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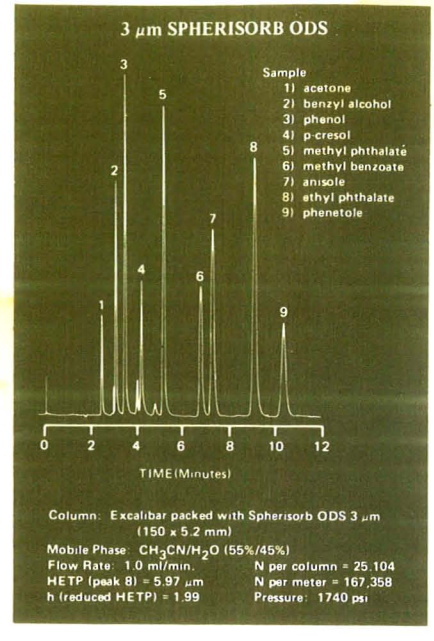

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JOURNAL OF LIQUID CHROMATOGRAPHY

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JOURNAL OF LIQUID CHROMATOGRAPHY

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JOURNAL OF LIQUID CHROMATOGRAPHY

Volume 4, Number 1, 1981

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Editorial

With this issue of The Journal, we start our fourth year of publication and, hopefully, our fourth year of service to the scientific community.

I take this opportunity to thank everyone who has helped me bring The Journal to this point, especially those of you who have served as members of the Editorial Board and those who have published herein.

We've experienced phenomenal growth, having started with 6 issues in 1978, 9 in 1979, and 12 in 1980. We've published special issues dedicated to specific topics, and these have been, apparently, well received by the scientific community. I have received numerous comments from readers that all boil down to, "give us more of the same"!

The year 1981 will certainly bring "more of the same." We will increase the size of each of the 12 issues by about 30 percent; and we will continue the publication of special topics issues during 1981.

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Water, %	0.005	0.002	0.003	0.003	0.002	0.01	0.006	0.003	0.003	0.003
Absorbance at 220 nm	<0.005	<0.005	<0.005	<0.005	<0.005	0.007	0.007	0.005	0.006	0.006
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UV Cut-off (nm)	188	188	189	189	189	189	189	188	188	188

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Acetonitrile
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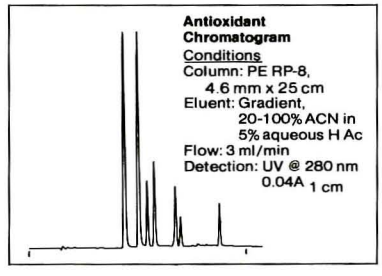

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(1), 1-12 (1981)

INVESTIGATION OF HYDROXY-TERMINATED LOW MOLECULAR WEIGHT POLYISOPRENES BY LIQUID CHROMATOGRAPHY METHODS

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Academy of Sciences, 162 06 Prague 6
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ABSTRACT

Model samples of low-molecular weight polyisoprenes and polyisoprenes containing one or two hydroxy endgroups were investigated by using various methods of liquid chromatography. Gel permeation chromatography (GPC) was employed in the determination of molecular weights and their distribution. The validity of the principle of universal calibration was confirmed. Under the given GPC experimental conditions separation was unaffected by the adsorption of hydroxylated polyisoprenes on the column packing used. The bonded -phase liquid chromatography method on columns with the amine phase allowed efficient separation to be achieved according to the content of functional groups. Since there also was separation according to molecular weights, the method allowed an estimate to be made of the molecular weights of fractions with various contents of hydroxy groups. The results obtained proved the possibility of a complex analysis of low-molecular weight polymers with functional groups, such as e.g. liquid rubbers; at the same time, the results of bonded-phase column separation indicate the possible use of this modern liquid chromatography method in the analysis of polymers.

INTRODUCTION

Polymers with functional endgroups have been widely used in polymer chemistry and technology. A typical example can be seen in, e.g., liquid rubbers. These materials exhibit two main different features compared with classical polymeric materials. Their molecular weights are rather low, i.e. several thousands or several tens of thousands of daltons; moreover, they contain various num-

bers of functional groups in their chain which to a large extent determine their physical and chemical character. Important parameters which must be known for the characterization of this type of polymers are average molecular weights and molecular weight distribution (MWD), as well as the distribution of functionality, i.e. the relative fraction of chains containing various numbers of functional groups. All these parameters may be determined by the methods of liquid chromatography. Gel permeation chromatography (GPC)is a convenient tool for the determination of molecular parameters and bonded-phase liquid chromatography for the separation according to the number of functional groups.

Several complicating factors arise in the application of GPC to the determination of MWD of these polyemers. With respect to the content of endgroups, separation may be complicated by undesired interactions of the solute with column packing, as has been observed earlier (1-3). Also, the effective hydrodynamic volume of chains with functional groups may differ from effective dimensions of chains without functional groups; this finding should be borne in mind in the interpretation of experimental data, especially if the principle of universal calibration is used (4). In addition, the validity of constants and exponents of the Mark-Houwink equation reported in the literature and needed for the interpretation of experimental data remains uncertain in the low-molecular weight range under investigation.

Two basic problems must be solved in the application of bonded-phase liquid chromatography, the separation properties of which have been demonstrated using low-molecular weight models of hydroxy and carboxy terminated chains (5). It is necessary to find a suitable system eluent-bonded phase, which would provide good resolution according to the number of functional groups even at a relatively high molecular weight of polymers undergoing separation. It is also important to suppress as much as possible the undesired influence of molecular weights or of their distribution on resolution, as this factor has already been shown to complicate separation in adsorption chromatography (6).

It has been an objective of this study to estimate the possibilities offered by the liquid chromatography methods outlined above for the determination of characteristic quantities, such as molecular weights or their distribution and the distribution of functionality. For this purpose we prepared model samples of low-molecular weight polyisoprene contanining a defined number of hydroxy groups, n = 0,1 and 2, in a sufficiently wide range of molecular weights, with the individual samples having a relatively narrow MWD.

MATERIALS AND METHODS

Preparation of Polymers

The polymers were prepared by the anionic polymerization with organolithium initiators (7,8) in toluene. Polyisoprene without the functional endgroup and α-hydroxypolyisoprene were prepared with n- and sec-butyllithium as initiator; α,ω-dihydroxypolyisoprene was prepared using the dilithiopolyisoprene oligomer obtained from the trans-stilbene-lithium adduct (9). The "living" polymer was transformed into a hydroxy terminated product by reaction with ethylene oxide (10, 11).

Gel Permeation Chromatography

The gel permeation chromatograph was built at this Institute. Four columns, 8 x 1200 mm in size, connected in series and packed with the styrene-divinylbenzene gel Styragel (Waters Associates Inc., Milford, Mass. USA) were used in the experiments, with a differential refractometer R-403 (Waters) serving as detector. Tetrahydrofuran (THF) was the eluent, flow rate 0.35 ml/min, at 25°C. The universal calibration curve (4) was constructed using a series of polystyrene standards (Waters) and the Mark-Houwink equation $[\eta] = 1.17 \times 10^{-4} M^{0.717}$ valid for linear polystyren in THF at 25°C (12). The molecular weights of polyisoprene samples under study with and without functional groups were calculated using GPC experimental data and the equation $[\eta] = 1.77 \times 10^{-4} M^{0.735}$ (13).

Vapour-Phase Osmometry

The measurements were performed with a Hitachi Perkin Elmer 115 osmometer (Hitachi Ltd., Tokyo, Japan) in toluene at 50°C in the concentration range 0.015-0.030 g/cm^3. The apparatus was calibrated by means of benzil and the real constant of the apparatus thus obtained was 1.2×10^4. The number average molecular weights values $\overline{M}_n$ were calculated from the reduced temperature difference extrapolated to zero concentration.

Membrane Osmometry

The measurements were performed with an automatic membrane osmometer (Hallikainen Instruments, Richmond, Ca., USA) at 30°C. The $\overline{M}_n$ values were calculated from the extrapolated reduced pressure to zero concentration.

Light Scattering

The measurements were performed in dried THF with a Photo-Gonio-Diffusometre FICA (Société Française D´Instruments de Controle et d´Analyse FICA, Le Mesnil Saint Denis, France) in vertically polarized light with the wavelength 546.1 nm in the angular range from 30° to 150°. Benzene with the absolute scattering value at 90° $R_{90} = 22.5 \times 10^{-6}$ cm^{-1} was used as standard. The experimental data were treated by the Zimm method, i.e. by the twofold extrapolation of K_c/R_θ to zero angle and zero concentration. Optically pure solutions in the concentration range 0.005-0.015 g/cm^3 were used. The refractive index increment measured with the Brice-Phoenix differential refractometer (Phoenix Precision Instruments, Gardiner, N.Y., USA) is 0.125 at 25°C and is independent of the number of functional groups in the polymer for n = 0,1,2.

Bonded-Phase Liquid Chromatography

All measurements were carried out with an HP 1084 B liquid chromatograph (Hewlett-Packard, Palo Alto, Ca., USA). A column 4.6x250 mm in size packed with silicagel modified with $-NH_2$ was used in the separation (Hewlett-Packard). The samples were dis-

solved to c. 0.05 g/cm^3 solutions in n-hexane and injected into the separation column in an amount of 20 microlitres. Various hexane-dichloroethane and hexane-isopropanol mixtures were used as eluents. The flow rate of the eluent was 2 ml/min., temperature 30°C. A Model 2025 differential refractometer (Knauer, Oberursel, FRG) was used for detection.

RESULTS AND DISCUSSION

Using the comparison between the average molecular weights calculated from GPC data by employing the universal calibration method and the values determined by absolute methods (cf. Table 1), it is possible to evaluate the validity of universal calibration and the adequacy of the given Mark-Houwink equation for polyisoprene also in the range of relatively low molecular weights. Simultaneously it is possible to determine the influence of functional hydroxy groups on GPC results. The resulting data show that under the chosen experimental conditions the influence (if any) of interactions of hydroxy groups with the column packing does not play any important role, and that the constants of the Mark-Houwink equation may be used in the molecular weight range under investigation (c. 3×10^3 - 4×10^4), regardless of the number of functional groups. Within the limits of experimental error, the weight average molecular weight values $\overline{M}_w$ calculated from GPC data are in good agreement with those obtained by light scattering.

No anomalies could be observed in $\overline{M}_w$ measurements by the light scattering method. The twofold extrapolation according to Zimm was linear (with a positive value of the second virial coefficient) despite the relatively high concentrations of investigated solutions, i.e. no aggregation trends could be observed. Also, it was not necessary to compensate the polyelectrolyte effect in samples with two functional groups.

The agreement of $\overline{M}_n$ values calculated from GPC and obtained by membrane osmometry is also very good. However, $\overline{M}_n$ values obtained by vapour-phase osmometry are lower in most cases than

TABLE 1

Molecular Weights of Polyisoprene Samples with Various Numbers of Hydroxy Endgroups

Sample	GPC data		Light scattering	Osmometry
	$\overline{M}_w$	$\overline{M}_n$	$\overline{M}_w$	$\overline{M}_n$
		Functionless		
PI-0-1	3 800	1 600		
PI-0-2	5 200	3 200		
PI-0-3	5 400	3 200		2 400[a]
PI-0-4	11 900	9 300	14 700	5 900[a]
PI-0-5	24 100	5 800	30 800	7 700[a]
		Monofunctional		
PI-1-1	3 500	2 600		1 700[a]
PI-1-2	3 500	2 500		
PI-1-3	7 300	5 700	8 500	5 000[a]
PI-1-4	12 000	9 600	-	-
PI-1-5	13 000	10 100	-	-
PI-1-6	13 600	10 400	-	-
PI-1-7	26 200	12 200	29 000	-
PI-1-8	33 200	22 900	-	22 600[b]
		Bifunctional		
PI-2-1	6 100	3 900	-	2 800[a]
PI-2-2	7 600	4 800	-	3 800[a]
PI-2-3	8 700	5 500	-	3 900[a]
PI-2-4	10 200	7 300	11 600	5 000[a]
PI-2-5	12 600	8 600	-	4 600[a]
PI-2-6	15 000	10 100	20 000	8 300[a]
PI-2-7	36 000	26 200	26 000	21 100[b]
PI-2-8	42 300	28 400	41 300	24 500[b]

[a]Values determined by vapour-phase osmometry.

[b]Values determined by membrane osmometry.

those calculated from GPC. This is probably due to the fact that the values obtained by osmometric measurements may be subjected to an error originating from two sources. Firstly, owing to the low real constant of the apparatus a high concentration of solutions (0.015-0.030 g /cm^3) must be chosen, when the concentration gradient is already being formed in the drop on the thermistor, which reduces the measured value. Secondly, it is probable that the polymers under study contain residual low-molecular weight components (catalysts, residual monomers, antioxidants and their transformation products), which again reduce the real average molecular weight of polyisoprene. Under these circumstances, the $\overline{M}_n$ values calculated from GPC data may be regarded as more reliable, because during GPC separation these low-molecular weight components are separated from the polymer itself, and the calculated $\overline{M}_n$ values are not affected by them.

Because of the good solubility of samples in nonpolar n-hexane, separation according to the number of functional groups was carried out on a column with the polar chemically bonded $-NH_2$ phase (LiChrosorb Si 100-NH_2). This column was used in all measurements.

The first successful separation was completed in the system hexane-dichloroethane 80/20 (v/v), where functionless and monofunctional polyisoprenes could be adequately separated in the molecular weight range under study, but the difunctional polymers were not eluted from the column under these conditions (Fig.1). A further increase in the elution power of the eluent, which should lead to the elution of difunctional polyisoprenes, would cause a considerable change in the thermodynamic quality of the solvent compared with pure n-hexane and consequently it would cause the precipitation of the polymers from solution and also lead to some difficulties in detection. For this reason, we looked for a system in which elution could be achieved for all the three types of polymers by adding a small quantity of a second component to n-hexane. The hexane-isopropyl alcohol system appeared to be a suitable choice. Fig.2 (3% v/v of isopropyl al-

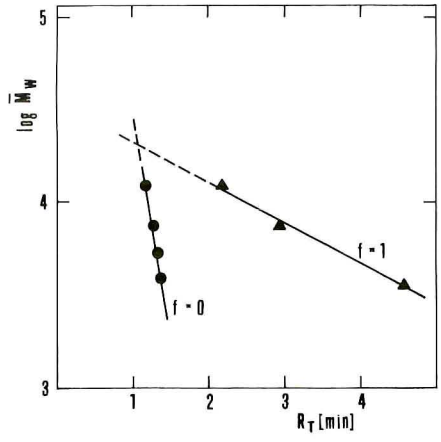

FIGURE 1. The dependence of retention times on molecular weights of samples of functionless and monofunctional polyisoprenes in the elution with hexane-dichloroethane mixture 80/20 (v/v). Conditions: cf. Experimental; f - number of functional hydroxy groups.

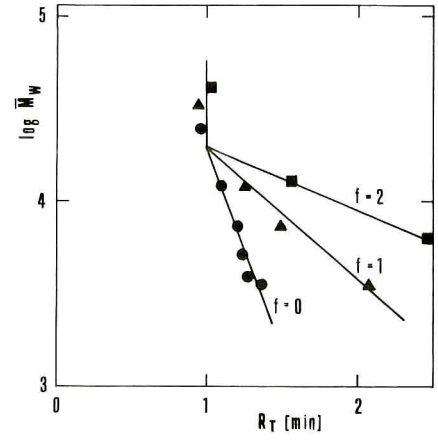

FIGURE 2. The dependence of retention times on molecular weight of polyisoprene samples with 0, 1 and 2 hydroxy endgroups in the elution with hexane-isopropyl alcohol mixture 97/3 (v/v).

cohol in hexane) shows the dependence of the retention data of functionless, monofunctional and bifunctional polyisoprenes on the logarithm of molecular weight. Under such conditions, a sufficient resolution of all three types of polyisoprene can be reached, which makes possible a quantitative evaluation of the distribution of functionality up to a molecular weight of c. 10 000. By lowering the isopropyl alcohol content to 1% (v/v), it is possible to improve the separation of functionless and monofunctional polyisoprenes also in the range of higher molecular weights (above 10 000), but bifunctional polymers are not eluted from the column any more (cf. Fig.3).

An interesting observation in all cases is the considerable dependence of retention times on molecular weight. The separation is consequently reduced to a certain range of molecular weights, but it makes possible not only the determination of the relative amount of polymers with various content of functional groups, but also of their approximate molecular weight. Polyisoprenes with molecular weights higher than approx. 18 000-20 000 cannot be

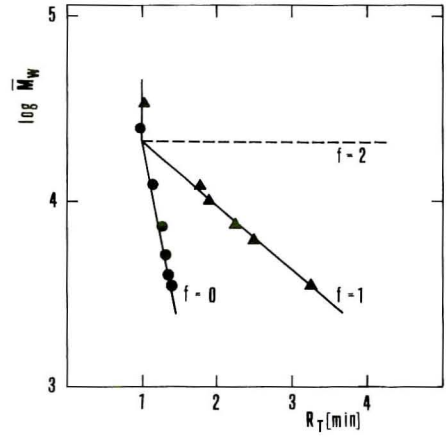

FIGURE 3. The dependence of retention times on molecular weight of polyisoprene samples with 0, 1 and 2 hydroxy endgroups in the elution with hexane-isopropyl alcohol mixture 99/1 (v/v).

separated under our experimental conditions. This limit of molecular weights coincides with the exclusion limit at GPC of polystyrene standards on a silicagel packed column with a similar porosity of 100 Å, as indicated by Fig.4. The likely explanation of this dependence consists in the simultaneous effect of the mechanism of steric exclusion. With increasing molecular weight of the polyisoprene samples, macromolecules penetrate into a smaller pore volume of the column packing, because part of the pores is inaccessible to them for steric reasons, and the surface undergoing interaction with the packing is therefore smaller. Another possible explanation is seen in the reduced chain polarity resulting from increased molecular weight, and consequently in the reduced interaction with the bonded phase which leads to a shorter retention time. Which of these causes is the real one could be decided by using a packing with such a large pore size that the effect of steric exclusion in the molecular weight range under study could not become operative. A study of these aspects is in progress.

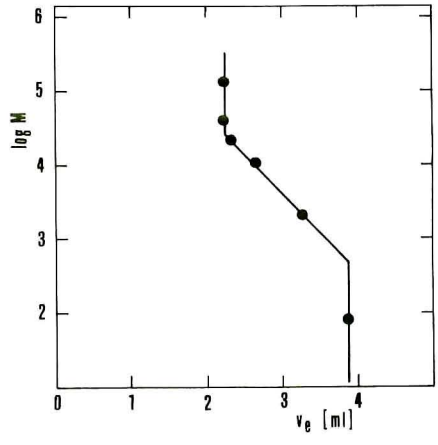

FIGURE 4. The calibration plot of a column packed with Lichrosphere 100 Å using polystyrene standards with tetrahydrofuran as eluent; flow rate of the elution agent 0.5 ml/min.

For a quantitative evaluation of GPC and bonded-phase liquid chromatography results obtained with refractometric detection, it is important to know if the refractive index of polyisoprenes under study depends on molecular weight and on the number of functional groups. Measurements with a differential Brice-Phoenix refractometer and chromatographic measurements have revealed that in the range of molecular weights and of the number of functional groups under investigation the refractive index increment remains constant within the limits of experimental error.

The results reported here show that the liquid chromatography methods may be successfully employed in the characterization of polymers bearing functional groups. GPC can be used in the determination of molecular parameters using the principle of universal calibration within the whole range of molecular weights under study (i.e. c. $3x10^3$-$4x10^4$ daltons). Using bonded-phase liquid chromatography, and under experimental conditions just described, it is possible to determine the quantitative participation of the individual functionalities up to molecular weights of c. 10 000, i.e. within the limits of most frequently occurring values in the case of liquid rubbers. The dependence of retention times on molecular weight makes also possible a direct estimate of molecular weight. It should be stressed that although these conclusions hold only for the hydroxy terminated polyisoprenes with the functionality n = 0-2 investigated in this study, they indicate at the same time the general possibility of use of liquid chromatography also in the study of other polymeric systems with functional groups.

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REVERSE-PHASE LIQUID CHROMATOGRAPHY OF PHENOL-FORMALDEHYDE ALL-ORTHO OLIGOMERS

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ABSTRACT

High Performance Liquid Chromatography was proven to be a powerful method for the separation and quantitation of ortho-linked Phenol-Formaldehyde oligomers. A homologous series of oligo [(2-hydroxyl-1,3-phenylene)methylene] s (dinuclear to octanuclear compounds) was analyzed. The samples were separated by reverse-phase chromatography and monitored at 280 nm. Optimum conditions were obtained on a μ-Bondapack C_{18} column employing isocratic ambient elution with a metanol/water 80:20 (v/v) mixture. Four reference mixtures of oligomers of known composition were used to assure the reliability of the method. Subsequent analysis of two samples of all-ortho novolac resins was performed in order to substantiate further the validity of the technique.

INTRODUCTION

Among the phenolic resins, Phenol-Formaldehyde (P-F) novolacs in which all phenolic nuclei are linked by an ortho-ortho methylene bridge, represent a class of substances of special interest and broad utility since it exhibits shortened curing periods over the conventional products(1).

In the past, GLC has been used extensively to separate quantitatively low molecular wieght P-F condensation products. This

technique, combined with other analytical methods (IR, UV, paper chromatography, GPC, ^{1}H NMR, and mass spectroscopy), provided structural information such as the ratio of ortho/para linkages and the number-average molecular weight, but failed to give the exact composition of the resin. Recently (2), ^{13}C NMR spectroscopy has proved to be a more powerful tool for the analysis of these resins. In this field, however, HPLC analysis has received only marginal attention and has been mainly applied to alkaline-catalyzed P-F condensates (3) or to individual phenolic compounds (4).

Our interest in the application of coordinating metal phenolates in the ortho-site specific P-F condensations (5) and in the control of practical syntheses of faster curing novolacs (6) prompted us to develop an accurate quantitative technique for the rapid characterization of resins of this type. This paper reports the quantitation of ortho-linked P-F oligomers and related resins by reverse-phase HPLC.

EXPERIMENTAL

Apparatus

HPLC was performed using a Model 6000A pump (Water Associates, Milford, Mass.), a U6K injector (Waters), and a Model 440 UV detector (Waters). The samples were separated at ambient temperature on a 30 cm x 3.9 mm i.d. μ-Bondapak C_{18} high efficiency column (Pat.No.27324, Waters) and monitored at 280 nm. The recorder was an OmniScribe (Houston Instruments, Austin, Texas) instrument. A mobile phase of 80% methanol in water with a flow rate of 2.0 ml/min was used throughout this work. Actual sample mass, injection volume, and other LC operating parameters are indicated in each chromatogram. All the samples were dissolved in methanol and filtered on Millipore FHLP 0.5 μm filters prior to the injection. Standard solutions of each oligomer were prepared at various concentrations in order to cover a range of 0.3 to 6.0 μg per injection.

Chemicals

The oligo[(2-hydroxy-1,3-phenylene)methylene]s dinuclear to octanuclear derivatives) used were prepared in our lab according to a previously described (5) ortho-specific P-F oligomerization with a purity of 99+% by HPLC. The resin NOVO-A was prepared in benzene from bromomagnesium phenoxide and paraformaldehyde (3:2 mol/mol); colorless homogeneous powder, m.p. 56-60 °C (6). The resin NOVO-B was prepared in xylene (5 mol) from phenol and paraformaldehyde (3:2 mol/mol); pale yellow glassy solid, m.p. 46-50 °C (7). RS-HPLC grade methanol, obtained from Carlo Erba, Milano, was used without further purification. Redistilled water (Carlo Erba) was filtered on Sep-Pak C_{18} cartridges and degassed immediately prior to use.

RESULTS AND DISCUSSION

A representative separation of a standard solution of di- to octanuclear P-F oligomers is illustrated in Figure 1. Optimum resolution of components and time of analysis were achieved on a μ-Bondapak C_{18} reverse-phase column employing isocratic ambient elution with a solvent mixture of methanol/water 80:20 (v/v) as described in the Experimental. In the range of 0.3 to 6.0 μg of injected sample the UV detector response at 280 nm was linear for all seven oligomers. The standard calibration curves showed excellent linearity with a correlation coefficient greater than 0.99.

The precision of the oligomer analysis is shown in Table 1. The average %RSD (relative std deviation) using peak area was 2.62% while no significant loss of precision was observed in the size ranging from 1.0 to 5.0 μg. The relative response factors were calculated using the mean peak area and were as follows: Di = 1.000, Tri = 1.009, Tetra = 0.998, Penta = 0.996, Hexa = 0.997, Hepta = 0.993, and Octa = 0.990.

There was an insignificant variation in response factors with a decrease of less than 0.01% for di- to octanuclear oligomers thus

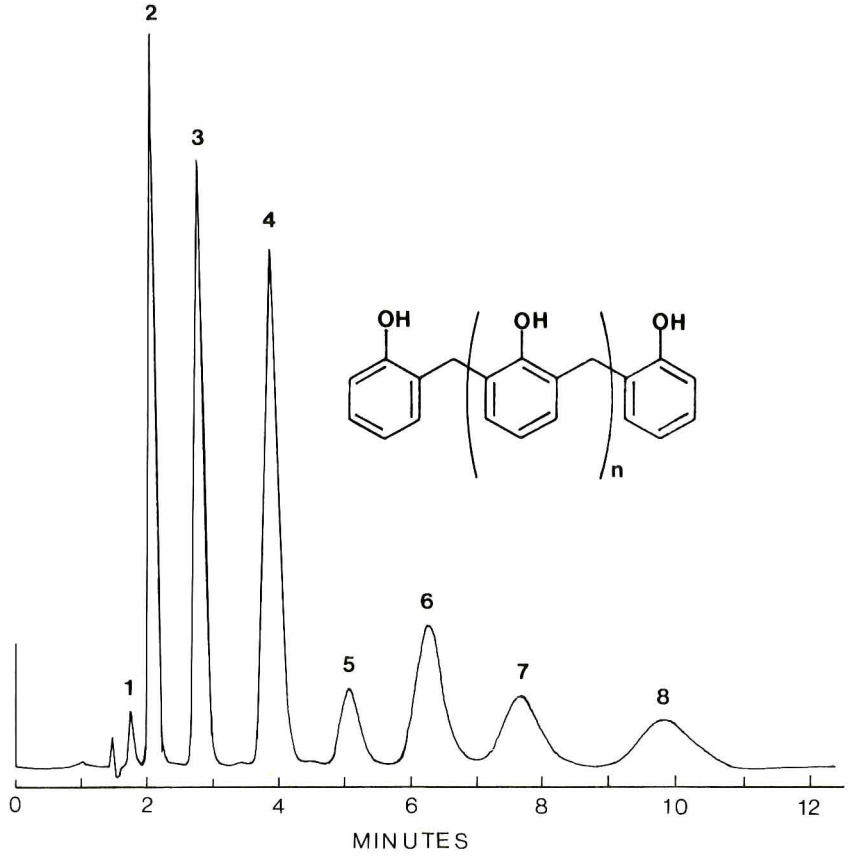

FIGURE 1

Chromatogram of a P-F oligomer reference mixture. Conditions: detector sensitivity 0.1 AUFS; back pressure 2800 psi; flow-rate 2.0 ml/min; chart speed 2.0 cm/min; peak identities: (1) phenol, (2) 1.01 µg Di (n = 0), (3) 1.25 µg Tri (n = 1), (4) 1.63 µg Tetra (n = 2), (5) 0.25 µg Penta (n = 3), (6) 1.00 µg Hexa (n = 4), (7) 0.54 µg Hepta (n = 5), and (8) 0.64 µg Octa (n = 6) in 12.5 µl methanol.

TABLE 1

Precision of Ortho P-F Oligomers

(3.66 µg sample)

Peak Areas (cm^2)

RUN NO.	Di	Tri	Tetra	Penta	Hexa	Hepta	Octa
1	12.196	12.010	11.685	12.120	11.788	11.968	12.120
2	12.204	11.940	12.180	11.170	11.968	12.030	12.150
3	12.015	11.967	12.035	12.056	12.312	12.110	11.935
4	11.969	12.453	12.852	11.960	12.272	11.652	11.023
5	12.246	12.469	12.240	11.936	12.156	11.354	12.411
6	12.302	12.090	12.300	12.044	11.932	11.561	11.832
7	11.790	11.697	11.542	12.289	12.042	12.211	11.598
8	12.401	11.942	12.420	12.456	12.213	11.813	12.232
9	12.601	12.014	12.354	12.198	12.456	12.223	12.010
10	11.940	12.551	11.890	11.988	12.234	12.322	12.036
Mean	12.166	12.281	12.148	12.122	12.137	12.087	12.045
Standard Deviation	± .215	± .334	±.490	±.355	±.235	±.365	±.240
% RSD	1.77%	2.71%	4.03%	2.93%	1.93%	3.01%	1.99%

offering a fortunate circumstance for our quantitation. Thus, for routine analyses, peak area should be the method of choice with no necessity of response correction for di- to octanuclear oligomers.

The use of a common calibration plot (slope 3.23 $cm^2/\mu g$; intercept 0.43 cm^2; correlation coefficient 0.998) in Figure 2, possible because of the negligible variance in the calibration constants of individual curves, is the most attractive feature of this method in its application to the quantitation of ortho-linked P-F oligomers. The most likely explanation for the near coincidence of all calibration plots lies in the well-known linear dependence of the molar absorptivity upon the number of phenolic units in the polyphenol oligomer series (8, 9). Assuming that in all oligomers there is, on the average, the same number of phenolic units per unit of weight,

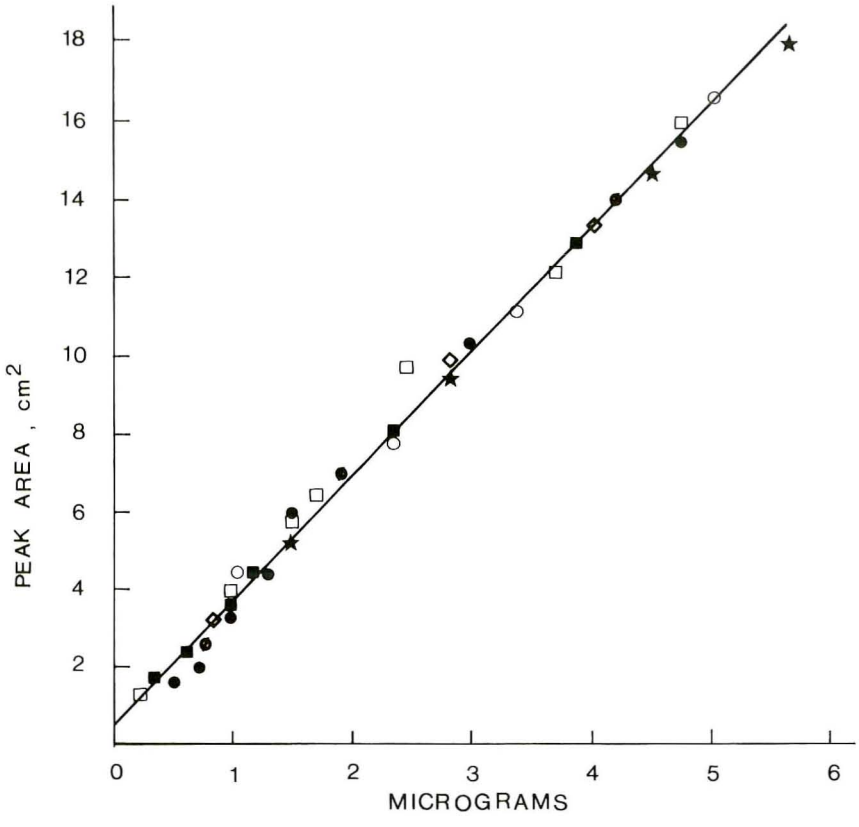

FIGURE 2

Load-response curve for di- to octanucelar P-F ortho-oligomers. ■ Di; ● Tri; □ Tetra, ○ Penta, ★ Hexa; ◇ Hepta; ø Octa. Solid line is a linear least-squares fit to data from 0.29 to 5.65 µg.

the UV absorption at 280 nm (λ_{max} for all oligomers) gives the same integrated value for unit of weight for each component whatever its molecular weight.

Analysis of four mixtures of all-ortho P-F oligomers of known composition, as summarized in Table 2, shows an average relative error of 4.02%.

TABLE 2

P-F Oligomer Mixture Composition

(Weight %)

	Di	Tri	Tetra	Penta	Hexa	Hepta	Octa
MIXTURE 1							
Certified	15.98	19.78	25.79	3.95	15.82	8.54	10.13
Experimental	16.27	20.57	26.63	3.82	15.31	8.34	9.41
Rel.Error (%)	1.81	3.99	3.26	3.29	3.22	2.34	7.11
MIXTURE 2							
Certified	14.36	16.52	23.34	4.49	14.36	16.16	10.77
Experimental	14.05	15.99	23.64	4.72	14.03	15.51	9.93
Rel.Error (%)	2.15	3.20	1.28	5.12	2.29	4.02	7.80
MIXTURE 3							
Certified	16.24	19.28	26.39	5.07	16.24	6.60	10.15
Experimental	16.08	20.10	27.39	5.27	16.83	6.28	9.04
Rel.Error (%)	0.98	4.25	3.78	3.94	3.63	4.84	10.93
MIXTURE 4							
Certified	18.31	18.31	32.04	1.33	18.31	4.94	6.77
Experimental	17.70	19.53	31.18	1.31	17.80	5.26	7.18
Rel.Error (%)	3.33	6.66	2.68	1.50	2.78	6.48	6.05

Average Relative Error, 4.02% (0.98% - 10.93%)

From the presented data it appears that the reverse-phase technique is especially appropriate for the analysis of identified components in novolac resins. The C_{18} column was able to separate all seven oligomers with both high speed and good resolution.

As an application of this procedure, analysis of two faster-curing novolac samples was carried out. Fingerprints obtained for NOVO-A and NOVO-B resins are compared in Figure 3. The compositions of the major oligomers are shown in Table 3.

