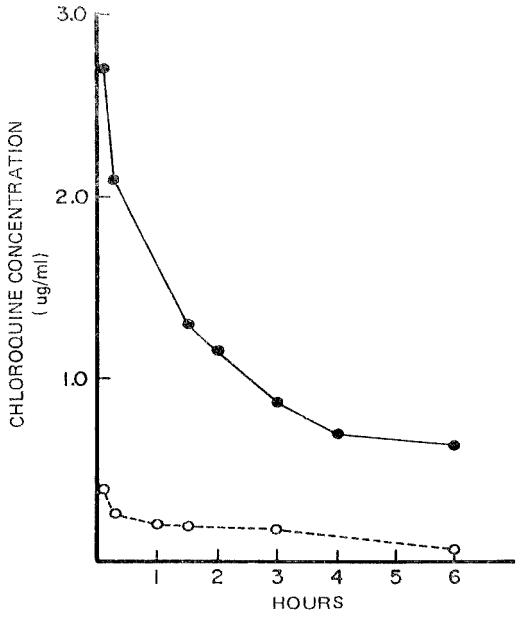

Figure 3 - Blood (●) and plasma (○) concentration-time profile for a rabbit receiving a 6.2 mg/kg intravenous dose of chloroquine.

and the metabolite concentrations remained relatively constant, ranging from 50-100 μg/ml, for 24 hr after dosing. Secondly, the data shown in Figure 2 indicate that chloroquine concentrations in whole blood samples are considerably higher than those found in corresponding plasma samples. This observation is consistent with previous work (5). Figure 3 represents blood and plasma chloroquine concentrations determined in another rabbit following a 6.2 mg/kg intravenous dose. The blood concentrations are approximately seven-fold greater than those found in plasma.

Previous studies in humans have indicated peak plasma chloroquine concentrations of approximately 80 ng/ml and 1 μg/ml following single dose administration of 300 mg of chloroquine base by oral and intravenous routes, respectively (1). Further, during chronic daily administration of 300 mg of chloroquine base, blood chloroquine concentrations ranged from approximately 150-500 ng/ml (3). Concentrations of chloroquine in the urine of these patients ranged from 17-20 μg/ml. Therefore the present assay, which is relatively rapid and is specific for chloroquine and its major metabolite, is applicable to pharmacokinetic studies in animals and man, as well as suitable for routine monitoring of patients who are receiving this drug.

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HPLC ASSAY FOR S-2-(3-AMINOPROPYLAMINO)ETHYL PHOSPHOROTHIOATE (WR 2721) IN PLASMA

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ABSTRACT

A specific HPLC assay has been developed for determination of the radioprotective drug WR 2721. The method is based on precolumn derivatization of plasma with fluorescamine, separation with a C-18 cartridge and detection by fluorescence. An external standard was used for calibration, and values were adjusted based upon recovery of added ^{14}C-labeled WR 2721. WR 2721 had a retention time of about 13 minutes using a mobile phase of acetonitrile/water (22:78), 0.01 M in dibutylammonium phosphate, at a flow rate of 2 mL/min. Sensitivity of the assay was characterized to 2 μg/mL, and detector response was linear over the range of 2 to 1100 μg/mL. The assay requires 90 μL of plasma and has a total chromatography time of about 45 minutes. 2-(3-Aminopropylamino)ethanethiol (WR 1065) and bis-[2-(3-aminopropylamino)ethyl]disulfide (WR 33278), metabolites of the drug, and a variety of primary amines were shown not to interfere with the assay. Suitability of this assay for pharmacokinetic studies was demonstrated in preliminary experiments with a beagle dog.

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INTRODUCTION

The chemical radioprotector S-2-(3-aminopropylamino)ethyl phosphorothioate (I) has been the object of intense study by research groups of the U.S. Army and the National Cancer Institute. An Army program established in 1959 found that the drug [labeled Walter Reed (WR)-2721] protects various tissues from radiation (1). It was also demonstrated that certain solid animal tumors are not protected, which suggested its use in radiotherapy (2).

$$H_2N{-}CH_2CH_2CH_2{-}NH{-}CH_2CH_2{-}S{-}R$$

I, WR 2721, R = $PO(OH)_2$
II, WR 1065, R = H
III, WR 33278, R = $SCH_2CH_2NHCH_2CH_2CH_2NH_2$

It is believed that in the body the drug is transported intact into the tissue where it is enzymatically cleaved to yield the thiol II (WR 1065) (3). Free radicals produced by radiation are then scavenged by interaction with the sulfhydryl (SH) group of II (4).

A major shortcoming of WR 2721 is that its activity after oral dosing is severely limited--probably due to premature cleavage of the phosphate group under the acidic conditions encountered immediately after oral dosing (5).

Attempted bioassay of WR 2721 has been hampered by several serious obstacles. The compound is acid-labile, has no convenient chromophore, has essentially no solubility in organic solvents and is extremely polar, capable of existing as a dipolar ion or a bis-dipolar ion. Lack of solubility precludes its extraction from biological fluids and its polarity places limits on the types of chromatographic systems which might be used to separate it from endogenous materials. To facilitate

pharmacokinetic studies of the drug, an analytical procedure capable of efficiently processing large numbers of samples is also an important consideration. An HPLC method which overcomes the stated problems and is capable of handling multiple samples is presented in this paper.

MATERIALS AND METHODS

Instrumentation

A Waters Associates Model 244 Liquid Chromatograph equipped with a Model 420 AC Fluorescence Detector and Data Module was employed. Excitation wavelength was 395 nm and emission wavelength was >460 nm. Samples were injected using a Rheodyne Model 7125 Injector fitted with a 20- or 50-μL loop. Separations were carried out on a Waters Associates RCM-100 Radial Compression Module fitted with a 100-mm x 8-mm cartridge filled with 5-μm spherical C-18 packing. The analytical column was protected with a Whatman, Inc. guard column filled with Waters Associates CoPell C-18 packing. The mobile phase was acetonitrile/water (22:78), 0.01 M in dibutylammonium phosphate (pH ~3) at a flow of 2.0 mL/min.

