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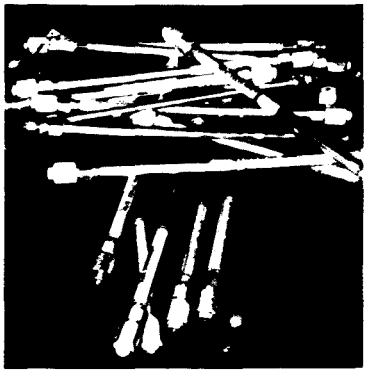

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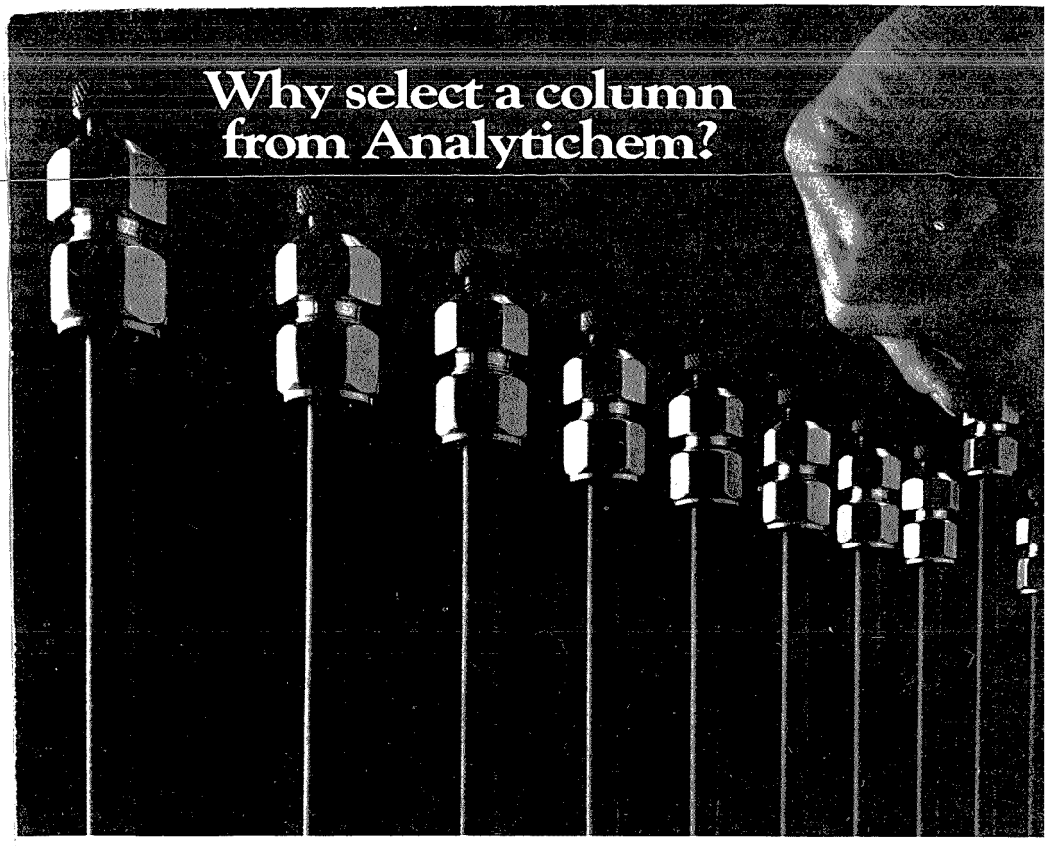

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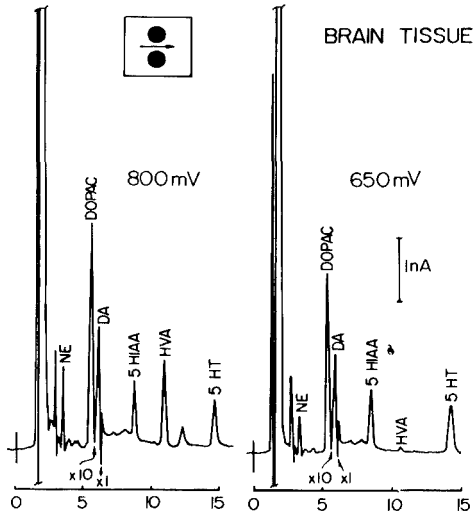

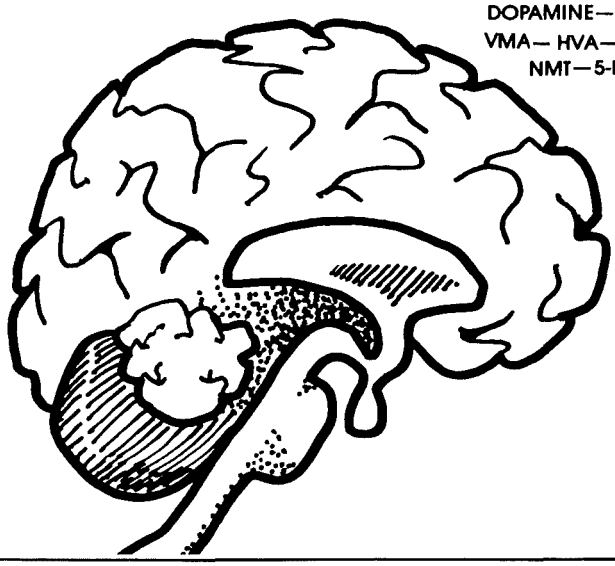

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POLYVINYLACETATE GEL PERMEATION CHROMATOGRAPHY OF H-COAL LIQUIDS AND MODEL COMPOUNDS. COMPARISON WITH RIGID GEL, REVERSED PHASE AND NORMAL PHASE CHROMATOGRAPHY

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ABSTRACT

This paper reports the application of polyvinylacetate gel (Fractogel) for size fractionation of the H-Coal liquids and model aromatic hydrocarbons. The four H-Coal liquids were each divided into three classes according to solubility in hexane, benzene, and pyridine. A model mixture of eight compounds when applied to the Fractogel column yielded four fractions; a similar performance was given by a prepacked, Toyo Soda microparticulate column containing styrene-divinylbenzene copolymer. Regarding the twelve H-Coal solvent fractions, the chromatograms obtained from the Fractogel column were analogous to those from the Toyo Soda column. By making further use of model compounds, the Fractogel results are compared with those obtained from reversed phase chromatography on a Partisil ODS column and those from normal phase chromatography on a LiChrosorb silica column. These comparisons reveal the usefulness in separating certain aromatic hydrocarbons by the Fractogel column. Furthermore, judging from the chromatograms of a hexane-soluble H-Coal fraction obtained by the reversed phase and normal phase methods, the ODS column will complement the Fractogel column in fractionating the H-Coal liquids.

INTRODUCTION

The liquefaction of coal to produce conventional refinery products represents a better use of the solid fuel. The H-Coal process is a coal dissolution process carried out in an ebullated

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bed reactor in the presence of hydrogen and a desulfurization and hydrogenation catalyst. The crosslinks in the coal particles, whether they are methylene, oxygen, sulfur, or nitrogen bridges, undergo thermal homolysis. Upon transfer of hydrogen to the radical sites, depolymerized coal units are formed which are separated into four product streams: atmospheric still overhead (ASO), atmospheric still bottom (ASB), vacuum still overhead (VSO), and vacuum still bottom (VSB). We have been involved in the characterization of these products (1), and have reported a structural study of the H-Coal liquids by the FT-NMR method (2) as well as a combined use of gel permeation chromatography (GPC) and vapor pressure osmometry for fractionation and molecular weight information (3). Although Anbar et al. (4) have shown by field ionization mass spectra of the H-Coal liquids that their molecular weights range from about 100-600, the applicability of gas or liquid chromatography for their complete analysis is doubtful because of the residue problem. Some of these liquids contain polymeric compounds which tend to be retained on the column. In this regard, GPC comes close to being a total chromatographic method for the coal liquids. There are several reports on GPC separation of various coal-derived liquids (5,6,7). The column packing most commonly employed is a styrene-divinylbenzene cross-linked polymer. It is used more often in the form of a rigid gel as found in the microparticulate, prepacked columns such as the μStyragel of Waters Associates (5) and the Toyo Soda TSK column (6) from Japan and distributed by Varian Associates. A soft gel such as the Bio-Rad Bio-Beads is also used in homemade, slurry-packed columns (7). This polystyrene copolymer, either as the rigid or the soft gel, has its limitations for analyzing coal liquids. For example, the prepacked column is costly, has low sample loading capacity, and subject to clogging by sample precipitation. The Bio-Beads gel is more economical but has been found to exhibit a non-size exclusion mechanism of separation (7), making it difficult to interpret the chromatogram of a highly heterogeneous coal liquid. We have been studying vinyl acetate

copolymer, the Merck Fractogel PVA 500, as column packing material for the analysis of the H-Coal liquids. We have found it useful both analytically and preparatively and have compared its performance with that obtained on the Toyo Soda TSK G-2000H_{10} column. By means of model hydrocarbons, the Fractogel separation has been compared with data obtained from chromatography on a reversed-phase octadecylsilyl (ODS) column and a normal phase silica gel column. The latter two methods have also been applied to a H-Coal liquid. In these separations, the Fractogel and ODS columns are complementary to each other.

MATERIALS AND METHODS

The H-Coal liquids were received from the University of Kentucky Institute for Mining and Minerals Research. The coal liquefaction was performed by the H-Coal process in the syncrude mode with reactor temperature at 450°C, exit reactor partial pressure of hydrogen at 2245 psig. The nominal boiling ranges of the liquids are: ASO C_4-200°C, ASB 200-350°C, VSO 350-520°C, and VSB is a chunky tar.

The instrument for chromatography consists of a M6000A pump, U6K injector and model 440 absorbance detector operating at 254 nm for analytical and 313 nm for preparative run. These components are products of Waters Associates, Milford, Mass. The Fractogel PVA 500, 32-63 μ, was purchased from MCB, Inc., Ohio. This gel was slurry-packed to 800 psig into a 610 mm x 7.8 mm stainless steel column; two columns were joined in series. It was eluted at 1 mL/min with chloroform-methanol 3:1. The rigid gel column, TSK G-2000H_{10}, 610 mm x 8 mm containing 10 μ particles, was purchased prepacked from Toyo Soda of Japan. The mobile phase for the TSK was straight chloroform at a flow rate of 1 mL/min. The theoretical plate count of the Fractogel column set was about 1500, while that of the TSK column was about ten times greater, as determined by injecting a 2% acetone solution. The samples for injection were prepared in the same solvent as the mobile phase used. The concentrations for the model hydrocarbons were 1 -10 x

10^{-3} M. For the H-Coal liquids, solutions of about 15 mg/mL were prepared for analytical runs, and about 20 times that for preparative separation.

For the reversed-phase column, Whatman Partisil ODS was packed into a 250 mm x 4.6 mm stainless steel column. It was eluted with acetonitrile-water 1:1 at 3 mL/min. The same kind of column was packed with Merck LiChrosorb Si60 for normal phase chromatography. It was eluted with n-hexane at 0.4 mL/min. Both columns contained 10 μ particles and showed a plate count of about 5000.

RESULTS AND DISCUSSION

Since application of the Fractogel polyvinylacetate column in coal liquid analysis was rather new, an attempt was made to understand the way by which the coal liquid components can be fractionated. For this purpose, eight model aromatic hydrocarbons were injected into the Fractogel column individually and also as mixtures. Their retention volumes are shown in Table 1 which are also compared with those obtained from the Toyo Soda column. As the Fractogel column is twice the length of the Toyo Soda column, the retention volumes from the Fractogel are about two times as large as those from the Toyo Soda. For the mixture of eight compounds, the Fractogel chromatogram as shown in Figure 1 reveals four bands: band 1 contains phenyltridecane, band 2 1,2-diphenylethane and triptycene, band 3 biphenyl and 1,5-dimethylnaphthalene, and band 4 contains benzene, naphthalene, and phenanthrene. The separation of this mixture on the Toyo Soda column is similar (see Figure 2), the main difference being that the second band in the Fractogel chromatogram is herein resolved into two peaks, with triptycene eluting first followed by 1,2-diphenylethane. The clean-cut resolution of phenyltridecane (MW 260) from trypticene (MW 254), a polycyclic, suggests that the separation is based on the length of the molecules. Likewise, 1,2-diphenylethane (MW 182) and phenanthrene (MW 178) are well separated based on a difference in the molecular length.

TABLE 1

Retention Volumes of Eight Model Aromatic Hydrocarbons from the Fractogel and Toyo Soda Column

Model Compounds	Molecular Weight	Retention Volume, mL	
		Fractogel	Toyo Soda
Phenyltridecane	260	21.3	13.0
Triptycene	254	26.7	14.2
1,2-Diphenylethane	182	27.7	14.6
1,5-Dimethylnaphthalene	156	31.0	15.8
Biphenyl	154	32.2	16.0
Phenanthrene	178	35.6	16.8
Naphthalene	128	35.8	17.0
Benzene	78	36.5	17.1

Hence, the lack of separation between 1,5-dimethylnaphthalene (MW 156) and biphenyl (MW 154) which are similar in length is not surprising. However, it is unexpected that the parent aromatic compounds of one, two and three rings can neither be resolved on the Fractogel nor on the Toyo Soda, the latter having about 10 times the theoretical plates of the former. The useful molecular weight range for the Fractogel column is about 100-600, while that of the Toyo Soda is about 100-2000. Phananthrene (MW 178) is not approaching the total permeation limit of either column and should be resolvable from benzene. Nevertheless, this does not detract from applying the Fractogel column in the fractionation of the H-Coal liquids. The pattern of separation is established in that the aromatic compound with long side chains will elute first while the unsubstituted will lump together and elute last.

It has been customary to classify coal liquids according to solubility (8): hexane soluble fraction (HSF) contains the oils, hexane-insoluble, benzene soluble fraction (BSF) contains the asphaltenes and benzene-insoluble, pyridine soluble fraction (PSF) contains the asphaltols. The molecular weights increase in this

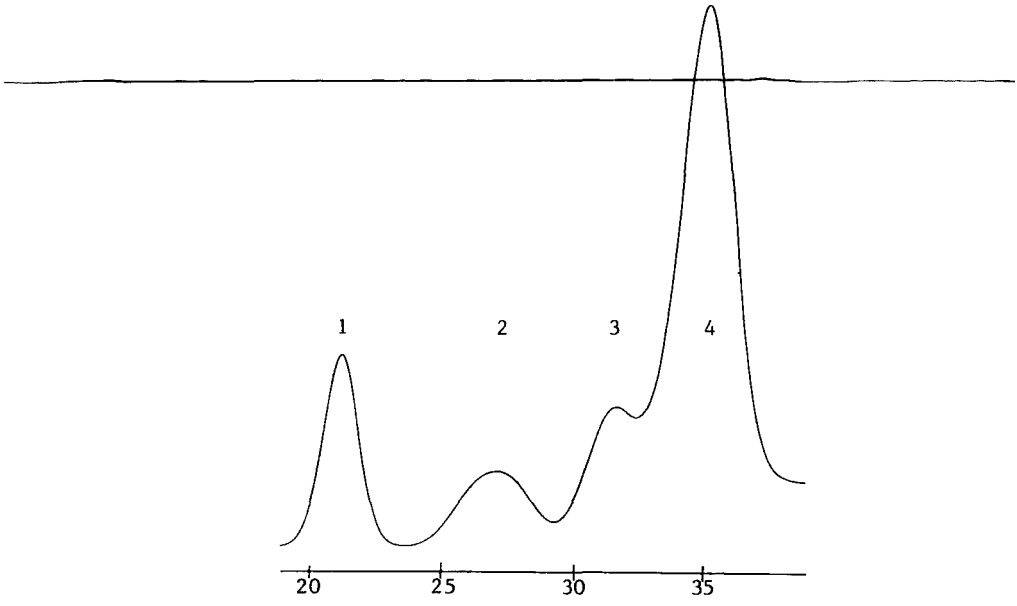

FIGURE 1. Separation of a mixture of eight model compounds as shown in Table 1 on a Fractogel column.

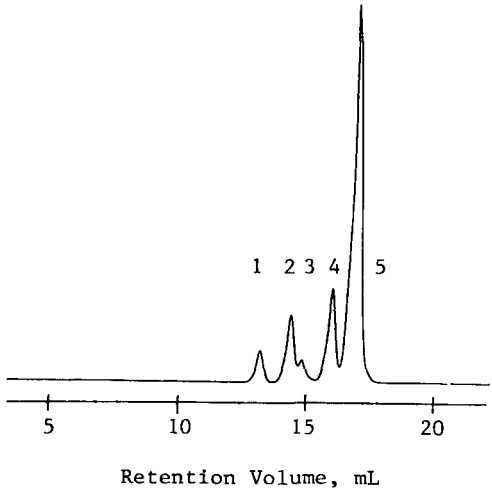

FIGURE 2. Separation of a mixture of eight model compounds as shown in Table 1 on a Toyo Soda Column.

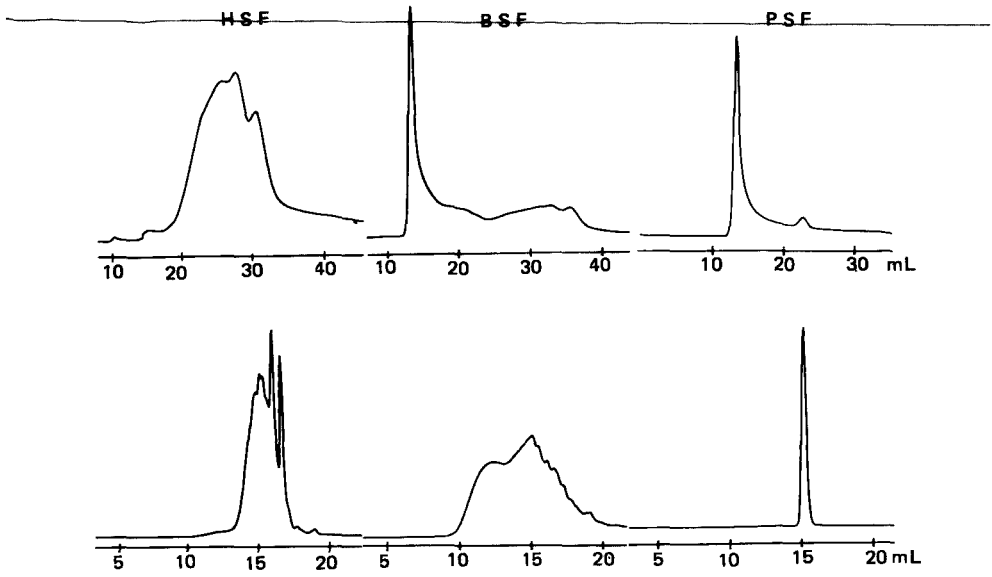

FIGURE 3. Gel Permeation chromatograms for the 3 ASO subfractions on Fractogel (top) and Toyo Soda column (bottom); retention volumes are shown.

order to about 1000 for most current liquefaction products. The four H-Coal liquids were solvent-fractionated accordingly, the HSF fractions of ASO, ASB, and VSO were more than 92 percent of the original mixture, while that of the VSB contained about 7 percent. The BSF ranged from 1 to 5 percent of the first three H-Coal liquids but amounted to about 50 percent of the VSB. The PSF was from 0.5 to 4 percent for the first three H-Coal liquids but was 42 percent of the VSB. These twelve solvent fractions were chromatographed on the Fractogel column set as well as on the Toyo Soda column for comparison. Figures 3, 4, 5, and 6 show the chromatograms for the three solvent fractions of ASO, ASB, VSO, and VSB, respectively, the top figures are the Fractogel chromatograms while the bottom are the Toyo Soda. Insofar as the HSF fractions are concerned, the Fractogel chromatograms show less well-resolved peaks than the Toyo Soda chromatograms. However,

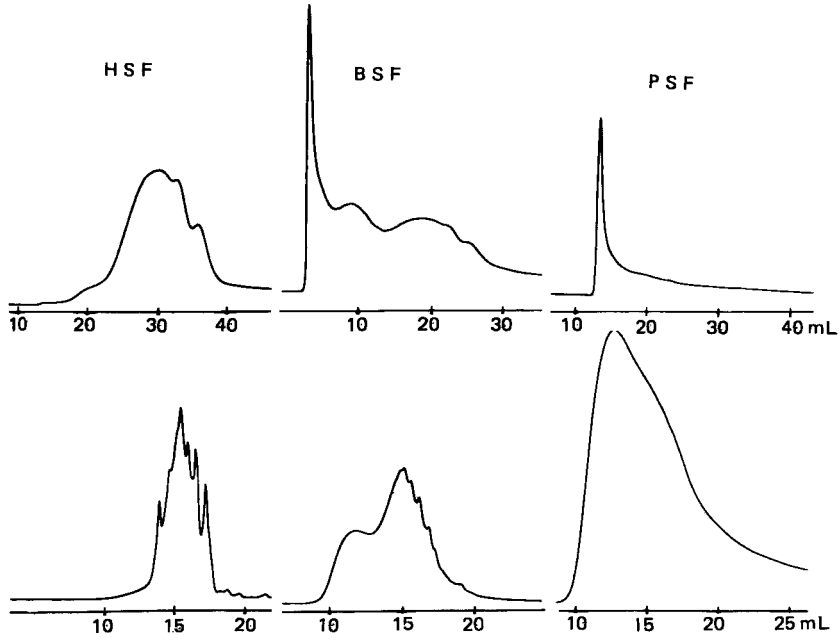

FIGURE 4. Gel permeation chromatograms for the 3 ASB subfractions on Fractogel (top) and Toyo Soda column (bottom); retention volumes are shown.

the bulk of their retention volumes are defined by the eight model compounds as shown in Table 1 for both the Fractogel and the Toyo Soda column. It appears that the phenyltridecane type is the largest size while benzene the smallest of the HSF components. In the case of the BSF and PSF fractions, the Fractogel chromatograms show a sharp peak at about 14 mL. Since the mobile phase contains 25 percent methanol in chloroform, the peak at 14 mL can be attributed to enlarged molecules resulting from solute-solvent interaction (5). It is plausible that the likes of phenolic groups and pyridine nitrogen in these fractions may form polymer-like molecules with methanol through hydrogen bonding. The absence of such a peak on the Toyo Soda chromatograms can be explained in terms of the exclusion limit of the Toyo Soda column

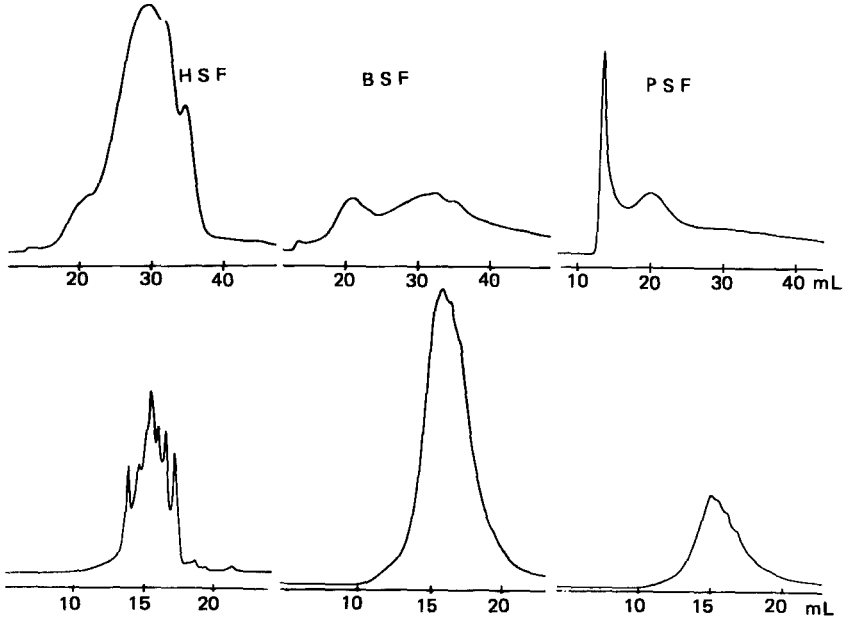

FIGURE 5. Gel permeation chromatograms for the 3 VSO subfractions on Fractogel (top) and Toyo Soda column (bottom); retention volumes are shown.

being three times as much as the Fractogel and that it was eluted with neat chloroform. Otherwise, the BSF and PSF chromatograms tend to show more distributions of larger molecules than the HSF, their separations being similar on both columns. The fine coal ash entrapped in the VSB fractions may account for the broad envelopes in all of the VSB chromatograms. Calibrations of these chromatograms to generate molecular weight information were carried out via preparative runs of the HSF on the Fractogel column. The fractions were subjected to vapor pressure osmometry for the determination of molecular weight. The latter, ranging from 627 for the exclusion fraction to 115 for the fraction at 32 mL, were plotted against the retention volumes of the collected fractions to generate the calibration curves. The molecular

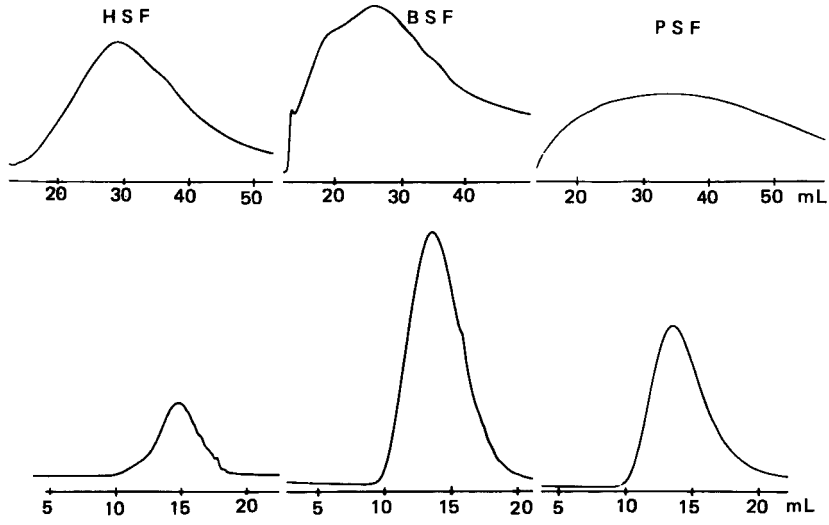

FIGURE 6. Gel permeation chromatograms for the 3 VSB subfractions on Fractogel (top) and Toyo Soda column (bottom); retention volumes are shown.

weight distributions of the H-Coal liquids have been reported (3), the number-average molecular weight distributions being in the range of 200-500 for the three solvent fractions. Since the Fractogel column can be loaded with about 20 mg of the coal liquid per injection to yield a chromatogram similar to that provided by the Toyo Soda column, and considering the low price and ease of packing of the gel, the Fractogel column has shown versatile use as both a preparative and an analytical column for the H-Coal liquids.

