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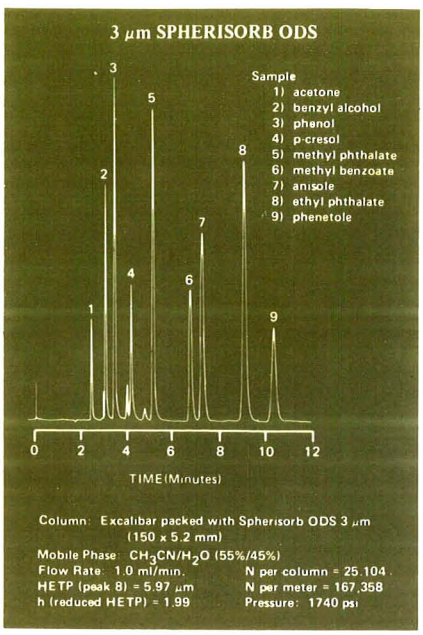

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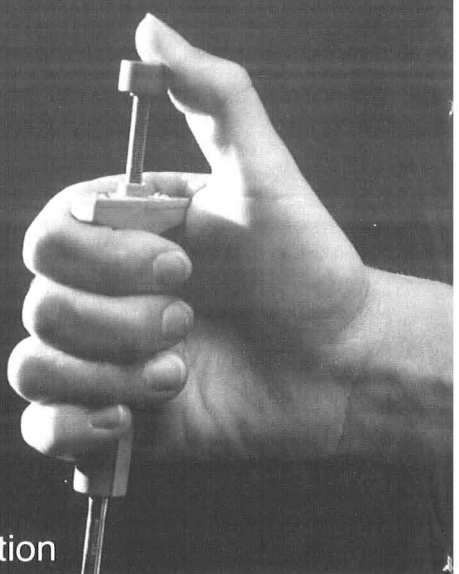

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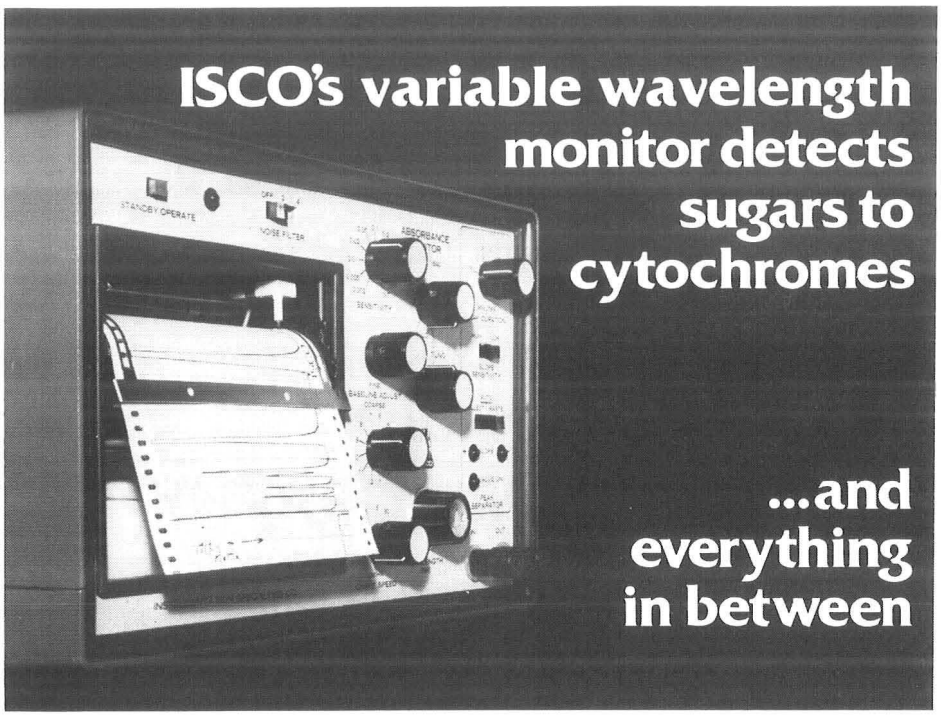

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OPTIMIZATION OF GPC EXPERIMENTS AS AN EFFECTIVE MEANS OF SIGNIFICANTLY ENHANCING THE RESOLUTION OF MULTICOLUMN SETS TO BE USED FOR ANALYZING SPECIFIC POLYMER SYSTEMS

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ABSTRACT

Problems concerning GPC band spreading effects and their elimination were discussed, reference being also made to particular requirements to be met in the case of copolymer studies. GPC analysis optimization experiments were performed in order to minimize instrumental spreading for a multicolumn set operated in carbon tetrachloride. Results of experiments carried out at mobile phase flow rates of 1.0 , 0.3 and 0.1 cm^3/min for narrow MWD solutes were used to compute the values of the degree of polydispersity and other MWD obtainable parameters determining the extent of spreading minimization attained. The GPC data generated for runs conducted at the lowest flow rate employed were shown to have been rendered sufficiently accurate for the raw chromatograms to represent the polymers being analyzed without the need of performing mathematical corrections for imperfect GPC resolution.

INTRODUCTION

Gel permeation chromatography (GPC) is today a widely recognized method of determining the molecular weight distribution (MWD) of polymers. In this technique

of instrumental analysis, the solvent flowing inside the GPC column set used constitutes the mobile phase to which is introduced a polymer solution forming a narrow rectangular band at the beginning of analysis. In the course of its migration in the column that band is gradually broadened as a result of processes taking place both inside the columns themselves and in the remaining elements of the chromatographic system(1,2).

Consequently, the MWD curves obtained for polydisperse polymer samples are generally too broad and somewhat skewed because the experimental chromatogram constitutes a composite picture of not only the MWD, but also of the superimposed instrumental spreading effects occurring in the chromatographic columns.

Distortion of experimental chromatograms due to the fact that the band broadening process interferes with the integrity of MWD information reflected by the GPC elution profile, is known to be highly detrimental to chromatographic resolution. Column dispersion effects are thus clearly the main source of errors in the quantitative interpretation of GPC analysis results leading to the determination of polymer molecular weights and MWDs(1-3).

To ensure accuracy and precision of experimental MWD information, the spreading effects resulting from specific features of individual experiments should be removed from the chromatograms, so as to be able to extract from them the desired intrinsic MWD characteristics of the analyzed polymer.

Since the magnitude of GPC peak dispersion effects depends on the rate of the mass transfer process occurring between the mobile phase(solvent) and the stationary phase(solvent trapped inside the pores of column packing), these phenomena are hence determined by such

difficult to measure and control factors as pore structure and pore size distribution, microscopic flow irregularities in the packed chromatographic columns, as well as by solvent viscosity and flow rate, polymer sample concentration, and other extracolumn effects (4-6).

As a result, mathematical methods of correcting chromatograms for imperfect resolution require the application of suitable experimental calibration procedures that are by no means trivial to perform, as discussed briefly later on.

And that is the reason why the question of running GPC experiments in conditions making possible a significant minimization of instrumental spreading is of great importance as far as obtaining more accurate and correct MWD information from GPC analyses is concerned.

This trend is reflected by pertinent studies carried out to date on that problem (7-9). As a matter of fact, even though the findings relating to, for instance, resolution enhancement by decreasing solvent flow rate are admittedly not new (10), the problem of improving GPC separation efficiency by optimizing the chromatographic system operating variables continues to receive constant attention, as exemplified by a recent paper of Cooper (11).

The foregoing considerations had induced us to undertake an experimental study aimed at minimizing peak dispersion effects for a column set to be used in our investigations of random copolymers by multiple detector GPC technique. The importance of correcting for imperfect resolution also the chromatograms of polymers for which there is no unique relationship between their size in solution and molecular weight has been indicated in a recent review article (12).

The experiments were performed in carbon tetrachloride, selected as the mobile phase on the basis of its

compatibility with one of the solute detectors, the use of the IR flow-through detector employed being limited to solvents transparent at the absorption wavelengths monitored in order to determine copolymer composition variations with respect to molecular weight (13-15).

In view of the relatively high viscosity of carbon tetrachloride (1.0 Cp at 20°C), its choice as GPC solvent constituted in our case an evident compromise between the attainable level of resolution and the amount of information to be obtained from the chromatograms, since solvent viscosity is a factor influencing directly the rate of solute permeation into the pores and hence controlling column dispersion (4-6).

Another aspect of the present work is concerned with the fact of its having been carried out using conventional GPC columns and packings (particle diameter 35 μm) known to yield significantly greater column dispersion effects than those produced by short columns with microparticulate packings (16).

In these conditions it seemed all the more worthwhile to perform the present GPC resolution enhancement study, as the expensive modern microparticulate packings requiring the use of costly high-pressure pumping equipment are not within everybody's reach.

EXPERIMENTAL

Apparatus and Operating Variables

The chromatographic analyses were performed using a Model 200 gel permeation chromatograph (maker: Waters Associates Inc.) equipped with a set of five conventional-type GPC columns (122 mm long; internal dia. 0.95 mm) packed with Styragel and Poragel gels with particle

diameter of 35 μm and pore sizes of: 10nm, 10^2nm, 10^3nm, $3x10^4$nm and 10^5nm. The Waters Associates R-400 series deflection-type differential refractometer and the Wilks Instruments MIRAN 1A infrared spectrometer were the solute detectors used.

Narrow molecular weight distribution (NMWD) polystyrene standards supplied by Waters Associates Inc. were used in the present work. Freshly distilled carbon tetrachloride (supplier: Polish Chemical Reagents POCh, Gliwice) was the GPC solvent employed.
The GPC experiments were conducted in an air-conditioned laboratory inside which a constant temperature of $21^{\circ}C \pm 1^{\circ}C$ was maintained.

As a considerable increase of chromatographic resolution may be attained by reducing solvent flow rate (7,9), it was decided to investigate thoroughly the effect of mobile phase flow rate on separation efficiency for a chromatographic system set up for a copolymer study, as already indicated in the introductory section. The experiments were therefore run at the following flow rates: 1.0 , 0.3 and 0.1 cm^3/min.

The multicolumn set was selected in such a way as to ensure the linearity of the GPC calibration curve in the molecular weight range of interest, which is in turn known to improve both resolution and accuracy of molecular weights calculated from experimental chromatograms (9,17,18). In order to improve the accuracy of GPC measurements at the low solvent flow rates applied, a suitably modified siphon was used to minimize solvent losses by evaporation (19). The retention volume of chromatographic peaks was determined by calculating the position of the first moment of the peak, known from theory to be independent of flow rate (2,6).

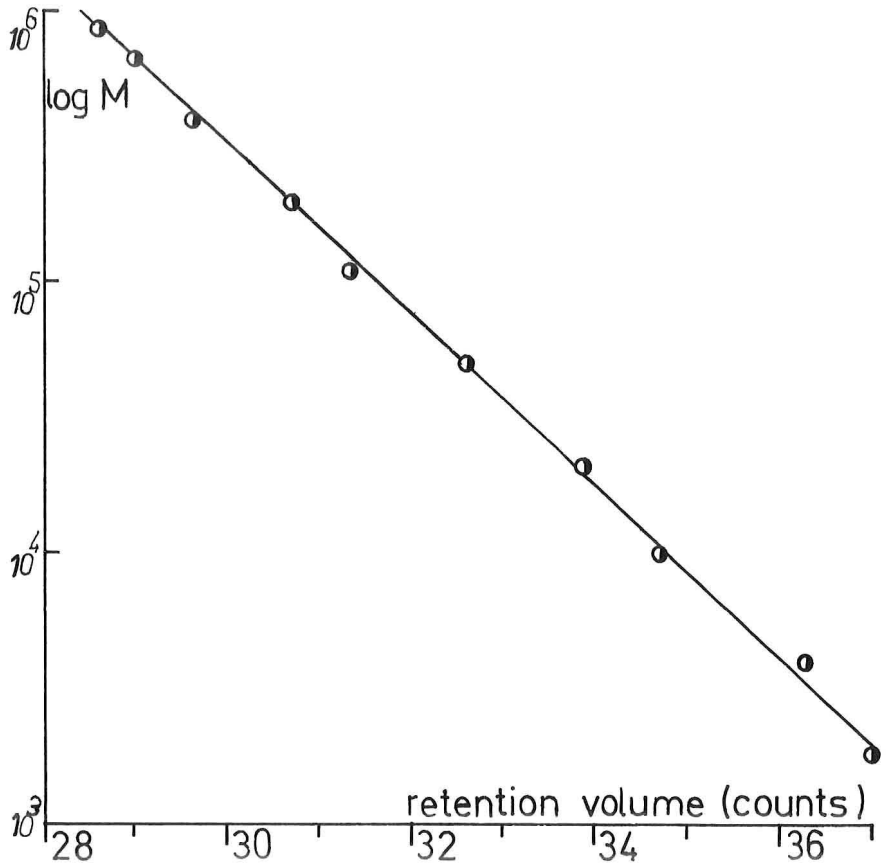

FIGURE 1 The linear calibration dependence obtained for the set of five conventional GPC columns used in the present work - column pore sizes: 10nm , 10^2nm , 10^3nm, $3x10^4$nm, and 10^5nm; GPC solvent: CCl_4 at 25°C; solvent flow rate: 0.1 cm^3/min .

Optimization of GPC Analyses

The percentage deviation of the degree of polydispersity of NMWD polystyrene standards, determined experimentally from uncorrected chromatograms $(M_w/M_n)_u$, from the "true" polydispersity value quoted for those standards by their supplier $(M_w/M_n)_t$ was selected as

a convenient criterion for estimating the effect of GPC solvent (CCl_4) flow rate on the separation efficiency of the multicolumn set employed.

This deviation, expressed overleaf as $\Delta(M_w/M_n)\%$, makes it possible to determine readily the attained resolution enhancement of the chromatographic system investigated, as the values of $(M_w/M_n)_u$ obtained from experimental chromatograms should be equal to their true values $(M_w/M_n)_t$ when the state of infinite resolution has been reached (20).

$$\Delta(M_w/M_n)\% = 100 \times \left[\frac{(M_w/M_n)_u - (M_w/M_n)_t}{(M_w/M_n)_t} \right] \qquad (1)$$

Additional criteria of assessing the attained extent of system resolution increase included the determination of the symmetrical spreading correction factor Λ (21) from uncorrected chromatograms recorded at the three solvent flow rates studied, and also the corresponding values of the specific resolution factor R_s, constituting a general measure of system efficiency (22). The symmetrical correction factor is given by the following relation (when $\Lambda \leqslant 1.05$ spreading effects can be neglected):

$$\Lambda = 0.5 \left[\frac{M_n(t)}{M_n(u)} + \frac{M_w(u)}{M_w(t)} \right] \qquad (2)$$

where :

$M_n(t)$, $M_w(t)$ - true values of the number and weight average molecular weights of the NMWD polystyrene standards

$M_n(u)$, $M_w(u)$ - values of the number and weight average molecular weights of the NMWD polystyrene standards computed from uncorrected chromatograms.

The specific resolution factor R_s was determined for the solvent flow rates studied using the relation given by Bly (22).

$$R_s = \frac{(V_2 - V_1)}{2(\sigma_1/d_1 + \sigma_2/d_2)\log(M_1/M_2)} \qquad (3)$$

where :

V_1, V_2 - retention volumes for standards with molecular weights M_1 and M_2 ,

σ_1, σ_2 - standard deviation of Gaussian peaks determined from experimental chromatograms of the NMWD samples and expressed in counts ,

d_1, d_2 - polydispersities of the NMWD samples "1" and "2", i.e. $(M_w/M_n)_t$

RESULTS AND DISCUSSION

Chromatographic analyses of a series of NMWD standards were carried out in conditions specified in the experimental section. The linear GPC calibration relationships found for the individual carbon tetrachloride flow rates studied were then used to determine the values of the optimization parameter $\Delta(M_w/M_n)\%$ and the molecular weights of the NMWD polystyrene standards analyzed. Computations were performed according to a program similar to that of Pickett et al.(23)

The values of the degree of polydispersity $(M_w/M_n)_u$ and of the optimization parameter $\Delta(M_w/M_n)\%$ calculated from raw chromatograms obtained for the NMWD polystyrene samples at the different flow rates studied, are listed in table 1.

The values of the optimization parameter $\Delta(M_w/M_n)\%$ computed for the three different flow rates investigated were plotted as a function of molecular weight of the NMWD polystyrene samples (Figure 2).

TABLE 1

Values of the Degree of Polydispersity (M_w/M_n) and of the Optimization Parameter $\Delta(M_w/M_n)\%$ Computed from Uncorrected Chromatograms of the NMWD Samples Analyzed at the GPC Flow Rates Studied (CCl_4 at 25°C, Flow Rates - 1.0, 0.3 and 0.1 cm^3/min)

Sample	Standard M_w	$(M_w/M_n)_t$	$(M_w/M_n)_u$			$\Delta(M_w/M_n)\%$		
			1.0	0.3	0.1	1.0	0.3	0.1
1.	867,000	1.122	2.082	1.406	1.313	85.56	25.31	17.02
2.	390,000	1.098		1.228	1.170		11.83	6.55
3.	200,000	1.036	1.370	1.164	1.107	32.23	12.35	6.85
4.	111,000	1.045	1.354		1.104	29.56		5.64
5.	50,000	1.041			1.095			5.18
6.	20,800	1.04	1.268	1.131	1.089	21.92	8.75	4.71
7.	10,000	1.041	1.254	1.144	1.122	20.46	9.9	7.78
8.	4,000	1.103	1.280	1.190	1.127	16.04	7.88	2.17
9.	2,100	1.12	1.513	1.282	1.184	34.82	14.46	5.71

N.B. The true values of the degree of polydispersity quoted in table 1 for samples 4, 8 and 9 are those reported for them by Ambler (24).

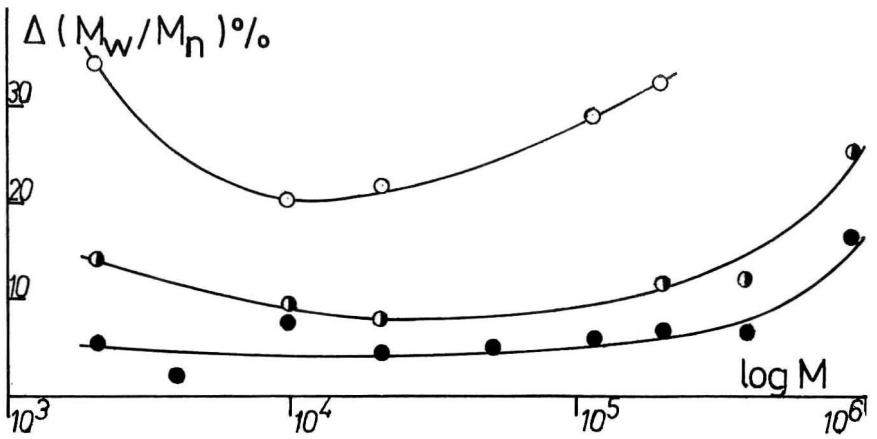

FIGURE 2 The experimental dependence of the optimization parameter M_w/M_n % on molecular weight, found for the different solvent flow rates investigated - CCl_4 at 25°C ; solvent flow rates: 1.0 cm^3/min - O , 0.3 cm^3/min - ◑ , 0.1 cm^3/min - ● .

The data listed in table 1 and depicted in figure 2 show clearly that for the lowest flow rate studied (0.1 cm^3/min) there has occurred a considerable reduction of the percentage deviation of the values of the degree of polydispersity determined from raw chromatograms $(M_w/M_n)_u$ from the true degree of polydispersity $(M_w/M_n)_t$ of the NMWD polystyrene standards chromatographed. The significant augmentation of GPC band broadening effects with molecular weight to be observed for the individual flow rates studied is in agreement with pertinent theoretical predictions (4-6, 25,26). Results of MWD analyses obtained for the NMWD samples studied were then utilized to determine the values of the GPC symmetrical spreading correction factor Λ (eqn.2) reflecting the band broadening effects present at the solvent flow rates investigated (21).

Values of the specific resolution factor R_s (22), constituting a qualitative indication of the overall separation efficiency of the chromatographic system, were also determined for the molecular weight range of interest. The obtained values of Λ and R_s were listed in tables 2 and 3.

It can be seen from table 2 that the experimental values of the correction factor Λ, calculated for the NMWD polymer samples studied at the GPC solvent flow rate of 0.1 cm^3/min, are less than 1.05 in the molecular weight range of interest.

This means that instrumental spreading effects have been minimized to such an extent that they may be neglected when interpreting GPC analysis results (8,21). That finding thus confirmed the conclusion drawn from the data listed in table 1 and figure 2, the results of R_s determinations indicating unequivocally the enhancement of overall separation efficiency.

Numerical values of the number (M_n) and weight (M_w) average molecular weights calculated for several NMWD polystyrene standards from chromatograms uncorrected for instrumental spreading effects are compared in table 4 with the corresponding M_n and M_w values quoted for those standards by their supplier.

The results listed in table 4 confirm the validity of observations already made, the improvement in the values of the number average molecular weights of the NMWD samples investigated becoming evident with the reduction of solvent flow rate.

The attained improvement of the accuracy of the numerical values of the number and weight average molecular weights is to be regarded as most satisfactory in the experimental conditions employed. It should be remembered that, in addition to GPC band broadening

TABLE 2

Values of the Symmetrical Spreading Correction Factor Λ Calculated for GPC Data Generated for the NMWD Samples at the Solvent Flow Rates Studied (CCl_4 at 25°C)

Sample M_w	Spreading Correction Factor 0.1 cm^3/min	0.3 cm^3/min	1.0 cm^3/min
390,000	1.033	1.058	
200,000	1.036	1.061	1.158
111,000	1.05		1.164
20,800	1.027	1.045	1.10
10,000	1.04	1.05	1.09
2,100	1.049	1.09	1.19

TABLE 3

Values of the Specific Resolution Factor R_s Determined at the Solvent Flow Rates Investigated (CCl_4 at 25°C)

Pairs of Standards M_{w1} , M_{w2}	Specific Resolution Factor R_s 0.1 cm^3/min	0.3 cm^3/min	1.0 cm^3/min
867,000 - 200,000	1.37	1.23	0.87
867,000 - 20,800	1.45	1.26	1.06
200,000 - 20,800	1.90	1.53	1.27
200,000 - 10,000	1.68	1.52	1.21
20,800 - 2,100	1.80	1.64	1.32
10,000 - 2,100	1.84	1.66	1.31

TABLE 4

Values of Number and Weight Average Molecular Weights of the NMWD Standards Computed from Raw Experimental Chromatograms Recorded at the GPC Solvent Flow Rates Studied (CCl_4 at 25°C).

Mol.wts.of Standards	GPC Solvent Flow Rate (cm^3/min) 0.1	0.3	1.0
773,000	668,000	671,000	478,000
867,000	877,000	944,000	995,000
355,000	353,000	330,000	
390,000	413,500	405,500	
111,000	108,500		93,700
111,000	119,700		127,000
20,200	18,100	20,400	17,700
20,800	19,700	23,100	22,500
1,950	1,870	1,850	1,530
2,100	2,200	2,370	2,320

effects, other sources of error are also to be reckoned with, e.g. errors involved with the choice of correct chromatogram baseline, or those committed when taking readings of chromatogram heights in the chromatographic data treatment stage (3,27).

The practical value of the experimental approach to GPC resolution optimization carried out to obtain more accurate MWD information may be best appreciated by considering briefly the main problems involved in the general mathematical approach to the elimination of band broadening effects.

The mathematical correction of GPC chromatograms for imperfect resolution requires the determination of the relation between the experimental chromatogram

$F(v)$ of the investigated polymer sample and the molecular weight distribution function $W(M)$ that would be obtained in the absence of column dispersion processes described by the instrumental spreading function $G(v,M)$. It is to be emphasized that the relation between these three functions, given by Tung in the form of the below quoted, familiar convolution integral equation (4) for GPC band broadening, implies that the spreading of a component is not affected by its own concentration and concentrations of other components in solution.

$$F(v) = \int_0^\infty W(M)\ G(v,M)\ dM \qquad (4)$$

where: M - molecular weight, v - retention volume, $G(v,M)$ - normalized instrumental spreading function, $F(v)$, $W(M)$ - experimental and spreading corrected chromatograms, respectively.

Extraction of the function $W(M)$ representing the corrected chromatogram requires the selection of the correct spreading function and the determination of numerical values of its parameters.

Numerous methods of solving the equation (4) with respect to $W(M)$ for a known spreading function have been presented in the literature (29,30), most of them tacitly relying on the assumption that the chromatogram of a polymer sample is a linear superposition of individual chromatograms corresponding to all components of the polymer investigated. Since the elution of each species is known to depend somewhat on the concentration of all other species present, the above assumption may not be explicitly correct over certain concentration and molecular weight ranges (32,33).

Moreover, the spreading function $G(v,M)$ parameters are known to depend on the columns used and on the sizes of polymer molecules. They are also determined

by operating conditions, exhibiting a certain dependence on flow rate and polymer concentration(33). The concentration dependence has not been yet elucidated adequately, and therefore spreading function treatments have been generally confined to the region of low concentrations in which concentration effects, and specifically species interactions, are sufficiently small to be neglected.

In the case of copolymers it is obviously necessary to exercise caution as far as the question of the absence of specific interactions is concerned(34). Should such interactions prove significant, it would be indeed difficult to maintain that the resolution factor(h) is really independent of polymer type, as asserted by Tung and Runyon in their treatment of the instrumental spreading problem(35).

It therefore becomes all the more important in the case of copolymers to ensure the attainment of an optimum overall resolution efficiency of the chromatographic system by appropriately optimizing experimental conditions.

The importance of such an approach to the minimization of GPC band broadening effects is further supported by the fact that the problem of choosing the correct spreading function has not yet been solved. In addition to the most often employed Gaussian model of the spreading function G(v,M) proposed by Tung et al.(36), numerous attempts have been made to apply more general, asymmetrical spreading functions with a larger number of parameters(37-42). The main difficulty encountered in this connection is due to the fact that the accuracy of both absolute and GPC techniques of measuring average molecular weights of polymers is insufficient to determine accu-

rately the parameters of the more complex GPC spreading functions (1,42).

It should be pointed out that the application of calibration standards whose molecular weights are known with an accuracy of ± 5 % has been thought until recently to be sufficient for determining the parameters of the spreading function characteristic for a given chromatographic system. That belief has been nevertheless contradicted by numerous literature data (1,37,43). The practical determination of the spreading function suffers chiefly from the lack of good experimental methods of obtaining the values of the number average molecular weight (M_n) over a wide range of molecular weights, so that the commercially available polymer standards have insufficiently accurate M_n values assigned to them (43).

Significance of the above fact should be realized fully when attempting to determine the spreading correction factor (h) either by the extremely tedious reverse-flow experiment (1,35,36) or by computational methods, such as those of Hamielec and Ray (44) or Balke and Hamielec (45).

CONCLUDING REMARKS

In general, it has become widely accepted that the quantitative interpretation of GPC data for the purpose of obtaining correct polymer molecular weight averages requires the application of rather elaborate data treatment procedures in order to deconvolute band broadening effects from experimental chromatograms. Such an attitude among GPC users does tend to limit the number of those who venture beyond the scope of routine GPC data interpretation, even though the

correction of raw chromatograms for those effects is well known to be indispensable for NMWD samples(36) and,as indicated by Kotaka and Donkai(46),the omission of band broadening correction may sometimes lead to serious errors also in the case of broad MWD polymer samples.In this situation,the present band broadening minimization study indicates clearly the immense practical value of GPC analysis optimization as an effective means of obtaining more correct and accurate MWD information.

In our case the choice of column length and packing porosity combination together with the application of a low solvent flow rate (0.1 cm^3/min)was found to result in a significant minimization of GPC spreading effects. The resultant increase of chromatographic system resolution was shown to produce such an improvement of the accuracy of GPC analyses that,in comparison with their true values,the values of average molecular weights of NMWD polymer samples computed from raw experimental chromatograms were found to be well within limits of experimental error(3,27).

It is to be further emphasized that the present study was effected for a chromatographic system operating in conditions generally considered as unfavourable to maximum resolution requirements,i.e.conventional type GPC packings with particle diameters of 35 μm and the viscous carbon tetrachloride as GPC solvent were employed.

At the same time,the increase of analysis time resulting from the application of low GPC solvent flow rate in the case of conventional column packings is thought to be offset entirely by the fact that more accurate GPC data are obtained without the necessity of resorting to the complex mathematical methods of eliminating GPC instrumental spreading effects.

Under these circumstances,the effectiveness of the optimization study reported herein is therefore all the more gratifying,and its results should prove particularly encouraging to those who wish to extract the maximum amount of information from their GPC experiments,but do not have access to modern microparticulate packings or special computer systems.

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ANALYSIS OF RIBONUCLEOTIDES BY REVERSE-PHASE HPLC USING ION PAIRING ON RADIALLY COMPRESSED OR STAINLESS STEEL COLUMNS

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ABSTRACT

A method is described for the analysis of 16 major nucleotides including NAD, IDP, GDP, cAMP, cGMP and succinyl AMP by reverse phase HPLC. The use of 65mM KH_2PO_4 at pH 3.2, low concentrations of the ion pairing reagent tetrabutylammonium phosphate and acetonitrile allowed the simultaneous separation of these nucleotides by isocratic or gradient elution in 18 and 28 minutes respectively. Stainless steel and radially compressed columns were compared and a similar separation profile was obtained. The latter columns increased retention and improved the efficiency of separation.

INTRODUCTION

Although several methods have been developed for the separation of nucleotides by HPLC, most have relied on the use of anion exchange chromatography (1-6). The introduction of reverse

phase columns has improved column stability and extended the technique to new applications. However, the nucleotides IDP, GDP, ADP, NAD and succinyl AMP were of special interest in our studies of helminth parasite metabolism and had not been adequately resolved or separated by either ion exchange or reverse phase chromatography. With the earlier technique IDP had not been fully resolved from ADP (1) and with the latter, Anderson and Murphy (7) could not demonstrate complete resolution of inosine, guanine and adenine nucleotides. Furthermore, IMP, GMP and ATP coeluted, as did, GTP and ITP, and GDP and IDP. Apart from these studies very little has been reported on the separation of IDP and succinyl AMP.

Hoffman and Liao (8) employed ion pair reverse phase chromatography, a relatively high concentration of tetrabutylammonium hydrogen sulphate (25 mM), various high ionic strength combinations of KH_2PO_4, NH_4Cl, CH_3COONH_4, CH_3COOH in the presence of 15 to 25% methanol. Moreover numerous pH conditions were required and the use of such mixtures is undesirable as precipitation is possible. More recently, Shaw et al (9) suggested the use of a combination of anion exchange and reverse phase columns for the the separation of major nucleotides. This system is more complex, time consuming and is unlikely to be versatile.

Using anion exchange HPLC, Riss et al (5) separated most major nucleotides, but NAD coeluted with AMP, and IDP and ITP were ignored. Furthermore, this analysis required approximately 60 minutes, an interrupted flow rate and discontinuous buffer gradient. On the other hand, Schwenn and Jender (10) used tetrabutylammonium hydroxide on a reverse phase C_{18} column to separate adenine nucleotides but required an extremely high pH (9.4 to 10). Unfortunately, high pH is known to dissolve the salica matrix of C_{18} columns and could mask the contribution of substituted groups on the purine or pyrimidine ring.

In the present study we investigated the ion pairing reagent tetrabutylammonium phosphate on reverse-phase stainless steel columns and the recently developed radially compressed cartridges (Waters Associates). The aim was to develop a simple, fast and practical method for the separation of adenine, guanine and inosine ribonucleotides under isocratic or gradient elution conditions.

MATERIALS AND METHODS

HPLC instrumentation

A Waters high pressure liquid chromatography system was used (Waters Associates, Milford, Mass., U.S.A.), which consisted of an M600A and

M45 solvent delivery system, model 440 absorbance detector, U6K Universal Liquid Chromatograph injector, model 660 solvent programmer and RCM-100 Radial Compression separation system. Absorbance was measured at 254nm and peak area and retention time were calculated using a Spectraphysics minigrator (Santa Clara, Cal., U.S.A.).

Columns

Two types of Waters reverse-phase columns, containing 10μ octadecysilane permanently bonded to unmodified silica were used, μ Bondapak C_{18} stainless steel columns (30 cm x 3.9 mm I.D.) and Radialpak A cartridges (10 cm x 8 mm I.D.).

Chromatographic conditions

The mobile phase was prepared by making a 65mM KH_2PO_4, 0.9 mM TBAP solution in glass distilled water with the pH adjusted to 3.2 All analyses were carried out at ambient temperature after filtration of solutions through a Millipore HAWP 0.45 μ filter (Bedford, Mass., U.S.A.). Isocratic or gradient elution modes were selected as required. Acetonitrile or methanol was used at 6% or less and all columns were equilibrated for at least 15 minutes. The radial compression module was used at 1600 to 2500 p.s.i. as recommended by the manufacturer. Column regeneration was achieved by washing with methanol, ethanol and then hexane.

Peak identification

Peaks were identified by observing retention time, addition of internal standards, absorbance ratios and with some nucleotides, by the enzymic shift method (2) or chemical hydrolysis of the pyrophosphate bond. Only those nucleotides of biochemical interest to us were subjected to enzymic identification.

Nucleotides and reagents

NAD and the mono-, di-, and tri-phosphate nucleosides of adenosine, cytidine, inosine and guanosine were purchased from Boehringer Mannheim, (Melbourne, Australia), succinyl AMP (adenyl succinic acid) from Calbiochem, (Sydney, Australia), and 3':5' cyclic AMP and GMP from Sigma Chemicals Co., (St. Louis, Mo., U.S.A.).

HPLC grade acetonitrile, hexane, methanol and TBAP as PIC Reagent A were obtained from Waters Assoc., Freon from DuPont Aust., (Sydney, Australia); tri-n-octylamine from ICN Pharm., N.Y., U.S.A. All other chemicals used were of analytical grade.

Preparations of standard solutions and tissue extract

The 0.1mM standard solutions were prepared by dissolving nucleotides in glass distilled water or the mobile phase buffer. The tissue used was adult Fasciola hepatica, which after collecting

from sheep liver was washed with saline solution at 37°C, homogenized in 10% TCA at 0°C and centrifuged at 3000 g in a refrigerated centrifuge. It was then filtered through a Millipore 0.45 μ filter. Neutralization and extraction of nucleotides was carried out according the Khym procedure (6) as optimized by Van Haverbeke and Brown (11). Some precipitation of the extract was observed on standing in ice or storage in liquid nitrogen, hence all solutions of standards and extracts were filtered again prior to HPLC analysis. The recovery of nucleotides by this procedure was periodically determined.