Reagents

Acetonitrile was purchased from Burdick and Jackson Laboratories, Inc. Dibutylamine and fluorescamine were obtained from Aldrich Chemical Company. Concentrated (1 M) solutions of dibutylammonium phosphate were prepared by titrating 12.9 g of dibutylamine to pH 2.5 with phosphoric acid and diluting to volume. Fresh solutions containing fluorescamine (5 mg/mL) were prepared weekly using reagent grade acetone which had been stored over 4A molecular sieves. WR 2721 trihydrate, Lot AU-BJ 09506AJ-68-2, was furnished in >99.0% purity by the Walter Reed Army

Institute of Research. Radiolabeled WR 2721, S-[2-(3-aminopropylamino)ethyl-1,2-^{14}C]phosphorothioate was obtained from Research Triangle Institute, Lot 3874-52; its specific activity was 86.0 μCi/mg and reported purity was >97.0%.

Standards containing WR 2721 were prepared by dissolution in 0.05 M sodium borate-potassium chloride pH 10 buffer.

Sample Preparation

Plasma (90 μL), 50 μL of a solution of the radiolabeled internal standard (1.40 μg/mL) and 160 μL of 0.05 M sodium borate-potassium chloride buffer were placed in a polyethylene vial and, while the mixture was being agitated using a vortex mixer (American Scientific Products), 200 μL of the fluorescamine reagent was added. After mixing for 60 s, the mixture was treated with an additional 200 μL of fluorescamine reagent and agitation was continued for 20-30 s. The resulting mixture was centrifuged at 1500 rpm for three minutes and an aliquot of the supernatant was injected onto the HPLC column. Each frozen sample was individually thawed, derivatized and immediately injected because both WR 2721 and its derivative decompose in plasma at room temperature.

Internal Standard

Plasma samples were spiked with increasing amounts of WR 2721, mixed with internal standard, derivatized and injected as described above. The following concentrations were used to construct a standard curve: 0, 1.89, 6.33, 11.9, 56.3, 112, 279, 556, 834, and 1110 μg/mL. The curve was constructed by fitting a regression line to the peak area versus concentration data after correction for recovery. Recovery was determined by adding 50 μL of a solution containing ^{14}C-labeled WR 2721 (activity 334 dpm/μL) to each sample vial prior to derivatization. After

derivatization, a 20- or 50-μL sample was removed from the reaction vial, mixed with scintillation fluid and counted using a Packard Tri-Carb Model 4530 scintillation counter. Similarly, either a 20- or 50-μL sample was analyzed using HPLC. Twelve 1-mL fractions of column effluent were collected starting three minutes prior to the elution time of WR 2721 and continuing for six minutes. Each vial, after addition of scintillation fluid, was counted for 10 minutes, and the recovered radioactivity was determined by summing the disintegrations observed in those fractions with activity greater than the background level. Recovery of WR 2721 was then determined for each assay using the expression: % recovery = (dpm recovered x 100)/dpm added. All calculations were corrected for contributions due to background. Counting efficiencies were determined via automatic external standardization. Recoveries obtained were between 35% and 55% (mean = 48%, n = 58).

Animal Dosing Experiments

A healthy, one-year old, AKC-registerable, male beagle dog weighing 12.7 kg was used in pilot dosing experiments to test the analytical method. The animal was dosed intravenously in the cephalic vein with a 0.9% saline solution containing 1.9 g (150 mg/kg body weight) of WR 2721. The infusion required two minutes. Blood samples (3 mL) were withdrawn into an EDTA Vacutainer® from a cannula placed in the jugular vein. Each sample was immediately chilled in an ice/water bath and then centrifuged. A 90-μL aliquot of the separated plasma was added to 50 μL of the internal standard, the mixture was agitated using a vortex mixer and was then quick-frozen in a dry ice/alcohol bath. Samples were stored at -20°C until time of analysis when they were thawed at room temperature and immediately treated as described in the Sample Preparation section. The drug was shown

to be stable in plasma up to about 30 days when stored at -20°C or lower, but was unstable at room temperature.

RESULTS AND DISCUSSION

Derivatization

Major obstacles in the development of the assay were the instability of WR 2721 in acidic media and its lack of a suitable chromophore. A pH-hydrolysis rate profile (Figure 1) demonstrates the lack of stability under acidic conditions. The data were generated by measuring the rate of appearance of WR 1065 (6) and calculating the rate of disappearance of WR 2721.

In simple aqueous systems underivatized WR 2721 has been analyzed by HPLC using separation on a Whatman PAC column (7) and UV detection at 204 nm, but this procedure was unsuitable for plasma analysis. Derivatization of the drug with fluorescamine allowed detection in the picomole range and at the same time modified its chromatographic behavior such that it was retained on reverse-phase columns.

It was found that precision was remarkably improved by a two-stage derivatization procedure (see Methods section). This may be attributed to the effect of the acetone solvent which, by protein denaturization, could release bound material. Pretreatment of plasma with methanol, ethanol or acetonitrile did not provide a satisfactory increase in precision.

Derivatization with o-phthalaldehyde was briefly investigated but was not further pursued when the derivative was found to be very unstable.

Separation

Figure 2 illustrates a typical chromatogram for a plasma sample spiked with WR 2721 at the 5 µg/mL level. Note that the

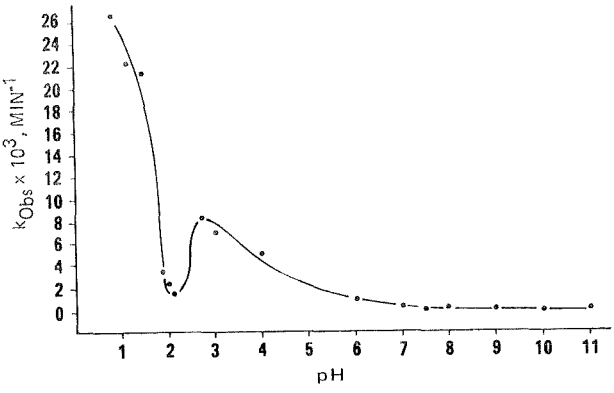

Figure 1. pH-Rate Profile for Hydrolysis of WR 2721 at 37°C

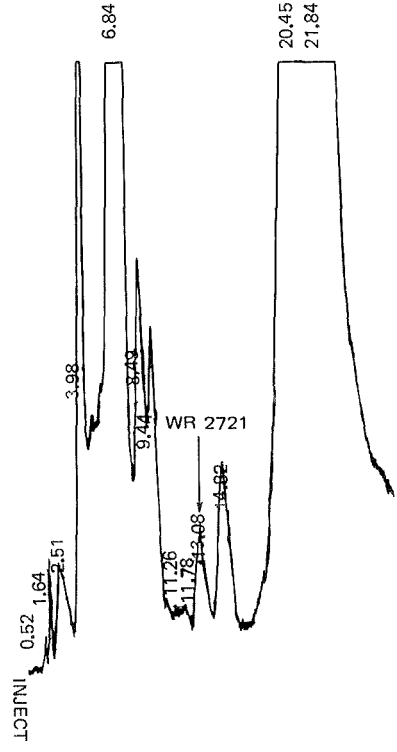

Figure 2. Chromatogram of Beagle Plasma Containing WR 2721 (5 μg/mL)