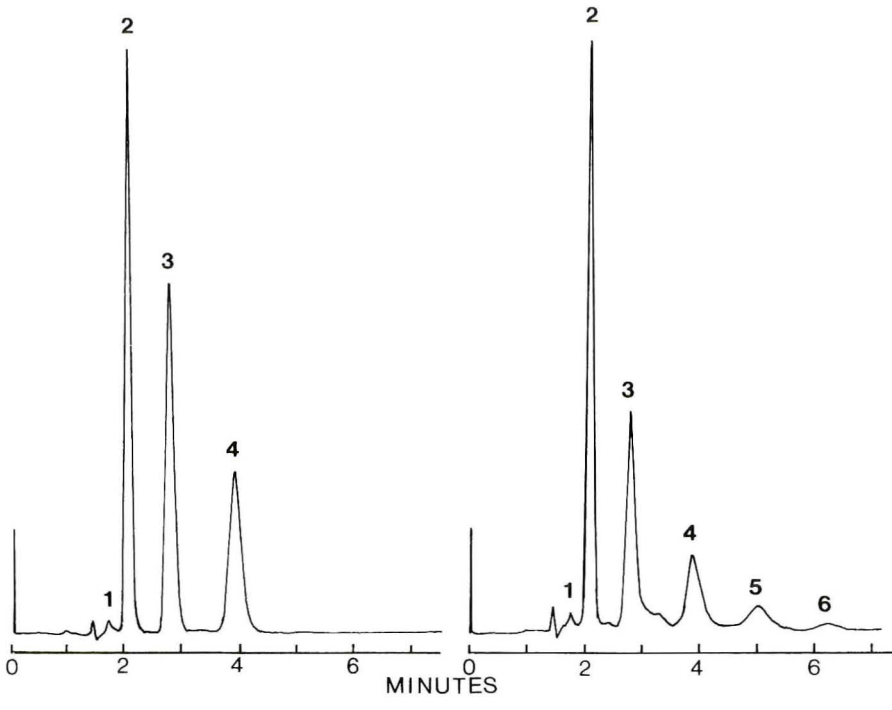

FIGURE 3

Separation of oligomers of all-ortho novolac resins. Resin NOVO-A (left), load 3 µg in 6 ml of methanol; resin NOVO-B (right), load 3 µg in 10 ml of methanol. Conditions and peak identities as in Figure 1.

The results of the HPLC runs for both samples agree,within the experimental error, with the values obtained by other techniques. From these and the above data it is clear that the application of this method to the isolation and quantitation of various polyphenol components is quite facile.

CONCLUSIONS

We have found a suitable HPLC procedure for the identification, separation, and quantitation of methylene-bridged polyphenol com-

TABLE 3
Major Oligomer Composition of
All-ortho Novolac Resins
(Weight %)

	Di	Tri	Tetra	Penta	Hexa
RESIN NOVO-A					
Composition %	39.84	33.53	26.06		
$\bar{M}n$(HPLC)	289.64				
$\bar{M}n$(^{1}H NMR)	280.60				
$\bar{M}n$(^{13}C NMR)	286.51				
$\bar{M}n$(Osmometry)	294.00				
RESIN NOVO-B					
Composition %	53.30	26.89	13.21	4.72	1.89
$\bar{M}n$(HPLC)	279.55				
$\bar{M}n$(^{1}H NMR)	271.40				
$\bar{M}n$ (^{13}C NMR)	275.60				
$\bar{M}n$(Osmometry)	283.00				

pounds occurring in all-ortho P-F novolac resins. Although only seven oligomers (di- to octanuclear) were considered, this was sufficient for the analysis of commercial resins with an average molecular weight ranging from 200 to 600 max. The technique had %RSD from 1.77% for di- to 4.03 for tetranuclear oligomer and the analysis had an average relative error less than 4.05% for analysis of known mixtures. The methodology reported here could be useful for the investigation of faster-curing P-F resins in which ortho-ortho methylenes are the sole or the largely predominant bridges.

ACKNOWLEDGMENTS

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(1), 23-29 (1981)

A THERMODYNAMIC MODEL FOR LIQUID-LIQUID CHROMATOGRAPHY WITH A BINARY MOBILE PHASE

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ABSTRACT

A simple model which previously led to successful predictive equations for gas-liquid partition coefficients on mixed stationary phases has been slightly modified to include liquid-liquid partitioning. Predictive expressions are developed for distribution coefficients determined from liquid-liquid chromatographic studies using a binary mobile phase. Limitations of the method are briefly discussed.

INTRODUCTION

Liquid-Liquid Chromatography (LLC) is a very powerful technique for chemical separations. This technique has not been applied to analytical determinations as frequently as the more modern method of gas-liquid chromatography. Recent improvements in column technology and instrumental design have led to a renewed interest in LLC. The desirable experimental features include; small (μl) samples, relatively short analysis times, a large selection of possible partitioning systems, and the ability to use nonvolatile solutes. Theoretically, there exist numerous combinations of binary immiscible phases which can be used in LLC. The number grows even larger if one considers the ternary (or higher order multicomponent) systems which can be mixed to form two immiscible liquid phases.

The ability to conveniently work at very low solute concentrations offers possibilities to determine thermodynamic properties through equations developed by Locke and Matire[1-2]. In this paper, we extend the considerations of Locke and Matire to include thermodynamic studies using a binary solvent as the mobile phase. In many instances the derived equations should provide an indication of phase compositions needed to achieve desired elution characteristics. Similar expressions developed specifically for gas-liquid chromatography have been shown to be beneficial in the selection of mixed stationary phases and in the study of associations complexes between the solute and one of the solvent components[3,4].

Relationship Between Solute Retention and Thermodynamic Properties.

The partitioning of a solute between two immiscible liquid phases provides the physical basis for liquid-liquid chromatography. As such, it is often convenient to define an experimental distribution coefficient as the ratio of solute concentration in each phase:

$$K_R = \frac{\text{concentration of solute in stationary phase}}{\text{concentration of solute in mobile phase}} \qquad (1)$$

For LLC it has been shown that solute retention volume (V_R) is related to the distribution coefficient through

$$V_R = \nu_m + K_R \nu_S \qquad (2)$$

where ν_S is the volume of the stationary liquid in the column and ν_m is the interstitial volume occupied by the mobile phase. To express retention measurements in a manner independent of experimental parameters, Littlewood *et al.*[5], suggested specific retention volume (V_g):

$$V_g = \frac{K_R}{\rho_S} \qquad (3)$$

where ρ_s is the density of the stationary phase.

The mathematical derivation of equation (2) assumes normal LLC operating conditions, and if one further requires liquid-

liquid partitioning to be the sole retention mechanism, then retention measurements can be related to thermodynamic quantities. The activity coefficient of a solute relative to Raoult's law, $\gamma_3^{\ell}(T,P)$, in a binary solution is defined by:

$$RT \ln \gamma_3^{\ell}(T,P) = \mu_3^{\ell}(T,P) - \mu_3^{o,\ell}(T,P) - RT \ln X_3^{\ell} \quad (4)$$

where $\mu_3^{o,\ell}(T,P)$ is the chemical potential of pure liquid solute at T and P, $\mu_3^{\ell}(T,P)$ is the solute chemical potential in the solution, and X_3^{ℓ} is the mole fraction of solute.

In liquid-liquid chromatography the condition for thermodynamic equilibrium is

$$\mu_3^{s}(T,P) = \mu_3^{m}(T,P) \quad (5)$$

in which $\mu_3^{s}(T,P)$ and $\mu_3^{m}(T,P)$ are the solute chemical potentials in the stationary and mobile phases respectively. Substitution of equation (5) into equation (4) with rearrangement yields:

$$\mu_3^{o,m}(T,P) - \mu_3^{o,s}(T,P) = RT \ln \frac{\gamma_3^{s} X_3^{s}(T,P)}{\gamma_3^{m} X_3^{m}(T,P)} \quad (6)$$

since both $\mu_3^{o,m}(T,P)$ and $\mu_3^{o,s}(T,P)$ represent the chemical potential of pure liquid solute at T and P, ($\lim_{X_3 \to 1} \gamma_3^{i} = 1$), their difference is zero. For simplicity we have elected to choose the column pressure (P) as our reference and interested readers are referred to an article by Locke and Matire if another reference state is preferred. In very dilute solutions the experimental distribution coefficient is expressed more conveniently as:

$$K_R = \frac{X_3^{s}\bar{V}_m^{o}}{X_3^{m}\bar{V}_s^{o}} \quad (7)$$

where $\bar{V}_i^{o}$ is the molar volume of pure liquid phase $\underline{i}$. Combination of equations (6) and (7) give:

$$\ln K_R^{o} = \ln \frac{\gamma_3^{m,\infty}(T,P)\ \bar{V}_m^{o}}{\gamma_3^{s,\infty}(T,P)\ \bar{V}_s^{o}} \quad (8)$$

the superscript ∞ denoting values at infinite dilution. Expressed in terms of specific retention volume, equation (8) becomes

$$\ln V_g = \ln \frac{\gamma_3^{m,\infty}(T,P)\ \bar{V}_m^o}{\gamma_3^{s,\infty}(T,P)\ M_s} \qquad (9)$$

in which M_s is the molecular weight of the stationary phase. These simple mathematical relationships enable a priori predictions of distribution coefficients and retention volumes for any system in which the activity coefficients are known or can be estimated from solution theories.

The Nearly Ideal Binary Solvent (NIBS) Approach to Liquid-Liquid Partition Coefficients in Non-Complexing Systems.

The Nearly Ideal Binary Solvent (NIBS) approach developed by Bertrand and co-workers has been shown to be quite successful in predicting heats of solution[6], gas-liquid partition coefficients[3], and solubilities[7,8] in systems containing only nonspecific interactions. Using a simple mathematical model for the excess Gibbs free energy of a multicomponent system:

$$\Delta\bar{G}^{mix} = RT \sum_{i=1}^{N} (X_i \ln \phi_i) + \Delta\bar{G}^{fh} \qquad (10)$$

$$\Delta\bar{G}^{fh} = (\sum_{i=1}^{N} X_i \bar{V}_i^o) \text{ all pairs}^{\Sigma} \phi_i\phi_i A_{ij} \qquad (11)$$

Acree and Bertrand developed a zero-parameter equation (Equation VV, reference 7) which predicts solubilities in 35 systems of non-specific interactions containing naphthalene, stannic iodide, iodine and benzil as solutes with an average deviation of 2.2% and a maximum deviation of 25%. This maximum deviation occurs in a system (Benzil-Benzene + Cyclohexane) in which complex formation has been suggested and if this system is excluded from calculations, the maximum deviation becomes 6%. The success of this model is even more remarkable if one realizes the mole fraction solubility of benzil changes by a factor of 14 in the carbon tetrachloride + n-hexane system[8].

Using the following thermodynamic definitions

$$\Delta\bar{G}^{fh} = RT \sum_{i=1}^{N} X_i \ln\gamma_i^{fh} \qquad (12)$$

$$\ln\gamma_i^{fh} = \ln\,(a_i/\phi_i) - (1- \frac{\bar{V}_i^o}{\bar{V}_M^o}) \tag{13}$$

$$(\gamma_i^{fh})^{\infty} = \lim_{X_i \to o} \gamma_i^{fh} \tag{14}$$

$$\gamma_3^{\infty} = (\gamma_3^{fh})^{\infty} \frac{\bar{V}_3^o}{\bar{V}_{solvent}^o} \exp\,(1- \frac{\bar{V}_3^o}{\bar{V}_{solvent}^o}) \tag{15}$$

the activity coefficient for a solute (relative to Flory-Huggins entropy) at infinite dilution in binary solvent mixtures (components 1 and 2)

$$\ln(\gamma_3^{fh})^{\infty} = \phi_1^o\, \ln(\gamma_3^{fh})^{\infty}_{x_1=1} + \phi_2^o \ln(\gamma_3^{fh})^{\infty}_{x_2^o=1} - \bar{V}_3^o\, (X_1^o\bar{V}_1^o+X_2^o\bar{V}_2^o)(RT)^{-1}\, \Delta\bar{G}_{12}^{fh} \tag{16}$$

where,

$$\phi_1^o = \frac{X_1^o\bar{V}_1^o}{X_1^o\bar{V}_1^o+X_2^o\bar{V}_2^o} \quad \text{and} \quad X_1^o = \frac{X_1}{X_1+X_2} \tag{17}$$

is shown to depend on a weighted average of the solute activity coefficient in each of the pure solvents $(\gamma_3^{fh})^{\infty}_{x_1=1}$, $(\gamma_3^{fh})^{\infty}_{x_2=1}$ and a contribution due to the "unmixing" of the solvent pair by the presence of the solute. Enhancement of the unmixing term by a large solute molecule can lead to predictions of maxima or minima in the thermodynamic properties of the solute.

Although the NIBS approximations for solute activity coefficients in binary solvent mixtures cannot be rigorously extended to liquid-liquid partitioning in systems of practical importance, expressions developed for "model" systems may possess predictive applicability[9]. The simplest system to consider involves the partitioning of a solute between two "completely" immiscible liquid phases where only nonspecific interactions are permitted. The mobile phase consists of a binary mixture of inert solvent molecules (completely immiscible with the stationary phase at all

binary compositions) and the stationary phase is such that the solute activity coefficients at infinite dilution $\gamma_3^{s,\infty}$ remains constant. The combinations of equations (8), (15) and (16) then gives:

$$\ln K_R^o = \phi_1^o \ln (K_R^o)_{x_1^o=1} + \phi_2^o \ln (K_R^o)_{x_2^o=1} - \bar{V}_3^o (X_1^o \bar{V}_1^o + X_2^o \bar{V}_2^o)(RT)^{-1} \Delta \bar{G}^{fh}(T,P) \quad (18)$$

With this equation retention volumes in pure solvents can be used to calculate solute distribution coefficients at infinite dilution in the pure solvents. These values can be combined with the excess free energy of the binary solvent mobile phase to predict solute distribution coefficients. The predictive ability of this equation will depend to a large extent on the complexity of the system under investigation. It is anticipated that this approach will provide reasonably accurate approximations for simple systems containing only nonspecific interactions and will fail in systems having either specific solute-solvent or solvent-solvent interactions. The extension of equation (18) to systems involving complexation between the solute and one component of the binary mobile phase should be similar to methods employed in gas-liquid chromatography to study association complexes.

Experimental LLC partition coefficinets were unavailable for comparison, but similar expressions based on mole fractions have been applied by Buchowski and Teperek[10] to infinite dilution partition coefficients of benzoic acid and o-nitroaniline in systems benzene + cyclohexane--water and isooctane + bromoform--water. This general approach if proven successful, will offer new possibilities to study association phenomenon for nonvolatile solute molecules.

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DETERMINATION OF VAPOR-PHASE CARBONYLS BY HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

Methods have been developed for the trapping and quantitative analysis of low molecular weight carbonyls in complex gas phase mixtures. Formaldehyde, acetaldehyde, acrolein, and acetone are separated as the 2-4-dinitrophenylhydrazones with a sensitivity of less than 10 ppb. The separation can be done on a variety of commercial C_{18} reverse-phase columns.

INTRODUCTION

Carbonyl compounds from one to four carbon atoms present a significant environmental hazard. Formaldehyde has recently been declared a carcinogen (by the National Cancer Institute); acetaldehyde and acrolein are well-established ciliatoxic and cytotoxic agents (2), and acetone has demonstrated toxicity although at somewhat higher levels. In addition, the Occupational Safety and Health Administration (OSHA) has established limits for exposure to formaldehyde (.5 ppm) and acrolein (.1 ppm) as well as acetone (500 ppm) (3). These levels require the development of specific and sensitive analytical methods.

Classically, carbonyl compounds have been determined using colorimetry. Derivatives such as oximes, semicarbazones, and phenylhydrazones are easily formed, and can be used as qualitative indicators of carbonyl content. However, the determination of specific carbonyls requires some separation system. With the exception of formaldehyde, the carbonyls of interest in this work are easily vaporized and thus amenable to gas chromatography. Several approaches have been in use for some time, including direct introduction of gas

samples with or without cryogenic focusing(4,5) and "purge and trap" injection of aqueous samples.

Gas chromatographic determination of these compounds is of limited utility, for several reasons. The inherent lack of sensitivity precludes the analysis of acrolein at subpart-per-million levels. Precision and accuracy are impaired due to volatility and reactivity considerations, and direct standardization is difficult. Thus, improvement of the methodology is imperative if the determination of these compounds is to be performed on a routine basis, as may be required under OSHA guidelines.

An alternative approach has been to take advantage of the reactivity of these compounds for the formation of absorbing derivatives, followed by separation using HPLC. The 2,4-dinitrophenylhydrazones are easily formed, stable, and highly absorbing. In addition, these derivatives are of low volatility, making sample concentration feasible. Finally, the carbonyls of interest have been separated on reverse-phase columns as the 2,4-dinitrophenylhydrazones (6).

The primary objective of this work has been to provide quantitative definition of the methodology, particularly with respect to trapping efficiency and recovery. This has been done by comparison with established gas chromatographic methods. A second objective has been to establish adequate separation systems with a variety of reverse phase columns, in order to provide a working guide for those interested in the determination. The third objective has been to demonstrate the utility of this approach using complex environmental samples.

MATERIALS AND METHODS

Reference samples of the carbonyls were obtained from commercial sources at the highest available purity. The 2,4-dinitrophenylhydrazine was obtained from J. T. Baker Chemical Company. All solvents were reagent grade or better, and were distilled in glass prior to use. Reverse phase columns were obtained prepacked from the various vendors.

Standard 2,4-dinitrophenylhydrazones were prepared in bulk form and recrystallized twice from hot methanol. The purity was checked by HPLC. The standards were dissolved in methanol on a weight/volume basis and stored frozen. Solutions prepared in this manner were stable for several months.

Trapping of the gas-phase carbonyls was accomplished by adding 20 ml of saturated 2,4-dinitrophenylhydrazine in 2 N HCl and 20 ml chloroform to a 1 L gas sampling flask. The flasks were then evacuated, and the carbonyls added. Cigarettes were smoked under standard analytical conditions (1 puff/minute, 35 ml puff volume, to a butt length of 23 mm) using a Filamatic single port smoker (7). Diesel fuel aerosols were introduced directly into the sampler.

High-pressure liquid chromatography was done using a variety of equipment. Injection was done either by use of a Rheodyne Model 7020 loop injector or a Waters WISP-710 Autosampler. Waters Model 6000A pumps were used, and detection was by UV with either a Waters Model 440 fixed wavelength detector or a Varian variable wavelength detector. All columns were C_{18} reverse-phase, obtained from various commercial sources.

RESULTS AND DISCUSSION

The separtion of the DNPHs of formaldehyde, acetaldehyde, acrolein and acetone was compared on a series of reverse phase columns (Table 1). All columns tested gave adequate separation for the compound of interest. With slight variations in the mobile phase composition, analysis times were roughly equivalent. We believe that any reasonably efficient C_{18} column will perform this separation. The degree of separation can be imrpoved by using weaker mobile phases at the expense of increased analysis time (Figure 1).

Detection can be performed at either 254 nm or 350 nm. For highest sensitivity with reasonably clean gas samples (filtered), 254 nm is best. However, for complex aerosols, better selectivity is obtained at 350 nm at about a 20% decrease in sensitivity. Fluorescence detection further improves the selectivity, but does not offer any significant increase in sensitivity. Thus, all experiments were carried out at 254 nm. The ultimate sensitivity (roughly equivalent for all carbonyls) is 10 ng injected on column.

The use of HPLC for the quantitative analysis of these compounds demands adequate conversion of the carbonyls to the derivatives during trapping. However, the extreme volatility and reactivity of the compounds makes assessment of the conversion efficiency quite difficult. For this reason, comparisons were made using cigarette smoke. The direct determination of these compounds by GC, even though suffering the limitations mentioned earlier, has been carried out in this laboratory for

TABLE 1

Conditions for the Separation of Carbonyls on Various Commercial C_{18} Columns

Column	Dimensions	Flow Rate	Mobile Phase (methanol/water)
Partisil-10-ODS-2 (Whatman)	2.5 cm x 4.6 mm	1.0 ml/min	60/40
Zorbax-ODS (Dupont)	2.5 cm x 4.6 mm	1.0 ml/min	70/30
HC-Sil-ODS (Perkin-Elmer)	2.5 cm x 4.6 mm	1.0 ml/min	65/35
Bio-Sil-HP-10 Reverse Phase (Bio-Rad)	2.5 cm x 4.6 mm	1.0 ml/min	60/40

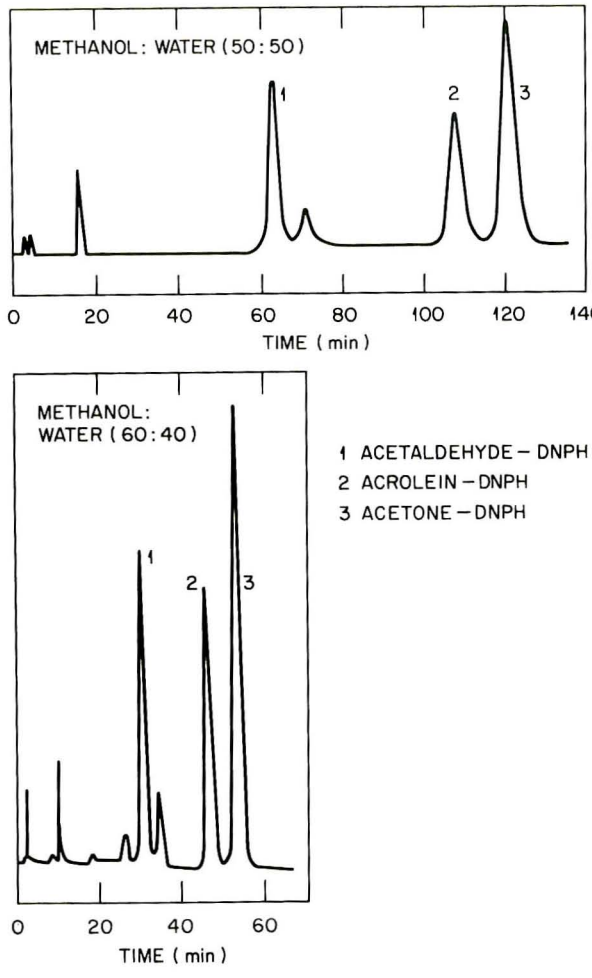

FIGURE 1. Effect of Mobile Phase on the Separation of Carbonyls. Column: Partisil 10 ODS-2. Flow Rate: 1 ml/min.

many years. The values for acetaldehyde and acrolein are thus well established, and recovery calculations based on known deliveries of reference cigarettes are possible (4).

For determination of trace levels of the carbonyls in ambient atmospheres, a flow-thru trapping system would be preferable. This type of system would allow the sampling of large volumes of air, with con-

centration of the carbonyls. We thus tested several trapping systems with varying sampling rates, reagent volumes, and sampling stages. However, under our conditions, we were not able to recover more than 20 to 40% of the acrolein from the gas stream. Under high flow conditions significant breakthrough occurred, even when additional traps were used. Under low flow conditions, the aldehydes apparently react to form higher molecular weight adducts (Figure 2) which are detected as later eluting peaks in the chromatogram. The best recoveries were obtained using liquid nitrogen cold traps with subsequent addition of the reagents, and even with these conditions the recovery was poor for acrolein.

Therefore, a closed trapping system was adopted which permitted the introduction of one liter of gas into an evacuated bulb containing the reagent (saturated 2,4-dinitrophenylhydrazine in 2 N HCl) and extract-

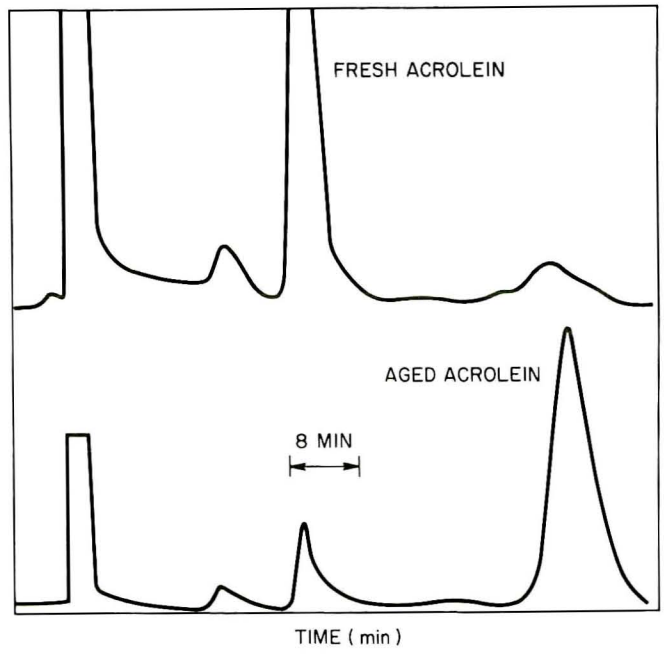

FIGURE 2. Effect of Ageing at Room Temperature on the Chromatogram of Acrolein-DNPH. Fresh Acrolein: Reagent stored at 10°F, derivatized. Aged Acrolein: Same reagent after standing at room temperature three days, derivatized.

ant ($CHCl_3$). After sampling, the bulb was shaken for 30 minutes, the organic layer was separated, and adjusted to a known volume. Recovery of the carbonyls using this system was of the order of 80% for acrolein, 90% for acetaldehyde, and 95% for acetone. The reproducibility was excellent. Further studies were performed in order to maximize recovery, and the best conditions were those listed.

The application of this methodology to the determination of carbonyls in cigarette smoke is shown in Figure 3. The results obtained

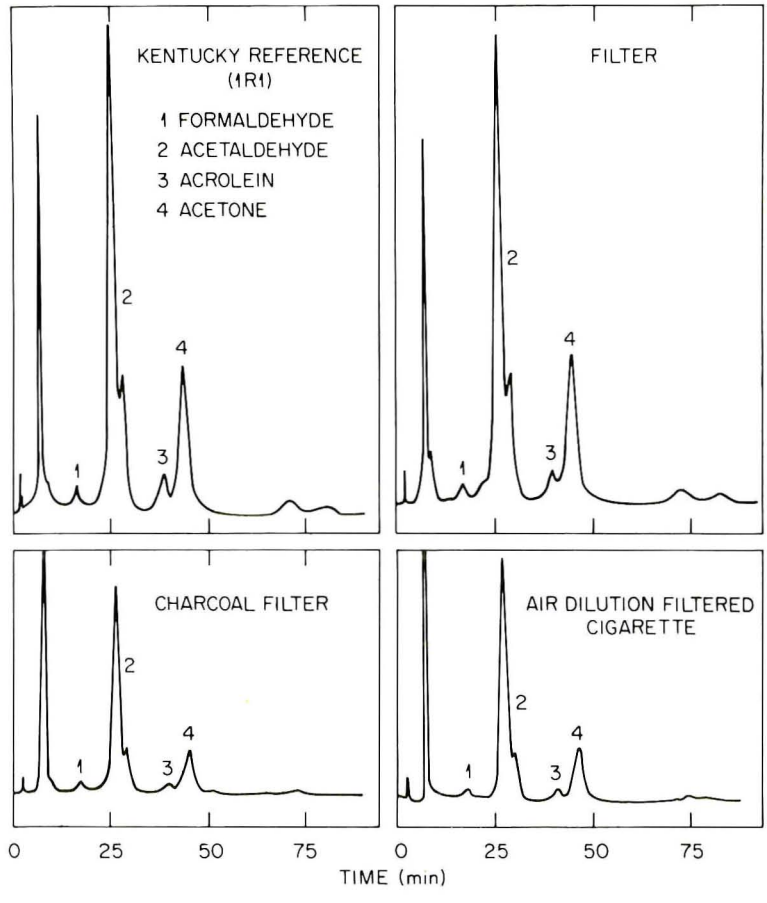

FIGURE 3. Carbonyl Profiles for Selected Cigarettes. Column: Partisil 10 ODS-2. Mobile Phase: Methanol:Water (60:40). Flow Rate: 1 ml/min.

were in good agreement with established values, indicating the quantitative reliability of the technique. Levels were highest in non-filter cigarettes and lowest in cigarettes using charcoal filters. Significant improvements over direct GC determination were made in sample throughput and sensitivity. Recent work has indicated that even the lowest commercial cigarettes (>.1 mg tar) contain detectable amounts of the carbonyls when measured in this way.

The extension of this methodology to other combustion mixtures is shown in Figure 4. Diesel fuel was aerosolized in a hot manifold and the resulting gases trapped. When no air was present (Figure 4a), the levels of the carbonyls were much lower than those found when air was present (4b), indicating the nature of formation of these compounds. While the presence of air increases the formation of all vapor phase carbonyls, acrolein and formaldehyde are markedly increased. Sampling of ambient air in a similar fashion resulted in a wide range of

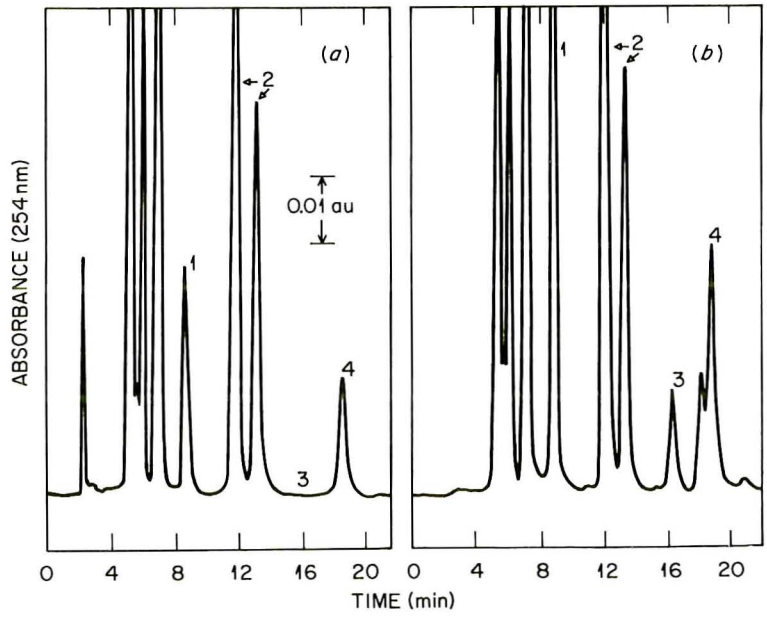

FIGURE 4. Carbonyl Profile of Diesel Fuel Aerosol. Curve numbers per Figure 3. Oxygen: (a) Absent, (b) Present. Column: Dupont Zorbax ODS. Mobile Phase: Methanol:Water (70:30). Flow Rate: 1 ml/min.

acrolein values, from 40 ppb to >50 ppm. Detection limits for acrolein using the current system are lower than the established OSHA exposure limits, permitting accurate assessment of occupational exposure.

In conclusion, quantitative trapping techniques combined with analysis by HPLC have been developed which provide enhanced sensitivity and increased sample throughput in the determination of the C_1-C_5 carbonyls. (Although not the purpose of this work, the techniques can also be extended to the analysis of higher molecular weight carbonyl compounds.) The determination provides sensitivity in excess of current OSHA standards, and should be of value to those concerned with compliance to workplace standards.

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OPTIMIZATION OF PEAK SEPARATION AND BROADENING IN AQUEOUS GEL PERMEATION CHROMATOGRAPHY (GPC). DEXTRANS

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ABSTRACT

Herein is reported an experimental optimization of the aqueous size-exclusion chromatography of dextrans on untreated CPG-10 glass packings. The molecular weight calibration curve was independent of ionic strength and there was no evidence of polymer adsorption on the glass packings. However, in the absence of salt in the mobile phase, chromatograms did show a high molecular weight shoulder which is attributed to small negative charges on the dextran molecules resulting in ion exclusion from the pores of the negatively charged glass. These high molecular weight shoulders were completely eliminated with the addition of a small amount of salt (e.g. 0.05 M $NaSO_4$). A proper choice of pore sizes was essential to obtain good separation with minimal peak broadening giving a linear molecular weight calibration curve with a wide separation range and a small correction for imprefect resolution. Corrections to M_N and M_W were generally less than 5%. To establish an optimal column combination, it is recommended that single columns containing packing with one pore size be employed to establish the performance of a particular sized pore before a column combination is chosen.

INTRODUCTION

Many investigations of the aqueous size-exclusion chromatography of dextrans have been published recently (1-11). One of

the earliest studies was that of Bombaugh et al. (11), who used water at 65°C and 1 ml/min as mobile phase and deactivated Porasil as packing. Qualitatively the chromatograms indicated excellent peak separation for the molecular weight range of 11,000-150,000. The chromatograms for the higher molecular weight standards had shoulders near the void volume. This might have been due to size exclusion or possibly ion exclusion. Unfortunately salt was not added to the mobile phase to suppress ion exclusion. A careful study of adsorption showed that it was negligible. Cooper and Matzinger (4) found using CPG packing (a single 4 ft x 3/8 in column containing 75 A, 240 A and 2000 A pore diameters) and mobile phases containing 0.01 M, 0.1 M and 1.0 M phosphate at pH = 7.0 that the molecular weight calibration curve was independent of ionic strength. They showed that CPG packing materials can exhibit ion exclusion for polyelectrolytes in low ionic strength media. Spatorico and Beyer (5) chromatographed dextrans on CPG-10 packings (5 columns: 1250 A, 670 A, 500 A, 190 A and 75 A, each column 5 ft x 0.17 in) using 0.2 M and 0.8 M Na_2SO_4 as mobile phase. They observed that the molecular weight calibration curve was independent of salt concentration and flowrate. Peak separations were good and the corrections to $\bar{M}_N$ and $\bar{M}_W$ for imperfect resolution were apparently quite small although data were not presented. Buytenhuys and Vander Maeden (9) chromatographed dextrans on Lichrospher packings (untreated silica micropacking with particle diameter of about 10 microns and 100 A, 300 A, and 500 A pores) using water and also 0.5 M sodium acetate (pH = 5) as mobile phases. The use of the salt eliminated the high molecular weight shoulder caused by ion exclusion. These authors suggest that dextrans may have a few negative charges. A similar explanation would apply for the CPG packings when water is used as mobile phase. Soeteman, Roels, Van Dijk and Smit chromatographed dextrans on treated Porasil with water as the mobile phase. They apparently did not observe any high molecular weight shoulders attributable to ion exclusion. It may be that the negative charge on the treated Porasil is negligible. Their

intrinsic viscosity measurements suggest that below a molecular weight of 50,000 the dextrans are essentially linear. At higher molecular weights the levelling off of intrinsic viscosity suggests that the molecules are highly branched. Treating the dextrans as linear up to a molecular weight of $\bar{M}_W$ = 532,000 has permitted accurate determinations of $\bar{M}_N$ and $\bar{M}_W$ by GPC by these workers. Perhaps the use of branched dextran standards to establish the molecular weight calibration curve has made the calculational procedure reasonable. It should be noted however that large errors in $\bar{M}_N$ and $\bar{M}_W$ would likely result if the calibration curve were used for an unknown dextran sample whose branching frequency were appreciably different.