In order to determine the scope of separation on the Fractogel column, Table 2 shows the performance in separating aromatic compounds by means of gel permeation chromatography (GPC), reversed phase chromatography (RPC), and normal phase chromatography (NPC), using the Fractogel, Partisil ODS, and LiChrosorb Si60 column, respectively. The Fractogel polyvinyl acetate column apparently separates by size exclusion, the

TABLE 2

Comparison of Separation of Aromatic Compounds by GPC[1], RPC[2], and NPC[3]

Aromatic Compounds	Retention Volume, mL GPC[1]	RPC[2]	NPC[3]
A. Benzene + 3 carbons			
1,2,3-trimethylbenzene	31.2	9.9	2.5
1,2,4-trimethylbenzene	33.0	11.5	2.5
n-propylbenzene	34.2	10.5	2.5
B. Benzene + 4 carbons			
durene	29.8	13.0	2.5
sec-butylbenzene	33.5	13.0	2.3
C. Benzene + 6 carbons			
1,3,5-triethylbenzene	24.3	21.0	2.3
hexamethylbenzene	29.4	31.0	2.4
D. Diaromatic with 12 carbons			
1,3-dimethylnaphthalene	33.4	14.8	2.4
1,5-dimethylnaphthalene	34.7	14.0	2.5
1,4-dimethylnaphthalene	35.0	14.3	2.5
1,8-dimethylnaphthalene	35.6	13.4	2.5
biphenyl	36.3	11.0	2.9
acenaphthylene	39.6	9.8	3.2

[1]Fractogel column eluted with chloroform-methanol 3:1

[2]Partisil ODS column eluted with acetonitrile-water 1:1

[3]LiChrosorb Si60 column eluted with n-hexane

Partisil ODS by reversed phase principle involving partition, adsorption, and surface tension (9), and the LiChrosorb silica gel column by adsorption principle. In terms of separating group A compounds in Table 2 which contain a benzene ring plus three carbons, GPC works as well as reversed phase, both of which are far superior to the normal phase chromatography. Likewise, for group C where compounds contain a benzene ring plus six carbons,

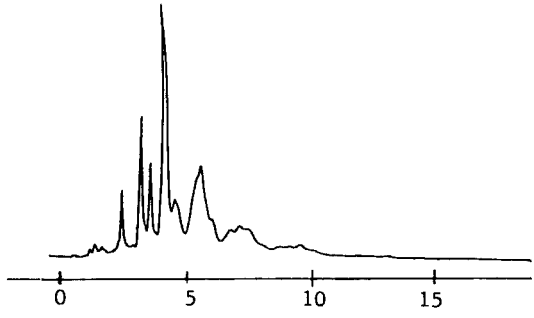

FIGURE 7. Reversed phase chromatogram of HSF of ASO on a Partisil ODS column.

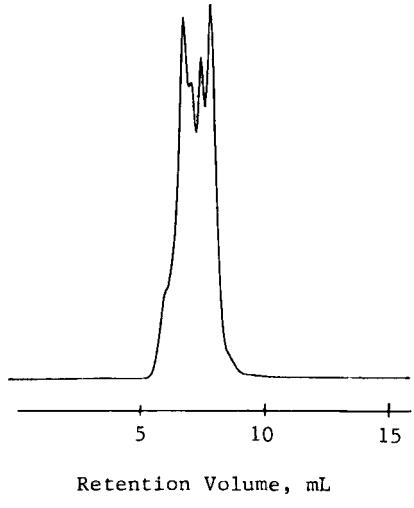

FIGURE 8. Normal phase chromatogram of HSF of ASO on a LiChrosorb Si60 column.

GPC and RPC are just as efficient while NPC is inadequate. For the two group B compounds, benzene plus four carbons, GPC appears to be superior to both RPC and NPC. The last group of compounds are diaromatics containing 12 carbons. In this case, reversed phase chromatography appears to be the best in separating them. Judging from these sets of model compound separations, it would seem that either GPC can be used alone to fractionate H-Coal liquids or that it can be used in combination with the reversed phase method. Figures 7 and 8 show the hexane soluble fraction of ASO when chromatographed by the reversed phase and the normal phase methods, respectively. The chromatogram obtained from the reversed phase ODS column shows a much better spread of the HSF than the silica, hence the ODS will complement the Fractogel column in fractionating the H-Coal liquids.

REFERENCES

1. This is part of a series of reports on the physicochemical properties of liquids derived from Kentucky coal. The research was supported by the Kentucky Institute of Mining and Minerals Research from 1977-1980. The chromatograms in this paper were run by Drs. C. M. Gladstone and M. M. Khan who worked on the project as research associates.

2. (a) Joseph, J. T. and Wong, J. L., Determination of Protonated and Quaternary Carbons in H-Coal Liquids from Quaternary Carbon Suppressed and Nonsuppressed CMR Spectra, Amer. Chem. Soc. Fuel Chem. Div. Preprints, 24, 317, 1979; (b) Joseph, J. T. and Wong, J. L., Fuel, 59, 777, 1980.

3. (a) Wong, J. L., Gladstone, C. M., and Joseph, J. T., Characterization of H-Coal Liquids by FT-NMR Spectroscopy and High Pressure Gel Permeation Chromatography, CHEMRAWN 1, Toronto, Abst. P-19, 1978; (b) Wong, J. L. and Khan, M. M., Gel Permeation Chromatography of H-Coal Liquids, Coal Symposium, Kentucky Academy of Sciences Meeting, 1979; (c) Khan, M. M., Molecular Weight Distributions and Size Fractionations of H-Coal Liquids, Fuel, 61, 553, 1982; (d) Wong, J. L. and Gladstone, C. M., Size Exclusion Chromatography of H-Coal Liquids on Silica, Polyvinylacetate and Polystyrene Columns, Fuel, 62, 000, 1983.

4. Anbar, M. and St. John, G. A., Characterization of Coal-Liquefaction Products by Molecular-Weight Profiles Produced by Field-Ionization Mass Spectrometry, Fuel, 57, 105, 1978.

5. Philip, C. V. and Anthony, R. G., Separation of Coal-Derived Liquids by Gel Permeation Chromatography, Fuel, 61, 357, 1982, and pertinent references cited.

6. Brown, R. S., Hausler, D. W., and Taylor, L. T., Gel Permeation Chromatography of Coal-Derived Products with On-Line Infrared Detection, Anal. Chem., 52, 1511, 1980.

7. Hausler, D. W., Hillgeth, J. W., McNair, H. M., and Taylor, L. T., Size Exclusion Chromatography for the Separation of Whole Coal Liquids, J. Chromatogr. Sci., 17, 617, 1979, and pertinent references cited.

8. (a) Farcasiu, M., Mitchell, T. O., and Whitehurst, D. D., Asphaltols--Keys to Coal Liquefaction, Chem. Tech., 7, 680, 1977; (b) Farcasiu, M., Fractionation and Structural Characterization of Coal Liquids, Fuel, 56, 9, 1977.

9. Horvath, C. and Melander, W., Liquid Chromatography with Hydrocarbonaceous Bonded Phases, J. Chromatogr. Sci., 15, 393, 1977.

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APPLICATION OF FLOW PROGRAMMING IN THE ANALYSIS OF DRUGS AND THEIR METABOLITES IN BIOLOGICAL FLUIDS

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ABSTRACT

An HPLC procedure using flow programming under isocratic elution conditions for determination of drugs and their metabolites in biological fluids is discussed. This technique was used in the analysis of triamterene and its metabolites in urine, chlorothiazide in plasma and hydrochlorothiazide in urine. Advantages of flow programming over conventional procedures such as isocratic elution and gradient elution are discussed.

INTRODUCTION

HPLC analysis employing isocratic elution conditions is the most common procedure used for the analysis of drugs and their metabolites in biological samples. Occasionally, a gradient elution technique is employed for complex samples that consist

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of components with a wide range of distribution coefficients. Gradient elution refers to the technique of increasing the solvent strength as the separation proceeds. It is used to decrease analysis time and optimize separations with respect to time and resolution. Flow programming, which involves changes in flow rate under isocratic conditions, even though known in principle, is not widely used. Application of flow programming under isocratic conditions for separation of drugs and their metabolites in biological fluids is briefly discussed here. The biological samples used were part of in-vivo bioavailability studies (1,2). The methods are described in brief, primarily to illustrate the use of flow programming.

EXPERIMENTAL

Chromatography was performed using Varian Model 5000 LC systems equipped with a Perkin-Elmer fluorescence detector or Varichrom variable-wavelength uv detector. Chromatographic separations were carried out using Varian MicroPak MCH type (reverse-phase) columns. All solvents used were HPLC grade. Biological samples used were part of in-vivo bioavailability studies (1,2).

Triamterene and triamterene sulfate in urine:

The urine sample was spiked with internal standard (3,5-dibromosalicylic acid) and injected directly onto an HPLC system equipped with a reverse-phase column and fluorescence detector.

The mobile phase consisted of 45% methanol in 0.1% aqueous potassium dihydrogen phosphate. The flow rate was increased from 1 ml/min to 3.5 ml/min over the first 10-minute period, and then lowered to 1.0 ml/min over the next 2-minute period. Under these conditions, the retention times for triamterene sulfate, 3,5-dibromosalicylic acid and triamterene were 2.8, 4.5 and 8.8 minutes, respectively.

Hydrochlorothiazide in urine:

The urine sample was spiked with internal standard (sulfadiazine) and injected onto an HPLC system equipped with a reverse-phase column and fixed-wavelength, 254 nm uv detector. The mobile phase consisted of 5% acetonitrile in 0.1% aqueous potassium dihydrogen phosphate. The flow rate was increased from 1 to 1.9 ml/min over the first 27 minutes and then lowered to 1 ml/min over the next 3 minutes. Under these conditions, the retention times for hydrochlorothiazide and sulfadiazine were 18 and 21 minutes, respectively.

Chlorothiazide in plasma

The plasma sample was spiked with internal standard (sulfathiazole) and mixed with 2 ml of acetonitrile. After centrifugation, the supernatant was separated, evaporated to dryness, reconstituted in 50 mcl of methanol, and 30 mcl was injected onto an HPLC system equipped with a reverse-phase column and a variable-wavelength uv detector set at 280 nm. The mobile phase was 4% methanol in 0.2% acetic acid. The flow rate

was increased from 1 ml to 3.4 ml/min over the first 21 minutes, then further increased to 5 ml/min over the next 2 minutes, and maintained at that flow rate for an additional 12 minutes. At the end of the run (35 minutes), the flow rate was returned to 1 ml/min. Under these conditions, the retention times for chlorothiazide and sulfathiazole were 13.5 and 26 minutes, respectively.

RESULTS AND DISCUSSION

In gradient elution, the column is subjected to the varying polarity of the eluent. This alters the behavior of the column, and at times, it does not return to its original condition at the beginning of the next sample analysis. This affects the reproducibility of the sample analysis. Flow programming under isocratic conditions, a new technique for separation of drugs and their metabolites in biological fluids, was developed to overcome some of the drawbacks of gradient elution. In flow programming, the flow of the isocratic solvent is increased (or decreased) over a period of time. Both the initial and final flow rates and the time interval are selected. The change in flow rate occurs linearly over the selected time interval. The column is always equilibrated with the solvent, thus giving more reproducible sample analysis.

It has been reported that for HPLC analysis of triamterene and its metabolites, the urine samples need to be analyzed once

for the parent drug and then a second time for the metabolites (3). Using flow programming, as described above, it has been possible to analyze for both triamterene and its major metabolite in a single injection.

Similarly, flow programming under isocratic conditions has been used successfully in the analysis of hydrochlorothiazide in urine and chlorothiazide in plasma.

Flow programming under isocratic conditions offers the following advantages:

a) drugs and metabolites which otherwise might require different analytical conditions and more than one sample injection can be analyzed in a single injection; b) the column is not exposed to different solvent conditions as in the gradient elution technique, and is thereby not subjected to 'shocks' during a run; c) the column is always equilibrated with the solvent and therefore no column regeneration is required; d) the chromatograms are more reproducible; e) the life of the column is extended; f) it is easy to operate; and g) it is suitable for use with all detectors, including refractive index detectors.

Thus, flow programming under isocratic conditions opens a new avenue for obtaining effective separation using HPLC.

REFERENCES

1. Shah, V.P., Walker, M.A., Prasad, V.K., Lin, J., Knapp, G. and Cabana, B.E. Abstract, Academy of Pharmaceutical Sciences, Am. Pharm. Asso., Washington, D.C., vol. 12, no. 2, p. 162 (1982).

2. Shah, V.P., Walker, M.A., Hunt, J.P., Prasad, V.K. and Cabana, B.E. Abstract, Academy of Pharmaceutical Sciences, Am. Pharm. Asso., Washington, D.C., vol. 11, no. 2, p. 98 (1981).

3. Lin, E.T., Sorgel, F., Hasegawa, J. and Benet, L.Z. Abstract, Academy of Pharmaceutical Sciences, Am. Pharm. Asso., Washington, D.C., vol. 10, no. 2, p. 120 (1980).

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THE EFFECT OF AMINE MODIFIERS ON THE CHROMATOGRAPHIC BEHAVIOR OF SALBUTAMOL ON REVERSED PHASE CHEMICALLY BONDED SILICA GEL

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ABSTRACT

The reversed phase chromatography of salbutamol in an aqueous phosphate buffer with quaternary and tertiary amine modifiers is described. The object was to control the retention and improve the asymmetry for use with electrochemical detection.

INTRODUCTION

Liquid chromatography with electrochemical detection is well suited to the separation and analysis of many bronchodilators in biological fluids. Electrochemical detection has the advantage of high sensitivity, a necessary requirement for this group of compounds. The mobile phase must be electrically conductive and an aqueous solvent without an organic modifier such as acetonitrile appears to provide improved sensitivity, lower background current and fewer problems.

However chromatographic peaks arising from compounds with a basic nitrogen are unsymmetrical and show extensive tails in reversed phase HPLC, particularly when the organic component of the solvent is present in low concentration (1).

Addition of organic amine compounds can improve the peak shape (1,2,3,4), and it may also be possible to make use of the concentration of the amine to adjust the retention of the compound of interest instead of adding an organic component such

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as methanol to the mobile phase. It is generally considered that tailing peaks arise from interactions between the solute and the adsorption sites on the silica matrix of the stationary phase. Apparently the organic amine preferentially occupies these active sites limiting the adsorption of solute. A wide range of amines have been used but this work examines the effects of tertiary and quaternary ammonium compounds on the reversed-phase chromatography of salbutamol using an aqueous mobile phase.

EXPERIMENTAL

Chemicals

Methanol was analytical grade (J. T. Baker, Phillipsburg, N.J. USA). Tetraethylammonium iodide (GPR, BDH), tetrapropylammonium iodide (Eastman Kodak), tetra-n-butylammonium iodide (GPR, BDH) and tetrapentylammonium iodide (Eastman Kodak) were converted into their hydroxides by precipitation of silver iodide with silver oxide and filtering. The iodide content was checked by measuring the absorbance at 254 nm. The quaternary ammonium hydroxide concentrations were confirmed by an acid-dye method (5). N,N-Dimethylhexylamine (DMHA), N,N-dimethyloctylamine (DMOA) and N,N-dimethyldecylamine (DMDA) were synthesized by a modified Eschwailer-Clarke reaction (6) and distilled. No impurities were found when examined by proton and carbon-13 NMR.

Salbutamol sulphate was of Pharmacopoeial grade and was supplied by Allen & Hanburys (Palmerston North, N.Z.).

All other substances were of analytical or reagent grade and used without purification.

Apparatus

A Waters Associates ALC/GPLC 244 liquid chromatograph with a Model 440 absorbance detector operating at 254 nm was used.

Column Packing Materials

The packing materials used were Zorbax CN (column I) and Zorbax ODS (columns II-V) (Du Pont Co. Wilmington, DE, USA), with mean particle diameters of 8 μm.

Column Tubing and Fittings

The columns consisted of 100mm x 4.6 mm i.d. or 150mm x 3.9mm i.d. (column V) stainless steel 316 tubing with a polished inner surface.

They were equipped with modified Swagelok compression fittings (#SS-400-6-1, Crawford Fitting Company, Cleveland, Ohio, USA). A 2µm removable frit (#716525, Alltech, Summer Hill, NSW, Australia) was placed at the outlet and an 8µm 316 stainless steel frit made from mesh (N. Greening Ltd, Warrington, U.K.) at the inlet. Teflon washers were inserted at each end.

Column Packing Technique

Columns were packed at 10,000 psi (except column I where the pressure was 2000 psi) using an upward slurry packing technique with the internal diameter of the reservoir being the same as the column. The slurry solvent was ethanol (95%)-n-propanol-toluene 1:1:1 (v/v) as described by Keller et al (7) for columns II-V and n-propanol for column I.

The quality of the columns was tested with anthracene and methanol-water (75/25) as eluent. The columns had a reduced plate height of less than 7.5 (8) and an asymmetry of less than 2.0 (except column I where the ASF was 4.0).

Asymmetry factors (ASF) were measured as follows: a perpendicular was drawn from the baseline to the vertex formed by the two peak tangent lines. A second line was drawn through the peak parallel to the baseline at 15% of the peak height. The ASF was calculated by dividing the length of the rear portion of this second line by the front part.

Chromatographic Conditions

Electrochemical detection of salbutamol tended to be more sensitive under acid conditions, however noisy traces attributed to the dissolution of iron (particularly from syringe needles) resulted. Thus ethylenediaminetetraacetic acid (10^{-4} M) was introduced to chelate any iron present and the optimum pH was

found to be 5.6. The solvent used in all experiments was sodium dihydrogen phosphate (0.1M) adjusted to pH 5.6 with sodium hydroxide (1M) plus 0.24 mmole of the appropriate amine (unless otherwise specified) per litre. The flow rate was 1.0 ml/min. Salbutamol (1mg/ml) dissolved in the mobile phase (less modifier) was injected in 10 ul amounts. Columns were taken as being equilibrated with the mobile phase when three replicate injections of salbutamol gave the same capacity factor (k'). The column void volume was estimated by an injection of sodium nitrate.

Procedures

a) Effect of tetra-n-butylammonium hydroxide (TBA) concentration on equilibration time and capacity factor.

The time taken for column (I) to equilibrate with the mobile phase was measured from the graph of the time of a salbutamol injection (the time was taken as zero when the mobile phase was switched from one without a modifier to one containing the appropriate concentration of TBA) versus the capacity factor of salbutamol for that injection. These plots showed a terminal straight line region (steady state conditions) and the time taken to reach this region was considered to be the equilibration time. The experiment was carried out with concentrations 0.03, 0.06, and 0.12 and 0.24 mM of TBA in the mobile phase and the equilibration time and capacity factor measured in each case.

b) Effect of amine modifiers on the k' & ASF of salbutamol.

Column V was equilibrated with mobile phase containing 0.12 mM of the appropriate amine and the capacity and asymmetry factors measured. The amines used were tetraethylammonium hydroxide (TEA), tetrapropylammonium hydroxide (TPrA), tetra-n-butyl ammonium hydroxide (TBA), tetrapentylammonium hydroxide (TPeA), dimethylhexylamine (DMHA), dimethyloctylamine (DMOA) and dimethyldecylamine (DMDA).

c) Adsorption of quaternary amine modifiers.

Three Zorbax ODS columns (II, III, V) were each equilibrated with a mobile phase containing the appropriate modifiers (TEA,

TPrA, TBA, TPeA). Methanol (100 ml) was then passed through (9) and collected. The methanol was evaporated and the residue dissolved in distilled water (10 ml). The concentration was measured by the method of Chatten and Okamura, using procedure c (5).

The concentration of amine modifier in the mobile phase used with column (V) was 0.12 mM. Columns (II) and (III) used a concentration of 0.24 mM.

Columns (III) and (V) had not been used with an amine modifier before whereas column (II) had been in use for two weeks with a quaternary ammonium salt.

After completion of these experiments, the equivalent amount of distilled water to two weeks use (6 litres) was passed through column (III). This column was then re-equilibrated with mobile phase containing 0.24 mM TBA and the amount of quaternary ammonium ion adsorbed was again estimated.

d) Adsorption of tertiary amine modifiers.

Column (V) was equilibrated with mobile phase containing 0.24mM of a tertiary amine modifier (DMHA, DMOA, DMDA), the modifier was eluted with methanol and an aqueous solution (200 ml) prepared as described for the quaternary amine modifiers.

The amount of amine absorbed in column (V) was measured by potentiometric titration which involved titrating a 10 ml sample with 1.0 mM hydrochloric acid and determining the end point with a pH meter (about 5.5).

The total amount of amine (amine + amine salt) was measured by gas chromatography using a 3 foot column of 10% carbowax 20M and 2% potassium hydroxide on Gas Chrom Q (Applied Science Laboratories, Inglewood, CA, USA), with the appropriate standards (ca 0.1 µg/ml).

e) Estimation of free silanol groups.