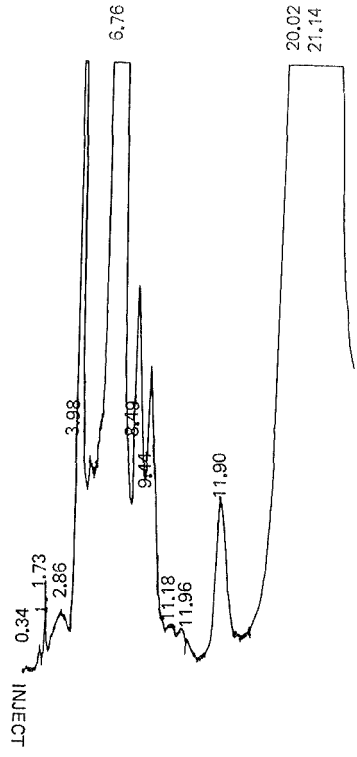

Figure 3. Chromatogram of Beagle Plasma (Blank)

peak of interest was well separated from those peaks due to endogenous materials, thought to be amino acids. A chromatogram of a plasma blank is shown in Figure 3.

The separation was performed under isocratic conditions to increase sample throughput and increase precision. As a result, less polar materials (including WR 1065 and WR 33278) were not eluted and, when allowed to remain, seriously degraded resolution. Flushing the column with methanol/water (70:30) at the end of each analysis day restored column resolution and increased its lifetime.

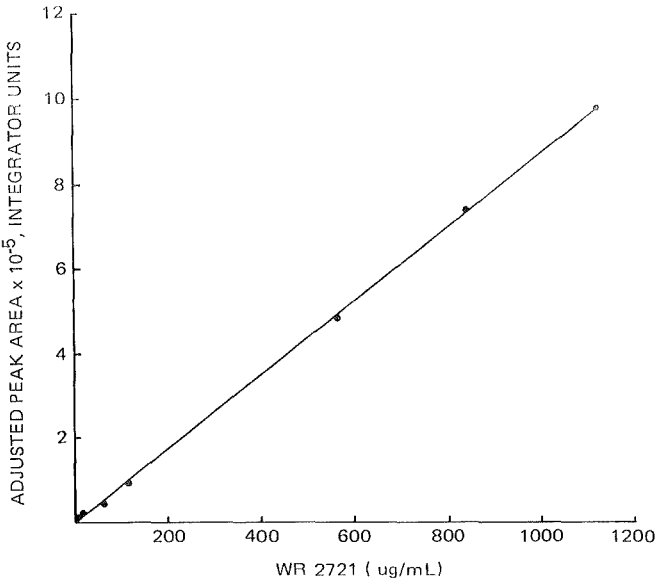

Figure 4. Standard Curve. Samples of beagle plasma spiked with WR 2721 and internal standard were derivatized and chromatographed. Points were corrected for recovery of internal standard.

Internal Standard

As can be seen in Figure 2, the complex chromatogram of derivatized plasma did not offer much hope of finding a suitable internal standard, i.e., one which would have similar behavior toward hydrolysis during sample storage and workup, and elute in a reasonable time in an unobstructed region of the chromatogram. A number of homologs of WR 2721, several other phosphorothioates and a number of amino acids were screened without success. Therefore, radiolabeled WR 2721 was added to plasma samples immediately after they were drawn and the column effluent was collected and counted to determine recovery. Excellent precision and accuracy values were obtained over the approximate range of 2 to 1100 μg/mL using this procedure.

TABLE 1
Precision of WR 2721 Analytical Method

WR 2721 Concentration, g/mL	Number of Replicates Analyzed, n	RSD (%)
1110	5	9.1
112	5	5.3
11.9	5	5.6
6.3	5	5.1
3.0	5	7.8
	Average =	6.6

TABLE 2
Accuracy of WR 2721 Analytical Method

Spike Level, g/mL	Measured Level, g/mL	% Deviation (D)
1.89	1.92	1.6
8.55	8.75	2.3
34.1	33.1	-3.0
78.6	75.9	-3.4
112	107	4.5
390	430	10.2
779	742	-4.8
1110	1050	-5.4

$$\text{Average Deviation} = \frac{|D|}{n} = 4.4$$

Linearity

Figure 4 depicts the relationship between the drug plasma levels and the peak area for derivatized WR 2721. Linear regression of peak area versus concentration gave a coefficient of determination (R^2) of 0.9969, with slope of 930 and intercept of -7684.

Precision and Accuracy

Precision of the method over the entire working range was determined by the analysis of replicate spiked samples. In

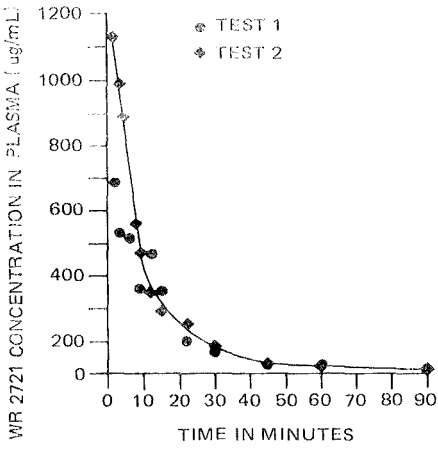

Figure 5. WR 2721 Concentration in Beagle Plasma as a Function of Post-Infusion Time

Table 1 the concentration, number of replicates analyzed and the relative standard deviation (RSD) for each data set is presented. Average RSD for the method was 6.6%.

Accuracy of the method for plasma concentrations ranging from 1.89 to 1110 g/mL was determined by the analysis of blind spiked plasma samples. Average deviation for eight determinations was 4.4%. Spike levels and measured levels are presented in Table 2.