The objective of the present investigation was to define a mobile phase and a combination of columns of CPG-10 packing for the efficient size separation of dextrans with corrections for imperfect resolution to $\bar{M}_N$ and $\bar{M}_W$ of less than about 5%.

EXPERIMENTAL

The polymers used in this study were a series of dextrans supplied by Pharmacia Fine Chemicals (Piscataway, N.J.). the molecular weight data supplied by Pharmacia are shown in Table 1.

The dextran standards are known to be highly branched, broadly distributed polymers. These standards are useful in an optimization study of peak separation and broadening. However, they should be used with caution for calibration purposes when dextran polymers with unknown branching characteristics are to be analyzed by aqueous GPC.

The liquid chromatograph employed in this study was a Waters Associates Model ALC/GPC 300 with a differential refractometer operated at room temperature. A 2 ml sample loop with polymer concentrations of 0.05-0.1 wt% and a 5 ml siphon were employed with mobile phase flowrates in the range, 1-8 ml/min. The columns were dry-packed with CPG-10 packing. Details of packing, column combination, mobile phase type and flowrate accompany the Figures showing the results of the investigation.

TABLE 1

Molecular Weights of Dextrans

Designation	$\bar{M}_N$	$\bar{M}_W$	$\bar{M}_W/\bar{M}_N$
T 2000	-	-	-
T 500	173.0	509	2.94
T 250	112.5	231	2.05
T 150	86.0	154	1.79
T 110	76.0	106	1.39
T 70	42.5	70	1.65
T 40	28.9	44.4	1.54
T 20	15.0	22.3	1.49
T 10	5.7	9.3	1.63

RESULTS AND DISCUSSION

The first mobile phase to be investigated was distilled water with no additives. Typical chromatograms obtained for the dextran standards in water are shown in Figure 1. A chromatogram for a high molecular weight nonionic polyacrylamide is also included to show the void volume. Most of the dextran chromatograms have a high molecular weight shoulder or are clearly bimodal. The shoulder or second peak is clearly not the result of solute exclusion from the largest pores on the basis of size as the retention volumes are considerably larger than the void volume. Also molecular aggregation is unlikely for branched dextrans. This phenomenon is observed with linear poly (vinyl chloride) synthesized at lower temperatures where syndiotactic sequences are of sufficient length to permit the formation of crystallites (12). These dissolve very slowly and are responsible for high molecular weight shoulders and bimodal chromatograms for PVC. The most likely explanation for the bimodal chromatograms for dextrans is ion exclusion. It is hypothesized that the dextran molecules have

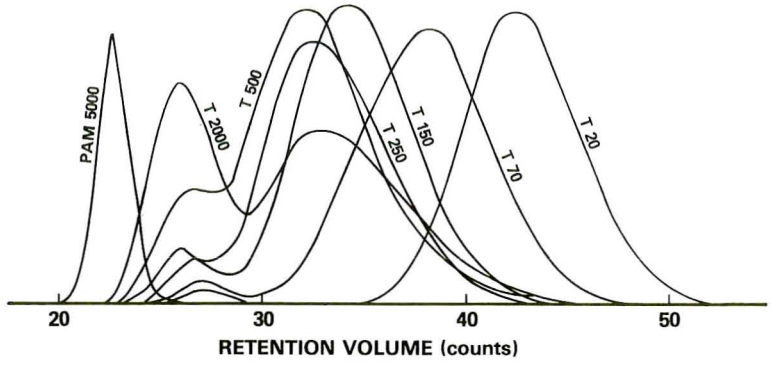
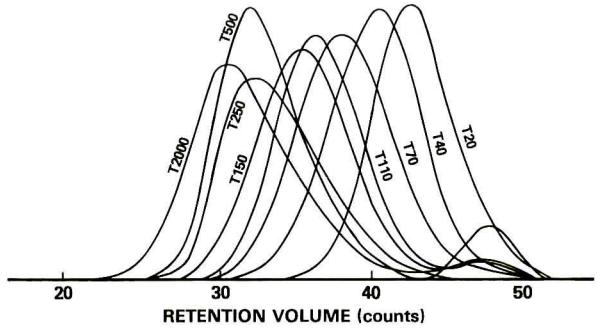


FIGURE 1: Chromatograms of dextrans on CPG 10 packing (one column, 4' x 3/8", of each of pore sizes: 125 A, 240 A, 370 A, 2000 A) in distilled water showing partial ion exclusion. PAM 5000 (a high MW polyacrylamide) shows void volume of column system.

a small negative charge and that the larger of these polymer molecules have substantially reduced available pore volume due to charge repulsion near the glass surface. Buytenhuys and Vander Maeden (9) have made a similar suggestion. The addition of salt to the mobile phase eliminates the bimodalities and gives unimodal peaks as shown in Figure 2a. Also shown is a salt peak due to ion inclusion. The electrolyte added to the mobile phase presumably screens the charge on the polymer molecules and compresses the electrical double layer associated with the glass surface. The available pore volume for the larger dextran molecules is thus increased. The molecular weight calibration curves obtained for water and two salt solutions as mobile phase are shown in Figure 2b. The column combination employed was the same for the data presented in Figures 1, 2a and 2b. It was observed that the peak retention volumes are independent of electrolyte concentration. Moreover, the addition of other additives including salts, acid, and, nonionic surfactants (e.g. Tergitol, a low MW polyether) also had no effect on elution volumes. This suggests that adsorption of dextran on the CPG surface is negligible. It was concluded

(a)



(b)

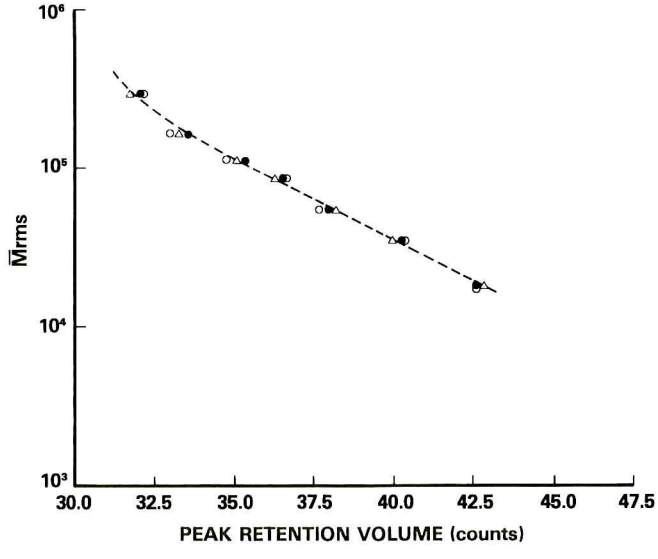


FIGURE 2: (a) Chromatograms of dextrans on CPG 10 packing (same columns as Figure 1) with addition of 0.05 M Na_2SO_4. A salt peak is now apparent. (b) MW calibration curves for dextrans in water (O), 0.1 M KBr (●) and 0.05 M Na_2SO_4 (Δ), showing lack of dependence on electrolyte type and concentration.

that an aqueous solution 0.05 M in Na_2SO_4 is as effective as any other mobile phase, and should be used for further development of a GPC system for these polymers.

The next step in system development was to optimize pore size selection to produce an effective column combination. The procedure used was to calibrate single columns containing one pore size or a relatively narrow pore size distribution. The results of these calibrations are shown in Figure 3. It is clear that pores of 1000 A or greater are too large to give appreciable peak

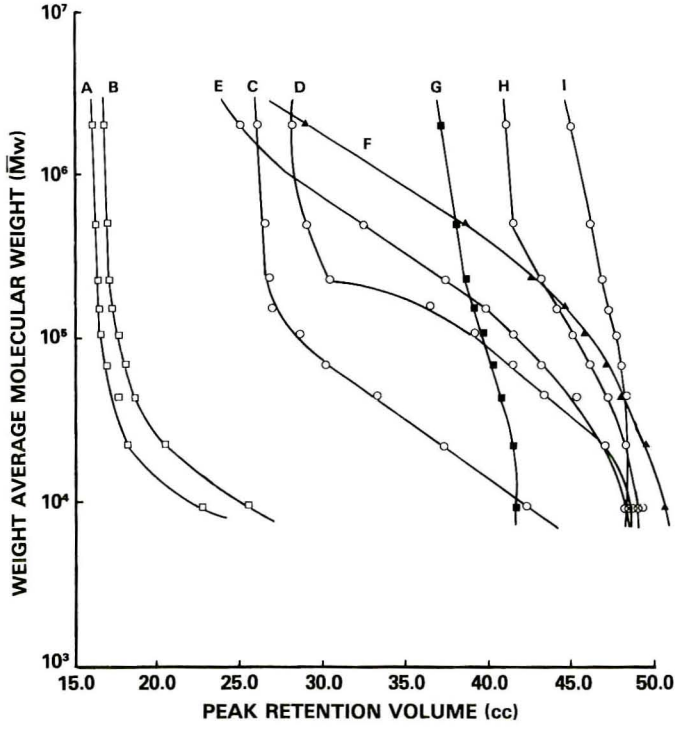


FIGURE 3: MW calibration curves for dextrans on CPG 10 using single columns (each 4' x 3/8") with 0.05 M Na_2SO_4 as mobile phase. A - 88 A, B - 120/88 A, C - 240/120 A, D - 370/327 A, E - 700/500/370 A, F - 727/700 A, G - 1000 A, H - 2000 A, I - 3000 A.

separation for the relatively small dextran molecules. It may be seen that the slopes of the molecular weight calibration curves for the 727/700 A, 700/500/370 A, 370/327 A, 240/120 A and 88 A columns are approximately the same in their linear regions and that there are no molecular weight gaps. This suggests that a column combination with one column of each of these pore sizes should give a linear molecular weight calibration curve having the same slope and a very wide molecular weight separation range. This is borne out by the molecular weight calibration curves shown in Figure 4. The true molecular weight calibration curve was

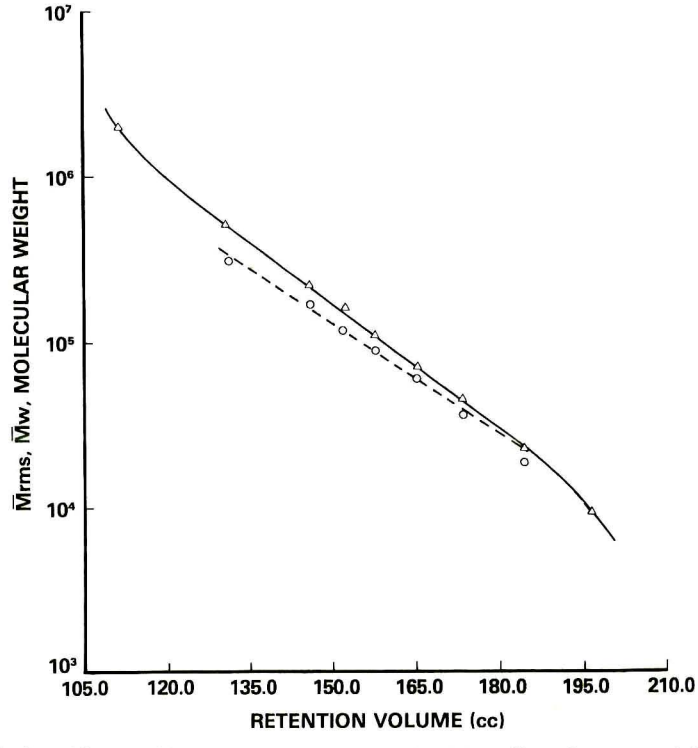


FIGURE 4: MW calibration curve for "optimum" column combination (88 A, 240/120 A, 370/327 A, 700/500/370 A, and 727/700 A) and mobile phase (0.05 M Na_2SO_4). O = M_{rms}; Δ = M_w; ---- theoretical calibration curve using two broad standard method.

obtained using the two broad standards method (13). It is of interest to note that for dextrans the use of the root mean square molecular weight ($M_{rms} = \sqrt{M_N \cdot M_W}$) gives a molecular weight calibration curve which is in good agreement with the true calibration curve. This would not be true in general for any molecular weight distribution but apparently works reasonably well for the dextran standards. The use of $\bar{M}_W$ obviously gives the incorrect calibration curve. The true molecular weight calibration curve was then used to calculate $\bar{M}_N$ and $\bar{M}_W$ using the chromatograms of the standards. These calculated molecular weight averages along with molecular weight averages provided by the supplier are shown in Table 2. The agreement is excellent for the intermediate molecular weight standards with poorer agreement at the high and low molecular weight ends where the calibration curve is non-linear.

TABLE 2

MW Data for Dextrans: GPC Values Compared to Values of Manufacturer

	Values Supplied by Manufacturer (Pharmacia)			GPC Values Uncorrected for Imperfect Resolution		
Sample	$\bar{M}_N \times 10^{-3}$	$\bar{M}_W \times 10^{-3}$	$\bar{M}_W/\bar{M}_N$	$\bar{M}_N \times 10^{-3}$	$\bar{M}_W \times 10^{-3}$	$\bar{M}_W/\bar{M}_N$
T 10	5.70	9.3	1.63	8.73	12.27	1.41
T 20	15.00	22.3	1.49	15.56	22.66	1.46
T 40	28.90	44.4	1.54	27.22	44.69	1.64
T 70	42.50	70.0	1.65	43.02	70.85	1.69
T 110	76.00	106.0	1.39	70.95	104.65	1.48
T 150	86.00	154.0	1.79	89.38	151.99	1.70
T 250	112.50	231.0	2.05	124.89	230.82	1.85
T 500	173.00	509.0	2.94	206.78	421.27	2.04

In summary it has been shown that addition of small amounts of electrolyte (e.g. 0.05 M $Na_2 SO_4$) to water eliminates high molecular weight shoulders from the chromatograms of dextrans on CPG-10 glass. Using this mobile phase a column combination having pore sizes in the range 88-727 A gives excellent resolution in the molecular weight range $22,000 < \bar{M}_w < 230,000$.

ACKNOWLEDGMENT

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THE PURIFICATION OF XANTHENE DYES BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A reliable method for the separation of fluorescein dyes from their impurities was developed using high performance liquid chromatography and involved a μBondapak C_{18} reverse phase column and mixtures of methanol and ammonium acetate buffer. This technique was used to verify the purity of commercial products as well as to aid in the development of an empirical theory related to retention of halogenated fluorescein dyes by reverse phase columns.

INTRODUCTION

Commercial preparations of halogenated fluorescein dyes exhibit varying degrees of purity. From this fact was borne the need for a rapid reliable technique to separate the dyes from impurities and to monitor the quality of the commercial dyes (1, 2). Open column and thin layer chromatography do not possess the separative capacity for this task (3). Reverse phase high performance liquid chromatography (HPLC) does provide the necessary capability.

MATERIALS AND METHODS

The dyes used in this study were a series of chemical compounds based on the structure of fluorescein (Fig. 1), and contained different degrees of halogenation of the ring systems. The Hilton-Davis Chemical Company provided fluorescein; 3', 4', 5', 6'-tetrachlorofluoran; 2, 4, 5, 7-tetrabromofluorescein (eosin yellowish); 2, 4, 5, 7-tetraiodofluorescein (erythrosin B); 2, 4, 5, 7-tetrabromo-3', 4', 5', 6'-tetrachlorofluorescein (phloxin B); 2, 4, 5, 7-tetraiodo-3', 4', 5', 6'-tetrachlorofluorescein (rose bengal); and 2, 4, 5, 7, 3', 4', 5', 6'-octabromofluorescein. 4, 5-Diiodofluorescein and 4, 5-dibromofluorescein were ob-

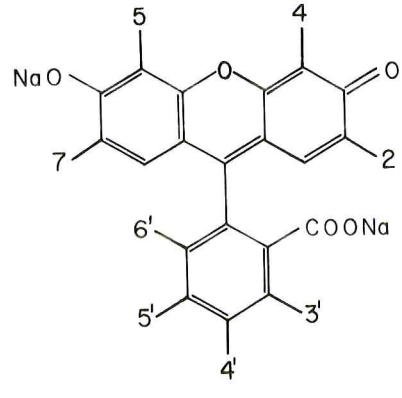

Figure 1

The basic structure for fluorescein and all dyes that are related by structure. Each available position is numbered.

tained from Eastman Chemical Company and 2, 7-dichlorofluorescein was obtained from Gallard-Schlessinger.

Tetrachlorofluoran was converted to tetrachlorofluorescein by addition to 0.1N NaOH before dilution with distilled water. Each dye was dissolved in distilled water, passed through a sample clarification kit equipped with a 0.45 μ filter, and introduced onto a Waters μBondapak C_{18} reverse phase column (3.9mm ID x 30cm) via a Waters U-6K injector. A mobile phase consisting of variable amounts of methanol (Burdick and Jackson) and ammonium acetate buffer (0.02M, pH = 3.5) was used at a flow of 2.0 ml/min.

Other equipment for the study included 2 Waters M6000A pumps, a Waters Model 660 Solvent Programmer, a Waters uv-visible Model 440 fixed wavelength detector, a Perkin-Elmer uv-visible Model 55 variable wavelength detector, and a Houston Ommniscribe recorder.

RESULTS AND DISCUSSION

The series of xanthene dyes was monitored for purity using HPLC. Excellent separations were obtained for each dye by varying the methanol concentration in the methanol-ammonium acetate elution solution. The range of methanol concentrations varied from a minimum of 46% methanol used for fluorescein to a maximum of 68% used for 3',4',5',6'-tetrachlorofluorescein.

Fig. 2 shows the HPLC traces of 4,5-diiodofluorescein (retention time = 13.5 min) and its impurities monitored at 4 wavelengths: 536nm, 520nm, 515nm, and 488nm. The absorbance maximum (λ_{max}) for 4,5-diiodofluorescein is 536 nm (Fig. 3). If each impurity is assumed to have the same λ_{max} and extinction coefficient (ε), the sample is shown to be no more than 80% pure. This is an invalid assumption as evidenced by the variation in peak heights for the impurities observed at the other wavelengths. Therefore, the sample may be much less pure than 80%, but in the absence of isolation and characterization of the λ_{max} and ε for

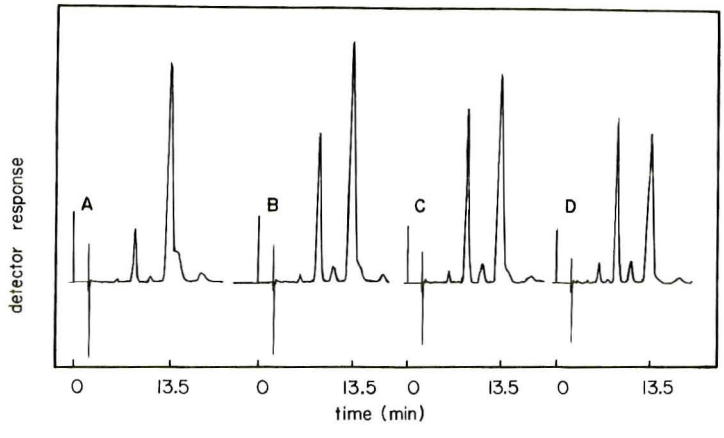

Figure 2

Commercially available diiodofluorescein chromatographed on reverse phase and detected at 4 specific wavelengths: curve A, 536nm; curve B, 520nm; curve C, 515nm; curve D, 488nm.

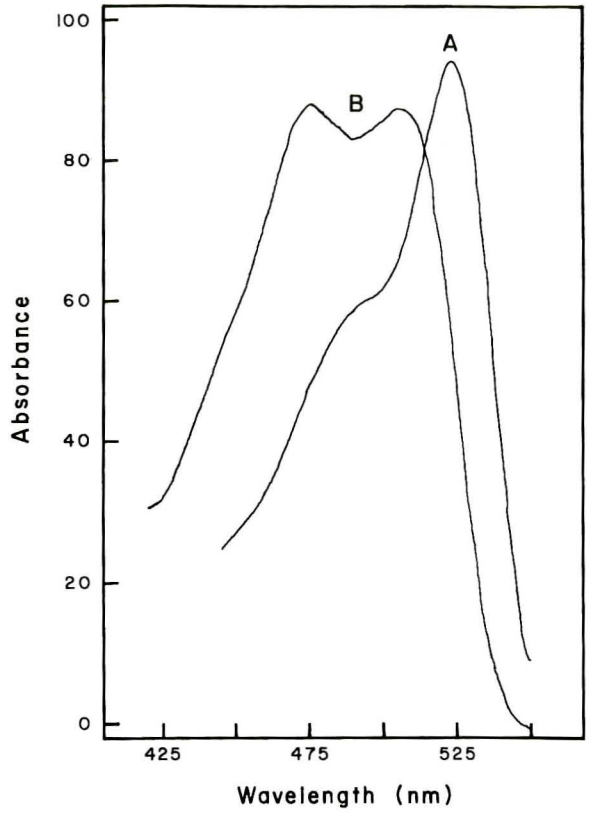

Figure 3

Absorbance maxima for 4, 5-diiodofluorescein (A) and one impurity (B) isolated from commercial diiodofluorescein.

each impurity, no more precise evaluation is possible. Corresponding data for the other xanthene dyes was also obtained.

Table 1 lists the dyes, the k' values, λ_{max}, the optimum solvent system, and maximum purity of each

Table 1

Dye	Solvent[1]	k'	λ_{max} (nm)[2]	Maximum % Purity
Fluorescein	46/54	7.14	478	99
4,5-Diiodofluorescein	60/40	7.01	536	80
4,5-Dibromofluorescein	52/48	21.44	517	80
2,7-Dichlorofluorescein	56/44	9.42	490	95
Erythrosin B	62/38	4.77	536	95
Phloxin B	64/36	5.41	545	95
Rose Bengal	66/34	5.67	555	85
3',4',5',6'-Tetrachloro-fluorescein	68/32	5.41	495	80
Octabromofluorescein	63/37	7.81	545	95
Eosin Yellowish	58/42	5.79	525	90

[1]Ratio of Methanol to Buffer in the solvent system.

[2]The λ_{max} was determined spectrophotometrically using 50% Methanol and 50% Buffer as the solvent.

dye based on the assumption of equal absorbance for each component at that wavelength.

With this knowledge, the necessity for further purification was obvious. Using optimum separation conditions, the presence of each dye, its purity, and some insight concerning the necessary scale-up for preparative HPLC, may be obtained.

A comparison of the retention times of the selected xanthene dyes within a single solvent system is shown in Table 2. Comparison of retention time to the type of halogen substituents, the degree of halogenation, and the location of the halogen substituents allowed an empirical theory to be developed to explain the retention observed.

Table 2

Dye	Functional Groups Lower Ring	Upper Ring	k'[1]
Fluorescein	-	-	1.31
Eosin Yellowish	-	4 Br	3.23
2,7-Dichlorofluorescein	-	2 Cl	4.26
4,5-Dibromofluorescein	-	2 Br	4.38
Erythrosin B	-	4 I	5.67
4,5-Diiodofluorescein	-	2 I	6.12
Phloxin B	4 Cl	4 Br	8.48
Octabromofluorescein	4 Br	4 Br	9.38
3',4',5',6'-Tetrachlorofluorescein	4 Cl	-	11.69
Rose Bengal	4 Cl	4 I	11.95

[1]The k' for each compound calculated in a solvent system consisting of 62% Methanol and 38% Buffer.

A comparison of the retention times of all of the other xanthenes to fluorescein (no halogens) indicated that the presence of any halogen on the molecule caused retention of the compound to be extended. Lower ring halogenation apparently had a greater effect on retention than halogenation of the upper rings. The 4 xanthenes containing halogen on the lower ring exhibited longer retention times. Further, rose bengal, containing 4 chlorine atoms on the lower ring, exhibited a retention time twice that of erythrosin B, which was identical save the lack of the chlorine atoms. Similarly, phloxin B had a retention time twice that of eosin yellowish.

Closer inspection indicated that a relationship based on specific halogens to retention times may be

made in the dyes exhibiting upper ring halogenation only. Considering the 3 dihalogenated xanthenes: diiodofluorescein, dichlorofluorescein, and dibromofluorescein, the elution order followed the order of increasing size. This relationship held for the two xanthenes with tetrahalogenated upper rings, eosin yellowish (4Br) and erythrosin B (4I), that were studied.

Detectable impurities have been found to elute both before and after the target peak of a dihalogenated xanthene. Theoretically, the elution order of xanthenes would be dependant upon the degree of halogenation. Thus, xanthenes exhibiting fewer than two halogens would elute prior to the dihalogenated xanthene and those exhibiting more than two halogens would elute after the dihalogenated xanthene. Therefore, the xanthene impurities in the diiodofluorescein shown in Fig. 2 would be fluorescein, iodofluorescein, triiodofluorescein, and/or tetraiodofluorescein. Other impurities would not be xanthenes.

CONCLUSION

A method for separation, purification, and quality control of xanthene dyes was developed. This HPLC technique proved to be a simple, accurate, and rapid method of monitoring the xanthenes that were routinely used in the experiments. A theory relating halogenation

of the xanthene ring to retention time in reverse phase HPLC was also developed.

ACKNOWLEDGEMENTS

The authors thank Dr. R. B. Koch, Dr. M. L. Salin, and Dr. R. P. Wilson for critically reveiwing this manuscript. The work was supported in total by funds made available by the Mississippi Agricultural and Forestry Experiment Station, MAFES Publication #4478.

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THE USE OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE STUDIES OF PIGMENT COMPONENTS FROM SERRATIA MARCESCENS 08 BEFORE AND AFTER HYDROGEN PEROXIDE OXIDATION

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ABSTRACT

A method to separate the pigment components of Serratia marcescens 08 by high performance liquid chromatography (HPLC) is described. By maintaining a small but constant amount of concentrated HCl in the mobile phase of 25% ethylene dichloride in methanol on a reverse-phase column (Lichrosorb RP-18), the pigment components were resolved and separated in about 5 min. This method allowed preparative isolation of the individual components for infrared spectroscopic characterization. The interrelationship of the components studied by hydrogen peroxide oxidation of the pigment extract and the HPLC profiles and infrared spectra of the oxidized products as well as those of the individual isolated fractions were investigated. It is suggested that components separated by this method and detected at 272 nm might be related to the parent pigment of prodigiosin.

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INTRODUCTION

Prodigiosin (2-methyl-3n-amyl-6-methoxy-prodigiosene) is the characteristic tripyrrole red pigment commonly produced by Serratia marcescens. It has been suggested that pigments extracted by organic solvents represent a mixture of prodigiosin related components which could be separated by thin-layer or column chromatography into several fractions with antibiotic properties[1]. Even in pigmentless strains, monopyrrole and dipyrrole precursors have been detected and isolated[2,3]. More recently, the availability of more sophisticated instrumentation such as high performance liquid chromatography (HPLC) allows clear demonstration of the considerable heterogeneity in the pigment extracts[4]. In spite of the limited success of the isolation of the mutants which produce these prodigiosin precursors, and the isolation of the precursors themselves, the biosynthetic pathways of prodigiosin remain unclear.

In this communication, we describe a method for the rapid separation of the major components of the pigment extract by reverse-phase HPLC. Successful preparative isolation of the components, along with hydrogen peroxide oxidation of the pigment extract provided the opportunity for us to study the interrelationship of the components. Our results seemed to indicate that for better separation of the components, it was necessary to control the amount of hydrogen chloride in the elution solvent. This factor is important because prodigiosin might occur as the salt of fatty acids[5] in close association with the lipid portion of cell membranes[6]. Our recent observed inhibitory of a cationic antibiotic, polymyxin B, on the pigment formation further supports this new finding[7].

MATERIALS AND METHODS

Serratia marcescens 08 was used in the study. The cells were grown in an enriched medium containing casamino acids, glycerol, sodium chloride and nutrient broth as described previously[8]. They were aerated at room temperature and harvested at the late log phase. The cells were centrifuged at 4 C, and the pigments were extracted by acetone, followed by partition with petroleum

ehter according to the method of Williams, et al.[9] The pigments in the petroleum extract were recovered by rotary evaporation in a stream of nitrogen.

A Spectra-Physics high performance liquid chromatograph (Spectra-Physics, Santa Clara, Calif. U.S.A.) equipped with a 740B pump and a Valco Model VU-6UH Pa-N60 injection valve was used for these separations. A variable wavelength detector (Perkin Elmer LC-55 Spectrophotometer with a Perkin Elmer Model 56 recorder attachment) was used at either 272 nm or 537 nm[10]. A 10 - µl sample loop was used for analytical separations and 50 - µl sample loop was used for preparative collection of the sample fraction components. The columns were a 25 cm x 4.6 mm i.d. stainless steel column packed with Lichrosorb RP-18 of 10 µm particle diameter and a 7 cm x 2.2 mm i.d. stainless precolumn packed with Polygosil 60-D 5 C-18 (Macherey-Nagel). The solvent used was 25% ethylene dichloride (Aldrich, high purity) in methanol (Fisher Certified) at a flow rate of 2 ml/min. To maintain a rapid and reproducible retention time, 10 µl of concentrated HCl were added to 1 liter of mobile phase (10 ppm of concentrated HCl). The HCl had a pronounced effect on the retention time of the major peak of the pigment components (prodigiosin parent peak) (see Results and Discussion). Increasing concentrations of HCl in the mobile phase decreased the retention time of this component (Figure 1). The 10 ppm of concentrated HCl gave the desired retention time of about 5 min for this component.

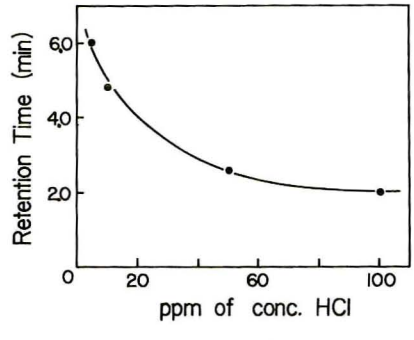

Figure 1. Effect of concentrated HCl on the retention time of prodigiosin component detected at 537 nm.

Oxidation of pigment extract by 30% hydrogen peroxide (Baker) was carried out according to the procedure of Payne, et al.[11] The pigment extract (400 mg) was dissolved in 10 ml of methanol, and enough 0.1 M NaOH was added to give a basic test with litmus paper. The 5 ml of 30% hydrogen peroxide was added and the mixture was stirred at 45 C. At intervals of 1.5 hr, 3.0 hr and 7 days, aliquots of the reaction mixture were taken and the methanol evaporated under nitrogen. Each aliquot was extracted several times with equal volumes of 1M HCl and chloroform; after separation, the chloroform phase was evaporated to dryness for HPLC, Infrared (Perkin Elmer Infrared Spectrophotometer) UV-Visible (Beckman Spectrophotometer Model ACTA VI) spectroscopic analysis.

RESULTS AND DISCUSSION

In an earlier attempt of applying HPLC to separate the pigment components in the chloroform-methanol extracts of S. marcencens, several not well resolved peaks were obtained[4]. There was no mention if the components were fully protonated (red acid form which absorbs at 535-540 nm) or nonprotonated (orange alkaline form which absorbs at 470 nm). Only a single wavelength (546 nm) was used to monitor the separation; therefore, nonpigmented UV-absorbing components escaped detection. In our study, we investigated first the relationship of the retention time of the major components at two wavelengths with ppm of HCl added in the eluting mobile phase. Figure 1 shows the decreasing of retention time with increasing concentration (in ppm) of HCl in the eluting solvent. By maintaining the amount of concentrated HCl at 10 ppm, we were able to ensure the well protonated form to be eluted in about 5 min. Furthermore, by monitoring the detector wavelengths at both 272 nm and 537 nm, it was possible with this system to detect prodigiosin (537 nm) and its metabolites and/or precursors (272 nm), Figure 2 shows the chromatograms of the pigment extract. When detected at 272 nm (Figure 2a), three peaks with retention times 1.6, 3.4 and 5.2 min were obtained. However, at 537 nm (Figure 2e), only one symmetrical peak was obtained. When the three peaks were collected

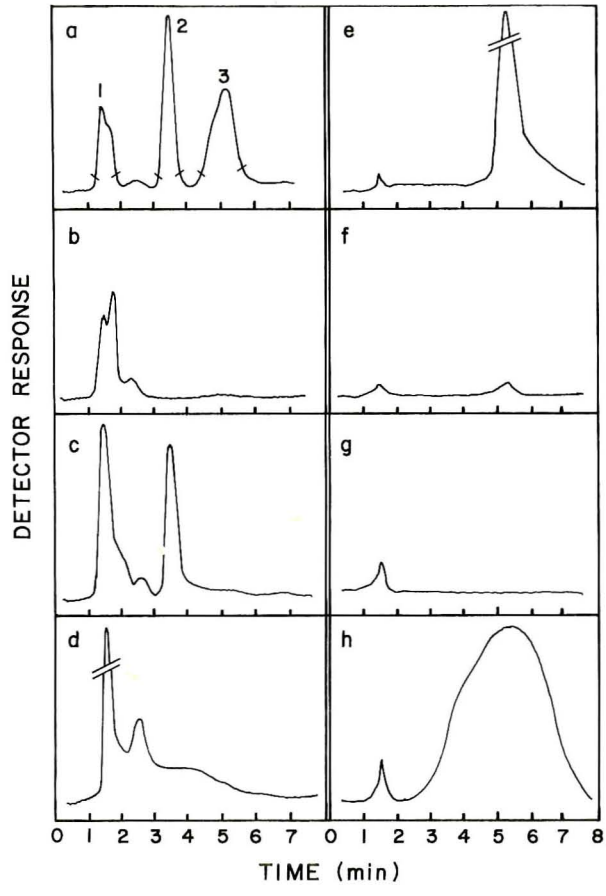

Figure 2. HPLC profiles of pigment extract from Serratia marcescens and its individual isolated components. (a) - (d) detected at 272 nm. (a) original pigment extract; (b) fraction 1; (c) fraction 2; (d) fraction 3; (e) - (h) detected at 537 nm. (e) original pigment extract; (f) fraction 1; (g) fraction 2; fraction 3.

by preparative technique and rechromatographed, fraction 1 (peak 1) showed that it contained only peak 1 (Figure 2b). The chromatograph of fraction 2 showed that it contained peaks 1 and 2 (Figure 2c), while that of fraction 3 showed that it contained peaks 1 and 3 (Figure 2d). When the IR spectra of the original extract and the three collected fractions were compared, it could be noted that they resembled closely those reported by Lynch, et al. as separated by column chromatography[12] and those by Button, et al. as separated by thin-layer chromatography[13]. All fractions except fraction 1 showed clearly defined peaks at 660, 750 and 1,210 cm^{-1}. A group of three peaks occurring at 2,875, 2,950 and 3,040 cm^{-1} was characteristic of the original extract and fraction 1, but to less extent of fractions 2 and 3 which had more pronounced hydrocarbon absorption bands (3,040 cm^{-1}). The absorption of 660 and 750 cm^{-1} probably showed C-H out of plane bending (n-amyl group of prodigiosin) and those at 2,875, 2,950 and 3,040 cm^{-1} indicated the presence of methylene and methyl groups. In all fractions, absorption in the ranges of 1,000 to 1,500 cm^{-1} and 3,000 to 3,100 cm^{-1}, characteristic of the pyrrole ring, was present. Overall, IR spectra of fractions 2 and 3 were almost identical. All collected fractions showed much structural similarity to the original extract with the exception that n-amyl group might be missing in fraction 1. Since fraction 1 was consistently present in the rechromatographed fractions 2 and 3, it is likely that it represents as the precursor or degradation product of fractions 2 and 3.