The method used is that described by Karch et al (11) and Tanaka et al (10). Column (IV) was new and had not been used with organic modifiers, column (III) was also new and had not been used with organic modifiers (except for procedure c), but

had the equivalent of two weeks use of distilled water passed through (6 litres). Column (II) had been well used with amine modifiers, as shown in table 3 in the extent to which the quaternary ammonium salts adsorbed.

RESULTS AND DISCUSSION

Although reversed phase coated silica is not quite so susceptible to attack by alkali as silica, quaternary ammonium compounds such as TBA can render a column useless within days (12). Hence it is desirable to maintain low TBA concentrations. However, at low solvent quaternary ammonium concentrations the time taken for the column to equilibrate with the solvent increases. This is shown in Table 1 along with the capacity factor for salbutamol after the column has equilibrated. The modifier TBA controls the retention and concentrations as low as 0.03 mM TBA are effective in improving the chromatography of salbutamol in aqueous solutions.

The effect of a series of quaternary ammonium compounds and tertiary amines on the asymmetry (ASF) and the capacity factors of salbutamol are shown in table 2.

Sokolowski and Wahlund (1) found that TBA and TPrA did not improve the ASF of a series of tricyclic antidepressants in 1:1 methanol, phosphate buffer pH 2-3.3 with 0.05M additive and Tilley-Melin et al (13) state that the adsorption sites for quaternary ammonium ions are not easily accessible to cations with bulky substituents such as TBA.

Our results (table 2) show that both TBA and TPrA do improve the ASF and there is an apparent trend towards the cations with bulky substituents showing the greatest improvement. An explanation for this apparently contradictory result is that the bulky quaternary ammonium ions favour the mobile phase when the methanol content is high and insufficient coverage of the active site occurs. In aqueous solvents hydrophobic bonding can be significant (14) and the greater the hydrophobicity of the quaternary ammonium ion, the higher the concentration in the

TABLE 1

The time taken for Column (I) to Equilibrate with the Mobile Phase containing Various Concentrations of TBA

TBA Concentration (mM)	Equilibration Time (min)	Capacity Factor
0	-	7.0
0.03	88	1.33
0.06	62	0.84
0.12	38	0.38
0.24	26	0.22

TABLE 2

The Effect of a Series of Amine Modifiers on the Retention and Asymmetry of Salbutamol

Modifier (0.12mM)	Capacity Factor	Asymmetry
None	86	7.6
TEA	47	3.2
TPrA	16	1.7
TBA	1.2	2.0
TPeA	1.0	2.0
DMHA	22	double peak
DMOA	2.6	2.2
DMDA	-0.6	1.7

stationary phase. The decreased retention is probably due to the stationary phase taking on cation exchange character.

Excessive tailing is caused by interaction with silanol groups on the stationary phase and it is likely that certain sites affect salbutamol more than others depending on their accessibility. Thus the modifier which has most effect would be the one which blocks those sites which affect salbutamol greatest. This would suggest that TPrA is effective at blocking those sites which cause tailing of salbutamol.

Peak tailing has been described by a two site theory (15) and in the case of reversed phase chromatography the second site is considered to be unprotected silanol groups (10). Under certain conditions the two sites can give rise to two peaks (16). DMHA is ineffective at covering sites active to salbutamol, presumably because either it has insufficient hydrophobic character and the concentration in the stationary phase is too low or it does not remain on the sites active to salbutamol for long enough.

Negative capacity factors as with DMDA have been explained as charge exclusion phenomena (17). When DMDA is adsorbed as the hydrogen phosphate the pores become electrically charged and tend to exclude similarly charged molecules making a portion of the solvent void volume inaccessible to salbutamol hydrogen phosphate.

The amounts of quaternary ammonium and tertiary amine adsorbed on reversed phase columns are shown in Table 3 and 4. As expected (18) the amount of quaternary ammonium ion adsorbed is greater with increasing hydrophobic chracter. However, if the concentration of quaternary ammonium ion in the mobile phase is halved the amount adsorbed does not change significantly (column (V) cf column (III)).

Iler (14), and Bijsterbosch et al (19) proposed that the mechanism of adsorption of the long chain aliphatic hydrocarbon amines to silica involves hydrophobic bonding. At low concentrations a single hydrophobic layer is adsorbed with the

TABLE 3

The Quantity of Quaternary Amine Modifier Adsorbed on Three Reversed Phase Columns

Quaternary Amine	Amount Adsorbed (mole x 10^{-5})		
	Column		
	III	V	II
TEA	0.03	0.17	0.51
TPrA	0.47	0.44	1.18
TBA	2.17	2.06	6.74
*TBA	3.89	-	-
TPeA	5.39	4.97	8.26

*This value was re-estimated after six litres of distilled water had been passed through Column (III).

nitrogen close to the silica surface and at higher concentrations a double layer forms. The second layer has the reverse orientation with the apolar chain towards the surface of the silica and hence the amine group has an associated counter ion.

The function of amine modifiers on reversed phase columns is to block sites that contribute to separation mechanisms other than liquid-liquid partition. Thus ideally a monolayer coverage with all sites occupied is required. If a bilayer is formed (with the amine group in the mobile phase), ion exchange as a separation mechanism would predominate and the separation characteristics of the column would change completely.

The adsorbed amine is eluted and measured by potentiometric titration. This gives an estimate of the proportion of monolayer formed (the amine contributing the double layer would be eluted as its phosphate salt and is not measured). Gas chromatography measures the total amine eluted and these results are shown in Table 4. The proportion of bilayer gives an estimate of the degree of ion-exchange character.

TABLE 4

The Extent and Type of Adsorption of Tertiary Amine Modifiers.

Tertiary Amine (0.24 mM)	Amount Absorbed		
	Total (mole x 10^{-5})	Monolayer (mole x 10^{-5})	Bilayer (percent of monolayer)
DMHA	3.49	3.58	0
DMOA	9.04	7.15	26
DMDA	24.9	17.2	45

Reversed phase packing materials are quite unstable towards quaternary ammonium compounds but less so towards the tertiary amines (1, 12). The amount of TBA adsorbed by the stationary phase was measured on a column previously unused (III) and a column used with a quaternary ammonium compounds in the mobile phase for two weeks (table 3, Column (II)). This column did not show a significant change in ASF or capacity factor for salbutamol when compared with the unused column. However, there is a considerable increase in the quantity of quaternary ammonium ions adsorbed for all the modifiers. Wehrli et al (12) have shown that the mechanism of breakdown of the column occurs by cleavage of the organic layer from the silicate particles and collapse of the silicate structure and thus presumably there would be more active sites for the quaternary ammonium ion. Water also hydrolyses silica and after the studies with the quaternary ammonium compounds, column (III) had six litres of water (the equivalent to two weeks use) passed through. The amount of TBA adsorbed increased but not to the same extent as column (II).

A method of estimating the polarity of a column, resulting from unshielded silanol groups is to measure the retention of small polar molecules in dry heptane (10,11). The more polar the column (i.e. the greater the number of available

TABLE 5

A Comparison of Three Columns for Free Silanol Groups

Column	State	Capacity Factor	
		Anisole	Methylbenzoate
IV	new	1.34	7.8
III	washed with water (61)	1.38	9.5
II	well used with amine modifiers	2.10	12.0

silanol groups) the more the solute is retarded. For a reversed phase column with no silanol groups the capacity factor should be one. The results for an unused column, a column which had used water as the mobile phase and a column which had used TBA, phosphate buffer as mobile phase are shown in table 5. The results confirm that less polar sites result from the use of an aqueous mobile phase than one containing TBA.

In summary the quaternary ammonium ions and tertiary amines studied are effective in controlling the ASF and retention of salbutamol. However the quaternary ammonium ions hydrolyse the silica stationary phase and are adsorbed on the resulting silanol groups. As the hydrophobicity of the tertiary amines increases a second layer is formed which can function in an ion exchange mode.

REFERENCES

1. Sokolowski, A. and Wahlund, K. -G., Peak tailing and retention behaviour of tricyclic antidepressant amines and related hydrophobic ammonium compounds in reversed-phase ion-pair liquid chromatography on alkyl-bonded phases, J. Chromatogr., 189, 299 (1980).

2. Westerlund, D. and Erixson, E., Reversed-phase chromatography of zimelidine and similar dibasic amines, J. Chromatogr., 185, 593 (1979).

3. Wahlund, K. -G and Sokolowski, A., Reversed-phase ion-pair chromatography of antidepressive and neuroleptic amines and related quaternary ammonium compounds, J. Chromatogr., 151, 299 (1978).

4. Bij, K.E., Horvath, C., Melander, W.R. and Nahum, A, Surface silanols in silica-bonded hydrocarbonaceous stationary phases. II. Irregular retention behaviour and effect of silanol masking, J. Chromatogr., 203, 65 (1981).

5. Chatten, L.G. and Okamura, K.O., Assay of quaternary ammonium compounds in various dosage forms by acid-dye method, J. Pharm. Sci., 62, 1328 (1973).

6. Icke, R.N., Wisegarver, B.B. and Alles, G.A., β-Phenylethyldimethylamine, Org. Syntheses, 25, 89 (1945).

7. Keller, H.P., Erni, F., Linder, H.R. and Frei, R.W., Dynamic slurry-packing technique for liquid chromatography columns, Anal. Chem., 49 1958 (1977).

8. Bristow, P.A. and Knox, J.H., Standardization of test conditions for high performance liquid chromatography columns, Chromatographia, 10, 279 (1977).

9. Tilly-Melin, A., Askemark, Y., Wahlund, K. -G, and Schill, G., Retention behaviour of carboxylic acids and their quaternary ammonium ion pairs in reversed phase chromatography with acetonitrile as organic modifier in the mobile phase, Anal. Chem., 51, 976 (1979).

10. Tanaka, N., Goodell, H, and Karger, B.L., The role of organic modifiers on polar group selectivity in reversed-phase liquid chromatography, J. Chromatogr., 158, 233 (1978).

11. Karch, K., Sebestian, I. and Halasz, I., Preparation and properties of reversed phases, J. Chromatogr., 122, 3 (1976).

12. Wehrli, A., Hildenbrand, J.C., Keller, H.P., Stampfli, R. and Frei, R.W., Influence of organic bases on the stability and separation properties of reversed-phase chemically bonded silica gels, J. Chromatogr., 149, 199 (1978).

13. Tilly Melin, A., Ljungcrantz, M. and Schill, G., Reversed-phase ion-pair chromatography with an adsorbing stationary phase and hydrophobic quaternary ammonium ion in the mobile phase. I., J. Chromatogr., 185, 225 (1979).

14. Iler, R.K., The Chemistry of Silica, Wiley-Interscience, New York (1979).

15. Giddings, J.C., Kinetic origin of tailing in chromatography, Anal. Chem., 35, 1999 (1963).

16. Villermaux, J., Deformation of chromatographic peaks under the influence of mass transfer phenomena, J. Chromatogr. Sci., 12,822 (1974).

17. Bidlingmeyer, B.A., Deming, S.N., Price, W.P., Sachok, B. and Petrusek, M., Retention mechanism for reversed-phase ion-pair liquid chromatography, J. Chromatogr., 186, 419 (1979).

18. Crommen, J., Fransson, B. and Schill, G., Ion-pair chromatography in the low concentration range by use of highly absorbing counter ions, J. Chromatogr., 142, 283 (1977).

19. Bijsterbosch, B.H., Characterisation of silica surfaces by adsorption from solution. Investigations into the mechanism of adsorption of cationic surfactants, J. Colloid Interface Sci., 47, 186 (1974).

20. Bidlingmeyer, B.A., Del Rios, J.K. and Korpl, J. Separation of organic amine compounds on silica gel with reversed-phase eluents, Anal. Chem., 54, 442 (1982).

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HPLC ISOLATION AND CHARACTERIZATION OF PENTACARBOXYLIC PORPHYRINS DERIVED FROM UROPORPHYRINOGEN III

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ABSTRACT

A reversed-phase HPLC system with 22% (v/v) acetonitrile in 1 M ammonium acetate buffer pH 5.16 as mobile phase on an ODS-Hypersil column is developed for the analysis, isolation and characterization of the pentacarboxylic porphyrins derived from uroporphyrinogen III. The results proved conclusively that enzymic decarboxylation of uroporphyrinogen III does not always begin at the ring D acetic acid group and proceeds in a clockwise manner as currently believed.

INTRODUCTION

Uroporphyrinogen III is the first cyclic tetrapyrrole formed in the biosynthesis of haem (1). It is converted into coproporphyrinogen III by stepwise decarboxylation of the side chain acetic acid groups and is catalysed by the enzyme uroporphyrinogen decarboxylase. It was proposed (2) that enzyme decarboxylation starts at the ring D acetic acid group and proceeds in a clockwise fashion through the acetic acid groups of ring A, B and C. To support this argument pentacarboxylic porphyrins in the urine of normal subjects and patients with porphyria cutanea tarda (PCT) were analysed by HPLC (3). The authors could not detect the pentacarboxylic porphyrin with the ring D acetic acid group intact (Fig.1) and concluded that the clockwise decarboxylation sequence must be correct.

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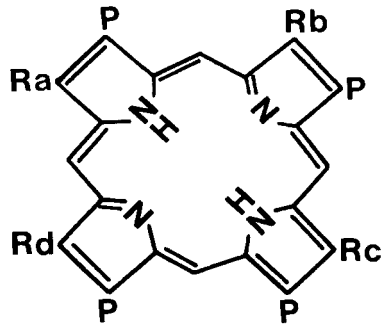


FIGURE 1. Structure of type III pentacarboxylic porphyrin isomers.
5 abc (Ra = Rb = Rc = Me, Rd = CH_2COOH);
5 abd (Ra = Rb = Rd = Me, Rc = CH_2COOH);
5 acd (Ra = Rc = Rd = Me, Rb = CH_2COOH);
5 bcd (Rb = Rc = Rd = Me, Ra = CH_2COOH).

This paper describes a highly efficient HPLC system for the detail analysis of pentacarboxylic porphyrins (Fig.1) dervied from uroporphyrinogen III. The results provide unequivocal evidences for the presence of naturally occurring pentacarboxylic porphyrin with the ring D acetic acid group unaffected by enzymic decarboxylation and therefore the existence of decarboxylation pathways other than that (2) currently believed.

EXPERIMENTAL

Materials and reagents

Type III pentacarboxylic porphyrin isomers and type I pentacarboxylic porphyrin were prepared by heating uroporphyrin III and I respectively in 0.3 M HCl at 160° for 2 h. Uroporphyrin I and III octamethyl esters were from Sigma Chemical Co., Poole, Dorset, U.K. The esters were hydrolysed in 25% (w/v) HCl at room temperature in the dark for 96 h.

Ammonium acetate, acetic acid, ethylenediamine tetraacetic acid (EDTA) ethyl acetate and hydrochloric acid were AnalaR grade from BDH Chemicals, Poole, Dorset, U.K.

Acetonitrile was HPLC grade from Rathburn Chemicals, Walkerburn, Peebleshire, U.K.

Extraction of porphyrins from urine

The porphyrins were extracted onto talc and esterified as previously described (4). The methyl esters were then hydrolysed in 25% (w/v) HCl for HPLC analysis.

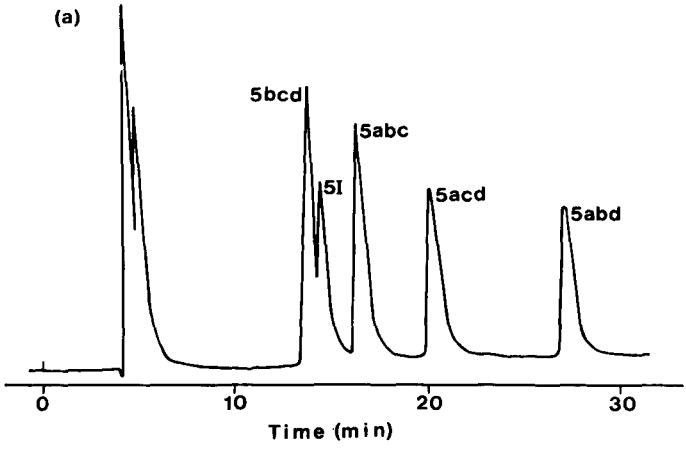
High-performance liquid chromatography

A Varian Associates (Walnut Creek, CA, U.S.A.) model 5000 liquid chromatograph was used. Injection was via a Rheodyne 7125 injector fitted with a 100 µl loop. A variable-wavelength U.V. detector (Varian UV-50) set at 404 nm or an LS-3 fluorescence detector (Perkin-Elmer, Beaconsfield, Bucks., U.K.) set at an excitation and an emission wavelength of 404 and 618 nm respectively, was used for detection.

The analytical separation was carried out on a 25 cm x 5 mm ODS-Hypersil column (Shandon Southern Ltd., Runcorn, Cheshire, U.K.) with 22% (v/v) acetonitrile in 1 M ammonium acetate buffer pH 5.16 as eluent. The mobile phase flow rate was 1 ml/min. Preparative HPLC was carried out on a 25 cm x 8 mm ODS-Hypersil column using the same mobile phase but with EDTA (100 ml/l) added to prevent the formation of metalloporphyrins. The flow rate was 3 ml/min. The isolated porphyrin fractions were washed with ethyl acetate. The aqueous solutions were adjusted to pH 3.0-3.2 and the porphyrins were extracted into ethyl acetate and were recovered by evaporation of the solvent.

RESULTS AND DISCUSSION

There are four possible type III pentacarboxylic porphyrins (Fig.1) that can be produced by enzymic decarboxylation of uroporphyrinogen III or by heating uroporphyrin III in acid. The separation of a standard mixture and of the corresponding porphyrins in the urine


(a)
5bcd
5I
5abc
5acd
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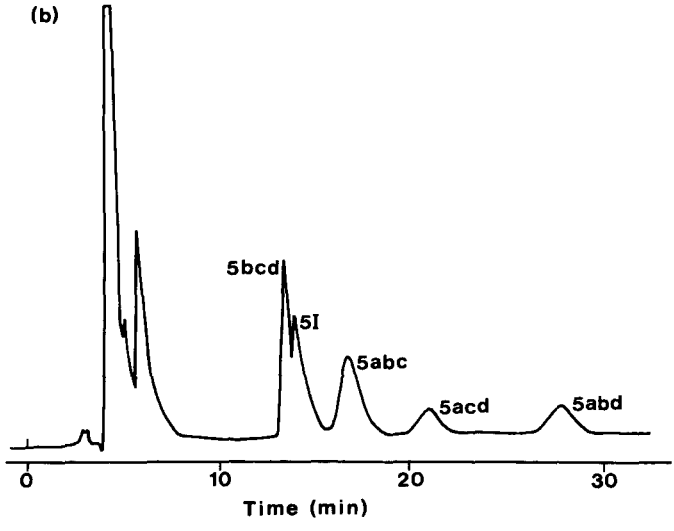
0
10
20
30
Time (min)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Time (min)

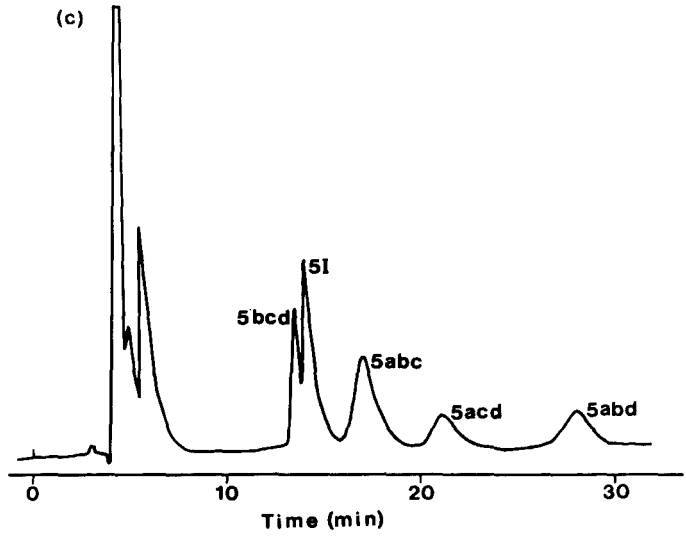

FIGURE 2. HPLC separation of pentacarboxylic porphyrin isomers. (a) Standard mixture; (b) normal urine extract; (c) PCT urine extract. Column, ODS-Hypersil; mobile phase, 22% (v/v) acetonitrile in 1 M ammonium acetate buffer pH 5.16; flow rate, 1 ml/min. 5 I is type I pentacarboxylic porphyrin. For other peak identification see FIGURE 1.

of normal subjects and PCT patients is shown in Fig.2(a,b and c). The detection of the pentacarboxylic porphyrin 5 abc in urine clearly demonstrates that porphyrins with the ring D acetic acid intact are definitely natural products. The enzymic decarboxylation of uroporphyrinogen III in man is therefore able to begin at the acetic acid groups of either ring A, B, C or D and is not restricted to ring D as reported (2). The most obvious reason for the different results obtained is that our HPLC system is highly efficient and is able to completely resolve the four type III isomers and the type I isomer. This was not possible with the system reported for the separation of pentacarboxylic porphyrin methyl esters (3).

The pentacarboxylic porphyrins in the urine were isolated by preparative HPLC and on decarboxylation by heating in 0.3 HCl at

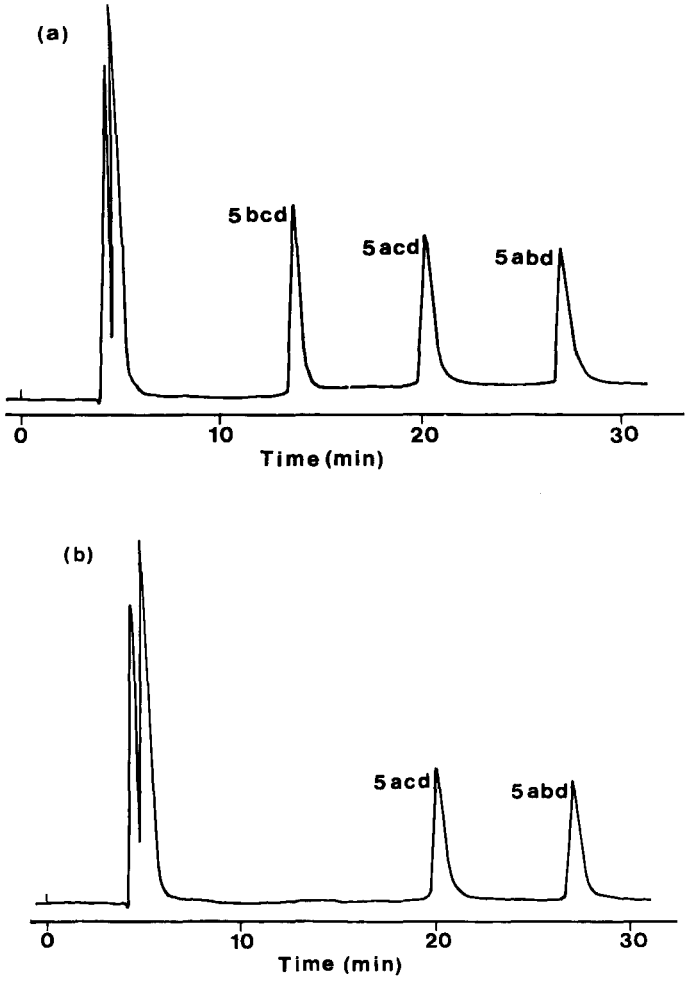

FIGURE 3. HPLC separation of pentacarboxylic porphyrins formed by decarboxylation of type III (a) Hepta- and (b) hexa-carboxylic porphyrins isolated from PCT urine. HPLC conditions as in FIGURE 2. For peak identification see FIGURE 1.