RESULTS AND DISCUSSION

Ion pair chromatography functions largely by exploiting the ionization characteristics of the compounds under investigation. Nucleotides are ionic species above pH3 and this property has allowed their separation initially by anion exchange and later by ion pair HPLC. Although ion pair partitioning is complex, some similarity can be assumed to exist between the phase equilibria of these two techniques (9, 12, 13). This similarity may be responsible for the apparent resemblence of some of the mobile phase buffers used in both cases.

Because aqueous buffers had been used with some success in anion exchange chromatography of

nucleotides (1-6) various solutions of phosphate, formate and acetate were investigated in the presence and absence of TBAP. Optimal conditions were greatly influenced by each of the mobile phase parameters. Phosphate in the presence of TBAP was found to be the most satisfactory buffer. Phosphate concentrations between 50 and 120 mM gave the best resolution, whilst the addition of TBAP at concentrations between 0.5 and 2.0 mM significantly increased resolution and retention time. However, concentrations higher than 2 mM increased retention without any apparent improved resolution.

Varying the pH considerably affected the resolution of closely related nucleotides. The resolution of IDP and GDP, for example, was lost above pH 3.9 and ADP and GDP coeluted at pH 2.7. For the nucleotide groups NAD, AMP, GMP and CTP, or IDP, GDP and ADP, optimal pH conditions were 3.1 to 3.4 at 60 to 65 mM KH_2PO_4 containing 0.8 to 1.5 mM TBAP. The increase in retention time, resulting from the addition of the ion pairing reagent, was overcome without loss of resolution by the addition of small proportions of methanol or acetonitrile. The optimal concentration of these organic solvents was dependent upon the nucleotides to be separated and the elution time required. Acetonitrile was found to maintain resolution better than methanol.

Suitable separations of nucleotides could be achieved with both, stainless steel and radially compressed columns. However, the latter columns required higher flow rates to achieve similar elution time but shorter time to equilibrate. The relative behaviour of the two types of columns was similar to the findings of Assenza and Brown (14). Under isocratic conditions elution time on the radially compressed columns could be more than twice that on stainless steel columns (compare Figures 1A and 1B)but the magnitude of this increase in retention was not constant. In the absence of acetonitrile mono- and diphosphate nucleosides and NAD could be readily detected in standard solutions or tissue extracts. However, under these conditions triphosphates were difficult to detect and quantitate because of long retention times (Figure 1A). On the other hand the presence of a low concentration of acetonitrile markedly reduced retention without significant loss of resolution (Figure 1C).

Simultaneous separation of nucleotides by gradient elution was also possible for both standard solutions and tissue extracts (Figure 2). However, any variations in the gradient conditions had noticeable effects on reproducibility and the elution profile of some nucleotides. For example, NAD was fully resolved in Figure 2A but coeluted with GMP in Figures 2B

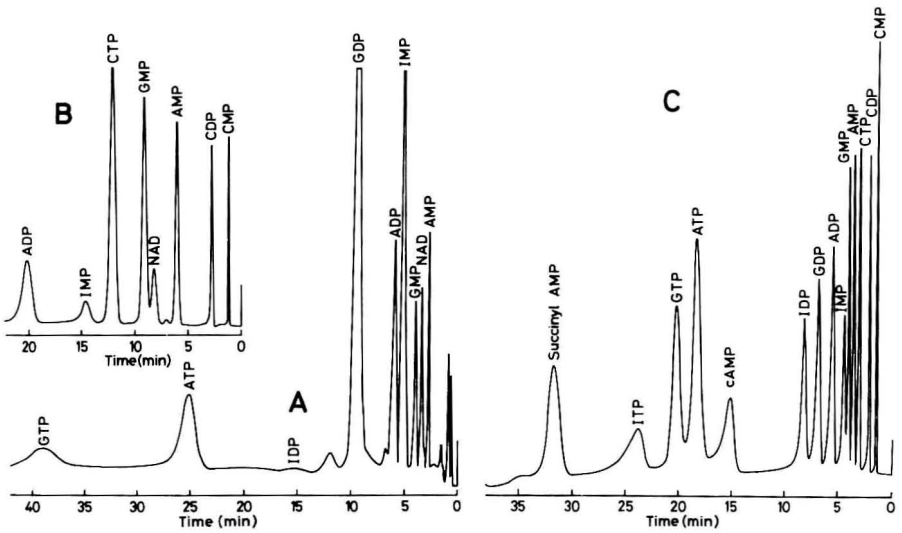

Figure 1: Isocratic elution in the absence (A + B) and presence (C) of acetonitrile using both columns and showing the difference in retention. Initial mobile phase, 65mM KH_2PO_4, 0.9 mM TBAP, pH 3.2.
A. Tissue extract with added GMP, IMP and GDP on μ Bondapak C_{18} column; flow rate 2.8 ml/min.
B. Separation of standard nucleotides on Radialpak A column; flow rate 3.5 ml/min.
C. Separation of nucleotide standards on Radialpak A column in the presence of 3.3% acetonitrile, flow rate 2.0 ml/min.

and 3B. The elution behaviour of CTP varied between isocratic and gradient conditions (compare Figures 1C and 3A with 2A and 3B).

The selection of an isocratic or gradient elution mode of separation was largely dependent on the type of nucleotides wanted, the time required to determine a suitable gradient or equilibrate the column, and the period for total elution. The contrast between these two modes is made in

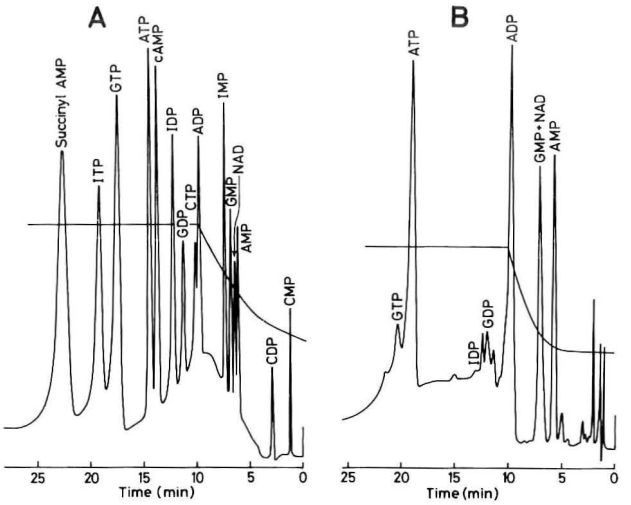

Figure 2: Gradient elution on Radialpak A column showing the difference in resolution of GMP and NAD caused by variations in acetonitrile conditions and gradient profile. Initial mobile phase as for Figure 1. A. Separation of 15 nucleotide standards with 0 to 5% acetonitrile gradient; flow rate 3.5 ml/min. B. Tissue extract with 1 to 6% acetonitrile gradient; flow rate 2.0 ml/min.

Figure 3 where isocratic elution of CTP, ATP, GTP, succinyl AMP, cAMP and cGMP was achieved in 18 minutes while gradient elution required 28 minutes. However, the latter gave better resolution of the mono- and diphosphate nucleosides, and the absence of significant base line fluctuation allowed the selection of high sensitivity attenuation.

The consistent increase in retention of nucleotides on the radially compressed column enhanced the resolution of CTP, AMP, NAD and GMP, or cAMP, cGMP, ATP and GTP. Varying the amount of acetonitrile and the flow rate allowed the

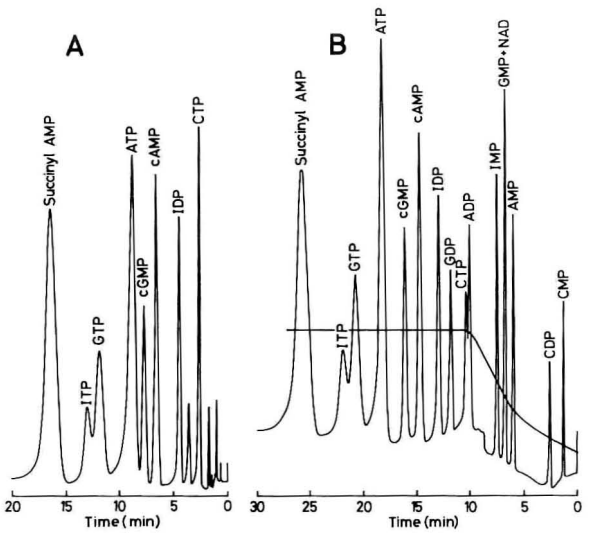

Figure 3: Isocratic versus gradient elution of standards including cAMP, cGMP and succinyl AMP in the presence of triphosphate nucleosides on Radialpak A column, initial mobile phase as for Figure 1. A. Isocratic elution with 5% acetonitrile; flow rate 2.5 ml/min. B. Gradient elution with 0 to 5% acetonitrile; flow rate 3.0 ml/min.

selective analysis of these related groups of nucleotides with very short elution time. This in turn eliminated peak broadening and made the detection of picamole levels possible.

From these observations it can be concluded that the use of relatively low ionic strength buffer, low concentrations of TBAP and acetonitrile and a pH of 3.1-3.4 gave good reproducibility, even with gradient elution, and permitted the analysis of very closely related nucleotides. Under these conditions low levels

of nucleotides in tissues can be determined isocratically or with gradient elution on stainless steel or radially compressed columns.

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APPENDIX

List of Abbreviations

ADP	Adenosine 5' -diphosphate
AMP	Adenosine 5'-monophosphate

ATP	Adenosine 5'-triphosphate
CDP	Cytidine 5'-diphosphate
CMP	Cytidine 5'-monophosphate
CTP	Cytidine 5'-triphosphate
GDP	Guanosine 5'-diphosphate
GMP	Guanosine 5'-monophosphate
GTP	Guanosine 5'-triphosphate
IDP	Inosine 5'-diphosphate
IMP	Inosine 5'-monophosphate
ITP	Inosine 5'-triphosphate
cAMP	Adenosine 3':5'-cyclic monophosphate
cGMP	Guanosine 3':5'-cyclic monophosphate
TBAP	Tetrabutylammonium phosphate
TCA	Trichloroacetic acid
NAD	Nicotinamide-adenine dinucleotide

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DETERMINATION OF PENICILLINASE-RESISTANT PENICILLINS IN SERUM USING HIGH-PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for the determination of methicillin, oxacillin, cloxacillin, dicloxacillin, and nafcillin in serum using high-pressure liquid chromatography (HPLC) is described. The drugs were extracted from serum using a two-step procedure employing acetonitrile followed by methylene chloride. The extraction procedure concentrated the antibiotics in a smaller volume which allows more accurate determinations of low serum levels. The treated sera were analyzed by HPLC on a reverse-phase column and detected by ultraviolet light absorption at 254 nm. Serum concentrations were measurable as low as 0.5 µg/ml. Recovery procedures showed less than 2.5% variation in peak heights when the antibiotics were extracted from different pools of serum. No interfering absorption was found in extracts of serum samples pooled from healthy volunteers, from a commercial source, or from two serum pools from patients receiving a variety of other drugs. Two spiked serum specimens prepared for each antibiotic were assayed four times by HPLC and by the microbiological agar diffusion method. No significant statistical differences between the methods were observed. Control materials were assayed for between-batch and within-batch reproducibility in the presence or absence of an internal standard. Results for between-batch reproducibility demonstrate CV's of about 5%. This procedure provides a sensitive, specific, accurate, and rapid method for determining antibiotic levels in routine clinical specimens.

INTRODUCTION

The penicillinase-resistant penicillins are semi-synthetic drugs consisting of bulky side groups attached to 6-aminopeni-

cillanic acid, the nucleus of penicillin. The side chains protect the β-lactam ring from the action of penicillinases. Methicillin was the first of the resistant penicillins developed. However, the drug is acid sensitive and cannot be given orally. Newer semi-synthetic drugs, the isoxazolyl penicillins (cloxacillin, oxacillin, and dicloxacillin) and the closely related nafcillin are both penicillinase resistant and relatively acid stable.

The toxicity of these antibiotics is similar to the other penicillins. Large doses of methicillin can result in neutropenia, bone marrow depression (1), nephropatny including hematuria, proteinuria, and renal insufficiency (2, 3, 4). The isoxazolyl penicillins may be hepato- (5) or neurotoxic (6). Since large doses of the antibiotics are frequently administered, rapid and accurate determinations of serum levels are clinically important especially when renal failure occurs.

High-pressure liquid chromatography (HPLC) has been successfully applied to the detection and quantitation of the penicillins in pharmaceutical preparations and in urine (7, 8, 9, 10, 11, 12, 13, 14, 15, 16). With few exceptions (17, 18), HPLC has never been used for the determination of the penicillins in biological fluids (serum, cerebro-spinal fluid, joint fluid, and pleural fluid). We have successfully applied HPLC to the clinical quantitation of the β-lactamase resistant penicillins in serum and CSF for one and a half years. This technique offers greater accuracy and speed in comparison to the standard microbiological determinations. Its major advantage over microbiological assays is the ability to quantitate one or more drugs given in combination without interference.

MATERIALS AND METHODS

Oxacillin, methicillin, cloxacillin, dicloxacillin, and nafcillin standard powders were obtained as their sodium salts from Bristol Laboratories (Syracuse, N.Y.). Stock antibiotic

solutions were prepared in water at a concentration of 1,000 μg/ml and stored at -20°C for no more than one month. Acetonitrile, methylene chloride, and ammonium acetate were of reagent grade. Water used in the procedure was purified by double distillation. Serum pools were prepared from specimens supplied by a local hospital laboratory, specimens received for fungal immunodiffusion by our laboratory, from a commercial source (Gibco; Grand Island, N.Y.), and from five healthy human volunteers. Serum from patients treated with the various antibiotics were obtained from four hospitals in the Detroit Medical Center.

Sample Preparation. Serum (0.4 ml) was mixed with an equal volume of acetonitrile and vortexed for 10 sec. The serum-acetonitrile slurry was placed on a shaker and slowly mixed for 15 min to facilitate protein precipitation and then centrifuged for 10 min at 3,000 x g at ambient temperature. The supernatant fluid was decanted to a clean screw-cap tube and 4.0 ml of methylene chloride was added. The sample was vortexed for 10 sec, shaken for 15 min and then centrifuged for 10 min at 3,000 x g. A 50 μl aliquot of the aqueous (upper) layer was injected with a microsyringe (#810; Hamilton Co., Reno, Nev.) for chromatography.

Chromatographic Conditions. Analyses were performed on a reverse-phase column (μ-Bondapak C_{18}; Waters Associates, Milford, Mass.) at ambient temperature. Separation was accomplished with a ALC/GPC 204 liquid chromatograph (Waters Associates, Milford, Mass.). It was equipped with a model 6000A solvent delivery system, a model U6K universal injector, and a model 440 absorbance detector. The eluant was monitored at 254 nm and the peaks recorded on a 10 mv chart recorder (Houston Instruments, Houston, Texas) at a chart speed of 0.5 cm/min. Reproducibility studies were completed using an automatic sample injector (Waters Intelligent Sample Processor (WISP) 710B; Waters Associates, Milford, Massachusetts).

The mobile phase consisted of a 62:28:10 mixture of distilled water, acetonitrile, and 0.2 M ammonium acetate, pH 5.6. This mobile phase was previously suggested for determinations of penicillin in pharmaceuticals (19). The buffer was filtered through a 0.5 μm filter (Fluoropore; Millipore Corp., Bedford, Mass.) and was deaerated with vacuum. The flow rate was 1.0 ml/min (approximately 900 psi) for oxacillin, cloxacillin, methicillin, and nafcillin, while the flow rate for dicloxacillin was 3.0 ml/min (approximately 2,800 psi).

Standards were prepared by adding aliquots of the antibiotic stock solutions to 1.0 ml of serum. The drug concentration was corrected for volume and expressed in μg/ml. Standard curves for each drug were generated by plotting peak height vs concentration of the antibiotic.

Microbiological Determination. The assay used for the drugs was a slight modification of the standard agar diffusion technique (20). The indicator organisms were Bacillus subtilis ATCC 6633 and Sarcina lutea ATCC 9341. Two vials of B. subtilis spore suspension (spore suspension # 2; Difco Laboratories, Detroit, Mich.) or 3 ml of an overnight culture of S. lutea were added to 100 ml of cooled (50°C) assay medium (Antibiotic assay medium No 5; Difco Laboratories, Detroit, Mich.). After mixing, 5 ml aliquots were transferred into sterile petri dishes (100 mm diameter) and allowed to solidify. The plates were stored in sealed plastic bags at 4°C until used. Plates containing B. subtilis spores were used up to one month after preparation while unused S. lutea plates were discarded after one week. Three dilutions of each antimicrobial agent were prepared in serum. Twenty sterile paper discs were placed in a sterile petri dish and four discs were inoculated with 0.02 ml of each dilution. Four discs were inoculated with the unknown sample and four with a 1:2 dilution of the unknown. Two discs of each concentration of antibiotic standard, an unknown, and a diluted unknown were placed on a seeded agar plate such that identical concentrations were

directly opposite one another. After 4 h of incubation at 37°C, the zone sizes were measured to the nearest 0.1 mm using a ruler and a stereo microscope. The unknown concentration was read from a standard curve generated by plotting log of the antibiotic concentration vs zone size in mm. The standards and unknowns were run concurrently on HPLC and by the microbiological method.

RESULTS

Choice of Absorbance Detector Wavelength. The ultraviolet absorbance maximums for the five drugs range from 226 - 245 nm. Since the chromatography unit was equipped with a fixed wavelength detector, 254 nm, the closest available wavelength to the maximums was used for all drugs.

Separation of the Drugs by Liquid Chromatography. A typical chromatogram of the drugs extracted from serum is shown in Fig. 1. The retention times for methicillin, oxacillin, cloxacillin, nafcillin, and dicloxacillin were 4.0, 5.7, 6.5, 9.0, and 11.7 min, respectively. Extraction of serum blanks and spiked serums showed that the methicillin peak came off with or very close to the serum peaks. A number of possible mechanisms to change the retention time of methicillin were investigated including alterations in the flow rate and adjustments in the concentrations of the mobile phase components. A decrease in the acetonitrile concentration from 28% to 20% increased the retention time to 6.6 min (Fig. 2) which satisfactorily separated the methicillin and serum peaks. The retention time for dicloxacillin was decreased to 6.8 min by increasing the flow rate of the original mobile phase to 3.0 ml/min.

Standard curves generated for each drug (Fig. 3A-E) passed through the origin and were linear up to 128 μg/ml, the maximum concentration tested. Drug concentrations as low as 0.5 μg/ml could be measured. We have experienced slight variations in the peak heights of the standard curve over a period of time. These

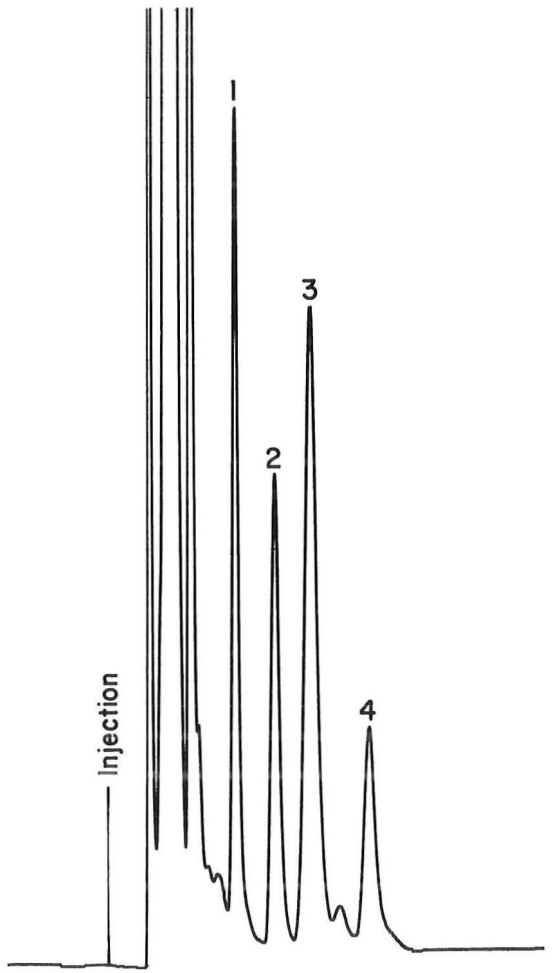

FIGURE 1

Elution profile of the penicillinase-resistant penicillins from a 50 µl injection of an extracted serum containing 20 µg/ml of each drug. The order of elution is: 1) oxacillin; 2) cloxacillin; 3) nafcillin; and 4) dicloxacillin. The flow rate was 1.0 ml/min and the attenuation was 0.02 absorbance unit full scale.

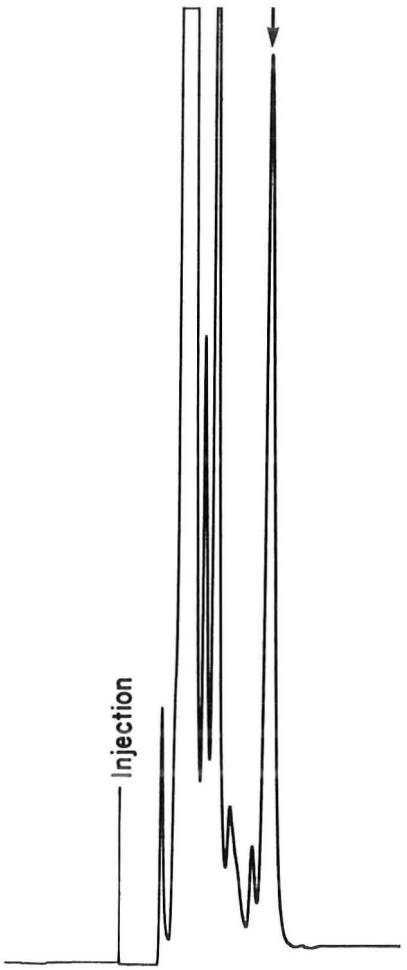

FIGURE 2

Chromatogram of methicillin (20 µg/ml) extracted from serum. The mobile phase was water, acetonitrile, and 0.2 M ammonium acetate, pH 5.6, (70:20:10) at a flow rate of 1.0 ml/min. The attenuation is 0.02 a.u.f.s.

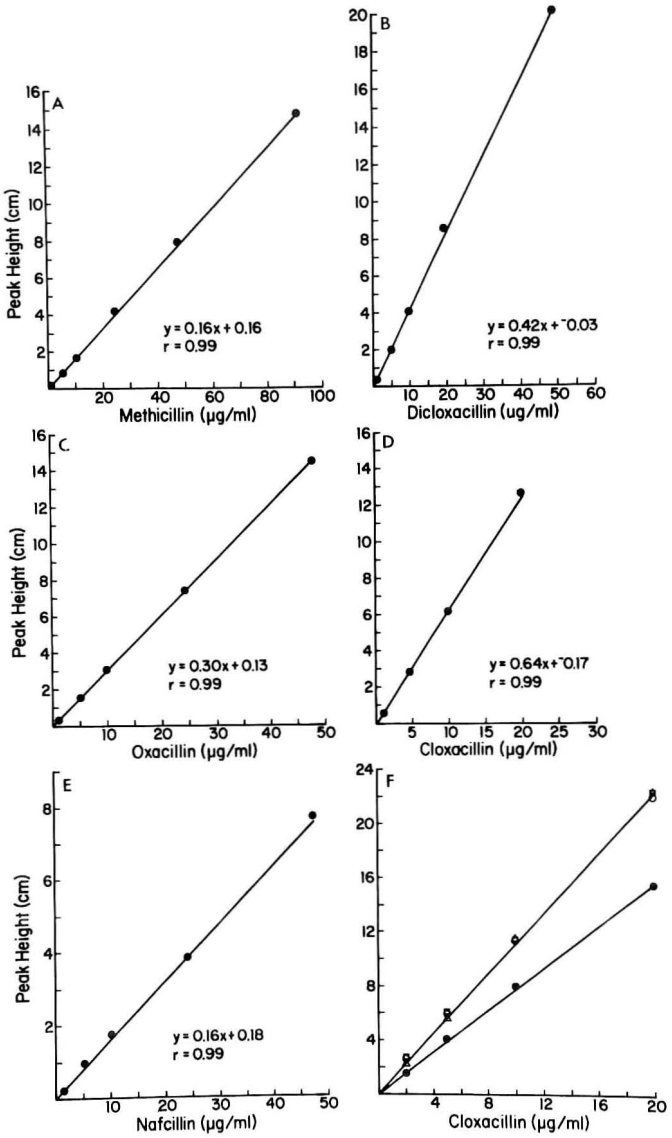

FIGURE 3

Standard curves for the drugs extracted from serum (A) methicillin, flow rate 1.0 ml/min, attenuation 0.1 a.u.f.s.; (B) dicloxacillin, flow rate 3.0 ml/min, attenuation 0.005 a.u.f.s.; (C) oxacillin, flow rate 1.0 ml/min, attenuation 0.1 a.u.f.s.; (D) cloxacillin, flow rate 1.0 ml/min, attenuation 0.02 a.u.f.s.; (E) nafcillin, flow rate 1.0 ml/min, attenuation 0.1 a.u.f.s. (F) Comparison of peak heights as a function of cloxacillin concentration in an aqueous control (•) and extracted from serum pools one (o), two (Δ), and three (□). The attenuation was 0.01 a.u.f.s.

differences may be due to a variety of factors. A change in the concentration or pH of the mobile phase components which causes an upward shift in the retention factor (k') of a drug would result in an increased peak width and a concomitant decrease in peak height. Conversely a decrease in k' would increase peak height and decrease peak width. Similarly as the column ages, decreased efficiency would also result in broader peaks with reduced heights. Despite the changes in peak height on different days, the values reported for the same serum unknowns were always within 5% of the actual concentration. Therefore, it was necessary to make a new standard curve each day. Ideally, the standards and the patient's serum should be extracted at the same time.

To demonstrate the reliability of this technique two 'unknowns' of each drug were prepared in serum by one investigator and assayed by the other. Each specimen was examined by HPLC and by the standard microbiological procedure. The mean of four determinations by HPLC and from four plates for each specimen by the microbiological method are presented in Table 1. There was close overall agreement between the two methods and differences were not statistically significant ($2P > 0.5$; paired t-test).

Recovery. Standard curves for the five drugs were generated by HPLC using aqueous drug solutions of various concentrations without any extraction. Identical amounts of drug were added to three different pools of human serum and extracted. Fig. 3F shows representative data for cloxacillin. In all cases the extracted drugs gave peak heights greater than those observed for the aqueous preparations. These findings indicate a relative concentration of the drugs in the aqueous layer during extraction.

Total protein content in each of the three serum pools was determined on a Technicon SMA 12/60 (Technicon Instruments Corp., Tarrytown, N.Y.). The protein concentrations for the pools were: 6.1, 6.5, and 7.0 g/dl respectively for pools 1, 2, and 3.

TABLE 1. Comparison of HPLC

Drug	Amount Added to serum (μg/ml)	Microbiological Assay: Average[a] Recovery (μg/ml) [Range]	Standard Deviation	Coefficient of Variation
Nafcillin	24.6	24.1 [20.3 - 29.5]	3.9	16.2%
	4.97	7.0 [5.9 - 7.5]	0.76	10.9%
Oxacillin	6.95	6.4 [5.9 - 7.1]	0.56	8.7%
	1.99	1.1 [1.0 - 1.1]	0.06	5.5%
Cloxacillin	6.36	7.0 [5.7 - 7.8]	1.12	15.9%
	12.67	11.5 [10.1 - 12.7]	1.1	9.6%
Methicillin	1.99	2.1 [1.9 - 2.4]	0.21	10.0%
	9.9	9.3 [9.2 - 9.3]	0.08	0.87%
Dicloxacillin	4.97	4.8 [4.7 - 4.9]	0.10	2.0%
	9.9	8.5 [6.5 - 10.5]	2.08	24.5%

a) average from four plates on different days
b) average from four injections on different days

and the Agar Diffusion Method

Method of Recovery				
		HPLC		
Recovery %	Average[b] Recovery (μg/ml) [Range]	Standard Deviation	Coefficient of Variation	Recovery %
99.2	23.4 [22.1 - 26.0]	1.74	7.4%	96.5
143	4.65 [4.6 - 4.7]	0.06	1.2%	94.9
91.9	6.71 [6.3 - 7.46]	0.65	9.7%	96.5
52.8	1.79 [1.6 - 2.09]	0.26	14.5%	89.7
111	6.11 [5.75 - 6.58]	0.34	5.6%	96.3
90.7	12.4 [11.1 - 13.2]	0.94	7.6%	97.6
103	1.90 [1.8 - 2.05]	0.11	5.5%	95.3
93.9	9.90 [9.4 - 10.2]	0.36	3.6%	99.9
95.9	4.58 [4.47 - 4.75]	0.13	4.2%	92.2
85.8	10.0 [9.5 - 10.5]	0.42	2.8%	101

Although there were some differences between the sera, the quantity of antibiotic extracted from each pool is almost identical. At antibiotic levels as high as 100 µg/ml, the differences in peak height for the three pools is less than 2.5%.

Reproducibility. Between-batch reproducibility was assessed by assaying control serums containing three different concentrations of oxacillin 10 times over a three month period. The control serums were also assayed 10 times within the same run to provide within-batch reproducibility data. At the same time, the need for an internal standard was assessed by adding 20 µl of nafcillin (4 µg/µl) to 1.0 ml of serum before extraction. Concentrations of the control serums were calculated in two ways: 1) a ratio of the peak height of oxacillin to the peak height of nafcillin in the serums were compared to a standard curve constructed in the same fashion; and 2) peak heights for oxacillin were compared to the standard curve constructed for oxacillin using peak heights. The concentration of oxacillin was corrected for the added volume of both oxacillin and nafcillin in constructing the standard curves and reading results of the data excluding the internal standard. These results are presented in Table 2 and 3. Results for the other four drugs are similar (data not shown).

Clinical Application. During the past year and a half, we have received approximately 200 patient serum, CSF and joint fluid specimens for assay of the drugs under consideration from hospitals in the Detroit Medical Center. A large number of these patients received a combination of antibiotics. Although this presents little difficulty for the HPLC assay, it makes comparison of HPLC and microbiological assays difficult. Some representative data for dicloxacillin assayed by both methods is shown in Figure 4. Similar results for the other drugs were observed (see discussion).

Interference Studies. We have not observed interfering peaks in the serums of approximately 200 patients receiving other drug therapy. In addition, when several other drugs were added to

TABLE 2

Results for Within-batch Reproducibility Experiments

With internal standard						Without internal standard					
Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)	Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)
3.0	10	3.0	0.19	6.3	99.0	2.9	10	2.9	0.15	5.2	97.6
17.0	10	17.1	0.27	1.6	101	16.4	10	16.3	0.28	1.7	99.7
47.0	10	47.9	0.48	1.0	102	44.0	10	43.9	0.71	1.6	99.6

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

TABLE 3

Results for Between-batch Reproducibility Experiments

With internal standard						Without internal standard					
Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)	Oxacillin concentration (μg/ml)	n	$\bar{x}$	SD	CV	average recovery (%)
3.0	10	2.9	0.25	8.4	97.6	2.9	10	2.8	0.18	6.3	95.6
17.0	10	16.6	0.71	4.3	97.6	16.4	10	15.9	0.55	3.5	96.9
47.0	10	46.3	1.69	3.7	98.5	44.0	10	42.5	1.53	3.6	96.5

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

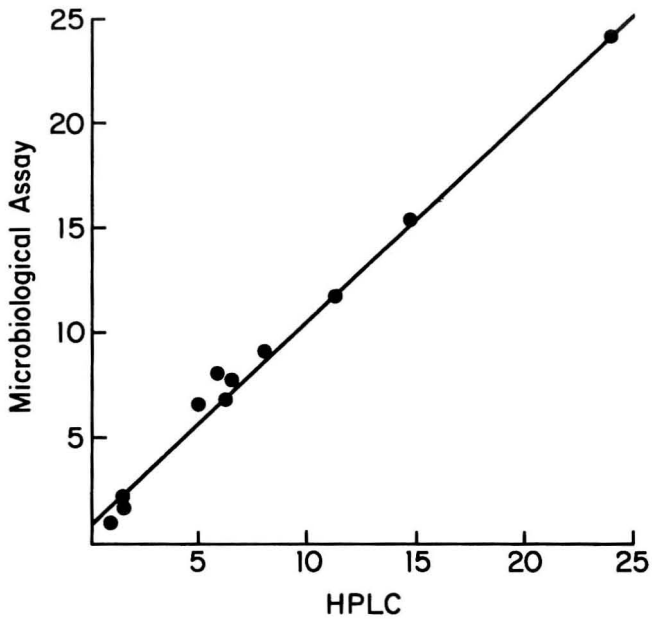

FIGURE 4

Patient comparison between levels (µg/ml) of dicloxacillin obtained with HPLC and the microbiological assay, with a correlation coefficient of 0.99, a slope of 0.97, and an intercept of 0.96.

serum and extracted none of them had retention times the same as any of the penicillinase-resistant penicillins. Those drugs showing no interference include: penicillin, ampicillin, ticarcillin, amoxacillin, mecillinam, pipericillin, carbenicillin, moxalactam, chloramphenicol, vancomycin, cephalothin, cefazolin, cephalexin, cephaloridine, cepharin, cepharadine, cefatoxamine, cefoxitin, cefamandole, co-trimoxazole, metronidazole, clindamycin, 5-fluoro-cytosine, theophylline, tobramycin, gentamicin, amikacin and ceforanide.

DISCUSSION

Antimicrobial agents are one of the most widely prescribed classes of drugs used in modern medicine. Dosage of the drugs is

usually tailored to the severity of the infection with more severe infections receiving larger doses. However, as the dosage increases, the possibility of adverse side affects also increases. Thus, it is appropriate to monitor antibiotic levels in patients with severe infections or renal insufficiency. All of the drugs discussed in this report have serum half-lives of under 1 h making it necessary to administer the drug frequently to maintain effective serum levels. With few exceptions, all of the techniques previously employed for determining the levels of the penicillinase-resistant antimicrobials require excessive time and may produce results of only moderate accuracy.