It has been suggested that prodigiosin could act as an auto-oxidizable electron acceptor[15]. In order to investigate this possibility and to correlate the chemical relationship of the individual isolated fractions, the pigment extract was submitted to hydrogen peroxide oxidation for various periods of time. The reaction products were characterized by HPLC and by IR spectroscopy. Figure 3 shows the chromatograms of the oxidation products. It can be seen that after 1.5 hr, peak 2 disappeared (Figure 3b) while after 3.0 hr, peak 3 disappeared (Figure 3g). As oxidation proceeded, there was an increase of

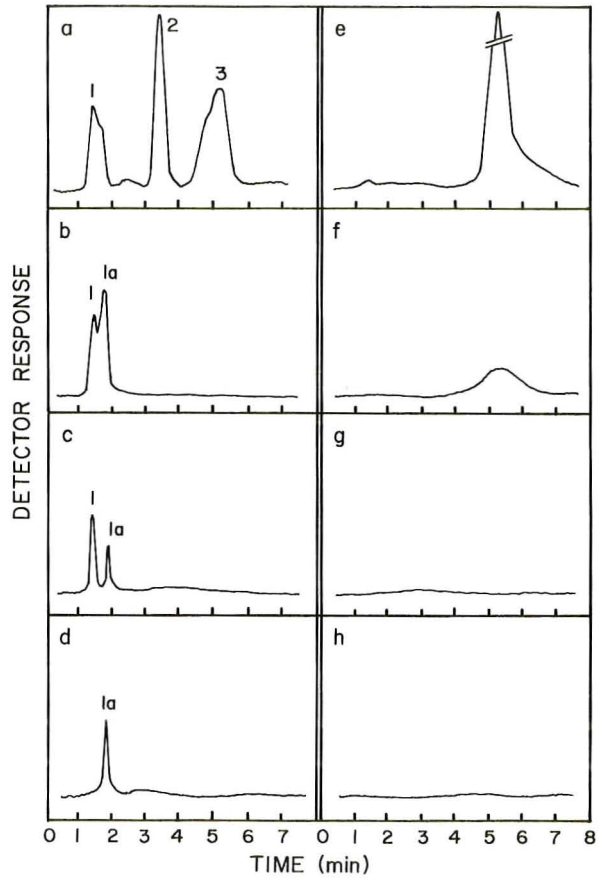

Figure 3. HPLC profiles of hydrogen peroxide oxidized products. (a) - (d) detected at 272 nm. (a) original pigment extract; (b) fraction after 1.5 hr oxidation; (c) fraction after 3.0 hr oxidation; (d) fraction after 7 days oxidation. (e) - (h) detected at 537 nm. (e) fraction after 1.5 hr oxidation; (f) fraction after 3.0 hr oxidation; (g) fraction after 7 days oxidation.

size of peak 1a while peak 1 (retention time 1.5 min) gradually decreased and, finally, completely disappeared at the end of the oxidation period (7 days) (Figure 3d). When the IR spectra of the final oxidation product and the isolated fraction 1 (without oxidation) were compared, they were quite similar, if not identical, to each other.

By maintaining a small but constant amount of concentrated HCl in the mobile phase, we have been able to develop a rapid method to separate the pigment components of S. marcescens 08 by HPLC. Detection at 537 nm or 272 nm enabled us to monitor the eluent for the protonated red form of prodigiosin or the nonpigmented but UV absorbing components, respectively. From the HPLC profiles and IR spectra of the collected fractions as well as those of the hydrogen peroxide oxidized products at various time intervals, we tend to conclude that the components separated by the HPLC method described in this study may be chemically and/or biosynthetically related to the parent pigment, prodigiosin. We intend to apply this method for the characterization of the pigment extracts of other strains of S. marcencens as well as those producing prodigiosin-like pigments.[15]

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AN INEXPENSIVE, ON-LINE DATA PROCESSING SYSTEM FOR GEL PERMEATION CHROMATOGRAPHY

By

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ABSTRACT

An inexpensive, on-line data processing system is described for the Waters 200 gel permeation chromatograph. The system consists of (a) interface chassis; (b) microprocessor with ∿3K memory and (c) a console device with cassette tapes for the storage of data and programs.

INTRODUCTION

Gel permeation chromatography is now a routine analytical procedure for the qualitative and quantitative analysis of polymeric materials. It provides an indirect measure for $\overline{M}w$, the weight average molecular weight, $\overline{M}n$, the number average molecular weight and MWD, the molecular weight distribution. All the three parameters are very important in understanding the physical behavior of polymers. The analyses generate raw data that must be laboriously manipulated and calculated to yield results required by the analyst. A number of commercial equipment are available with microprocessors to handle the data. The purpose of this report is to describe an inexpensive on-line data processing system for gel permeation chromatography which can be assembled with some basic knowledge of computer hardware and software. Greggs (1) and MacLean (2) have described data processing systems. The

latter uses PEP-2 hardware and software which are proprietary to the Perkin Elmer Corporation.

THE HARDWARE

The hardware consists of three subsystems: (a) the GPC interface, (b) Microcomputer, and (c) the Terminal-Storage unit.

The GPC Interface consists of four subsystems:

1. Buffer-Signal Conditioner or Level Converter.
2. Sample and Hold.
3. Analog to Digital Converter.
4. Clock and Control.

The level converter proved to be the most difficult problem. The analog signal obtained from the refractive index detector of the Waters GPC 200 consisted of a 0-100 millivolt differential DC signal which was superimposed on a 3-5V peak-to-peak AC signal at approximately 60 Hz. A differential input operational amplifier configuration was chosen to eliminate the common mode AC voltage, and to provide a high impedance input buffer. (Figure 1). The amplifier now in use provides an input independence of 10^{11} ohms. This amplifier provides sufficient isolation so as not to effect the signal to the recorder.

After the buffering stage the signal is fed into another operational amplifier providing a gain of 100, to bring the signal into the 0-10V DC range required by the A to D converter.

Due to an imbalance in the differential common mode signal, a high amount of 60 Hz noise was still passed to the buffer stage. This noise was eliminated by providing a 1 second constant for the output amplifier, thus averaging out the 60 Hz noise.

The analog to digital converter board is in two parts, the sample and hold (S & H) and the analog to digital converter (ADC). Connections

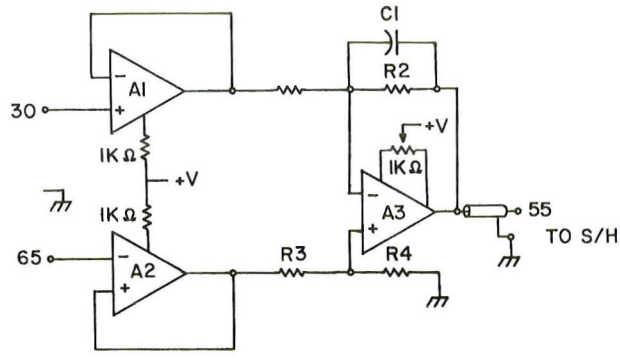

Figure 1. Differential input operational amplifier.

between the buffer amplifier and the sample and hold and the analog to digital converter should be made with a shielded cable.

The sample and hold module (Figure 2) on computer command, holds the value of the input signal constant long enough for the ADC to convert it into digital format. The ADC converts the analog signal into a binary coded decimal (BCD) digital signal on command from the computer (Figure 3).

The clock and control board contains a crystal controlled clock that operates at 3.5794 MHz at an accuracy of .005%. The clock is divided to several frequencies (60 Hz, 10 Hz, 1 Hz, .1 Hz). The .1 Hz frequency is used by the system for determining the sampling rate (Figure 4).

All of the above-mentioned boards can be consolidated into a single board. The component layout is not critical and interconnections are made directly without the use of optoisolators since all power supplies share a common ground reference.

The computer is a unit manufactured by TECHNICO and based on the Texas Instruments TMS 9900 Microprocessor. This is a 16 BIT Processor with a powerful mini computer instruction set, and in the present configuration can be upgraded to mini computer capabilities with "plug-in boards."

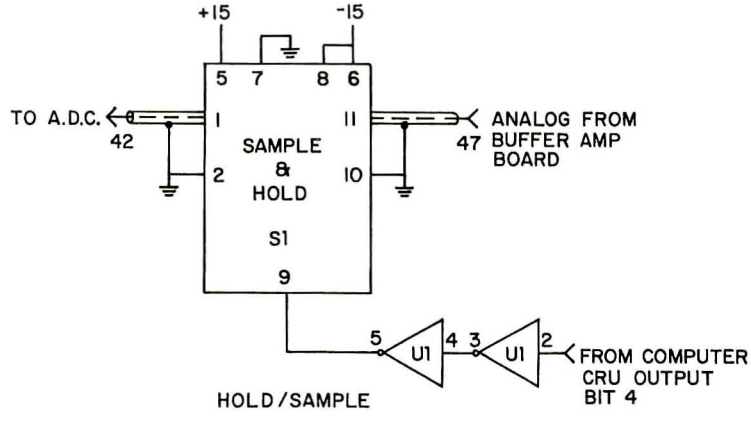

Figure 2. Sample and hold module.

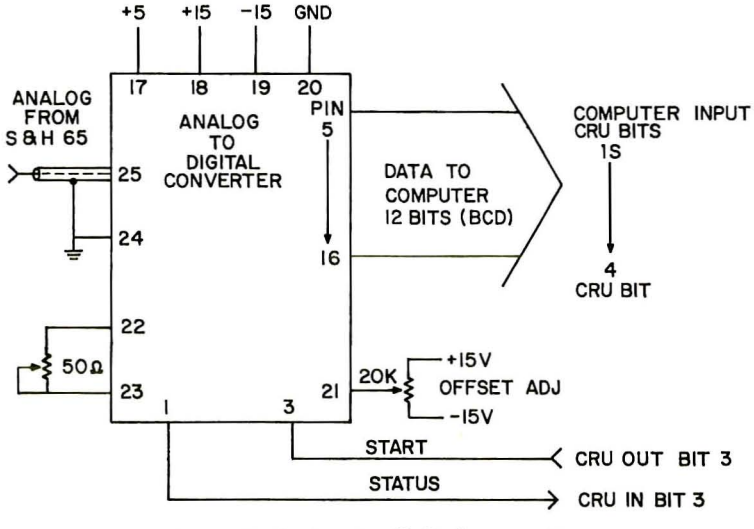

Figure 3. Analog-to-digital converter.

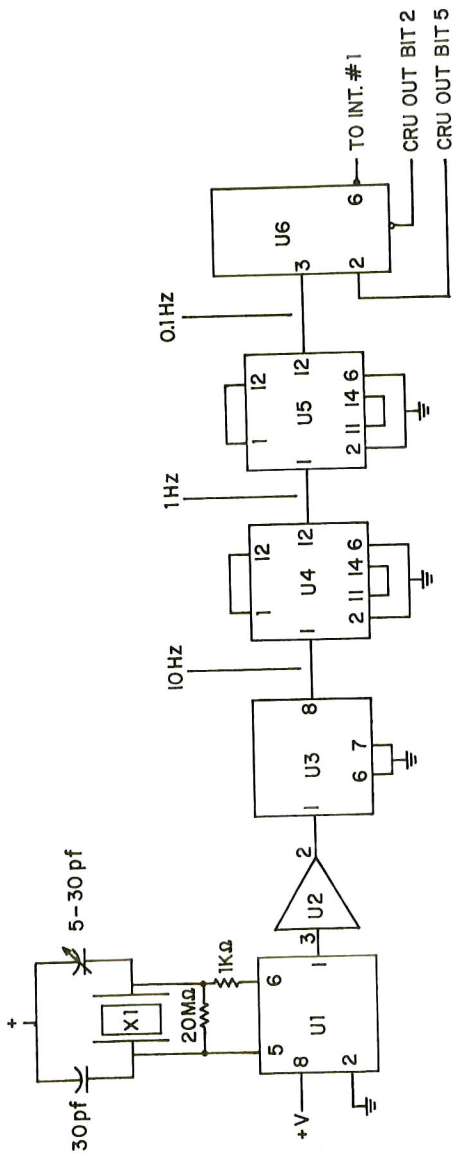

Figure 4. Clock and control board.

The computer is a complete one board unit with all memory and interfaces needed for stand along operation. It is controlled by Texas Instruments Silent 700 terminal with twin cassette tape drives and automatic device control (ADC) option. The ADC allows the computer to control the tape units (on, off, read, write and rewind). The tapes are used for program and data storage during acquisition. All status and warning messages are printed on the terminal during the acquisition process.

TMS 9900 DATA ACQUISITION PROGRAM

The program begins by resetting the A to D converter clock and injection flipflops and placing the S & H in the hold mode. It also initializes various registers, pointers, and outputs as program identification message. In the next step a message goes out requesting the number of counts per sample. On receipt of a character it is converted to binary and the program waits for another character. On receipt of the second character, a check is made to see if it is a carriage return. If not, the character is converted to binary and added to the first number which has then been multiplied by 10. This is now multiplied by 30 (points per count) and stored for later use. If a carriage return was encountered, it means that the first number was multiplied by 30 and stored as above.

The program now requests a command and on receipt of a character determines whether it is a "go," "stop," or an illegal character. If the character is valid, the computer outputs the rest of the command word and branches to the appropriate routine. If the command is "go" the program outputs "working" and enables the inject and interrupt flipflops. The program then returns to wait for a command. If it is a stop command, interrupts are disabled and an "EOT" command is written on tape, next "data scan complete" is put out after which the program restarts.

The interrupt handler takes over if the interrupts are enabled and the clock times out. After entering the handler further interrupts are

disabled and the data interrupt flipflog is reset and disabled. Next the program checks to see if an injection has occurred. If it has, a "-1" is printed on the tape to signal injection (all data is positive). The number of points is incremented to reflect the new total for the additional scan. Next the sample and hold is placed in the hold mode and the A to D converter is started. When the converter has completed its task the data is read, converted to A.S.C.I.I., and written on tape. The total number of points is decremented by one and checked to see if the last point has been taken. If the number of points equals Ø, an EOF is written to tape, and the program restarts. If the number of points is nòt equal to Ø, the sample and hold is set to sample and all the flipflops are enabled. Program control then reverts to the point of interruption. Flow charts provided in Figures 5, 6, and 7 describe the steps involved in data acquisition.

The original data reduction program was written in BASIC. This was modified to allow it to select the desired data out of the stored sample runs. The program requests the data file, checks to see if it exists. Next the program requests the number of counts per sample and the desired scan number. The data is read into the program after it is cleared of noise spikes and the valid counts are found. The program then displays the number of the smallest count and will provide a crude plot if desired, to facilitate picking the desired peak. The peaks' boundary points are entered along with any scale multiplier. A baseline correction is performed and the calculation continues as in the original program.

A typical plot of the GPC chromatogram is presented in Figure 8. The corresponding data sheet is presented in Table 1. A series of narrow distribution standard polystyrenes were run. Table 2 shows Mw, Mn and MWD data obtained by normalizing the distribution curves and using simple integration steps manually. These are compared to values obtained by

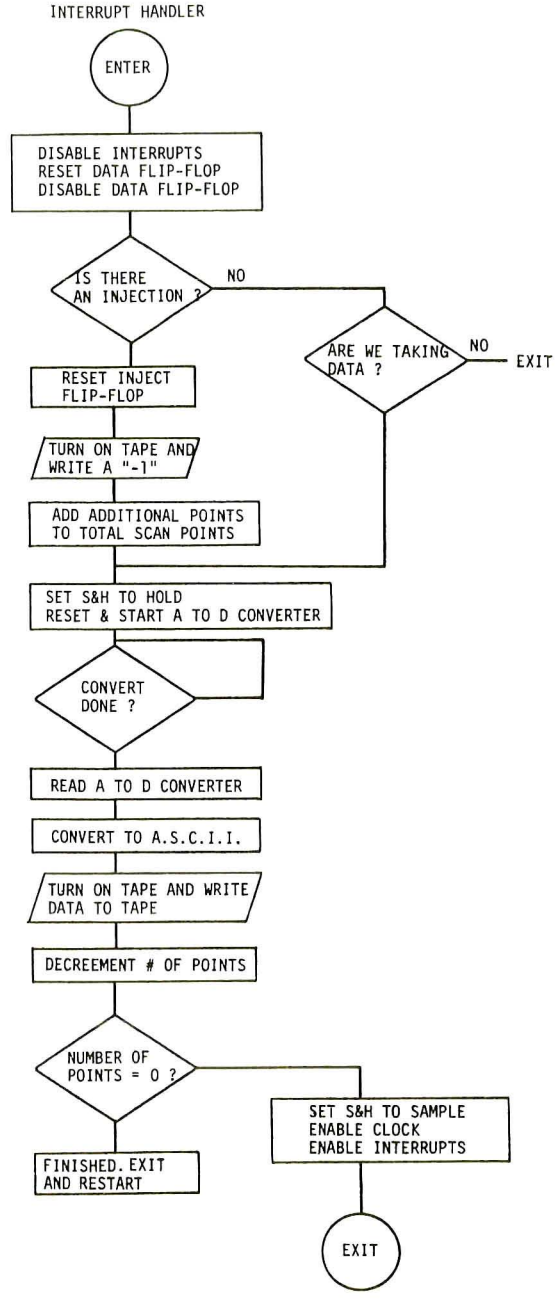

Figure 5. Program flow chart - Interrupt handler.

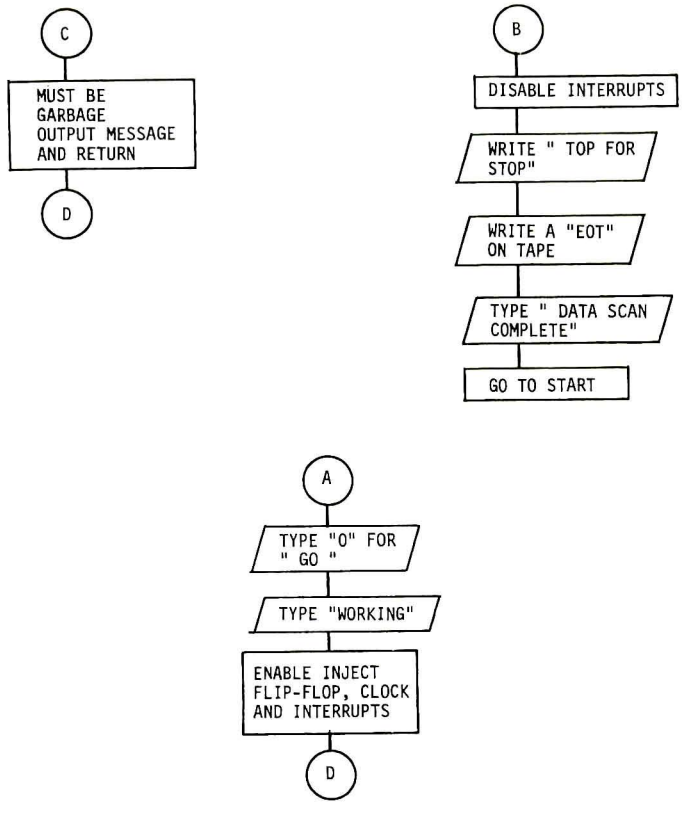

Figure 6. Program flow chart - Auxilliary routines.

the data processor. The agreement is quite good except for the two high molecular weight polystyrenes, presumably due to excessive axial dispersion and skewing effects.

CONCLUSION

A simple, inexpensive on-line data processing system is described for the Waters 200 Gel Permeation Chromatograph. This system can be assembled with only a basic knowledge of computer hardware and software and saves considerable amount of time spent for data manipulation.

Interface chassis and the microprocessor together cost $800.

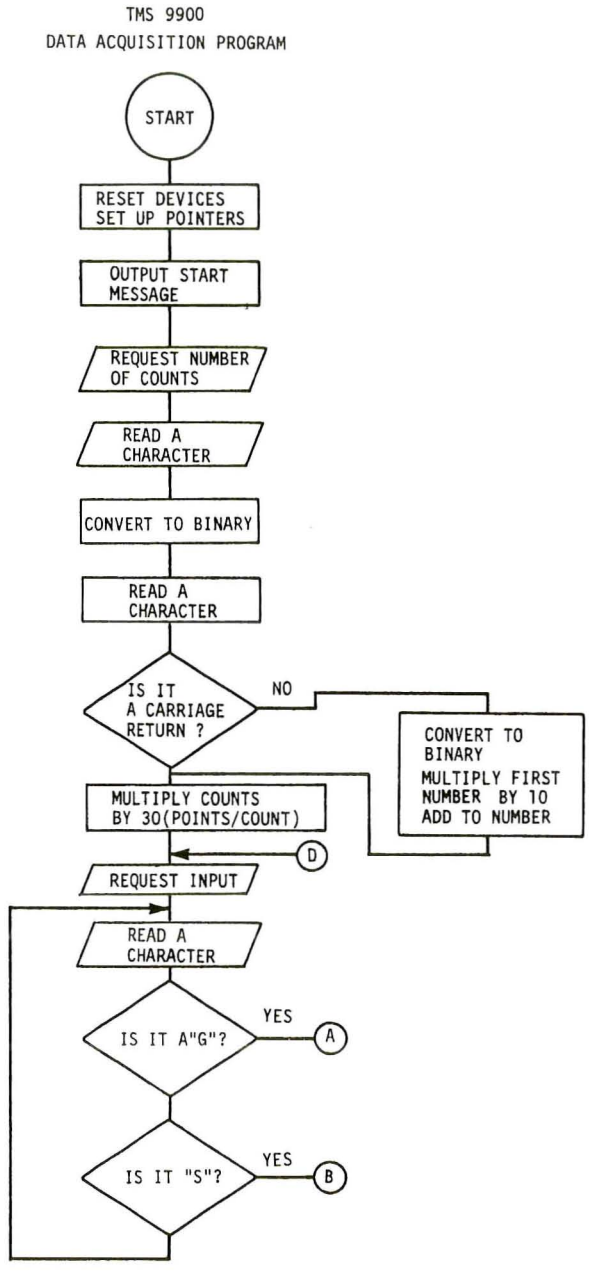

Figure 7. Program flow chart - Data acquisition program.

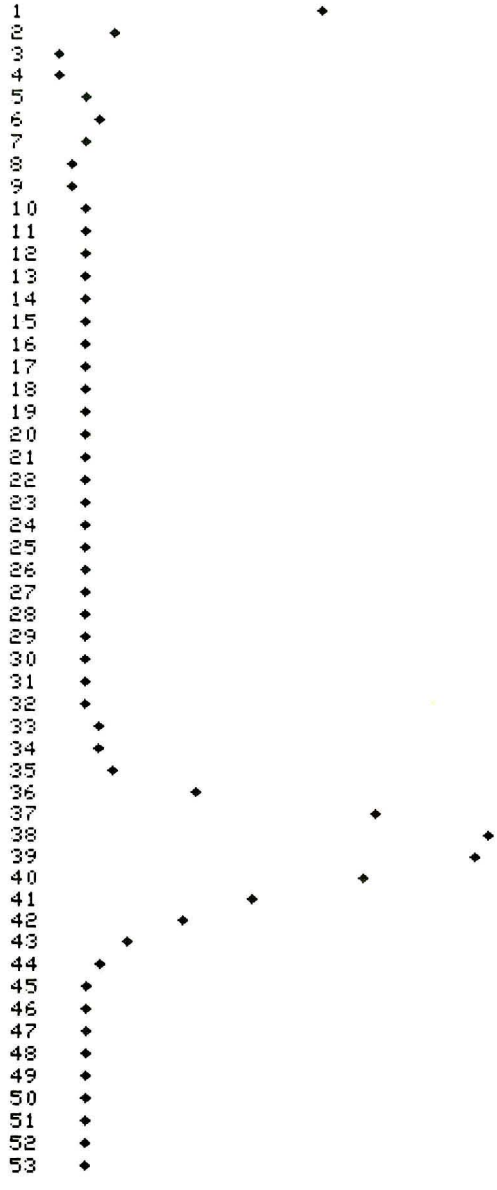
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Figure 8

GEL PERMEATION CHROMATOGRAPHY DATA SHEET

NOV 06 1978

SAMPLE NO:
?1
ANALYST:
?

COLUMNS: 10 TO 6TH,5TH,4TH,3RD,500,60 SOLVENT: CHCL3

FLOW RATE: 1 ML/MIN ROOM TEMPERATURE

COUNT	AI	HEIGHT	HIXAI	HI/AI
33	16341	0	0	0
34	9609	3.58333	34432.2	3.72914E-04
35	5268	16.1667	85166.0	3.06884E-03
36	3268	91.7500	299839.	2.80753E-02
37	2022	251.333	508196.	.124299
38	1200	345.917	415100.	.288264
39	702	327.500	229905.	.466524
40	405	238.083	96423.7	.587860
41	230	139.667	32123.3	.607246
42	152	81.2500	12350.0	.534539
43	93	33.8333	3146.50	.363799
44	57	9.41667	536.750	.165205
45	35.6000	-2.22045E-16	-7.90479E-15	-6.23721E-18
	TOTALS=	1538.50	1.71722E+06	3.16925

RESULTS:

ANGSTROM WEIGHT AVE MOL SIZE= 1116
ANGSTROM NUMBER AVE MOL SIZE= 485
MOL WGT DIST= 2.30

TABLE 2

GEL PERMEATION CHROMATOGRAPHIC DETERMINATION OF POLYSTYRENE STANDARDS

Material Specification†			Manual Measurement			Computer Measurement		
Mw	Mn	MWD	Mw	Mn	MWD	Mw	Mn	MWD
4000	3570	1.12	3895	3280	1.18	4100	3403	1.21
17500	*	*	18368	15621	1.17	16933	14350	1.81
50000	47000	1.06	47191	40508	1.17	48134	40221	1.20
110000	*	*	109962	93111	1.18	107543	95694	1.12
233000	*	*	242064	184787	1.31	233337	190814	1.22
390000	383000	1.02	366827	260300	1.41	347434	26334	1.23
1800000	1780000	1.01	1937700	687600	2.82	1826591	782813	2.33

†Data obtained from Pressure Chemical Co., Pittsburgh, Pa.

*Not specified.

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SYNTHESIS AND ION-EXCHANGE PROPERTIES OF TANTALUM SELENITE AND ITS USE FOR THE SEPARATION OF METAL IONS BY ION-EXCHANGE COLUMN CHROMATOGRAPHY

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ABSTRACT

A new inorganic ion-exchanger tantalum selenite has been synthesized by mixing 0.10M Ta_2O_5 and 0.10M Na_2SeO_3. The ion-exchange properties, chemical stability, TGA and IR absorption have been studied. Some industrially important separations of Fe^{3+} from Mn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, VO^{2+}, Al^{3+} and Ba^{2+} from Ca^{2+}, Sr^{2+}, Mg^{2+} have been achieved on the columns of tantalum selenite. Separation of Fe^{3+}-Ni^{2+} can be applied to separate and determine Fe^{3+} from nickel electroplating bath.

INTRODUCTION

Out of the various inorganic ion-exchangers studied recently, the tantalum based ion-exchangers have received scant attention. Studies on tantalum pentoxide have been reported by Abe and Ito (1) and Chidley, et al. (2). Studies on tantalum phosphate by Kraus and Phillips (3), tantalum antimonate by Qureshi,et al.(4) and tantalum arsenate by Rawat and Mujtaba (5) have also been reported. Furthermore, only few studies have been reported on selenite based inorganic ion-exchangers. Costa and Jeronimo (6) prepared zirconium selenite and used for the separation of 1B group metal ions using paper chromatographic technique. Qureshi, et al. (7,8) prepared titanium selenite and stannic selenite and separated metal ions by ion-exchange column chromatography. A search of literature showed that no such studies have been reported on tantalum selenite. Therefore, the studies on tantalum selenite were explored in the field of ion-exchange

chromatography by achieving the selective separation of Fe^{3+} from a number of common interferring metal ions on the column of tantalum selenite. The separation can be applied to the determination of Fe^{3+} content as impurity in nickel electroplating solutions.

MATERIALS AND METHODS

Reagents and Apparatus

Tantalum pentoxide (Fluka), sodium selenite (B.D.H.) were used. A temperature controlled SICO shaker, Bausch and Lomb Spectronic 20 and Elico pH meter model Li-10 were used for shaking, spectrophotometric and pH measurements respectively. For IR studies spectromom 2000 (Budapest) infrared spectrophotometer was used.

Synthesis

The tantalum selenite samples were prepared by mixing 0.1M tantalum pentoxide and 0.1M sodium selenite solution in the ratio 1:1 under conditions given in Table 1. The sodium selenite solution was added to tantalum pentoxide solution and desired concentration of acid was adjusted by adding aqueous ammonia. Sample 3 was prepared by

TABLE 1

Synthesis and Properties of Tantalum Selenite

Sample No.	Conditions of synthesis		Properties		
	Conc.of H_2SO_4 at which exchanger prepared	Temperature at which samples prepared	Appearance of exchanger in H^+ form	Ion-exchange capacity meq/g for H^+	Composition Ta:Se
1	4.50M	25.0°C	Light red	1.18	1:1.50
2	4.50M	60°C	Light grey	0.96	1:1.45
3	4.50M	Refluxed at 100°C	Light grey	0.50	1:1.38
4	0.10M	25°C	Light red	0.89	1:1.15

refluxing the mother liquor of sample 1 for 24 hours at 100°C. The precipitate was kept overnight with mother liquor. It was filtered off, washed with deminaralized water and dried in oven at 40°C. The dried product broke into small granules when immersed in water. The granules were then dried and placed in 2.0M HNO_3 for 24 hours with occasionally shaking and intermittent changing of acid to convert them into hydrogen form. The chemical composition of the products synthesized was determined by estimating tantalum by Pyrogallol method (9) and selenium by sulpherdioxide method (10).

RESULTS AND DISCUSSION

The condition of synthesis and some basic properties of different sample of tantalum selenite are summarized in Table 1.

Ion-exchange capacity

The ion-exchange capacity of Tantalum selenite (Sample 1) was also determined for different uni and divalent metal ions by column operation and was found to be 1.12, 1.19, 1.45, 1.10, 1.05, 1.12 and 1.30 for Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} respectively. The exchange capacity by gravimetric procedure (11) was also measured as 1.12, 1.18, 1.22 and 1.53 for Mg^{2+}, Ca^{2+}, Sr^{2+} and Ba^{2+} respectively. A comparison of these results with the exchange capacity by column procedure indicates that the exchange capacity by gravimetric procedure is slightly higher than the column procedure capacity. This is due to some adsorption of metal ions on the exchanger (12).

Dissolution of tantalum selenite

To determine the solubility of tantalum selenite in different solvents, 0.5 g of exchanger was taken with 50 ml of the solution concenred at room temperature for 6 hours. After removing the undissolved material tantalum and selenite were determined in the filtrate colorimetrically with pyrogallol (9) and diaminobenzidine (13) respectively. The results show that the amount of tantalum and selenium dissolved in 50 ml is negligible in demineralized water, $NaNO_3$, NH_4NO_3, HNO_3, H_2SO_4, HCl, alcohol and acetic acid. The dissolution of tantalum selenite is upto certain extent in NH_4OH, NaOH and oxalic acid.

The results of Table 1 further reveal that the ion-exchange capacity and chemical stability are considerably affected by the

conditions of preparation. The results are in agreement with the results of Nancollas (14) who found that the method of preparation of amorphous ion-exchanger has a considerable effect on composition and the degree of hydration. These two factors are responsible for the ion-exchange capacity and the chemical stability of the product.

Ion-exchange Potentiometric Titrations

The pH titration of sample 1 were performed by the method of Topp and Pepper (15) with LiOH, NaOH, KOH and aqueous NH_3 with their respective salts of known concentrations. The backward pH titrations were also performed by taking 50 ml NaOH 0.1M with the addition of 0.1M HNO_3 and water to make up the volume 100 ml. After shaking for four hours and the pH was measured. The effect of hydroxyl ion on the pH of equilibrating solutions are given in Figs.1 and 2. These results show that tantalum selenite in hydrogen form behaves as a monobasic acid. The results in Fig.2 forward and backward titration curve for Na^+ ions suggest a reversible behaviour of the exchanger, as a very small hysteresis loop is observed. The concentration of NaOH was 0.1M in which material dissolves negligibly.

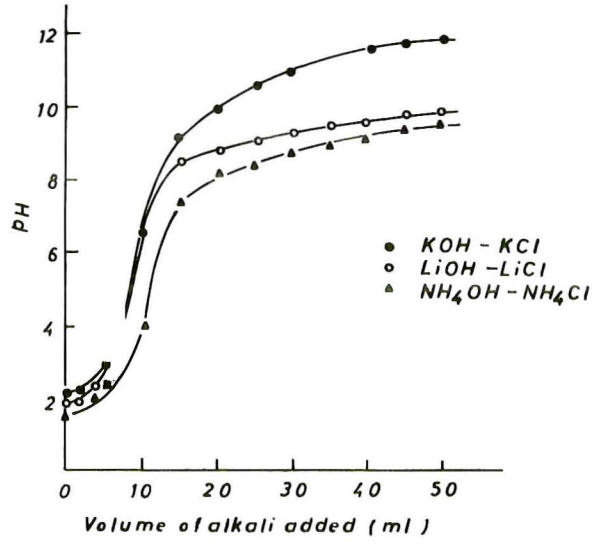

Figure 1 Potentiometric titration for tantalum selenite.