200° for 2 h each gave the same product, coproporphyrin III. They are therefore isomers. The porphyrin 5 abc can be positively identified by comparing the partial decarboxylation products formed by heating uroporphyrin III and the heptacarboxylic porphyrin III isolated from PCT urine which is known to be a pure compound with the ring D acetic acid decarboxylated (5). On partial decarboxylation of uroporphyrin III in 0.3 M HCl at 160° for 2 h all four possible pentacarboxylic isomers were formed (Fig.2a). The decarboxylation of the heptacarboxylic porphyrin, on the other hand, produced only 5 bcd, 5 acd and 5 abd (Fig.3a). The 5 abc peak is therefore easily identified.

The porphyrin 5 bcd can be similarly identified by partial decarboxylation of the hexacarboxylic porphyrin with the ring A and D acetic acid groups decarboxylated. This compound can be isolated from PCT urine or faeces by preparative HPLC (5) and on heating in 0.3 M HCl at 160° for 1 h gave 5 abd and 5 acd (Fig.3b), thus allowing the assignment of the 5 bcd peak.

The remaining 2 peaks, 5 abd and 5 acd, were assigned by their chromatographic behaviours. 5 abd was more strongly retained than 5 acd because the three side chain Me-groups are closer to each other and this imparts a stronger hydrophobic interaction with the C_{18} stationary phase.

The partial decarboxylation of uro-, hepta- and hexa-carboxylic porphyrins followed by HPLC separation and isolation is a simple way of obtaining pure pentacarboxylic porphyrin isomers important for biochemical studies. It is common for the enzyme uroporphyrinogen decarboxylase to be assayed using synthetic 5 abd as the substrate (6). The availability of this compound, however, is limited to a few chemical laboratories interested in its synthesis. The present system is thus invaluable for the preparative isolation of 5 abd and its isomers.

REFERENCES

1. Battersby, A.R., Fookes, C.J.R., Gustafson-Potter, K.E., McDonald, E. and Matcham, G.W.J., J. Chem. Soc.Perkin I., 2427, 1982.

2. Jackson, A.H., Sancovich, H.A., Ferramola, A.M., Evans, N., Games, D.E., Matlin, S.A., Elder, G.H. and Smith, S.G., Phil. Trans. R. Soc., Lond. Ser. B, 273, 191, 1976.

3. Smith, S.G., Rao, K.R.N. and Jackson, A.H., Int. J. Biochem., 12, 1081, 1980.

4. Dowdle, E.B., Goldswain, P., Spong, N. and Eales, L., Clin. Sci., 39, 147, 1970.

5. Lim, C.K., Rideout, J.M. and Wright, D.J., Paper presented at the VIIth International Symposium on Column Liquid Chromatography, Baden-Baden, May 3-6, 1983.

6. De Verneuil, H., Sassa, S. and Kappas, A., J. Biol. Chem., 258, 2454, 1983.

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STUDIES ON STEROIDS CLXXXII. DETERMINATION OF 6β-HYDROXYCORTISOL IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A new sensitive method for the determination of 6β-hydroxycortisol in urine by high-performance liquid chromatography with fluorescence detection has been developed. 6β-Hydroxycortisol and its C-6 epimer (internal standard) were transformed quantitatively into the 21-(9-anthroyl) derivatives when treated with 9-anthroyl nitrile in the presence of triethylamine in acetonitrile. The resulting fluorescent esters were readily separated on a Cosmosil 5SL column using ethyl acetate/hexane (2:1) as a mobile phase with a detection limit of 25 pg. The efficient clean-up was achieved by the combined use of Bond Elut and Clin Elut cartridges. The present method is applicable to the quantification of 6β-hydroxycortiol in human urine with satisfactory accuracy and precision.

INTRODUCTION

6β-Hydroxycortisol is a polar unconjugated metabolite of cortisol in human urine (1). The urinary excretion is significantly elevated in the newborn, pregnancy and cancer as well as in humans who administered drugs such as diphenylhydantoin, phenobarbital and o,p'-DDD. Several methods which involve radioimmunoassay (2,3) and enzyme immunoassay (4), have already been

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developed for the determination of 6β-hydroxycortisol in biological fluids. For this purpose high-performance liquid chromatography (HPLC) has also been employed (5). The procedure, however, is still unsatisfactory with respects to the sensitivity and simplicity. The present paper deals with a new method for the determination of 6β-hydroxycortisol in urine by HPLC with fluorescence detection using 9-anthroyl nitrile as a pre-column labeling reagent.

MATERIALS AND METHODS

Apparatus

The apparatus used was a Waters 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) equipped with a Hitachi 650-10 LC fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan) (excitation wavelength 360 nm; emission wavelength 460 nm). The test samples were applied to the chromatograph by a U6K sample loop injector (Waters Assoc.) with an effective volume of 2 ml. The Cosmosil 5SL (5 μm) (150 mm x 4 mm i.d.) (Nakarai Kagaku Co., Kyoto, Japan) and LiChrosorb RP-18 (5 μm) (125 mm x 4 mm i.d.) (E. Merck AG, Darmstadt, F.R. Germany) columns were used under ambient conditions.

Chemicals and Reagents

6β-Hydroxycortisol and 6α-hydroxycortisol were prepared in these laboratories by the known methods and purified by repeated recrystallization. 9-Anthroyl nitrile was synthesized in the manner described in the previous paper (6). All other chemicals employed were of analytical-reagent grade. Solvents were purified by distillation prior to use. The Clin Elut CE1000M and Bond Elut C_{18} 607101 cartridges were purchased from Analytichem International, Inc. (Harbor City, CA, U.S.A.). A Bond Elut cartridge was washed successively with ethanol (10 ml) and water (10 ml) before use.

Derivatization with 9-Anthroyl Nitrile

To a test sample was added a solution of 9-anthronyl nitrile (100 μg) in 5% triethylamine/acetonitrile (100 μl), and the whole was kept at room temperature for 1 hr. After removal of the solvent with an aid of nitrogen gas stream, the residue was redissolved in acetone (100 μl), mixed with hexane (2 ml), and heated at 70-80°C for a few minutes to remove acetone. The solution was applied to a Clin Elut cartridge which was previously impregnated with 0.1 N NaOH (0.3 ml). The cartridge was washed with hexane (4 ml) to remove the excess reagent, and the desired fraction was eluted with dichloromethane (4 ml). The eluate obtained was dried in vacuo and redissolved in ethyl acetate (0.5 ml). A 10 μl aliquot of the solution was injected into the chromatograph.

Procedure for the Determination of 6β-Hydroxycortisol in Urine

A urine sample (0.5 ml) was pipetted into a test tube containing a known amount of 6α-hydroxycortisol (ca. 50 ng) in phosphate buffer (pH 7) (1 ml). The mixture was applied to a Bond Elut cartridge impregnated with phosphate buffer (pH 7) (1 ml). The cartridge was washed successively with water (3 ml) and 5% ethanol (3 ml), and the desired fraction was eluted with 70% ethanol (2 ml). The eluate obtained was dried in vacuo and redissolved in ethyl acetate (0.3 ml). The solution was applied to a Clin Elut cartridge impregnated with 15% ammonium carbonate (0.3 ml). The desired fraction was eluted with ethyl acetate (4 ml). The eluate obtained was subjected to pre-column derivatization followed by HPLC in the manner described above.

RESULTS AND DISCUSSION

In the previous papers we reported the reactivities of 9-anthroyl nitrile and 1-anthroyl nitrile toward various hydroxyl groups on the steroid nucleus (6,7). The former reagent is capa-

ble of reacting selectively with the primary hydroxyl groups at C-21 in cortisol and cortisone in triethylamine/acetonitrile. Therefore, 9-anthroyl nitrile appeared to be also promising for derivatization of 6β-hydroxycortisol. Initially, the suitable condition for acylation of 6β-hydroxycortisol with 9-anthroyl nitrile in triethylamine/acetonitrile was investigated (Figure 1). The excess of the reagent which would disturb the chromatogram, could be efficiently removed by passing the reaction mixture through a Clin Elut cartridge. The effects of concentration of triethylamine in acetonitrile and reaction time on the formation of the ester were examined. The reaction rate was significantly influenced by the concentration of triethylamine where the maximum yield was obtained with 5% triethylamine in acetonitrile. In addition, the formation of the ester increased with the reaction time up to 60 min and reached a plateau as illustrated in Figure 2.

The HPLC separation of derivatized 6β-hydroxycortisol and 6α-hydroxycortisol was more satisfactorily achieved on a normal phase column (Cosmosil 5SL) using ethyl acetate/hexane (2:1) as a mobile phase rather than on a reversed phase column (LiChrosorb RP-18).

A calibration curve was constructed by plotting the ratio of peak area of 6β-hydroxycortisol to that of 6α-hydroxycortisol (internal standard) against the amount of the former where a linear response was observed in the range of 50 to 400 ng of 6β-hydroxycortisol.

The combined use of Bond Elut and Clin Elut cartridges proved to be effective for the clean-up of urinary 6β-hydroxycortisol. As illustrated in Figure 3, the desired 6β-hydroxycortisol was recovered at the rate of 95% when eluted with 2 ml of 70% ethanol after washing with 5% ethanol. The typical chromatograms of 6β-hydroxycortisol together with 6α-hydroxycortisol are shown in Figure 4. A known amount of 6β-hydroxy-

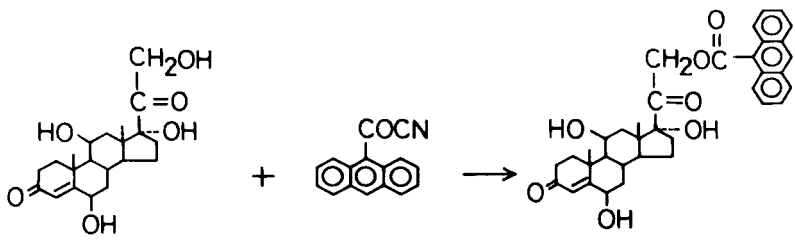

FIGURE 1 Reaction of 6β-Hydroxycortisol with 9-Anthroyl Nitrile

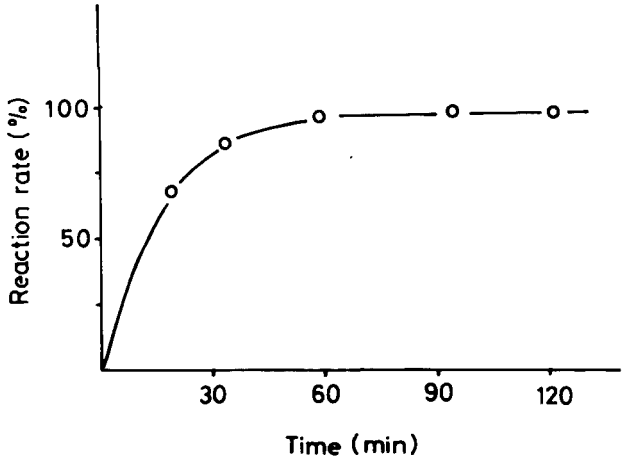

FIGURE 2 Time Course for Derivatization of 6β-Hydroxycortisol with 9-Anthroyl Nitrile

cortisol was added to human urine specimens at two levels and their recovery rates were determined by the standard procedure. It is evident from the data in Table I that the satisfactory results are obtainable by the proposed method.

The present method was then applied to the determination of 6β-hydroxycortisol in urine specimens collected from seven male healthy volunteers. As listed in Table II, the urine levels of 6β-hydroxycortisol were observed in the range previously estimated by the enzyme immunoassay method (4).

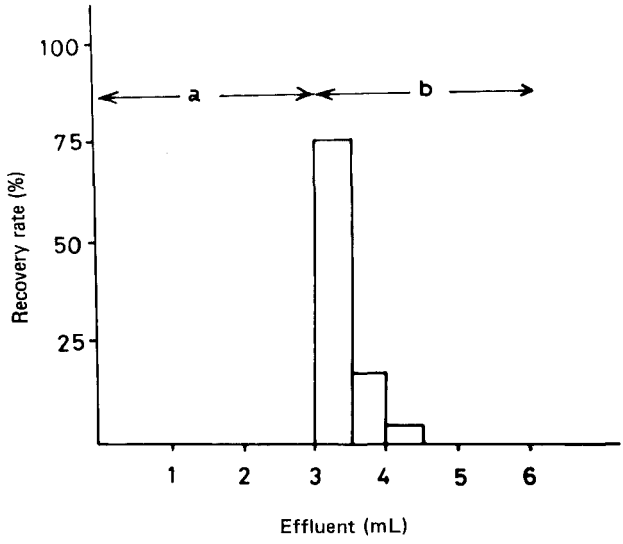

FIGURE 3 Elution Pattern of 6β-Hydroxycortisol on a Bond Elut Cartridge
Eluent: a) 5% ethanol; b) 70% ethanol.

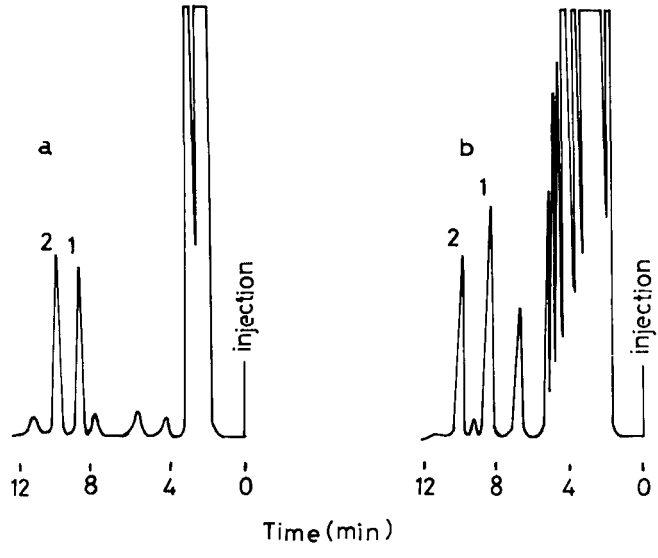

FIGURE 4 Chromatograms of 6β-Hydroxycortisol and 6α-Hydroxycortisol Derivatives formed with 9-Anthroyl Nitrile
a) Standard sample; b) urine sample. 1: 6β-hydroxycortisol, 2: 6α-hydroxycortisol (internal standard).

TABLE I
Recovery of 6β-Hydroxycortisol added to Human Urine

Urine	Added (ng/0.5 ml)	Expected	Found	Recovery (% ± S.D.*)
86	50	136	137	101 ± 1.6
193	100	293	270	92 ± 2.1

* n=8

TABLE II
Urine Levels of 6β-Hydroxycortisol in Healthy Male Volunteers (μg/24 hr)

Subject	A	B	C	D	E	F	G	Mean
	407	254	503	212	278	219	246	303 ± 45

TABLE III
The k' Values and Peak Area Ratios of 6β-Hydroxycortisol in Human Urine to Internal Standard

	A		B		C	
	S	U	S	U	S	U
6β-Hydroxycortisol	0.77	0.77	0.79	0.79	0.84	0.84
6α-Hydroxycortisol	0.80	0.80	0.83	0.83	0.86	0.86
Peak area ratio (6β/6α)	1.32	0.82	-	-	1.32	0.80

S: standard sample, U: urine sample.
Conditions: A) Cosmosil 5SL, ethyl acetate/hexane (2:1), 1 ml/min; B) Cosmosil 5SL, ethyl acetate/chloroform (3:4), 1.5 ml/min; C) LiChrosorb RP-18, methanol/water (3:1), 0.6 ml/min.

The disadvantage of HPLC in structural elucidation, because the information provided is insufficient, has already been pointed out. In order to solve these problems, several methods have already been devised. Previously, we demonstrated an attempt for unequivocal characterization of bile acid sulfates in bile by HPLC using mobile phases of varying pH (8). In this study, a similar approach was done for structural characterization of 6β-hydroxycortisol in urine. The eluate corresponding to the peak on the chromatogram was collected and, after the addition of the derivatized 6α-hydroxycortisol, was subjected to HPLC under three different conditions. As listed in Table III, k' values of 6β-hydroxycortisol in human urine were completely identical with those of the authentic sample. Moreover, the peak area ratio of the two epimers showed the same value under two different HPLC conditions. These results imply that the present method undergoes no interferences with coexisting substances and is favorable for the determination of 6β-hydroxycortisol in urine.

The newly developed method for the determination of 6β-hydroxycortisol in biological fluids is highly sensitive, convenient and may be useful for monitoring the effects of hormones and drugs acting as inducers of microsomal enzymes.

ACKNOWLEDGEMENTS

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REFERENCES

1) Frantz, A.G., Katz, F.H. and Jailer, J.W., 6β-Hydroxycortisol and other polar corticosteroids: Measurement and significance in human urine, J. Clin. Endocrinol. Metab., 21, 1290, 1961.

2) Kishida, S. and Fukushima, D.K., Radioimmunoassay of 6β-hydroxycortisol in human plasma and urine, Steroids, 30, 741, 1977.

3) Park, B.K., A direct radioimmunoassay for 6β-hydroxycortisol in human urine, J. Steroid Biochem., 9, 963, 1978.

4) Hosoda, H., Sakai, Y. and Nambara, T., A direct enzyme immunoassay of 6β-hydroxycortisol in human urine, Chem. Pharm. Bull., 29, 170, 1981.

5) Roots, I., Holbe, R., Hovermann, W., Nigam, S., Heinemeyer, G. and Hildebrandt, A.G., Quantitative determination by HPLC of urinary 6β-hydroxycortisol, an indicator of enzyme induction by rifampicin and antiepileptic drugs, Eurp. J. Clin. Pharmacol., 16, 63, 1979.

6) Goto, J., Goto, N., Shamsa, F., Saito, M., Komatsu, S., Suzaki, K. and Nambara, T., New sensitive derivatization of hydroxysteroids for high-performance liquid chromatography with fluorescence detection, Anal. Chim. Acta, 147, 397, 1983.

7) Goto, J., Saito, M., Chikai, T., Goto, N. and Nambara, T., Studies on steroids CLXXXVII. Determination of serum bile acids by high-performance liquid chromatography with fluorescence labeling, J. Chromatogr., in press.

8) Goto, J., Kato, H., Saruta, Y. and Nambara, T., Studies on steroids CLXX. Separation and determination of bile acid 3-sulfates in human bile by high-performance liquid chromatography, J. Chromatogr., 226, 13, 1981.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF ANDROGEN ACETATES

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ABSTRACT

More than twenty $C_{19}O_2$ androgen acetates were chromatographed by both normal-phase (silica) and reversed-phase (C_{18}) HPLC. These two HPLC systems together with normal-phase and reversed-phase HPLC of free androgens have made the separation of various $C_{19}O_2$ androgen isomers possible. 3,17-Diacetoxyandrostane derivatives, in general, are more polar in normal-phase HPLC when the 3-acetoxyl group is equatorial than when it is axial. However, 3-acetoxyandrostan-17-one derivatives are more polar in normal-phase HPLC when the 3-acetoxyl group is axial than when it is equatorial. 17α-acetoxyandrostane derivatives in general are more polar than their 17β-analogs in both normal-phase and reversed-phase HPLC.

INTRODUCTION

High-performance liquid chromatography (HPLC) of androgens has been reviewed recently [1,2,3]. They were chromatographed as either free androgens or their derivatives. Androgen derivatives used were 2,4-dinitrophenylhydrazine derivatives[4], benzoates, p-nitrobenzoates[5], sulfates, glucuronides[6] and acetates[7,8]. Gorog and Herenyi[7] have separated epimers of ethynodiol diacetate by

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reversed-phase HPLC. Holder et al.[8] have used reversed-phase HPLC for the trace analysis of trenbolone acetate and trenbolone.

We have recently reported both normal-phase and reversed-phase HPLC of 69 underivatized free androgens including $C_{19}O$, $C_{19}O_2$ and $C_{19}O_3$[9] for the study of the metabolism of 4-[4-^{14}C] androstene-3,17-dione ($C_{19}O_2$). These two HPLC systems complement each other, but still are not good enough for the identification of the radioactive metabolites, because some androgens do not separate well in both systems. We have previously used both normal-phase and reversed-phase HPLC and co-crystallization to constant specific radioactivity to identify the reduction products of 4-[4-^{14}C]progesterone in pea plants[10]. That work required 10 mg of each reference compound for co-crystallization. In the study of the metabolism of 4-[4-^{14}C]androstene-3,17-dione in cucumber plants[11], we were limited by the scarcity of some androgens.

In this communication, both normal-phase and reversed-phase HPLC of androgen acetates are described. These two HPLC systems together with both normal-phase and reversed-phase HPLC of free androgens have made the identification of the radioactive metabolites of 4-[4-^{14}C]androstene-3,17-dione in cucumber plants possible without using co-crystallization[11]. Ten radioactive metabolites have been identified including the major metabolite, testosterone. This communication is limited to $C_{19}O_2$ androgens, because no significant radioactivities of $C_{19}O$ and $C_{19}O_3$ androgens metabo-

lized from 4-[4-^{14}C]androstene-3,17-dione were detected by the HPLC of free androgens in cucumber plants[11].

METHODS

The HPLC system consisted of a pump (Model 110A, Altex, Berkeley, California) with a high sensitivity pressure filter (Altex), a sample injector (Model 7125, Rheodyne, Cotati, California) with a loop volume of 1 ml, a variable wavelength UV-VIS detector (Model 155-30, Altex) and a recorder (Model 385, Linear, Irvine, California). The normal-phase column was a 25 cm x 0.46 cm stainless-steel chromatography tube, packed with Zorbax BP-SIL (silica; 7-8 μm; DuPont, Wilmington, Delaware). The reversed-phase column had the same dimensions, but it was packed with Zorbax BP-ODS (C_{18}; 7-8 μm; DuPont). The columns were packed in this laboratory[12].

Androgen acetates were made by acetylation of free androgen (1-5 mg) with 0.5 ml pyridine and 0.25 ml acetic anhydride (Acetylation Kit, Applied Science Laboratories, State College, Pennsylvania). The UV detector at 200 nm was used for androgen acetates, because the λ_{max} of 5α-androstane-3β,17β-diol is 200 nm. About 2 μg of the Δ^4- or Δ^5-androgen acetates and about 200 μg of the other androgen acetates were analyzed by HPLC. The chromatographic conditions are given in figure legend and Table 1.

RESULTS AND DISCUSSION

The results are summarized in Table 1. Systems 1 and 2, the normal-phase and reversed-phase HPLC of free androgens, have been

TABLE 1

HPLC Retention Times (min.) Of $C_{19}O_2$ Androgens
Hydroxyl groups are indicated by α and β, depending on orientation at C-3 and C-17. However, at C-5, α and β are used to designate the orientation of hydrogen. Keto groups are indicated by O, and double bonds by Δ. Free androgens were chromatographed in Systems 1 and 2 (published previously[9]), acetates in all other systems. System 1, normal-phase column, hexane-ethanol (97:3); System 2, reversed-phase column, methanol-water (7:3); System 3, normal-phase column, hexane-ethanol (998:2); System 4, normal-phase column, hexane-ethanol (995:5); System 5, normal-phase column, hexane-ethanol (99:1); System 6 (see Fig. 1), reversed-phase column, methanol-water (9:1); System 7, reversed-phase column, methanol-water (8:2). Capacity factors, $k' = t-t_o/t_o$, t_o = 1.60 min (normal-phase), t_o = 1.24 min (reversed-phase).