Currently, several methods for the determination of antimicrobials exist. Enzymatic assays and radioimmunoassays have been developed that are rapid and specific (21, 22), but they are rather expensive and at the present time are not available for the penicillins. All the other methods presently used suffer from major disadvantages. The turbidimetric (23, 24), potentiometric (25) and agar diffusion methods are subject to interference caused by other antibiotics and they lack a uniform procedure for all the antibiotics.

HPLC has been applied to the quantitation of penicillins in pharmaceuticals. One procedure for the quantitation of penicillin in biological fluids does exist (17), however the drug is broken down with acid and detected by post-column derivatization. Our procedure requires no derivatization and detects the presence of the intact drug. Soldin et al. (18) have recently described an HPLC procedure for cloxacillin and nafcillin. This procedure utilizes an internal standard and monitors each drug at a different wavelength using a variable wavelength detector. There are no significant differences in between-day coefficients of variation or sensitivity between the two procedures. However, we can quantitate all five drugs at a single wavelength on a less expensive detector. Their procedure requires vacuum evaporation and reconstitution of a chloroform extract with approximately a 95% recovery of the drugs. The extraction procedure we describe

requires no evaporation and also concentrates the antibiotics in a smaller volume which allows accurate determination of low serum levels. This concentration is a logical consequence of the extraction procedure. The acetonitrile precipitates the proteins and extracts the lipids resulting in a reduced volume and thus increasing concentration. A significant increase in drug concentration occurs during the methylene chloride extraction step. Acetonitrile is miscible in methylene chloride, while the aqueous material in serum is practically insoluble. Thus the acetonitrile is effectively removed by the methylene chloride while the antibiotic is concentrated in the upper aqueous layer.

The presence of interfering substances in patient material is always a matter of concern. Despite the fact that no interference occurs when the antibiotics are extracted from normal serum, serum from medicated patients and from pooled sera may contain interfering substances. In our experience with clinical specimens, we found only one specimen we were unable to assay due to interference. This patient was an extreme case of renal failure. Interference in the standards can be detected by making a blank of each new lot of pooled serum or by finding a standard curve that does not pass through the origin. In the case of the patient's serum, there is no practical way to determine interference. This problem can, however, be minimized in two ways: 1) adequate communication between the physician and the laboratory with respect to any and all drug therapy will allow the laboratory to rule out interference by other drugs; and 2) when sufficient patient material is provided (2.5 ml), the standard curve can be constructed by adding antibiotic to aliquots of the patient material. All the samples are extracted and the peak height of the drug in the patient's sample with no added antibiotic is subtracted from those samples containing added antibiotic. A standard curve is then constructed from this data and the antibiotic concentration in the patient's serum read from the graph.

Several procedures routinely use an internal standard to increase accuracy. The addition of internal standard prior to extraction assumes correction for errors in sample and standard manipulation. Although this compensation may not be identical for the internal standard and the compound of interest due to physio-chemical properties, it is assumed that a constant proportion of each is extracted. Tables 2 and 3 clearly show that the addition of an internal standard does not significantly alter the accuracy or precision of this procedure. It would appear that the internal standard may be of some benefit with low drug concentrations. On the whole, an internal standard increases sample preparation and chromatography time and raises the possibility of interference at a second point in the analysis. We do not see the need for an internal standard in this procedure, however, if one is desired, one of the other antibiotics not used in the assay may be included for this purpose. We have found that the addition of a Chaney adaptor to the microsyrine helped to standardize the sample injection, thus increasing precision. For repeated injection of the same sample or group of samples, the automated injection system offers excellent precision.

Active metabolites of the isoxazolyl penicillins are known to be present in both urine and serum (26). These metabolites possess antibacterial activity of approximately the same order of magnitude as the parent compounds. In urine, metabolite activity compared to total antimicrobial activity ranged from 20% for oxacillin to 10% for flucloxacillin with cloxacillin and dicloxacillin being intermediate. However, following a 2 g i.v. injection of cloxacillin the metabolite activity at 90 min was 20% in urine, but only 9% in the serum. Thus it would appear that the metabolite is of lesser importance in the serum. This would also explain the close overall agreement we have observed between HPLC and microbiological methods (Fig. 4). The separation of these metabolites was accomplished by reverse-phase thin layer chromatography (27) and their separation by HPLC was

recently described (28). At the present time, quantitation and separation of these metabolites in routine clinical specimens is not practical for three reasons: 1) the in vivo activity of these metabolites is unknown; 2) the data suggests that their concentrations may be rather insignificant with the possible exception of patients in renal failure; and 3) sufficient quantities of metabolite for standard curve construction with routine specimens is not available.

Column life is a practical consideration with any procedure. Using the procedure outlined above we are able to make approximately 1,500 injections of extracted serum during the lifetime of a column. We have found that the use of a column guard and regular column regeneration greatly extend column life.

In the legend presented for the antibiotics, we have attempted to indicate which antibiotic levels and attenuations will be most useful for routine clinical specimens. Although these levels will be the most frequently encountered, higher levels can be expected in patients with severe infections or in renal failure. For example, we have seen methicillin levels in excess of 200 µg/ml and nafcillin levels in excess of 100 µg/ml.

The data presented demonstrates the efficacy of HPLC for routine clinical testing of antibiotic levels in human serum. We have also applied this procedure to cerebro-spinal, pleural, and joint fluids. Its sensitivity of less than 0.5 µg/ml and accuracy of greater than 95% is more than adequate for routine testing.

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PAIRING ION EFFECTS IN THE REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PEPTIDES IN THE PRESENCE OF ALKYLSULPHONATES*

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ABSTRACT

The influence of pH and the concentration of the two lipophilic pairing ions, hexylsulphonate and camphor-10-sulphonate, on the retention of a group of small peptides to chemically bonded hydrocarbonaceous, microparticulate silicas has been further investigated. With low pH aqueous methanol mobile phases containing various concentrations of these surface active anions, the capacity factors of unprotected and C-protected peptides show similar dependencies on the concentration of the pairing ion. Column selectivity becomes essentially independent of pairing ion concentration above ca 25mM. At higher pH values, the influences of pairing ion interactions on peptide retention appear to diminish due to competing protic equilibria.

INTRODUCTION

Reversed phase high performance liquid chromatography (RP-HPLC) has gained wide popularity over the past decade.

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* High Performance Liquid Chromatography of Amino Acids, Peptides and Proteins, Part XXXIII. For Part XXXII see ref [1].

In part, the versatility of the technique resides in its ability to resolve ionic or ionisable solutes using aqueous mobile phases, based in many cases on hydro-organic solvent mixtures and combinations of compatible buffer reagents. Secondary chemical equilibria established between the polar solute molecules and these components present in the mobile phase can dramatically influence their retention behaviour on hydrocarbonaceous silicas. Manipulation of protonic equilibria is one way by which the retention of ionised compounds can be varied on these non-polar supports. Control over selectivity can also be achieved by means of suitable interactive lipophilic or hydrophilic ions added at an appropriate concentration to the aqueous mobile phase. In this circumstance, which is commonly referred to as 'ion-pair' reversed phase chromatography, the retention of ionised solutes can be augmented or attenuated depending on the polarity of the added buffer ion. With lipophilic ions, adsorption of the ion onto the hydrophobic surface of the column packing material can occur and this will result in the stationary phase effectively behaving as a dynamic solvent-generated ion-exchanger. With hydrophilic ions, adsorption to the hydrophobic surface of the column packing material is much less favoured and ion-pair formation may take place in the aqueous mobile phase followed by sorption of the solvated complex to the non-polar stationary phase surface. A variety of retention mechanisms have been proposed [2-6] to account for the influence of these competing secondary equilibria on the chromatographic behaviour of ionised solutes such as catecholamines and polar pharmaceuticals.

Similar concepts have been recently applied to the analysis and purification of peptides from synthetic and natural sources. Because of the flexibility which can be achieved over retention

and selectivity with interactive electrolyte systems, the use of RP-HPLC has consequently become very popular in peptide and protein chemistry. These areas of application have recently been reviewed [7-9]. The main objective of the present study was to further elucidate the influence of protonic and pairing-ion interactions on the retention of ionised peptides to chemically bonded hydrocarbonaceous stationary phases. We restricted this study to mobile phases containing low concentrations of alkyl-sulphonates in aquo-methanol mixtures over the range pH 2.5-7.0 and relatively simple peptides, some with their C-terminus protected.

EXPERIMENTAL

Apparatus:

All the data were collected under isocratic elution conditions using a Waters Associates (Milford, Mass., U.S.A.) HPLC system which included a M6000 A solvent delivery system, an U6K universal liquid chromatograph injector and a M450 variable wavelength UV monitor coupled to a Rikadenki dual channel recorder. The μBondapak C_{18} columns (10μm, 30cm x 4mm I.D.) were purchased from Anac (NZ) Ltd. Sample injections were made with Pressure-Lok liquid syringes, series B110, from Precision Sampling (Baton Rouge, La., U.S.A.).

Chemicals and Reagents:

All solvents were Analar grade and purified prior to use as described previously [10]. The amino acids and peptides used in this study were obtained from Sigma Chem. Co. (St Louis, Mo., U.S.A.) and Vega Biochemicals (Tucson, Ariz., U.S.A.). All amino acids except glycine were of the L-configuration. The alkylsulphonic acids were obtained from B.D.H. (Poole, Great Britain) or prepared from the corresponding alkylbromide and anhydrous sodium sulphite [11]. Orthophosphoric acid,

LINEAR SEQUENCES OF PEPTIDES USED IN THE PRESENT STUDY*

No.	Peptide	No.	Peptide
1.	G.	7.	V.L.
2.	G.G.	8.	F.L. amide
3.	A.G.	9.	A.K.
4.	G.F.	10.	G.L.Y.
5.	G.F. amide	11.	G.G.Y. amide
6.	R.F.	12.	R.F.A.

* The one letter code for the amino acids is as given by M.O. Dayhoff in Atlas of Protein Sequence and Structure (National Biomedical Research Foundation, Silver Spring, Md., U.S.A., 1972), G = gly, A = ala, F = phe, R = arg, V = val, L = leu, K = lys, and Y = tyr.

sodium hydroxide and sodium dihydrogen phosphate were obtained from May and Baker (Dagenham, Great Britain).

Methods:

All chromatograms were carried out at ambient temperature (ca 18^{o}). All peptides were made up in the eluent under study. Bulk solvents were degassed by sonication and the appropriate mobile phases prepared and equilibrated to operating conditions as reported previously [12]. All columns were equilibrated to new eluent conditions for at least 100 column volumes. Sample sizes were generally 10μg peptide material injected in 5- or 10-μl volumes. The capacity factors were calculated as reported previously [10] and the data analysed using a non-linear least squares fit programme developed for the ligand

adsorption model. Two modified subroutines "Miniz" and "Fun" were bound to a BMD 07R programme (Biomedical Computer Programs, University of California) to determine the curves of best fit from the non-linear least squares analysis on a Burroughs 6700 Computer. Copies of the modified subroutines of the programme appear in the appendix.

RESULTS AND DISCUSSION

At low pH, an unprotected peptide with only a single N-terminal amino group will exist in solution effectively as the zwitterionic species, HP_i, and as the positive ion, $H_2P_i^+$. The corresponding C-protected peptide will be present only in the protonated form, $H_2P_i^+$, under these conditions. The capacity factor for a C-protected peptide on a hydrocarbonaceous stationary phase with a mobile phase containing a surface active alkylsulphonate, e.g. sodium hexanesulphonate(Na^+Hex^-), can be given by

$$k' = \psi \cdot K_e \frac{[Na^+Hex^-]_s}{[Na^+]_m} \qquad \ldots.(1)$$

where ψ is the phase ratio, $[Na^+Hex^-]_s$ and $[Na^+]_m$ the concentrations of the bound sodium hexanesulphonate and sodium ions in the mobile phase respectively and K_e is the equilibrium binding constant for an ion-exchange event, i.e.

$$K_e = \frac{[H_2P_i^+Hex^-]_s [Na^+]_m}{[Na^+Hex^-]_s [H_2P_i^+]_m} \qquad \ldots.(2)$$

Similarly, the capacity factor for a diprotic unprotected peptide can be expressed in the form

$$k' = \psi \left[K_{e_1} + \frac{K_{e_2} K_{a_1}}{[H^+]} \right] \frac{[H^+]}{[H^+] + K_{a_1}} \cdot \frac{[Na^+Hex^-]_s}{[Na^+]_m} \qquad \ldots.(3)$$

where K_{e_1}, K_{e_2} are the equilibrium binding constants for the charged and zwitterion species respectively and K_{a_1} the dissociation constant for the ionisation event concerned. Alternative forms of equations 1 and 3 can be derived from the explicit hetaeric model of Horvath and coworkers [3,13], e.g. in the circumstance where the chromatographic process is represented by the limiting case of dynamic ion exchange, the capacity factor is given by

$$k' = \psi \frac{K_0[Hex^-]_s + K_1K_4[Hex^-]_m[Hex^-]_s}{(1 + K_1[Hex^-]_m)(1 + K_2[Hex^-]_m)} \quad ....(4)$$

where $[Hex^-]_s$ and $[Hex^-]_m$ are the maximum surface concentrations of bound hexanesulphonate and the concentration of hexanesulphonate in the eluent, K_0 and K_1 are the equilibrium constants for the binding of the peptide and hexanesulphonate to the stationary phase surface, K_2 and K_4 are the equilibrium constants for ion-pair formation in the mobile phase and dynamic ion-exchange complex formation at the surface of the stationary phase respectively.

The effect of ionisation on the capacity factor for a C-protected monoprotic peptide under these conditions can be expressed as

$$k' = \frac{k_0 + k_1 \frac{[H^+]}{K_{a_1}}}{1 + \frac{[H^+]}{K_{a_1}}} \quad ....(5)$$

where k_0 and k_1 are the limited capacity factors of the unionised and positively charged species and K_{a_1} is the protonic dissociation constant. In a similar fashion, the capacity factor of a diprotic peptide is given by

$$k' = \frac{k_o + k_{-1}\frac{K_{a_2}}{[H^+]} + k_1\frac{[H^+]}{K_{a_1}}}{\frac{K_{a_2}}{[H^+]} + \frac{[H^+]}{K_{a_1}} + 1} \qquad \ldots.(6)$$

where k_o, k_{-1} and k_1 are the limiting capacity factors of the zwitterionic, anionic and cationic forms of the unprotected diprotic peptide and K_{a_1} and K_{a_2} are the first and second acid dissociation constants respectively.

Under constant low pH elution conditions, eqns 1-4 predict that the capacity factor for both unprotected and C-protected peptides will initially increase and then slowly decrease with increasing pairing ion concentrations, provided $K_1[Hex^-]_m$ and $K_2[Hex^-]_m$ are both of the order of unity at the highest pairing ion concentration. Thus a parabolic dependence of the capacity factor on pairing ion concentration is anticipated provided complex exchange processes [3] do not occur. An approximately parabolic dependence will also occur if the pairing ion forms, at a sufficiently high concentration, micelles into which the solutes can partition. In the case where one of the terms in the demoninator of eqn 4 vanishes, e.g. when binding of the pairing ion to the non-polar stationary phase is unfavoured and $K_1[X^-]_m$ is small, the plot of k' versus $[X^-]_m$ is expected to take the form of a rectangular hyperbola. Furthermore, as the pH is increased at constant pairing ion concentration, the capacity factors of unprotected peptides are anticipated to initially fall, reach minima values proximal to the pI values and then slowly increase at higher pH values. The capacity factors of C-protected peptides on the other hand should increase more rapidly at higher pH values.

Figure 1 shows the retention behaviour of the peptides listed in the Table as a function of the sulphonate concentra-

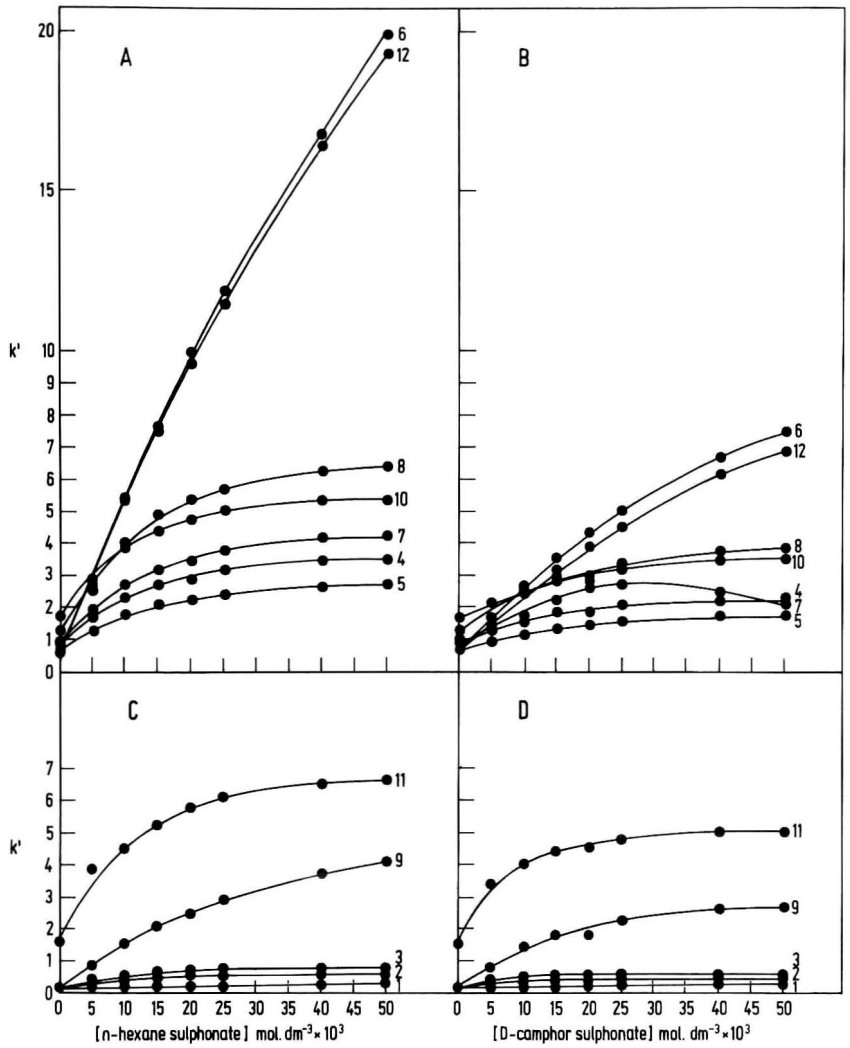

Figure 1. Dependence of the capacity factors of protonated peptides on the concentration of pairing ion in the mobile phase. Chromatographic conditions: column μBondapak C_{18}; flow rate, 2ml/min; temperature, 18°; mobile phases, (A) and (B) 25% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 10M NaOH to pH 3.0, and (C) and (D) 5% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 100mM NaOH to pH 3.0 containing various concentrations of the pairing ion reagents. Peptide key given in the Table.

tion for two alkylsulphonates in aqueous methanol eluents containing 50mM NaH_2PO_4, pH 3.0. These results obtained with hexanesulphonate and camphor-10-sulphonate are consistent with earlier observations [2,6-8,11,12,14] on the effect of anionic and cationic lipophilic reagents on peptide and amino acid retention to alkylsilicas. These previous studies have demonstrated the difficulty in making unambiguous interpretations of such chromatographic data in terms of either an exclusively ion-pair or a dynamic liquid-liquid ion-exchange mechanism because the formal dependence of retention on the lipophilic ion concentration in the mobile phase is the same in both cases. As noted above, the retention mechanism is dependent on a variety of experimental parameters and may change upon varying the mobile phase water content or, for that matter, differ slightly from one lipophilic counterion to another or from one peptide to another. Both of the alkylsulphonates used in the present study are, however, known to act as surface active anions as revealed by their adsorption isotherms which are of the L- or H-type and obey the relationship explicit to the Freundlich equation, i.e.

$$[X^-]_s = a\,[X^-]_m^b \qquad \ldots.(7)$$

According to the ligand adsorption model [2,3], the general form of the capacity factor dependence on $[X^-]$ can be expressed by the relationship

$$k' = (k_o + \beta[X^-])\cdot(1 + K_1[X^-])^{-1}\cdot(1 + K_2[X^-])^{-1} \qquad \ldots.(8)$$

where k_o is the capacity factor of the solute in the absence of a pairing ion and the meaning of β depends on the underlying physicochemical equilibria controlling retention, i.e. for dynamic liquid-liquid ion exchange $\beta = K_1K_4$ and for ion pair formation followed by distribution to the stationary phase $\beta = K_2K_3$ where K_3 is the ion pair equilibrium distribution

constant. Under chromatographic distribution conditions which involve only ion pairs, the dependence of the capacity factor for ionised solutes on pairing ion concentration can be expressed in terms of a modified form of equation 8, namely,

$$k' = (k'_0 + K_2K_3[X^-])\cdot(1 + K_2[X^-])^{-1} \quad ....(9)$$

Based on data obtained with 3-nitrobenzoic acid and sodium cromoglycinate as model solutes and alkylbenzyldimethylammonium chlorides as pairing ions, Riley et al. [15] concluded that ion-pairing in the mobile phase followed by distribution to the stationary phase was the dominant retention mechanism. Furthermore, this group demonstrated that the bulk phase liquid-liquid distribution coefficients for ion-pair association, K_D, are linearly related to the chromatographic ion-pair distribution constants, K_3, viz.

$$K_{3,i} = a_iK_{D,i} + b_i \quad ....(10)$$

where a_i and b_i are the slope and intercept coefficients for solute i.

Analysis of the retention data for compounds (1) - (12) as a function of pairing ion concentration in terms of equations 7 and 8 was carried out on a Burroughs 6700 computer using a BMD computer programme modified to determine curves of best fit from the non-linear least squares analysis. In all cases better fit of the experimental data to the relationship inherent in the three parameter equations, i.e. eqn 8, was obtained. Although this circumstantial evidence may favour the involvement of dynamic ion exchange processes at the surface of the stationary phase as has been proposed in several studies [5,6,12-14], precise data on the magnitude of the association constants for protonated peptides and alkylsulphonates are required from suitable extrachromatographic experiments before such a postulated mechanism could be unequivocally substantiated.

With the peptides studied the trend was apparent with k´ (hexanesulphonate)>k´(camphor-10-sulphonate) although selectivity factors were similar. In Figure 2 are shown plots of $\Delta\alpha/\alpha$ versus pairing ion concentration. Above about 25mmol/l, the

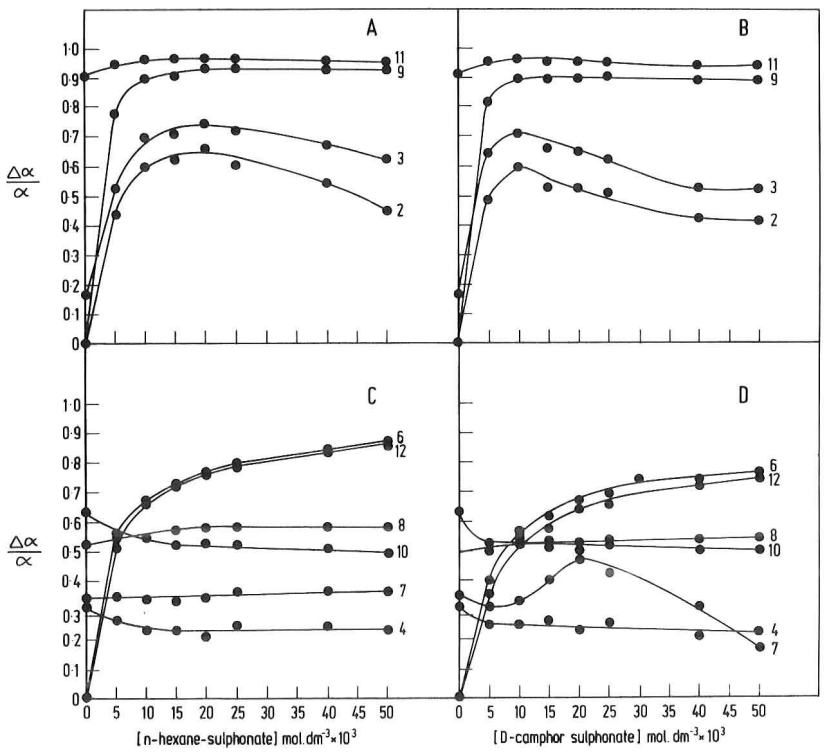

Figure 2. Plots of the dependence of selectivity parameters of protonated peptides on the concentration of pairing ion in the mobile phase. Chromatographic conditions: column, μ-Bondapak C_{18}; flow rate, 2ml/min; temperature, 18°; mobile phases, (A) and (B) 5% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 10M NaOH to pH 3.0 and (C) and (D) 25% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 with 100mM NaOH to pH 3.0 containing various concentrations of the pairing ion reagent. The α value of peptides (1) and (5) were taken as unity in the calculation of the $\Delta\alpha/\alpha$ values. Peptide key given in the Table.

selectivity coefficients become effectively independent of both the hexylsulphonate and the camphor-10-sulphonate concentration for these peptides, i.e. above this limiting value the capacity factors but not selectivities can be increased by appropriate increases in the pairing ion concentration. Similar observations have been made [12] for the retention behaviour of larger peptides on reversed phase silicas in the presence of lipophilic ions. The observed isocratic elution order for the peptides under the low pH, pairing ion conditions was consistent with the current concept that peptide selectivity in RP-HPLC to a large extent reflects differences in their interfacial hydrophobic contact areas. These contact area parameters can be related [2] to the relative hydrophobicities of the individual peptides. The side chain functionality, positional array and extent of ionisation all make significant contributions to the overall hydrophobicity of a peptide under these low pH and pairing ion conditions. As can be seen from Figures 1, 3 and 4 the capacity factors of the glycinyl peptides do not show a pronounced dependency on pairing ion concentration or on pH under the conditions examined, a result which suggests that the peptide chain may make only a small contribution to the sorption phenomena of peptides chromatographed under these conditions.

Shown in Figure 3 are typical plots of k′ versus pH for several peptides chromatographed on a μBondapak C_{18} column with a phosphate based eluent. Two types of retention behaviour are evident from these plots. The first type, shown by the C-protected peptides, is characteristic of weak bases. As the pH increases, the charged ammonium moieties of the C-protected peptide deprotonate. With a reduction in the extent of ionisation, the k′ values of these peptides increase. The observed dependency of k′ on pH shown, for example, by peptide (8) is in good agreement with eqn 5, which predicts that plots

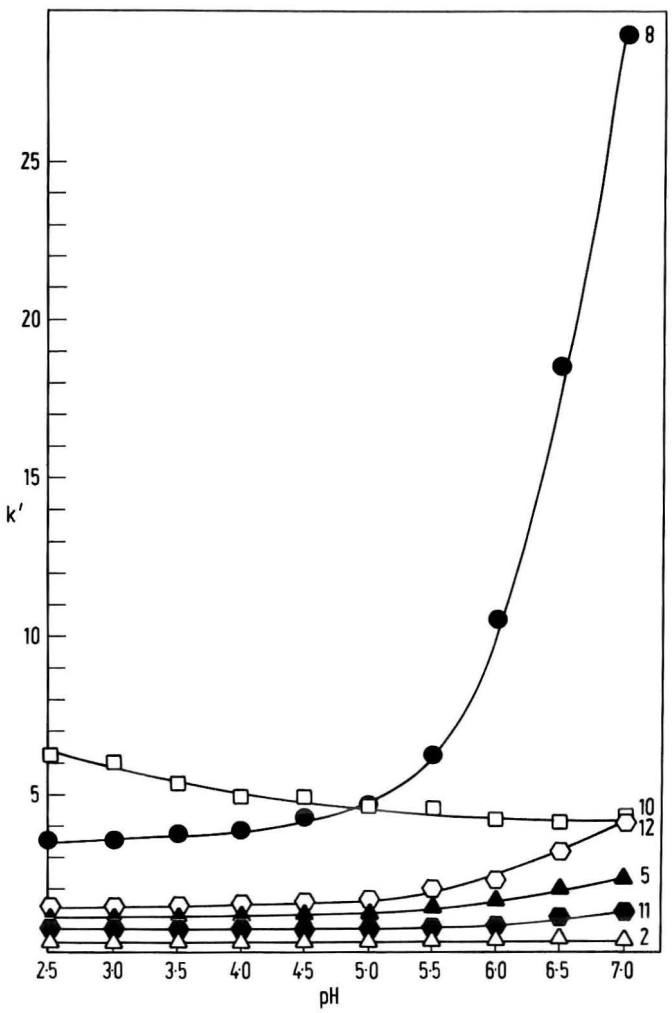

Figure 3. Capacity factors for several peptides on a μ-Bondapak C_{18} column as a function of pH. The eluent was 5% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4 titrated with 10M NaOH over the range pH 2.5-7.0; flow rate 2ml/min; sample size, 5μg/5μl injections. Peptide key given in the Table.

of k′ versus pH for such weak monoprotic bases should be sigmoidal with the inflexion pH value corresponding to the pK_{a_1} value. Because of the instability of hydrocarbonaceous bonded silicas above ca pH 7.5 it was not possible to complete the pH titration although the trend is evident. Similar arguments can be applied to the treatment of k′ versus pH plots of N-protected peptides and other weak acids where the reverse situation exists, e.g. k′ increases with decreasing pH. With the small unprotected peptides, such as gly-leu-tyr and arg-phe-ala competing retention contributions due to the protonic equilibria of the amino and carboxyl groups will tend to be counterbalanced in the pH region proximal to the isoelectric point of the peptide and this will lead to maximal ionisation and minimum retention. At pH values higher than the pI, the retention behaviour of unprotected peptides will increasingly reflect the contribution made by the free amino group to the retention process. With polyprotic peptides, the effect of additional ionogenic centres on the dependence of k′ on pH can be accomodated in expanded expressions similar to eqn 6 with addition k'_n, K_{a_n} terms included for each additional ionogenic centre. In general k′ values will reflect the extent of ionisation of the solute, i.e. as the extent of ionisation increases, k′ will decrease.

Shown in Figure 4 are representative data on the influence of pH on the k′ values of unprotected and C-protected peptides at constant hexanesulphonate molarity of 10mM. At otherwise constant mobile phase composition, the pH strongly influences the retention of both classes of peptides under these conditions. As anticipated on the basis of eqns 2 and 3, the k′ values of all the peptides initially decreased when the pH was increased from pH 3.0. Comparable decreases in k′ values with increasing pH has also been observed with amino acids under similar

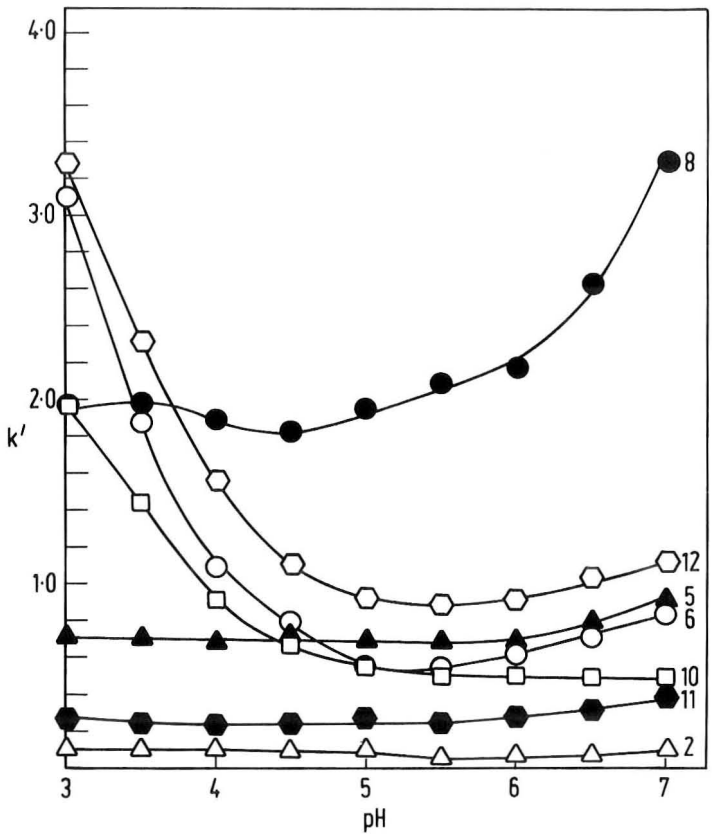

Figure 4. Capacity factors for several peptides on a μ-Bondapak C_{18} column as a function of pH in the presence of a low concentration of pairing ion reagent. The eluent was 20% methanol-water-50mM NaH_2PO_4-15mM H_3PO_4-10mM sodium hexane-sulphonate, titrated with 10M NaOH over the range pH 2.5-7.0; flow rate, 1ml/min; sample size 5μg/5μl injections. Peptide key given in the Table.

conditions with dodecylsulphonate system [14]. Above ca pH 5.0, pairing ion interactions appear to play less significant roles with the k′ values of the various peptides generally increasing with an elution order comparable to that observed with the corresponding phosphate based mobile phase with the

pairing ion deleted. As has been discussed elsewhere [8,12], the k′ versus pH plots determined in the presence of low concentrations of lipophilic pairing ions, can be used to assess the homogeneity of a particular peptide over a very wide range of ionisation conditions. Major selectivity changes can be easily achieved and related to the physicochemical basis of the retention mechanism under these conditions. Since several competing secondary chemical equilibria are simultaneously modulated in a controlled manner, excellent resolution can be achieved for very closely related peptides by the judicious choice of both the pH and pairing ion condition. Similar criteria can be proposed for the assessment of polypeptide and protein homogeneity and have been employed for the reversed phase HPLC analysis of the tryptic maps of proteins, including pituitary protein hormones [10,16,17].