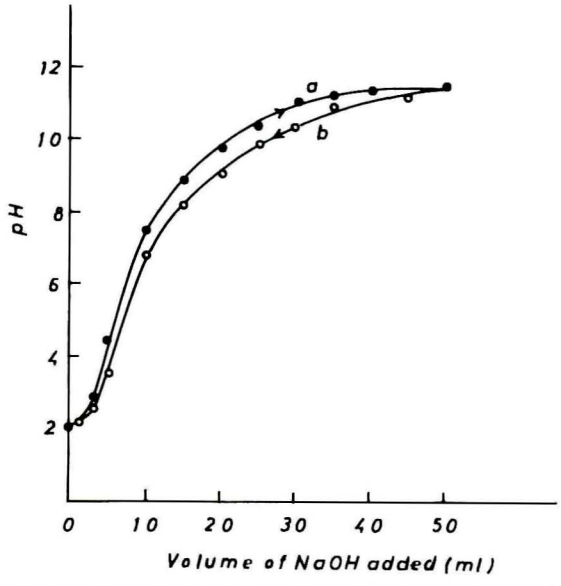

Figure 2 Potentiometric titration for tantalum selenite

'a' Forward, 'b' Backward with 0.1M NaOH and 0.1M NaCl.

IR Absorption Studies

IR spectra of tantalum selenite in hydrogen form were measured in nujol media. The results show that the tantalum selenite gives the characteristic peaks. A broad peak in the range 2900-3600 cm^{-1} with a maxima at 3400 cm^{-1} represents the interstitial water free water with nujol. The strong peak at 1650 cm^{-1} corresponds to the deformation vibration of interstitial water and of OH groups. The weak peaks are at 1150 cm^{-1} and 1050 cm^{-1} and very strong peak is the region 650-900 cm^{-1}. These peaks are due to the stretching vibration of the M-O bond i.e. Ta-O and Se-O. Some of the peaks in this IR spectrum resembles with the IR studies of sodium selenite performed by Millar and Wilkins (16).

Thermal Treatment

To examine the effect of drying temperature on the ion-exchange properties of the material, the sample was dried at various temperatures in a muffle furnace for 2 hours. A decrease in the

ion-exchange capacity with increasing temperature is plotted in Fig.3. Thermogravimetric analysis of sample 1 in hydrogen form was performed at a rate of 5 °/min. The results of TGA show that the weight loss of the sample in H^+ form upto 250 °C is due to the removal of external water molecules in the structure. Above this temperature upto 500 °C the weight loss is nearly constant. It shows that there is no loss of other water molecules. A sharp increase in weight loss is observed when the temperature is raised upto 850 °C. Significant structural changes must occur over this temperature range and condensation may take place i.e. may be the formation of oxides.

The effects of the drying temperature on the ion-exchange capacity are given in Fig.3. These results indicate that this ion-exchange material can be used upto 100 °C without much loss of ion-exchange capacity. This ion-exchanger, therefore, possesses the higher capacity in comparison to other selenites and tantalum based exchangers. Above 100 °C temperature the capacity decreases continuously upto 700 °C.

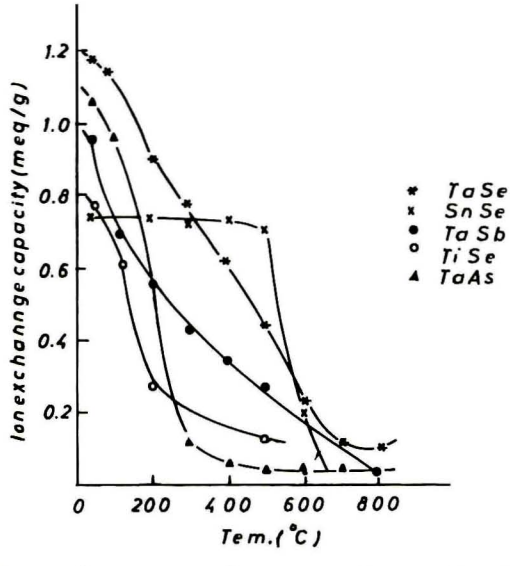

Figure 3 Ion-exchange capacity of various selenites and tantalum based exchangers as a function of drying temperature.

Distribution Studies

Kd values of metal ions were determined by batch process on sample 1 in different systems

$$Kd = \frac{50\ (I-F)}{0.5 \times F}\ ml\ gm^{-1}$$

where I is the volume of 0.002M EDTA used to titrate cation solution initially and F is the EDTA volume needed for cation titration after equilibrium. The total volume of equilibrating solution was 50 ml and 0.5 g exchange was taken. The results are given in Table 2.

Separations

Some analytically important and industrially useful separations were achieved on the basis of Kd values of metal ions.

TABLE 2

Distribution coefficients of metal ions on tantalum selenite

Cation	DMW	0.1M $NaNO_3$	0.1M NH_4NO_3	1.0M HNO_3	0.1M HNO_3	0.01M HNO_3	0.001M HNO_3	0.0001M HNO_3
Ba^{2+}	2210.0	1400.0	1462.0	62.0	328.4	800.0	1180.0	2150.0
Sr^{2+}	8.2	0.0	5.0	2.0	2.6	2.6	7.0	7.0
Mg^{2+}	1.4	1.0	7.0	0.0	4.0	6.5	15.9	16.5
Ca^{2+}	0.0	0.0	0.0	0.0	0.0	0.0	1.5	1.5
Cu^{2+}	22.0	18.0	18.0	2.0	3.0	13.0	17.0	18.0
Mn^{2+}	0.0	0.0	6.0	4.0	6.0	24.0	27.0	27.0
Ni^{2+}	2.4	0.0	2.5	1.0	2.5	2.5	2.5	5.0
Co^{2+}	5.6	4.0	8.2	0.0	1.5	3.8	8.0	15.0
Al^{3+}	21.0	20.0	4.4	0.0	3.0	5.0	6.0	8.0
Fe^{3+}	T.A.	T.A.	1550.0	2.0	7.5	115.0	455.0	780.0
VO^{2+}	30.0	49.0	3.6	7.0	18.0	32.0	40.0	43.0
Zn^{2+}	0.0	9.0	2.0	0.0	6.0	11.0	19.0	19.0

T.A. = Total Adsorption

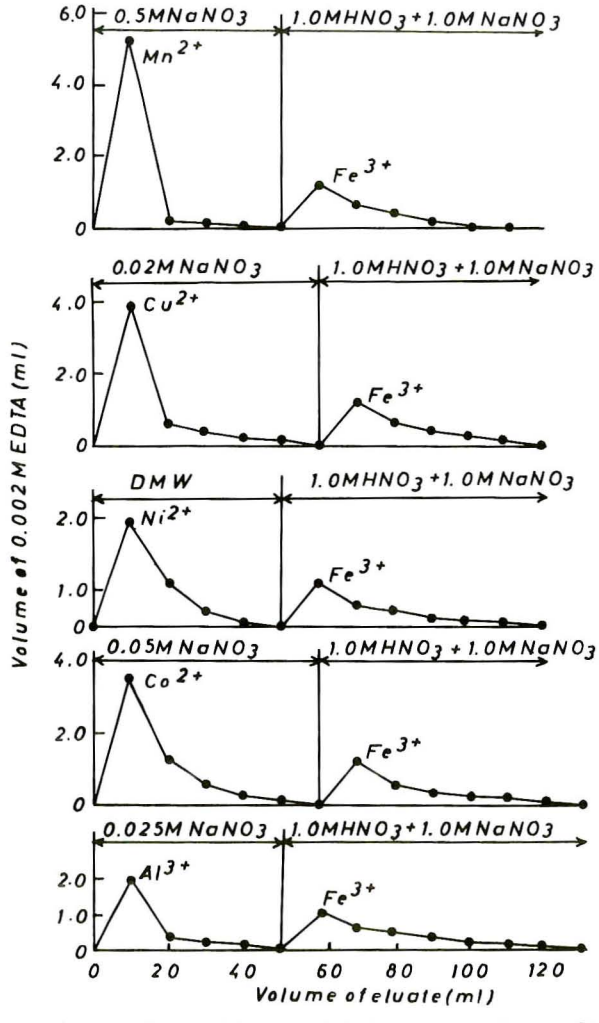

Figure 4a Separations achied on tantalum selenite.

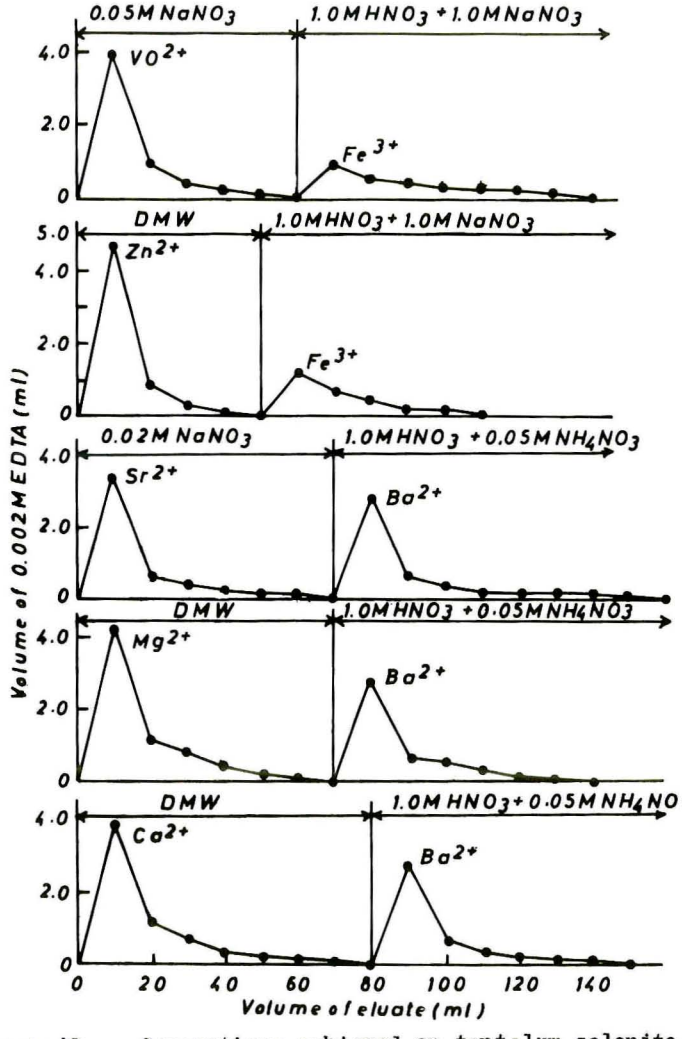

Figure 4b Separations achieved on tantalum selenite.

The separations were performed using the ion-exchange column chromatographic technique. The order of elution and eluents are presented in Fig.4. In order to explain the effectiveness of tantalum selenite for the separation of Fe^{3+} and Ba^{2+} was made. It was found that the selenites of Mg, Ca, Sr, Ni, Cu, Co, Mn, Zn and Al have been prepared by mixing the carbonates of the respective metal ions with selenious acid (17). Except ferric selenite and barium selenite all are soluble in water as well as in dilute mineral acids. Therefore ferric and barium should be adsorbed more strongly than the remaining cations under study. Hence elution of Ma, Ca, Sr, Ni, Cu, Co, Mn, Zn and Al has been made with demineralized water or 0.5M sodium nitrate while the elution of ferric and barrium with 1.0M nitric acid adding 1.0M sodium nitrate or ammonium nitrate. The elution of ferric and barium is not sharper than other metal ions also for the same reason.

The industrial utility of these separations was checked by separating Fe^{3+} from Ni^{2+} in a electroplating bath solution. The presence of Fe^{3+} in the electroplating bath solution of nickel produces black spots on electroplated shiny surfaces. It was found that for two samples taken from the same electroplating bath the iron(III) content was 189 and 201 µg in 2 ml bath solution.

ACKNOWLEDGMENT

The authors are grateful to Prof.W.Rahman for providing research facilities. One of us (K.P.S.M.) is also thankful to C.S.I.R. India for financial assistance.

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DETERMINATION OF THEOPHYLLINE IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

We describe a rapid and highly specific method for determining theophylline in plasma. Following addition of ammonium sulfate and β-Hydroxy-ethyl-theophylline as internal standard, theophylline is extracted into a mixture of chloroform hexane (70 : 30) and evaluated by high performance liquid chromatography, using Microporasil "Waters" 10 µm as stationnary phase and N-hexane-ethanol (76 : 24) mixture as mobile phase. Absorption at 280 nm is monitored. The method has a good precision (coefficients of variation between 3 % and 4 % for 1 mg/l and 10 mg/l) and its sensitivity is about 0.25 mg/l. No interferences from endogenous compounds, metabolites of theophylline, or from drugs commonly co-administrated with theophylline have been encountered. This technique can be used in analytical toxicology, and also for therapeutic controls and pharmacokinetic studies.

INTRODUCTION

The determination of theophylline in blood is of interest for both the therapeutist and the toxicologist for several reasons. This medicine, used in the treatment of asthma and, more recently, that of apnea in new-born children, actually requires precise monitoring of its concentrations in plasma in order to best match the therapeutics to the individual clinical response. In addition, the therapeutic index is narrow due to the fact that the toxic and efficient doses are close to one another.

Since the findings of Schack and Waxler (1), based on the use of ultraviolet spectrophotometry, technology for the determination of theophylline in blood has considerably progressed. Thus, besides a few immuno-assays (2), most of the methods have relied on gas chromatography (3-5) and high pressure liquid chromatography (HPLC), the only two techniques fulfulling the desired specificity and sensitivity criteria.

The gas chromatographic techniques are applied after extraction by several solvents. They include the use of an internal standard and, in most cases, the formation of one derivative. Usually, detection is performed by flame ionization or, at times, by electron capture. Sheehan and al. (6) have recommanded its use in combination with mass spectrometry. These processes are rather easy to implement but prove inadequate, as a rule, due to the lengthy operations they require, particularly at the extraction and derivative formation stages.

Using the liquid chromatography techniques, the preliminary extraction is simplified even eliminated. One resorts either to adsorption (7-9) or to reverse phase chromatography (10-21) or to chromatography by ion-pair (22-23). According to the apparatus, the UV spectrometry detectors use a fixed (254 nm) or variable wavelength (in this latter case they operate on 275 or 280 nm) and also electrochemical detection (24-25) is employed. In view of the foregoing, we have preferred high performance liquid chromatography with internal calibration and detection by UV spectrometry at 280 nm. The method we have developed is presented below.

MATERIALS and METHODS

Apparatus and Operating Conditions

A Hewlett-Packard HPLC Model 1084 B with automatic injector and recorder/integrator 79850 ALC H.P. terminal is used. The

chromatograph is equiped with a column (30 cm long, 3.9 mm, µPorasil, 10 µm, Waters Associates).

The mobile phase is a mixture Hexane/Ethanol (76 : 24) with a flow rate of 1.5 ml/mn. Absorbance is monitored at 280 nm.

A Mass Spectrometer, Model 5980 A, with data system 5934 A (Hewlett-Packard) was also used to establish identity and purity of the theophylline liquid chromatography peak.

Reagents

All solvents and reagents (ethanol, n-hexane, chloroform and ammonium sulfate) are analytical grade.

The stock solutions of theophylline (N° T 1633 crystallin anhydrous "Sigma Chemical Company"), β-hydroxy-ethyl-theophylline ("Boehringer Ingelheim"), 1-methyl-xanthine (N° 69720 "Fluka"), 3-methyl-xanthine (N° 69722 "Fluka"), and 1,3-dimethyl-uric acid (N° D 2889 "Sigma Chemical Company"), prepared in ethanol, contain 1 mg per ml. Standard solutions are prepared by dilution in the same solvent.

Operating Procedure

The blood samples are collected in oxalated tubes (Venoject T 200 x F 105) and then centrifuged for 15 min at 2000 rpm and + 4° C ; at this time, the plasma should immediately be frozen until analysis.

Insert 0.5 ml of plasma into a centrifuge tube of suitable volume and stopper. Add 0.5 g of ammonium sulfate, 2 ml of double-distilled water and 1 to 10 µg of internal standard (according to the anticipated theophylline quantity to be determined in the sample) Mix the contents on a vortex-type mixer for 15 s after each new addition of a different product. Add 15 ml of the chloroform-hexane mixture then mechanically agitate for twenty minutes. Centrifuge at 4000 rpm during 5 minutes at + 4° C. Recover the organic phase, dry-evaporate at 60° C under nitrogen stream and pick up the residue in 100 µl of ethanol while vortex-mixing for 15 s. Then inject a 20 µl sample of this solution into the chromatograph for analysis.

Under the conditions defined above, the retention times of the theophylline (I) and the internal standard (II) were 6.88 and 10.33 minutes, respectively (Figure 1).

The ratio of the I/II peak areas were calculated with an integrator. Refer to calibration curves (Figures 2-3) plotted from I/II peak area ratios obtained after sample analysis of test sample plasmas to which increasing quantities of theophylline were added (0.25 to 2.00 µg/ml or 2.5 to 40 µg/ml) and a constant quantity of internal standard (1 µg or 10 µg) as well.

RESULTS and DISCUSSION

Internal standard

β-hydroxyethyltheophylline was chosen as an internal standard. This substance is structurally very similar to theophylline ; their maximum absorption wavelength is the same and their extraction conditions are alike. In addition, β-hydroxyethyl-theophylline is neither a drug nor a theophylline metabolite.

Specificity

Maximum absorbance of theophylline occurred at 272 nm with the instruments used. However we have preferred to operate at 280 nm since the base line is better at this wavelength and the technical capabilities are improved. Actually, between 270 and 275 nm, interferences due to substances existing in the plasma have been noticed. This phenomenum is particularly apparent whenever the theophylline quantities to be determined are small.

No interferences were observed from uric acid or the theophylline metabolites (1 methylxanthine, 3 methylxanthine and 1.3 dimethyl uric acid). Their retention times are 3.40, 7.53, 8.68 and 21.39 minutes, respectively.

In regards to specificity, no interference exists from caffeine or theobromine, even if the levels of these components are significant (Figure 4). The same holds true for

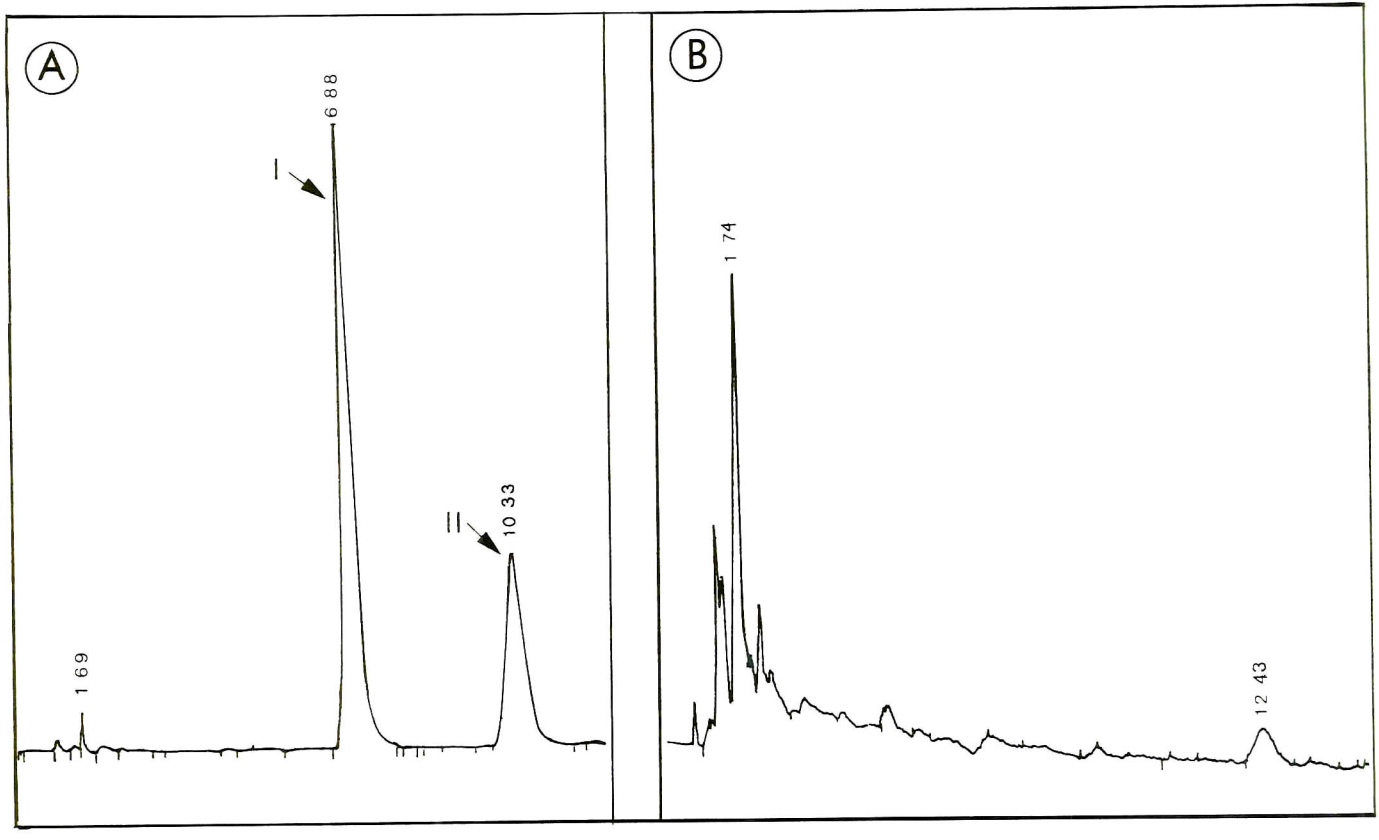
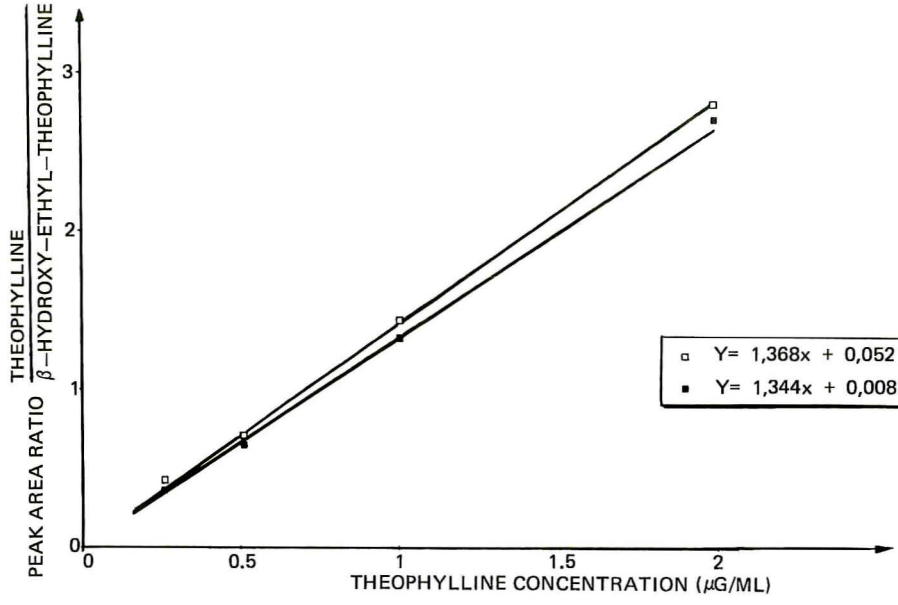


FIGURE 1. (A): Chromatogram of a patient's plasma, with internal standard, containing 12 µg/ml of Theophylline. I, theophylline; II, β-hydroxy-ethyl-theophylline (internal standard). (B): Plasma control after extraction.



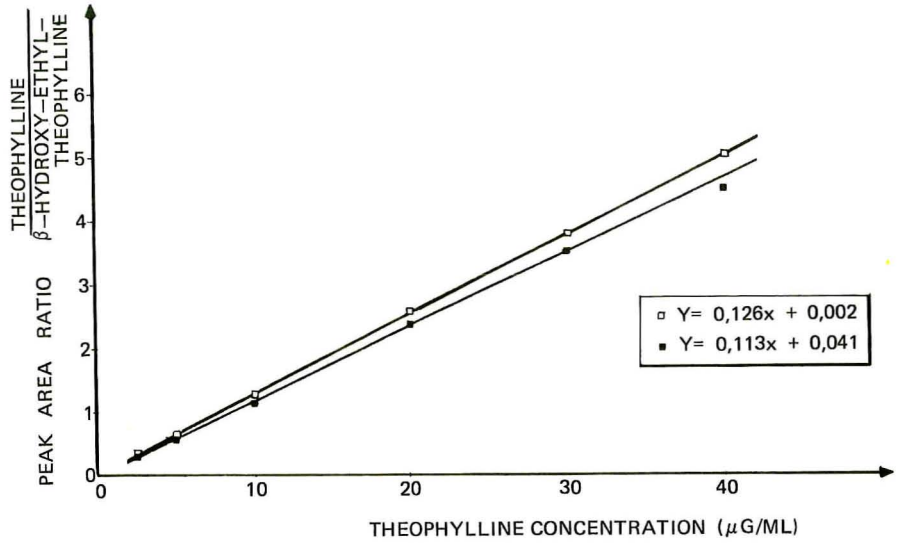
2. Standard curves for the quantitative analysis and determination of the relative recovery to the internal standard of Theophylline with low values

□ Theophylline and Internal Standard were chromatographed directly (r = 0,99)

■ Both Theophylline and Internal Standard were extracted from plasma (r = 0,99).

drugs such as salbutamol (Ventoline^{R}) and terbutaline (Bricanyl^{R}) used as relays for theophylline and corticoids. But we note an interference with Bactrim^{R} (association of sulfamethoxazole and trimethoprim).

Moreover, plasma from a patient has been checked using mass spectrometry to show that the liquid chromatography peak with a retention time of 6.88 minutes is actually imputable to the theophylline itself by its molecular ion (M-181) (Figure 5). For this work, the drug-containing fraction was collected and introduced into the mass spectrometer via the direct insertion probe after evaporation of the solvent.



3. Standard curves for the quantitative analysis and determination of the relative recovery to the internal standard of Theophylline with high values

   □ Theophylline and Internal Standard were chromatographed directly (r = 0,99)

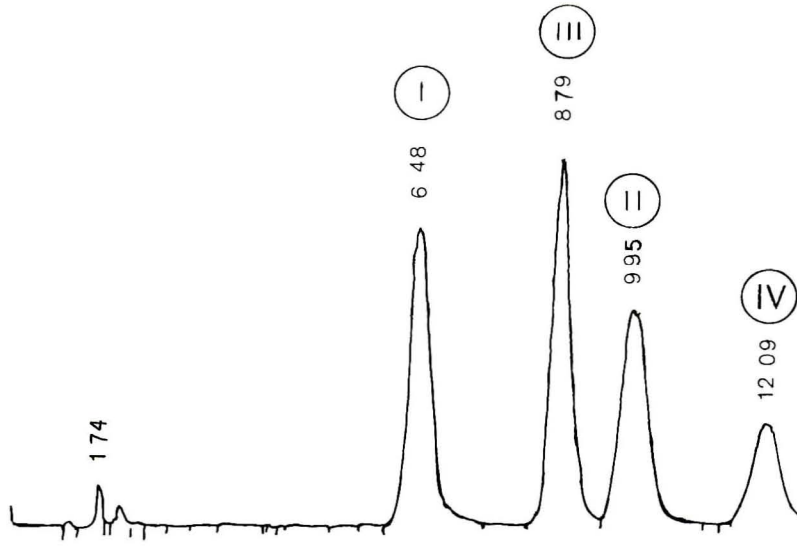
   ■ Both Theophylline and Internal Standard were extracted from plasma (r = 0,99)

Extraction Procedure

The performance of the proposed method is influenced by the extraction pH and the nature of the solvents. The best results have been obtained by performing the extraction with the chloroform/hexane mixture (7/3) at plasma pH, in presence of ammonium sulfate which precipitates serum proteins and reduces the significance of the interferences above mentioned. Under these conditions, the percent recovery determined for theophylline quantities from 0.25 to 40 µg was approximately 94.

Sensitivity, Reproducibility and Accuracy

Similarly, for routine assays, the quantitative limit of sensitivity is about 0.25 mg/liter.



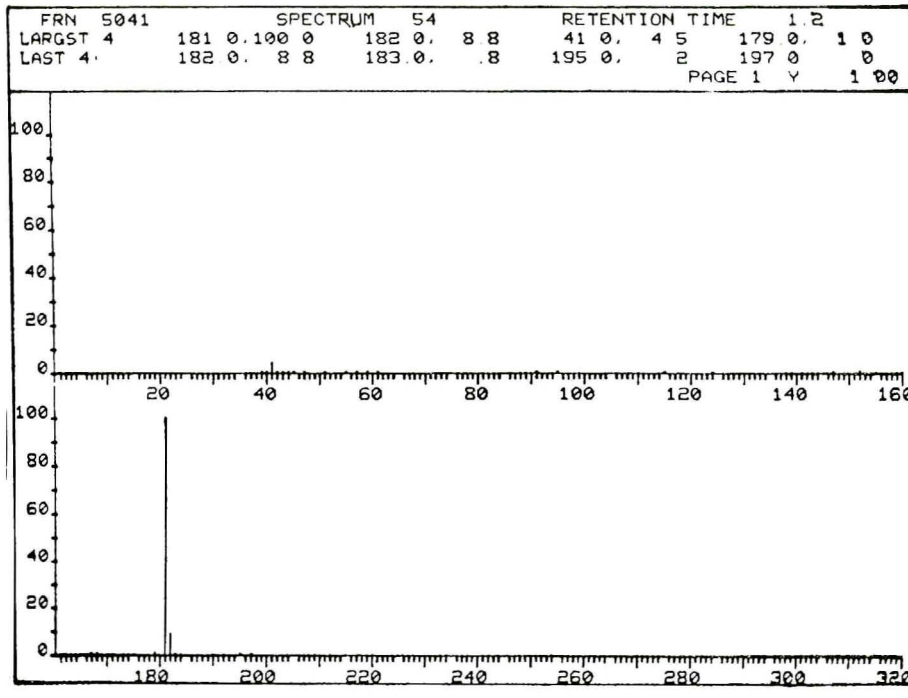
4. Chromatogram of a mixture of xanthines
I, theophylline ; II, Internal Standard ; III, caffeine ; IV, theobromine

Repeatability was investigated by analyzing a plasma pool containing 0.5 µg theophylline and 0.5 µg internal standard per 0.5 ml. The coefficient of variation within tests, for 6 successive extractions and assays was 3 %. With larger quantities (5 µg theophylline and 5 µg internal standard) in 0.5 ml plasma and 7 successive operations it was 4 %.

Injection repeatability was also considered. By injecting the same ethanol solution of theophylline and internal standard (1 and 1 µg), 6 times, the coefficient of variation was 1 %.

APPLICATIONS

The proposed technique is useful in toxicology, especially for the diagnosis of possible theophylline overdosage. It can also serve for performing various assays in the therapeutic



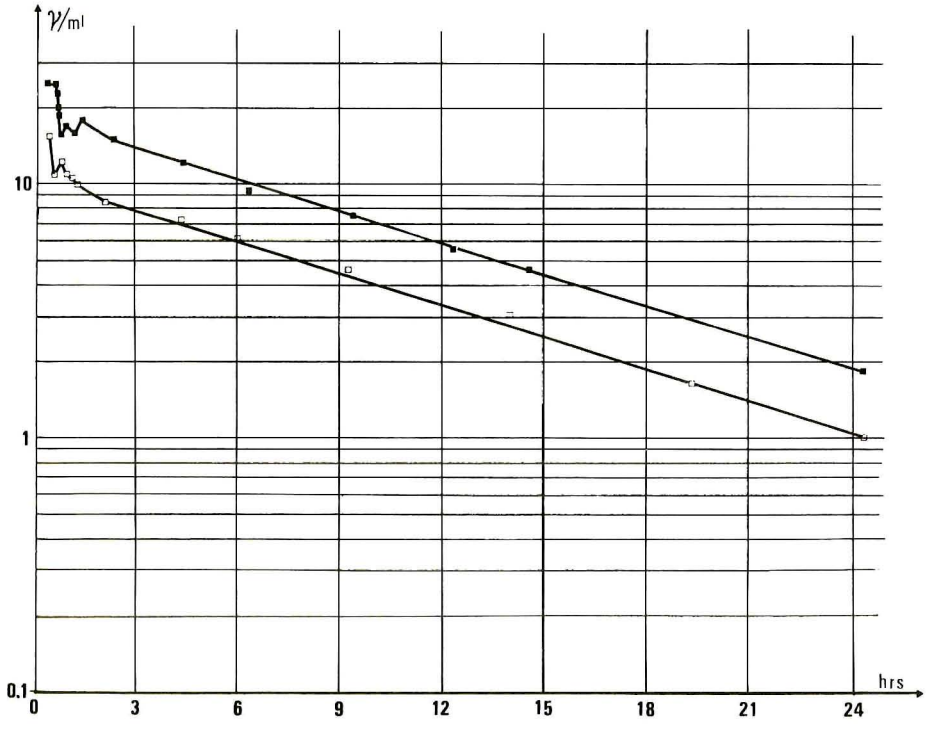
5. Mass spectrum for collected theophylline fraction (chemical ionisation mode ; $(M + 1)^+ = 181$) via the direct insertion probe into the mass spectrometer

and pharmacovigilance fields on both adults and children. Thus, as a routine procedure, it will be easy to check that the theophylline level is within the limits of the range generally recognized as efficient, between 10 and 20 mg/liter, and does not exceed the 25 mg/liter threshold beyond which toxicity would exist. The good sensitivity of the process also enables extension of its use to pharmacokinetics research.

We have studied the decrease, with time, of theophylline plasma levels of a patient having severe asthma crises, who had received, successively, at 72 h intervals, 240, then 480 mg by short infusion (15 mn). This application established the kinetic linearity in the zone of therapeutic concentrations.

The erratic aspect of the plasma concentration decreases between 15th mn and 1st H derives from a phenomenum of theophylline absorption on the catheter during the infusion. For the two dosages, 240 and 480 mg, the half-life varies from 9 to 7 h and plasmatic clearance from 2.2 to 2.5 l/h (Figure 6).