No.	Substituents				Systems						
	3	4	5	17	1	2	3	4	5	6	7
1	O	-	β	O	7.5	15.5	-	-	-	-	-
2	O	-	Δ	O	8	17.5	-	-	-	-	-
3	O	-	α	O	8	17.75	-	-	-	-	-
4	O	-	β	α	8.5	23	-	17	7.5	-	11.5
5	β	-	β	O	10	14.25	-	12.5	-	-	12.5
6	O	-	α	α	10.25	18	-	31	9.5	-	12.5
7	α	-	α	O	12.5	16.25	-	14.5	-	-	14.25
8	β	-	Δ	O	12.5	11.25	-	11	-	-	12.75
9	O	-	α	β	12.5	16	-	15	-	-	14.75
10	O	-	β	β	12.5	15.75	-	16.25	-	-	13.25
11	β	-	α	O	13	14.75	-	11.5	-	-	15
12	α	-	β	O	13	16.25	-	10.5	-	-	12
13	β	-	β	β	16.5	11.75	15	-	-	9	-
14	α	-	α	β	18	16.75	12.5	-	-	9	-
15	β	-	β	α	19.25	16	15	-	-	7.75	-
16	O	Δ	-	O	20	11.5	-	-	-	-	-
17	β	-	Δ	β	20	10.25	19.5	5	-	9.25	-
18	β	-	α	β	20	13.5	22	-	-	10.5	-
19	β	Δ	-	β	20.25	9.75	20.75	-	-	8	-
20	α	-	α	α	20.25	33	14.75	-	-	8.25	-
21	β	-	Δ	α	20.5	11.25	23.5	5.5	-	7	-
22	β	-	α	α	20.5	14.5	20.75	-	-	7	-
23	O	Δ	-	α	21.5	12	-	-	18.5	-	9.75
24	α	-	β	α	24	32	21.25	-	-	7	-
25	α	-	β	β	24	15.5	13	-	-	9	-
26	O	Δ	-	β	25.75	10.75	-	-	16.75	-	9.75

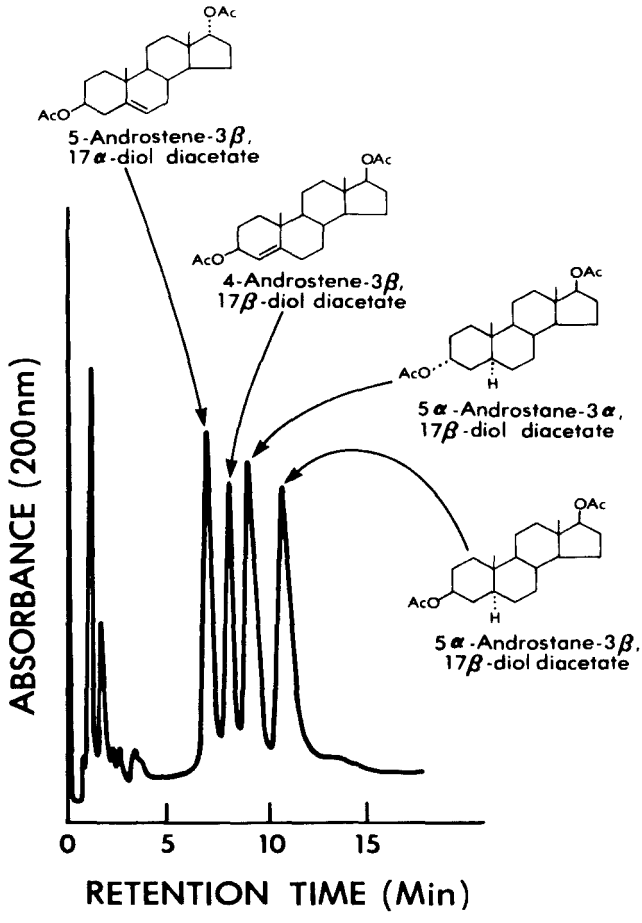

FIGURE 1. Reversed-phase chromatogram of androstane-3,17-diol diacetates. Between 1 µg (5-androstene-3β,17α-diol diacetate) and 30 µg (5α-androstane-3β,17β-diol diacetate) of diacetates, dissolved in 100 µl of methanol, were chromatographed on a column of Zorbax BP-ODS, 25 cm x 0.46 cm. Eluent, methanol-water (9:1); flow rate, 2 ml/min; pressure, 500 p.s.i.; UV detector, 200 nm, range 0.5; recorder, speed 12 cm/h, span 10 mv.

published[9]. The retention times (min) in Table 1 have never varied more than 5% except at System 3 where the eluent used contained very small amount of ethanol in hexane. The HPLC of androgen acetates (Systems 3-7, Table 1) showed that 17-acetoxy-4-androsten-3-ones (compounds 23 and 26, Table 1) are more polar than mono-acetoxy-monoketo-androstane derivatives (compounds 4-12) and that mono-acetoxy-monoketo-androstane derivatives are more polar than diacetoxyandrostane derivatives in both normal-phase and reversed-phase HPLC. Apparently, in these HPLC systems, a conjugated keto group is more polar than an isolated keto group and an isolated keto group is more polar than an acetoxyl group.

3,17-Diacetoxyandrostane derivatives, in general as shown in Table 2, are more polar in normal-phase HPLC when the 3-acetoxyl group is equatorial than when it is axial. This is similar to the results for 3-hydroxyandrostane derivatives[9]. However, 3-acetoxyandrostan-17-one derivatives, as shown in Table 2, are more polar in normal-phase HPLC when the 3-acetoxyl group is axial than when it is equatorial. Normal-phase HPLC, in general, separates pairs of equatorial 3-acetoxy- and axial 3-acetoxy-androstane derivatives better than reversed-phase HPLC. However, reversed-phase HPLC separates pairs of equatorial 3-hydroxy- and axial 3-hydroxy-androstane derivatives better than normal-phase HPLC except for the pairs of 5-epimers[9].

17α-Acetoxyandrostane derivatives, in general, are more polar than their 17β-analogs in both normal-phase and reversed-

TABLE 2

Relative Polarities Of Androgen Acetates
> Indicates that the first group of steroids is, as a rule, more polar than the second. N = normal-phase, R = reversed-phase, e = equatorial, a = axial, + indicates that the rule shown in each heading is obeyed, - indicates that it is violated, = indicates that analogues are inseparable. The superior HPLC system for each group of analogue separations is underlined. The compound numbers are the same as those in Table 1.

Comparison between 3-acetoxy(e) and 3-acetoxy(a)							
3α(e),5β>3α(a),5α		N	R	3β(e),5α>3α(a),5α		N	R
12	7	-	+	11	7	-	-
24	20	+	+	18	14	+	-
25	14	+	=	22	20	+	+
3β(e),5α>3β(a),5β		N	R	3α(e),5β>3β(a),5β		N	R
11	5	-	-	12	5	-	+
18	13	+	-	24	15	+	+
22	15	+	+	25	13	-	=
3β(e),Δ^5>3α(a),5α		N	R	3β(e),Δ^5>3β(a),5β		N	R
8	7	-	+	8	5	-	-
17	14	+	-	17	13	+	-
21	20	+	+	21	15	+	+
3β(e),Δ^4>3α(a),5α		N	R	3β(e),Δ^4>3β(a),5β		N	R
19	14	+	+	19	13	+	+

Comparison between 17α-acetoxy and 17β-acetoxy							
17α>17β		N	R	17α>17β		N	R
6	9	+	+	21	17	+	+
4	10	+	+	22	18	-	+
15	13	=	+	23	26	+	=
20	14	+	+	24	25	+	+

phase HPLC as shown in Table 2. However, 17α-hydroxyandrostane derivatives are less polar than their 17β-analogs in reversed-phase HPLC[9]. Androgen acetates inseparable by normal-phase HPLC can often be separated by reversed-phase HPLC and vice versa. These two HPLC systems together with normal-phase and reversed-phase HPLC of free androgens have made the separation of various $C_{19}O_2$ androgen isomers possible.

ACKNOWLEDGEMENTS

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REFERENCES

1. O'Hare, M. J. and Nice, E. C., Analysis of Steroid Hormones in Adrenal and Testicular Cells and Tissues, in: Chromatographic Science Series Vol. 16, Steroid Analysis by HPLC, Kautsky, M. P., ed., Marcel Dekker, New York, 1981, p. 277.

2. Redel, J. J. and Capillon, J., Separation of Steroid Epimers by HPLC, in: Chromatographic Science Series Vol. 16, Steroid Analysis by HPLC, Kautsky, M. P., ed., Marcel Dekker, New York, 1981, p. 343.

3. Heftmann, E. and Lin, J. T., Steroid Analysis by High-performance Liquid Chromatography, J. Liquid Chromatogr. 5 (Suppl. 1), 121, 1982.

4. Fitzpatrick, F. A., Siggia, S. and Dingman, J., High Speed Liquid Chromatography of Derivatized Urinary 17-Keto Steroids, Anal. Chem., 44, 2211, 1972.

5. Fitzpatrick, F. A. and Siggia, S., High Resolution Liquid Chromatography of Derivatized Non-ultraviolet Absorbing Hydroxy Steroids, Anal. Chem., 45, 2310, 1973.

6. Lafosse, M., Keravis, G. and Durand, M. H., Etude par Chromatographie Liquide Haute Pression de 17-Cetosteroides Libres et Conjugues, J. Chromatogr., 118, 283, 1976.

7. Gorog, S. and Herenyi, B., Analysis of Steroids XXX. Simultaneous Determination of α- and β-Ethynodiol Diacetate by High-pressure Liquid Chromatography. J. Chromatogr., 152, 240, 1978.

8. Holder, C. L., Blakemore, W. M. and Bowman, M. C., Trenbolone Acetate and Trenbolone: Trace Analysis in Animal Chow, Wastewater and Human Urine by High Pressure Liquid Chromatography and Electron Capture Gas Chromatography, J. Chromatogr. Sci., 17, 91, 1979.

9. Lin, J. T. and Heftmann, E., Comparison of Adsorption and Reversed-phase Partition High-performance Liquid Chromatography for the Separation of Androgens, J. Chromatog., 237, 215, 1982.

10. Lin, J. T. and Heftmann, E., Sterospecific Reduction of Progesterone by Pisum sativum, Phytochemistry, 20, 1017, 1981.

11. Lin, J. T., Palevitch, D. and Heftmann, E., Reduction of 4-Androstene-3,17-dione by Growing Cucumber Plants, Phytochemistry, in press.

12. Lin, J. T., Heftmann, E. and Hunter, I. R., High-performance Liquid Chromatography of the Reduction Products of Progesterone, J. Chromatog., 190, 169, 1980.

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A SIMPLE ASSAY OF 3-METHOXY-4-HYDROXYPHENYLETHYLENEGLYCOL IN CEREBROSPINAL FLUID BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

An improved method for the quantitation of 3-methoxy-4-hydroxyphenylethyleneglycol in cerebrospinal fluid is described. Sample cleaning was done by SEP PAK C_{18} Cartridge prior to the MHPG assay by high-performance liquid chromatography with electrochemical detector. The results are in good agreement wilth the GC/MS method. The average recovery is 68.3±4.1%, within run coefficient of variation of 3.8% and day to day of 7.2%. The method is simple, sensitive and accurate and can be used for routine work.

INTRODUCTION

3-Methoxy-4-hydroxythenylethylene glycol (MHPG or HMPG) is the major metabolite of norepinephrine. Its level in biological fluids and tissues has been measured in depression, anxiety and pain (1-13), obesity, hypertension and mania (14), sleep disorder (15), Gilles de la Tourette's syndrome (16) and tumors such as pheochromocytoma, neuroblostoma and ganglioneuroma (17). In plasma and urine large quantities of this metabolite exist mostly as sulfate and glucoronate conjugate. In amniotic fluid about 40% is free (18). Because of its poor volatility and thermal instability its assay involves deconjugation, extraction, addition of

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internal standard, and derivatization. Presently the assay is done by flourimetry (19), high-performance liquid chromatography (20-24), gas chromatography (25-28) and GC/mass spectrometry (29-33).

Low levels of MHPG are present in cerebrospinal fluid existing almostly completely in the free state. The compound has been assayed by direct injection into HPLC column after sample cleaning by SEP PAK C_{18} cartridge. We have simplified this HPLC assay of cerebrospinal MHPG considerably, while achieving higher sensitivity and reproductivity.

MATERIALS AND METHOD

Chemicals

3-methoxy-4-hydroxyphenylethylene glycol piperazine salt was purchased from Sigma Chemical C0. (St. Louis, MO, U.S.A.). SEP PAK C_{18} Cartridge was obtained from Waters Associates, Inc. (Milford, MA, U.S.A.). All solvents, buffer components and chemicals were of analytical reagent grade. Water was deionized and then double distilled in glass.

Apparatus

The HPLC system is made of four components. M-45 solvent delivery system (Waters Associates, Inc.); Model 7125 injection valve (Rheodyne Inc., Cotati, CA, U.S.A.). Biophase ODS 5 um C_{18} reverse phase column (Bioanalytical systems, West Lafayette, IN, U.S.A.) with an in-line guard column of 5 um RP-18 (Brownless Labs, Santa Clara, CA, U.S.A.) and LC-3 detector with a TL-5 glassy carbon electrode (Bioanalytical systems). The mobile phase consists of 0.009 mol/l citric acid and 0.089 mol/l sodium acetate buffered to pH 5.1 and contains 3% methanol. It was degassed by filteration under vacuum through a millipore 0.2 um membrane.

Procedure

The SEP PAK C_{18} Cartridge was activated by passing through 5 ml of methanol under vacuum. It was then washed with 10 ml of water to make certain

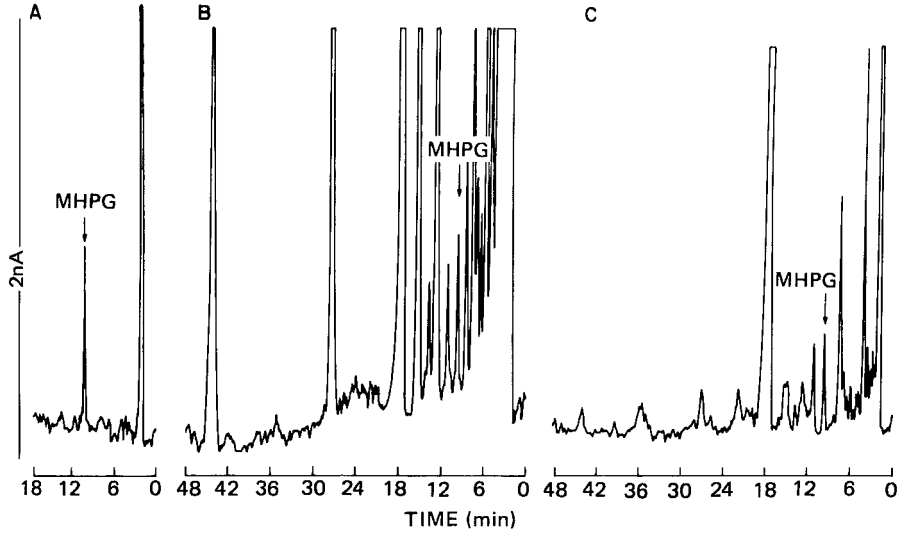

Fig. 1. Chromatograms of (A) MHPG standard (2 ng), (B) Direct injection of CSF sample, (C) CSF sample after SEP PAK cleaning. Chromatographic conditions as described under experimental.

that no methanol was left in the cartridge. The CSF sample (1 ml kept in ice) was then filtered slowly through the cartridge and washed wilth 5 ml. water. A second wash with 0.2 ml mobile phase containing 50% methanol was performed. The MHPG was then elected from the SEP PAK with 1 ml of mobile phase containing 50% methanol into a 50 ml round bottomed flask with a standard joint. The contents of the flask were then evaporated at 35°C to dryness (five minutes) on a rotary evaporator. The residue was redissolved in 0.5 ml mobile phase and aliquots were injected into the HPLC column.

Standard solutions of 5 to 20 ng MHPG piperazine salt in water were prepared and passed through SEP PAK, as were the CSF samples. The same cartridge was regenerated everytime before filtering the next sample by washing it with 5 ml of methanol and then with 10 ml of water. A standard curve was drawn with peak

Table I

MHPG in CSF (ng/ml)

Patient Number	GC/MS	HPLC
1	12.5	13.0
2	13.3	14.2
3	14.4	13.2
4	14.5	15.3
5	19.0	18.7
6	12.8	13.4
7	10.7	11.5
8	12.8	11.5
9	21.6	24.0
10	16.5	15.0
Mean	14.8	15.0
S.D.	3.3	3.8

heights against different amount of MHPG injected. The GC/MS assay was done according to B. Sjoquist et al. (30).

RESULTS AND DISCUSSION

Typical chromatograms are shown in fig. 1. The retention time for MHPG is ten minutes. The results are compared with the GC/MS assay and are shown in table 1. They are in excellent agreement. The sensitivity is 1.6 ng/ml CSF and can be improved using more than 1 ml of CSF. The recovery from SEP PAK C_{18} Cartridge is 68.3$\pm$4.1% ($M\pm S.D.$, N=10) for 5 to 100 ng/ml of MHPG. The within

day coefficient of variation is 3.8 % and day to day variation is 7.2% for an average value of 13.8 ng/ml of pooled CSF samples (N=15). As shown in fig. 1

SEP Pak cleaning removes many of the unwanted materials from the samples thus reduces column contamination and achieve base line separation. Since the sample is reconstituted in the mobile phase before injection into the column, the peak shape remains the same irrespective of the volume injected. On the average ten samples can be assayed in a day. The same SEP PAK can be used for several samples. The method is very simple, sensitive, inexpensive and accurate.

REFERENCES

1. K.L. Davis, L.E. Hollister, A.A. Mathé, B.M. Davis, A.B. Rothpearl, K.F. Faull, J.Y-K. Hsieh, J.D. Barchas and P.A. Berger, Am. J. Psychiatry, 138, 1555-1561, 1981.
2. L.E. Hollister, K.L. Davis, J.E. Overall, and T. Anderson, Arch. Gen. Psychiatry, 35, 1410-1415, 1978.
3. A.J. Gelenberg, C.J. Gibson, and J.D. Wojcik, Psychopharmacology Bulletin, 18, 7-18, 1982.
4. B.E. Leonard, Neurochem. International, 4, 339-350, 1982.
5. G. Curzon, Psychological Medicine 12, 465-470, 1982.
6. N.G. Ward, V.L. Bloom, J.F. Fawcett, and R.O. Friedel, J. Nervous and Mental Disease, 171, 55-58, 1983.
7. T.W. Uhde, L.J. Siever, R.M. Post, D.C. Jimerson, J.P. Boulenger and M.S. Buchsbaum, Psychopharmacology Bulletin, 18, 129-132, 1982.
8. R.C. Veith, R.J. Bielski, V.L. Bloom, J.A. Fawcett, N. Narasimhachari, and R.O. Friedel, J. Clin. Psychopharmacology, 3, 18-27, 1983.
9. S. Wilk, B. Shopsin, S. Gershon, and M. Suhl, Nature, 235, 440-441, 1972.
10. R.M. Post, E.K. Gordon, F.K. Goodwin, and W.E. Bunney, Science, 179, 1002-1003, 1973.

11. P. Vestergaard, T. Sorenson, E. Hoppe, O.J. Rafaelsen, C.M. Yates, and N. Nicolaou, Acta Psychiat. Scand. 58, 88-96, 1978.

12. H. Agren and L. Oreland, Psychiatry Research, 7, 245-254, 1982.

13. J.J. Schildkraut, Pharmakopsychiat, 15, 121-127, 1982.

14. J.W. Maas, S.E. Hattox, N.M. Greene and D.H. Landis, Science, 205, 1025-1027, 1979.

15. M.M. Amin, R. Khalid and P. Khan, Int. Pharmacopsychiat, 15, 81-85, 1980.

16. V.K. Yeragani, M. Blackman, and G. B. Baker, J. Clin. Psychiatry, 44, 27-29, 1983.

17. A.M. Krstulovic, M. Zakaria, K. Lohse, and L.B. Dziedzic, J Chromatogr, 186, 733-748, 1979.

18. L.M. Dziedzic, S.W. Dziedzic, S. Cerqueira, and S.E. Gitlow, Clin. Chim. Acta, 125, 291-297, 1982.

19. R.K. Sarani, R.C. Sahuja, N.N. Gupta, M. Hasan, K.D. Bhargava, K. Shankar and K. Kishor, Science, 200, 317-318, 1978.

20. A.M. Krstulovic, L.B. Dziedzic, S.W. Dziedzic, and S.E. Gitlow, J. Chromatogr., 223, 305-314, 1981.

21. G. Santagostino, P. Frattini, S. Schinelli, M.L. Cucchi, and G.L. Corona, J. Chromatogr., 233, 89-95, 1982.

22. P. Frattini, G. Santagostino, M.L. Cucchi, G.L. Corona, and S. Schinelli, Clin. Chim. Acta, 125, 97-105, 1982.

23. H. Ong, F.C. Antonini, N. Yamaguchi and D. Lamontagne, J. Chromatogr., 233, 97-105, 1982.

24. A.J. Cross and M.H. Joseph, Life Science, 28, 499-505, 1981.

25. H. Dekirmenjian and J.W. Maas, Anal. Biochem., 35, 113-122, 1970.

26. S. Wilk, K.L. Davis, and S.B. Thacker, Anal. Biochem., 39, 498-504, 1971.

27. A.E. Halaris, E.M. Demet, and M.E. Halari, Clin. Chim. Acta, 78, 285-294, 1977.

28. C.M. Davis and D.C. Fenimore, Anal. Biochem., 106, 517-523, 1980.

29. F. Karoum, H. Lefevre, L.B. Bigelow, and E. Costa, Clin. Chim. Acta, 43, 127-137, 1973.

30. B. Sjoquist, B. Lindstrom, and E. Anggard, J. Chromatogr., 105, 309-316, 1975.

31. J.W. Maas, S.E. Hattox, D.H. Landis, and R.H. Roth, Brain Research, 118, 167-173, 1976.

32. D.C. Jimerson, S.P. Markey, J.A. Oliver, and I.J. Kopin, Biomed. Mass. Spec., 8, 256-259, 1981.

33. J.J. Warsh, D.D. Godse, S.W. Cheung, and P.P. Li, J. Neurochem., 36, 893-901, 1981.

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LIQUID CHROMATOGRAPHIC ANALYSIS OF PENTAZOCINE AND TRIPELENNAMINE IN COMBINATION

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ABSTRACT

A reverse phase and normal phase liquid chromatographic procedure is described for the separation of pentazocine and tripelennamine. The isocratic methods use dual wavelength detection at 254, 280, and 313 nm.

INTRODUCTION

Pentazocine and tripelennamine is a combination of drugs which has been encountered in many parts of the United States as an intravenous substitute for heroin. Pentazocine, the active drug ingredient in Talwin tablets, is a potent analgesic with comparisons of analgesic properties made to codeine (1). Tripelennamine is an ethylenediamine class of antihistamine producing somnolence in a fair proportion of patients (2). Recently, tripelennamine has been encountered in counterfeit Quaalude preparations.

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The combined intravenous abuse of pentazocine and tripelennamine is referred to on the streets as "T's and Blue's" or "T's and B's." The "T's" is derived from the trade name of the 50 mg Talwin tablet and "Blue's" from the blue color of the PBZ or tripelennamine tablet. As early as 1973 in the state of Alabama Talwin tablets were documented as an intravenous drug of abuse when several deaths were documented as the result of pulmonary complications due to binder in the tablets. The combined abuse of pentazocine and tripelennamine apparently began about 1977, mainly in Chicago and St. Louis (3,4), and then spread throughout the Midwest (5).

Numerous items of drug paraphernalia suspected of containing pentazocine and tripelennamine have been submitted to this agency for analysis. High pressure liquid chromatography (HPLC) has proven to be an effective screening method for the presence of the two drugs. Reverse phase HPLC is a useful technique for screening paraphernalia when the suspected drugs are dissolved in an aqueous medium for injection. Thin layer chromatography (6) and gas chromatography/mass spectrometry (7) have been used for the simultaneous detection of pentazocine and tripelennamine. In a recent publication Monforte et al. (8) described a

procedure for the combined detection of pentazocine and tripelennamine by thin layer chromatography (TLC), gas chromatography (GC), ultraviolet spectrophotometry (UV), and spectrofluorometry in biological specimens. However, HPLC data was not included. This paper identifies the HPLC methods which have proven effective in identifying pentazocine and tripelennamine in combination.