ACKNOWLEDGEMENTS

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APPENDIX 1: SUBROUTINE MINIZ

```
0       $SET SEPARATE                                                                   00001000
1            SUBROUTINE MINIZ(X,A,P,NV,NC,NP1,TOL,EPS,EE,ND,MXIT,LLK,P0,P1,IW)  00001100
2       C       SUBROUTINE MINIZ FOR BMDX85                    APRIL  4, 1966       00002000
3            DIMENSION A(NP1,NP1),X(NV,NC),P(100),FP(100),D(100),P0(100),P1(100  00003000
4           1),B(10)                                                                00004000
5            DIMENSION PP0(100)                                                     00005000
6       C                                                                           00006000
7       C                                                                           00007000
8            P(4)=X(2,1)                                                            00008000
9            WRITE(6,999) P(4)                                                      00008100
10       999 FORMAT(9HKVALUE = F5.2)                                                00008200
11      C                                                                           00008300
12           IF(IW.EQ.0)C=1.0                                                       00009000
13           NP=NP1-1                                                               00010000
14           MNAIL = 10                                                             00011000
15           NAIL = 0                                                               00012000
16           DO 557 I=1,NP                                                          00013000
17       557 PP0(I)=P(I)                                                            00014000
18      C                                                                           00015000
19           EPS1=5.*EPS+1.                                                         00016000
20           WRITE(6,23)(P0(I),I=1,NP)                                              00017000
21        23 FORMAT('1MINIMA',15X,1P9E12.4/(22X,1P9E12.4))                          00018000
22           WRITE(6,22)(P1(I),I=1,NP)                                              00019000
23        22 FORMAT('0MAXIMA',15X,1P9E12.4/(22X,1P9E12.4))                          00020000
24           WRITE(6,195)                                                           00021000
25        195 FORMAT('0ITERATION    ERROR     PARAMETERS'/14X,'MEAN'/14X,'SQUARE  00022000
26          1')                                                                     00023000
27       194 LLL=5                                                                  00024000
28           LIT=0                                                                  00025000
29           HU=NC                                                                  00026000
30           H=0.                                                                   00027000
31           LLLL=5                                                                 00028000
32           E0=7.2E65                                                              00029000
33           DO 556 I=1,NP                                                          00030000
34       556 P(I)=PP0(I)                                                            00031000
35           FNC=NC-NP                                                              00032000
36       29  DO 85 I=1,NP1                                                         00033000
37           DO 85 J=1,I                                                            00034000
38       85  A(I,J)=0.                                                             00035000
39           DO 2 L=1,NC                                                            00036000
40      C    CALL FUN(F(L),FP,X(1,L),P,LLK)                                         00037000
41           CALL FUN(Q,FP,P,X(1,L))                                                00038000
42           FP(NP1)=X(ND,L)-Q                                                      00039000
43           IF(IW.NE.0)C=X(IW,L)                                                   00040000
44           IF(C.LT.(1.E-20)) GO TO 2                                              00041000
45           DO 1 I=1,NP1                                                           00042000
46           FPIC=FP(I)*C                                                           00043000
```

```
      DO 1 J=1,I                                                        00044000
    1 A(I,J)=A(I,J)+FPIC*FP(J)                                          00045000
    2 CONTINUE                                                          00046000
      EE=A(NP1,NP1)/FNC                                                 00047000
    6 IF(EE.LE.E0) GO TO 871                                            00048000
      NAIL=NAIL+1                                                       00049000
      IF(NAIL.GT.MNAIL) GO TO 871                                       00050000
      DO 872 I=1,NP                                                     00051000
      P(I)=PP0(I)+D(I)/2.                                               00052000
  872 D(I)=D(I)/2.                                                      00053000
      GO TO 29                                                          00054000
C                                                                       00055000
C                                                                       00056000
  871 WRITE(6,198)LIT,NAIL,EE,(P(I),I=1,NP)                             00057000
  198 FORMAT(I6,1X,I2,1X,1P10E12.4/(22X,1P9E12.4))                      00058000
      NAIL=0                                                            00059000
 8191 LIT=LIT+1                                                         00060000
      CALL STEP(A,D,P,P0,P1,NP1,TOL)                                    00061000
      IF(LIT.GT.MXIT) GO TO 200                                         00062000
      IF(EE.EQ.0.) GO TO 470                                            00063000
      PC=1.                                                             00064000
      IF(E0.NE.7.2E65) PC=ABS((E0-EE)/EE)                               00065000
      E0=EE                                                             00066000
      IF(PC.LT.EPS) GO TO 100                                           00067000
      LLL=LLLL                                                          00068000
  121 DO 28 I=1,NP                                                      00069000
      PP0(I)=P(I)                                                       00070000
   28 P(I)=P(I)+D(I)                                                    00071000
      GO TO 29                                                          00072000
  200 WRITE (6,201)                                                     00073000
  201 FORMAT(30H0THE PROCESS IS NOT CONVERGING)                         00074000
      GO TO 470                                                         00075000
  100 LLL=LLL-1                                                         00076000
      IF(LLL.NE.0) GO TO 121                                            00077000
  470 DO 778 I=1,NP                                                     00078000
  778 FP(I)=SQRT(ABS(A(I,I)*EE))                                        00079000
      WRITE (6,779) (FP(I),I=1,NP)                                      00080000
  779 FORMAT('0ASYMPTOTIC STANDARD DEVIATIONS OF THE PARAMETERS'//      00081000
     X(10X,1P10E12.4))                                                  00082000
      DO 841 I=1,NP                                                     00083000
      FP(I)=SQRT(ABS(A(I,I)))                                           00084000
      DO 841 J=1,I                                                      00085000
  841 A(J,I)=A(I,J)                                                     00086000
      WRITE(6,774)                                                      00087000
  774 FORMAT('0ASYMPTOTIC CORRELATION MATRIX OF THE PARAMETERS')        00088000
      L1=0                                                              00089000
  371 L0=L1+1                                                           00090000
      L1=MIN0(NP,L1+10)                                                 00091000
      WRITE(6,372)(L,L=L0,L1)                                           00092000
  372 FORMAT('0',10I12)                                                 00093000
      DO 842 I=1,NP                                                     00094000
      K=0                                                               00095000
```

APPENDIX 1 (Continued)

```
      DO 373 J=LO,L1                                                    00096000
      K=K+1                                                             00097000
      IF(FF(I).EQ.0.0.OR.FF(J).EQ.0.0.OR.A(I,J).EQ.0.0)GO TO 843        00098000
      B(K)=A(I,J)/(FF(I)*FF(J))                                         00099000
      GO TO 373                                                         00100000
  843 B(K)=0.0                                                          00101000
  373 CONTINUE                                                          00102000
  842 WRITE(6,374)I,(B(J),J=1,K)                                        00103000
  374 FORMAT(I4,2X,10F12.5)                                             00104000
      IF(L1.NE.NP)GO TO 371                                             00105000
 1515 WRITE(6,553)                                                      00106000
  553 FORMAT('1CASE                    F          Y-H  STANDARD        VARIABLES00107000
     1 /30X,'DEVIATION'/30X,'OF ESTIMATE')                              00108000
      DO 551 L=1,NC                                                     00109000
      SUEST=0.0                                                         00110000
      CALL FUN(Q,FF,F,X(1,L))                                           00111000
      DO 24 I=1,NF                                                      00112000
      DO 24 J=1,NF                                                      00113000
   24 SUEST=SUEST+FF(I)*FF(J)*A(I,J)                                    00114000
      SDEST=SQRT(SUEST*EE)                                              00115000
      T=X(ND,L)-Q                                                       00116000
  551 WRITE(6,552)L,Q,TT,SDEST,(X(I,L),I=1,NV)                          00117000
  552 FORMAT('1X,I4,10F12.5/(41X,7F12.5))                               00118000
      RETURN                                                            00119000
C                                                                       00120000
      END
```

END OF LIST

APPENDIX 2: SUBROUTINE FUN

```
      $SET  SEPARATE                                                    00120000
            SUBROUTINE FUN(F,D,P,X)                                     00121000
      C       SUBROUTINE FUN FOR TEST PROBLEM OF BMDX85                 00122000
            DIMENSION X(1),D(3),P(4)                                    00123000
            Q=P(4)                                                      00123100
            F=(Q+P(1)*X(1))/(1+P(2)*X(1))                               00124000
            D(1)=X(1)/(1+P(2)*X(1))                                     00125000
            D(2)=(-X(1)*(Q+P(1)*X(1)))/ ((1+P(2)*X(1))**2)              00126000
      C     D(3)=(-X(1)*(Q+P(1)*X(1)))/((1+P(2)*X(1)) * ((1+P(3)*X(1))**2))  00127000
            RETURN                                                      00128000
            END                                                         00129000

 ** END OF LIST **

      $SET  SEPARATE                                                    00120000
            SUBROUTINE FUN(F,D,P,X)                                     00121000
      C       SUBROUTINE FUN FOR TEST PROBLEM OF BMDX85                 00122000
            DIMENSION X(1),D(3),P(4)                                    00123000
            Q=P(4)                                                      00123100
            F=(Q+P(1)*X(1))/((1+P(2)*X(1))*(1+P(3)*X(1)))               00124000
            D(1)=X(1)/((1+P(2)*X(1))*(1+P(3)*X(1)))                     00125000
            D(2)=(-X(1)*(Q+P(1)*X(1)))/((1+P(3)*X(1)) * ((1+P(2)*X(1))**2))  00126000
            D(3)=(-X(1)*(Q+P(1)*X(1)))/((1+P(2)*X(1)) * ((1+P(3)*X(1))**2))  00127000
            RETURN                                                      00128000
            END                                                         00129000

 ** END OF LIST **
```

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REVERSED PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHIC SEPARATION OF PRECOCENES-I, -II, ANTIJUVENILE HORMONES AND THEIR DERIVATIVES

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ABSTRACT

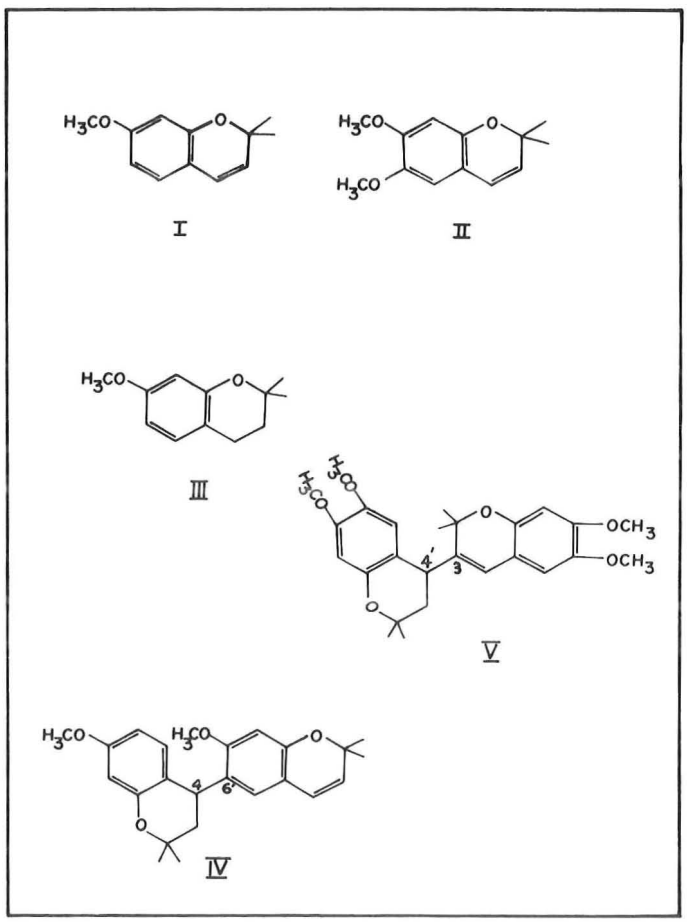
HPLC separation of precocene-I, precocene-II, dihydroprecocene-I, precocene-I dimer and precocene-II dimer has been carried out under varying conditions of eluent concentration for MeOH: H_2O system. It is observed that the mixture of above compounds is adequately resolved using MeOH: H_2O (3:1).

INTRODUCTION

Certain chromene derivatives namely precocenes-I (I), and -II (II) isolated from the plants of genus Ageratum have been found to exhibit antijuvenile hormone activity (1). These have been termed as precocenes due to their ability to bring about precocious metamorphosis in young larval stages of the test insects, resulting thereby into unviable or moribund miniature adults. Precocenes contribute a new type of insect growth regulators and have been referred to as fourth generation insecticides. It has been suggested that the epoxides of precocenes formed during metabolic activity might be the species responsible for their anti-JH activity (2). Therefore the study of mode of action of these compounds and anti-JH activity of derivatives of these compounds becomes very important. However in the biological work as stated above one always comes across nanogram amounts of the metabolites. The detection of

these by any other analytical tool would be extremely difficult. Hence, the separation and detection of these compounds by HPLC technique becomes indispensable.

The five precocenes, namely precocene-I, precocene-II, dihydro-precocene-I (III), precocene-I dimer (7, 7'-dimethoxy, 2,2,2', 2'-tetramethyl-3' (4')-dihydro 6'-4-bichroman (IV) and precocene-II dimer (6,7,6',7'-tetramethoxy-2,2,2',2' tetramethyl-3' (4')-dihydro 3-4' S-bichroman) (V) were chosen as model compounds for their separation studies. These were found to exhibit a reasonably strong UV absorption and could be detected in low concentration, using a UV detector (280 nm).



MATERIALS AND METHODS

Chemicals and Reagents

Precocenes-I and -II were isolated from Ageratum conyzoides plants (3). Precocene-II dimer was synthesized by methods reported earlier (3), while, precocene-I dimer was synthesized by an alternative method (4). All of these were dissolved in methanol. Methanol (spectroscopic grade) and acetone (analytical reagent grade) were purchased from E. Merck (India).

Apparatus

A Waters Associates Instrument, Model ALC/GPC 244, equipped with a Model 6000 A solvent delivery system, U6K injector and Model 440 detector was used.

A μ Bondapak C_{18} column (stainless steel 300 mm x 3.9 mm I.D.) with particle size (10 microns) was purchased from Waters Associates. The dead volume of column between the point of injection and the UV detector was found using acetone.

Analytical conditions

Various mixtures of methanol and water were used as mobile phases. One pump was employed to pump water and the other for methanol, the percentage of each being controlled by the programmer. Prior to the analysis, the column was washed for half an hour with methanol (flow rate 1 ml/min.).

RESULTS

The separation of precocenes and their derivatives was carried out with good resolution using μ Bondapak C_{18} column and MeOH: H_2O eluent system. Fig. 1 shows separation for MeOH:H_2O (3:1) eluent system. The retention times (R_t), capacity factors (k') and separation factors (α) for the above eluent system are recorded in Table 1. The capacity factors are calculated with acetone as the reference, as it absorbs in the UV and has no retention on the column.

Similarly, these compounds showed clean separation with CH_3CN: H_2O (3:2) as eluent system. The retention times (R_t), capacity factors (k') and separation factors (α) are shown in Table 2.

Effect of solvent strength on retention time.

The variation of retention time versus the polarity of the eluent was also investigated. Five concentrations of the eluent methanol and

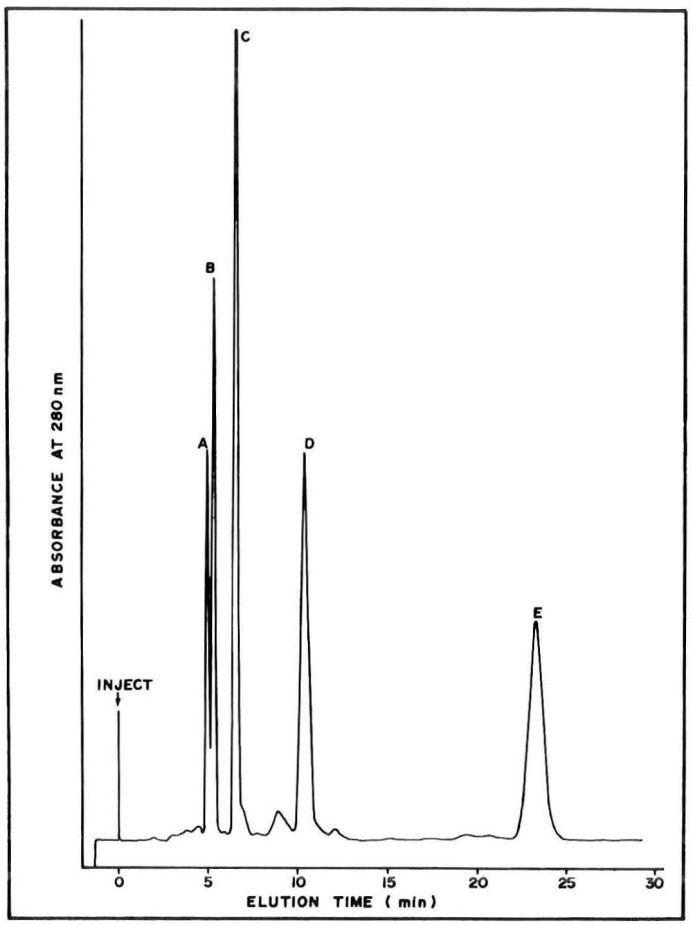


FIGURE 1 :- HPLC separation of precocene-I, precocene-II, dihydro precocene-I and dimers of precocene-I and -II at 280 nm with a μBondapak C_{18} column and MeOH:H_2O (3:1) at a flow rate of 1 ml/min. Chromatogram represents a standard solution containing known quantities of the following in the 20λinjection : Precocene-I, 4 μg (C), precocene-II, 5 μg (B), dihydroprecocene-I, 4 μg (A), dimer of precocene-I, 10 μg (E) and dimer of precocene-II, 7 μg (D).

TABLE I

RETENTION TIMES, CAPACITY FACTORS AND SEPARATION FACTORS FOR PRECOCENES USING MeOH:H_2O (3:1 V/V) AS ELUENT. FLOW RATE = 1 ml/min. DEAD TIME = 3.25 min.

Precocene	Retention Time (min)	k'	α
Dihydro-precocene-I	5.0	0.54	1.17
Precocene-II	5.3	0.63	1.46
Precocene-I	6.25	0.92	2.26
Precocene-II-dimer	10.0	2.08	2.92
Precocene-I-dimer	23.0	6.08	

TABLE II

RETENTION TIMES, CAPACITY FACTORS AND SEPARATION FACTORS FOR PRECOCENES USING $H_2C{:}CH_3CN$ (2:3 V/V) AS ELUENT. FLOW RATE = 1 ml/min. DEAD TIME = 3.25 min.

Precocenes	Retention Time (min.)	k'	α
Dihydro precocene-I	5.02	0.54	1.5
Precocene-II	5.9	0.81	1.56
Precocene-I	7.38	1.27	1.81
Dimer of precocene-II	10.72	2.23	2.71
Dimer of precocene-I	23.52	6.24	

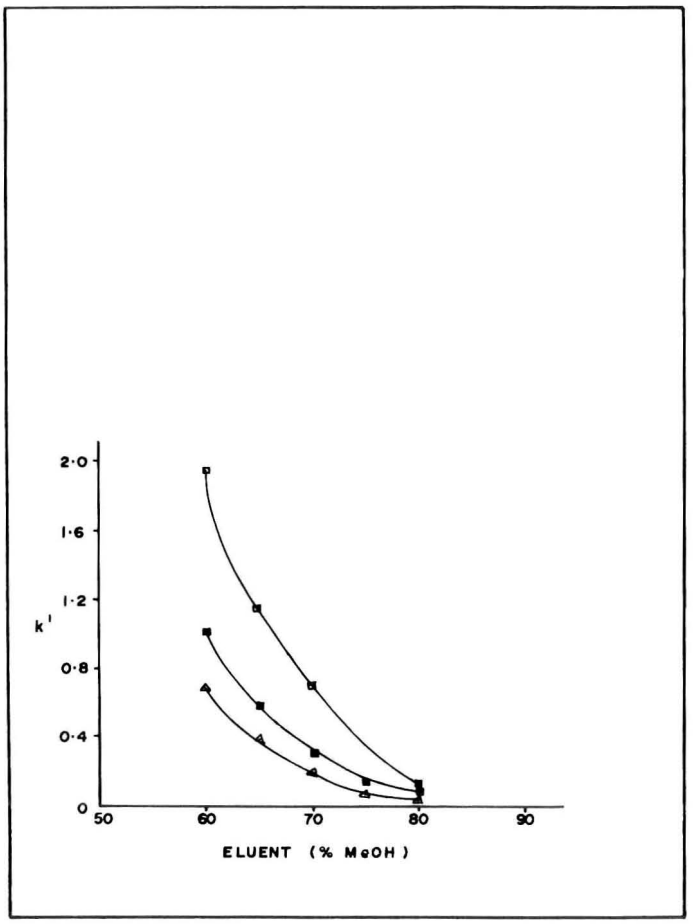


FIGURE 2 :- Effect of polarity of eluent on the capacity factor of precocenes, at a flow rate of 1.5 ml/min. The symbols in the figure are: -□- = I, -■- =II, -△- =III.

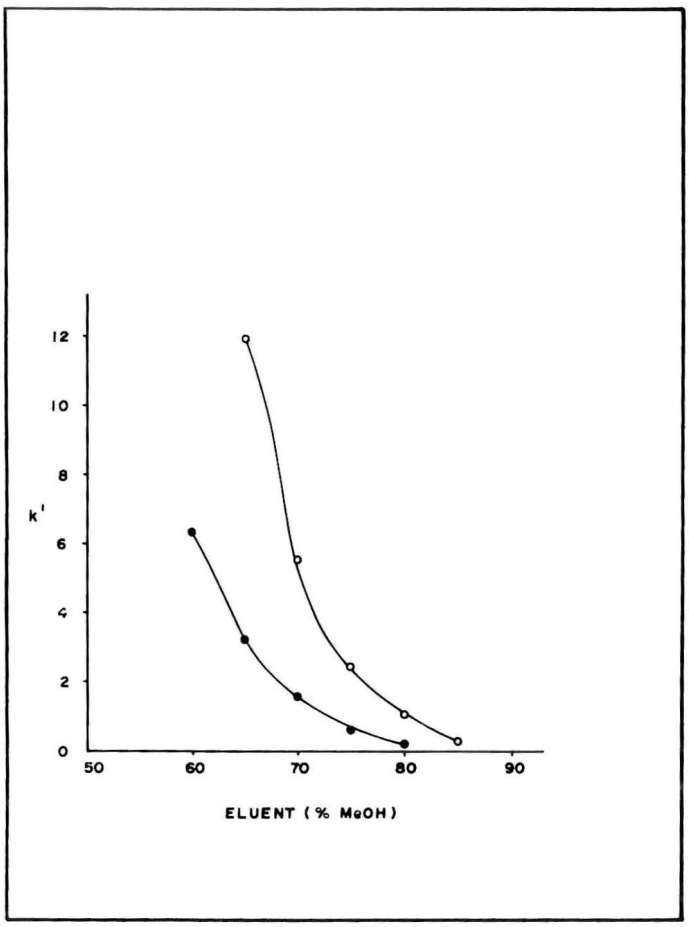


FIGURE 3 :- Effect of polarity of eluent on the capacity factor of precocene dimers at a flow rate of 2 ml/min. The symbols in the figure are:

—○— = IV, —●— = V.

water (20, 25, 30, 35, 40) were taken for this purpose. The capacity factors of these compounds were calculated and are plotted against polarity of the eluent (Figs. 2 and 3). It was found that k' decreased with increasing concentration of methanol in the eluent.

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THIN-LAYER CHROMATOGRAPHIC SEPARATION OF SOME ALLIED METAL IONS AND THEIR DETERMINATION BY RING COLORIMETRY

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ABSTRACT

Suitable methods have been worked out for the separation and determination of various groups of similar metal ions in mixtures, employing a combination of thin-layer chromatography and ring colorimetry. Solvent mixtures employed were : n-butanol + 12N HCl + dioxan (5:1:4); n-butanol + 6N HCl + dioxan (5:1:4); hexanol + dioxan + acetic acid + water (160:10:2:40); methanol + water (9:1 and 7:3). The following mixtures of cations were separated by TLC and the separated constituents were subsequently determined by ring colorimetry : Fe(III)- Co(II)- Cu(II)- Pb(II)- Ni(II); Pt(IV)- Co(II)- Cu(II)- Pd(II)- Ni(II); Ag(I)- Ni(II)- Co(II)- Fe(III); Cu(II)- Ni(II)- Bi(III); Cu(II)- Bi(III)- Hg(II).

INTRODUCTION

The work on the applications of thin-layer chromatography (TLC) in quantitative inorganic analysis has been sparse (1). Here, the separation and determination of some inorganic ions have been achieved by using a combination of TLC and ring colorimetry (2). The procedure has been found to be useful and has an

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added advantage that only inexpensive equipment is needed.

The cations studied in the present communication were : Al(III), Ag(I), Cu(II), Co(II), Ni(II), Cd(II), Hg(II), Bi(III), Fe(II), Fe(III), Mn(II), Zn(II), Mg(II), Pd(II), Pt(IV), Rh(III), Ru(III), and UO_2(VI), and the following combinations of developing solvents were used :

(a) n-Butanol + 12N HCl + dioxan (5:1:4)
(b) n-Butanol + 6N HCl + dioxan (5:1:4)
(c) Hexanol + dioxan + acetic acid + water (160:10:2:40)
(d) Methanol + water (9:1)
(e) Methanol + water (7:3)

A number of solvents and acid in various concentration were employed and only the above mentioned combinations have been found to be satisfactory.

EXPERIMENTAL

Metal Solutions :

0.1 Mol dm^{-3} stock solutions of the following were prepared using reagent grade chemicals (BDH or Johnson Matthey, London, England), standardized by the estimation of the metal contents and diluted as necessary:

Nitrates of silver(I), mercury(II), bismuth(III), uranyl(VI), and magnesium(II); chlorides of nickel(II), cobalt(II), copper(II), iron(II), iron(III), platinum (IV), cadmium(II), zinc(II), aluminium(III), manganese(II), palladium(II), ruthenium(III) and rhodium(III).

Chromogenic Reagents :

Diethyldithiocarbamate (DDTC) (BDH AnalaR); 0.1% w/v in ethanol

p-Dimethylaminobenzylidene rhodamine (BDH AnalaR): 0.1% w/v in ethanol

1-(2-pyridylazo)-2-naphthol (PAN; Koch-Light, Coinbrook, England): 0.1% w/v in ethanol

8-Hydroxyquinoline (BDH AnalaR): 2% w/v in chloroform.

Developing Solvents :

Suitable mixtures of n-butanol, dioxan, hexanol, methanol, acetic acid and hydrochloric acid (all BDH AnalaR).

Adsorbents :

Silica gel G (E. Merck, Darmstadt, FGR) and cellulose (Joseph Crosfield and Sons, Coinbrook, England).

Apparatus and Procedure :

TLC equipment kit (Toshniwal, New Delhi, India) was used. The adsorbents were spread on the glass plates (20 X 20 cm^2) as a slurry using an applicator supplied with the equipment. The plates were kept horizontally in the drying racks, dried in air and finally at 110° for 2 hr. They were then stored in desiccators. The plates were examined before use and those with uniform layers were used.

In these studies, solutions of 0.01 mol dm^{-3} concentration were used. In the cases of mixtures, equal volumes of solutions 0.01 mol dm^{-3} were mixed.

The spots of the mixtures were applied to the plates with the help of a self-filling micropipette and allowed to dry in air. The plates were placed with an edge dipped about 2 cm in the solvent and placed vertically in a rectangular glass chamber (21 X 21 X 9 cm^3) covered with ground glass. The chamber had been previously saturated with the vapour of the corresponding solvent.

After the desired migration of ions, the plates were dried in air, examined in visible and ultraviolet light after spraying the appropriate chromogenic reagents. The distances were measured to obtain L_E (leading edge) and T_E (tailing edge). R_f values were obtained from :

$$R_f = \frac{L_E + T_E}{2 \times \text{distance moved by the solvent}}$$

The various ions were identified on the plates as indicated in Table 1.

TABLE 1

Reagents for Identification

Treatment	Observation	Metal Ion
Sprayed with 8-hydroxyquinoline, exposed to NH_3 and held under ultraviolet light.	Dark Spot	UO_2(VI)
	Yellow fluorescent spot	Al(III)
Sprayed with a 1:1 mixture of PAN and p-dimethylaminobenzylidene rhodamine and viewed in :		
(i) Visible light	Red Spot	Mn(II), Ni(II), Cu(II), Zn(II), Cd(II), Hg(II).
	Blue Spot	Co(II), Pd(II), Pt(IV).
	Gray Spot	Fe(II), Fe(III).
	Yellow Spot	Ag(I).
(ii) Ultraviolet light	Dark Spot	Ru(III), Rh(III).
Sprayed with DDTC and viewed in visible light	Green Spot	Pb(II), Bi(III).

For the solvent mixture (a) and (b), the coating material was cellulose, while for the other combinations of solvents i.e. (c), (d) and (e), silica gel was employed. The thickness of the adsorbent layer was 0.2 mm, which was found suitable. The developing time of run was 45 min with solvents (a) and (b), while with (c) the plates were developed for 1.45 hr. The time of development with mixtures (d) and (c) were 30 min. In all the cases, one drop of 0.01 mol dm^{-3} metal solution was used. The R_f values for the individual metal ions are reported in Table 2.

Quantitative Separation by Ring Colorimetry (3)

Table 2 helped in finding the suitable possible combinations of metal ions for quantitative separa-

TABLE 2

R_f values of Various Metal Ions

Metal Ion	Solvent mixture				
	(a)*	(b)*	(c)†	(d)†	(e)†
Al(III)	0.19	0.14	...	...	...
Ag(I)	0	0	...	...	...
Cu(II)	0.78	0.67	0.38	0	0
Co(II)	0.66	0.27	0.07	0	0.09
Ni(II)	0.13	0.13	0.95	0.06	0.10
Cd(II)	...	...	...	0.15	0.17
Hg(II)	...	...	...	0.91	0.15
Bi(III)	...	...	...	0.37	...
Fe(II)	0	0	0	0	0
Fe(III)	0.96	0.89	0	...	...
Mn(II)	0.26	0.22	...	...	...
Zn(II)	0.91	0.87	...	...	...
Mg(II)	0.12	0.095	...	...	...
Pd(II)	...	...	0.62	...	0.16
Pt(IV)	...	...	0	...	...
Rh(III)	...	...	0	...	...
Ru(III)	...	...	0	...	...
UO_2(VI)	...	...	0	...	0.47

*Adsorbent : Cellulose

†Adsorbent : Silica Gel

tions. In the following mixtures, the metal ions were quantitatively estimated by ring colorimetry after they were separated on thin layer chromatograms. They were:

1. Fe(III)- Co(II)- Cu(II)- Pd(II)- Ni(II)
2. Pt(IV)- Co(II)- Cu(II)- Pd(II)- Ni(II)
3. Ag(I)- Ni(II)- Co(II)- Fe(III)
4. Cu(II)- Ni(II)- Bi(III)
5. Cu(II)- Bi(III)- Hg(II).

The mixtures were spotted on the plates as usual and chromatograms were run using the following solvents (Table 3) :

After the run, the duplicates (or guides) were treated with detecting reagents to locate the spots, while the original chromatogram (not treated with reagent) was used for the determinations. The separated substances (located by comparison with the guide) were scarpped off quantitatively, with the help of a spatula, into a sintered glass funnel (porosity G4). Each lot of scrapping was washed carefully with 1 cm^3 of 0.5 mol dm^{-3} HCl using a few drops each time and received on a circular Whatman No.1 filter paper (diameter 55 mm), and treated as usual on the ring oven for the transportation to the ring zone. The standard rings were obtained separately for comparison by ring colorimetry. The amount of metal ion separated

TABLE 3

Solvents Employed

Solvent Mixture	Mixture of Ions
(c)	Fe(III)- Co(II)- Cu(II)- Pd(II)- Ni(II) Pt(IV)- Co(II)- Cu(II)- Pd(II)- Ni(II)
(a)	Ag(I)- Ni(II)- Co(II)- Fe(III)
(e)	Cu(II)- Ni(II)- Bi(III)
(d)	Cu(II)- Bi(III)- Hg(II).

on the TLC plate was known by using the visual comparison of the test rings with standard scales as usual.

Some of the typical results of determinations are shown in Tables 4 to 8.

It has been found that the determinations were possible down to the following limits when present in a few drops of the solutions : Fe(II) 0.84 µg; Ni(II) 0.88 µg; Co(II) 0.89 µg; Cu(II) 0.95 µg; Ag(I) 1.06 µg;

TABLE 4

Quantitative Separation of the Mixture :
Fe(III)- Co(II)- Ni(II)- Cu(II)- Pd(II)

Ion	Taken µg	Found µg	No. of determinations	Error(%)	Standard deviation(%)
Fe(III)	1.68	1.60	4	-4.7	0.60
Co(II)	1.77	1.72	4	-2.9	0.38
Ni(II)	1.76	1.77	4	+0.5	0.17
Cu(II)	1.90	1.90	4	0	0.24
Pd(II)	3.19	3.19	4	0	0.14

Developing Solvent : Hexanol + dioxan + acetic acid + water (160:10:2:40)

Adsorbent : Silica Gel Time of Run : 1.45 h.

TABLE 5

Quantitative Separation of the Mixture :
Ni(II)- Co(II)- Cu(II)- Pd(II)- Pt(IV)

Ion	Taken µg	Found µg	No. of determinations	Error(%)	Standard deviation(%)
Ni(II)	1.76	1.78	4	+1.1	0.16
Co(II)	1.77	1.75	4	-1.1	0.10
Cu(II)	1.90	1.90	4	0	0.08
Pd(II)	3.19	3.20	4	+0.30	0.08
Pt(IV)	5.85	5.80	4	-0.85	0.21

Developing Solvent : Hexanol + dioxan + acetic acid + water (160:10:2:4)

Adsorbent : Silica Gel Time of Run : 1.45 h.