Using this method, we could individualize theophylline posology, by a previous pharmacokinetical identification, in view of increasing drug efficacity and decreasing toxic effects (26).



6. Theophylline plasma levels after I.V. infusion during 15 mn.

Dose administrated (□—□ = 240 mg ; ■—■ = 480 mg)

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ASSAY OF BRAIN TOCOPHEROLS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The four natural tocopherols were separated using a μ-Bondapak-NH_2 column. For the analysis of brain tocopherols 5,7-dimethyltocol was used as an internal standard. α-Tocopherylquinone and other tocopherols than α-tocopherol were not detected. Rat cerebral cortex and cerebellum contained 19.3 μmol/g and 11.2 μmol/g of α-tocopherol, respectively. A chromatographic system with a reversed-phase column proved less suitable.

INTRODUCTION

Enhanced lipid peroxidation has been implicated as a mechanism in various types of tissue damage, *e.g.* during incomplete ischemia in brain (1). The peroxidative damage of brain membrane lipids during *in vitro* incubation has been characterized in this laboratory (2, 3). So far the role of tocopherol in these processes remains unclear. To some extent, this is due to the lack of rapid and sensitive analytical methods for studies of brain tocopherol levels and metabolism. Methods involving thin-layer chromatography and gas chromatography are often laborious, and tocopherols are very susceptible to oxidation if several steps of purification are involved. High performance liquid chromatography (HPLC) is

better suited to tocopherol analysis, especially since it employs specific detection methods and the exposure of tocopherols to air can be minimized.

Methods for the separation of α-tocopherol from serum or animal feed by reversed phase HPLC have previously been reported (4, 5, 6). Chromatography of other tocopherols have not been described except for the comigration of β- and γ-tocopherol (4). With Corasil columns instead, all four tocopherols have been resolved (7, 8, 9) and also the four tocotrienols (8). The objective of the present investigation was to compare different HPLC procedures for the separation of brain tocopherols and their metabolites such as α-tocopherylquinone.

EXPERIMENTAL

Apparatus

Waters Associates high performance liquid chromatography system (Model U6K injector, 6000A solvent delivery system, 440 UV absorbance detector) was used. The absorbance was measured at 254 nm and 280 nm.

Column Packing Materials

The packing material was Nucleosil 10 C18 (Macherey-Nagel & Co, Düren, West Germany, art. nr. 71215) with a mean particle diameter of 10 μm. A prepacked bonded amine phase column, with a mean particle size of 10 μm, μ-Bondapak-NH_2 (84040 Water associates, Milford, USA) was also used.

Column Tubings and Fittings

The Nucleosil column consisted of 6.4 mm O.D. x 200 mm length of 316 stainless steel tubing. The internal diameter was 5.0 mm. It was equipped with Parker-Hannifin compression fittings. Thin stainless steel mesh discs were placed at both ends of the column (part no. 206, hetp, Sutton, England). The μ-Bondapak-NH_2 column had the dimensions 300 x 4 mm I.D. in stainless steel. The loop

injector and detector were connected to the column via 1/16" O.D. (0.23 mm I.D.) stainless steel tubing.

Column Packing Technique

The column was packed according to the upward slurry packing technique (10). The Nucleosil 10 C18 material was slurried in chloroform and packed with acetone.

Chemicals

Solvents. n-Hexane, HPLC-grade, was obtained from Rathburn Chemicals (Walkersbrum, Peeblesshire, Scotland), methanol, p.a., from May & Baker (Dagenham, England) and ethanol, spectrographic grade, from Svensk Sprit AB (Sweden). Glass-distilled water was used.

Tocopherols. α-Tocopherol was obtained from Merck (Darmstadt, West Germany), 5,7-dimethyltocol from Koch-Light (Colnbrook, UK) and other tocopherols and α-tocopherolacetate from Hoffman-LaRoche (Basel, Switzerland). α-Tocopherylquinone was prepared from α-tocopherol according to Nair and Machiz (11).

Reagents. L(+)-Ascorbic acid, p.a., was from Merck (Darmstadt, West Germany) and tetra-n-butylammonium-hydroxide from BDH Chemicals Ltd (Poole, England).

Procedure

Animals. Male Wistar rats were fed a commercial pellet diet (Astra-Ewos, Sweden) containing 40 mg vitamin E/kg according to the manufacturer. Rats weighing 275-350 g were decapitated into liquid nitrogen. The brains were chiselled out during intermittent irrigation with liquid nitrogen and stored at -80°C until extraction.

Extraction procedures. A modification of a published procedure (12) was used. About 100 mg of the brain sample was put into a 10 ml homogenizer tube with a mixture of 1 ml 10 % aqueous solution of ascorbic acid, 1 ml ethanol and 2 ml hexane. 5,7-Dimethyltocol

was added as an internal standard (2.27 μg/sample). The mixture was homogenized for 3 min with a knife homogenizer (MSE 431) at the highest speed. The solution was transferred to a 15 ml centrifuge tube and was centrifuged at +4° C for 10 min at 10 000 rpm. The hexane phase was transferred to a test tube and was kept on ice until analyzed. Alternatively, the sample could be stored under nitrogen at -20°C for at least two weeks without loss of α-tocopherol. Fifty μl of the sample was injected into the chromatograph when the straight phase column was used. When the reversed phase column was used the extract was taken to dryness under nitrogen and the residue was dissolved in methanol.

Chromatographic conditions. All chromatographic experiments were performed at ambient temperature (approx. 20°C). The nonpolar C18-column was used with methanol/water, 98:2, as mobile phase. In some experiments tetra-n-butylammoniumhydroxide (0.25 mM, pH 7.8) was added to the mobile phase. The NH_2-column was used with n-hexane containing 0.8 % ethanol as mobile phase. The flow in the reversed phase system was 2.0 ml/min and in the straight phase system 1.0 ml/min.

RESULTS AND DISCUSSION

The Reversed Phase System (Nucleosil 10 C18)

In this system separation between α-tocopherol, β- + γ-tocopherol and δ-tocopherol was obtained (Fig. 1) in agreement with previous work (4, 6). The separation was improved if tetra-n-butylammoniumhydroxide was added to the methanol/water mobile phase, but changes in the methanol/water proportions or in the amount of quartenary amine did not give separation of β- and γ-tocopherol.

Another disadvantage with this system was that UV-absorbing substances in brain extracts migrated close to α-tocopherol. This probably explained the higher α-tocopherol content measured for rat brain using a nonpolar C18-column compared to the results obtained using a polar NH_2-column (Table 1). Furthermore, the

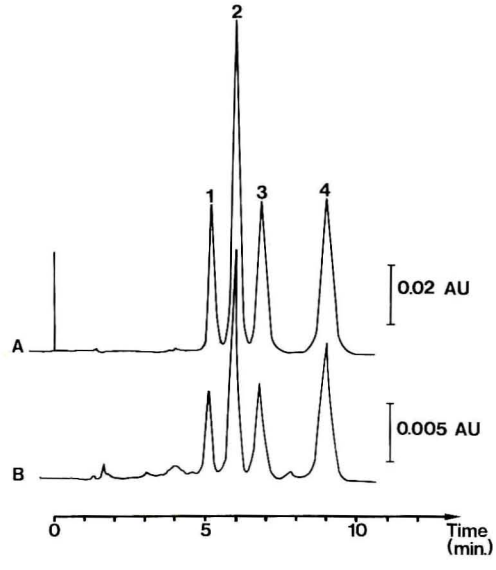

FIGURE 1. Separation of tocopherols using the reversed phase system. Nucleosil 10 C18 with methanol:water (98:2) as mobile phase. The flow rate was 2.0 ml/min. A is the absorption curve at 280 nm and B is the curve at 254 nm for the same sample. The peaks are: 1, δ-tocopherol; 2, β- + γ-tocopherol; 3, α-tocopherol; 4, α-tocopherolacetate. AU, absorbance units.

TABLE 1

Concentration of α-Tocopherol in Rat Brain Determined by HPLC. Data are expressed as μg/g wet tissue and are means ± S.E.

	Straight phase system	Reversed phase system
Cerebral cortex	19.3 ± 0.9 (n=9)	23.2 ± 0.6 (n=3)
Cerebellum	11.2 ± 0.3 (n=4)	-

redissolving of the brain lipids in the mobile phase prior to chromatography was incomplete. This chromatographic system is therefore not recommended.

The Straight Phase System (μ-Bondapak-NH_2)

Apart from reversed phase partition systems, HPLC of tocopherols has mainly been performed with silica columns *e.g.* Corasil. Chemically bonded polar phases have not been much used. Therefore, a column with a chemically bonded primary amine was tried (Fig. 2). All four tocopherols were resolved and in addition 5,7-dimethyltocol and α-tocopherylquinone could be separated from α-tocopherol. The degree of separation of different tocopherols obtained with μ-Bondapak-NH_2 (Fig. 2) was equal or superior to that previously reported for Corasil (7-9). α-Tocotrienol was, however, incomplete-

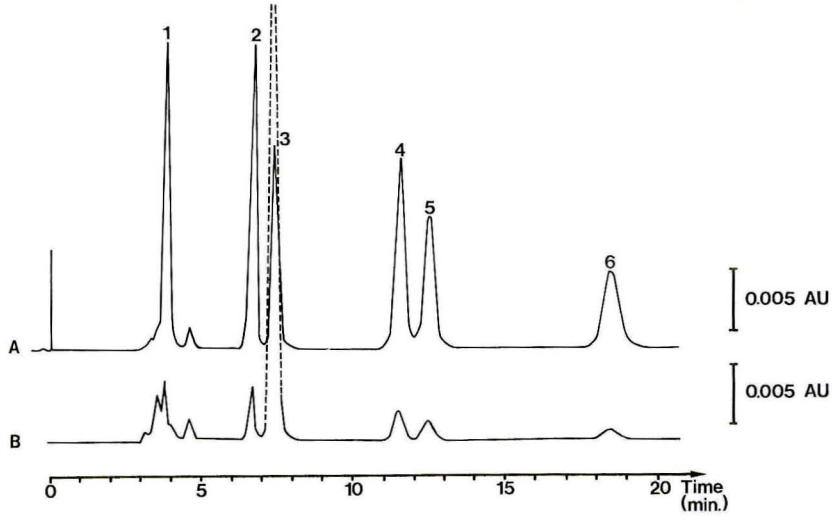

FIGURE 2. Separation of tocopherols using the straight phase system, μ-Bondapak-NH_2 with hexane : ethanol (99.2:0.8) was mobile phase. The flow rate was 1.0 ml/min. A is the absorption curve at 280 nm and B is the curve at 254 nm for the same sample. The peaks are: 1, α-tocopherolacetate; 2, α-tocopherol; 3, α-tocopherylquinone; 4, β-tocopherol; 5, γ-tocopherol; 6, δ-tocopherol.

ly resolved from α-tocopherol. The retention time of 5,7-dimethyltocol was close to that of α-tocopherol. This indicates that the number of methyl groups adjacent to the 6-hydroxyl group is important for the separation.

The effect of varying the proportion of ethanol in the mobile phase was studied (Fig. 3). At lower ethanol concentrations 5,7-dimethyltocol and α-tocopherylquinone were best resolved, but

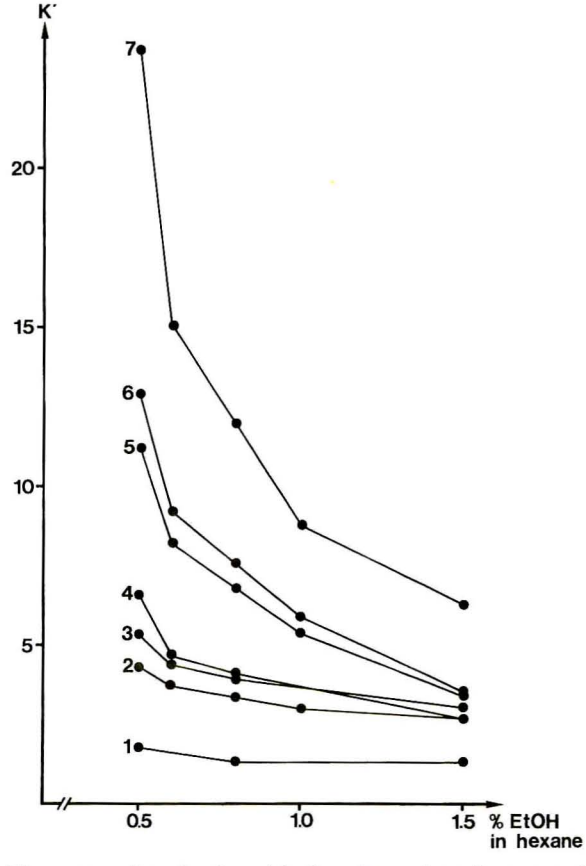

FIGURE 3. The capacity factor k' for tocopherols as a function of the amount of ethanol in the mobile phase in the straight phase system. 1, α-Tocopherolacetate; 2, α-tocopherol; 3, 5,7-dimethyltocol; 4, α-tocopherylquinone; 5, β-tocopherol; 6, γ-tocopherol; 7, δ-tocopherol.

under these conditions a disturbing peak broadening of α-tocopherol occurred, and therefore hexane:ethanol (99.2:0.8) was routinely used as mobile phase. A typical analysis of an extract from rat cerebral cortex in this system is shown in Fig. 4. Other tocopherols than α-tocopherol were not detected.

Quantitative Analysis

For the determination of α-tocopherol in brain, 5,7-dimethyltocol or α-tocopherolacetate were added as internal standards during the extraction. The linearity of the analysis was checked by chromatography of equal volumes of reference solutions containing identical amounts of 5,7-dimethyltocol and different amounts

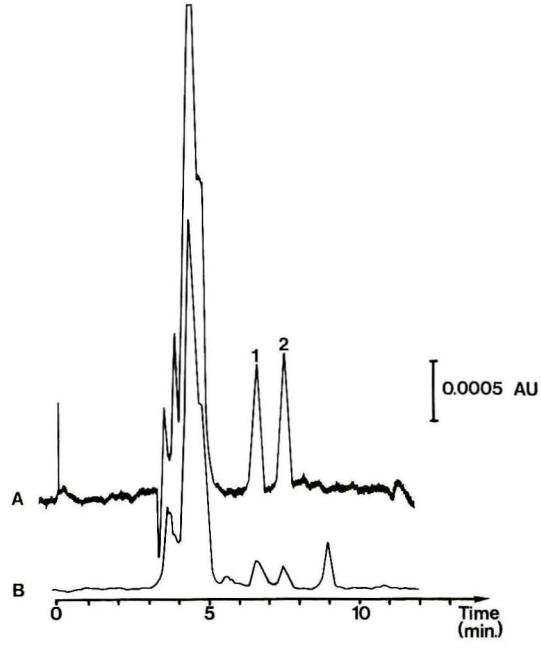

FIGURE 4. A chromatogram of an extract from rat cerebral cortex. For conditions see Fig. 2. The peaks are: 1, α-tocopherol; 2, 5,7-Dimethyltocol (internal standard).

of α-tocopherol (Fig. 5). Peaks were quantitated by peak height measurement and the standard curve was used for the quantitation of tocopherol in brain extracts. A similar curve was obtained using α-tocopherolacetate as internal standard. 5,7-Dimethyltocol was the most suitable internal standard. It is chemically similar to, and migrates close to α-tocopherol. However, it is difficult to acquire, and it is badly resolved from α-tocopherylquinone. Since UV-absorbing substances in brain extracts interfered with the quantitation of α-tocopherolacetate, this substance could not be used as internal standard. However, it may be a convenient internal standard if a fluorescence-detector is used.

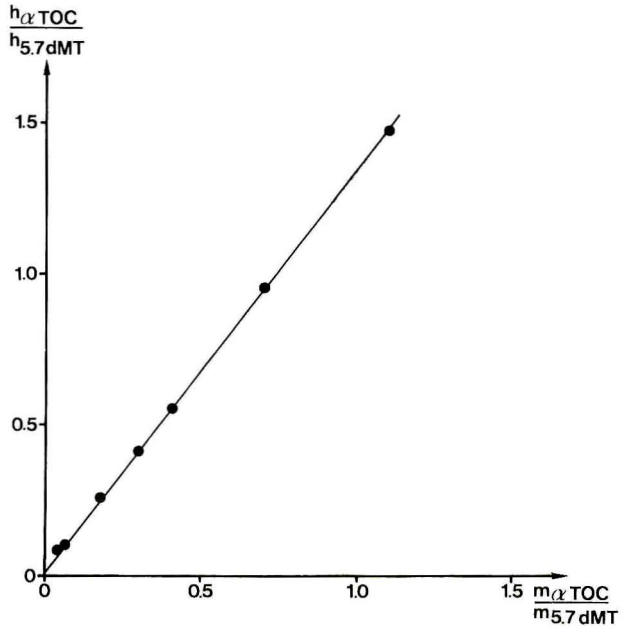

FIGURE 5. Standard curve used for quantitation of α-tocopherol with 5,7-dimethyltocol as internal standard. The reference solutions contained 2.27 μg 5,7-dimethyltocol and 0.13-2.50 μg α-tocopherol per ml of hexane. For chromatographic conditions see Fig. 2. Vertical axis, ratio of peak heights; horizontal axis, mass ratios.

Accuracy

The identity of α-tocopherol in brain extracts was established as follows. The k'-value of standard α-tocopherol was equal to that in brain extracts. α-Tocopherol added to brain extracts comigrated with the endogenous α-tocopherol and it was also quantitatively recovered (Table 2). The absorption of the eluent was routinely measured at both 280 and 254 nm. The ratio A_{280}/A_{254} was 5.11 ± 0.06 (mean ± S.E., n=8) for standard α-tocopherol and 5.12 ± 0.18 (n=14) for brain α-tocopherol.

Brain extracts were routinely analyzed twice and the coefficient of variance for duplicate analysis of α-tocopherol was 2.3 % (n=18). When duplicate samples of cortex from the same brain were analyzed, the coefficient of variance was 10.7 % (n=10). One sample, analyzed six times, had a ratio of α-tocopherol/5,7-dimethyltocol of 1.19 ± 0.006 (mean ± S.E.).

Brain Tocopherol Concentration

Rat cerebral cortex and cerebellum were analyzed (Table 1). As mentioned above analysis in the reverse phase system gave somewhat higher values. Cerebellum contained less α-tocopherol than cerebral cortex, which agrees with the recent report of Vatassery and Younoszai (13). Our values are higher, which proba-

TABLE 2

Recovery of α-Tocopherol Added to Extraction Mixtures in the Presence or Absence of Brain Tissue
Data are expressed as mean ± S.E.

Expt	Brain present	Amount of added α-tocopherol (μg)	Increment in α-tocopherol amount (μg)
I	+	1.17	1.18 ± 0.02 (n=4)
II	-	2.34	2.33 ± 0.05 (n=4)

bly is due to differences in rat diets. This point remains to be elucidated since data on dietary vitamin E was not given in the cited report (13).

In studies on α-tocopherol metabolism it is necessary to detect different metabolites. α-Tocopherylquinone could be separated from α-tocopherol in the straight phase system and some other oxidation products, probably dimers, could also be resolved.

α-Tocopherylquinone was badly resolved from 5,7-dimethyltocol but occurrence of α-tocopherylquinone in brain extracts would easily have been detected since the A_{280}/A_{254} ratio differed between the two compounds. 5,7-Dimethyltocol had a A_{280}/A_{254} ratio of 6.5 while the ratio for α-tocopherylquinone was 0.3. So far we have not detected any oxidation products in brain extracts.

It is possible that the NH_2-groups on the column could form stable Shiffs bases with the carbonyl group in the quinone, giving a false low value for α-tocopherylquinone in the brain extracts. In order to investigate that, a column with a bonded nitrile group (Nucleosil, 5 CN, Macherey-Nagel & Co., Düren, West Germany, art. nr. 71216) was used. There were no signs of a quinone peak in the brain extracts with this column either.

These data indicate that the straight phase system (μ-Bondapak-NH_2) was superior to the reversed phase system for the separation of tocopherol and related substances. Another advantage of the former system is that the extract can be injected onto the column directly without evaporation.

In summary, this report describes a rapid, sensitive technique for the analysis of brain tocopherols. The main advantages compared to previous studies are the rapid freezing of tissue in liquid nitrogen, minimizing postmortal changes, the use of different internal standards, and the high chromatographic resolution.

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CEFOPERAZONE IN SERUM

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ABSTRACT

A new HPLC method for the determination of Cefoperazone in serum is described.

INTRODUCTION

Cefoperazone[2] is a new semi-synthetic cephalosporin derivative with broad antibacterial spectrum against aerobic and anaerobic gram positive and gram negative organisms including "Enterobacteriaceae", indole positive "Proteus" and "Pseudomonas aeruginosa".

It is resistant to inactivation by penicillinase and very stable in the presence of cephalosporinase with wide substrate profiles (1).

A rapid HPLC method for the determination of Cefoperazone in serum is described after extraction with SEPPAK cartridges filled with a reverse phase of octadecylsilane (μBondapak C_{18}/Porasil ^{R}B).

MATERIALS AND METHOD

A Hewlett-Packard high-performance liquid chromatograph 1084B and a reverse phase column (μBondapak C_{18} , 10 μm particle size, 250mm x 4,6mm i.d.) were used.
This chromatograph is equiped with a variable wavelength detector (HP 79875 A) and connected to a LC Terminal integrator (HP 79850 B).

SEPPAK cartridges were purchased from Waters Associates (part n°51910). Cefoperazone was supplied by Pfizer Belgium and Methanol P.A. purchased from Merck.

A stock solution of Cefoperazone was prepared in bidistilled water at the concentration of 500 μg/ml and diluted in water or serum in order to prepare a standard curve from 1 to 10 μg/ml.

After prewashing the cartridge with methanol and water, pour 2 ml of sample (or standard) on it, using a syringe, and wash with water to elute the substances unadsorbed on the phase. Thereafter elute the adsorbed Cefoperazone with twice 1 ml of methanol-water (1/1) and inject twenty-five microliters onto the column using methanol-water(1/1) as mobile phase at a flow rate of 1,5 ml/min.(resulting pressure 120 bars). Retention time is 2.30 min. and detection is made at 228 nm.

The concentrations of Cefoperazone were determined using the standard curve assayed following the described method. The detection limit is 0,25 µg/ml.

RESULTS AND DISCUSSION

A standard curve was made at concentrations of 1 - 2 - 3 - 5 - 7,5 and 10 µg/ml.(n=5 for each concentration) (TABLE 1). A graph of the peak area vs. concentration gives a straigh line with a coefficient of correlation of 0,9994.

Recovery from aqueous solutions of Cefoperazone after SEPPAK is 99,6% ± 3,8% (TABLE 2).

Pure serum blank was not adsorbed onto the SEPPAK as controlled by a 97% recovery in the eluate,

TABLE 1

Standard Curve of Cefoperazone

Concentration (µg/ml)	Mean area (range)
10	24792 (24870-25470)
7,5	19200 (19070-19270)
5	12230 (12030-12390)
3	6983 (6916-7300)
2	4624 (4261-4688)
1	2474 (2320-2543)

TABLE 2

Recovery from Aqueous Solutions of Cefoperazone

Concentration (µg/ml)	N	Mean recovery (SD) (%)
4	9	101,6 (± 5,8)
2	5	98,5 (± 2,6)
1	5	98,8 (± 3,0)

measured by a Biuret reaction. Further washing the SEPPAK with methanol-water (1/1) did not result in a peak on the chromatogram that could interfere with Cefoperazone determination.

Recovery from serum solutions of Cefoperazone after SEPPAK was 92% (n = 4 for each concentration from 1 to 10 µg/ml.).

The method described is easy, rapid and reproducible. The limit of sensitivity (0,25 µg/ml.) is adequate for clinical use: A.F.Allaz et al. gave Cefoperazone 2 g every 12 hours I.V. to healthy subjects and measured at 10 hours after the injection a concentration of 3,8 µg/ml (range: 1,5 to 10 µg/ml.) using a biological assay (2).

The SEPPAK extraction method allows the separation from biological fluids of hydrophilic drugs which cannot be extracted by organic solvents (3,4).

Therefore it is very useful and could find wide applications in pharmaceutical and pharmacological analysis.

ACKNOWLEDGMENTS

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(2): Sodium 7-[D(-)-α-(4-ethyl-2,3-dioxo-1-piperazinecarboxamido)-α-(4-hydroxyphenyl)acetamido]-3-[(1-methyl-1H-tetrazol-5-yl)thiomethyl]-3-cephem-4-carboxylate.

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HIGH-RESOLUTION ANION-EXCHANGE CHROMATOGRAPHY OF ULTRAVIOLET-ABSORBING CONSTITUENTS OF HUMAN ERYTHROCYTES

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ABSTRACT

The analysis of nucleic acid components of human erythrocytes was achieved by high-resolution anion exchange chromatography using a column packed with a macroreticular anion-exchange resin and linear gradient elution with ammonium acetate solution, pH 4.5. On the chromatogram of trichloroacetic acid extracts from human erythrocytes, at least 50 components were detected as ultraviolet-absorbing constituents. On assignment of the chromatographic peaks they were found to be nucleic acid components. The analysis was achieved in 120 min and the elution time of guanosine 5'-triphosphate was 64.3 min.

INTRODUCTION

We previously reported that the separation of ultraviolet (UV)-absorbing constituents of body fluids, such as urine (1,2), blood plasma or serum (3) and hemodialysate (3), can be achieved by chromatography using a macro-

reticular anion-exchange resin. Blood cells are also an important part of the blood and contain literally hundreds of organic compounds. Particularly, a great number of nucleotides are known in the blood cells (4,5). The tremendous advance in studies of free nucleotides is correlated with the introduction and development of ion exchange chromatography (6). Microreticular (7-10) or pellicular (11-15) ion-exchange resins were employed for the separation of nucleic acid components, but they were not satisfactory in term of resolution and time for separation. Therefore, we tried to use a macroreticular anion-exchange resin column for the separation of nucleic acid components in human erythrocytes.

EXPERIMENTAL

Reagents. Analytical grade ammonium acetate, acetic acid and trichloroacetic acid (TCA) were purchased from Wako Pure Chemical Industries (Tokyo, Japan). The reference compounds, cytosine, cytidine, uridine, uracil, thymidine, thymine, adenosine, adenine, guanosine, guanine, inosine, hypoxanthine, xanthine, riboflavin, 5'-CMP, 5'-CDP, 5'-UMP, 5'-UDP, 5'-AMP, 2'-AMP, 5'-ADP, 5'-ATP, 5'-IMP, 5'-IDP, 5'-GMP, 5'-GDP, 5'-GTP, FMN, FAD and 3',5'-cyclic AMP were purchased from Wako. β-NAD, β-NADH, β-NADP, UDPG, UDPGA, 5'-TMP, 5'-TDP, 5'-TTP, 5'-XMP and 3',5'-cyclic GMP were purchased from Sigma Chemicals (St.Louis,

Mo., U.S.A.), and 5'-CTP, 5'-UTP and 5'-ITP were purchased from P.L. Chemicals Inc., (Milwaukee, Wis., U.S.A.).

Apparatus. The instrument for the analysis was a Hitachi high-speed liquid chromatograph (model 634, Hitachi Co., Tokyo, Japan). It consisted of a gradient device, a high pressure pump, a sample injection valve, an anion exchange resin column, a double-beam spectrophotometer, a 10-mV data recording device and a circulating water bath. Stopped-flow scanning spectrophotometry was performed with a scan-speed of 60 nm/min and a slit-width of 4.0 nm for scanning from 340 to 220 nm.

The strongly basic macroreticular anion-exchange resin, Diaion CDR-10 (control #520000), is a polystyrene-divinyl-benzene copolymer having quaternary ammonium groups and a particle size distribution of 5 to 7-um. This resin was obtained from Mitsubishi Chemical Industries (Tokyo, Japan). Macroreticular resins have a porous structure that allows the rapid diffusion of ions or molecules into the resin beads (16). Diaion CDR-10 have a higher cross linkage (35 %), a larger surface area (100 m^2/g), a higher pore capacity (0.27 me/mL) and a larger pore diameter (400 A) than those of microreticular resins (17,18).

Procedures.

Sample preparation. Fresh heparinized blood from a normal healthy adult was centrifuged for 5-min at 3,500

r.p.m. at 5°C. The plasma and white blood cells were pipetted out, and the settled layer was used as the red blood cell (erythrocyte) sample. The preparation of TCA extracts of erythrocytes was performed by the method of Miech (19). One milliliter of whole blood or 0.5-mL of erythrocytes was pipetted up, and added dropwise to 2-mL of cold 12% TCA aqueous solution and then stirred rapidly with a Vortex mixer. After centrifugation for 5-min at 3,500 r.p.m., the upper layer was separated through a filter (0.22-um pore size, Millipore type GS), then 100 or 50-uL was injected onto the column.

Preparation of anion-exchange resin column. A stainless steel column (50-cm in length and 4-mm I.D.) was packed with the Diaion CDR-10 as described by Scott and Lee (20). The column was packed using 6.0 M ammonium acetate buffer (pH 4.5) and run for 1-hr at 15 MPa. The column was connected to a circulating water bath maintaining the column temperature within ±1°C of the selected setting.

Anion-exchange chromatography. A blood sample was introduced onto the column. The constituents of the sample were eluted with a linear ammonium acetate gradient at an average flow-rate of 0.70 mL/min. The linear acetate gradient was formed with a two-chamber gradient generator placing 30-mL of distilled water in the first gradient

chamber, and 30-mL of 6.0 M ammonium acetate buffer (pH 4.5) in the second chamber. The column temperature was raised from 22 to 70°C over the first 30 min, and then maintained at 70°C to the end of the run. Due to the increase in the column temperature, the inlet pressure changed from 10.5 to 7.0 MPa over the first 25 min, and then on introduction of the acetate gradient, the inlet pressure changed from 7.0 to 13.0 MPa to the end of the run. The column effluent was monitored with a double beam spectrophotometer operated at 254 nm.

RESULTS

Analyses of whole blood and blood plasma by the direct injection method.

50-uL of whole blood or 100-uL of blood plasma was directly chromatographed on the macroreticular anion exchange resin column. UV-absorbance chromatograms are shown in Figure 1 (whole blood) and Figure 2 (plasma). The UV-absorbing constituents of plasma have three protein peaks (eluted at 3.0, 4.1 and 22.2 min) and one uric acid peak (eluted at 32.0 min)(3). While the chromatogram of whole blood showed many peaks, the majority of them came from blood cells since the plasma have only four main peaks.

We have usually analyzed body fluids by the direct injection method, especially when rapid treatment of

sample was required. However, the direct injection of whole blood samples caused the clogging of the column and increased pressure. After ordinaly analyses of five to ten samples, regeneration of the column was required. For the regeneration, 2.0 M sodium hydroxide aqueous solution was run through the column for 1-hr at room temperature and at a flow-rate of 0.5 mL/min.

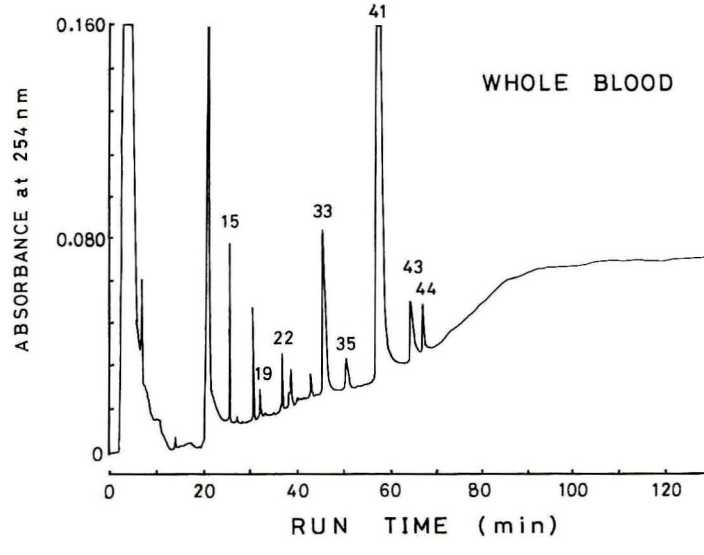

FIGURE 1. Chromatogram of human whole blood with the direct injection method.
Run conditions: column, 50 x 0.4 cm I.D., packed with Diaion CDR-10; eluent, ammonium acetate buffer, pH 4.5, varying concentration from 0 to 6.0 M by linear gradient; temperature, increasing from 22 to 70°C over the first 30-min, then 70°C to the end of the run; average flow rate, 0.70 mL/min; average pressure, 10.5-MPa; sample volume, 50-uL.

Analysis of the TCA extracts from whole blood and from erythrocytes.

A chromatogram of the TCA extracts from whole blood is shown in Figure 3. 50-uL of the TCA extracts is equivaleht to about 17-uL of original whole blood. The chromatogram shows about 50 peaks.