EXPERIMENTAL

Reagents and Chemicals

Pentazocine hydrochloride and tripelennamine hydrochloride were obtained from their respective manufacturers and used without further purification. All solvents were HPLC grade except diethylamine which was reagent grade and were purchased from Fisher Scientific Co., Fair Lawn, NJ.

Instrumentation

The liquid chromatograph consisted of a Waters Associates (Milford, MA) Model 6000 A pump, Model U6K injector, Model 440 UV detector with dual wavelength accessory capable of operation at 254, 280, and 313 nm, and a Houston Instrument (Austin, TX) OmniScribe dual pen recorder.

Chromatographic Procedures

Reverse phase separations were carried out on a 30 cm x 3.9 mm id μBondapak C_{18} column (Waters Associates) at ambient temperature. The analytical column was preceded by a 7 cm x 2.1 mm id guard column dry packed with CO:Pell ODS (Whatman Inc., Clifton, NJ). Powdered samples (10 mg each) of the two drugs were dissolved in HPLC grade methanol and chromatographed using a mobile phase (solvent system A) of pH 3.0 phosphate buffer - HPLC grade methanol - HPLC grade acetonitrile (10+3+1). The pH 3.0 phosphate buffer was prepared by mixing 9.2 g monobasic sodium phosphate (NaH_2PO_4) in 1 L double distilled water and adjusting the pH to 3.0 with 2N H_3PO_4. The mobile phase flow rate was 2.0 mL/min and the detector was operated at 0.2 AUFS. Absorbance ratios were calculated from the average peak height measurements of a minimum of 3 injections for each drug tested.

Normal phase separations were carried out using a 30 cm x 3.9 mm id μPorasil column (Waters Associates) at ambient temperature. The analytical column was preceded by a 7 cm x 2.1 mm id guard column dry packed with HC Pellosil (Whatman Inc., Clifton, NJ). The mobile phase (solvent system B) was a mixture of cyclohexane-methylene chloride-methanol-diethylamine

(450+40+10+0.5). The mobile phase flow rate was 1.5 mL/min and the detector was operated at 0.2 AUFS. Absorbance ratios were calculated in the same manner as described above.

RESULTS AND DISCUSSION

The UV absorption spectra of pentazocine and tripelennamine have proven useful in screening various samples suspected of containing this combination of drugs. Pentazocine exhibits absorption maxima at approximately 280 and 222 nm in aqueous acidic medium while tripelennamine absorbs at approximately 314 and 240 nm. Monforte et al. (8) presented a detailed description of the ultraviolet spectra of these drugs when present in combination in an earlier publication. The absorption properties of these two drugs make them likely candidates for separation by HPLC with ultraviolet detection.

The chromatographic properties of pentazocine and tripelennamine were examined in reverse phase and normal phase systems. The reverse phase separation was maximized using an isocratic solvent system of pH 3.0 phosphate buffer-methanol-acetonitrile (10+3+1). The separation from a representative sample which was removed from a bottle cap is shown in Figure 1. The

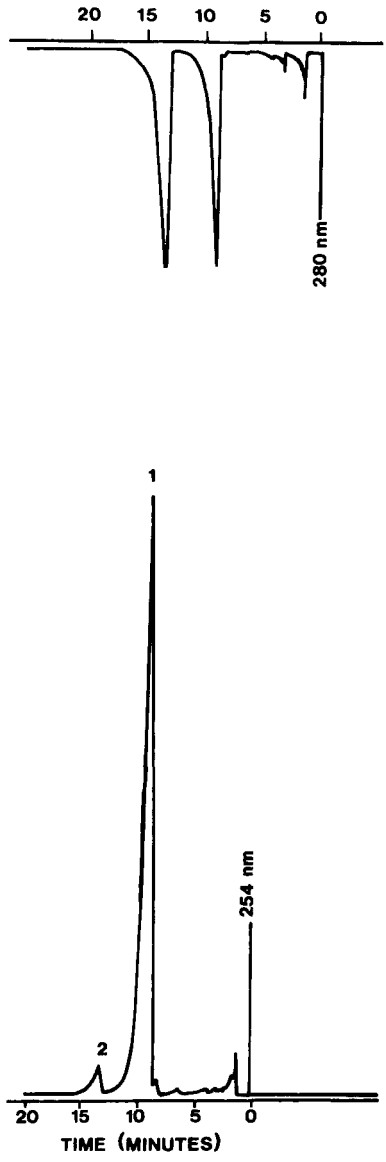

FIGURE 1.

Reverse phase liquid chromatogram of (1) tripelennamine and (2) pentazocine at 254 and 280 nm. Mobile Phase, Solvent System A.

highly acidic mobile phase produces good resolution and sufficient solute retention. Under these conditions pentazocine (pKa 8.76) and tripelennamine (pKa 4.2, 8.71) should exist exclusively as the protonated amine.

The addition of acetonitrile to the mobile phase rather than using pH 3.0 phosphate buffer-methanol (5+2) decreased the elution time of tripelennamine and pentazocine approximately 3 min and 5 min respectively as well as improved the symmetry of the peaks. Figure 2 illustrates the improved peak symmetry and retention times using a combination of methanol and acetonitrile in the mobile phase.

The normal phase separation of pentazocine and tripelennamine was accomplished using an isocratic solvent system of cyclohexane-methylene chloride-methanol-diethylamine. Figure 3 illustrates the separation of a representative sample removed from a bottle cap.

Further proof of the identity of pentazocine and tripelennamine may be obtained from a ratio of absorbances at various wavelengths. Baker et al. (9) have used the A_{254}/A_{280} ratio to determine the identity of drugs having similar elution characteristics in an HPLC system. However, the interlaboratory use of

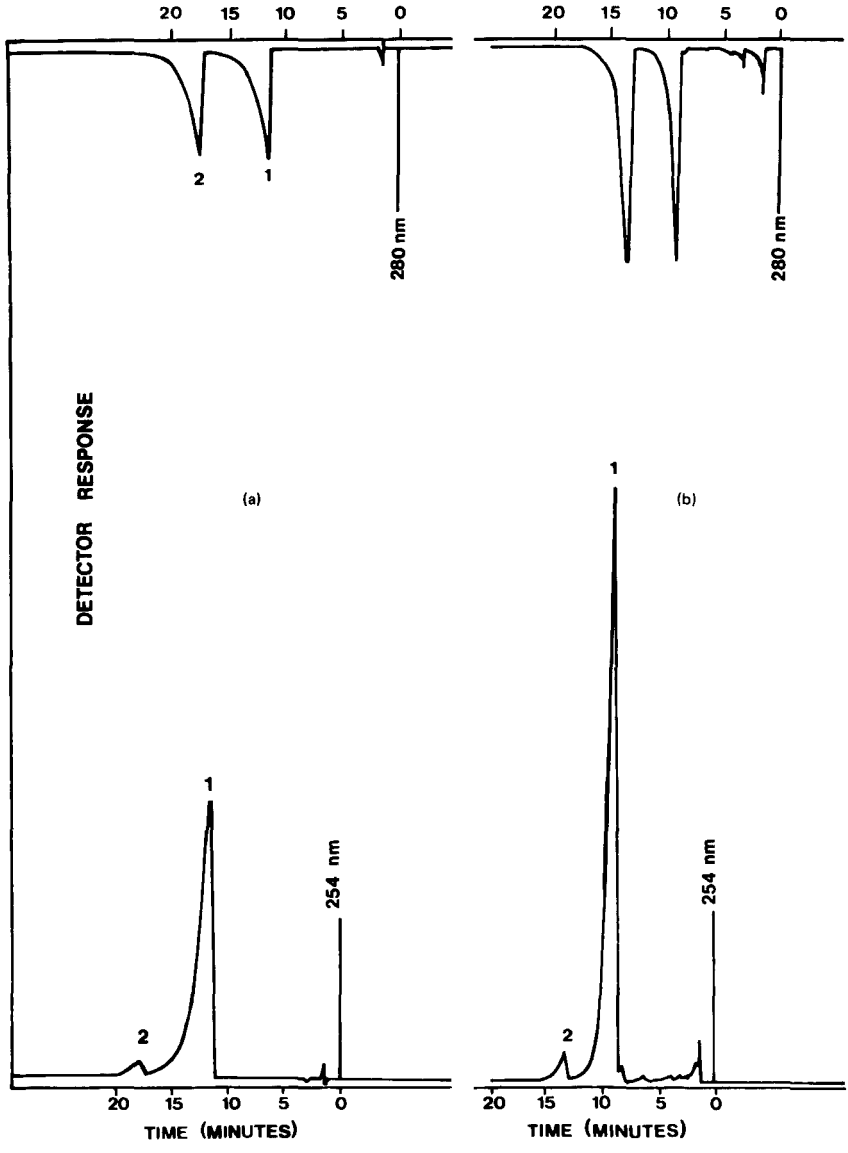

FIGURE 2

Reverse phase liquid chromatograms of (1) tripelennamine and (2) pentazocine at 254 and 280 nm. A, pH 3.0 phosphate buffer - methanol (5+2). B, pH 3.0 phosphate buffer - methanol - acetonitrile (10+3+1).

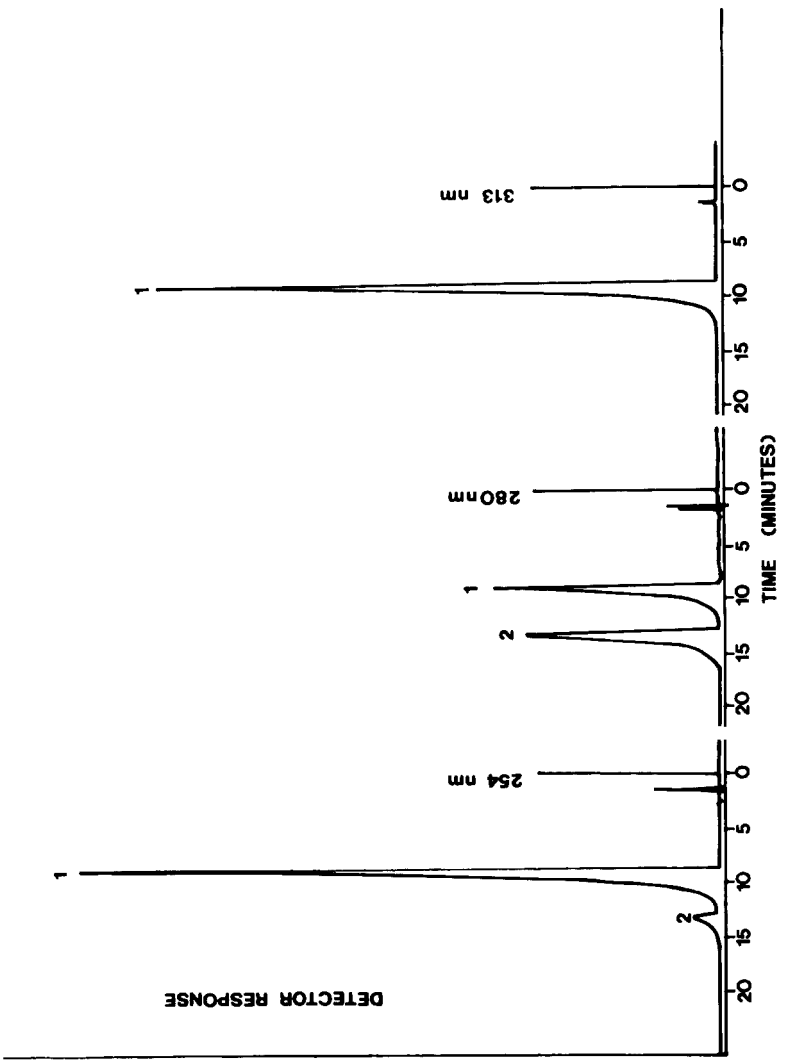

FIGURE 3

Reverse phase liquid chromatograms of (1) tripelennamine and (2) pentazocine illustrating the differences in absorbance at 313, 280, and 254 nm. Mobile Phase System A.

TABLE 1

Retention and Absorbance Data
for Pentazocine and Tripelennamine

Solvent System	Drug	Retention Time (min.)	Absorbance Ratios A_{254}/A_{280}	A_{254}/A_{313}	A_{280}/A_{313}
A	Tripelennamine	9.0	2.78	1.15	0.40
A	Pentazocine	13.6	0.15	----	----
B	Tripelennamine	3.7	14.20	4.91	0.35
B	Pentazocine	9.0	0.11	----	----

these ratios should be approached with caution. Variations may occur in the absorbance ratios because molar absorptivity and the wavelength of maximum absorbance vary with solvent composition, pH, and other factors (10). Absorbance ratios should be determined in an individual chromatographic system using reference standards. Table 1 gives retention data and absorbance ratios of pentazocine and tripelennamine which were determined in this study.

Figure 4 illustrates the variation in absorbance of the two drugs in the reverse phase system at 254, 280, and 313 nm. Since pentazocine exhibits no appreciable absorbance at 313 nm in the solvent systems investigated, this wavelength is of no value in the identification of pentazocine. A compromise wavelength which affords sufficient sensitivity for simultaneous detec-

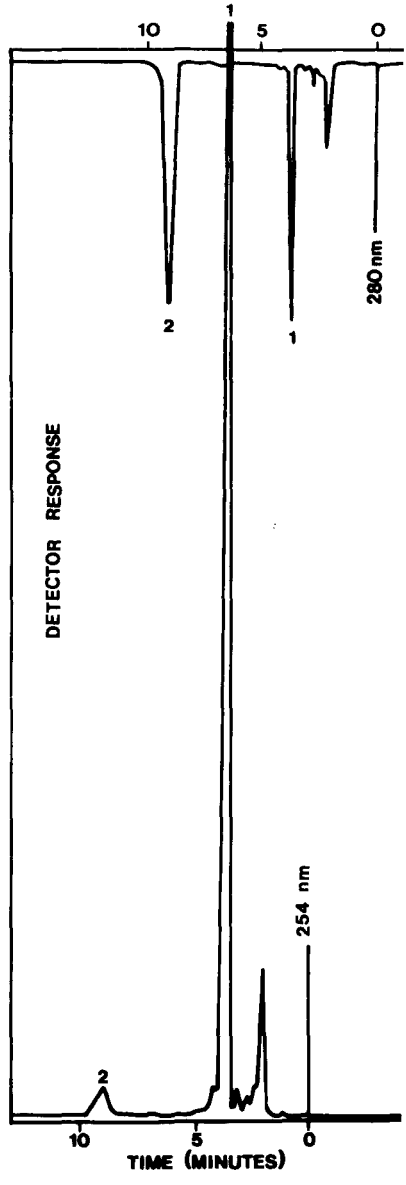

FIGURE 4

Normal phase liquid chromatogram of (1) tripelennamine and (2) pentazocine at 254 and 280 nm. Mobile Phase, Solvent System B.

tion of the two drugs utilizing a fixed wavelength detector is 280 nm. In most items of paraphernalia examined to date, the ratio of pentazocine and tripelennamine has varied. However, the majority of the items contain a greater quantity of pentazocine than tripelennamine confirming the findings of Monforte et al. (8).

REFERENCES

1. Physicians' Desk Reference, Medical Economics Company, Oradell, NJ, 37th Ed., 1983.
2. Goodman, L. S. and Gilman, A., The Pharmacological Basis of Therapeutics, 4th Ed., Macmillan, New York, NY.
3. Showalter, C. V. and Moore, L., JAMA, 239, 1610, 1978.
4. Poklis, A., JAMA, 240, 10, 1978.
5. DeBard, M. L. and Jagger, J. A., Clin. Tox., 18, 1117, 1981.
6. Kaistha, K. K. and Tadrus, R., J. Chromatogr., 155, 214, 1978.
7. Reid, R. W., and Gerbeck, C. M., Clin. Chem., 27, 10, 1981.
8. Monforte, J. R., Gault, R., Smialek, J., and Goodin, T., J. For. Sci., 28, 90, 1983.

9. Baker, J. K., Shelton, R. E., and Ma, C. Y., J. Chromatogr., 168, 417, 1979.

10. Conners, K. A., A Textbook of Pharmaceutical Analysis, 2nd Ed., John Wiley and Sons, New York, NY, 1975.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PENICILLINS FOLLOWING DERIVATIZATION TO MERCURY-STABILIZED PENICILLENIC ACIDS

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ABSTRACT

A high-performance liquid chromatographic, pre-column derivatization procedure is described, allowing the determination of natural and semi-synthetic penicillins by absorption at 325nm. Derivatization with an imidazole/mercuric chloride reagent is followed by reverse-phase chromatography of the resulting mercury-stabilized penicillenic acids on an octadecylsilane, chemically-bonded stationary phase. Gradient elution is employed with a mobile phase containing organic modifier, buffer and ethylenediaminetetraacetic acid. Pre-column reaction conditions including time and temperature are examined. Accuracy, reproducibility and detection limits of the method are discussed. The technique is applied to the analysis of fermentation media following sample preparation by centrifugation and deproteinization.

INTRODUCTION

The efficacy of penicillin and its derivatives has resulted in their widespread use for many years. With all such antibiotics, assay systems are required which enable accurate and precise quantitation.

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Several physico-chemical techniques for the determination of penicillins have been developed (1). These include the iodometric titration (2), and colorimetric assays such as the hydroxylamine (3) and molybdoarsenic acid-mercuric chloride methods (4). The isomerism of penicillins to their corresponding penicillenic acids which absorb at 325nm, has also been used in the determination of β-lactams (5). However, many of these assay systems are severely limited in their application. The methods often lack intrinsic sensitivity or fail to distinguish individual penicillin levels, when two or more β-lactams are present. Also, the occurrence of interfering components in biological media such as serum or microbial fermentation broths often produce misleading results due to lack of reagent specificity and/or the presence of high background readings.

The development of high-performance liquid chromatography (HPLC), has enabled the rapid, sensitive and quantitative determination of many antibiotics, including penicillins (6).

An extensive range of stationary and mobile phases has been reported for the analysis of β-lactams (7-9). Optimum chromatographic conditions appear to have been achieved using reverse-phase systems, often employing ion-pairing reagents to increase the capacity factor of highly polar compounds (10). Many HPLC detection methods are now available (11), although the most widely used is U.V. absorption. Detection of penicillins by this technique is usually performed at 254nm or 220nm. However, our interest lies in the microbial biosynthesis of β-lactams (12-14), and we have found the presence of interfering components in complex fermentation media to be problematic when using these wavelengths.

A derivatization procedure was therefore considered, to enable detection of penicillins at a longer wavelength, there-

by reducing interference from other media constituents. Such problems have been overcome to some extent, by use of post-column derivatizing agents, including fluorescamine (15) and o-phthaldialdehyde (16). However, both reagents are limited to the detection of β-lactams possessing a primary amino function.

Treatment of penicillins with an imidazole/mercuric chloride reagent was investigated as a possible pre-column derivatization method. The slow reaction rate prevented the use of post-column derivatization.

The reaction is highly specific, and the resulting penicillenic acids, after stabilization as mercury (II) complexes, can be detected at 325nm (17) following HPLC.

EXPERIMENTAL

Materials and Reagents

Acetonitrile was HPLC S grade and obtained from Rathburn Chemicals (U.K.). Other mobile phase constituents, including ethylenediaminetetraacetic acid (disodium salt), and sodium dihydrogen orthophosphate were of "analar" grade and supplied by B.D.H.(U.K.).

Reagent grade imidazole was supplied by Sigma, London, (U.K.), whilst the mercuric chloride and hydrochloric acid (concentrated) used in the pre-column derivatization reagent were of "analar" grade and obtained from B.D.H.(U.K.).

β-lactamase Type I, used in the preparation of fermentation broth "controls", was obtained from Sigma, London, (U.K.).

Penicillins K (sodium salt), N (barium salt), V, G (potassium salts), X (zinc salt) and methicillin (sodium salt) were kindly donated by Ciba-Geigy (Switzerland) and Glaxo (U.K.).

Apparatus and Operating Conditions

The solvent delivery system used consisted of two, double reciprocating pumps (model No. 750/04; Applied Chromatography

Systems, U.K.) controlled by a high pressure mixing unit (model No. 750/36; Applied Chromatography Systems, U.K.), enabling gradient formation. Injections were made using a Rheodyne valve (model No. 7125; Anachen, U.K.) fitted with a 20µl loop. Chromatography was performed on a 20 x 4.6 mm ID analytical column slurry-packed (18) with 5µm Spherisorb C18 (Cat. No. S5ODS2; Phase Separations, U.K.). A 4 x 10 mm ID guard-column was fitted prior to and in series with the analytical column and was tap-packed (18) with 5µm Spherisorb C18.

U.V. detection was carried out at 325nm, using a variable wavelength detector (Spectromonitor III; Laboratory Data Control, U.S.A.) fitted with a 12µl flow cell. Integration of peak areas and data handling was performed by a Hewlett-Packard reporting integrator (model No. 3390A; Hewlett-Packard, U.S.A.).

The mobile phase was prepared by mixing various proportions of CH_3CN with an aqueous solution containing O.01M NaH_2PO_4 and O.01M EDTA. The solvent was then adjusted to pH 6.5 with 2.0M NaOH. Degassing was performed by the continual passage of helium through the solution.

The imidazole/$HgCl_2$ reagent was prepared in plastic-capped, glass vessels by dissolving 4.125g of imidazole (recrystalized twice form propan-2-ol) in 2.5ml of distilled water. After addition of 1.0ml of HCl, 0.5ml of $HgCl_2$ (0.11M) were added, and finally a futher 1.5ml of HCl.

Pre-Column Reaction System

To each 1ml sample aliquot, 0.1ml of the imidazole /$HgCl_2$ reagent (prepared as previously described) was added. The reaction mix was then placed, for the period of time described, in a water bath thermostatically controlled to the required temperature. Samples were then rapidly cooled to room temperature by immersion in an ice bath and injected onto the column.

Sample Preparation

Preparation of fermentation broths involved centrifugation, (10mins. at 8,000xg) following adjustment of the pH to 7. The supernatant was then removed, and subjected to deproteinization by the method described by Rudrick and Bawdon (19), involving protein precipitation by acetonitrile, subsequent to re-extraction with methylene chloride. After final centrifugation (15mins. at 8,000xg) and removal of the aqueous layer, the sample was then treated with the derivatization reagent, as described above.

Using an analytical microsyringe, fermentation broths were spiked, prior to deproteinization, with standard solutions of the penicillins. These were prepared daily by dilution with distilled water to the required concentration from a stock of 1mg/ml.

"Control" samples were prepared by addition of 50µl of β-lactamase (final concentration 4 units/ml) to 1ml of the fermentation broth. Following incubation at 25°C for 2 hours, samples were then treated by deproteinization and pre-column derivatization as previously described.

RESULTS AND DISCUSSION

Detection Wavelength

Detection of the penicillenic acids which were produced by reaction with imidazole and stabilized by complexation with mercury (II), was carried out at 325nm. Cuvette studies had previously shown this to be the absorbance maximum of these derivatives (20). This was confirmed by using a "stop-flow" HPLC technique which produced an absorption band centred at 325nm.