TABLE 6

Quantitative Separation of the Mixture :

Fe(III)- Co(II)- Ni(II)- Ag(I)

Ion	Taken µg	Found µg	No. of determi-nations	Error(%)	Standard deviation(%)
Fe(III)	1.68	1.63	4	-2.9	0.24
Co(II)	1.77	1.75	4	-1.1	0.08
Ni(II)	1.76	1.78	4	+1.1	0.19
Ag(I)	3.24	3.21	4	-0.92	0.20

Developing Solvent : n-Butanol + 12N HCl + dioxan (5:1:4)

Adsorbent : Cellulose Time of Run : 45 min

TABLE 7

Quantitative Separation of the Mixture :

Ni(II)- Cu(II)- Bi(III)

Ion	Taken µg	Found µg	No. of determi-nations	Error(%)	Standard deviation(%)
Ni(II)	1.76	1.78	4	+1.1	0.14
Cu(II)	1.90	1.91	4	+0.52	0.12
Bi(III)	6.27	6.23	4	-0.63	0.19

Developing Solvent : Methanol + water (9:1)

Adsorbent : Silica Gel Time of Run : 35 min

TABLE 8

Quantitative Separation of the Mixture :

Cu(II)- Hg(II)- Bi(III)

Ion	Taken µg	Found µg	No. of determi-nations	Error(%)	Standard deviation(%)
Cu(II)	1.90	1.90	4	0	0.08
Hg(II)	6.03	6.06	4	+0.5	0.36
Bi(III)	6.27	6.23	4	-0.63	0.16

Developing : Methanol + water (7:3)

Adsorbent : Silica Gel Time of Run : 30 min

Pd(II) 1.6 µg; Pt(IV) 2.93 µg; Bi(III) 3.1 µg; U(VI) 3.6 µg.

The total time required for separation and determination of the constituents in the above combinations was about 50-90 min including the time required for the preparation of the standard scales.

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HPLC URINARY ORGANIC ACID PROFILING: ROLE OF THE ULTRAVIOLET AND AMPEROMETRIC DETECTORS[a]

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ABSTRACT

The profiling of urinary organic acids is an important aspect of the diagnosis of inborn metabolic disorders. The carboxylic acids of interest may contain additional functional moities such as phenyl, hydroxyl, oxo, etc. The state-of-the-art method of organic acid analysis is by GC/MS. Prior to GC/MS analysis, the carboxylic acids must be isolated from urine by extraction or ion exchange chromatography and made volatile by derivatization. This is a lengthy procedure that does not lend itself to rapid analysis. We have developed a rapid procedure for the profiling of urinary α, B-unsaturated, aromatic and α-ketocarboxylic acids.

Urine containing an internal standard, 3-hydroxy-4-methoxybenzyl alcohol, is filtered through a 0.3 um Millipore filter and injected on to an HPX-87 organic acid HPLC column (Bio-Rad). The mobile phase, 4.5 mN H_2SO_4, is passed through the column at 0.8 ml/min. Detection is effected by an UV detector at 200 nm in series but upstream from an electrochemical detector with a glassy carbon working electrode at +1.15V vs. an Ag/AgCl reference electrode. At this electrode potential, phenolic, methoxyphenyl, eneolic and α-ketocarboxylic acids are oxidized and can be electrochemically detected with a glassy carbon electrode.

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a. Presented in part to the Seventh Annual Detroit Anachem Symposium, October 16, 1980, Wayne State University, Detroit, MI.

b. To whom inquiries should be addressed.

Results (Rf and peak height ratio) from a urine sample are compared to a group of normal urine samples. Peaks of abnormal size and/or Rf are isolated and rechromatographed on a C_{18} HPLC column using 1% CH_3CN-0.1 M phosphate, pH 2.5. This 2-dimensional map is used in the probable identification of the peak.

INTRODUCTION

The identification and quantitation of urinary carboxylic acids is an important part in the diagnosis of metabolic disorders[1-6]. The state-of-the-art technology for the separation, identification and quantitation of urinary organic acids involves gas chromatography/mass spectrometry[1,3,5,7-10]. Gas chromatographic/mass spectrometric urinary carboxylic acid analyses are not applicable to the routine analysis of a large number of urine samples because of the long sample preparation time. What is needed is a screening process that enables one to identify rapidly a urine sample as normal or abnormal with regard to the carboxylic acids present. Abnormal urine samples could then be completely analyzed by gas chromatography/mass spectrometry. We have developed a rapid screening procedure for urinary carboxylic acids that involves neither sample extraction nor sample derivatization. This procedure is applicable to α,β-unsaturated acids, oxalic acid, phenolic acids, eneolic acids, aromatic acids and α-ketocarboxylic acids.[11]

METHODS

Equipment:

Varian 5000 HPLC with a Bio-Rad Aminex HPX-87 cationic exchange column or a Whatman PXS 10/25 ODS reverse-phase column was

used for HPLC analysis. An Hitachi UV spectrophotometer equipped with an Altex flow cell and a Bioanalytical Systems LC-4 amperometric detector with a glassy carbon electrode were used for detection. Both detectors were connected to Linear recorders.

Materials:

3-Hydroxy-4-methoxybenzyl alcohol and all carboxylic acids were purchased either from Sigma Chemical Company or Aldrich Company and were used without further purification. Distilled and deionized water was further glass distilled from alkaline permanganate and was passed through a 0.22 um Millipore filter prior to use.

Method:

Urine Organic Acid Profile: To 0.20 ml of urine was added 25.0 ul of a 1.8 mg/ml solution of 3-hydroxy-4-methoxybenzyl alcohol. The sample was passed through a 0.3 um Millipore filter and 10 ul of the filtrate was injected on to the cation exchange column via a loop injector. The mobile phase, 4.5 mN H_2SO_4, was passed through the HPX-87 analytical column at a flow rate of 0.8 ml/min. at ambient temperature. A dual detection system employing a 200 nm ultraviolet spectrophotometric detector in series with but upstream from a glassy carbon electrode amperometric detector at +1.15V vs. an Ag/AgCl reference electrode was used. For the ultraviolet and amperometric chromatograms, each peak was assigned an Rf value and a peak height ratio relative to the internal standard.

Eight control urine samples from normal infants from 0-6 months of age were analyzed. From the control samples, we determined an average peak height ratio for each Rf value for both the ultraviolet and amperometric chromatograms. The average peak height ratio ± SD defined normal at each Rf in each chromatogram.

Heart Cutting:[16] Metal guard columns (7.0 cm x 0.3 cm) were dry packed with Whatman CO: Pell ODS reverse-phase material. Three columns were connected to positions 1-3 on an Altex 6-way valve. The inlet to the 6-way valve was connected to the outlet from the ultraviolet detector. Each guard column was washed with 2-30 ml of the mobile phase before being used to isolate a component from the chromatogram. To isolate a specific component, the eluant from the ultraviolet detector was diverted through one of the guard columns as the component reached its maximum peak height on the ultraviolet recorder. Passage through the desired guard column was continued for 0.5 - 1.5 min. depending upon the size of the eluting component. As many as three components could be isolated per chromatographic run.

2-Dimensional HPLC Analysis: A guard column containing the isolated component was inserted into the loop injector valve as a replacement for the 10 ul loop. To the guard column was added 10 ul of a solution that consisted of 25.0 ul of 3-hydroxy-4-methoxybenzyl alcohol in 200 ul of H_2O. The contents of the guard column was injected on to an analytical Whatman Partisil PXS 10/25 ODS column. The isocratic mobile phase, 0.1 M phosphate, pH 2.5

plus 1% CH_3CN, was pumped through the system at 1 ml/min. The components were detected at 200 nm and their Rf values calculated.

HPLC Estimation of Orotic Acid: To 0.20 ml aliquots of H_2O was added 25.0 ul of a 1.8 mg/ml 3-hydroxy-4-methoxybenzyl alcohol solution and:

a. 10.0 ul of a 5.75 umole/ml orotic acid solution.
b. 25.0 ul of a 5.75 umole/ml orotic acid solution.
c. 50.0 ul of a 5.75 umole/ml orotic acid solution.

Each aliquot was analyzed by ion exchange HPLC using 4.5 mN H_2SO_4 at a 0.8 ml/min. flow rate. Ultraviolet detection at 200 nm was used. The orotic acid peak height/internal standard peak height ratio afforded a straight line, $y=1.68x$ ($r^2=0.99$) when graphed against the orotic acid concentration. From this working curve, the orotic acid concentration of a urine sample could be estimated from the initial 200 nm chromatogram.

Spectrophotometric Estimation of Orotic Acid: The procedure of Harris and Oberholzer[12] was used for the estimation of orotic acid in urine. The least squares working curve for this procedure was $y=0.67x$ ($r^2=1.00$).

RESULTS AND DISCUSSION

A block diagram of the HPLC system employed in the urinary organic acid screening procedure is shown in Figure 1. Because of the chemical reactions involved in the amperometric oxidation at the glassy carbon electrode, the amperometric detector must

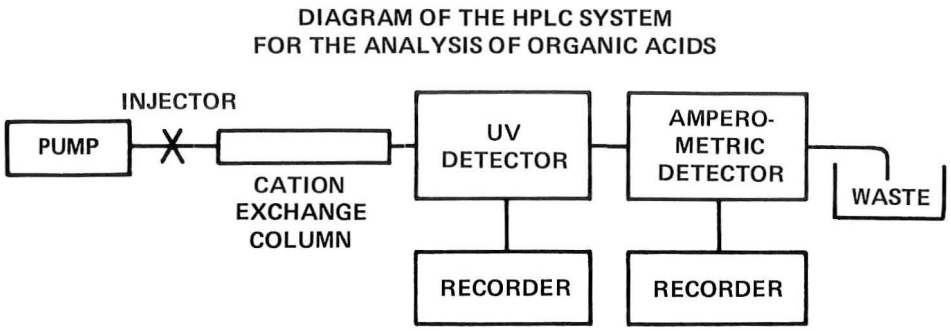

Figure 1. Block diagram of the HPLC system employed in the urinary organic acids screening.

be downstream from the ultraviolet detector. The cation exchange HPLC column, at ambient temperature, affords adequate resolution of the urinary acids.

The standard organic acid screening procedure utilizes the UV detector at 200 nm and the amperometric detector at +1.15V vs. an Ag/AgCl reference electrode. At 200 nm, the carboxyl C=O moiety undergoes $n \rightarrow \pi^*$ absorption[13]. This transition is very weak for saturated carboxylic acids[13] ($\epsilon \approx 50$) and is very strong for α, B-unsaturated carboxylic acids[13] ($\epsilon \approx 10^4$). Since metabolic abnormalities generally result in urinary carboxylic acid concentrations that are 10 - 1000 times their normal value,[14,15] abnormal concentrations of saturated carboxylic acids should be detectable at 200 nm. At +1.15V, phenolic, eneolic, oxalic and α-ketocarboxylic acids are all detectable[11].

To obtain a normal urinary organic acid profile, we utilized the results from the urine of eight healthy, normal infants (0-6

months). For each urine sample, every peak in the 200 nm and +1.15V chromatogram was assigned an Rf value and a peak height/internal standard peak height ratio. The average values from these normal infants were tabulated (Table 1) and utilized as a normal urinary organic acid profile. Any peak height ratio outside the $\bar{x} \pm$ SD range was considered to be abnormal as was any peak found at an Rf value not found in our normal profile.

In Figure 2, we show the urinary profile of a critically ill two week old infant with the urea cycle defect, citrullinemia. Peak 1 (Rf 0.11) is the only abnormal component in this profile and is only abnormally large in the 200 nm ultraviolet chromatogram (peak height ratio 2.98 vs. a normal value of 0.30). Peak 1 in the +1.15V amperometric chromatogram is of a normal size (peak height ratio of 0.08 vs. a normal peak height ratio of 0.14). Based upon the Rf values of some of the known urinary organic acids (Table 2), peak 1 could be orotic acid, cis-aconitic acid, α-ketoglutaric acid or pyruvic acid. Orotic acid and cis-aconitic acid do not undergo oxidation at the glassy carbon electrode at a potential of +1.15V while both α-ketoglutaric acid and pyruvic acid do undergo oxidation. The normal peak height ratio of peak 1 in the +1.15V amperometric chromatogram eliminated α-ketoglutaric acid, pyruvic acid and any other α-ketocarboxylic acid from consideration. Both orotic acid and cis-aconitic acid are detectable at 230 nm and have a peak height 230 nm/peak height 200 nm ratio of 0.28. At 280 nm orotic acid has a substantial ultraviolet absorptivity while cis-aconitic has essential-

TABLE 1

Normal Infant Urinary Organic Acid Profile

Rf	200 nm Ave Pk Ht Ratio + SD	Rf	+1.15V Ave Pk Ht Ratio + SD
0.08	2.50	0.08	0.86 ± 0.37
0.09	0.47 ± 0.14	0.09	0.17 ± 0.09
0.10	0.35 ± 0.21	0.10	0.03
0.11	0.30 ± 0.18	0.11	0.14
0.12	0.10 ± 0.06	0.12	
0.13	0.06	0.13	
0.14	0.06 ± 0.05	0.14	0.12 ± 0.13
0.16	0.48 ± 0.57	0.16	0.01
0.17	0.03	0.17	0.19 ± 0.11
0.18	0.10 ± 0.08	0.18	0.08
0.19	0.07 ± 0.07	0.19	0.06 ± 0.05
0.20	0.05 ± 0.02	0.20	0.01
0.23	0.05	0.23	
0.25	2.79 ± 1.52	0.25	1.35
0.28	0.04 ± 0.02	0.28	
0.30	0.03 ± 0.02	0.30	0.03
0.33	0.01	0.33	
0.36	0.02	0.36	0.05 ± 0.06
0.37	0.03	0.37	0.06
0.38	0.03	0.38	0.08 ± 0.06
0.39	0.03 ± 0.02	0.39	0.10 ± 0.04
0.40		0.40	0.04 ± 0.03
0.42		0.42	0.02
0.44	0.04	0.44	0.16
0.50	0.02	0.50	0.02 ± 0.02
0.54	0.02 ± 0.01	0.54	0.01
0.56		0.56	0.02
0.58	0.01	0.58	
0.60		0.60	0.02
0.66	0.02 ± 0.01	0.66	0.07 ± 0.03
0.68	0.02	0.68	
0.70	0.10	0.70	0.28
0.72	0.06 ± 0.02	0.72	0.13 ± 0.06
0.74	0.08	0.74	
0.76	0.05	0.76	
0.78	0.15	0.78	
0.81	0.20 ± 0.17	0.81	
0.86	0.04	0.86	0.04 ± 0.03
0.88	0.02	0.88	0.03

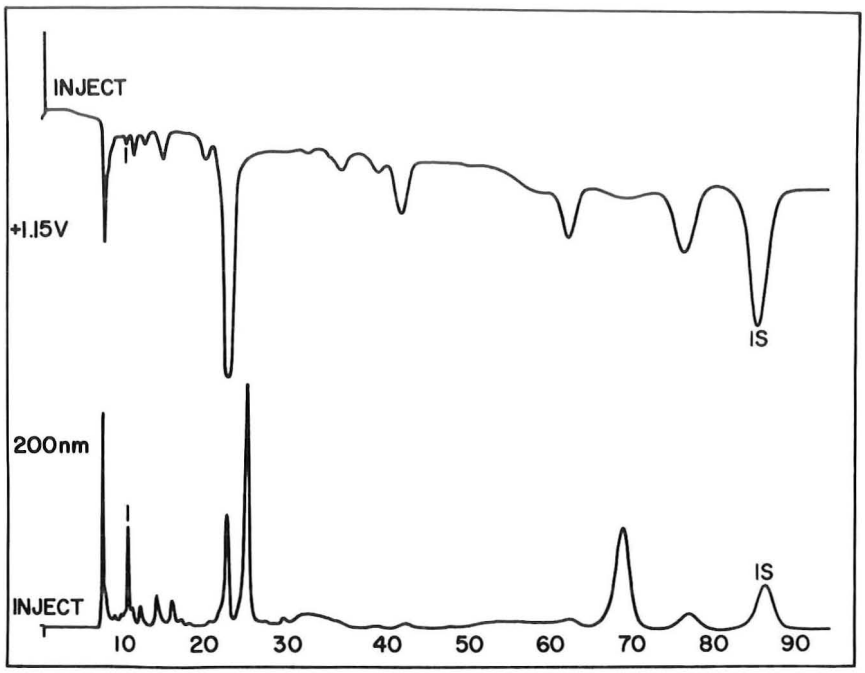

Figure 2. Amperometric (upper) and ultraviolet (lower) urinary organic acid chromatograms from a critically ill two week old infant. Internal standard (IS) is 3-hydroxy-4-methoxybenzyl alcohol. HPLC conditions are explained in the Methods section. Time scale is in minutes.

ly no absorptivity. For orotic acid, the peak height 280 nm/peak height 200 nm ratio is 0.68 while for cis-aconitic this same ratio is 0.01. Peak 1 was found to have a peak height 280 nm/peak height 200 nm ratio of 0.75. Peak 1 was thus thought to be orotic acid. HPLC quantitation of orotic acid (see Methods) in this urine sample yielded an orotic acid concentration of 1.68 umole/ml. The spectrophotometric[12] quantitation of orotic acid in this urine sample yielded an orotic acid concentration of 1.96 umole/ml. These values

TABLE 2

Rf Value and Amperometric Activity of Standard Organic Acids

Acid	Rf	Amperometric Activity(V)
α-Keto-B-methylthiobutyric acid	0.08	+1.15
Methylcitric acid	0.09	Inactive
Oxalic acid	0.07-0.09	+0.86
Uric acid	0.09	+0.86
Orotic acid	0.11	Inactive
Pyruvic acid	0.11	+1.15
α-ketoglutaric acid	0.12	+1.15
Cis-aconitic acid	0.12	Inactive
α-ketoisovaleric acid	0.13	+1.15
α-ketobutyric acid	0.14	+1.15
Cis-oxalacetic acid	0.14	+1.15
Ascorbic acid	0.15	+0.86
α-ketovaleric acid	0.16	+1.15
α-ketocaproic acid	0.16	+1.15
α-ketoisocaproic acid	0.17	+1.15
p-Hydroxyphenylpyruvic acid	0.21	+0.86
Acetic acid	0.22	Inactive
Vanillic acid	0.25	+0.86
B-phenylpyruvic acid	0.27	+1.15
Homogentisic acid	0.37	+0.86
VMA	0.39	+0.86
3,4-dihydroxyphenylacetic acid	0.46	+0.86
p-Hydroxyphenyllactic acid	0.48	+0.86
Mandelic acid	0.48	Inactive
HMPG	0.57	+0.86
o-hydroxyphenylacetic acid	0.57-0.58	+0.86
B-methylcrotonic acid	0.62	Inactive
Catechol	0.66	+0.86
p-hydroxyphenylacetic acid	0.69	+0.86
Hippuric acid	0.81	Inactive
HVA	0.87	+0.86
Aspirin	1.16-1.20	+1.15
5-HIAA	1.46	+0.86
o-hydroxyhippuric acid	1.48	+0.86

are approximately 100 times higher than the normal orotic acid concentration in infants that are two weeks old[12].

α-Ketocarboxylic acids undergo electrochemical oxidation at the glassy carbon electrode at +1.15V but do not undergo oxidation

at +0.86V.[11] Urine samples that have peaks of abnormal size which because of their Rf are suspected of being due to an α-ketocarboxylic acid are amperometrically profiled at 0.86V. Peak 2, Rf 0.16, in Figure 3 was present in the +1.15V chromatogram but was absent from the +0.86V chromatogram. Heart cutting[16] (see Methods) on to a 10 um C_{18} reverse phase packed mini-column effected the

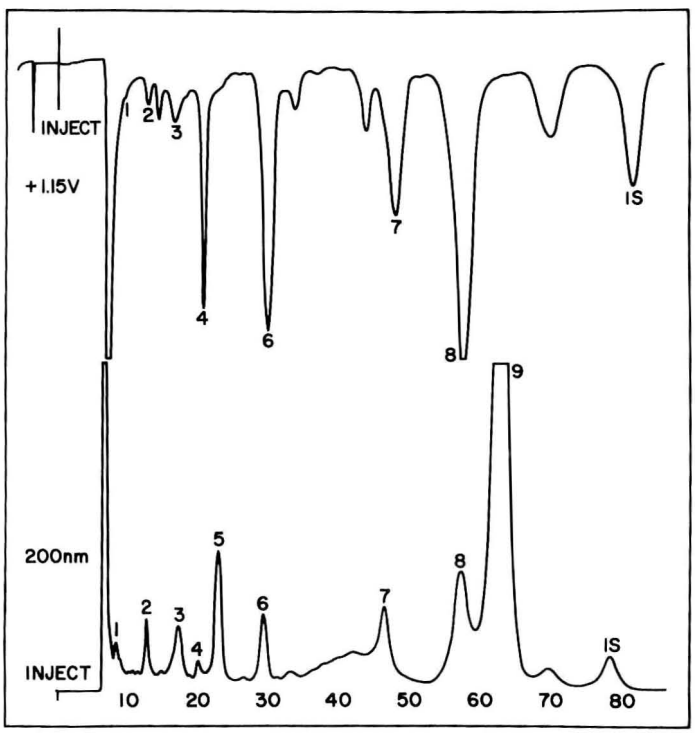

Figure 3. Amperometric (upper) and ultraviolet (lower) urinary organic acid chromatograms from a sick two year old who does not have a recognizable metabolic disease but whose urine does contain many large peaks. The numbered peaks are all larger than our standard of normal. Internal standard (IS) is 3-hydroxy-4-methoxybenzyl alcohol. Time scale is in minutes.

isolation of peak 2. Peak 2 and internal standard were rechromatographed on a 25 cm analytical C_{18} column and were detected spectrophotometrically at 200 nm. The resulting 2-dimensional map (Rf ion exchange 0.16, Rf reverse-phase 0.48) was compared with the 2-dimensional maps of authentic α-ketocarboxylic acids (Table 3). Peak 2 was thus identified as α-ketoisocaproic acid. In our system, heart cutting and 2-dimensional mapping was found to be viable only for peaks of medium to large peak heights that were well separated from neighboring components.

This method offers several advantages over the currently available procedures for identification of urinary organic acids. (1) The initial capital investment for the HPLC equipment is a small fraction of that required for a GC-MS system adequate for this usage. (2) The maintenance and supply requirements are minimal and can be performed by the operator. (3) Finally, the

TABLE 3

2-Dimensional Maps of Some α-Ketocarboxylic Acids

Phase 1: Cation Exchange Column.
Phase 2: Reverse-phase C_{18} Column.

Compound	Rf_1	Rf_2
p-Hydroxyphenylpyruvic acid	0.21	0.42
α-Ketocaproic acid	0.16	0.54
α-Ketobutyric acid	0.14	0.20
α-Ketoisocaproic acid	0.17	0.46
α-Ketovaleric acid	0.16	0.29
Pyruvic acid	0.12	0.15
Oxalacetic acid	0.15	0.16
α-Ketoisovaleric acid	0.12	0.23
α-Ketoglutaric acid	0.11	0.17

range of inborn errors potentially detectable is quite large. Known standards have been chosen representative of a number of inborn errors including: propionic acidemia (methylcitrate); oxalosis (oxalic acid); Lesch-Nyhan syndrome (uric acid); urea cycle enzymopathies (orotic acid); lactic acidoses (pyruvic acid); branched chain ketoaciduria (α-ketoisocaproic; α-ketomethylvaleric; α-ketoisovaleric); phenylketonuria (phenylpyruvic); tyrosinemia (p-hydroxyphenylactic); alkaptonuria (homogentisic); B-methylcrotonyl C_oA carboxylase deficiency (B-methylcrotonic acid). Further refinement in chromatographic procedure should result in extension of this method to the detection of metabolites characteristic of other inborn errors.

ACKNOWLEDGEMENT

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JOURNAL OF LIQUID CHROMATOGRAPHY, 4(9), 1601-1607 (1981)

PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ISOLATION OF THE TRUE PROAZULENES FROM ARTEMISIA ARBORESCENS L.

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ABSTRACT

The use of the preparative medium-pressure liquid chromatography (prep-MPLC) and the preparative high-pressure liquid chromatography (prep-HPLC) techniques for the separation of the main true proazulenes from a purified extract of *Artemisia arborescens* L. are reported and discussed.

INTRODUCTION

The isolation of unstable substances from natural sources using conventional column chromatography is usually hard and often accompanied by extensive loss of material.The use of preparative medium-pressure liquid chromatography (prep-MPLC) and preparative high-pressure liquid chromatography (prep-HPLC),minimizing the contact between the stationary phase and the substances to be separated,proves very useful with this regard.

We report here an example dealing with the use we made of this techniques during the isolation of the true proazulenes from *Artemisia arborescens* L.

Although this plant has been known for a long time to yield a dark blue oil (Jona,1914) due to the presence of chamazulene (Meisels and Weizmann,1953),the principles responsible for the formation of this compound during the conditions of hydro-distillation had never been isolated (Šorm and Doleiš,1965).

MATERIALS AND METHODS

Sample preparation

A purified chloroform extract of *A. arborescens* was obtained using a general method previously described (Nano *et al.*,1980).

Pre-separation of the proazulenic fraction

A sample (10 g) of the purified chloroform extract of the plant was pre-separated by means of prep-MPLC into nine fractions using a Prep-LC/System 500 A Waters,equipped with two Prep PAK-500/Silica columns.As a mobile phase 6 l of a mixture of acetone:n-hexane (1:5) technical grade was used.The refractive index detector was set at x 20 relative response;the flow rate at 300 ml/min;chart speed 1 cm/min.The TLC control of the fractions obtained was performed on 5 x 7.5 cm,layer 0.2 mm,HPTLC-Alufolien Kieselgel 60 F 254 chromatoplates (Merck),using as eluent the system acetone:n-hexane (1:3.5) (1 ascent at 20 °C).The spots on HPTLC plates were detected by spraying with EP reagent (Stahl,1969).The HPLC control of the separated fractions was run on a Perkin-Elmer Series 3B liquid chromatograph;the eluted components were monitored with a LC-75 variable wavelength detector (160-600 nm) equipped with a LC-75 Autocontrol.The column used was a Hibar LiChrosorb RP 18 5 μm (Merck,12.5 x 0.4 cm I.D.).The separations reported were achieved under the following conditions: mobile phase 55% acetonitrile (LiChrosolv,Merck) in water purified by a MILLI-Q-System

(Millipore);flow rate,1.5 ml/min;temperature,28 °C;wavelength,242 and 209 nm;chart speed,0.5 cm/min. Graphs were generally obtained with an attenuation setting corresponding to 0.32 AUFS on a 10 mV Perkin-Elmer 561 recoder.

Lactone A

A crystalline compound (200 mg) was obtained from fraction n°6 by crystallization from EtOAc.

Lactone B

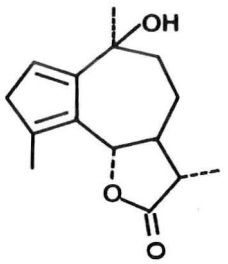
TLC analysis showed that fraction n°5 was a mixture of lactone A and another EP-positive compound.They were separated by prep-MPLC using the same conditions as above,and 60 mg of another crystalline compound were obtained.

Separation of lactone A into its two constituents

200 mg of lactone A were separated by prep-HPLC using a Perkin-Elmer Series 3B liquid chromatograph,equipped with a LC-75 spectrophotometer detector and recorder unit.A high efficiency preparative liquid chromatography stainless steel column (Perkin-Elmer,25 x 1.6 cm I.D.) packed with LiChrosorb RP 18 10 µm (Merck) was used.The preparative separations were achieved under the following conditions:eluting solvent,55% acetonitrile (LiChrosolv,Merck) in water purified by a MILLI-Q-System (Millipore);flow rate,30 ml/min;temperature,25 °C;chart speed,0.5 cm/min;wavelength,209 nm. Five injections of 100 µl,in a normal volume loop (175 µl) were made.Graphs were obtained with an attenuation setting corresponding to 1.024 AUFS on a 10 mV recorder.

Identification of compounds

Compounds were identified according to their physical and spectral properties;full data will be published elsewhere.



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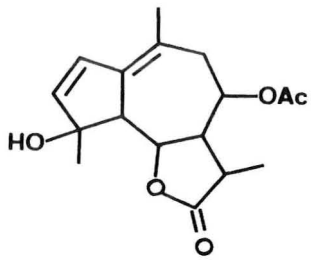

MATRICIN

STRUCTURE 1

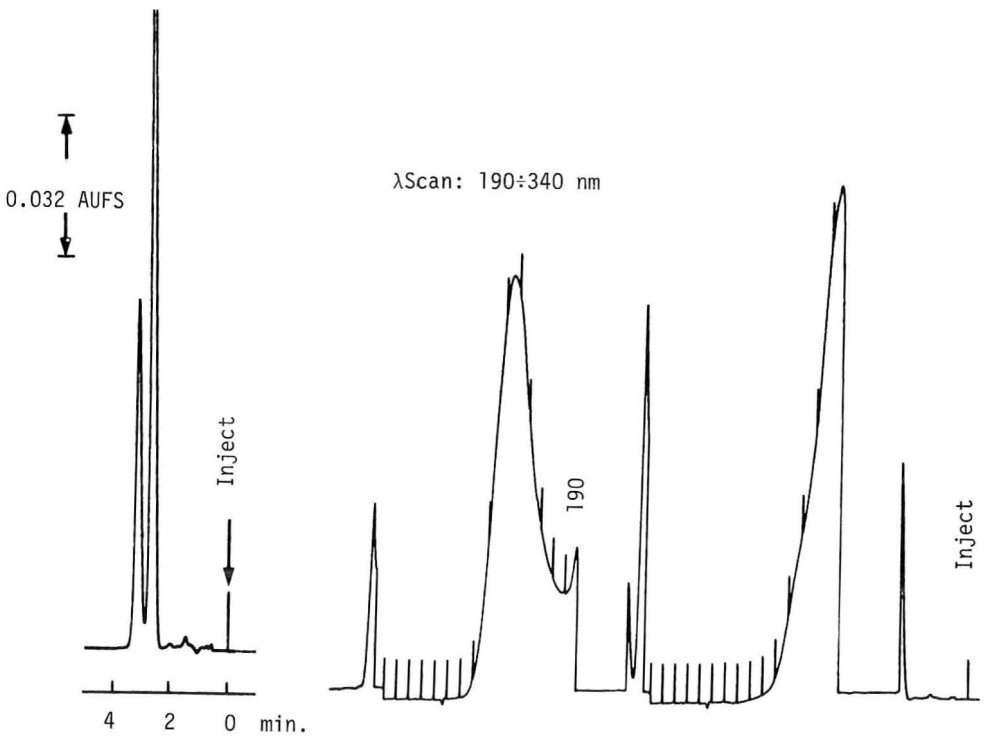

FIGURE 1

HPLC chromatograms illustrating the resolution of lactone A in two sequiterpene lactones and their U.V. spectra.
Column:Hibar LiChrosorb RP 18 5 µm (12.5 x 0.4 cm I.D.);mobile phase: acetonitrile:water (55:45 v/v);flow rate:1.5 ml/min;wavelength:209 nm; temperature:28 C;chart speed:0.5 cm/min.The U.V. spectra were performed in λ-SCAN MODE from 190 to 340 nm.

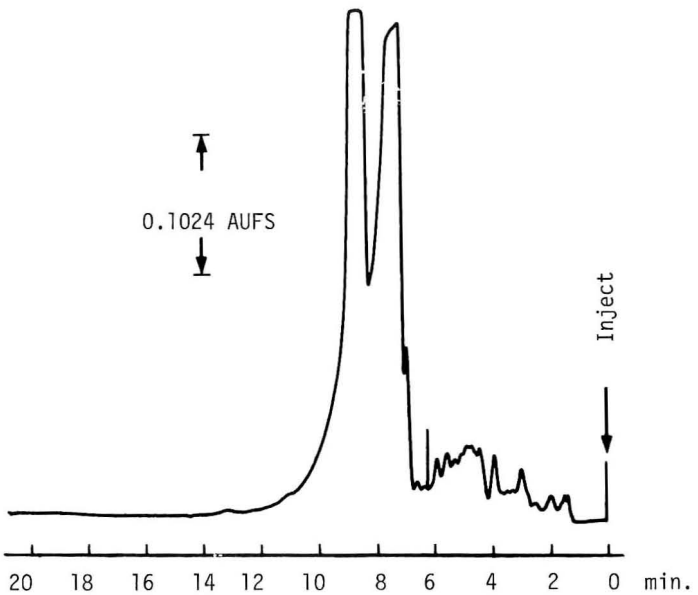

FIGURE 2

Preparative HPLC profile of the separation of lactone A into its two constituents.
Column:Perkin-Elmer (25 x 1.6 cm I.D.) packed with LiChrosorb RP 18 10 µm (Merck);mobile phase:acetonitrile:water (55:45 v/v);flow rate: 30 ml/min;wavelength:209 nm;temperature:25 C;chart speed:0.5 cm/min.

RESULTS AND DISCUSSION

Using prep-MPLC two crystalline proazulenes were obtained from the plant extract:lactone B was identified as artabsin (Vokáč *et al.*,1969)while lactone A,corresponding to the most EP-positive spot of the extract , was shown by spectral analysis (^{1}NMR,MS) to be a practically equimolecular mixture of two sesquiterpene lactones.Lactone A was apparently unitary,with a definite m.p. and a single spot,turning blue in a few hours due to *in situ* decomposition,in every chromatographic system we tested.The U.V. spectrum of this compound showed,besides a higher absorbition at 242 nm,a lower peak at 209 nm.The HPLC analysis run at

this lower absorbition,gave a slight separation into two peaks;this separation was then optimized allowing us to obtain two baseline resolved peaks having distinctive U.V. spectra (Fig. 1).The analytical conditions of separation were transferred to preparative scale (prep-HPLC). Both components of the crystalline mixture had a relatively high extinction coefficient.As the selected wavelength was optimal for separation, and in order to prevent the U.V. detector overloading,it was decided to inject small aliquots (100 µl) of the sample mixture instead of injecting a higher volume sample and setting the U.V. detector to another wavelength.In this way we were able to obtain in a good purity two sesquiterpene lactones (Fig. 2);one of them was identified as matricin (Čekan *et al.*,1956),while the structure of the second compound as well as the stereochemistry of matricin will be the subject of a separate paper.

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QUANTITATION OF P-AMINOHIPPURIC ACID AND N-ACETYL-P-AMINOHIPPURIC ACID FROM BLOOD BY HPLC

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ABSTRACT

P-aminohippuric acid, N-acetyl-p-aminohippuric acid, and p-aminobenzoic acid can be separated by a reverse-phase, isocratic high-pressure liquid chromatographic procedure in less than 4 minutes. The eluent is 10 mM sodium phosphate buffer, pH 3.5, containing 30% methanol. Detection is by absorbance at 270 nm; quantitation is accomplished by peak height. Linear response was obtained from 3 to 1600 nmoles of each compound in aqueous standards and in blood deproteinized with perchloric acid.