BIOLOGICAL APPLICATION

Plasma levels of WR 2721 were monitored after two intravenous dosings of a beagle dog. As shown in Figure 5, the experiments gave essentially the same profile; from an initial level of approximately 1000 μg/mL, the drug level approached the assay sensitivity (~2 μg/mL) within 90 minutes. Samples taken after longer periods did not decay to the zero level and work is in progress to increase sensitivity and to determine whether a low level (<1 μg/mL) interference was present.

SUMMARY

A procedure for the analysis of WR 2721 contained in plasma has been developed which requires only a fast derivatization reaction and HPLC separation. The method has been tested in pilot dosing experiments with a beagle dog in which drug levels have been measured from 2 to 1100 μg/mL. Work is in progress to extend the sensitivity of the method and to allow the use of a nonradiolabeled internal standard.

ACKNOWLEDGEMENT

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QUANTITATIVE DETERMINATION OF IRON AND ALUMINIUM IN SOME ALLOYS AND SILICATE ROCKS AFTER A CATION EXCHANGE SEPARATION ON ZIRCONIUM(IV) PHOSPHO AND SILICO ARSENATES

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ABSTRACT

A rapid and quantitative method has been developed for the analysis of some iron and aluminium based alloys and silicate rocks using zirconium(IV) based arsenophosphate and arsenosilicate cation exchangers. The method is simple, reproducible and precise with a standard deviation $< 3\%$, for the direct determination of iron and aluminium in rocks and alloys. The low standard deviation values suggest that the method should be useful for the standardization purposes.

INTRODUCTION

Analysis of alloys and rocks is important in chemical technology as the presence of various constituents play a vital role in their applications. Although several papers have earlier been published in this field using well known analytical techniques (1-5), the ion-exchange technique is more useful as it gives fast separation of the ionic species present. However such studies have been made mostly on organic

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resins (6,7) probably because of their excellent reproducibility and stability.

Inorganic ion-exchangers are well known for their high selectivity for metal ions and stability at elevated temperatures (8). Zirconium(IV) arsenophosphate (ZAP) and Zr(IV) arsenosilicate (ZAS) prepared in these laboratories (9,10) possess exceptionally good chemical stability and reproducibility in ion-exchange behaviour which improve further for ZAP on heating. A possibility of using these materials for the quantitative separation of metal ions from their synthetic mixtures and from some real samples has already been explored earlier in these laboratories (10). The present article summarizes our efforts for a quantitative separation of aluminium and iron from some alloys and rocks on their columns.

EXPERIMENTAL

Chemicals and Reagents

Zirconyl chloride used in these studies was of J.T.Baker Chemical Co.Philipsburg (USA), while tri-sodium orthophosphate was of BDH, Poole (London). Di-sodium arsenate and sodium silicate were E-Merck (Dermstadt) products and all other reagents and chemicals were of analaR grade.

Apparatus

A pye unichem model SP-2900 atomic absorption spectrophotometer was used for the quantitative determination of various elements present in rocks and alloys.

Synthesis and Ion-Exchange Capacity of ZAP and ZAS

These materials were synthesized by the methods reported earlier (9,10) and were thermally treated by putting them at various temperatures for one hour each in a muffle furnace. They were washed thoroughly with dil.HNO_3 and then with demineralized water (DMW) till the effluents were free of any metallic or non metallic impurities as tested by atomic absorption spectrophotometry. The Na^+-ion-exchange capacities of various samples thus obtained are reported in Table 1. On this basis a heated phase of ZAP upto 200°C (α-ZAP) and normal ZAS were used for further studies.

Distribution Studies

The distribution coefficients (Kd) for various metal ions were determined as usual by the batch process on α-ZAP and ZAS (10). Table 2 shows a comparative statement of these values in DMW and HNO_3.

TABLE 1

Ion-Exchange Capacity of ZAP and ZAS After Thermal Treatment

Heating temperature (°C)	Ion-exchange capacity (meq./dry g.)		% Retention in ion-exchange capacity	
	ZAP	ZAS	ZAP	ZAS
45	0.94	1.30	100.0	100.0
100	0.95	1.30	101.1	100.0
200	1.03	1.25	109.6	96.2
400	0.94	0.46	100.0	35.4
600	0.84	0.20	89.4	15.4

TABLE 2

Kd values of Various Metal Ions on α-ZAP and ZAS in DMW and HNO_3 Media

Metal ions	DMW		0.01M HNO_3		0.1M HNO_3		1M HNO_3	
	α-ZAP	ZAS	α-ZAP	ZAS	α-ZAP	ZAS	α-ZAP	ZAS
Ba(II)	1900	110	83	105	65	91	0	0
Cd(II)	5400	8030	1750	186	507	130	0	0
Pb(II)	5650	5430	360	1580	268	1460	0	0
Mg(II)	4400	367	20	77	3	63	0	0
Sr(II)	3150	218	0	35	0	3	0	0
Hg(II)	326	52	30	58	20	41	0	0
Mn(II)	3530	69	52	13	45	9	0	0
Ca(II)	3700	314	0	81	0	36	0	0
Zn(II)	3280	1000	28	57	8	36	0	0
Ni(II)	5150	8000	68	206	20	190	0	0
Co(II)	5150	600	5	700	5	409	0	0
Cu(II)	3250	85	126	2	3	0	0	0
Sn(II)	1800	61	2	1	0	0	0	0
Fe(II)	3900	3850	3900	293	630	210	0	0
Al(II)	3900	4350	3900	3880	320	320	0	0

Analysis of the Samples for Iron and Aluminium

A. Preparation of the Standard Solutions:

The standard solutions were prepared as follows:

Synthetic Alloy Samples

Various metallic solutions were mixed in certain ratios so that they correspond to the actual metallic proportions in the standard alloys.

Standard Alloy Samples

An accurately weighed amount of the alloy was dissolved in a minimum amount of aquaregia followed by the dilution to a desired volume with DMW.

Rock Samples

Twenty milliliters of 15% NaOH were heated in a Ni-crucible until melted and then fused with 100 mg. of the sample for 5-6 minutes at dull red heat ($\sim 600^{\circ}C$). The melt was cooled and 100 ml of DMW was added. After keeping overnight the liquid was transferred to a 1 liter volumetric flask containing 40 ml of 1:1 HCl and the volume made upto the mark with DMW.

B. Separation and Determination of Fe(III) and Al(III)

It was done as follows:

Two grams of the 60-100 mesh sized particles of the ion exchanger in H^+-form were packed in a glass tube having an internal diameter ~ 0.6 cm and fitted with glass wool at the bottom. The sample solution (1-5 ml) was evaporated to almost dryness to remove the excess acid and the residue was dissolved in a small amount (1-3 ml) of DMW which was then loaded on the column.