A chromatogram of the TCA extracts from erythrocytes is shown in Figure 4. 100-uL of the TCA ectracts from erythrocytes is equivalent to about 20-uL of original erythrocytes and to about 40-uL of original whole blood. This chromatogram also shows about 50 peaks, but there is

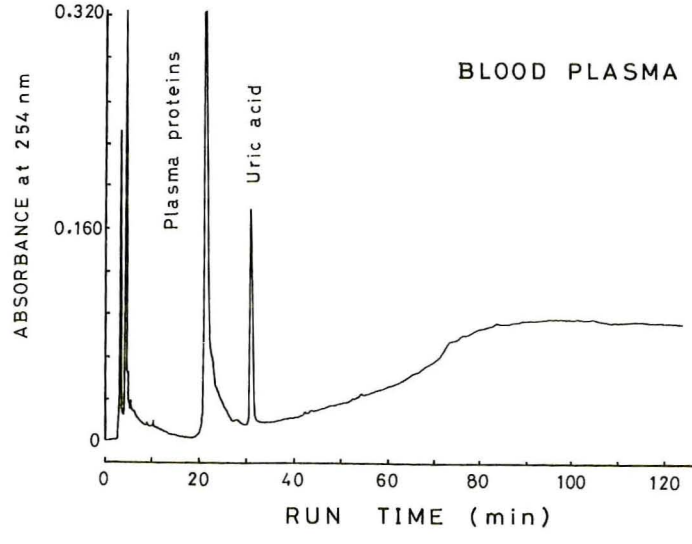

FIGURE 2. Chromatogram of human blood plasma with the direct injection method.
Run conditions were the same as in FIGURE 1.
Sample volume: 100-uL.

no peaks of uric acid. The difference, between Figure 3 and Figure 4, correspond to the chromatogram of TCA extracts from plasma. Another noticeable difference between the TCA extracts from whole blood and from erythrocytes is in the quantities of UV-absorbing constituents when calibrated to the whole blood. The content of triphosphate nucleotides (No.41 ATP and No.43 GTP) are higher and the content of nucleosides (No.8), monophosphate (No.19 AMP and No.22 GMP) and diphosphate (No.33 ADP and No.35 GDP) nucleotides are lower in whole blood than in erythrocytes. These difference could

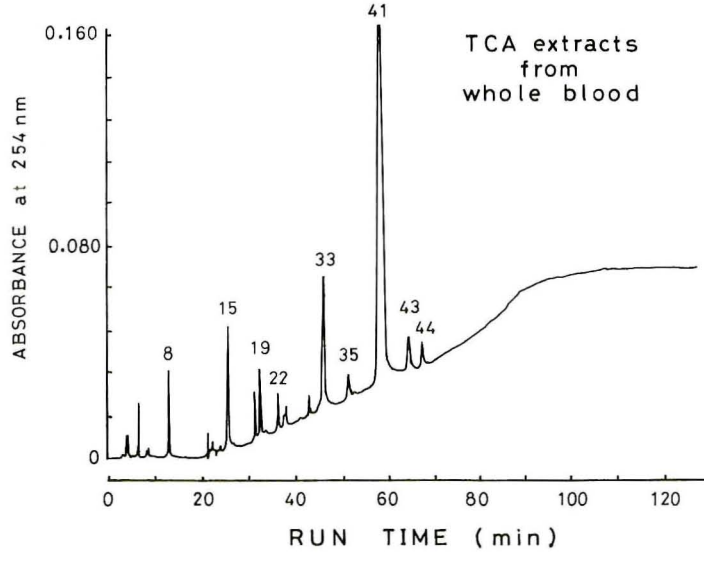

FIGURE 3. Chromatogram of TCA extracts from whole blood. Run conditions were the same as in FIGURE 1. Sample volume: 50-uL.

be explained by the rapid degradation of labile triphosphates and the production of di- and monophosphates even in the process of separation of erythrocytes and plasma. Because of this, direct extraction from whole blood is recommended for the determination of the true profile of nucleotides in blood cells.

Assignment of the chromatographic peaks.

The assignment of peaks in a chromatogram was performed in the following way: (1) by comparing the retention time of a peak to those of the standard compounds

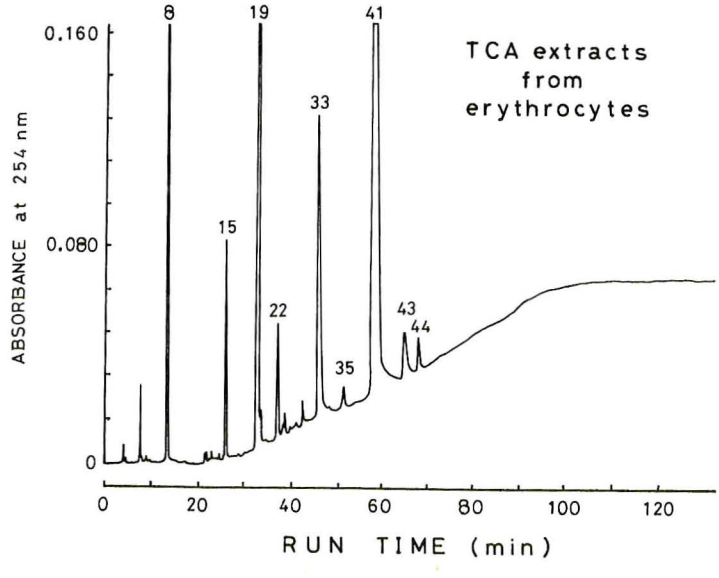

FIGURE 4. Chromatogram of TCA extracts from erythrocytes. Run conditions were the same as in FIGURE 1. Sample volume: 100-uL.

(2) by injecting the standard compounds along with the sample; (3) by measuring the UV-spectrum of a peak at the peak maximum by stopped-flow scanning spectrophotometry. The estimation of retention times of nucleic acid components and related compounds was repeated and mean values are given in Table 1. A representative chromatograms of a mixture of standard compounds is shown in Figure 5. The elution order of the nucleic acid bases was cytosine, uracil, thymine, adenine and guanine. This elution order was observed also in the case of nucleosides, cytidine, uridine, thymidine, adenosine and guanosine, and in the case of mono-, di- and triphosphate nucleotides. Cyclic 3',5'-AMP or cyclic 3',5'-GMP has a larger retention time than 5'-AMP or 5'-GMP, respectively. This elution order corresponds with that suggested in the case of N-bases and nucleosides by Singhal and Cohn (21) and in the case of nucleotides by Brown (12). The retention time of β-NADH, FAD and β-NADP in the chromatography using the macroreticular anion-exchange resin are remarkably different from those in the pellicular anion-exchange resin (12).

For the determination of the UV-spectrum, the flow was stopped at a peak maximum and the spectrum was measured. Then, when the absorbance came down to the base-line, again, the flow was stopped and the spectrum was measured. The latter was used as a control for the preceding peak, and the difference spectrum was made. The UV-spectra of

TABLE I

Retention times of standard nucleic acid components and related compounds.

N-bases and Nucleosides			Nucleotides								
			mono-phosphates			di-phosphates			tri-phosphates		
	peak number	*retention time(min)*		*peak number*	*retention time(min)*		*peak number*	*retention time(min)*		*peak number*	*retention time(min)*
Cytosine	1	5.5	NAD	15	25.5	UDPG	27	39.5	NADP	37	52.0*
Cytidine	2	8.1	(DPN)			NADH	28	41.0*	(TPN)		53.6*
Uridine	3	9.0	5'-CMP	16	28.4	(DPNH)		41.6*	5'-CTP	38	54.9
Uracil	4	11.0	5'-UMP	17	31.5	5'-CDP	29	42.0	5'-UTP	39	57.0
Thymidine	5	13.1	5'-TMP	18	32.0	FAD	30	45.1	5'-TTP	40	58.0
Thymine	6	14.0	5'-AMP	19	33.0	5'-UDP	31	45.5	5'-ATP	41	58.4
Adenosine	7	15.5	5'-IMP	20	34.1	5'-TDP	32	45.8	5'-ITP	42	60.2
Inosine	8	17.1	2'-AMP	21	34.9	5'-ADP	33	46.2	5'-GTP	43	64.3
Hypo-xanthine	9	17.5	5'-GMP	22	38.0	5'-IDP	34	48.0	unknown	44	67.0
Adenine	10	19.0	3',5'-cyclic-AMP	23	40.0	5'-GDP	35	51.8			
Xanthine	11	21.5				UDPGA	36	54.8			
Ribo-flavin	12	23.8	FMN	24	42.8						
			3',5'-cyclic GMP	25	43.1						
Guanosine	13	34.0									
Guanine	14	35.0	5'-XMP	26	44.9						

* The two retention data for NADH and NADP were considered to originate from the mixture of 2'- and 5'- of adenosine of these standards.
** Standard compounds were dissolved in distilled water and separated by HPLC.

peak No. 41 and 43 in Figure 4 are shown respectively in Figure 6. The wavelength of the absorbing maximum of the difference spectrum of other peaks are listed in Table 2 and they were good agreement with those of the standard compounds (22).

DISCUSSION

The nuclectide profiles of the whole blood and erythrocytes of humans and other animals were previously reported by Brown (5). She used pellicular anion-exchange

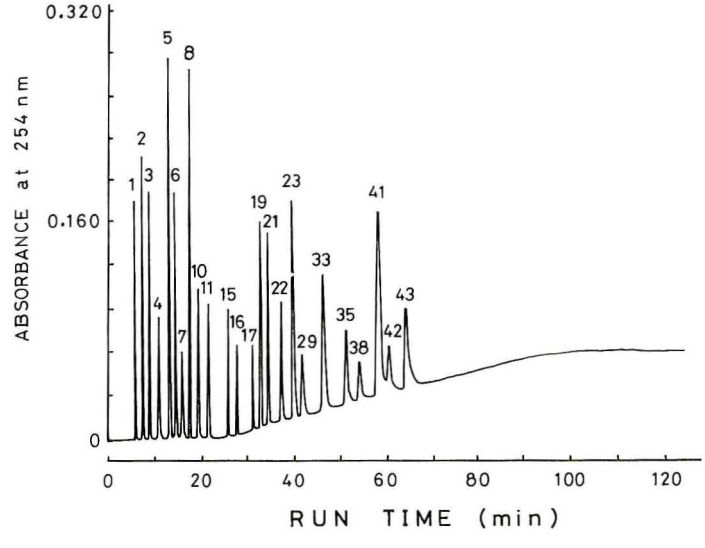

FIGURE 5. Representative chromatogram of nucleic acid components and related compounds. Run conditions were the same as in FIGURE 1. Sample: 100-uL of a mixture of standard compounds containing 2.5 ug/100-uL (except for 5 ug/100-uL of 5'-ATP and 5'-GTP).

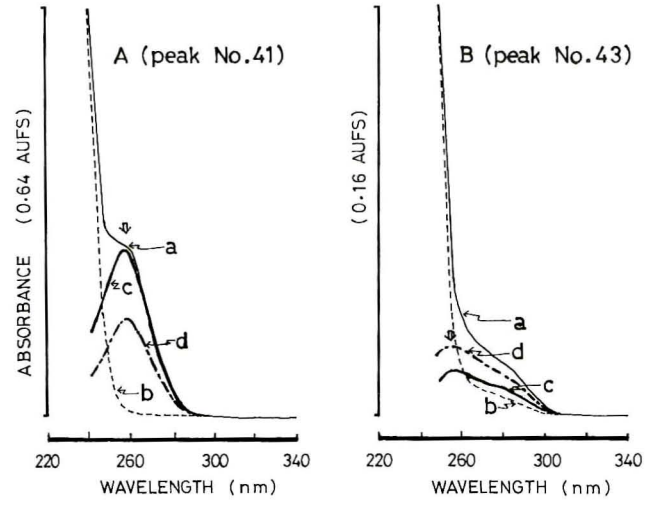

FIGURE 6. UV-spectra of peaks by stopped-flow scanning spectrophotometry.
A (peak No.41) and B (peak No.43).
Curve a (——): UV-spectrum of the peak at peak maximum, curve b (----): UV-spectrum of control, curve c (—·—): the difference spectrum (curve a minus curve b), and curve d (——): the difference spectrum of a standard compound under the same conditions as for curve a.
⇩ indicates absorbance maximum.

TABLE 2

The Wavelength of The Absorbance Maximum of The Difference Spectrum of Peaks

Peak number	*Wavelength* (λ_{max} nm)	*Assignment of the peak*
8	250	Inosine
15	258	NAD(DPN)
19	257	5'-AMP
22	255	5'-GMP
33	257	5'-ADP
35	255	5'-GDP
41	257	5'-ATP
43	255	5'-GTP

resins for the separation of nucleotides and microreticular resins for the separation of purine and pyrimidine bases and nucleosides. However, chromatography using a pellicular anion-exchange resin is lower in ion-exchange capacity and in resolution (13-15), and chromatography using microreticular resins requires a relatively longer separation time resulting lower sample throughput rates (8-10) compared with the present system. Furthermore, the overall analysis of nucleic acid components, including N-bases, nucleosides and nucelotides, could not be achieved with conventional anion-exchange resin column. Thus, the chromatography using the macroreticular anion-exchange resin described above showed better separation of nucleic acid components and related compounds.

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DETERMINATION OF THIAMPHENICOL IN SERUM AND CEREBROSPINAL FLUID WITH HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A procedure for quantitation of thiamphenicol in serum and cerebrospinal fluid was developed using high-pressure liquid chromatography. The drug was extracted from biological samples with methanol and separated by reverse-phase high-pressure liquid chromatography. Detection and subsequent quantitation were performed at 254 nm by on-line ultraviolet spectrophotometry. After the intramuscular administration of a single dose of 1 g of thiamphenicol to a patient, a poor transmission of the drug across the hematoencephalic barrier was demonstrated by this assay.

INTRODUCTION

The properties of thiamphenicol (TAP), a broad spectrum antibiotic with an antimicrobial spectrum similar to that of chloramphenicol, would be particulary useful nowadays. This antibiotic is a more soluble weak base (pKa $\pm$ 7.2) than chloramphenicol, it is only slightly bound to plasma's proteins ($\pm$ 10 %), and is not inactivated in the body by metabolic processes (1) (2) (3) (4) (5).

The difference in toxicity of these two products would be an important indication for the use of thiamphenicol. Only a few

results have previously been reported concerning the plasma kinetics and on the crossing of thiamphenicol to the cerebrospinal fluid (6) (7) (8) (9) (10) (11) (12). Several methods have been described for the determination of TAP in biological materials, including microbiological assay, colorimetry and gas chromatography with various detectors (13) (14) (15) (16).

The microbiological assay, in which a cup-plate method is used with pasteurella bovisepticus and sarcinea lutea as test organisms, is inaccurate when other antibacterial agents are administered together and when thiamphenicol is metabolized in active compounds. The colorimetric procedure is time consuming and lacks sensivity. The gaz chromatographic assay with different modes of detection seems to be time consuming due to the extraction and derivatization procedures that are required. High-pressure liquid chromatography (HPLC) has been largely used for determination of chloramphenicol (17) (18) (19). The chemical analogy of these two antibiotics, which differ only by a group on the benzene ring and allowsa more lipophilic comportment for chloramphenicol, indicates by their partition coefficient, the possibility of a similar behavior in high-pressure liquid chromatography (20) (21) (22). In the present study, this technique offers an opportunity to detect thiamphenicol in plasma and cerebrospinal fluid. This method is as rapid as the methods for chloramphenicol, and has the same specificity and accuracy. A concentration of 0.5 µg thiamphenicol per ml could be measured on sample as small as 100 µl. Thiamphenicol glycinate is totally hydrolysed in thiamphenicol alcohol in plasma and could not be determinated in this case.

MATERIAL AND METHODS

a) Chromatographic equipment

A Waters ALC/GPC 204 liquid chromatograph was used (Waters Associates, Paris, France). It consisted of a model 6000 solvent delivery system, and U6K universal injector and a model 440 U.V. absorbance detector. Absorbance was recorded on a 10 mV chart recorder.

b) Solvents and standards

Freshly distilled deionized water used throughout the procedure. Methanol was analytical grade (Merck, Darmstadt, Germany). Thiamphenicol alcohol was kindly donated by CLIN-MIDY, Paris, France.

c) Chromatographic eluent

The mobile phase consisted of a mixture of water and methanol (80 : 20, V/V), passed through a 0.6 µm filter (Millipore Corp., Bedford, Mass., U.S.A.) and deaerated by ultrasonics.

d) In vitro samples

Serum Thiamphenicol alcohol stock solution (100 $\mu g.ml^{-1}$) was directly prepared in pooled human plasma and congealed in aliquots at - 80°C. An aliquot was decongealed and diluted in pooled human plasma before use.

CSF Thiamphenicol alcohol stock solution (10 $\mu g.ml^{-1}$) was directly prepared in pooled human CSF and congealed in aliquots at - 80°C. It was decongealed before and diluted with CSF for standardisation.

e) In vivo samples

One patient was given thiamphenicol glycinate in physiological saline (equivalent to 1 g of thiamphenicol) intramuscularly. Blood and CSF were drawn prior to injection and at various times up to 12 hours. All the more different hospitalized patients were taking of blood for a test one hour after intramusculary injection of one g of thiamphenicol glycinate. An aliquot of each sample was dosed one day after experiment and others aliquots were stored at -30°C and - 80°C up to three months before analysis. No signs of decomposition were observed and identical determinations were found.

f) Chemical assay

- Extraction

  100 µl of methanol was added to 100 µl of plasma or CSF and standard solutions. The samples were mixed, then centrifuged for 10 minutes (at 2000 g) and the supernatant

passed through 0.22 µm filters and injected into the chromatograph.

- Chromatography

A reverse phase system was chosen to quantitate thiamphenicol. 100 µl of the extract was injected on to a µ-Bondapack C-18 (Waters) and eluent pumped through at 1 ml/min. The absorbance detector was set at 254 nm at a sensivity of 0.005 absorbance units full scale. Similar results was obtained at 268 nm at a sensivity of 0.01 absorbance units full scale. Quantitation was based on peak heights recorded. Two standard curves were used : One on plasma and the other for CSF.

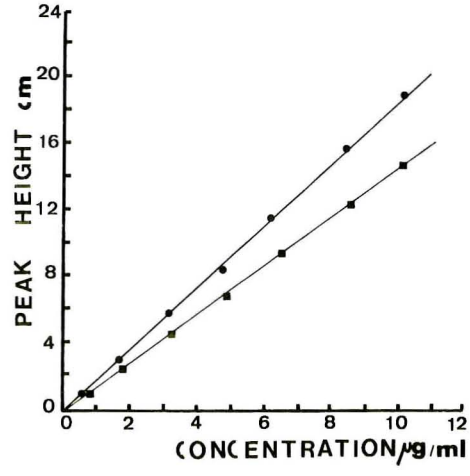

FIGURE 1

Standard curve for serum ■ and CSF ● thiamphenicol determinations. Chromatographic peak height expressed as a function of concentration in serum.

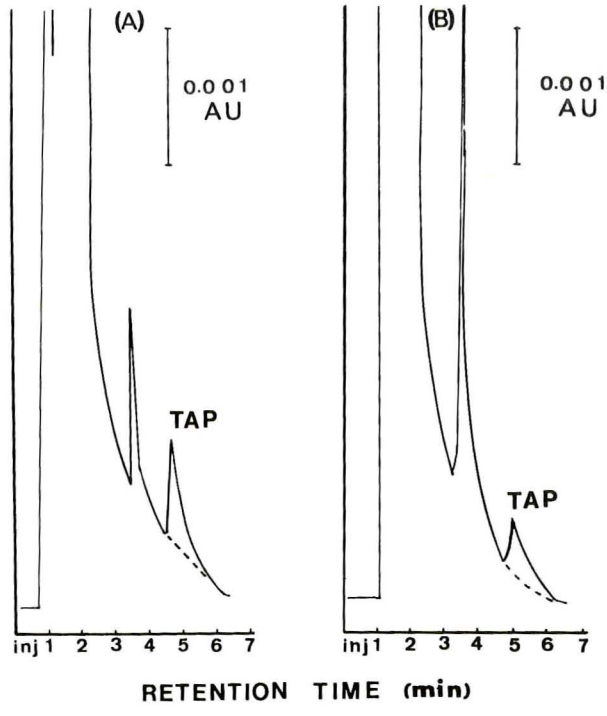

FIGURE 2

Chromatographic separation of thiamphenicol (TAP) in extract of serum (A) and of cerebrospinal fluid (CSF-B). The dashed lines are for the blanks of serum and CSF.
Colum : μ bondapak C 18, 30 cm x 4 mm I.d (Waters Associates).
Mobile phase : (water : methanol, 80 : 20, V/V).
Flow rate : 1 ml/min. Sample volume : 100 μl for serum extract and for CSF extract.

RESULTS

Chromatographic separation

With the conditions described, thiamphenicol had a retention time of 5 minutes and chloramphenicol had a retention time of 17.5 minutes in the same system. Thiamphenicol in plasma and CSF extract had the same retention time ; it was the same in aqueous solutions

of the drug. No interfering peaks were observed in chromatogramms of plasma or CSF. The more polar metabolized forms of thiamphenicol were eluted in the first peaks due to plasma or CSF and were undetectable for this reason.

Recovery

By assaying in vitro samples of known concentrations against standard curves obtained from pool control samples, recovery from serum and CSF was calculated and found to be quantitative.

By assaying in vivo samples of elevated concentration obtained from patients at the first hour after injection, recovery was also found to be quantitative for serum.

Sensitivity

A thiamphenicol concentration of 0.5 µg/ml serum of CSF could be accurately determined (peak height 5 mm).

TABLE I

Amount added to serum (µg/ml)	Amount added to CSF (µg/ml)	Amount measured* (µg/ml)	Recovery
1		0.97	97
2		1.95	97.6
5		4.9	98
8		7.83	97.9
10		10.15	101.5
	1	0.98	98
	2	1.97	98.7
	5	4.95	98.9
	8	7.92	99
	10	10.10	101

Recovery of Thiamphenicol from Serum and CSF

*Each value represents the mean of duplicate analyses.

TABLE II

Recovery of thiamphenicol from serum by dilution

	Serum of patients (µg/ml)	Dilution	Amount measured* (µg/ml)	Recovery
Serum A	5.4	0	5.4	100.0
		1/2	2.6	96.2
		1/4	1.2	80.9
Serum B	6.3	0	6.9	100.0
		1/2	3.0	95.2
		1/4	1.5	95.2
Serum C	10.4	0	10.4	100.0
		1/2	5.1	98.0
		1/4	2.5	96.2

*Each value represents the mean of duplicate analyses.

Precision

Intra-assay variation was determined by assaying a serum sample in two separate runs on the same day. For a concentration of 5 µg/ml the variation coefficient was 2.8 % and for 8 µg/ml it was 3 % and 0.9 % for 12 µg/ml. Interassay variation was calculated by dividing a serum sample into 5 portions which were stored at - 30°C and - 80°C and assayed at different weeks during a five weeks period. For a concentration of 5 µg/ml, the variation coefficient (with one standard deviation) was 3.1 % and at a concentration of 12 µg/ml it was 1.5 %.

Patient values

The kinetics of thiamphenicol in serum showed a period of elimination of 5 1/2 hours if we used a monoexponential representation for one patient ; his intramuscular injection (1 gram of thiamphenicol) gives a maximum value of 5.4 µg/ml ; in cerebros-

pinal fluid the values of the concentration were very low (0.6 to 1.2 µg/ml). Two other patients with therapeutic doses (1 g, IM) didn't show thiamphenicol in cerebrospinal fluid.

DISCUSSION

The procedure described above is more specific and sensitive than the widely used colorimetric and microbiological methods ; it is easier to perform than the gas chromatographic assay. This method is also less time consuming ; the largest step was the centrifugation following extraction. The limit of sensivity could be decreased by means of two processes : First, a larger sampling volume (1 ml serum) and ethyl-acetate extraction (2 ml) followed by evaporation and redissolution in mobile phase (50 µl) before injection ; secondly, the wavelenght must be set at 268 nm at

TABLE III

Analysis of clinical samples from three patients receiving thiamphenicol therapy (1 g, I.M. at t = 0).

	Time of prelevment after I.M. injection (hours)	Amount* measured in serum	Amount* measured in CSF
Patient A	1	5.28	0.7
	3	4.32	0.6
	6	2.40	0.9
	9	1.76	0.8
	12	1.52	0.8
Patient B	1	10.40	-
Patient C	1	6.30	-

*Each value is the mean of duplicate analyses. For patient B and C the limit of detection was lowed to 0.1 µg/ml in CSF sample and no thiamphenicol was measured.

0.01 full scale sensitivity ; this procedure was not useful for our clinical samples where serum or cerebrospinal concentrations of thiamphenicol were above the limit of sensivity. The metabolism of thiamphenicol as previously quoted is not significant and direct determination of thiamphenicol seems to be sufficient. In spite of this fact, samples could be dansylated or silylated and separated in a reverse phase system and its metabolites (glucuroconjugated and acylamine) quantitated. The first results obtained by this method, show a poor transfer of the drug into the CSF and, for two patients, thiamphenicol was respectively 6.3 and 10.4 µg/ml in serum after (1 gram intramusculary injection) one hour ; no thiamphenicol in CSF was detectable at the same time. These facts could be explained by the polarity of this molecule in comparison of the polarity of chloramphenicol which enters investigate into CSF in a significant proportion. Indeed, we need to further investigate this subject. This simple method seems to be very useful for such studies.

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DETERMINATION OF VITAMIN D IN MULTIVITAMIN PREPARATIONS BY HPLC

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ABSTRACT

A selective extraction for vitamin D was achieved from oils, dry concentrates and multivitamins without the need for saponification. This was accomplished by utilizing SEP-PAK cartridges as an alternative to the pre-column clean-up required in the standard vitamin D assay. The final LC analysis was streamlined by using Radial Compression Technology in place of the conventional analytical LC column.

INTRODUCTION

Use of High Performance Liquid Chromatography has been investigated as an approach to the determination of vitamin D_2 (D_3) in various feedstuffs and vitamin preparations. HPLC provides a greatly improved vitamin D assay by making it more rapid and specific than conventional methods; however, nearly every method relies on a complicated, time-consuming extraction and sample preparation procedure prior to injection onto the HPLC column.

In general, extraction of vitamin D out of its matrix has been a major problem, as vitamin D is present in very small amounts in formulations containing large amounts of vitamin A and, also, substantial amounts of other fat soluble vitamins.

Any method for vitamin D analysis requires accurate quantitation that is applicable to all types of multivitamin preparations including resins, oils, dry concentrates and gelatin protected beadlets.

Recent applications of HPLC to the determination of vitamin D include analysis from gelatin-protected beadlets (1). Vitamin D has been separated from its photochemical isomers (2), from inactive forms out of resins, oils and dry concentrates by Hofsass et al. (3), Oscada et al. (4), and deVries et al. (5).

Analysis of vitamin D has been accomplished from livestock feeds by HPLC (6, 7). Various reverse phase systems have been reported for analyzing vitamins A, D and E simultaneously from multivitamin tablets (8, 9) and from animal feeds (10).

Although HPLC has been found to be the most specific method for determining bioactive isomers only (3), not all systems distinguish active from inactive isomers. The presence of intermediate irradiation products in many preparations may result in higher than actual vitamin D potency in cases where there is no distinction made between them and active vitamin D. A comparison of the A.O.A.C. chemical method and the A.O.A.C. biological method shows this, indeed, to be the case (2).

A method has been developed which involves dissolution of vitamin D by homogenization and followed by extraction from other sample components by means of SEP-PAK cartridges. A normal phase HPLC system is used in the final analysis as in the work of deVries et al. (5) which separates pre-D and cis-D from all inactive isomers. This allows quantitation based on the sum of these two forms providing there are no compounds in the sample matrix with similar extraction and retention characteristics.

An interference, determined to be an impurity from vitamin A, was sometimes encountered when wavelength ratioing of the 280nm to 254nm detector response revealed an incorrect ratio in the cis-D peak. This interference was removed by employing a different solvent and simple extraction procedure that will be discussed below.

MATERIALS

Reagents and Solvents

LC grade n-hexane, tetrahydrofuran and methanol were used (Waters Associates, Milford, MA). Spectrograde ethyl acetate and

LC grade diemthyl sulfoxide were obtained from Burdick and Jackson, Muskegon, Michigan. Ethanol (85%), completely denatured, and normal amyl alcohol, AR, obtained from Mallinckrodt, St. Louis, Missouri.

Crystalline butylated hydroxy toluene (BHT), cholcalciferol and ergocalciferol were obtained from Sigma Chemical Company, St. Louis, Missouri. SEP-PAK C_{18} and SEP-PAK Silica cartridges (Waters Associates, Milford, MA).

System suitability standard used to check column efficiency was an oil containing approximately 2mg each of vitamin D_3 and tachysterol and 0.2mg each of pre-vitamin D_3 and 5,6-trans vitamin D_3 per gram of oil. System suitability standard and Δ4,6=cholestadienol were obtained from Philips-Duphar, Amsterdam, Holland.

Preparation of Standard Solutions

a. Vitamin D_3 Standard Solution - 50mg of cholcalciferol was dissolved in 100ml hexane and 10ml of this solution was diluted to 100ml with hexane in volumetric flask.

b. Internal Standard Solution - 15mg of Δ4,6-cholestadienol was placed in 200ml volumetric flask and diluted to volume with hexane.

   1. Procedure A: 50mg of Δ4,6-cholestadienol was placed in a 50ml volumetric flask and diluted to volume with 50:50 Methanol/THF.
   2. Procedure B: 10ml of internal standard solution was diluted to 100ml with hexane.

Determination of D_3 Response Factor

Pipet 4.0ml of D_3 standard solution and 10ml of internal standard solution into a 100ml volumetric flask and dilute to volume with hexane. Inject 200ul and determine peak heights of the Vitamin D_3 and internal standard. Calculate vitamin D_3 response factor by the equation:

$$F_D = (P_{ir} \times W_r \times V_{ir}) \div (P_r \times W_{ir} \times V_r)$$

Where P_{ir} = internal standard peak height

P_r - vitamin D_3 peak height

W_{ir} = weight of internal standard in mg (15)

W_r = weight of vitamin D_3 in mg (50)

V_{ir} = final volume in ml of internal standard (2,000 ml)

V_r = final volume in ml of vitamin D_3 (25,000 ml)

Determination of pre-D_3 Response Factor

Pipet 5.0ml of D_3 standard solution into a 100ml amber volumetric flask, add 20mg BHT crystals, replace air by nitrogen, heat 3/4 hour in subdued light in a 90°C waterbath and cool. Add 10ml internal standard solution and dilute to volume with hexane. Inject 200ul and measure peak heights of pre-D_3, D_3 and internal standard.

Calcualte D_3 content (in % amount in unheated soln) $(F_D \times P_D \times V_r \times W_{ir}) \div P_{ir} \times V_{ir} \times W_r) \times 100 = q\%$. Calculate pre-vitamin D content by 100 - q% = p%. Calculate pre-D response factor as follows:

$$F_{pre} = (P \times (P_{ir} \times V_{ir} \times W_r) \div 100 \times P_{pre} \times V_{pre} \times W_{ir})$$

Where F_D = Vitamin D response factor

P_D = peak height of vitamin D_3 in heated solution

P_{pre} = peak height of pre-vitamin D in heated solution

P_{ir} = peak height of internal standard in heated solution

W_r = weight D_3 in mg (50)

W_{ir} = weight internal standard in mg (15)

V_r = final volume in ml vitamin D in heated solution (20,000 ml)

V_{ir} = final volume in ml internal standard in heated solution (2,000 ml)

V_{pre} = final volume in pre-D in ml preD in heated solution (20,000 ml)

Calculate Conversion Factor

$F = F_{pre}/FD$

This conversion factor must be determined for each new column and should be checked routinely.

HPLC Chromatographic Procedure

All analyses were performed with a Waters Associates Model ALC/GPC 244 liquid chromatograph equipped with a Model 440 UV detector monitoring at 254nm and 280nm simultaneously. Waters' Model 730 Data Module was used to integrate all peaks and calculate results.

All separations were achieved using a RCM-100 Radial Compression Module (Waters Associates, Milford, MA) equipped with a Radial-PAK B normal phase chromatographic cartridge. The cartridge was equilibrated with n-hexane containing 0.35% n-amyl alcohol for approximately 2 hours at a constant flow of 5.0 ml/min before analysis.

The system suitability standard was injected to check column efficiency before any analyses were run. Resolution of $\geq$ 0.8 of

pre-vitamin D from trans-vitamin D and $\geq$ 1.0 of cis-D from tachysterol were requirements to assure accurate quantitation of bioactive isomers only. Figure 1 shows a chromatogram of the system suitability standard obtained with the Radial-PAK B cartridge used. Retention of pre-D should be approximately 7 minutes and cis-D approximately 13 minutes. This test was repeated on a weekly basis and each time a new cartridge was used.

Sample Preparation

Dry concentrates, resins and oils - an amount of sample containing 50,000 I.U. vitamin D is accurately weighed.

Multivitamins from 5-10 tablets, accurately weighed, are coarsely ground.

Procedure A: Sample was transferred to a homogenization flask with the aid of 20ml ethanol, homogenized 5-10 minutes (time depends on type of sample matrix), centrifuged and a 2ml aliquot was transferred into a vial and 75ul of internal standard solution #1 was added followed by 1ml water. This mixture was pumped

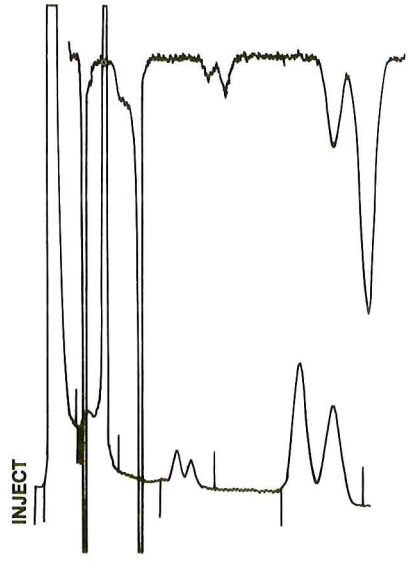

Figure 1. HPLC of system suitability standard on the Radial-PAK B cartridge used.

through a prepared C_{18} SEP-PAK, which was then flushed with 10ml of a 25/25/50 Methanol/THF/H_2O solution. Vitamin D, pre-D and internal standard were then eluted with 10ml methanol (100%) and the resultant eluate evaporated to dryness over sodium sulfate. The residue was redissolved in 5ml hexane and 200ul were injected. Figure 2 shows an oil analyzed in this manner.

Procedure B: Sample was transferred to a homogenization flask with the aid of 20ml DMSO, homogenized 5-10 minutes, centrifuged and 2ml were then transferred to a vial, 5ml of internal standard solution #2 was added and shaken vigorously for 30 seconds. The vial was placed in ice-cooled water and 1ml of ice-cooled water was added to the vial. After 1 minute the vial was shaken vigorously for another 30 seconds, 2ml of the hexane layer was separated and pumped across a silica SEP-PAK and the SEP-PAK was flushed with 3ml of 85/15 hexane/ethyl acetate. Vitamin D, pre-D and internal standard were eluted with 5ml of 80/20 hexane/ethyl acetate and evaporated to dryness. The residue was redissolved in 2ml hexane and 200ul was injected. Figure 3 shows an oil analyzed as described.

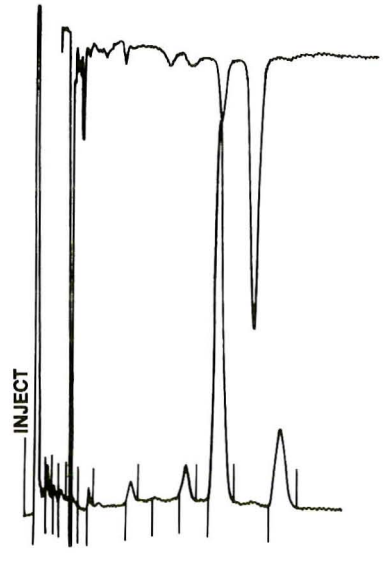

Figure 2. HPLC of an oil containing 40,000 I.U./gram Vitamin D prepared by Procedure A.