INTRODUCTION

Interest in p-aminohippuric acid (PAH) for assessment of effective renal filtration of plasma or blood, for determination of blood flow, and for assessment of renal damage has led to a variety of methods for quantitating PAH from biological fluids. Bratton and Marshall (1) used N-(1-naphthyl)-ethylenediamine dihydrochloride to react with diazotizable aryl amines such as PAH to form azo dyes, which then were quantitated colorimetrically.

Their method subsequently was modified (2). Brun (3) suggested a colorimetric method utilizing p-dimethylaminobenzaldehyde that forms a Schiff base when reacted with primary aromatic amines. Waugh and Beall (4) deproteinized and acidified samples of plasma in one step and modified the procedure of Brun. All these methods lack reagent stability and specificity and require careful timing between addition of reagents (5).

N-acetyl-p-aminohippuric acid (aPAH), the major metabolite of PAH in several species (6, 7, 8), can be detected by colorimetric procedures only after removing the N-acetyl group by acid hydrolysis before analysis for PAH. Hydrolysis of N-acetyl metabolites to primary aromatic amines is not always specific, however, in producing the desired compound (9). Therefore, secondary decomposition products must be considered when colorimetric, chemical, or separation techniques are employed after hydrolysis.

High-pressure liquid chromatography (HPLC) has been used to quantitate PAH by UV-absorbance (9, 10, 11) or by electrochemical detection (5). HPLC methods offer advantages because aPAH can be detected in samples, along with PAH, without prior hydrolysis, which removes a major potential source of error in blood-flow determinations where PAH is used as the marker. However, previous HPLC methods have poor resolution, require expensive electrochemical detectors, require elution times of at least 10 min., or use 254 nm absorbance for detection. Possible interference from other blood constituents could occur at 254 nm, and PAH, aPAH, and p-aminobenzoic acid (PAB) have greater absorbance at 270 nm (present report).

We developed a rapid, sensitive method of PAH and aPAH determination so that they could be used to accurately determine blood flow in animals to measure fluxes of metabolites in and out of organs. With PAB as an internal standard, the method

quantitates PAH and aPAH in less than 4 min. by using a reverse-phase column, isocratic mobile phase, and UV-absorbance detection.

MATERIALS AND METHODS

Analyses were performed on a Waters Associates HPLC system equipped with a model 6000A pump, a U6K continuous-flow injector, and an RCM-100 radial compression module containing a Microbondapak C^{18} Radial Pak cartridge. The mobile phase of 30% methanol (V/V) in 10 mM NaH_2PO_4 buffer was mixed and the pH adjusted to 3.5. Degassing and particle removal was by vacuum filtration through a 0.45-μM Millipore filter. Eluent flow of 2.5 ml/min. produced pressure of 800 to 1000 psi. Detection was at 270 nm by a Perkin-Elmer model 55 variable-wavelength spectrophotometer. Recordings were with a Houston Instruments Omniscribe recorder set at 0.1, 1.0, or 10.0 mV, providing 0.003, 0.03, or 0.3 absorbance units full scale.

Lyophilized PAH from Sigma Chemical Co. was neutralized with NaOH so that standard solutions had a pH of approximately 7.0. aPAH was prepared from PAH (12), and purity was monitored by HPLC and by a melting point between 198 and 200° C. PAB was obtained from J. T. Baker Chemical Co. Aqueous standards of PAH, aPAH, and PAB were prepared, adjusted to pH 7.0 with NaOH, and injected as 25-μl aliquots. Peak height was used for quantitation.

Whole-blood and plasma samples were prepared by mixing 2.5 ml of 1.6 μmoles/ml PAB internal standard with 5 ml of blood or plasma. To this mixture, 5 ml of 1.0 M perchloric acid was added and placed in ice for 10 min. After centrifugation to precipitate protein, the supernatant was filtered through tissue paper to another tube, and 1.25 ml of 2.0 M KOH was added to precipitate unreacted perchlorate. After centrifugation to remove potassium perchlorate, the supernatant was transferred to a vial that was capped and stored at 4° C. Unpublished experiments have shown that

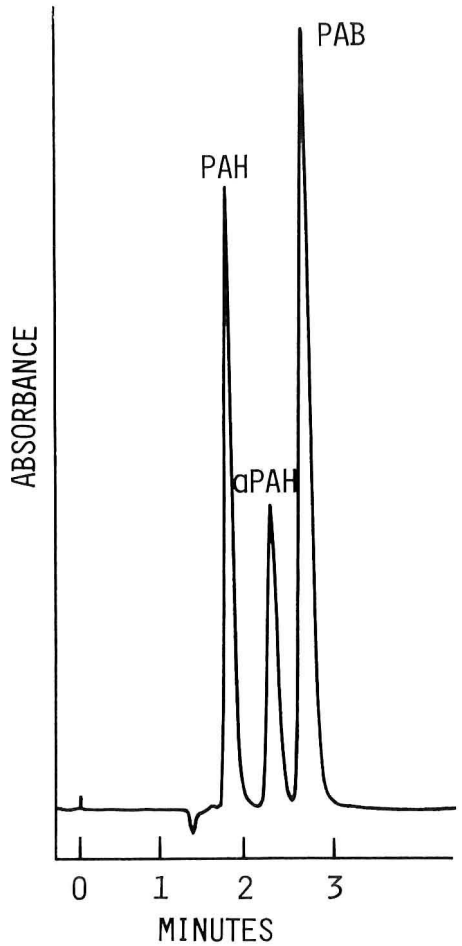

Figure 1. Chromatogram of a 20 µg/ml standard of PAH, aPAH, and PAB. Chromatographic conditions: Microbondapak C_{18} Radial PAK cartridge (10 cm x 0.8 cm i.d.); mobile phase of 30% methanol in 0.01 M NaH_2PO_4, pH 3.5 with a flow rate of 2.5 ml/min.; injection volume of 25 µl; detection at 270 nm.

the compounds in these basic supernatants are stable at 4° C for 1 month and longer. Within 24 hours, 25-µl aliquots were injected onto the HPLC column, and PAH and aPAH were quantitated by using PAB as an internal standard. Perchloric acid as a deproteinizing

agent gave quantitative recovery and baseline resolution of PAH, aPAH, and PAB. Sulfosalicyclic acid, $BaOH/ZnSO_4$, and trichloroacetic acid did not.

RESULTS AND DISCUSSION

A chromatogram of aqueous standards of PAH, aPAH, and PAB is shown in figure 1. Baseline resolution was achieved for all three compounds from 3 to 1600 nmoles. Linearity of response was maintained when the recorder was at 0.1, 1 or 10 mV full scale, which provides the detection range necessary for quantitation of PAH, and aPAH in blood-flow experiments. Figure 2 depicts the

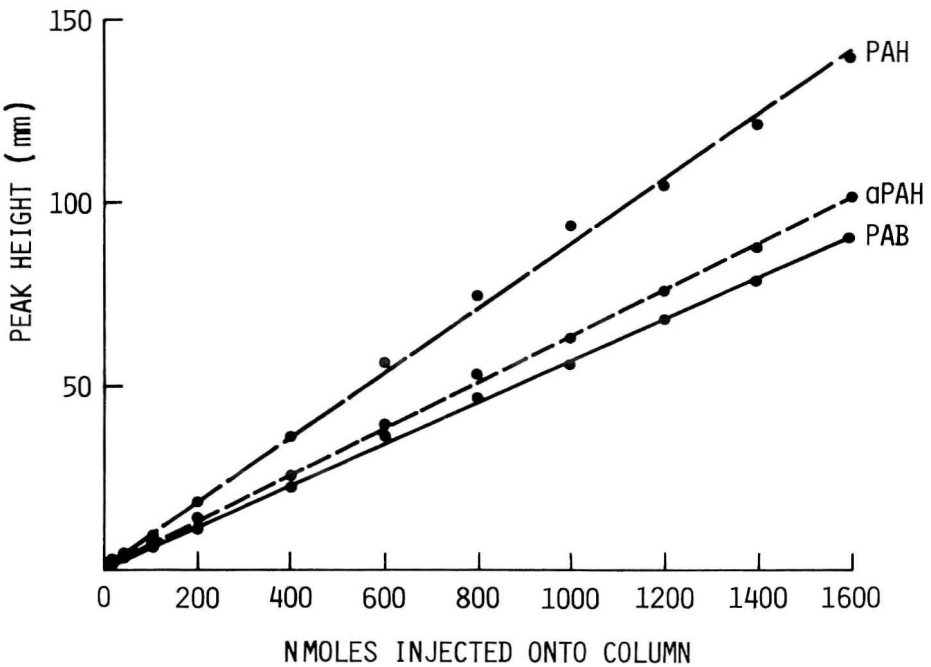

Figure 2. Standard curve of PAH, aPAH and PAB. Separate solutions of each compound were prepared in various concentrations and injected as 25 µl aliquots. The lines represent least-square estimates of the linear equations: PAH, Y=0.087X + 2.02; aPAH, Y=0.063X + 1.07; and PAB, y=0.56x + 1.0 (X=nmoles injected, Y=mm peak height).

TABLE 1.

Peak Height Ratios of PAH:PAB and aPAH:PAB from Standards

Amount Injected (nmoles)	PAH:PAB Ratios*	aPAH:PAB Ratio*
20	1.45 (0.06)	0.98 (0.03)
40	1.46 (0.02)	1.00 (0.04)
100	1.61 (0.01)	1.12 (0.02)
200	1.53 (0.01)	1.21 (0.01)
400	1.58 (0.01)	1.12 (0.01)
600	1.57 (0.01)	1.11 (0.01)
800	1.58 (0.004)	1.12 (0.006)
1200	1.54 (0.002)	1.12 (0.002)
1400	1.54 (0.003)	1.12 (0.003)
1600	1.55 (0.004)	1.13 (0.004)
	$\bar{x}$ = 1.54 (0.05)	1.10 (0.07)

*± Standard Error

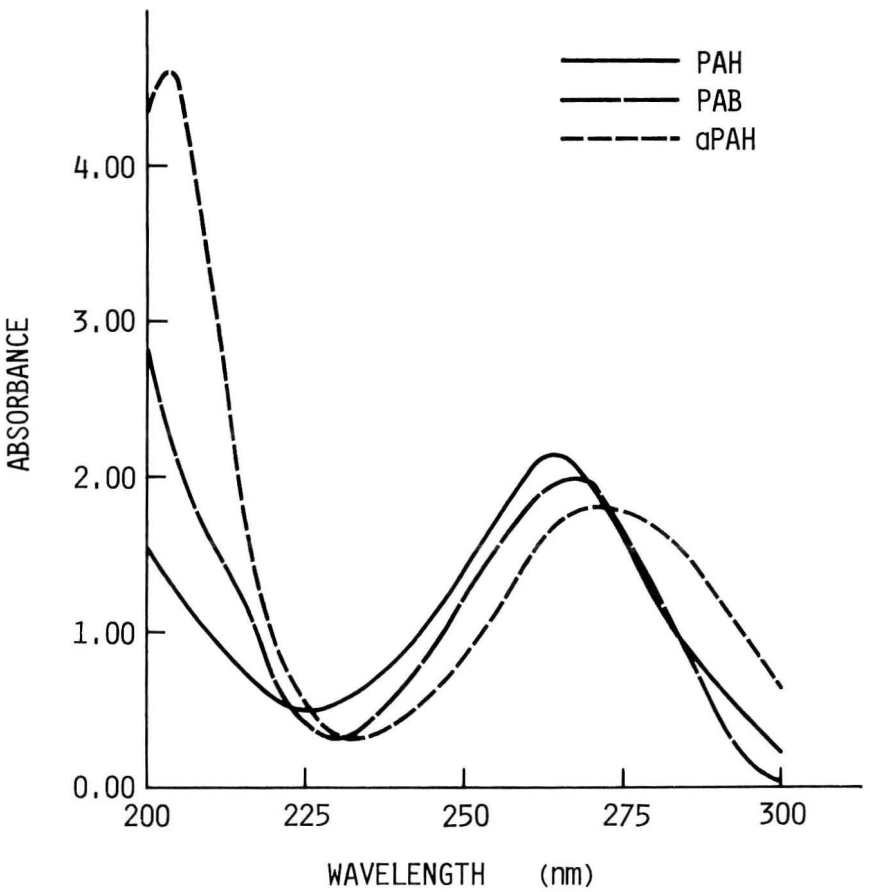

Figure 3. Absorption spectra of equal-molar concentration solutions of PAH, aPAH, and PAB. The pH of the solutions was 3.5.

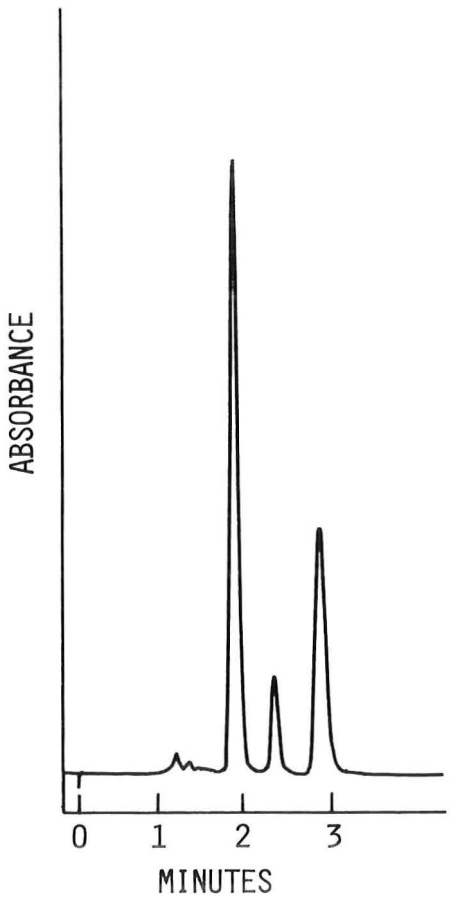

Figure 4. Chromatogram of a perchloric acid filtrate of whole blood. The steer was injected with 7 mg/kg of PAH and aPAH 15 minutes prior to the blood sampling.

linearity of detector response over an extended standard curve. For blood-flow experiments, the 10-mV recorder setting can give adequate sensitivity to quantitate the concentrations found in blood. ^{14}C-PAH and ^{14}C-aPAH confirmed 98 to 101% recovery in the two respective peaks.

Peak-height ratios for PAH:PAB and aPAH:PAB were essentially constant over a wide range of concentrations. Table 1 depicts

this constancy over a 500-fold concentration range. The constant ratios allow use of PAB as an internal standard for PAH and aPAH quantitation and negate the need for quantitative transfers of supernatants and filtrates during sample preparation.

Use of 270 nm maximizes aPAH detection and offers advantages over 254 nm used previously (9, 10). Interference from other compounds is minimized, and absorbance of PAH, aPAH, and PAB is greater (figure 3).

Figure 4 shows a chromatogram of PAH, aPAH, and PAB from a perchloric acid filtrate of whole blood taken from a steer injected with 7 mg/kg of body weight of PAH and aPAH 15 minutes preceeding blood sampling. There is consistent peak resolution and no interference from other blood constituents.

The adaptability of our HPLC procedure provides an attractive method for quantitation of PAH, aPAH, and PAB in wide concentration ranges in whole blood. Its use for determination of PAH in blood-flow experiments removes questionable pre-analysis sample treatment and thereby is more accurate and dependable than previously described methods.

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ASCORBIC ACID DETERMINATION BY HYDROPHOBIC LIQUID CHROMATOGRAPHY OF THE OSAZONE DERIVATIVE. APPLICATION TO THE ANALYSIS OF AQUEOUS HUMOR (*)

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ABSTRACT

The dinitrophenylhydrazine colorimetric method for the analysis of ascorbic acid has been converted to a high-performance liquid chromatography (HPLC) procedure. The bis-(dinitrophenyl) hydrazone resulting from the reaction of ascorbic acid and dinitrophenylhydrazine is separated on a reversed-phase system from other components in the reaction mixture and used for quantitation purposes. The specificity of the analysis is thus greatly improved and its sensitivity is brought down to the picomol level. The HPLC procedure described here is therefore specially suitable for the analysis of very small volume samples when high specificity and sensitivity are of prime importance. With this method, as in the original colorimetric procedure, it is also possible to specify

* A preliminary report of this work was presented at the 1980 Annual Meeting of the Association for Research in Vision and Ophthalmology (ARVO), Orlando, Florida.

the contribution of the reduced and oxidized forms of ascorbic acid to the total amount present in the sample. Basically, the same approach could be advantageously used to improve other colorimetric methods of analysis. Human aqueous humor samples taken from senile cataract patients at the time of surgery show variable but significant amounts of oxidized ascorbic acid. This oxidized fraction, therefore, should not be neglected as is frequently done when the ascorbic acid system of the aqueous humor is studied.

INTRODUCTION

The potential role of ascorbic acid in biological systems has stimulated wide multidisciplinary interest in this compound over the years. It is not, then surprising that a variety of now classical analytical methods exist for its quantitation (1-3) and that improved procedures have recently been reported in the literature (5-7). Methods (4, 5, 8) based upon reactions that permit measurement of both reduced and oxidized forms of ascorbic acid are of special significance because of the ability of ascorbic acid to participate in biological oxidation-reduction systems. In order to better evaluate the possible role of ascorbic acid in such systems, it is important to define, when a particular system is analyzed, the fraction of total ascorbic acid present in the oxidized or in the reduced form and how these fractions might be affected under variations imposed upon the system.

These analytical requirements are fulfilled by the widely used original or modified method of J.H. Roe (4, 9, 10) based upon the reaction of 2, 4-dinitrophenylhydrazine (DNPH) with oxidized as-

corbic acid to form the bis (dinitrophenyl) hydrazone (osazone) derivative. We have now adapted this colorimetric method to high-performance liquid chromatography (HPLC), the osazone product being isolated on a C^{18}-hydrophobic column.

The HPLC method has been applied to study the ascorbic acid system in samples of human aqueous humor from cataract patients.

MATERIALS AND METHODS

High Performance Liquid Chromatography (HPLC). A model ALC/GPC-204 Liquid Chromatograph with a fixed wavelength (254 nm) UV-monitor (Waters Associates, Inc., Milford, Mass.) was used for this work.

Solvents and Reagents. Acetonitrile (ACN) was obtained from Waters Associates. HPLC grade ethyl acetate (EtOAc) and spectranalyzed methanol (MeOH) were from J.T. Baker (Phillipsburg, NJ). Water for HPLC was prepared with a combined Milli-RO/destillator/Milli-Q/0.45 μm ultrafiltration membrane system (Millipore Corp., Bedford, Mass.). The aqueous part of the ACN buffer to be used in HPLC was filtered through a Millipore membrane (0.45 μm, HAWP 04700) before mixing it with the solvent.

Ascorbic acid was obtained from Sigma Chemical Co. (Saint Louis, Missouri). Meta-phosphoric acid (HPO_3); 2,4-dinitrophe-

nylhydrazine (DNPH); bromine (Br_2); thiourea and sulfuric acid were from J.T. Baker.

UV-Visible Spectrophotometry. A Perkin-Elmer model 552 Spectrophotometer (Norwalk, CT) was used for recording the UV-Vis absorption spectra shown in this report.

Preparation of samples. Ascorbic acid standard solutions were prepared in 5% meta-phosphoric acid and kept in the refrigerator for periods not greater than one week.

Aqueous humor samples were obtained by corneal puncture in patients ready to undergo cataract surgery. Aliquots of 20 µl were immediately added to 0.5 ml of 5% meta-phosphoric acid.

Derivatization of ascorbic acid. A. Common procedure. To 0.5 ml of 5% HPO_3, 20 µl of a standard solution of ascorbic acid or 20 µl of aqueous humor were added.

Ascorbic acid present in these samples was oxidized with 0.5 µl of Br_2. Excess Br_2 was removed by bubbling air through the solution until clear. Total (oxidized + reduced) ascorbic acid in the sample is obtained from this analysis.

Duplicate samples were also prepared which were not submitted to Br_2 oxidation. Therefore only oxidized forms of ascorbic acid already present in the sample will be detected in this analysis. Reduced ascorbic acid in the sample can be calculated by difference between those two determinations.

After adding to the tubes 0.5 ml of 1% thiourea in 5% HPO_3 and 20 µl of 2% DNPH in 9N H_2SO_4, the reaction between DNPH and oxidized ascorbic acid was allowed to proceed for 4 hrs. at 37°C. The acid solution of DNPH was prepared and filtered just before the analysis (Millipore FHLP01300 membrane, 0.45 µm, mounted on a plastic Swinnex).

The red osazone formed was extracted in 0.5 ml of EtOAc and aliquots of 5 µl of this extract were directly injected into the chromatograph. Alternatively, the EtOAc extract was separated from the aqueous phase underneath, kept at -25°C and HPLC analyzed next day. Unless otherwise stated, the common procedure just described was used throughout this work.

B. Microprocedure. Microassays were also performed in 1.0 ml Reacti-Vials (Pierce, Rockford, Ill.) containing 5 µl sample and 50 µl 5% HPO_3. Br_2 oxidation was carried out in this case by adding to the vial 5 µl of a freshly prepared solution of Br_2 (2 drops in 5 ml 5% HPO_3). After waiting for 10 min., 50 µl of 1% thiourea and 10 µl of 0.4% DNPH in 9N H_2SO_4 were added. Incubation conditions were as before. The mixture was extracted with 75 µl of EtOAc and 5 µl of this extract were injected into the column.

HPLC Conditions. A µ-Bondapak-C^{18} column (Waters Associates, 3.9mm × 30cm, 10 µ) was used for the analytical separation of the osazone of ascorbic acid from other components in the EtOAc extract.

The eluant solvent was 50% acetonitrile containing 10mM sodium acetate buffer, pH 4.1. Flow rate was 1.0 ml/min. The recorder was set at a chart speed of 0.2 in/min. The effluent was monitored at 254nm. The sensitivity setting of the monitor depended on the amounts of ascorbic acid to be analyzed.

Purification of the Osazone of Ascorbic Acid. The osazone of ascorbic acid was prepared in mg amounts by scaling up the common procedure described above. The red precipitate was collected and briefly washed with water and EtOH over a Millipore 0.45 um filter HAWP 04700 and then was redissolved in EtOAc.

Recrystallization of DNPH. DNPH was dissolved to saturation in warm EtOH. The solution was allowed to slowly cool down. Crystals formed were recovered by filtration, washed with cold EtOH and dried.

RESULTS

The osazone of ascorbic acid prepared as indicated in the previous section was dissolved in EtOAc and used to establish the HPLC conditions to be followed in the determination of ascorbic acid.

Figure 1 shows representative HPLC profiles of EtOAc extracts of actual samples. Profile A was obtained when the sample was either 5% HPO_3 or reduced ascorbic acid with the Br_2 step omitted

from the procedure. Profile B represents the typical chromatogram obtained when oxidized ascorbic acid is produced in the sample by Br_2 or when it already exists as such in the sample. The elution time of peak 6 in profile B (about 9 min) corresponds to that of the purified osazone of ascorbic acid.

Profile A shows an almost flat baseline at the level of peak 6 when the monitor is set at a relatively low sensitivity (.02 Absorbance Units Full Scale, AUFS). A small background peak which coelutes with the osazone becomes apparent at higher sensitivities (profile D, dotted line). The elution time of the osazone in D is about 7.5 min.

Figure 1 also indicates that the osazone peak is the only evident product of the reaction between oxidized ascorbic acid and DNPH because all the other peaks in profile B are also present in profile A.

It was possible to identify the source and, in some cases, the nature of peaks 1-5 and 7 by successively excluding or adding components to the derivatizing mixture and by studying the UV-Visible spectra of the isolated HPLC peaks.

Thiourea and EtOAc contribute to form peak 1. Peak 2 and partially peaks 4 and 5 are due to impurities in EtOAc.

Peak 3 corresponds to unreacted DNPH. Peak 7 and most of peaks 4 and 5 are due to components present in the acidic solution

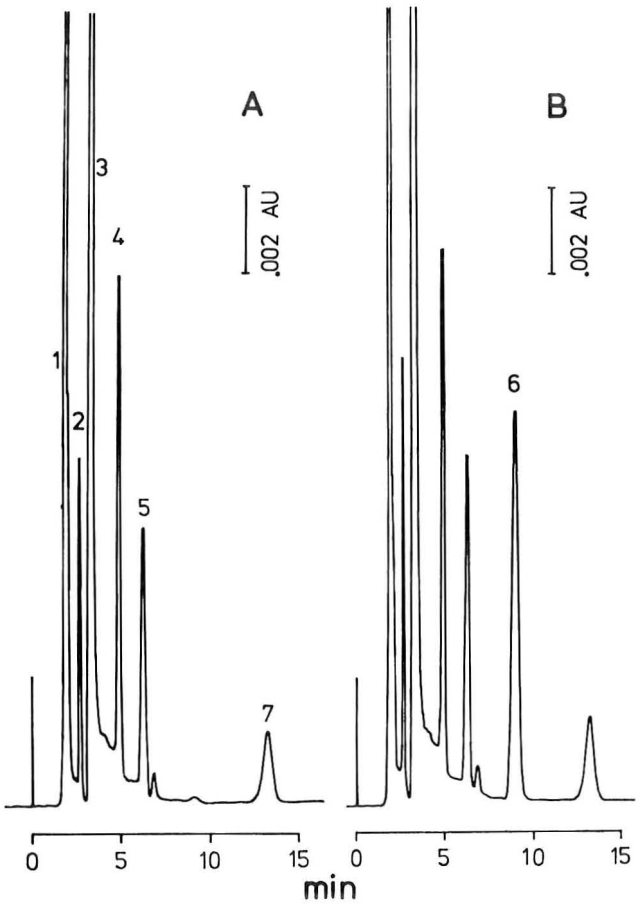


FIGURE 1A & B

of DNPH. Two commercial preparations of DNPH were tested and both showed these additional components. Although they represent a small fraction relative to DNPH (Fig.1C) peaks 4, 5 and 7 become very apparent at high sensitivities (Fig.1D). The recrystallization of DNPH, as described in the METHODS section, above, did not result in the long-term dissapearance of these extra peaks.

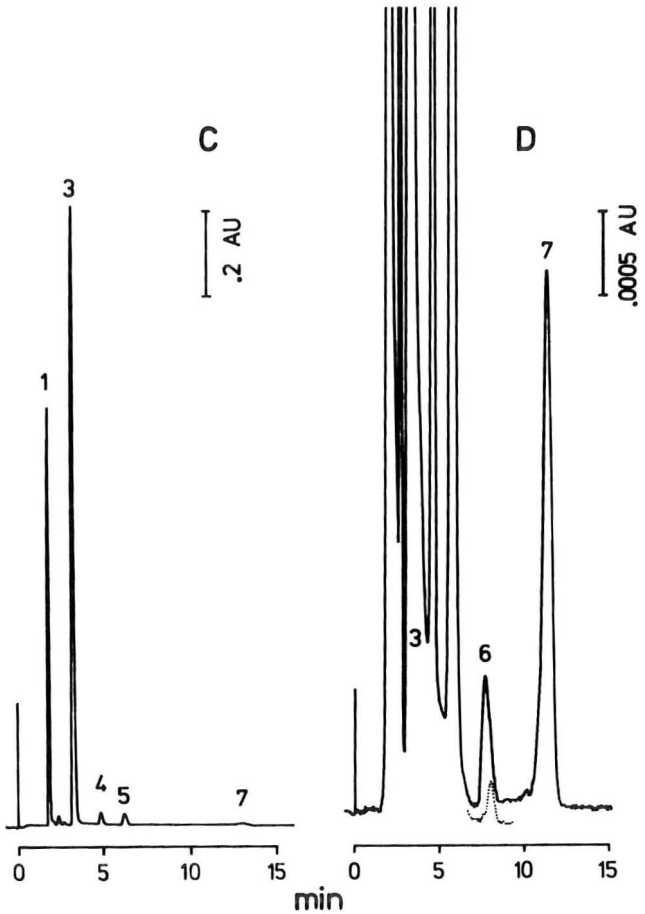


FIGURE 1

Representative HPLC profiles of the method described in the text. A and C are blank samples. B and D correspond respectively to 1.1 and 0.1 nanomols of ascorbic acid injected into the column.

Figure 2A (curve a) shows the absorption spectrum of the EtOAc-extracted derivatization mixture in the absence of ascorbic acid. This spectrum is dominated by DNPH (no osazone has been produ-

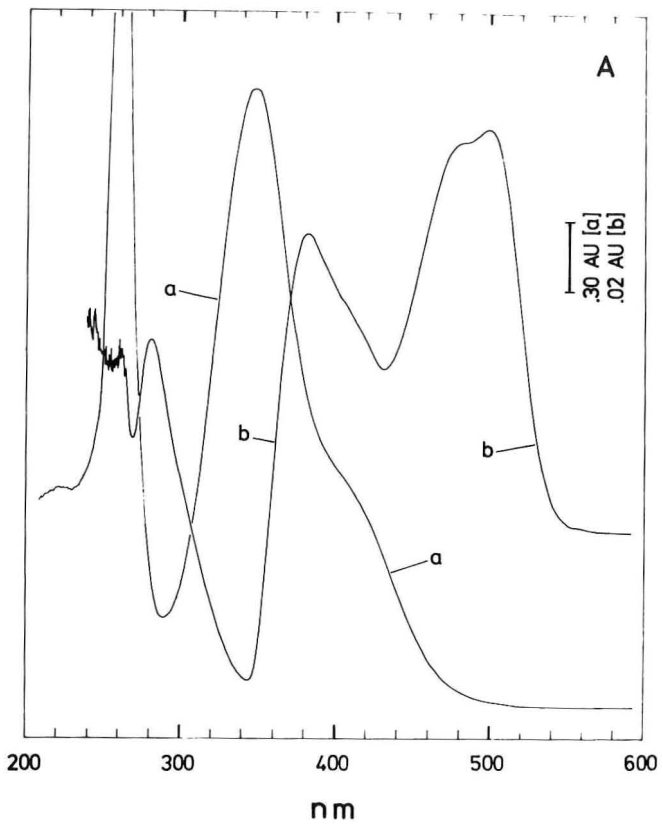


FIGURE 2

UV-Vis absorption spectra of the samples described in the text after properly diluted: EtOAc extracts (A), osazone of ascorbic acid (B) and HPLC peaks (C).

ced) with a peak of absorption at about 350 nm. When the osazone of ascorbic acid has been formed in the derivatizing mixture, a new component, absorbing at 480-500 nm appears which is best seen in the differential spectrum of the same figure (curve b). The

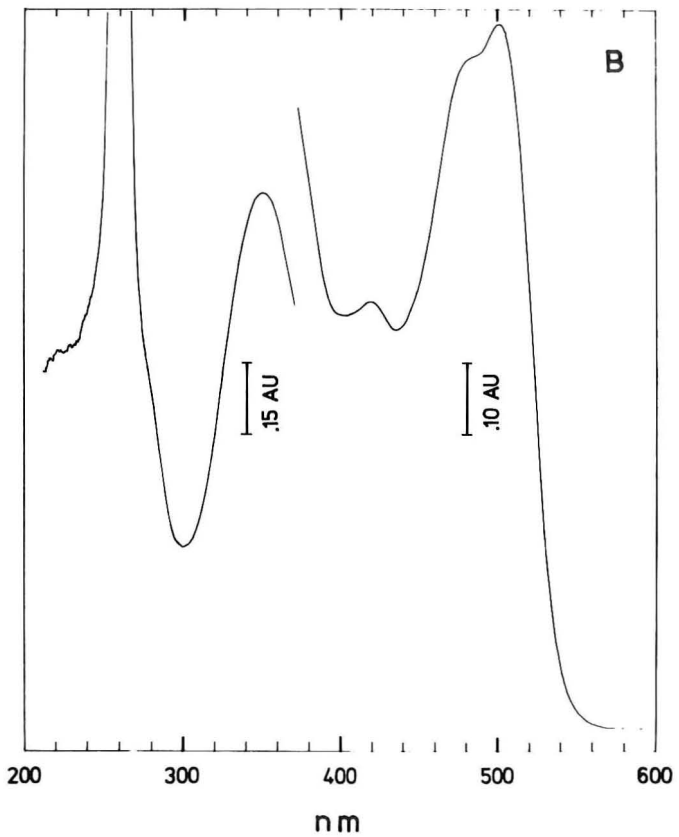


FIGURE 2B

double absorption band at 480 and 500 is characteristic of the osazone as shown by the absorption spectrum of the purified compound in Fig. 2B. The osazone also exhibits and absorption maximum at 350 nm. In the differential spectra of Fig. 2A (curve b) the latter has been distorted, however, probably because of the simultaneous dissappearance of DNPH to form the osazone.

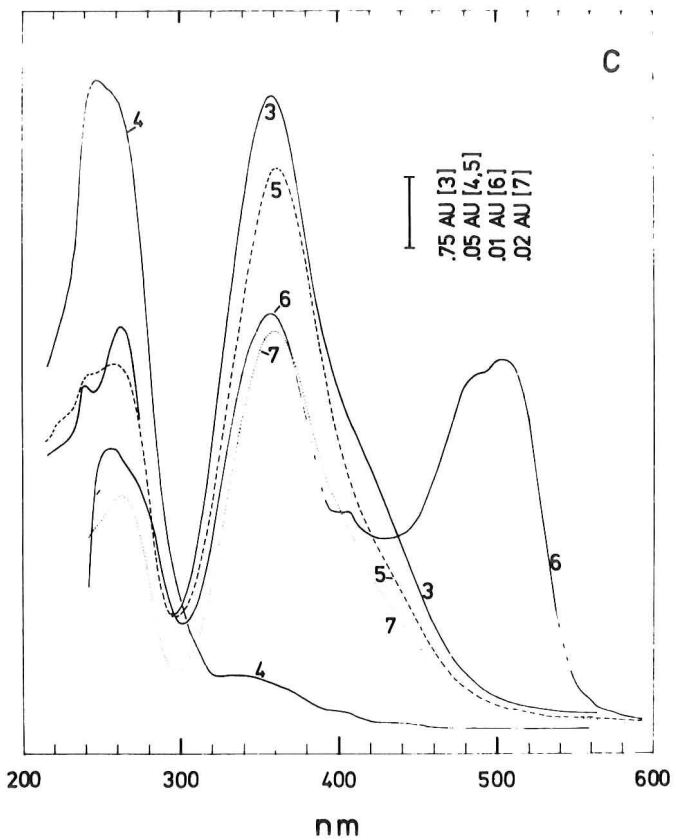


FIGURE 2C

Some of the peaks isolated by HPLC were also analyzed for light absorption. For this the chromatographic conditions were manipulated to obtain baseline peak resolution of the components eluted before peak 6. The UV-Vis spectra of peaks 3 to 7 are shown in Fig. 2C. We mentioned already that peaks 5 and 7 are closely associated with DNPH. They also show essentially the same spectra as DNPH (peak 3) with absorption maxima at about

360 nm. It should be noted the red-shift of about 10 nm in the absorption maxima of DNPH that has ocurred when the ACN buffer is used, instead of EtOAc, as the solvent.