TABLE 3

Quantitative Separation of Iron/Aluminium from Some Synthetic Alloys Using α-ZAP and ZAS Columns

Synthetic alloy and its composition (μg) per ml of the solution		Aluminium/iron obtained* in the effluent (μg)				% Error				% Standard deviation			
		α-ZAP		ZAS		α-ZAP		ZAS		α-ZAP		ZAS	
		Al	Fe	Al	Fe	Al	Fe	Al	Fe	Al	Fe	Al	Fe
Dow metal	(Al 40,Mn 15,Mg 945)	41.2	-	40.3	-	+3.00	-	+0.75	-	0.50	-	0.90	-
-do-	(Al 120,Mn 10,Mg 870)	118.3	-	122.4	-	-1.42	-	+2.00	-	0.80	-	0.95	-
Aluminium-zinc binary alloy	(Al 750,Zn 250)	745	-	743	-	-0.67	-	-0.93	-	1.40	-	2.30	-
-do-	(Al 950,Zn 50)	939	-	941	-	-1.16	-	-0.95	-	2.10	-	1.60	-
Aluminium bronze	(Al 100,Cu 900)	99.5	-	101.5	-	-0.50	-	+1.50	-	0.46	-	1.20	-
-do-	(Al 120,Cu 875,Sn 5)	122.4	-	123.0	-	+2.00	-	+2.50	-	0.75	-	1.20	-
Magnalium	(Al 850,Mg 150)	843.6	-	847.6	-	+0.75	-	-0.28	-	1.80	-	2.80	-
-do-	(Al 900,Mg 100)	890.9	-	904.2	-	-1.01	-	+0.47	-	1.30	-	0.30	-
-do-	(Al 925,Mg 75)	932.4	-	918.9	-	+0.80	-	-0.66	-	0.65	-	0.54	-
Y-alloy	(Al 940,Ni 20,Mn 10,Cu 50)	937.3	-	946.1	-	-0.29	-	+0.65	-	0.70	-	0.46	-
Duralumin	(Al 950,Mn 5,Mg 6,Cu 40)	944.9	-	959.1	-	-0.54	-	+0.95	-	0.15	-	0.42	-
Manganese steel	(Fe 879,Mn 120)	-	887.2	-	884.2	-	+0.93	-	+0.59	-	1.03	-	0.94
-do-	(Fe 849,Mn 150)	-	841.6	-	853.6	-	-0.87	-	+0.54	-	1.20	-	0.58
Nickel steel	(Fe 944,Ni 36,Mn 18)	-	921.8	-	-	-	-2.35	-	-	-	0.66	-	-
Chrome steel	(Fe 965,Mn 19,Cr 15)	-	957.0	-	-	-	-0.83	-	-	-	0.85	-	-
-do-	(Fe 960,Mn 19,Cr 20)	-	949.9	-	-	-	-1.05	-	-	-	0.30	-	-
Invar	(Fe 620,Ni 360,Mn 19.5)	-	609.8	-	-	-	-1.65	-	-	-	1.40	-	-
Stainless steel	(Fe 734,Ni 80,Mn 5,Cr 180)	-	730.5	-	-	-	-0.48	-	-	-	1.98	-	-
Nichrome	(Fe 120,Ni 750,Mn 20,Cr 110)	-	119.2	-	-	-	-0.67	-	-	-	0.21	-	-

*Average value of four replicates.

TABLE 4

Quantitative Separation of Iron from Some Standard Steel Samples Using ∝-ZAP Columns

Steel analysed	Volume of the stock solution loaded (Ml)	Elements present as per the standard composition of the steel (µg)						Iron determined in the effluent*** (µg)	% Error	% Standard deviation
		Fe	Cr	Ni	Cu	Mn	Si			
AISI-303*	1.0	222.80	56.52	26.69	-	4.71	1.57	230.46	+3.44	0.17
	2.0	445.60	113.04	53.38	-	9.42	3.14	454.40	+1.97	0.94
	2.5	557.00	141.30	66.725	-	11.775	3.925	566.40	+1.69	0.81
AISI-347*	1.0	219.40	56.52	31.40	-	4.71	1.57	225.13	+2.61	0.96
	2.0	438.80	113.04	62.80	-	9.42	3.14	446.26	+1.70	1.26
	2.5	548.50	141.30	78.50	-	11.775	3.925	554.43	+1.08	0.57
	3.0	658.20	169.56	94.20	-	14.13	4.71	626.42	-4.83	0.44
Inconel-600**	1.0	66.60	155.80	769.3	1.6	2.0	-	64.80	-2.70	0.75
Inconel-800**	1.0	451.4	209.90	319.8	2.9	8.1	-	450.20	-0.27	0.58

*A.I.S.I. Standard Steels.

**Huntington Alloy Products, Division of International Nickel Co., U.S.A.

***Average value of five replicates.

TABLE 5

Quantitative Separation of Aluminium and Iron from Various U.S.G.S. Standard Rocks Using α-ZAP and ZAS Columns

Rock analysed	Standard composition of the rock for Al and Fe per 5 ml of the stock solution (μg)		Al_2O_3 and Fe_2O_3 obtained* in the effluent (μg)				% Error				% Standard deviation			
			α-ZAP		ZAS		α-ZAP		ZAS		α-ZAP		ZAS	
	Al_2O_3	Fe_2O_3	Al_2O_3	Fe_2O_3	Al_2O_3	Fe_2O_3	Al_2O_3	Fe_2O_3	Al_2O_3	Fe_2O_3	Al_2O_3	Fe_2O_3	Al_2O_3	Fe_2O_3
G-2	77.00	13.45	78.00	13.80	79.00	18.80	+1.30	+2.60	+2.59	+2.60	1.94	0.65	1.64	1.06
AGV-1	85.95	38.90	85.40	39.20	87.00	38.50	-0.64	+0.77	+1.22	-1.02	1.38	1.97	0.94	1.10
BHVO-1	68.50	60.00	69.45	61.00	71.00	59.25	+1.39	+1.67	+3.64	-1.25	2.99	2.99	1.89	2.10
BCR-1	68.60	67.05	69.45	69.00	69.80	68.10	+1.24	+2.91	+1.74	+1.56	1.75	1.86	2.14	1.40
PCC-1	3.65	41.40	3.57	40.35	3.60	40.70	-2.19	-2.54	-1.36	-1.69	0.47	1.31	0.68	0.95

*Average values of five replicates.