Chromatographic Conditions

Fig. 1 indicates a typical chromatogram obtained following pre-column derivatization of four β-lactams with the

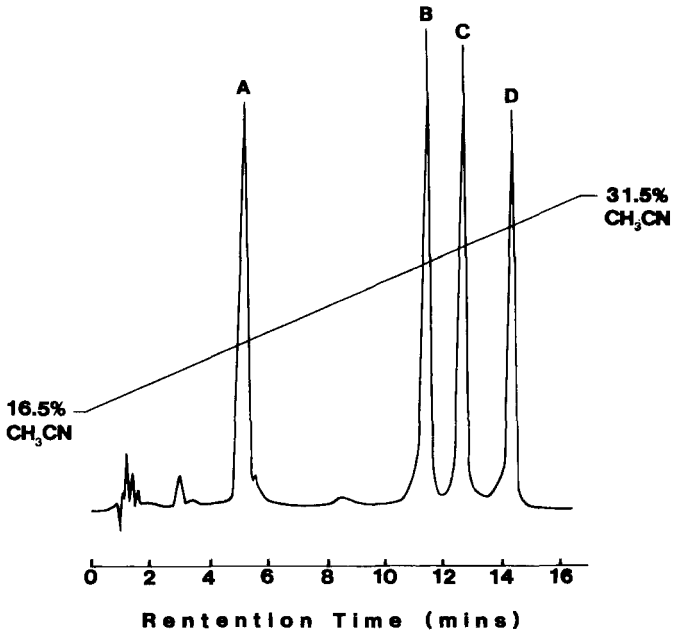

FIGURE 1

HPLC of penicillin X (50µg/ml) (A), methicillin (75µg/ml) (B), penicillin G (50µg/ml) (C) and penicillin V (60µg/ml) (D), following pre-column derivatization with the imidazole/$HgCl_2$ reagent, at 50°C for 50 minutes. Chromatographic conditions were as described in the text, using a gradient of acetonitrile as illustrated. Flow rate was 2.0ml/min. with a detection wavelength of 325nm, and an attenuation of 0.05 AUFS. Injection volume: 20 µl.

imidazole reagent. Chromatography of the products was optimized at a pH of 6.5 and by the inclusion of EDTA in the mobile phase. Although the role of the polyaminocarboxylic acid is unclear, its exclusion resulted in very broad, unsymmetrical peaks. It seems likely that the high complexing ability of EDTA is of importance as its substitution with succinic or citric acid produced poor chromatographic results. Replacement with trans-1,2-diaminocyclohexanetetraacetic acid however,

gave similar results to those obtained with EDTA. The complexing agent may act by removing excess mercury (II) ions or by forming a more stable penicillenic acid-mercury-EDTA complex.

Pre-Column Reaction Conditions

The effects of pre-column reaction time and temperature were investigated by HPLC analysis of the reaction products. Fig. 2 illustrates the results obtained with four, naturally-occurring β-lactams.

At 50°C, the rate of formation of the mercury-stabilised penicillenic acids was slower than at higher temperatures. After 50 mins. the maximum sensitivity for all four penicillins was obtained and longer periods of incubation at this temperature produced no significant increase of decrease in the peak areas.

Raising the incubation temperature to 70°C or 80°C resulted in not only an increase in the rate of formation of the penicillin derivatives but also their decay rate. At 70°C, slightly higher sensitivities than at 50°C were achieved for all penicillins studied. However, the optimum incubation time varied slightly with different penicillins (25-35mins.) and was more critical than at 50°C. For temperatures in excess of 70°C, the maximum sensitivity achievable, irrespective of incubation time, was always lower than that for derivatization at 50°C or 70°C. In further studies, the pre-column reaction conditions of sample incubation for 50 minutes at 50°C, were therefore chosen to provide maximum sensitivity and product stability.

Reaction of the imidazole/$HgCl_2$ reagent with penicillin K resulted in a derivative with relatively low intrinsic sensitivity, whilst with penicillin N the reaction proved completely unsuccessful. As both these β-lactams possess an aliphatic side-chain, it may be that the rate of product

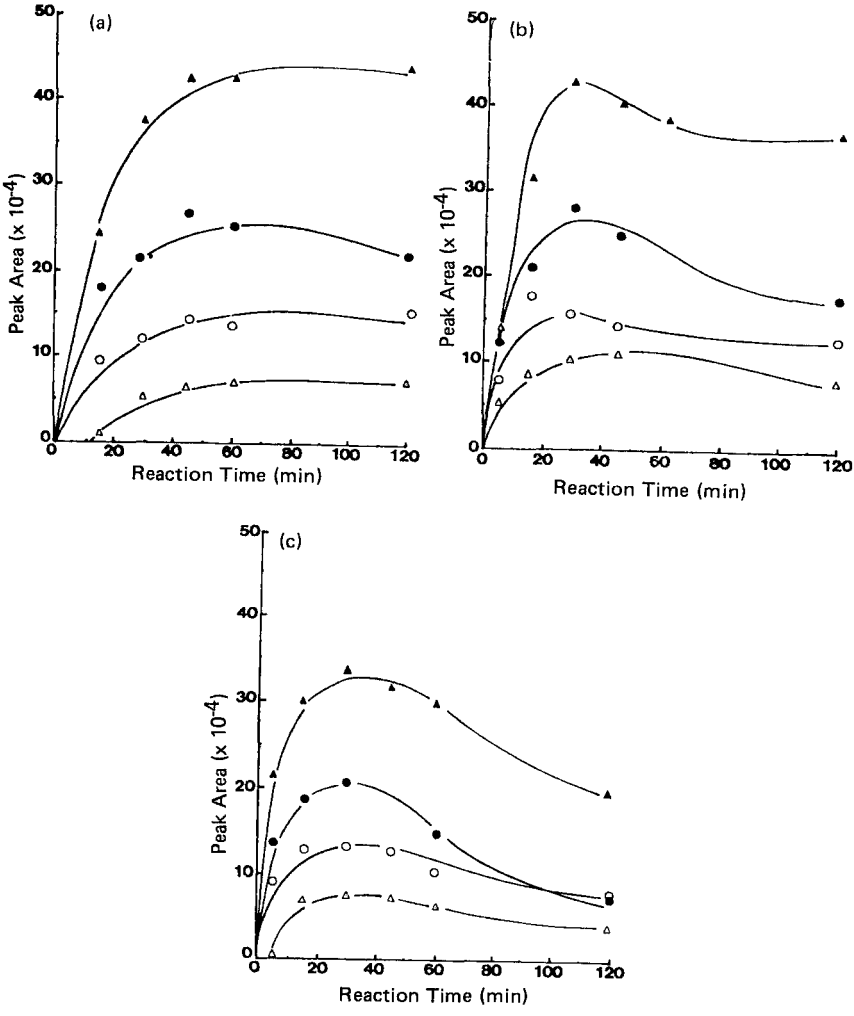

FIGURE 2

The effects of reaction time on the pre-column derivatization of penicillin X (o), penicillin V (●), penicillin K (Δ) and penicillin G (▲), at 50°C (A), 70°C (B) and 80°C (C). Chromatographic conditions were as described under Fig.1.

decay is reduced by aromatic stabilization of the oxazolone ring in derivatives of penicillin G, V, X and methicillin.

Substitution of mercury in the reagent with iron (II), zinc (II) or nickel (II) failed to produce an absorption at 325nm, following penicillin derivatization. However, with copper the reaction yielded two major products, detectable at this wavelength, and resolved by HPLC. Although the use of other metals has yet to be investigated, it is clear that at the present time, mercury, producing one, stable reaction product, is most suited to the assay system.

Accuracy and Reproduceability

During these studies chromatographic and pre-column reaction conditions were maintained as described under Fig. 1.

Linearity of the method with respect to concentration of penicillins X and G was checked. Serial dilutions of each β-lactam between 0 and 50µg/ml were prepared in triplicate, and, following pre-column derivatization, 20µl of each solution injected onto the column. Peak areas were used to construct calibration lines, giving a correlation coefficient of 0.997 for penicillin X and G. Gradients of 14959 area units/µg/ml for penicillin X and 15443 area units/µg/ml of penicillin G were obtained, by regression analysis. Good day-to-day reproducibility was obtained by repeating the calibration lines on several different occasions. Reproducibility of a 20µl repeated injection (n=10) of penicillin X (25µg/ml) gave a 2% error from the mean.

Accuracy was investigated by spiking fermentation media with known concentrations of penicillin G. Using penicillin X as an internal standard at 10µg/ml, and following sample treatment as described under apparatus and operating conditions, the ratio of peak areas was used to calculate a theoretical penicillin G concentration. The results are listed in Table 1.

TABLE 1

DETERMINATION OF PENICILLIN G IN FERMENTATION MEDIA

SAMPLE No.	PENICILLIN G CONCENTRATION (μg/ml) THEORETICAL	EXPERIMENTAL	ERROR (%)
1	17.0	18.0	5.5
2	4.5	4.3	4.4
3	24.0	22.5	6.25
4	37.5	35.2	6.1
5	50.0	48.9	2.2

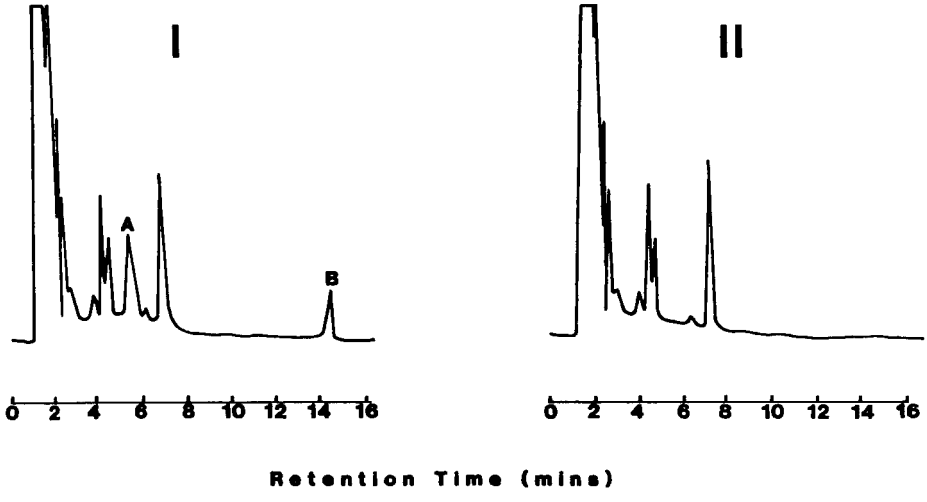

FIGURE 3

(I) HPLC analysis of a typical fermentation broth containing penicillins X (5μg/ml) (A) and G (5μg/ml) (B), following deproteinization and pre-column derivatization with imidazole/$HgCl_2$.

(II) Chromatography of a fermentation broth "control", resulting from β-lactamase treatment of the sample shown in Fig. 3 (I).

Derivatization and chromatographic conditions were as described under Fig. 1.

Sensitivity and Detection Limits

Using 20µl injections, detection limits of 1µg/ml were achieved for penicillin X and penicillin G, in samples of fermentation media. Although higher sensitivities may be obtained by use of larger injection volumes, 20µl was used to prevent possible overloading of the column. Fig. 3A illustrates a typical fermentation broth, containing penicillins X and G (both 5µg/ml), whilst Fig. 3B indicates the use of β-lactamase treatment in preparation of a "control" sample.

Sample preparation by deproteinization was necessary to ensure linearity and high levels of detection. However, this treatment was found to be unnecessary in batch reactions where absorbance at 325nm was measured in a cuvette. A possible explanation to these somewhat contradictory results may lie in reaction of the highly reactive N-penicilloylimidazole intermediate of the imidazole catalysed isomerism, with free amino and thiol groups of proteins (21). The product, whilst still containing the molecular structure necessary for U.V. absorption at 325nm, will undoubtedly have different chromatographic characteristics to the "free" penicillenic acid.

CONCLUSIONS

The pre-column derivatization procedure described allows the rapid and selective determination of semi-synthetic and naturally produced penicillins in a variety of biological media, including microbial fermentation broths. Although the procedure appears inapplicable to β-lactams such as penicillin N, the possibility exists for detection of 6-amino penicillenic acid by its prior acylation.

The method may be used for the determination of known penicillins and also has potential value for the detection of novel penicillins, due to the specificity of the reagent.

ACKNOWLEDGEMENTS

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REFERENCES

(1) Hughes, D. W., Vilim, A. and Wilson, W. L., Can. J. Pharm. Sci., 4, 97, 1976.

(2) Goodall, R. R. and Davies, R., Analyst (London), 86, 326, 1961.

(3) Ford, J. H., Anal. Chem., 19, 1004, 1947.

(4) Holm, K. A., Anal. Chem., 44, 795, 1972.

(5) Brandriss, M. W., Denny, E. L., Huber, M. A. and Steinman, H. G., Antimicrob. Agents Chemother., 626, 1962.

(6) Lebelle, M. J., Lauriault, G. L. and Wilson, W. L., J. Liquid Chromatogr., 3, 1573, 1980.

(7) Buhs, R. P., Maxim, T. E., Allen, N., Jacobs, T. A. and Wolf, F. J., J. Chromatogr., 99 , 609, 1974.

(8) White, R. E. and Fox, M., J. Antibiot., 35 , 1538, 1982.

(9) Miller, R. D. and Neuss, N., J. Antibiot., 29, 902, 1976.

(10) White, R. E. and Zarembo, J. E., J. Antibiot., 34, 836, 1981.

(11) Hamilton, R. J. and Sewell, P. A., Introduction to High Performance Liquid Chromatography, Chapman and Hall, New York, 1982.

(12) Makins, J. F., Allsop, A. and Holt, G., Adv. in Biotech., 3, 51, 1982.

(13) Macdonald, K. D. and Holt, G., Sci. Progr. (London), 63, 547, 1976.

(14) Normansell, P. J. M., Normansell, I. D. and Holt, G., J. Gen. Microbiol., 112, 113, 1979.

(15) Crombez, E., Van der Eken, G., Van den Bossche, W. and De Moerloose, P., J. Chromatogr., 177, 323, 1979.

(16) Rogers, M. E., Adlard, M. W., Saunders, G. and Holt, G., J. Chromatogr., 257, 91, 1983.

(17) Bundgaard, H., Tetrahedron Lett., 48, 4613, 1971.

(18) Snyder, L. R. and Kirkland, J. J., Introduction to Modern Liquid Chromatography, Wiley, New York, 1979.

(19) Rudrik, J. T. and Bawdon, R. E., J. Liquid Chromatogr., 4, 1525, 1981.

(20) Bundgaard, H. and Ilver, K., J. Pharm. Pharmacol., 24, 790, 1972.

(21) Bundgaard, H., Dansk. Tidsskr. Farm., 46, 29, 1972.

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LIQUID CHROMATOGRAPHIC ANALYSIS OF PENTOBARBITAL

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ABSTRACT

A method is described for a one step acetonitrile precipitation of serum or plasma and subsequent analysis of pentobarbital by reverse phase HPLC. The results of using two internal standards, N,N-Diethyl-m-toluamide and 5-(p-Methylphenyl)-5-phenylhydantoin are compared. Internal standard is added to serum (as little as 25 μL) and vortex-mixed with acetonitrile followed by centrifugation. An aliquote of the supernatant is analyzed on a C18 reverse phase column eluted with metanol/0.05 M $(NH_4)_2HPO_4$, pH 8/water (55/20/25, v/v/v). The effluent is monitored at 220 nm.

INTRODUCTION

Several disorders have been clinically treated using pentobarbital. These include Reye's syndrome (1), head injury (2) cerebral ischemia (3,4) and metabolic coma (5). Serious side effects can result from extended therapy or overdose making it necessary to have a quick plasma assay available. Pentobarbital has been assayed by GLC (6-8), however these procedures require both extraction and derivatization. HPLC drug screening procedures, which include pentobarbital, have been published (9-11) but no quantitative results were reported for pentobarbital. During the course of this study two quantitative HPLC methods have appeared (12,13) both utilizing extraction and reconcentration prior to analysis. The internal standard 5-(p-Methyl-phenyl)-5-phenylhydantoin has been used in the HPLC analysis of other barbiturates and

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drugs (11,14). We reported previously on the use of N,N-Diethyl-m-toulamide as an internal for acetaminopen and salicylate quantitation by HPLC (15).

In this paper we describe a quick one step precipitation procedure for the analysis of pentobarbital by reverse phase HPLC and compare the use of the aforementioned internal standards, which differ greatly in price.

MATERIALS AND METHODS

Instrumentation

A constant volume liquid chromatograph from Waters (Milford, MA.) consisting of a model 6000A solvent delivery system, a U6K sample injector and a model 450 detector (interfaced with a Waters 730 Data System) set at 220 nm was used. The detector was set at 0.04 for routine analysis. The column was a 4.5 x 150 mm prepacked 5 micron C18 from IBM Instruments (Wallingford, CT.).

Reagents

Methanol (MeOH) and acetonitrile (ACN) were HPLC grade obtained from Fisher Scientific (Detroit, MI.), as was $(NH_4)_2HPO_4$. Pentobarbital and internal standard (IS) 5-(p-Methylphenyl)-5-phenylhydantoin (5-MPPH) were obtained from The Anspec Co., Inc. (Ann Arbor, MI.). The IS N,N-Diethyl-m-toluamide was obtained from Chemical Dynamics Corp. (South Plainfield, NJ.).

A standard solution of pentobarbital was prepared by dissolving 40 mg in a liter of MeOH. This solution was found to be stable for up to two months at -20°C. Most of the experiments were carried out using a plasma standard solution prepared by dissolving 40 mg of pentobarbital in 2 ml MeOH and diluting this to a liter with drug free plasma obtained from our Blood Bank. This solution was then aliquoted in 500 µL amounts stored at -20°C until used. These aliquots have been stable for 3 months. The various dilutions were made from these aliquots by the addition of drug free plasma.

A stock IS solution (250 mg/L) of 5-(p-Methylphenyl)-5-phenylhydantoin was prepared in MeOH and is stable for about 1 month at 4°C. A 10-fold dilution working IS was prepared daily.

The IS N,N-Diethyl-m-toluamide (50 mg/L) was also prepared in MeOH and has been stable at room temperature for several months.

The mobile phase consisted of MeOH/0.05 M $(NH_4)_2$ PO_4, pH 8/H_2O (55/20/25 v/v/v).

Procedure

Add 200 µL of ACN and 50 µL of IS to 100 µL of serum or plasma in a 10 x 75 mm glass test tube. Vortex-mix for 15 sec., followed by a 2 min. centrifugation (2500 r.p.m.). An aliquot of the supernatant (usually 20 µL) was injected into the chromatograph and the column eluted with mobile phase at a flow rate of 1.3 mL/min. We have scaled this procedure down to using 25 µL of sample with no observed differences.

Quantification of the samples was carried out by the Waters 730 Data System following calibration with a 20 µg/mL standard plasma solution. Calculations were performed using the peak height calibration mode.

For routine analysis of patient samples the 20 µg/mL standard solution is used to calibrate the Data Module and high and medium level controls (Utak Lab, Saugus, CA.) are used as a procedure control.

RESULTS AND DISCUSSION

Figure 1 shows several typical HPLC chromatograms obtained using our described procedure. Figure 1A is the drug free plasma. Figure 1B is our plasma standard solution containing 20 µg/mL pentobarbital (RT = 6.21) and N,N-Diethyl-m-toluamide (RT = 9.58) as IS. Figure 1C is the plasma standard solution containing 10 µg/mL pentobarbital (RT = 6.21) and 5-(p-Methylphenyl)-5-phenylhydantoin (RT = 8.04) as IS. Figure 1D is the Utak control containing

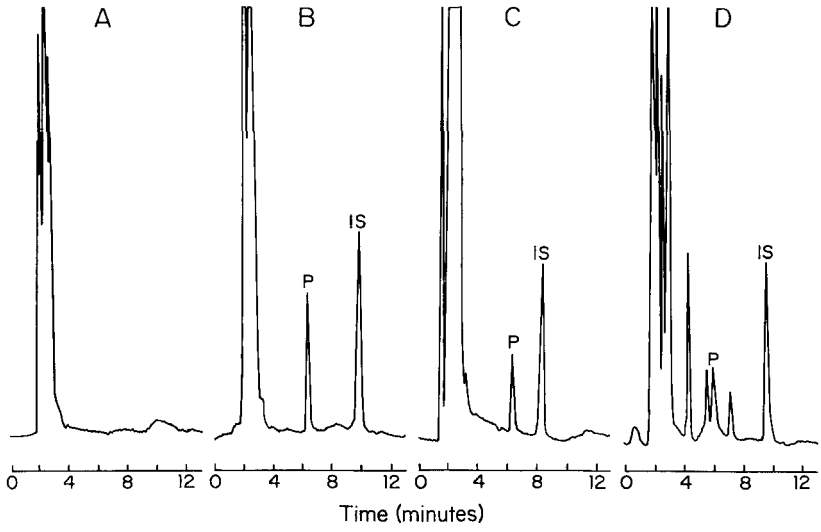

Figure 1. Typical chromatograms, obtained using the procedure given herein. (A) drug-free plasma; (B) control serum containing 20 μg/mL pentobarbital (P) and NNDET internal standard (IS); (C) control serum containing 10 μg/mL pentobarbital and 5-MPPH as IS; (D) Utak control containing 12 μg/mL pentobarbital and NNDEMT as IS. Other components include phenobarbital (RT = 3.10), butabarbital (RT = 4.38), amobarbital (RT = 5.68) and secobarbital (RT = 7.18).

12 μg/mL pentobarbital (RT = 6.18) and N,N-Diethyl-m-toluamide (RT = 9.51) as IS.

Figure 2 shows standard curves which were obtained by manual measurements and plotting peak height ratios (pentobarbital/IS) vs concentration of pentobarbital. As can be seen the response is essentially linear over the entire range tested (5 μg/mL to 40 μg/mL) using either IS.

Recovery and Linearity

Recoveries were assessed by comparing data obtained from a 100 μL aliquot of pentobarbital standards in MeOH to data obtained from drug free plasma which was adjusted to various levels of pentobarbital. These results are given in Table I. Essentially complete recovery was obtained up to a concentration

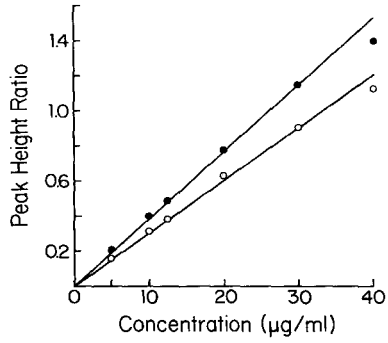

Figure 2. Pentobarbital standard curves obtained by manual calculation of peak height ratios, (●) with 5-MPPH as the IS and (○) with NNDEMT as IS. Each point is the average of 5 determinations.

of 30 µg/mL with slightly higher values being obtained using NNDEMT as IS. While the values obtained for the 40 µg/mL level are acceptable they were lower than the others, again NNDEMT gave higher recoveries. The lower recoveries of the uppel level pentobarbital explains why these levels don't fall exactly on the standard curve (Figure 2).

Accuracy and Precision

The within-run accuracy and precision was evaluated by applying the method 7 times to our plasma standards containing 5 µg/mL and 20 µg/mL of pentobarbital. The results are given in Table II. As can be seen there were slight but insignificant differences between the two internal standards. Day to day precision was assessed by assaying the 20 µg/mL plasma standard over a period of a week. The overall average of the mean coefficient of variation was 6.32% using NNDEMT as IS and 6.75% using 5-MPPH as IS.

Application

Table III shows some typical results from assays of various commercial serum controls, with and without added pentobarbital, and patient samples.

TABLE I

Recovery of Pentobarbital From Plasma.

Comparison Using Two Internal Standards

Concentration	NNDEMT	5-MPPH
µg/mL	%Recovery*	% Recovery*
5	102.1 (5.7)	98.4 (6.3)
10	99.5 (4.9)	96.3 (5.8)
30	97.3 (4.3)	93.4 (4.9)
40	89.0 (3.9)	80.3 (4.6)

*Values given as mean (S.D.), n = 5.