Peak 6, as expected, shows both a maximum at about 360 nm and the double absorption maxima at 480 and 500 nm of the osazone. The latter maxima have not been affected by the change in the polarity of the environment.

Only the height of peak 6 is linearly related to the concentration of ascorbic acid originally present in the sample. Excellent linearity (correlation coefficient, 0.996) was found for sample concentrations of ascorbic acid up to 5.68 mM (Fig.3) which corresponds to 1.136 n moles injected into the chromatograph when the analysis is started with 20 µl samples. Most points in figure 3 are mean values of at least two independent determinations, and generally showed standard deviations (S.D.) of 7% of the mean.

Samples of aqueous humor taken from patients at the time of cataract surgery were analyzed for ascorbic acid with the present procedure. Table 1 shows the individual data obtained.

DISCUSSION

Roe's colorimetric procedure for the determination of ascorbic acid is based upon the reactions shown in Scheme 1.

Oxidized forms of ascorbic acid (DHA and DKG) are able to react with DNPH to form the same water-insoluble osazone (1),

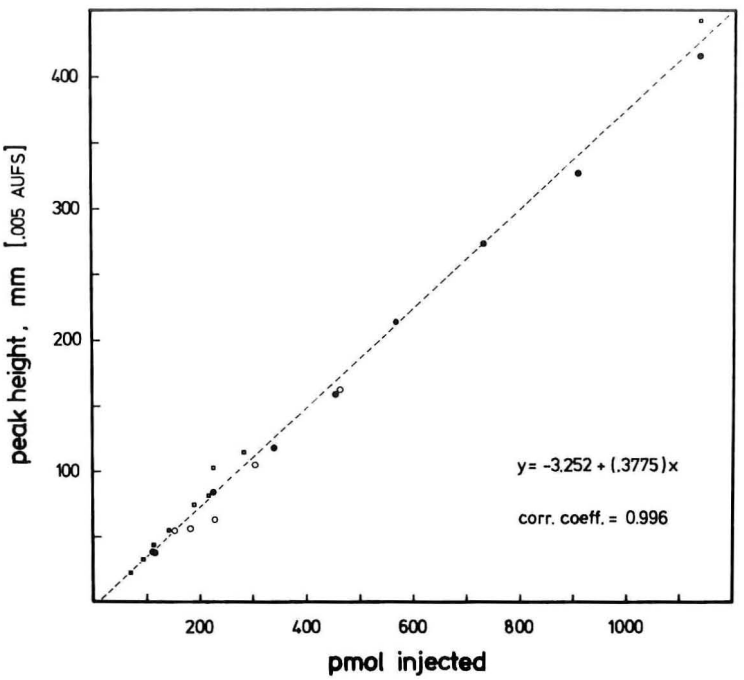


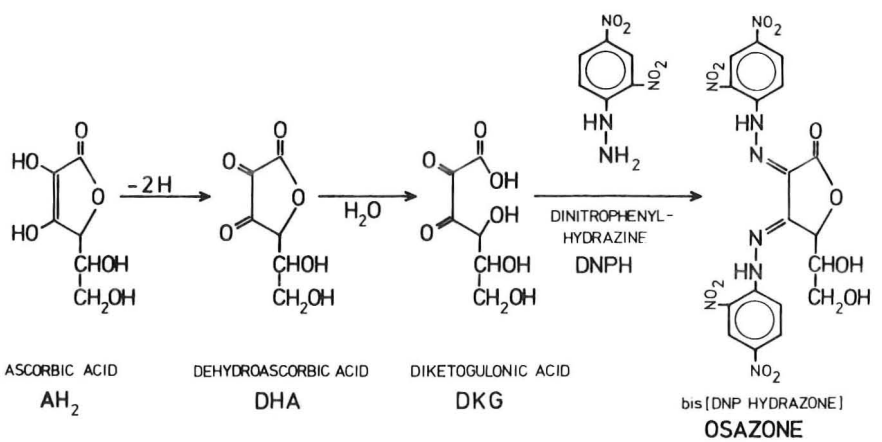
FIGURE 3

Correlation between the height of the osazone peak and the amount of ascorbic acid injected into the column. Squares and solid circles represent data obtained with the common HPLC procedure described in the text (20 µl samples). Empty circles represent preliminary data obtained with the microassay procedure (5 µl samples). Original peak heights were converted to a full scale sensitivity range of 0.005 absorbance units.

TABLE 1

Ascorbic Acid in the Aqueous Humor of Cataract Patients

Sample Identification	Ascorbic Acid		
	Total mM	Oxidized mM(%)	Reduced (%)
8036	1.020	.181 (17.7)	82.3
8037	.700	.133 (19.9)	81.0
8043	1.900	.088 (4.6)	95.4
8053	1.750	.297 (17.0)	83.0
8060	1.156	.130 (11.2)	88.8
8061	1.730	.369 (21.3)	78.7
8068	2.035	-	-
8076	1.464	.113 (7.7)	92.3
8084	1.960	.213 (10.9)	89.1
Averages	1.524	.190 (12.5)	87.5



SCHEME 1

Main reaction sequence in the dinitrophenylhydrazine (DNPH) based methods for the determination of ascorbic acid. Reduced ascorbic acid (AH_2) is oxidized under relatively mild conditions to dehydroascorbic acid (DHA). The latter, in an acidic aqueous medium is spontaneously converted to diketogulonic acid (DKG), the only species probably able to react with DNPH to form the osazone derivative (1).

while reduced ascorbic acid (AH_2) is not. If ascorbic acid in a sample is submitted to oxidation (Norit, Br_2, Cu^{2+}, etc.) a value for total ascorbic acid in that sample is obtained (AH_2+DHA+DKG). If oxidation of the sample is omitted, the amount of DHA + DKG in the sample can be determined. AH_2 is given by the difference between those two determinations.

Although, in this work, no attempt was made to obtain individual values for DHA and DKG, this is possible by choosing analytical conditions that specifically reduce DHA to AH_2 without DKG being affected (1,10).

The coupling of oxidized ascorbic acid and DNPH is carried out in Roe's based procedures under acidic conditions (about 1.5 N H_2SO_4) and in the presence of thiourea to avoid unwanted oxidation of ascorbic acid during the incubation time (4). A great molar excess (about 300 times minimum) of DNPH over ascorbic acid has been generally employed (1-3). An incubation temperature of 37°C is recommended to reduce interferences by sugars (11).

In the colorimetric method the final step consists of dissolving the osazone by adding H_2SO_4 to a final concentration of about 15 N (1-3). This results in a molecular rearrangement of the osazone, spectrally revealed by a red shift of 20 nm that occurs upon acidification. This step would also help to destroy small amounts of

sugar osazones formed during the derivatization (11). The colored solution is read at 520 nm.

In order to improve the specificity of the method when low levels of ascorbic acid are measured relative to blank values or to the levels of possible interferring substances (H_2O_2, sugars and their degradation products, other unidentified osazone-forming compounds) (2,11), several modifications have been made to the original procedure, such as isolating the insoluble osazone by filtration and then dissolving it in a smaller volume (12); or introducing a thin layer or column chromatographic step aimed to specifically purify and then quantitate the osazone of ascorbic acid (2). These additional steps, added to the long incubation period required to form the osazone result in extremely time-consuming methodology. Loss of material may also occur.

We have adapted the colorimetric procedure to a HPLC method. The reaction mixture containing the osazone is extracted into EtOAc and the components are separated on a C^{18} μ-Bondapak hydrophobic column. This way of finishing Roe's procedure results in a great increase of its specificity and reduces the chances of interference because of the resolving power inherent to the HPLC step added to the method. Also considerable amount of time is saved in comparison to the use of other types of chromatography.

For the common HPLC procedure described here we used a lower concentration of H_2SO_4 during the derivatization step (about 0.17 N) as well as a lower molar ratio between the reactants. A minimum 20-fold excess of DNPH over ascorbic acid was used. The linear relationship between ascorbic acid concentration and the amount of osazone formed under those conditions was preserved and less acid and DNPH entered the column. With the procedure described for 20 µl samples the detection of about 50 picomol of ascorbic acid injected into the HPLC system is possible. This compares very well with the most sensitive methods available (6).

Because in our common procedure only 1/100 of the EtOAc extract is actually injected into the column we prepared a microassay requiring less volume of sample (5 µl) which, on the other hand, was extracted with proportionally less volume of EtOAc so as to increase the amount of osazone in the 5 µl aliquot injected into the column. The coupling mixture used in the microassay contained a DNPH/ascorbic acid molar ratio of about 28 and H_2SO_4 was 0.75 N. When the former was lowered to 15 and the normality of H_2SO_4 was reduced to 0.16 N the linear relationship (Fig.3) between ascorbic acid in the sample and the height of the osazone peak obtained with the microassay was lost.

We had no option with the instrumentation available to us but to monitor the eluate from the HPLC column at 254 nm when this work was done. However, the eluate should have been ideally monitored at 500 nm, based upon the absorption spectrum of the osazone (Fig. 2B). Waters Associates Product Department has just developed to our request a 500 nm conversion kit not available before that allows the Waters 440 Absorbance Monitor to be used at the wavelength required by this method.

When the HPLC procedure for ascorbic acid determination was preliminarily applied to human aqueous humor samples from cataract patients significant amounts of oxidized ascorbic acid (DHA+ DKG) were detected in most cases. Because care was taken to immediately protect reduced ascorbic acid in those samples with 5% HPO_3 it does not appear justifiable to neglect this fraction when aqueous humor samples are studied, at least if they proceed from cataract patients. The possible correlation that might exist between oxidized ascorbic acid and the intensity or type of cataract is being investigated.

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RAPID ANALYSIS OF 6-DIAZO-5-OXO-L-NORLEUCINE (DON) IN HUMAN PLASMA AND URINE

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ABSTRACT

A paired-ion, reversed phase high pressure liquid chromatography (HPLC) procedure is described for the analysis of DON in human plasma and urine. Plasma proteins are removed by centrifugal membrane filtration, and the filtrate is injected directly onto an octadecylsilane column. The DON is eluted in a mobile phase consisting of 5 mM 1-heptanesulfonic acid, pH 2.4. Eluting material is monitored at 280 nm and 254 nm. The lower limit of sensitivity in plasma is 0.1 μg/ml.

INTRODUCTION

Renewed interest in the glutamine antagonist, 6-diazo-5-oxo-L-norleucine (NSC-7365; DON), has resulted from observations of its chemotherapeutic activity against human tumor lines implanted into nude mice and its activity in human clinical trials (1,2). The new clinical trials of this agent employ an intermittent, relatively high intravenous dose (2) whereas many of the

earlier trials utilized an oral preparation of the drug given on a chronic schedule (3). DON presents a problem to the analytical chemist since it is relatively unstable at extremes of temperature and pH (4). Furthermore, it is an amino acid analog and many methods for its selective extraction and analysis are complicated by the high levels of chemically-similar substances present in biological fluids. Fortunately, the compound has appreciable ultraviolet absorbance at 274 nm (reported $E^{1\%}_{1cm}$ =683, reference 4). We have taken advantage of this property of DON in developing the paired-ion, reversed phase HPLC method of analysis reported herein.

MATERIALS AND METHODS

The bulk chemical and pharmaceutical preparations of DON were supplied by the Pharmaceutical Resources Branch, Division of Clinical Investigation, National Cancer Institute, National Institutes of Health, Bethesda, MD. These materials were found to be at least 95% pure by ultraviolet absorbance and HPLC analysis. L-Heptanesulfonic acid, sodium salt, was a product of Eastman Kodak Co., Rochester, NY. Formic acid, 95-97%, was obtained from Aldrich Chemical Co., Milwaukee, WI. Deionized, glass distilled water which had been passaged through 0.2 μ membrane filters was used for the preparation of all solutions.

Blood samples (~2 ml) from the patient described were obtained from an indwelling catheter previously placed in the supe-

rior vena cava for other purposes. Plasma was obtained by centrifugation at 1000 xg for 10 min in a desk-top clinical centrifuge. One ml of the plasma was placed in a Centriflo CF-25 cone (25,000 MW cut-off, Amicon Corp., Lexington, MA), and the sample was centrifuged for 30 min at 900 xg in a Beckman Model J2-21 Centrifuge at 4^{o}. The filtrate was injected onto a μBondapak C_{18} column (4mm x 30 cm; Waters Associates, Milford, MA) via a Rheodyne Injector (Model 7120, Rheodyne Incorporated, Berkeley, CA). The sample was eluted at 2 ml/min using a solution of 5mM 1-heptanesulfonic acid, adjusted to pH 2.4 with formic acid. A Laboratory Data Control (Riviera Beach, FL) Series 7800 Liquid Chromatograph was used. Detection of eluting materials was accomplished by ultraviolet absorbance at 254 nm (UV III Monitor) and at 280 nm (spectromonitor II). The flow of either of two Constametric Pumps was driven by a Chromatography Control Module II (CCM). The output of the two detectors was plotted and integrated simultaneously by the CCM, using the machine language programmed features of the instrument. The linear regression analysis and statistical calculations were performed using the BASIC programmable features of the CCM microprocessor as supplied by Mr. Mike Tarter, Laboratory Data Control, Rivera Beach,FL.

Urine samples were collected, placed on ice and an aliquot of the pretreatment (control) sample was "spiked" with

a working standard amount (10 μg/ml) of DON. Five ml aliquots of the urine samples were passaged through Sep-pak cartridges (Waters Associates) which had previously been washed with 5 ml of methanol, followed by at least 10 ml of distilled, deionized water. The urine samples were then filtered through 0.45 μ disposable membrane filters (Model SLHA 025 0S, Millipore Corp., Bedford, MA) and stored at -20° until analysis by HPLC. The informed consent of parents and patients was obtained for drug administration and for the sample collections from blood and urine.

RESULTS AND DISCUSSION

The reversed-phase, ion-pairing approach to HPLC is proving to be a very fruitful means of analysis of chemicals (5), particularly of drugs since many are either weak acids or weak bases. For analysis of DON, 1-heptanesulfonic acid was found to be suitable as an ion-pairing reagent (Figure 1). In experiments not shown, DON could be analyzed in plasma filtrates by elution of two reversed phase columns with a solution of 10 mM KH_2PO_4, pH 5.5; however, under these conditions separation of uric acid from DON was inadequate for drug measurement at levels less than 1 μg/ml. Retention of DON, but not uric acid, was increased by substituting 1-heptanesulfonic acid at pH 2.4 as the eluant as illustrated in Figure 1. Detection of DON at 254 nm and at 280 nm provides additional confirmation of the identity of the DON peak. Further confirmation can be made utilizing DON's temper-

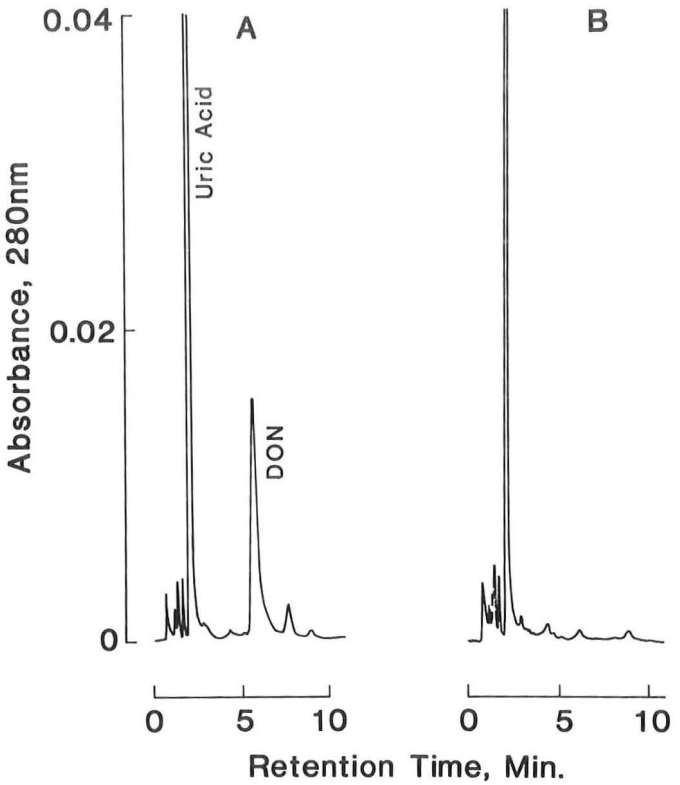

FIGURE 1. Representative separation of DON in human plasma. After removal of plasma proteins by membrane filtration, 50 µl of plasma containing 4 µg/ml of DON was injected onto the HPLC system described in Materials and Methods. A = sample prior to heat treatment. B = same sample after heating on a boiling water bath for 5 min.

ature instability, i.e., boiling the sample for 5 min eliminates the UV absorbing peak characteristic of DON (Figure 1).

A representative standard curve for DON in plasma is shown in Figure 2. Although the determination of DON in plasma samples containing 0.2 to 1.6 µg/ml is shown, additional determinations confirm linearity of detection to levels as

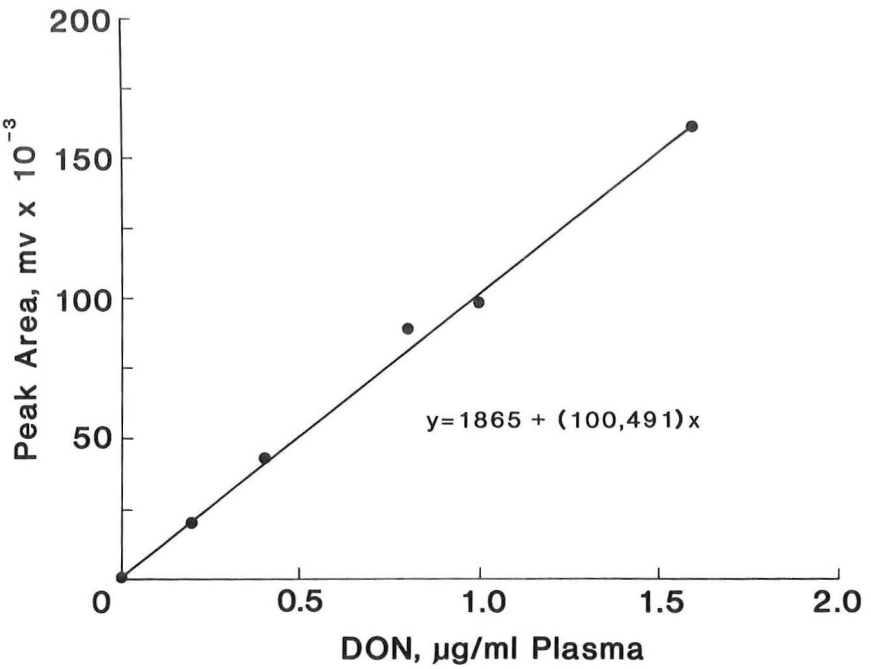

FIGURE 2. Standard curve for the HPLC analysis of DON in human plasma. The areas under the UV absorbing peaks at 280 nm are plotted at various known amounts of DON added to pooled human plasma. Qualitatively similar, but quantitatively different, curves were generated at 254 nm (data not shown). The equation shown is the least squares linear regression of the data.

high as 50 µg/ml. Table 1 presents data which indicates the quantitative recovery of DON during the membrane filtration step to remove plasma proteins, i.e., DON is not bound to plasma proteins. Also, the reproducibility of measurements of DON added to plasma is indicated in Table 1. DON was found to be stable in plasma prior to or after centrifugal membrane filtration when frozen at -20^{o} for 1 week.

TABLE 1

Recovery of DON from Human Plasma Using a Membrane Filtration Method to Eliminate Proteins

Sample No.	DON, μg/ml	% Recovery
1	5.39	100
2	5.94	111
3	5.53	103
4	5.01	93
Mean value ± S.E.	5.47 ± 0.19	102 ± 3.7

DON was added to pooled human plasma to give a final concentration of 5.37 μg/ml before removal of plasma proteins and assay by HPLC as described in Materials and Methods.

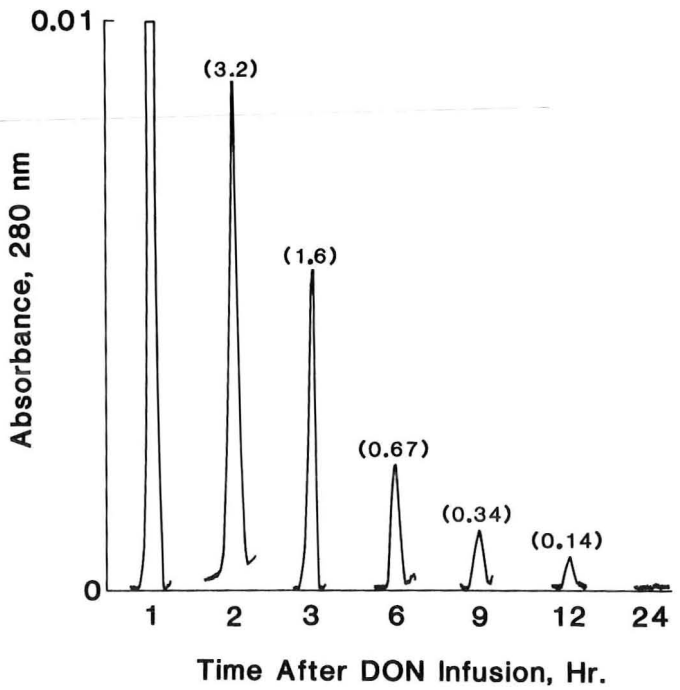

FIGURE 3. Disappearance of DON from the plasma of a child receiving 150 mg/m^2 as a 15 min infusion. The DON peak monitored at 280 nm is shown. The numbers in parenthesis are the integrated values for DON in plasma in μg/ml.

Application of the HPLC method for analysis of DON in plasma of a patient treated with the drug is given in Figure 3. The patient received 165 mg of DON (150 mg/m^2) as an i.v. infusion during a 15 min interval. Considerably less than 2 mg were excreted in the urine during the subsequent 24 hr collection interval in other pediatric patients treated with this dose (data not shown). The lower limit for detection of DON in urine is approximately 1 μg/ml.

In summary, previously reported methods for the analysis of DON in biological fluids have included microbiological (6,7), spectrophotometric (8) and HPLC after derivatization (9). Although the limits of sensitivity for some of these assays are adequate for the relatively-high doses being used in the current Phase I trials of the drug, the ease of sample preparation and the speed of the present method are important considerations for those interested in the clinical analysis of this rather labile chemical.

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DETERMINATION OF RESERPINE IN PHARMACEUTICAL FORMULATIONS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

High performance liquid chromatography was employed in the assay of reserpine in tablet formulations. A reverse-phase RP-8 column was used and reserpine was separated from its major degradation products and quantitated by peak-height measurement using ultraviolet detection at 254 nm and fluorescence at 330 nm excitation. Tablets analysed were within the official limits even after more than ten years following manufacture.

INTRODUCTION

The official method (1) for the determination of reserpine in tablets involves reaction of the chromatographically extracted reserpine with nitrous acid to form a colored solution which is then measured spectrophotometrically in the visible region. The major source of light absorption by the sample solution is due to the formation of the major solid-state degradation product of reserpine, 3,4-dehydroreserpine[1]. 3,4-dehydroreserpine (apple-green fluorescent), 3,4,5,6-tetradehydroreserpine (bright-blue fluorescent) and 3-isoreserpine (non-fluorescent) have been identified by Wright and Tang (2) in U.V. irradiated solutions of reserpine in methanol and chloroform. These authors also claim to have identified 3,4-dehydro-

reserpine and 3-isoreserpine (but not 3, 4, 5, 6-tetradehydroreserpine) in all commercially available brands of reserpine tablets examined by solvent extraction and T.L.C. analysis. The procedure incorporates a blank to compensate for absorbance due to degradation products present in the sample.

Prior to the advent of high performance liquid chromatography (HPLC), analysis of reserpine formulations was based either on direct U.V. determination or oxidation of reserpine followed by U.V. or fluorimetric estimation (3-8).

Horriberg, Stewart, Smith and Hester (9) have applied reverse phase HPLC successfully to the analysis of reserpine in multicomponent dosage forms but the retention times were quite long and gave considerable band spreading in some instances. It was also noted that reserpine was very sensitive to the pH (>8) of the solvent system.

Butterfield et al. (10) used a forward phase HPLC system to analyse reserpine and hydrochlorothiazide in two-component formulations and suggested that their method could be applied to single component formulations. However their method does not permit detection of 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine as these compounds were retained on the column. As well, the identification and presence of 3-isoreserpine was ascertained solely on the basis of retention behaviour in one system. Elution of both 3-isoreserpine and reserpine is very rapid as supported by very low k' values. Furthermore, monitoring by the HPLC system employed in the present study, a reserpine solution in tetrahydrofuran at room temperature for more than 5 days under laboratory light showed no 3-isoreserpine. The formation of 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine could be easily followed with time; initially, 3,4-dehydroreserpine is the major product of decomposition but 3,4,5,6-tetradehydroreserpine eventually gains ascendancy. Although the possibility of other factors relating to the other drug component or excipients may be of relevance to this issue, the efficacy of the identification of 3-isoreserpine based solely upon retention time in one system must be viewed with suspicion.

EXPERIMENTAL

Materials: Reserpine was obtained from two sources 1) U.S.P. Reference Standard 2) Working standard was from the Aldrich Chemical Co. Sodium phosphate, certified A.C.S. (Fisher Scientific, Fair Lawn, N.J., U.S.A.). Methanol and ethyl acetate were HPLC grade.

3-Isoreserpine: 3-Isoreserpine was prepared according to MacPhillamy et al. (11)

3,4-Dehydroreserpine and 3,4,5,6-Tetradehydroreserpine: These compounds were prepared by the procedure of Wright and Tang (2).

Extraction solvents[2]: The extraction solvent system consisted of water saturated with ethyl acetate and ethyl acetate saturated with water. Saturation was achieved by mixing in a large separatory funnel approximately equal amounts of both solvents, shaking for 10-15 minutes and allowing the system to stand until both layers became clear (preferably overnight).

Solutions:

a) Internal standard solution: A solution containing approximately 20 mg (roughly equivalent to 1 drop) of propiophenone in 500 ml of water-saturated ethyl acetate was used; at this concentration, a peak corresponding to 60% full scale deflection was obtained. Although this solution was found to be stable over a period of 1 month, a fresh solution was made weekly.

b) Reserpine standard solution: Approximately 25 mg of reserpine bulk drug (Aldrich) was weighed accurately and dissolved in 100 ml of the internal standard stock solution. This solution was prepared fresh daily.

Chromatographic parameters:

Apparatus: A Laboratory Data Control HPL Chromatograph (LDC, Division of Milton Roy Co., Riviera Beach, Florida) fitted with a Valco

valve and a 20 µl injection loop was used. An LDC (range 0.01 aufs) variable wavelength detector (set at 254 nm) was connected in series with a Schoeffel FS970 fluorescence detector (set at 330 nm, 0.05 µA range, sensitivity 6.8, time constant 0.5 sec) fitted with a Corning 7-51 excitation pre-filter and a 470 nm cut-off emission filter.

Column: The 4.6 mm i.d. x 25 cm stainless steel columns used contained 10 µm Lichrosorb sorbant coated with chemically bonded octylsilane (RP8, Brownlee Laboratories, Santa Clara, Calif., U.S.A.).

Recorders: Chromatograms were recorded on a Honeywell Electronik 196 and a Pharmacia Fine Chemicals model 410.

Integrator: Calculations of peak height were done manually or by using a Minigrator (Spectra-Physics, Santa Clara, Calif., U.S.A.) capable of measuring peak heights, set at attenuation 4.

Mobile phase: A 50:50 V/V mixture of methanol and sodium phosphate monobasic 0.05M in water (6.9 g of the phosphate dissolved in 1 liter of water, pH 4.5) was used. The solvent was filtered through a millipore filtering apparatus using glass fiber filters (Reeve-Angel, Whatman, Inc. Claxton, N.J. U.S.A.). The flow of the mobile phase was 2.0 ml/min at a pressure of approximately 2000 psi.

Analysis of Pharmaceuticals: Tablet-composite samples were prepared by grinding manually using a mortar and pestle, 25 tablets for a 0.25 mg dosage and 50 tablets for a 0.10 mg dosage, mixing until a uniform powder was obtained. An aliquot equivalent to .25 mg or .10 mg of reserpine was accurately weighed (equivalent to the amount of reserpine in 1 tablet) and the powder transfered to a 13 x 100 mm culture tube (Canadian Laboratory Supplies, Montréal, Québec, Canada) fitted with a teflon-lined screw cap. 1.0 ml of water (ethyl acetate saturated) and 1.0 ml of the internal standard solution were added to each individual sample. Samples were then extracted using an Evapo-Mix apparatus (Buchler Instruments, Fort Lee, N.J., U.S.A.) for 20 minutes and centrifuged for 2 minutes at 2000 rpm

(Safety Centrifuge, Fisher Scientific). Ten microliters of the ethyl acetate supernatant was injected after a small portion of this layer was transfered with a Pasteur pipette to a suitable container. Quantitation was by the peak height ratio between the sample and the internal standard. Fig. 1.

Calibration of the working standard: In order to establish the purity and integrity of the reserpine bulk drug, a working standard solution was compared with similar solutions of reserpine using the U.S.P. reference standard. Triplicate weighings of approximately 50 mg of reserpine bulk drug and the U.S.P. reference standard (dried 3hrs at 60°) were made up in 25 ml ethyl acetate containing the internal standard. From these solutions 1 ml aliquots were taken and diluted to 10 ml with internal standard solution. Duplicate 10 µl injections and subsequent ratio comparison of peak heights for reserpine and the internal standard in both of these prepared solutions gave a percentage value of 97.75%. Relative standard deviations of the peak height ratios for the Official Standard and working standard were ±1.4% and .8% respectively.

Calculations:

% found =

$$\frac{PHR_{SPL}}{PHR_{STD}} \times \text{Wt of STD} \times \frac{\text{Wt. tab}}{\text{Wt of SPL}} \times \frac{1}{\text{label claim (mg)}} \times \text{STD purity}$$

where:

PHR_{SPL} = peak height ratio of sample to internal standard.

PHR_{STD} = peak height ratio of standard to internal standard.

Wt of STD = weight of the standard in mg.

Wt tab. = average weight of tablet analysed in mg.

Wt of SPL = weight of the sample in mg.

STD purity = percentage value of the working standard as determined by calibration of the working standard.

RESULTS AND DISCUSSION

Reserpine formulations were assayed using a reverse phase mode and intact reserpine measured directly by ultra violet absorbance detection.

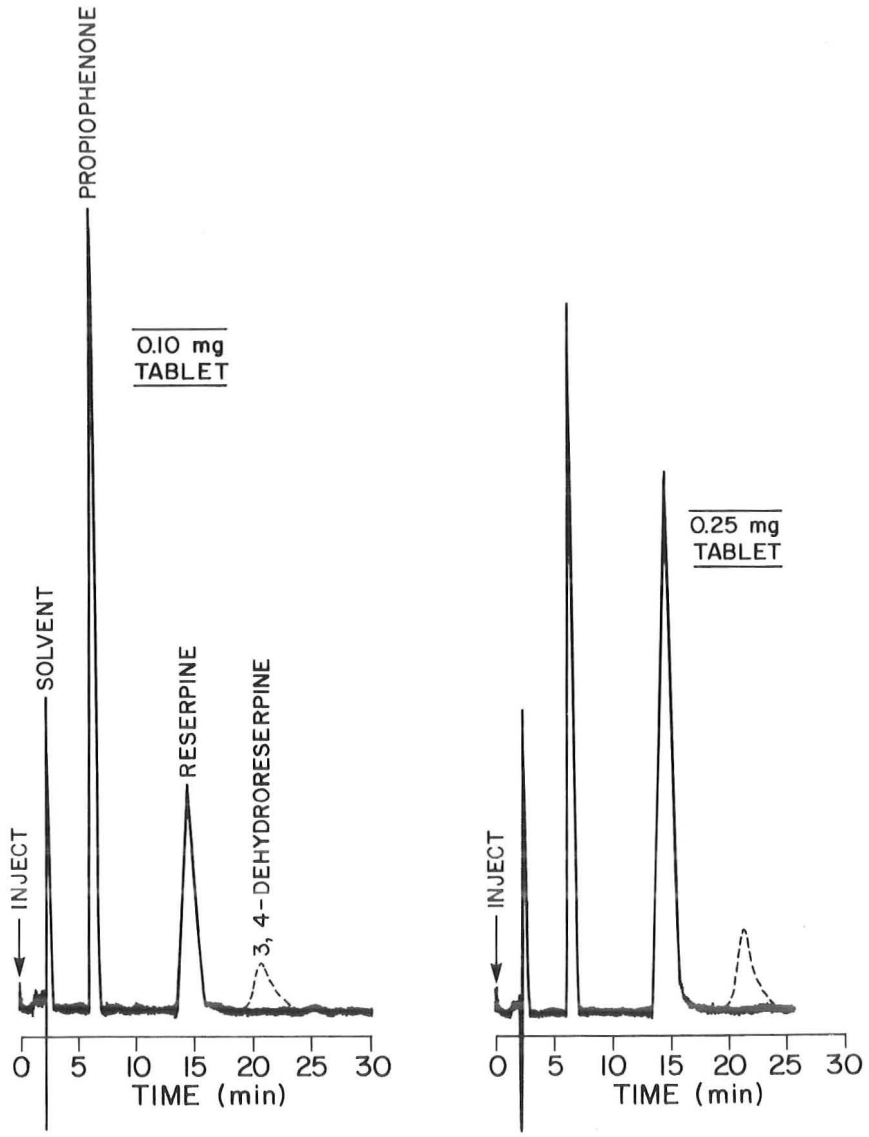

FIGURE 1

Typical chromatograms obtained when the equivalent of one tablet is extracted using water saturated ethyl acetate and 10 ul of supernatant injected on RP-8 column. (————) UV detection at 254 nm. (---------) fluorescence detection, excitation wavelength set at 330 nm, and 470 nm cut-off filter was used.