All the elements except Al and Fe were eluted out either in DMW or 0.01M HNO_3 (max.volume ~150 ml). These metals were then leached out with 1M HNO_3 (max. volume ~100 ml) and determined quantitatively by atomic absorption spectrophotometry. The observations are summarized in Tables 3-5.

RESULTS AND DISCUSSION

The essential feature of these studies is to use inorganic ion exchangers for the analysis of some alloys and silicate rocks. As it is clear from Table 2, the distribution behavior of ZAP is significantly affected on heating. The heated phase of this material (α-ZAP) becomes highly selective for Al(III) and Fe(III). Also, zirconium(IV) arsenosilicate preferentially holds these two ions (10). This property of these two ion-exchangers has been successfully utilized for the separation and quantitative determination of Al(III) and Fe(III) in some alloys and silicate rocks. When a solution of these samples (synthetic or real) is passed through the ion-exchange column with a very slow rate, only Al(III) and Fe(III) ions are retained and others are completely excluded by the column simply in DMW or 0.01M HNO_3. They are then eluted out in 1M HNO_3. (Tables 3-5). The method is quite simple and requires much less time as compared to the classical methods. Furthermore, quite a large number of samples can be analyzed using a single column because these materials have shown the excellent reproducibility in their ion exchange behavior, and then ion exchange capacity is not affected even after several

recycling processes. Since ZAS has a high Kd value for Ni(II) ions it could not be used for the separation of iron in the nickel containing steels. For such analyses, however, α-ZAP is quite suitable (Tables 3,4).

ACKNOWLEDGMENT

The authors are thankful to Prof. Mohsin Qureshi for research facilities and encouragement. Dr. S.C.D. Sah, Director, Wadia Institute of Himalayan Geology, Dehradun (India) is thanked for the instrumental facilities. The financial assistance provided by the C.S.I.R. (India) is gratefully acknowledged.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 6(8), 1547 (1983)

BOOK REVIEW

WILSON & WILSON'S COMPREHENSIVE ANALYTICAL CHEMISTRY VOLUME XIV: ION EXCHANGERS IN ANALYTICAL CHEMISTRY. THEIR PROPERTIES AND USE IN INORGANIC CHEMISTRY, M. Marhol, Elsevier, Amsterdam, $127.75 (US).

This book comprises three parts. Part 1 introduces some of the basic concepts of ion exchange, the skeletal network, inorganic functional groups, selectivity, swelling, etc. The theoretical treatment of the exchange process is adequate. Part 2 has useful information on practical procedures for the characterization of ion exchange resins. the subject of instrumentation for ion exchange chromatography receives very light treatment and the little information that is supplied is quite out of date. Most of Part 2 is devoted to a recitation of procedures where ion exchangers are used in essentially batch methods to effect separations. This section, too, is rather out of date.

Since the book covers references up to 1978, it misses recent developments in inorganic chromatography that are having a major impact on inorganic analysis. Some of the applications raise questions as to their purpose. For example, "preparation of dilute carbonate-free solutions of sodium or potassium hydroxides" requires as a first step the conversion of a strong base anion exchanger to the hydroxide form with carbonate-free sodium hydroxide. What is the point of this exercise?

Part 3 has a wealth of tables on cation and anion distribution coefficients in a great variety of media, diffusion coefficients, types of functionality, trade names, etc. This is, in my opinion, the most valuable part of the book and should be useful to those who regularly use ion exchange resins in their work.

Hamish Small
Dow Chemical USA
Midland, Michigan

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(8), 1549-1550 (1983)

BOOK REVIEW

ADVANCES IN THIN LAYER CHROMATOGRAPHY - CHEMICAL & ENVIRONMENTAL APPLICATIONS, J. C. Touchstone, ed., Wiley-Interscience, New York, 1982, 521 pp., $55.00 (US).

In comparison to gas chromatography, the development of the thin layer technique has been relatively modest. Nevertheless, major improvements have been introduced also in this relatively simple technique which help to retain its position in analytical chemistry and to utilize more fully its advantages. Among the recent developments in TLC, the introduction of narrow-classified normal and reversed-phase sorbents, and modifications of the development technique (including pressurized chambers) should be mentioned as well as the increasing use of auxilliary equipment such as densitometers (cf. for instance, ref. 1-3).

The book edited by J. C. Touchstone is an illustration of these trends. It contains 41 communications and lectures presented during the Second Biennial Symposium on Thin Layer Chromatography held in December, 1980 at the University of Pennsylvania School of Medicine. As a typical proceedings volume, it is composed of a number of reviews on various aspects of TLC, systematic investigations of methodological problems (especially quantitative scanning with photometric or radiometric detectors) and brief reports of current work in clinical and environmental analysis. Most of the papers are concerned with quantitative evaluation of the thin-layer chromatograms.

The review articles discuss actual directions of development of TLC: applications of chemically bonded (silanized) adsorbents, preparative TLC, ion exchange systems in clinical analysis; the role of solvent type on selectivity is discussed. A series of investigations are concerned with the application of new scanning equipment for quantitative evaluation of chromatograms and systematic analysis of various groups of substances - steroids, lipids, mycotoxins, sulfonamides, bile acids, pesticides, polycyclic aromatic hydrocarbons.

Novel techniques of TLC are illustrated in the papers. For instance, in a study on the analysis of mycotoxins, it is demonstrated that the combination of multiple and continuous

development permits to improve greatly the separation and quantitative analysis of complex mixtures. Several examples of automation of analysis are given.

The content of the book is a good representation of the present state of thin layer chromatography. It will be useful to those interested in quantitative clinical and environmental analysis and biomedical research.

1. A. Zlatkis and R. E. Kaiser, eds., High Performance Thin Layer Chromatography (HPTLC), Elsevier, Amsterdam, 1978.

2. W. Bertsch, S. Hara, R. E. Kaiser, A. Zlatkis, eds., Instrumental HPTLC, Dr. Alfred Huthig Verlag, Heidelberg, 1980.

3. V. G. Berezkin, A. S. Bochkov, Quantitative Thin-Layer Chromatography, Instrumental Methods, (in Russian), Nauka, Moscow, 1980.