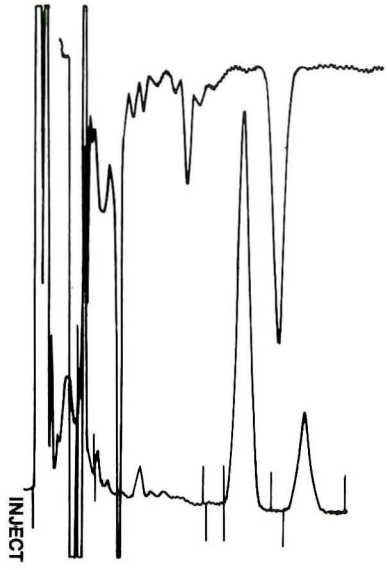

Figure 3. HPLC of an oil containing 40,000 I.U./gram Vitamin D prepared by Procedure B.

Calculation

D potency in I.U./gram in sample is calculated using the following equation:

$$P_D + (P_{pre} \times F) \times F_D \times W_{ir} \times V_s \times 40{,}000 \div (P_{ir} \times W_s \times V_{ir})$$

Where P_{ir} = peak height of internal standard in sample
P_D = peak height of pre-vitamin D in sample
P_{pre} = peak height of pre-vitamin in sample
F - conversion factor
F_D = response factor of vitamin D_3
W_{ir} = weight internal standard in mg (15)
W_s = weight of sample in grams
V_s = final volume in ml of sample solution (50ml)
V_{ir} = final volume in ml of internal standard (20,000ml)
40,000 - number of I.U. vitamin D per mg in USP reference std.

RESULTS AND DISCUSSION

The HPLC conditions used permit the resolution of vitamin D_3 (D_2) from all inactive isomers which may be present, such as irrad-

iation by-products or breakdown products formed as a result of accelerated storage conditions (i.e. elevated temperature or direct light).

Results of analyses performed on three different vitamin types (dry concentrate, oil, gelatin encapsulated multivitamin) by Procedure A compated very well with those obtained by the proposed A.O.A.C. method (Fig. 4). A problem was encountered, however, in the analysis of certain types of dry multivitamin formulations, which gave unusually high vitamin D potency. Employing wavelength ratioing of the 280nm - 254nm detector response, an incorrect ratio revealed an interference in the cis-D peak. (The proper wavelength ratio for the vitamin D standard should be determined for each set of filters and be less than that at 254nm resulting in a ratio < 1.0.) This interference, which appears to be an impurity in the vitamin A, has a larger absorbance at 280nm giving a ratio > 1.0.

This difficulty with multivitamin analysis resulted in the development of Procedure B using DMSO as the dissolution solvent which does not extract the interfering substance and results in the correct wavelength ratio in the final analysis.

Because no saponification step is necessary, the actual ratio of pre-vitamin D to vitamin D is preserved. This method is also applicable to oils, dry concentrates, gelatin encapsulated or dry multivitamins.

Results of AOAC Method and Radial Compression Techniques

Type of Sample	Concentration Range	AoAc Method	Radial Compression Technique
Concentrated Oil	40,000 I.U./gm (± 30%)	31,666 35,310	41,042 42,319
Dry Powder	100,000 I.U./gm (± 30%)	88,833 90,504	90,624 94,331
Dry Powder	50,000 I.U./gm (± 30%)	49,629 48,942	49,296 48,238
Multi-Vitamin	400 I.U./Cap. (± 50%)	752 677	576 646

Figure 4. Results of AOAC method and Radial Compression Techniques

Time required for smaple preparation has been reduced from 2-3 hours to 15 minutes, enabling 20-30 samples to be run per day by either procedure. A problem remains, however, in the quantitation of pre-vitamin D in some types of multivitamins. Figure 5 shows a typical multivitamin analyzed by Procedure B. The vitamin D is well separated and may be accurately quantitated in all cases; however, the pre-vitamin D region of the chromatogram is crowded with compounds which obscure the pre-D peak. Work is currently in progress on a method to separate pre-D from these interferences.

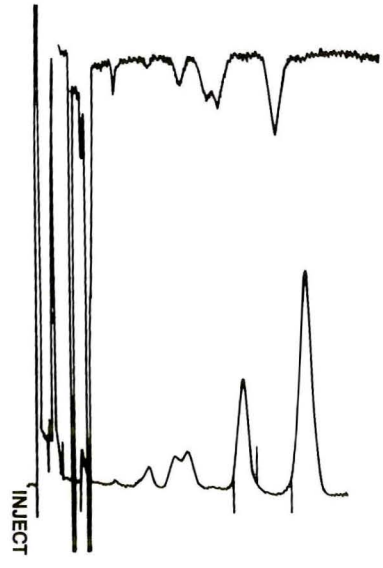

Figure 5. HPLC of a typical multivitamin analyzed by Procedure B.

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AN IMPROVED ANALYSIS OF THE PHEROMONE 3-METHYL-2-CYCLOHEXEN-1-ONE IN A CONTROLLED RELEASE FORMULATION BY USING LIQUID CHROMATOGRAPHY

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ABSTRACT

A method was developed for estimating the quantity of the pheromone 3-methyl-2-cyclohexen-1-one in a controlled release formulation. The thermal method measured the amount of releasable pheromone remaining in the formulation by using liquid chromatography with a variable wavelength ultraviolet detector. This method is 150 to 250 times more sensitive than the liquid chromatographic method used previously.

INTRODUCTION

During the evaluation of a controlled release formulation of 3-methyl-2-cyclohexen-1-one (MCH), the antiaggregation pheromone of the bark beetle *Dendroctonus pseudotsugae* Hopkins, it was necessary to estimate the quantity of the pheromone available for releasing that remained in the formulation. An accelerated release method was needed to remove and collect the remaining formulated pheromone for analysis. The pheromone was estimated previously by using a method of high performance liquid chromatography (1). The method, however, was not sufficiently sensitive because of a limitation imposed by a fixed wavelength ultraviolet detector at 254 nm that did not allow for a minimum of MCH less than 1 μg.

This paper reports on a thermal method for rapid release of the formulated pheromone and the analysis of it by high performance liquid chromatography in two modes, by using a variable wavelength ultraviolet detector. The method was developed for estimating quantities of MCH in the nanogram range.

EXPERIMENTAL

Apparatus

A Waters Associates (Milford, Massachusetts) (2) ALC 200 liquid chromatographic instrument equipped with a model U6K injector was used. The detector was a Tracor Instrument (Austin, Texas) model 970, set from 224 to 235 nm. The columns used were 2 mm x 61 cm packed with Corasil-1 (Waters Associates) and 3.9 mm x 30 cm packed with μ-Bondapak C_{18} (Waters Associates). A pre-column (Altex #255-56, Altex Scientific Inc., Berkeley, CA) was used in the reverse-phase column. The apparatus was operated at ambient temperature.

The heating apparatus for the formulation was a glass tube that was fitted with a nitrogen gas inlet at one end and connected with a piece of Tygon^T tubing at the other end to a smaller glass tube. The smaller glass tube was stuffed with glass wool at the outlet end and contained from 1.5 to 2 g of an absorbing medium. The larger tube was wrapped with a heating tape, and was connected together with a thermocouple to a recording temperature controller (Figure 1).

Materials

3-Methyl-2-cyclohexen-1-one was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wisconsin as 98% MCH. The controlled release MCH formulation was on a polyamide matrix, as described by Furniss (3). The absorbing medium was Porapak-QS/80-100 mesh (Waters Associates). The solvents used were dioxane (Mallinckrodt 4951), trimethylpentane (Mallinckrodt 6051), methanol (MCB MX684), and distilled water.

Procedure

A small quantity (0.5 g) of the controlled release formulation of MCH estimated to contain about 2% of the pheromone was placed in a porcelain combustion boat. The boat was placed in the heating tube, the tube heated to 150-160°C, and nitrogen was swept through the tube at 25 to 50 cm^3/min. Heating and sweeping were continued for 12 hours, an interval found to be sufficient for removing all of the releasable pheromone at the prescribed temperature. The Porapak containing tube was removed and eluted with either dioxane-

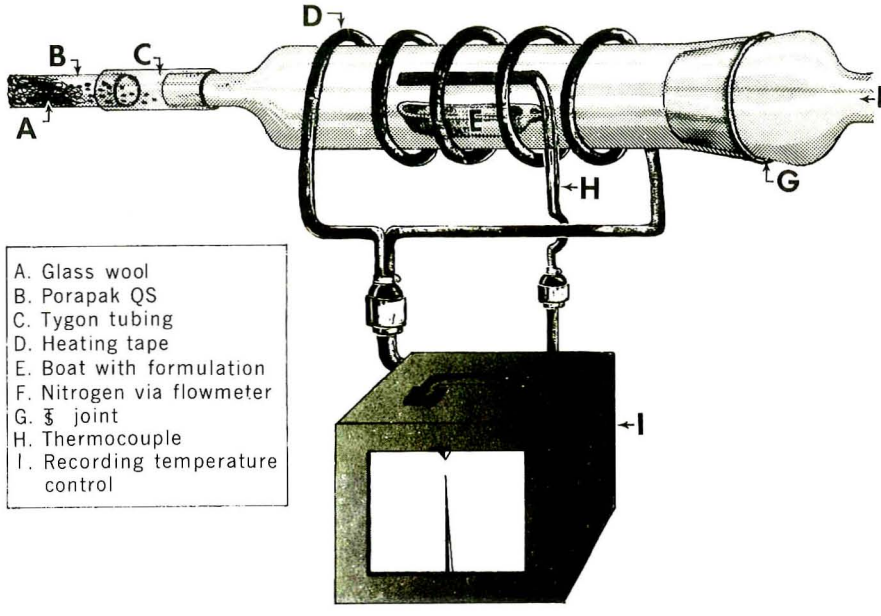

FIGURE 1. The thermal elution apparatus used to heat the controlled release formulation.

trimethylpentane (3:7) or methanol, depending on the phase type of the column to be used. Sufficient solvent was passed through the tube to fill a 25-ml volumetric flask.

For normal-phase chromatography, samples of 1 to 25 µl were injected into the Corasil-1 column and were eluted with a mixture of dioxane-trimethylpentane (3:7) at a flow rate of 2 ml/min. The quantities of MCH were estimated from a calibration on the basis of peak height. Analyses of 30 to 200 ng MCH per injection were made at an absorbance unit, full scale (AUFS) setting of 0.04 at 235 nm. When analyzing smaller amounts of MCH, the detector was set at 224 nm by using a calibration curve at this wavelength.

For reverse-phase chromatography, samples of 1 to 25 µl were injected into the µ-Bondapak C_{18} column and were eluted with a mixture of methanol-water (1:1) at a flow rate of 1 ml/min. The

MCH was estimated from a calibration curve on the basis of peak height. From 50 to 250 ng MCH were analyzed at an AUFS setting of 0.08 at 235 nm. Smaller quantities were analyzed at lower settings.

RESULTS AND DISCUSSION

Unlike the MCH released from the polyamide matrix at ambient temperature (1), the pheromone released at elevated temperatures was accompanied by interfering peaks in the normal phase (Figure 2). These peaks probably resulted from a decomposing matrix, or excess monomers in the polymer, or both. The peaks were not noticed in reverse-phase chromatography where a precolumn was used (Figure 3). Reverse-phase chromatography was also less critical of solvent impurities than was a normal phase. Both methods were equally sensitive: at an AUFS of 0.01 at 224 nm, a 4 ng-sample gave a peak

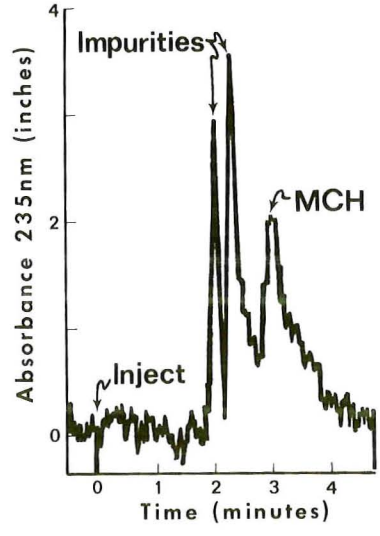

FIGURE 2. Chromatogram of MCH released at elevated temperature from polyamide matrix. UV detector at 0.04 AUFS. Mobile phase: dioxane-trimethylpentane (3:7). Flow rate: 1 ml/min. Corasil-1 2 mm x 61 cm column. 10-inch, 10-millivolt recorder at 0.5 inch/min.

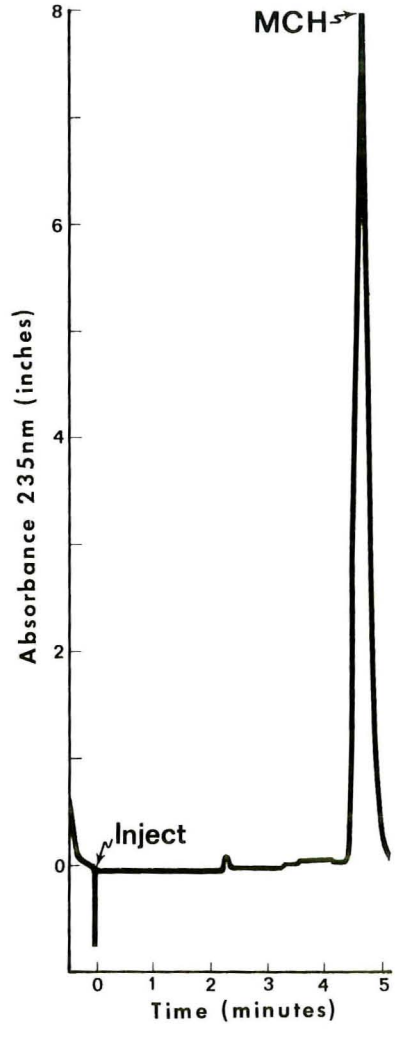

FIGURE 3. Chromatogram of MCH released at elevated temperature from polyamide matrix. UV detector at 0.08 AUFS; mobile phase: methanol-water (1:1); flow rate:/ml/min.; μ-Bondapak C_{18} 3.9 mm x 30 cm column; quantity MCH 0.55 μg; recorder: 10-inch, 10 millivolt at 0.5"/min.

of 2 cm on a 25-cm, 1-millivolt recorder. The signal-to-noise ratio was *ca.* 4:1.

This method of removing and estimating the quantity of MCH from a controlled release formulation compares favorably with that of the solvent extraction method developed by another laboratory. The results were within 0.05% (typical analysis of MCH in matrix was 1.3%) of each other (4).

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2. Trade names and commercial products or enterprises are mentioned solely for information. No endorsement by the U. S. Department of Agriculture is implied.
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DETERMINATION OF SERUM CHLORAMPHENICOL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

by

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ABSTRACT

A simple single extraction procedure for the analysis of serum chloramphenicol levels by high performance liquid chromatography (HPLC) is described. Serum is mixed with buffer and extracted with ether, which is then evaporated. The residue is dissolved in the eluting solvent and analyzed on a reverse-phase column. The eluting solvent is methanol/distilled water (50/50, V/V) and the effluent is monitored at 280 nm. Serum samples as small as 50 µl can be used.

INTRODUCTION

High performance liquid chromatography (HPLC) is establishing itself as a clinical tool for the quantitation of drugs in biological fluids. The simplicity of sample preparation and the fact that, in many cases, the drug can be analyzed without synthesizing a derivative makes HPLC a rapid method of analyses. Chloramphenicol, while it is an effective antibiotic, does have several serious side effects (1). Thus, in those patients receiving the drug there is a necessity to monitor its concentration in the serum. The procedure described here can be carried out in less than one hour and is capable of measuring less than 1 µg of chloramphenicol per sample.

MATERIALS AND METHODS

Instrumentation

The chromatograph was a Model 110 pump and a Model 7120 Rheodyne sample injector. (The Anspec Co., Ann Arbor, Michigan). A Model UA-5 dual beam multiple wavelength dector, with 5 µl cells (Instrument Specialties Co., Lincoln, NB.), was used at a sensitivity setting of 0.10 and a wavelength of 280 nm. The column was a prepacked Partisil PXS 10/25 ODS-2 (Whatman, Clifton, NJ). A 10" recorder (Linear Instruments, Irvine, Ca.) was used at a 100 mv setting.

Reagents

Diethylether and methanol-analytical grade.

Mephenesin (3-(2-methylphenoxy)-propane-1, 2-diol) and chloramphinicol-obtained from Sigma Chemical Co., St. Louis, MO. Both were pure based on HPLC.

Bromocresol green-prepared by dissolving 0.1 g in 250 ml of distilled water containing 1.43 ml of 0.1N NaOH.

Tris buffer-prepared by dissolving 12.11 g of Tris (hydroxymethyl) aminomethane in 1 liter distilled water and adjusting the pH to 10.4 with 0.8N NaOH.

Internal Standard-prepared by dissolving mephenesin in methanol to a concentration of 0.2 mg/ml.

Mobile phase-methanol/distilled water (50/50, V/V), degassed under vacuum.

Procedure

The extraction procedure is a modification of that suggested by Thies and Fischer (2). The experimental sample (200 µl) is placed in a 5 ml Mini-Vial (The Anspec Co. Inc., Ann Arbor, MI). The mephenesin internal standard (20 ug) and 100 µl of Bromocresol green solution are added. The addition of Bromocresol green makes it easier

to define the aqueous phase and does not interfer with the assay. The Tris buffer (1 ml) is added and the contents of each Mini-Vial mixed, followed by the addition of 2 ml of diethyl ether. Each Mini-Vial is then shaken vigorously for 1 minute and centrifuged at 3000 rpm for 3 minutes. Following centrifugation the lower, blue, aqueous phase is aspirated away. This is accomplished using a disposable Pasteur pipette attached to rubber tubing, which can be squeezed to stop aspiration. We routinely let a small amount of the upper ether phase get into the pipette to ensure complete removal of the aqueous phase. Since the calculations are based on peak height ratios to the mephenesin internal standard this slight loss of the ether phase is of no consequence. The remaining ether phase is then evaporated at 35°C under a stream of nitrogen. The residue is dissolved in 25 µl of mobile phase and duplicate 10 µl aliquots are injected into the chromatograph. The mobile phase of methanol/distilled water (50/50, V/V) is delivered at a flow rate of 2 ml/min.

For routine daily analysis of plasma samples we use two control serum samples (a high and a low) prepared by adding chloramphenicol to pooled samples of serum previously found to be free of the drug or any interfering ether soluble substances. The high level sample containing 55 mg/L has given a peak height ratio (chloramphenicol/mephenesin) of 4.49 ± 0.31 (S.D., n = 25) and the low level sample containing 13.6 mg/L has given a peak height ratio of 1.03 ± 0.02 (S.D., n = 25). We have found no change in these ratios over a 2 month period with storage at 5°C.

RESULTS AND DISCUSSION

Typical HPLC chromatograms obtained by our procedure are shown in Figure 1. As can be seen good separation is achieved between

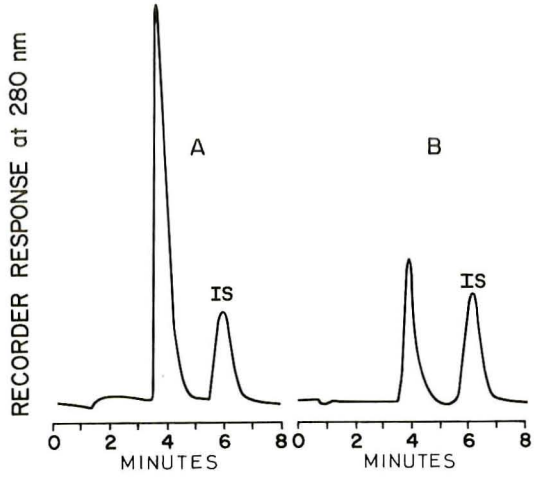

FIGURE 1

Typical chromatograms showing the separation of chloramphenicol and mephenesin internal standard (IS). The chromatogram in A is from a 55 mg/L prepared control serum. Chromatogram B was obtained from the serum of a patient under drug therapy.

chloramphenicol (elution time 4 minutes) and mephenesin (elution time 6.5 minutes). Figure B is a typical serum sample and shows the absence of any interfering substances. For comparison, the level of chloramphenicol in the sample in Figure 1B was calculated to be 15.7 mg/L.

Figure 2 is a typical standard curve and shows the linearity obtained by our procedure. One can extend the sensitivity into the ng level by adjusting the detector sensitivity range or changing the recorder mv range. However, the range of the standard curve given in Figure 2 is adequate for routine clinical samples.

We have assessed the recovery of chloramphenicol by comparing peak height ratios of standard solutions of chloramphenicol and

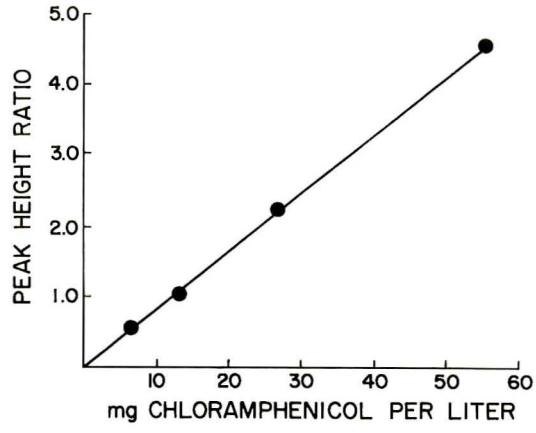

FIGURE 2

Plot showing the linearity of peak height ratio of chloramphenicol to mephenesin versus the concentration of chloramphenicol.

mephenesin in methanol/water (50/50, V/V) injected directly into the chromatograph to peak height ratios obtained on the same samples following the use of our procedure. The recoveries were 96.9 ± 0.2 (S.D., n = 6) and 101.2 ± 0.1 (S.D., n = 6) for samples of 10 mg/L and 50 mg/L respectively.

The assay described here can conveniently be scaled down to as little as 50 µl of sample, thus making it quite applicable to pediatric patients.

Others (2) have shown that the buffer used in this assay does not release chloramphenicol from its glucuronide conjugate which would result in erroneously high values. These same workers have also shown that no interfering metabolites of chloramphenicol are formed.

Other methods of assay for chloramphenicol have been published. These include microbiological (3,4) colorimetric (5,6) enzymatic (7),

fluorometric (8) and gas chromatographic (9). However, these methods either lack specificity or are time consuming.

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LC NEWS

SLIDER VALVE operates like a toggle switch, is chemically inert. It has zero dead volume, ie., no mixing cavities. Applications include solvent selection, fraction collection, reference cell flushing, and others. Rheodyne, Inc., JLC/81/1, P.O. Box 996, 6815 S. Santa Rosa Ave., Cotati, Ca, 94928, USA.

NEW MEDIA FOR HPLC - a 5 micron totally porous spherical silica provides optimum pore diameter and narrow pore size range for minimal peak tailing. Jones Chromatography, Inc., JLC/81/1, P.O. Box 12147, Columbus, OH, 43212, USA.

HPLC SOLVENT DELIVERY SYSTEM, SP-8700, incorporates a new design which results in high reliability and performance. A fast microprocessor-controlled A/D feedback system gives extremely precise flow control and eliminates need for a pulse dampener. Spectra-Physics, JLC/81/1, 2905 Stender Way, Santa Clara, CA, 95051, USA.

IMPROVED ANION EXCHANGE material has greater hydrophylic character and higher capacity, thus reducing the tendency for reverse-phase adsorption. It is also less susceptible to attack by base than previous materials, thereby increasing the operational pH range from less than 1 up to 9. The Separations Group, JLC/81/1, 16640 Spruce St., Hesperia, CA, 92345, USA.

HPLC PACKING MATERIALS AND COLUMNS is a brochure/technical manual that describes analysis of a variety of substances by HPGPC, aqueous GPC, LLC, LSC, IEC, etc. Numerous chromatograms are presented with details of experimental conditions. Toyo Soda Mfg. Co., Ltd., JLC/81/1, Toso Bldg, 1-7-7 Akasaka, Minato-ku, Tokyo 107, Japan.

PREPARATIVE LC/UV DETECTOR is described in Bulletin 80-800. A brief introduction to Beer's law is given, followed by applications to the fractionation of phthalate esters, subst'd benzaldimines, and subst'd prophenones. Gow-Mac Instrument Co., JLC/81/1, Central Jersey Industrial Park South, Kearney St - Bldg 26E, Bridgewater, NJ, 08807, USA.

SAMPLE PREPARATION DEVICE - Florisil Sep-Pak cartridge is for streamlined cleanup, isolation and concentration of chlorinated pesticides, and related compounds, typically from foods and environmental samples. Waters Associates, Inc., JLC/81/1, 34 Maple St., Milford, MA, 01757, USA.

CHROMAFILE is a chromatography reference card file that has four major sections: PHLC, TLC, CC, GLC. Applications subsections include pharmaceuticals, biologicals, pesticides, etc. Whatman, Inc., JLC/81/1, 9 Bridewell Place, Clifton, NJ, 07014, USA.

COLUMN FOR CARBOHYDRATE, GLYCOL, POLYOL separations contains an optimally cross-linked resin, provides thru-column speed and separation. It is particularly well suited for separation of lower oligomers. A total aqueous system is used for most separations. Applications include analysis of wood pulp hydrolyzates, corn syrups, cane syrups, beer, gasohol, wine, and others. Hamilton Co., JLC/81/1, P.O. Box 10030, Reno, NV, 89510, USA.

LOW PRESSURE LC CATALOG contains technical information on Cheminert accessories. Common applications include protein characterization, amino acid chromatography, and nucleotide analysis. Laboratory Data Control, Inc., JLC/81/1, P.O. Box 10235, Rivera Beach FL, 33404, USA.

NEW HPLC INVERTED FITTINGS feature improved plate count and peak shape, minimized dead volumes, removable frits, and improved mechanical behavior. Chrompak Nederland B.V., JLC/81/1, P.O. Box 3, 4330 AA Middelburgh, The Netherlands.

DERIVATIZATION CHART FOR CHROMATOGRAPHY provides full details of derivatization methods. Phase Separations, Ltd., JLC/81/1, Deeside Industrial Estate, Queensferry, Clwyd, Great Britain.

HPLC COLUMN PACKING BROCHURE includes an overview of column packing methods, slurry packing method, procedures, column conditioning technique and references to safety precautions. Phase Separations, Ltd., JLC/81/1, Deeside Industrial Estate, Queensferry, Clwd, Great Britain.

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LC CALENDAR

1981

February 2-9	"First Chem'l. Eng. Exhibition - KORCHEM", KOEX Exhibition Center, Seoul, Korea. Contact: Int'l. Chem. Eng. Exhibition, KOEX Exhibition Center, Seoul, Korea.
March 9-13	"Pittsburgh Conference on Anal. Chem. & Appl. Spectroscopy", Convention Hall, Atlantic City, NJ USA. Contact: The Pittsburgh Conference, Mrs. Linda Briggs, 437 Donald Rd., Pittsburgh, PA 15235, USA.
March 29-April 3	"National Am. Chem. Soc. Meeting", Atlanta, GA, USA. Contact: Am. Chem. Soc., 1155 Sixteenth Streeet, NW, Washington, DC 20036, USA.
March 29-April 3	"Advances in Separation Technology", Nat'l ACS Meeting, Atlanta, GA, USA. Contact: N. Li, Exxon Res. & Eng. Co., P. O. Box 8, Linden, NJ 07036, USA.
March 29-April 3	"Chromatographic Separations of Coal-Derived Materials", "Nat'l ACS Meeting, Atlanta, GA USA. Contact: L. Taylor, Chem. Dept., Virginia Polytechnic Inst. & State Univ., Blacksburg, VA 24601, USA.
March 29-April 3	"Standardized Materials for Chromatography", Nat'l ACS Meeting, Atlanta, GA USA. Contact: L. S. Ettre, Perkin-Elmer Corp., Main Avenue, Norwalk, CT 06856, USA.
April 28	"Detectors in Chromatography", Chrom. & Electrophoresis Grp. The Royal Society of Chemistry, College of Technology, Southend, U.K. Contact: Dr. D. Simpson, Anal. for Industry, Bosworth House, High Street, Thorpe-le-Soken, Essex CO 16 OEA, U.K.
May 11-15	"5th International Symposium on Column Liquid Chromatography", Avignon, France. Contact: G. Guiochon, Lab de Chim. Anal. Phys., Ecole Polytechnique, Rte. de Saclay, 91128 Palaiseau, France.
May 17-19	"Symposium on Environmental and Industrial Applications of LCEC and Voltammetry", Indianapolis, Indiana, USA. Contact: LCEC Symposium, 1205 Kent Avenue, West Lafayette, IN 47906, USA.
May 20-22	"Symposium on the Anal. of Steroids", sponsored by The Hungarian Chemical Society, Eger, Hungary. Contract: Prof. S. Gorog, Hungarian Chem. Soc., 1061 Budapest VI, Anker koz 1, Hungary.
June 4-5	"4th World Chromatography Conference", Aerogolf Sheraton Hotel, Luxembourg. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.
June 22-26	"4th Int'l Symposium on Affinity Chromatography and Related Techniques", Katholieke Universiteit, Nijmegen, The Netherlands. Contact: Dr. T.C.J. Gribnau, Organon Scientific Development Group, P.O. Box 20, 5340 BH OSS, The Netherlands.

July 20-24 "Second International Flavor Conference", National Hellenic Research Foundation, Athens, Greece. Contact: Dr. S.J. Kazeniac, Campbell Institute for Food Research, Campbell Place Camden, N.J. 08101, USA

August 23-28 "National Am. Chem. Soc. Meeting", New York, NY, USA. Contact: Am. Chem. Soc., 1155 Sixteenth Street, NW, Washington, DC 20036, USA.

September 20-25 "8th Annual FACSS Meeting", Philadelphia, PA USA. Contact: R. A. Barford, USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118, USA.

October 1-2 "Japan Conference on Chromatography", Palace Hotel, Tokyo, Japan. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7, Canada.

October 4-9 "Symposium on Novel Separation Processes", at the 2nd World Congress of Chemical Engineering, Montreal, Canada. Contact: Congress Secretariat, 151 Slater Street, Suite 906, Ottawa, Ont., Canada, K1P 5H3.

October 12-15 "EXPOCHEM '81", Houston, TX. Contact: Dr. A. Zlatkis, Chem. Dept., University Houston, Houston, TX 77004, USA.

November 19-20 "1981 International Chromatography Conference", Carillon Hotel, Miami Beach, Florida, USA. Contact: V.M. Bhatnagar, Alena Enterprises of Canada, P.O. Box 1779, Cornwall, Ontario, K6H 5V7.

1982

March 28-April 2 "National American Chem. Soc. Meeting", Las Vegas, NV USA. Contact: A. T. Winstead, Am. Chem. Sco., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

June 28-30 "Analytical Summer Symposium", Michigan State Univ., East Lansing, MI USA. Contact: A. I. Popov, Chem. Dept., Michigan State Univ., East Lansing, MI 48824, USA.

July 12-16 "2nd Int'l Symposium on Macromolecules", - IUPAC, University of Massachusetts, Amherst, Massachusetts, USA.

August 15-21 "12th Int'l Congress of Biochemistry", Perth, Western Australia. Contact: Brian Thorpe, Dept. of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia.

September 12-17 "National American Chem. Soc. Meeting", Kansas City, MO USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

1983

March 20-25 "National American Chem. Soc. Meeting", Seattle, WA USA. Contact: A. T. Winstead, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC 20036, USA.

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2. Title and Authors: The entire title should be in capital letters and centered on the width of the typing area at least 2 inches (5.1 cm) from the top of the page. This should be followed by three lines of space and then by the names and addresses of the authors in the following way (also centered):

A SEMI-AUTOMATIC TECHNIQUE FOR THE SEPARATION AND DETERMINATION OF BARIUM AND STRONTIUM IN SURFACE WATERS BY ION EXCHANGE CHROMATOGRAPHY AND ATOMIC EMISSION SPECTROMETRY

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Utah Biomedical Test Laboratory
520 Wakra Way
Salt Lake City, Utah 84108

3. Abstract: Three lines below the addresses, the title ABSTRACT should be typed (capitalized and centered on the page). This should be followed by a single-spaced, concise, abstract comprising less than 10% of the length of the text of the article. Allow three lines of space below the abstract before beginning the article itself.

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5. Paragraphs should be indented five (5) typewriter spaces.

6. Acknowledgment of collaboration, sources of research funds, and address changes for an author should be listed in a separate section at the end of the paper.

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2. In general, the chapter title and the abstract, as well as the tables and references, are typed single-spaced. All other text discussion should be typed 1½-line spaced, if available, or double-spaced. Prestige elite characters (12 per inch) are recommended, if available.

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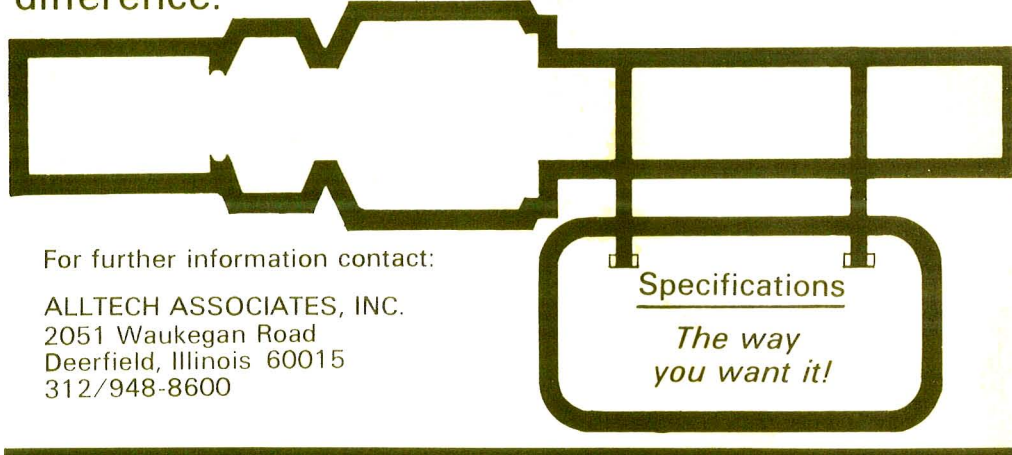

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