The use of a buffered water:methanol system in reverse phase obviates the necessity of a very complex solvent system (such as employed by Butterfield et al.) (10) which renders the latter procedure less easily applicable to routine use. In addition, the HPLC assay here presented permits direct observation of reserpine degradation products, as well as 3-isoreserpine. Fig. 2.

Reserpine tablets from various manufacturers were analysed and results compared with analytical data obtained by the official method. The sample lots consisted of three different age groups, namely, very old (>10 years), old (>3 years) and recent (<1 year).

The HPLC method for reserpine assay is shown to be rapid, precise and accurate. In addition, no interference from formulation excipients is observed. The relative standard deviations for representative samples assayed five times were between 1 and 2 percent (Table 1).

The reproducibility of the chromatographic system was assessed by injecting six 10 µl aliquots of an extracted composite sample; the relative standard deviation based on peak height ratio was 0.8%.

Linear response was determined using a stock solution of reserpine (Aldrich Chem. Co.) in 100 ml of water, saturated with ethyl acetate containing the internal standard, propiophenone. Volumes of 0.25, 0.50, 1.0 1.5, 2.0 and 4.0 ml were made up to volume with internal standard solution in a 10 ml volumetric flask. 10 µl of each solution was injected onto the column. Linearity was obtained for a concentration range of 0.250 µg to 4.0 µg/10 µl when peak height ratios were plotted against concentrations. The coefficient of correlation,(R^2) for 7 determinations, was 0.9998 (Y = 30.967 x + .242).

Extraction time was determined by extracting two weighed samples of a representative formulation for 20 minutes and 40 minutes, respectively. The peak height ratio gave a relative standard deviation of 0.8%, in accordance with the reproducibility of the system; maximum extraction efficiency was attained within 20 minutes. Also, injection of the aqueous layer of extracted samples gave no U.V. light absorption. Initially a Roto-Rack (Fisher Scientific Model 343) was used to tumble the sample tubes for various lengths of time. It was found, however, in some instances that

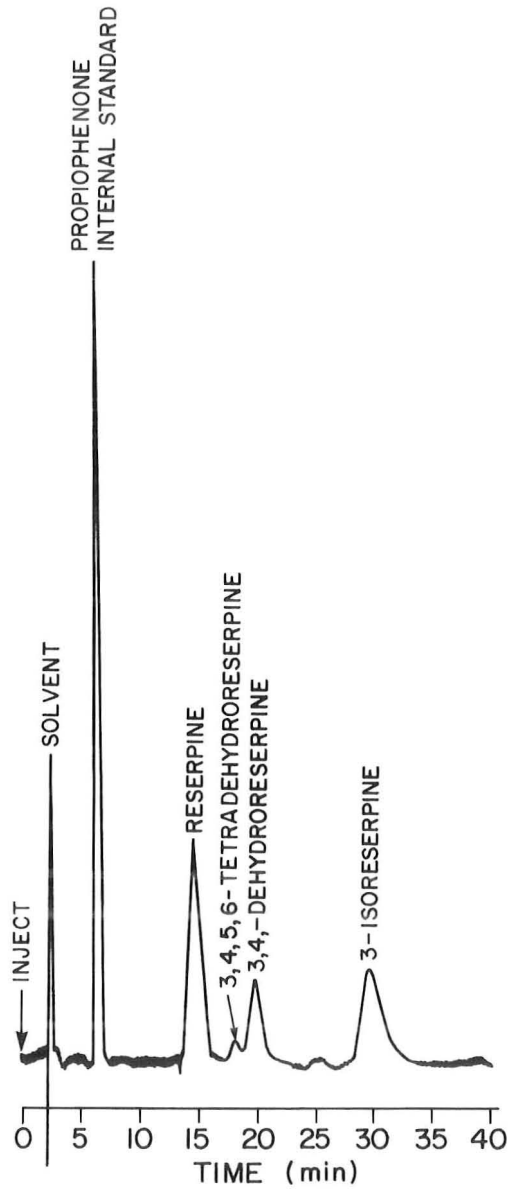

FIGURE 2

Separation of Reserpine and its degradation products on an RP-8 column using methanol:.05M aqueous phosphate buffer 50:50 and a flow rate of 2.0 ml/min. Detection is at 254 nm.

TABLE 1.
Results of Reserpine Formulation Assay

Reserpine Sample Formulation	Age (years)	Label Claim (mg)	% Label Claim USP ASSAY	% Label Claim HPLC ASSAY	RSD, %*
1 new	<1	.25	97.4	97.9	1.04
2 new	<1	.10	105.0	105.8	1.58
3 old	>3	.25	97.1	96.9	1.07
4 old	>3	.10	97.3	96.5	1.52
5 very old	>10	.25	94.4	93.0	2.05

*Calculated on the basis of 5 determinations for each sample.

time of extraction was not the only significant factor in extraction efficiency. Erratic extraction efficiency may indicate the need for more vigorous agitation of the extraction medium. The use of a Vortex or Evapo-Mix apparatus -instead of the Roto-Rack- for agitation of some samples resolved this problem.

The addition of water in the first step of the extraction procedure is a significant aid; failure to do so often produced cloudy solutions and led to erratic results when such solutions were analysed. Water facilitates disintegration of the solid and promotes extraction of water-soluble dyes and excipients[3]. Also maximum extraction of reserpine into the ethyl acetate layer and minimum carry-over of water-soluble material was ensured by the use of a mutually pre-saturated ethyl acetate -water extraction solvent.

Adjustment of the mobile phase by addition of a third solvent permitted greater separation between 3,4,-dehydroreserpine and 3,4,5,6,-tetradehydroreserpine in some cases, but reserpine was invariably not as well resolved in such instances; it was therefore decided that optimal separation of degradation products would be sacrificed in favour of better resolution of reserpine. Use of short connecting tubes between the U.V. detector and the fluorescence detector permitted simultaneous determination of reserpine and 3-isoreserpine, by U.V. and 3,4,-dehydroreserpine and 3,4,5,6,-tetradehydroreserpine, by fluorescence. Even though 3,4-dehydroreserpine and 3,4,5,6,-tetradehydroreserpine are well separated

TABLE 2.
Retention Time and k' Values*

Compound	Retention time in seconds	* k'
Propiophenone	400	3.9
Reserpine	910	10.2
3,4,5,6-Tetradehydroreserpine	1081	12.5
3,4-Dehydroreserpine	1200	14.0
3-Isoreserpine	1777	21.0

*Similar k' values were obtained on two other RP-8 columns.

from reserpine, the amounts present in the formulations examined gave little or no U.V. absorbance; the presence of these degradation products was therefore monitored by fluorescence at 330 nm, the excitation peak maximum wavelength for 3, 4, 5, 6-tetradehydroreserpine.

Precise individual determination of 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine is not yet possible but efforts are continuing to determine the chromatographic characteristics of these compounds and in particular the assessment of the extent of 3,4-dehydroreserpine oxidation to 3,4,5,6-tetradehydroreserpine during formulation work up and chromatography; the objective is to establish a fluorimetric assay method for low levels of reserpine degradation products. The results of this continuing effort will be reported in due course. It is recommended that in order to ensure reliability of the separation and HPLC system when analysing tablets on a routine basis when only a U.V. detector is available, that a mixture of reserpine and lumireserpine be injected occasionally to verify k' values, and the methanol content of the mobile phase adjusted accordingly.

The results herein reported indicate that the reserpine content in a variety of tablets on the Canadian market is still within acceptable official limits even after more than ten years following manufacture.

FOOTNOTES

1 Thin-layer chromatography (TLC) has shown that the reaction product contains a number of minor components (so far unidentified) in addition to 3,4-dehydroreserpine and 3,4,5,6-tetradehydroreserpine (lumireserpine).

2 The use of chloroform as solvent (as in the U.S.P. procedure) was avoided because reserpine was observed to undergo ready oxidation in chloroform solution unless care is taken to exclude light and oxygen. Also, its use would necessitate the pipetting of a subnatant solution in separation of the extract from the aqueous layer.

3 It was found that in one case that it was necessary to filter the supernatant through a small pledget of glass wool to obtain a clear upper layer.

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ANALYSIS OF THE CARBAMATE INSECTICIDES ALDICARB AND CARBARYL IN FORMULATIONS UTILIZING A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM WITH AN ON-LINE INFRARED DETECTOR

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ABSTRACT

An aldicarb (2-methyl-2-(methylthio)propanal, O-[(methylamino)-carbonyl]oxime) granular formulation with and without added carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) as an internal standard and a carbaryl (1-naphthalenyl methylcarbamate) wettable powder and liquid suspension formulations both with and without added methiocarb (3,5-dimethyl-4-(methylthio)phenyl methylcarbamate) as an internal standard were analyzed by utilizing a high-performance liquid chromatographic system operating in the normal phase with a Partisil column and mixtures of acetonitrile:dichloromethane:heptane as the mobile phase. An on-line infrared detector equipped with a flowcell was used. Analyses were conducted at ambient temperature. The commonly used calibration and quantitation techniques were also compared.

INTRODUCTION

The N-methylcarbamate compounds comprise a major class of insecticides. Although carbamates have been used for a long time in pest control operations, analytical methodology for their quantitation is still in the state-of-the-art relative to other classes of established pesticides. Gas chromatography (GC) generally has failed to qualify as a direct analytical tool due to the unfavorable chemical and physical properties of the carbamate insecticides

and frequently to the lack in their chemical structure of elements amenable for their detection by the generally used GC detectors. The available analytical methodologies, as well as the problems associated with them, have been reviewed by Dorough & Thorstenson (1), Magallona (2), and recently by Seiber (3).

The rapid development of high-performance liquid chromatography (HPLC) into a powerful analytical tool for trace analysis has led to a rapidly increasing number of applications to pesticide analysis in the last five years. HPLC has become a promising alternative for the compounds troublesome to analyze under GC conditions. The major drawbacks in HPLC analysis of pesticides today arise from a need for a detection system which is easily operated on the routine basis, trouble free and selective. The most commonly used detector in HPLC analytical systems is the ultraviolet (UV) detector. Moye (4), after reviewing the applications of HPLC in pesticide residue analysis, reported that in more than 85% of the cases, UV was used as the detection system; therefore except for special cases of relatively clean extracts, very extensive sample cleanup is required. Post-column hydrolysis and fluorogenic labeling has been applied by Krause (5) for the residue analysis of N-methylcarbamates.

Infrared (IR) spectroscopy has had a long history of use in pesticide analysis before the advent and wide use of GC, and later as a GC detector for on-the-fly GC effluents. The inherent low sensitivity of the conventional IR spectrophotometers and the constantly increasing emphasis on the development of more sensitive detection systems in pesticide residue analysis has diminished the use of IR. Fourier transform IR (FT-IR) interferometers as GC detectors have overcome the sensitivity deficiencies and have permitted the use of valuable IR spectral information for the positive identification of the sought analytes. However, the high cost of FT-IR instrumentation restricts its use for routine pesticide analysis. The utilization of an IR photometer on-line to the HPLC eluate via a flowcell for the analysis of pesticides was first applied for the formulation analysis of the pyrethroid insecticides

(6,7). The system was demonstrated to be very efficient in terms of selectivity, reproducibility and reliability for the analysis of emulsifiable and aerosol concentrates by direct injection on the HPLC after appropriate sample dilutions. The main problem was the relatively low sensitivity which can be either a curse or a blessing depending on one's objective and analytical problem. If it is desired to apply the system to residue analysis, the sensitivity problem can be resolved by increasing the injection volume and/or the amount of the extracted sample. Preliminary research in applications to residue analysis has shown that very concentrated solutions of a variety of plant extracts do not interfere with the analysis. A minimal cleanup is required only for the protection of the HPLC column.

The application of the HPLC-IR system for the analysis of carbamates is demonstrated below with the analysis of two commonly used carbamate insecticides, aldicarb and carbaryl. The commonly used calibration and quantitation techniques are also compared.

EXPERIMENTAL

Reagents and Solvents

Analytical grade (99.9%) aldicarb and carbaryl were obtained from Union Carbide Corporation. Analytical grade (99%) carbofuran was purchased from Chem Service and methiocarb (97.9%) was obtained from Mobay Chemical Corp. An aldicarb granular formulation and two carbaryl formulations, a wettable powder (80% wt/wt) and a liquid suspension (4 lb/gal) were donated by Dr. G. Carman (Department of Entomology, University of California, Riverside). All solvents were "distilled-in-glass" from Burdick & Jackson Laboratories, Inc. Solvents were filtered through 25-grade glass filter (Schleicher & Schuell) and degassed by shaking under reduced pressure just prior to use. A mixture of acetonitrile:dichloromethane:heptane was used as the mobile phase in the ratio of 15:30:55 (v/v/v) for carbaryl analysis and 20:40:40 (v/v/v) for aldicarb analysis. The appro-

priate mobile phase composition was controlled by a solvent programmer operated under isocratic conditions and constant flow rate of 1.5 mL/min.

Instrumentation

The HPLC system consisted of two Waters Associates Model 6000A pumps controlled by a Waters Associates Model 660 solvent programmer. A Rheodyne (Cotati, CA) 7125 valve injector equipped with a 20-μL loop was used. A Foxboro Wilks Miran-1A (El Monte, CA) variable wavelength IR filter-type photometer was used as the detection system; a 1.5-mm light path and 4.5-μL capacity BaF_2 flowcell was used. A 5 cm x 4.6 mm i.d. Whatman HC Pellosil guard column was used to protect the 25 cm x 4.6 mm i.d. Whatman 10-μm Partisil analytical column. Column fittings were of low dead-volume unions and 0.25 mm i.d. tubing was used to made the necessary connections.

Sample Preparation

Formulations were extracted according to the official AOAC methods (8) with minor modifications. A 1.2 g sample of aldicarb granular formulation was extracted by mechanical shaking with 100 mL of dichloromethane for 2 h (extract I) or Soxhlet-extracted at 5 cycles/h with 80 mL of dichloromethane for 3 h (extract II) and 4 h (extract III). The Soxhlet extracts were made up to 100 mL final volume with dichloromethane. A 10 mL fraction of each extract was filtered through 25-grade glass filter and was subjected either directly to HPLC analysis or after the addition of carbofuran internal standard to 7 mL of filtered extract and dilution to 10 mL by the addition of dichloromethane (diluted fraction); final carbofuran concentration of 3 mg/mL.

A 3.125 g sample of carbaryl wettable powder was extracted by mechanical shaking for 30 min in 50 mL of 10% acetonitrile in chloroform (v/v). The suspension was centrifuged for 5 min at 2000 rpm. The volume of the supernatant was recorded and a 10 mL fraction was filtered through 25-grade glass filter. One mL of the

filtrate was diluted to 25 mL by the addition of mobile phase and subjected directly to HPLC analysis or after the addition of methiocarb internal standard to give a final concentration of methiocarb of 1 mg/mL. A 0.5 mL (0.6 g) sample of the liquid suspension carbaryl formulation was extracted by mechanical shaking for 30 min with 100 mL of chloroform after the addition of 20 g of anhydrous Na_2SO_4. A fraction of the supernatant was filtered and directly analyzed by HPLC or after dilution of 7.5 mL to 10 mL with mobile phase with the simultaneous addition of methiocarb to give a final methiocarb concentration of 1 mg/mL.

The injection volume was kept constant at 20 μL by using the loop-filling technique. At least three replicate injections were made of each analyzed sample. All standard solutions for both aldicarb and carbaryl analyses were made in the corresponding mobile phases.

Calibration Methods and Quantitation

Both the external and internal standard calibration methods were used. Carbofuran was used as the internal standard at the final concentration of 3 mg/mL for the analysis of aldicarb, and methiocarb at 1 mg/mL for the analysis of carbaryl. Therefore, two calibration curves were made for each compound, one by plotting peak heights in cm *vs.* μg of the analyte, and the other by plotting the ratio, (peak height of the analyte)/(peak height of the internal standard), *vs.* μg of the analyte. The calculations were made on the basis of the corresponding calibration factors (slope of the calibration curves) expressed either as peak height in cm/μg of analyte (Method A), or the ratio of peak heights (analyte/internal standard) /μg of the analyte (Method B), or a one point calculation of the calibration factor (Method C). In the last method a standard curve was not constructed but the calculations were based on the response of a standard solution of the analyte of a certain concentration. This last standard solution was selected from among the ones used for the above-mentioned calibration curves and contained the appro-

priate standard at a concentration within ±1 µg/uL of the unknown sample.

RESULTS AND DISCUSSION

Carbamates with insecticidal activity are either aryl or oxime N-methylcarbamates. A common feature of the IR spectrum of all carbamates is the presence of the strong carbonyl absorption band in the area of 5.7 to 6.3 µm. Preliminary studies on the chromatographic behavior of the carbamate insecticides in normal phase liquid-solid chromatography (LSC) on a silica gel column showed that all compounds can be separated and eluted from the column with solvent combinations which are relatively transparent at the carbonyl absorption range.

The ultimate purpose of a trace analysis is the identification and quantitation of the sought analyte(s). In LC quantitative analysis, sources of error are: sampling, choice of the chromatographic conditions, detection, calibration of the method, and measurement (9). With the use of microvalve injectors, the sampling error can be minimized. The choice of the proper chromatographic conditions for a specific separation is an important factor. A brief review of the literature shows that certain groups of compounds are preferentially analyzed by one specific chromatographic mode. Furthermore, the selection of the proper mobile phase composition is critical to obtain the desired separation since undetected or unsuspected overlapping peaks can create erroneous quantitative results. Snyder & Kirkland (9) list the desired characteristics of an ideal LC detector. Measurements can be made either by measuring peak areas or peak heights depending on the conditions. It is generally believed that calibration methods with internal standards can increase the precision of the analysis. This is true if a pretreatment or derivatization of the sample is involved. However, too much weight is given to the use of the internal standard independent of the other analytical conditions. Publications appear which present methodologies which utilize internal standards without

justifying its presence; additional effort is required in finding a chemically and chromatographically appropriate and readily available compound to serve as the internal standard for a specific separation. It is also possible to increase the precision error of an analysis due to the possible low resolution of the internal standard from the compound(s) of interest, the interactions of the internal standard with coextractives or primary metabolites of the pesticide, and the differential stability in solution of the internal standard and the sought analyte even under refrigerated conditions as in the case with the N-methylcarbamates. Furthermore, one can actually increase the measurement error since one has to measure two peaks instead of one. Therefore, the different calibration and calculation methods commonly used in quantitative LC were compared here in terms of accuracy and reproducibility.

Aldicarb. A sample chromatogram of an aldicarb formulation extract is shown in Figure 1 and the results of the analysis are given in Tables 1 and 2. The analysis was conducted three times, spaced every other day. The measurements were made according to the three methods as explained in the experimental section. The calibration factors based on standard curves for each date of analysis were: 0.121, 0.124 and 0.133 cm/μg of aldicarb without an internal standard and 0.021, 0.021 and 0.022/μg of aldicarb in the presence of the internal standard. There is a small but consistent increase of the slope of the standard curve in the first set of values apparently due to the concentration of the standard solutions because of the high volatility of the solvent. This increase is not prominent in the second case since the presence of the internal standard compensates for the small changes in the aldicarb concentrations. In Table 1 are shown the results of the analysis of the undiluted aldicarb extract (without internal standard). The calculations were made according to Method A and C. The mean values considering the three dates as replicates for the three extracts are: 12.5±0.3, 12.3±0.2 and 12.5±0.3 from Method A and 12.6±0.6,

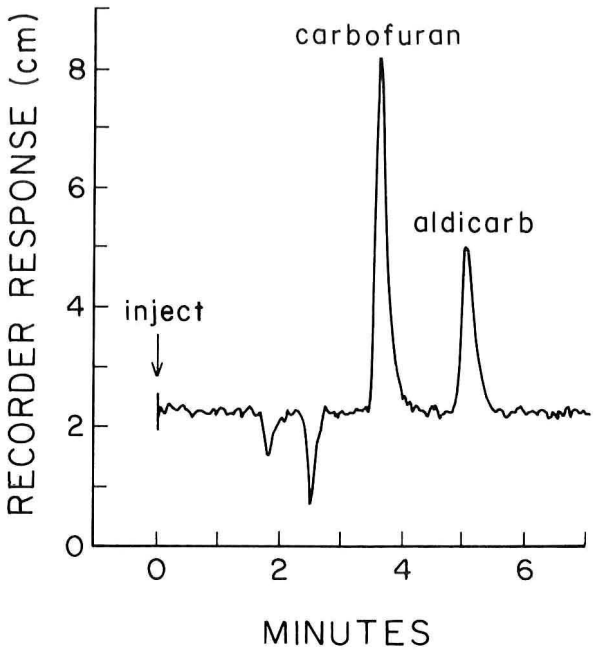

FIGURE 1

IR liquid chromatogram obtained for a 20-μL injection of an aldicarb granular formulation extract containing 3 mg/mL carbofuran as the internal standard. The IR detector was operated at 5.75 μm and 0.1 AUFS with a 1.5-mm path length, 4.5-μL capacity BaF_2 flowcell. The mobile phase consisted of acetonitrile:dichloromethane:heptane (20:40:40). Flow rate was 1.5 mL/min.

12.4±0.5 and 12.6±0.6 from Method C. Although the mean values obtained by either method of calibration and from all three extracts are not statistically significant at the 1% level, Method A appears to be more reliable (lower variance) for day to day analysis than Method C which showed a variance per date more than twice greater than from Method A for these three extracts analyzed.

In Table 2 are shown the results of the analysis of the diluted aldicarb extracts with the internal standard. Method C showed the smallest variance per date and Method A the highest for extract I. The order was reversed in extract III whereas in extract II Method

TABLE 1

Results, Expressed as % by Weight, of the Analysis of Aldicarb Extracts from a Granular Formulation[a/]

	Undiluted fraction without internal standard					
	Extract I		Extract II		Extract III	
Date	A	C	A	C	A	C
1	12.4	12.5	12.4	12.5	12.4	12.5
2	12.8	13.2	12.4	12.9	12.8	13.2
3	12.2	12.0	12.0	11.9	12.2	12.0
Mean	12.5	12.6	12.3	12.4	12.5	12.6
Std. Dev.	0.3	0.6	0.2	0.5	0.3	0.6
Variance	0.09	0.34	0.05	0.24	0.09	0.34

Grand Mean: 12.5±0.4

[a/] IR detector operated at 5.75 μm and 0.1 AUFS.

TABLE 2

Results, Expressed as % by Weight, of the Analysis of Aldicarb Extracts from a Granular Formulation[a/]

	Diluted fraction with carbofuran as the internal standard								
	Extract I			Extract II			Extract III		
Date	A	B	C	A	B	C	A	B	C
1	12.6	12.7	12.5	12.8	12.9	13.7	12.8	13.6	12.4
2	13.0	13.1	12.9	12.2	13.4	12.6	12.6	13.4	13.3
3	13.0	13.1	12.8	12.7	13.4	12.6	12.1	12.7	11.9
Mean	12.8	13.0	12.7	12.6	13.5	12.9	12.5	13.2	12.9
Std. Dev.	0.2	0.2	0.2	0.4	0.3	0.6	0.4	0.5	0.9
Variance	0.06	0.05	0.04	0.13	0.08	0.40	0.15	0.23	0.72
Grand Mean:	12.9±0.5								

[a/] IR detector operated at 5.75 μm and 0.1 AUFS. Each value is the mean of three injections.

B showed the smallest variance and Method C the highest. Applying the Bartlett's test of the homogeneity of variance, it was found that the pooled variance of all three methods over extractions for both diluted and undiluted samples were not statistically significant. Considering the pooled variance per date for each method separately over all extractions, there were not statistically significant differences at the 1% level for both diluted and undiluted extracts. Duncan's multiple range test of dates for each method showed dates not significantly different for Method A but they were different for Methods B and C. However, averages were taken over the three extracts.

Carbaryl. Two formulations of carbaryl were analyzed, a wettable powder and an aqueous suspension. Sample chromatograms of the analysis of both formulations are shown in Figure 2, and results of the analysis in Table 3. The analysis was repeated the next day. The

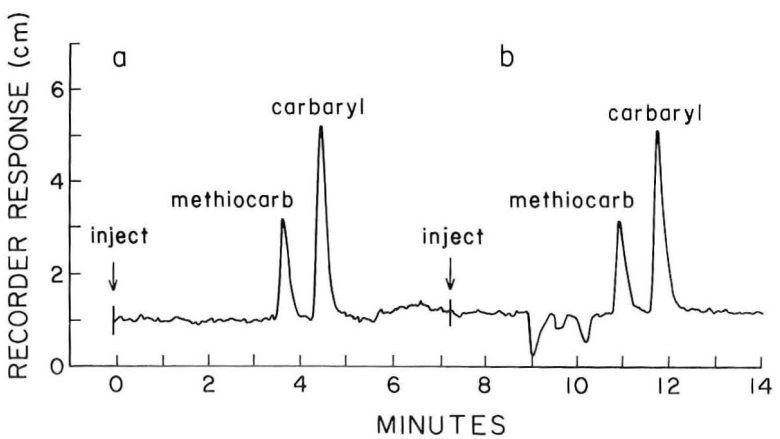

FIGURE 2

IR liquid chromatograms obtained for 20-µL injections of carbaryl formulation extracts: (a) wettable powder and (b) liquid suspension; each containing 1 mg/mL methiocarb as the internal standard. The IR detector was operated at 5.75 µm and 0.1 AUFS with a 1.5-mm path length, 4.5-µL capacity BaF_2 flowcell. The mobile phase consisted of acetonitrile:dichloromethane:heptane (15:30:55). Flow rate was 1.5 mL/min.

TABLE 3

Results, Expressed as % by Weight, of the Analysis of Carbaryl Extracted from a Wettable Powder and a Liquid Suspension Formulation[a/]

	With methiocarb as an internal standard					
	Wettable Powder			Liquid Suspension		
Date	A	B	C	A	B	C
1	77.5	77.6	78.9	43.3	43.3	45.0
2	77.5	77.6	78.9	43.0	41.6	42.6
Mean	77.5	77.6	78.9	43.2±0.2	42.4±1.3	43.8±1.7

[a/] IR detector operated at 5.75 μm and 0.1 AUFS.

calibration factors were: 0.102 and 0.111 cm/μg of carbaryl for each date of analysis and 0.05/μg carbaryl for both dates in the case where the internal standard was included. Again, the small increase of the slope in the first case is probably due to the concentration of the standard solutions whereas this change is compensated for by the presence of the internal standard. No statistical analysis was made for this set of results due to the small number of values, except for calculations of mean values and standard deviations per date for each method of measurement. The relatively high standard deviations of Methods B and C over dates in the case of the analysis of carbaryl liquid suspension is believed to be due to the difference in stability of carbaryl and methiocarb in the extraction solvent.

CONCLUSIONS

An HPLC system operated in the normal phase with a silica gel column and an on-line infrared detector has been demonstrated to be an easily operated, trouble-free system and has been successfully applied for the analysis of aldicarb and carbaryl in formulations. No interference from the formulation coextractives was present. The selected mobile phase combinations are relatively transparent in the 5.7 to 5.75 μm region of the infrared spectrum where these carbamates show their strongest absorption band and still have the proper strength and selectivity to separate and elute the analyzed carbamates in less than 6 min. Any calibration method can be used for the quantitative measurements since no statistically significant differences were found in the results obtained by either method. Therefore the selection has to be made on the basis of simplicity and the number of pitfalls associated with each method depending on the analytical problem.

ACKNOWLDEGMENTS

We thank Carol Adams for conducting the statistical analyses. Research was partially supported by Western Region Research Project W-45.

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BOOK REVIEW

"The LDC Basic Book on Liquid Chromatography,"
S. B. Schram
Milton Roy Company, St. Ptersburg, Florida, 1980
114 pp. + 3 appendices

This book represents an extremely basic and superficial coverage of HPLC. It will provide an elementary introduction for the novice to this technique who has had no previous experience. The chapters, which are well written as far as they go, include

Introduction, which briefly discusses what is HPLC and its various modes
Techniques of HPLC, where each mode is illustrated
Components of a Liquid Chromatograph
Principles of a Chromatographic Separation
Separation Mechanisms
HPLC Hardware Component Selection, covered in only 6 pages
Getting Started
Quality Control and Trouble Shooting
Qualitative and Quantitative Analysis of the Chromatogram
Safety

In most cases topics are treated in a page or two that normally require entire chapters to cover adequately in other books. Also, some of the less important topics are given an inordinate amount of space. The book is probably worth its $2.95 list price, but just barely.

JZ

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LC NEWS

CERTIFIED ANALYSIS TLC PLATES are offered for which the efficiency, resolution, and overall chromatographic performance are given for the specific plate. It is reported that this eliminates the plate itself as a variable in the TLC separation. Every significant parameter affecting performance is quantified on the Certificate, including physical characteristics, development data, conditions of development, R_f values, densitometry conditions, etc. Whatman, Inc., JLC/81/9, 9 Bridewell Place, Clifton, NJ, 07014, USA.

UV DETECTOR FOR HPLC provides variable wavelength control from 190 nm to 400nm. Flowcells are interchangeable for use in standard HPLC, low pressure LC, and microbore column LC methods. Can be operated at sensitivities as high as 0.01 AUFS. Kratos, Inc., JLC/81/9, 24 Booker Street, Westwood, NJ, 07675, USA.

CHROMATOGRAPHY CATALOG features new products such as FOXY, the smart fraction collector, a linear fraction collector, a memory module interface, and others. ISCO, Inc., JLC/81/9, P. O. Box 5347, Lincoln, NB, 68505, USA.

ANALYSIS OF URINARY INDOLES by HPLC-fluorescence is described in a recent applications note. Detection of catechols and indoles at sub-picomole levels is performed by fluorescence/HPLC. Elevated levels of these substances and their metabolites can be biochemical markers of disease states. Varian Instrument Group, JLC/81/9, 10060 Bubb Road, Cupertino, CA, 95014, USA.

CONTROL MODULE with optional data reduction and printer/plotter is available to remotely control HPLC equipment. Up to 10 different gradient programs can be stored in the memory simultaneously. Each program can control up to three pumps to generate $e^{1/5}$ to e^{5} for each one. Micromeritics, Inc., JLC/81/9, 5680 Goshen Springs Road, Norcross, GA, 30093, USA.

RADIOACTIVITY DETECTOR FOR LIQUID CHROMATOGRAPHY is a dual channel, microprocessor controlled device using either liquid or solid scintillation counting techniques. It is factory set to count Tritium and ^{14}C, but can be used to detect other isotopes such as ^{36}Cl, ^{32}P, and ^{125}I. It operates in real time by generating a histogram as peaks elute from the column and it eliminates scintillator vials by using an injected liquid scintillent and on-the-fly detection. The Anspec Co., Inc., JLC/81/9, P. O. Box 7044, AnnArbor, MI, 48107.

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LC CALENDAR

1981

July 13-17	Workshop: "Checking Foodstuffs for Trace Organics", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
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.
August 30-September 5	"XI Int'l Congress, IV European Congress of Clinical Chemistry", Vienna, Austria. Contact: 11th Int'l Congress of Clinical Chem., P. O. Box 105, A-1014 Wien, Austria.
September 7-10	"4th Int'l Bioanalytical Forum", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
September 10-12	Workshop: "Some Approaches to the Anal. of Biological Specimens", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
September 15-19	Workshop: "Introduction to Determination of Drugs in Biological Fluids", Guildford, U.K. Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Inst., Univ. of Surrey, Guildford, GU2 5XH, U.K.
September 20-25	"8th Annual FACSS Meeting", Philadelphia, PA USA. Contact: R. A. Barford, USDA, 600 E. Mermaid Lane, Philadelphia, PA 19118, USA.
September 28-October 1	"Chromatography-81: Int'l. Symposium on Advances in Chromatography" Barcelona, Spain. Contact: Dr. A. Zlatkis, Chem. Dept., University of Houston, Houston, TX 77004, USA.
September 30-October 2	Introduction to Liquid Chromatography, Santa Clara, CA, USA, Contact: G. Gilfillan, Hewlett-Packard Co., 1501 Page Mill Road, Palo Alto, CA 94304, 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 4-7 — ASTM-E19 Committee Meeting, Bar Harbor Hotel, San Diego, CA, USA. Contact: ASTM, 1916 Race St., Philadelphia, PA 19103, USA.

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

October 22-23 — LC/MS Workshop, sponsored by International Assoc. of Environmental Analytical Chem., Palais des Congres, Montreux, Switzerland. Contact: Prof. R. W. Frei, Free University, De Boelelaan 1083, 1018WV Amsterdam, The Netherlands.

October 27-29 — "Petroanalysis-81", Cumberland Hotel, Marble Arch, London, England Contact: Miss I.A.McCann, Inst. of Petroleum, 61 New Cavendish St., London, W1M 8AR, England

November 16-17 — International Symposium on HPLC of Proteins & Peptides, Washington, D.C. Contact: S. E. Schlessinger, Int'l. Symp. on HPLC of Proteins & Peptides, 400 E. Randolph, Chicago, IL 60601, 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.

November 23-25 — 2nd International Congress on Analytical Techniques in Environmental Chemistry, Barcelona, Spain. Contact: Expoquimia, Plaza de Espana, Barcelona-4, Spain.

November 26-27 — Workshop: Chem. & Anal. of Hydrocarbons in the Environment, Barcelona, Spain. Contact: J. Albaiges, Expochimia, Plaza de Espana, Barcelona-4, Spain.

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.

April 14-16 — "12th Annual Symposium on the Anal. Chem. of Pollutants", Amsterdam, The Netherlands. Contact: Prof. R. W. Frei, Congress Office, Vrije Universiteit, P. O. Box 7161, 1007-MC Amsterdam, The Netherlands.

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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May, 1981
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