Edward Soczewinski
Medical Academy
Lublin, Poland

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(8), 1551-1552 (1983)

LC NEWS

HPLC FILTER is made of a new fluoropolymer membrane and is housed in solvent resistant polypropylene. It is pressure rated at 75 psi and is available in a 0.45 micron pore size. Gelman Sciences, Inc., JLC/83/8, 600 S. Wagner Rd., Ann Arbor, MI, 48106, USA.

SEPARATIONS TECHNOLOGY FOR PHARMACEUTICAL PROCESSING is subject of a brochure that includes 38 applications involving membrane filtration, ion-exchange, reverse osmosis, ultrafiltration and HPLC. A flow diagram illustrates what separations problems can be solved in three specific categories: fine chemical manufacture, product formulation, and product packaging. Millipore Corp., JLC/83/8, 80 Ashby Rd., Bedford, MA, 01730, USA.

FLAME IONIZATION DETECTOR FOR LC is said to overcome general limitations of prior "universal" LC detectors. A rotating disc, "ringed" by a fibrous quartz belt, conducts samples to a dual-flame ionization detector for analysis. Volatile solvent is vaporized and removed by vacuum. Solutes are carried into the FID, and residual sample is then removed by hotter hydrogen/oxygen cleaning flames. Tracor Instruments, JLC/83/8, 6500 Tracor Lane, Austin, TX, 78721, USA.

HPLC REAGENTS & COLUMNS are described in a new brochure. Included are buffers, ion pair reagents, derivatizing reagents, hardware, and solvents. Fisher Scientific Co., JLC/83/8, 711 Forbes Ave., Pittsburgh, PA, 15219, USA.

LAB AUTOMATION SYSTEM FOR SAMPLE PREPARATION combines robotics and lab stations to automate procedures. The controller interfaces the operator with the robot. Software is menu-based and uses familiar laboratory terms. Zymark Corp., JLC/83/8, Zymark Center, Hopkinton, MA, 01748, USA.

APPLICATIONS GUIDE deals with sample preparation, highlighting background, principles, and techniques of solid phase extraction. The guide contains over 40 detailed procedures for preparing environmental, pharmaceutical, biological, food, and cosmetic samples, such as priority pollutants, crude oil, trace metals, aflatoxins, steroids, etc. J. T. Baker Chem. Co., JLC/83/8, 222 Red School Lane, Phillipsburg, NJ, 08865, USA.

FERMENTATION BROTH ANALYSIS by ion chromatography permits determination of anions, cations, organic acids, and transition metals. Many broths can be injected directly following filtration. Dionex Corp., JLC/83/8, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

HPTLC/TLC BIBLIOGRAPHY SERVICE is available free of charge. Publications may be included by mailing to the publisher. Applied Analytical Industries, Inc., JLC/83/8, Route 6, Box 55, Wilmington, NC, 28405, USA.

DETERGENT REMOVING GEL is an affinity chromatographic support that selectively removes detergents from protein solutions, with proteins being recovered in virtually 100% yields. The support can be regenerated for repeated use. Pierce Chemical Co., JLC/83/8 P. O. Box 117, Rockford, IL, 61105, USA.

AUTOMATED LC SAMPLE PREP/INJECTION SYSTEM combines solid-phase sample preparation and syringeless injection into a single operation. Samples are extracted by sorbent cassettes that selectively isolate and concentrate compounds of interest. Analytichem, International, Inc., JLC/83/8 24201 Frampton Avenue, Harbor City, CA, 90710, USA.

ANALYSIS OF FATTY ACIDS in cooking oils, lipid extracts, etc. necessitate an efficient separation approach prior to their quantitation in the laboratory. Previously accomplished by gas chromatography with derivatization, high resolution results are obtained with HPLC, particularly in analyses of rape seed oil and tall oils. Free acids are determined at the microgam level, and derivatized acids are determined at the nanogram level. Pye Unicam, Ltd., JLC/83/8 York Street, Cambridge CB1 2PX, England.

CHROMATOGRAPHY CATALOG introduces a new range of computer products designed for the chromatography laboratory, and many new HPLC packing materials. Alltech Associates, Inc., JLC/83/8 2051 Waukegan Rd., Deerfield, IL, 60015, USA.

GLASS LAYER-COATED STEEL COLUMNS are available in 250, 125, 50, and 25 mm lengths (4 mm ID). The inert characteristics of the column are maintained via use of ceramic/teflon seals. The columns are said to be ideal for separations that require short columns to achieve separations in seconds vs. minutes. EM Science, Inc., JLC/83/8 480 Democrat Rd., Gibbstown, NJ, 08027, USA.

SINGLE COLUMN ION CHROMATOGRAPHY is the subject of a free newsletter which includes articles on applications, methods optimization, and system troubleshooting. SCIC is the new technique that applies HPLC technology to the analysis of dissolved ionic substances. Wescan Instruments, Inc., JLC/83/8 3018 Scott Blvd, Santa Clara, CA, 95050, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(8), 1553-1556 (1983)

LC CALENDAR

1983

JUNE 27 - JULY 1: 3rd Symp. on Separation Sci. & Technol. for Energy Applications, Gatlinburg, Tenn. Contact: A. P. Malinauskas, Oak Ridge National Lab., P. O. Box X, Oak Ridge, TN, 37830, USA.

JULY 11-14: IUPAC Prague Meeting on Macromolecules, Prague. Contact: PMM Secretariat, Inst. of Macromolec. Chem., 16206 Prague 6, Czechoslovakia.

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 OBV, United Kingdom.

JULY 27-30: 3rd Int'l. Flavor Conf., ACS, The Corfu Hilton, Corfu, Greece. Contact: S. S. Kazeniac, Campbell Inst. for Food Res., Campbell Place, Camden, NJ, 08101, USA.

AUGUST 10-12: 22nd Canadian High Polymer Forum, Univ. of Waterloo, Canada. Contact: A. Garton, NRC of Canada, Div. of Chem., Ottawa, Ont., Canada, K1A OR6.

AUGUST 14-19: 25th Rocky Mountain Conference, Denver Convention Complex, Denver, Colorado. Contact: E. A. Brovsky, Rockwell International, P. O. Box 464, Golden, CO, 80401, USA.

AUGUST 15-19: Coal Science: 1983 Int'l Conference, Pittsburgh, PA. Contact: N. Maceil, JWK Int'l Corp., 275 Curry Hollow Road, Pittsburgh, PA, 15236, USA.

AUGUST 22-26: 7th Australian Symposium on Analytical Chemistry, Adelaide, Australia. Contact: D. Patterson, AMDEL, P.O.Box 114, Eastwood S.A. 5063, Australia.

AUGUST 26 - SEPTEMBER 2: Int'l. Symp. on Solvent Extraction, Denver, CO. Contact: D. Nowak, AIChE, 345 E. 47th St., New York NY, 10017, USA.

AUGUST 28 - SEPTEMBER 2: 11th World Petroleum Congress, London. Contact: Amer. Petrol. Inst., 2101 L St., N.W., Washington, DC, 20037, USA.

AUGUST 28 - SEPTEMBER 2: ACS 186th Nat'l Meeting, Washington, DC. Contact: A. T. Winstead, ACS, 1155 16th St., NW, Washington, DC, 20036, USA.

AUGUST 29 - SEPTEMBER 2: 4th Danube Symposium on Chromatography & 7th I'nt'l. Sympos. on Advances & Applications of Chromatography in Indudtry, Bratislava, Czech. Contact: Dr. J. Remen, Anal. Sect., Czech. Scientific & Technical Soc., Slovnaft, 823 00 Bratislava, Czechoslovakia.

SEPTEMBER 25-30: Federation of Anal. Chem. & Spectroscopy Societies (FACSS) Conf., Philadelphia. Contact: M. O'Brien, Merck, Sharp & Dohme Res. Labs., West Point, PA, 19486, USA.

OCTOBER 2-6: 97th Annual AOAC Meeting, Shoreham Hotel, Washington, DC. Contact: K. Fominaya, AOAC, 1111 N. 19th St., Suite 210, Arlington, VA, 22209, USA.

OCTOBER 3-5: Chemexpo '83, Harbor Castle Hilton Hotel, Toronto, Ont., Canada. Contact: ITS Canada, 20 Butterick Rd., Toronto, Ont., Canada, M8W 3Z8.

OCTOBER 3 - 6: Advances in Chromatography: 20th Int'l Symposium, Amsterdam, The Netherlands. Contact: A. Zlatkis, Chem. Dept., University of Houston, Houston, TX, 77004, USA.

OCTOBER 12-13: 8th Annual Baton Rouge Anal. Instrum. Disc. Grp. Sympos., Baton Rouge, LA. Contact: G. Lash, P. O. Box 14233, Baton Rouge, LA, 70898, USA.

OCTOBER 12-14: Analyticon'83 - Conference for Analytical Science, sponsored by the Royal Society of Chemistry and the Scientific Instrument Manufacturers' Ass'n of Great Britain, Barbican Centre, London. Contact: G. C. Young, SIMA, Leicester House, 8 Leicester Street, London WC2H 7BN, England.

NOVEMBER 3-4 ACS 18th Midwest Regional Meeting, Lawrence, Kansas. Contact: W. Grindstaff, SW Missouri State Univ., Springfield, MO, 65802, USA.

NOVEMBER 9-11: ACS 34th SE Regional Meeting, Charlotte, NC. Contact: J. M. Fredericksen, Chem. Dept., Davidson College, Davidson, NC, 28036, USA.

NOVEMBER 14-16: 3rd Int'l. Sympos. on HPLC of Proteins, Peptides and Polynucleotides, Monte Carlo, Monaco. Contact: S. E. Schlessinger, 400 East Randolph, Chicago, IL, 60601, USA.

NOVEMBER 16-18: Eastern Analytical Symposium, New York Statler Hotel, New York City. Contact: S. David Klein, Merck & Co., P. O. Box 2000, Rahway, NJ, 07065, USA.

1984

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

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

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

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

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

1985

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

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

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

1986

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

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

1987

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

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

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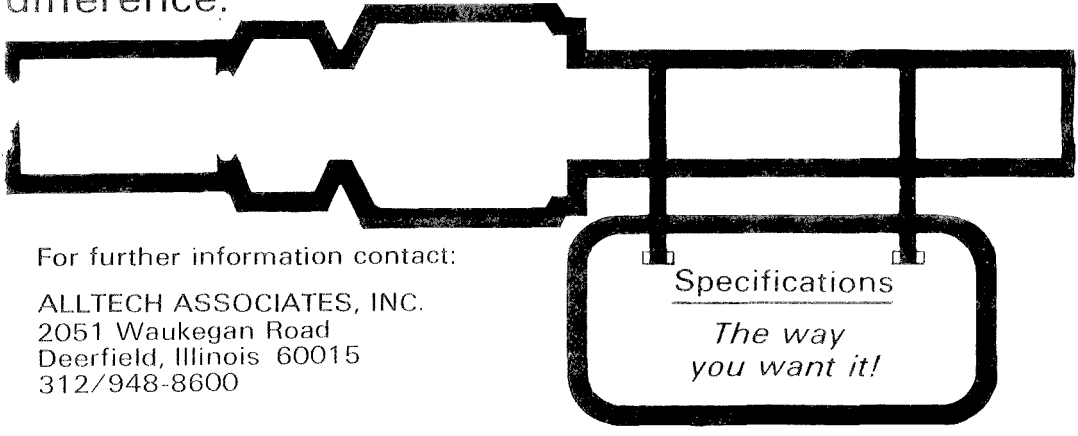

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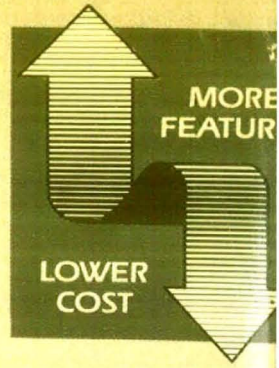
advances the art of fraction collecting...

again.

Announcing the 201-B Microprocessor Controlled Fraction Collector

We took our popular 201 Fraction Collector and added more features to make fraction collecting even more versatile and affordable than ever. The 201-B has all the features of the original 201, plus...



- New peak detection algorithm combines slope with level detection.
- New program mode lets you include peak, time and drop selections within the collection window.
- New user-selectable, free vessel programming allows cc lection into vessels of virtually any size or shape.
- New multicycle mode for repetitive collections of the same or different samples.
- New editing capabilities of operational parameters during a run.

In all there are twelve collection modes and eight rack configurations for collection into microtiter plates, scintillation vials, 13 and 18 mm tubes and a multipurpose rack.
And, our new lower price of $1950* makes the easy-to-use collector easy to own.
Contact us for complete information.

*For U.S. shipments on

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