TABLE II

Accuracy and Precision of Pentobarbital Assay

	5 µg/mL		20 µg/mL	
Measured Values*	NNDEMT	5-MPPH	NNDEMT	5-MPPH
Concentration	5.12	4.87	20.34	19.43
S.D.	0.23	0.26	1.37	1.34
C.V.	3.3	3.4	6.78	6.69
S.E.M.	0.08	0.09	0.52	0.51

*n = 7 in all cases

TABLE III

Results of Assaying Various Samples

Sample	µg/mL Pentobarbital Expected	Found NNDEMT	Found 5-MPPH
Ortho I[1]	0	0	0
Ortho I + 10 µg/mL	10	10.19 9.97	9.84 9.76
Ortho II[1]	0	0	0
Ortho II + 10 µg/mL	10	9.94 10.06	9.23 9.56
Tox Control Mid[2]	4	3.96 4.25	3.94 3.87
Tox Control High[2]	12	11.53 11.87	11.23 11.57
Patient 1 Patient 2 Patient 3	6.2[3] 12.8 15.6	6.01 12.32 15.73	5.81 11.94 14.87

[1]Ortho Diagnostics, Raritan, NJ.. [2]Utak Lab., Saugus, CA. [3]Assayed by BioScience Laboratories, Farmington Hills, MI.

Our 20 µg/mL plasma standard was used to calibrate the Data Module. As would be expected from the previous discussed results either IS gave comparable results with 5-MPPH yielding consistently slightly lower pentobarbital levels. Table IV lists some commonly used drugs which have been shown not to interfere with the present assay for pentobarbital.

Based on the assessed accuracy and precision, linearity and simplicity this present method is easily applicable to a clinical laboratory. The small sample size requirements make this an especially useful method to a pediatric service laboratory.

TABLE IV

Some Common Drugs Not Interfering with Pentobarbital Assay

Acetominophen	Methyprylone
Amobarbital	Mephobarbital
Aprobarbital	Meprobamate
Barbital	Phenobarbital
Butabarbital	Propoxyphene
Diazepam	Salicylate
Diphenylhydantoin	Secobarbital
Methaqualone	

Both of the IS investigated appeared to work equally well. The decision as to which one to use may be based on economic mattaers. The cost of 100 g of NNDEMT is $6.50 whereas the same amount of 5-MPPH would be over 3000 times this amount.

REFERENCES

1. Frewen, T.C., Swedlow, D.B., Watcha, M., Raphaely, R.C., Godinez, R.I., Heiser, M.S., Kettrick, R.G., and Bruce, D.A.: Outcome in severe Reye syndrome with early pentobarbital coma and hypothermia. J. Pediatr. 100, 663(1982).

2. Marshall, L.F., Smith, R.W., and Shapiro, H.M.: The outcome with aggressive treatment in severe head injuries. Part II: Acute and chronic barbiturate administration in the management of head injury. J. Neurosurg. 50, 26(1979).

3. Breivik, H., Safar, P., Sands, P., Fabritius, R., Lind, B., Lust., P., Mullie, A., Orr, M., Renck, H., and Snyder, J.V.: Clinical feasibility trials of barbiturate therapy after cardiac arrest. Crit. Care Med. 6, 228(1978).

4. Belopavlovic, M., and Buchthal, A.: Barbiturate therapy in the management of cerebral ischeaemia. Anesthaesia 35, 271(1980).

5. Marshall, L.F., Shapiro, H.M., Rauscher, A., and Kaufman, N.M.: Pentobarbital therapy for intracranial hypertension in metabolic coma. Crit. Care Med. 6, 1(1978).

6. Fiereck, E.A., and Tiety, N.W.: A gas-chromatographic method for separating and measuring barbiturates and glutethimide in blood. Clin. Chem. 17, 1024(1971).

7. Greeley, R.H.: New approach to derivatization and gas-chromatographic analysis of barbiturates. Clin. Chem. 20, 192(1974).

8. Hulshoff, A., VanDerHouwen, O., and Barends, D.M.: The determination of pentobarbital and other barbiturates in plasma by gas-liquid chromatography with on-column and pre-column butylation. Anal. Chim. Acta 105, 139(1979).

9. Kabra, P.M., Stafford, B.E., and Marton, L.J.: Simultaneous measurement of phenobarbital, phenytoin, primidone, ethosuximide, and carbamazepine in serum by high-pressure liquid chromatography. Clin. Chem. 23, 1284(1977).

10. Gill, R., Lopes, A., and Moffat, A.C.: Analysis of barbiturates in blood by high-performance liquid chromatography. J. Chromatog. 226, 117(1981).

11. Kabra, P.M., Koo, H.Y., and Marton, L.J.: Hypnotics and Sedatives. In "Liquid Chromatography in Clinical Analysis" Kabra, P.M. and Marton, L.J., eds. Humana Press, Clifton, N.J. 1981, p. 223.

12. Shin, G.K., and Nemoto, E.M.: Simple, rapid and sensitive reversed-phase high-performance liquid chromatographic method for thiopental and pentobarbital determination in plasma and brain tissue. J. Chromatog. 227, 207(1982).

13. Gupta, R.N., Smith, P.T. and Eng, F.: Liquid-chromatographic determination of pentobarbital in plasma with use of a resin column and an alkaline mobile phase. Clin. Chem. 28, 1772(1982).

14. Kabra, P.M., Gotelli, G., Stanfill, R., and Marton, L.J.: Simultaneous measurement of phenobarbital, diphenylhydantoin, and primidone in blood by high-pressure liquid chromatography. Clin. Chem. 22, 824(1976).

15. Ferrell, W.J., and Goyette, G.W.: Analysis of acetaminophen and salicylate by reverse phase HPLC. J. Liquid Chrom. 5, 93(1982).

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DETERMINATION BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF STABILITY OF TETRAHYDRO-β-CARBOLINES AT DIFFERENT AMBIENT TEMPERATURES

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ABSTRACT

To determine the stability of tetrahydro-β-carboline compounds over time and at different temperatures, a reversed-phase high pressure liquid chromatography system with electrochemical detection was utilized. Noreleagnine (1,2,3,4-tetrahydro-β-carboline) and tetrahydroharman (1-methyl-1,2,3,4-tetrahydro-β-carboline) were dissolved in water or ascorbate (0.1 mg/ml) vehicle and stored at -20°C, 22°C, or 37°C for one, seven or 12 days. After each solution was injected in the column in a concentration of 400-600 ng/10 µl, peak height values were obtained for the compound under each condition. Analysis of percent recovery showed that the two β-carbolines were relatively stable with a maximal degradation of 14% occurring only at the 12-day assay interval. These results suggest that this class of compound can be used in pharmacological studies in which they can be dispensed from a mini-pump implanted in tissue. Further, an HPLC system with electrochemical detector provides a valid and reliable procedure for quantification of indoleamine-aldehyde condensation products.

INTRODUCTION

Tetrahydro-β-carboline (THBC) compounds belong to a class of tricyclic structures which can be formed *in vivo* as a result of a con-

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densation reaction between an indoleamine and an aldehyde (1,2). Certain of the THBCs have been found to exist in the rat's brain (3,4) and are now recognized as normal constituents of human plasma and platelets (5). Because a THBC reportedly can modify brain 5-hydroxytryptamine (6,7), these compounds have been the focus of numerous medical and biological investigations including those involving affective disorders, schizophrenia, alcoholism and neurotransmitter activity (8,9,10).

One of the THBCs, noreleagnine, has been implicated in the mechanism underlying alcohol drinking (11), but the general role of other β-carbolines in the excessive intake of this drug is not yet known. For example, the consumption of alcohol in the laboratory rat reportedly declines following noreleagnine (1,2,3,4-tetrahydro-β-carboline) administered peripherally (12) but increases after intracerebroventricular infusion of the product over 12 days. (13). This latter study was replicated with two β-carbolines, tetrahydroharman (1-methyl-1,2,3,4-tetrahydro-β-carboline) and noreleagnine which were infused continuously into the cerebral ventricle through the use of an osmotic mini-pump implanted in the rat's neck (14). Although mass spectrometry was used to verify the presence of the compound in a given pump, the actual stability of the β-carboline compounds over a prolonged period remains uncertain. To illustrate, we have found recently that other amine-aldehyde condensation products are unstable over time, particularly at a temperature analogous to a body temperature of 37°C (15).

A high pressure liquid chromatography (HPLC) assay has been developed to separate noreleagnine from brain tissue (16). The purpose of the present investigation, therefore, was to (1) develop a method using HPLC with electrochemical detection for the determination of β-carbolines in a fluid medium; (2) quantify the rates of degradation of the β-carboline com-

pounds; (3) ascertain the effect of different ambient temperatures on the decomposition of these compounds; and (4) determine whether an antioxidant such as ascorbic acid, which is typically added to solutions used for pharmacological studies, would influence the degradation of a β-carboline.

MATERIALS AND METHODS

The HPLC system was comprised of a single pump (Altex Model 110, Solvent Metering Pump) with a pulse damper (Bioanalytical Systems) and a syringe loading sample injector (Rheodyne Model 7120). A C_{18} reversed-phase column (3.9 mm i.d. x 300 mm μBondapak, Waters) protected by a pre-column filter (Rheodyne) was fitted into the system. A glassy carbon electrochemical detector cell, a TL-8A thin layer transducer (Bioanalytical Systems), was coupled to a model LC4 amperometric detector (Bioanalytical Systems). The electrode potential was set at +0.85V using a silver-silver chloride electrode as a reference, and the level of sensitivity of the detector was set at 10 nA/V. A strip chart recorder (Fisher Recordall, Series 500) connected in parallel with a plotting integrator (Hewlett Packard Model 3390A) completed the system.

Mobile Phase

The mobile phase consisted of 0.1 M sodium acetate, 0.15 M acetic acid and 20% (v/v) methanol with a pH of 4.4. The mobile phase was first passed through a double filter (0.3 μm Gelman A/E glass fiber filter and 2-3 μm Whatman #5 filter) and then degassed by sonication. The flow rate of the mobile phase through the column was maintained at 1.5 ml/min.

Sample Preparation

The β-carbolines tested were noreleagnine (1,2,3,4-tetrahydro-β-carboline) and tetrahydroharman (1-methyl-1,2,3,4-tetrahydro-β-carbo-

line). Each compound was weighed accurately to 10^{-3} mg using a Cahn 21 Automatic Electrobalance. Two samples of each compound were prepared: one was dissolved in glass-distilled water and the second in glass-distilled water containing 0.1 mg/ml of ascorbic acid.

The concentration of the β-carboline solutions was 400 or 600 ng/10 µl. Noreleagnine was readily solubilized in either medium by sonication; however, samples of tetrahydroharman required sonication and gentle warming. The solutions were tested immediately after their preparation and then multiple aliquots of each were maintained subsequently in sealed vials at one of three temperatures: 22°C (laboratory room); 37°C (water bath); and -20°C (freezer). Following their preparation, these aliquots were tested at intervals of one, seven and 12 days. As a control for daily variation in performance of the chromatography system, the aliquots maintained at -20°C were thawed briefly and tested also at these three intervals so that their peak values could serve as the respective baseline values. Each sample was injected from a 50 µl Hamilton syringe onto the column in a volume of 10.0 µl. An injection of each sample was then repeated at least three times in order to verify the reliability of the data collected. If a given peak response was not consistent, a further series of injections were made. Glassware and syringes were sonicated in chromic acid and rinsed repeatedly in glass-distilled water to avoid possible contamination of a sample.

Reagents

L-ascorbic acid and HPLC grade methanol were purchased from Fisher Scientific, whereas anhydrous sodium acetate and glacial acetic acid were obtained from Mallinckrodt Chemical. Noreleagnine was obtained from Sigma Chemical Company and tetrahydroharman was obtained from ICN Pharmaceuticals, Inc.

RESULTS

The percent recovery of the two β-carbolines was calculated from the integrated peak height values as compared with aliquots of the standard sample of each compound prepared for use on the respective test day. Each value is thus expressed as a mean ± standard error of the percent recovery for each sample tested against the standard for that given time period.

Table 1 presents a comparison of the percent recovery of both noreleagnine (NORL) and tetrahydroharman (THH) at a room temperature of 22°C as measured at one, seven and 12 days after their preparation. The results of the degradation of both compounds prepared in water and ascorbate are also shown in Table 1. It is evident that essentially no substantial cumulative degradation occurred over time under either condition even though by the 12th day the recovery of NORL, contrasted with the control value, was reduced to 93% and 89% in the water and ascorbate vehicles, respectively. The degradation of THH in water was virtually absent over the 12-day period; that is, THH in the ascorbate vehicle declined to the 91% level on day 7, but was quantified at 98% on day 12.

Essentially the same result was obtained when NORL and THH were stored at 37°C. Once again, NORL when prepared in the ascorbate vehicle, appeared to be the more labile of the two compounds, but yet exhibited a degradation of only 14% below the control value. However, THH remained relatively stable over the entire 12-day interval independent of the carrier vehicle in which it was dissolved.

Representative records of the HPLC tracings are shown in Fig. 1 for NORL tested in both of the carrier vehicles at 22°C and 37°C. Similarly, Fig. 2 illustrates typical HPLC tracings for the THH compound again analyzed under the same conditions as those of NORL. In all cases, the

TABLE 1

PERCENT RECOVERY OF NORELEAGNINE (NORL) AND TETRAHYDROHARMAN (THH) IN DISTILLED WATER OR ASCORBIC ACID VEHICLE AT ROOM TEMPERATURE OF 22°C

	WATER			ASCORBIC ACID		
	Time Elapsed (Days)			Time Elapsed (Days)		
COMPOUND	1	7	12	1	7	12
NORL	98.7±1.0 n=4	95.0±2.4 n=7	92.8±4.6 n=6	98.2±1.8 n=4	94.4±3.6 n=6	88.8±3.6 n=6
THH	94.1±2.8 n=5	98.8±0.6 n=6	95.8±1.9 n=6	93.8±1.1 n=5	91.3±3.2 n=6	97.8±1.0 n=6

TABLE 2

PERCENT RECOVERY OF NORELEAGNINE (NORL) AND TETRAHYDROHARMAN (THH) IN DISTILLED WATER OR ASCORBIC ACID VEHICLE AT 37°C

	WATER			ASCORBATE		
	Time Elapsed (Days)			Time Elapsed (Days)		
COMPOUND	1	7	12	1	7	12
NORL	97.6±0.8 n=4	96.6±1.9 n=6	94.1±3.7 n=6	99.0±0.5 n=4	95.8±2.4 n=6	86.3±3.6 n=5
THH	97.9±1.2 n=4	99.2±0.8 n=6	98.0±1.1 n=6	92.5±3.1 n=4	97.0±1.9 n=6	98.9±0.9 n=6

peak of the solvent front produced by ascorbate was always readily discernible from the respective β-carboline peak.

DISCUSSION

The results of this study show that a relatively straightforward analytical method employing HPLC with an electrochemical detector can be utilized for the assay of THBC compounds in terms of their stability under different physical conditions. The mobile phase established for this experiment provided reliable measures of the compounds over a two-

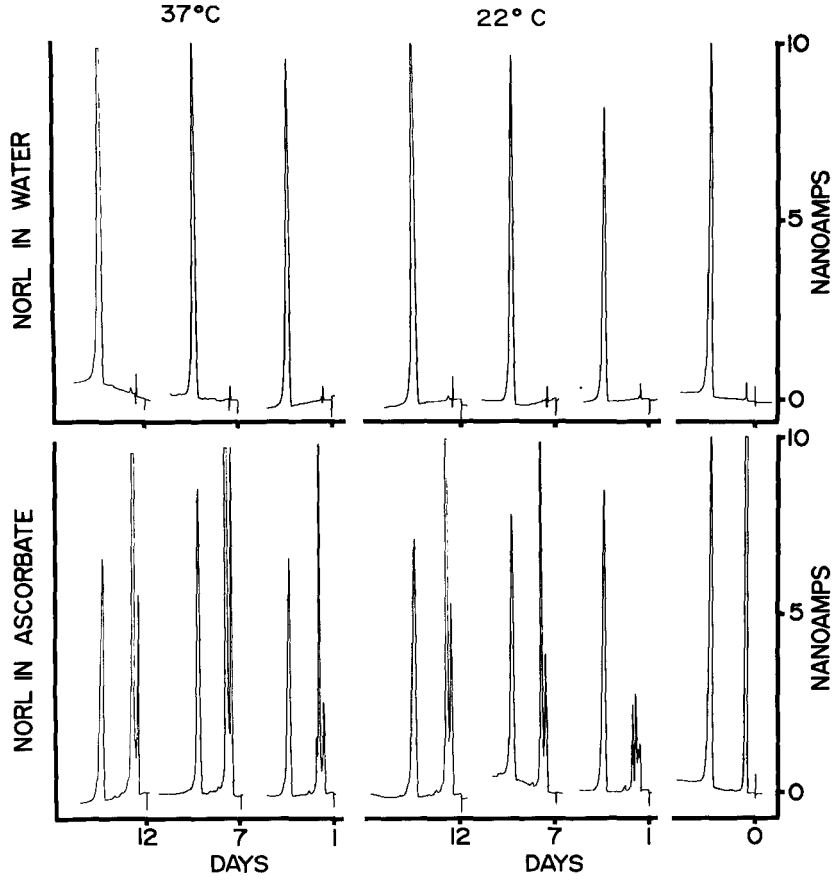

FIGURE 1: Chromatographic tracings of noreleagnine (NORL) dissolved in water or aqueous ascorbate (0.1 mg/ml). Samples were stored at either 37°C or 22°C and analyzed at one, seven and 12 days. The zero time represents the chromatograph of the freshly prepared sample. Peak height was measured in nanoamps.

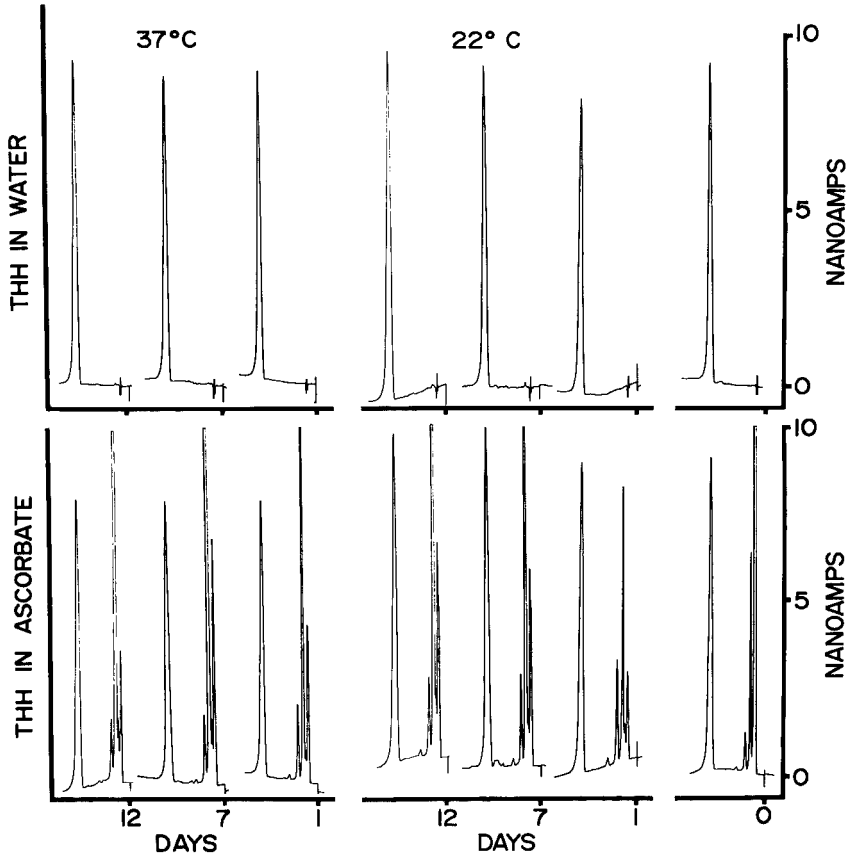

FIGURE 2: Chromatographic tracings of tetrahydroharman (THH) dissolved in water or aqueous ascorbate (0.1 mg/ml). Samples were stored at either 37°C or 22°C and analyzed at one, seven and 12 days. The zero time represents the chromatograph of the freshly prepared sample. Peak height was measured in nanoamps.

week period. Although a slight inconsistency was observed in the day-to-day column-detector sensitivity during this 12-day interval, quantification of a compound's activity was nevertheless readily attainable.

The relative stability of the two β-carbolines indicate that for pharmacological studies (10) the problem of degradation under physiological conditions is unlikely to be an experimental issue. Further, the absence or presence of ascorbate in the test medium also is not a factor in terms of the temporal integrity of these compounds, although the largest amount of decomposition occurred in the vehicle containing ascorbate. However, other condensation products, such as certain tetrahydroisoquinolines, do in fact require an antioxidant such as ascorbate to prevent their rapid degradation (15). Therefore, since the ascorbate does not appear to be necessary as an antioxidant, its deletion from a β-carboline solution used in an *in vivo* experiment is recommended, particularly since the acid may exert an effect on its own.

The results of this experiment also demonstrate that the two β-carbolines tested are equally stable at 22°C and 37°C. Since the elevated temperature did not compromise the stability of the compounds, the utilization of a mini-pump containing a β-carboline and implanted sub-dermally (14) in an animal with a body temperature of 37°C can be encouraged. According to our findings, the use of these compounds in a study in which repeated injections are required at either room (22°C) or body temperature over an interval such as two weeks would thus be a valid approach.

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REFERENCES

1. McIsaac, W.M., Formation of 1-methyl-6-methoxy-1,3,4,5-tetrahydro-β-carboline under physiological conditions, Biochim. Biophys. Acta, 52, 607, 1961.

2. Hsu, L.L., Formation of 1,2,3,4-tetrahydro-β-carboline and methylene-β-phenylethylamine from 5-methyltetrahydrofolate and amines in tissues from developing rat brain, Life Sci., 19, 493, 1976.

3. Shoemaker, D.W., Cummins, J.T. and Bidder, T.G., β-carbolines in the arcuate nucleus, Neuroscience, 3, 233, 1978.

4. Rommelspacher, H. and Greiner, B., Formation of β-carbolines: A new metabolic pathway of indoles in the brain of rats, Naunyn-Schmiedeberg's Arch. Pharmacol., 302, R60, 1978.

5. Bidder, T.G., Shoemaker, D.W., Boettger, H.G., Evans, M. and Cummins, J.T., Harman in human platelets, Life Sci., 25, 157, 1979.

6. Buckholtz, N.S. and Boggan, W.O., Effects of tetrahydro-β-carbolines on monoamine oxidase and serotonin uptake in mouse brain, Biochem. Pharmacol., 25, 2319, 1976.

7. Komulainen, H., Tuomisto, J., Airaksinen, M., Kari, I., Peura, P., and Pollari, L., Tetrahydro-β-carbolines and corresponding tryptamines: In vitro inhibition of serotonin, dopamine and noradrenaline uptake in rat brain synaptosomes, Acta Pharmacol. et Toxicol., 46, 299, 1980.

8. McIsaac, W.M., Taylor, D., Walker, K.E. and Ho, B.T., 6-methoxy-1,2,3,4-tetrahydro-β-carboline - a serotonin elevator, J. Neurochem., 19, 1203, 1972.

9. Kari, I., Rapakko, S. and Airaksinen, M.M., Effect of some β-carbolines on phenylethylamine and apomorphine stereotypies in rats, Pharmac. Biochem. Behav., 12, 979, 1980.

10. Myers, R.D. Pharmacological effects of amine-aldehyde condensation products. In: Alcohol Tolerance and Dependence, Rigter, H., and Crabbe, J., eds., Elsevier, The Netherlands, 1980, p. 339.

11. Myers, R.D. and Melchior, C.L., Differential actions on voluntary alcohol intake of tetrahydroisoquinolines or a β-carboline infused chronically in the ventricle of the rat, Pharmac. Biochem. Behav., 7, 381, 1977.

12. Messiha, F.S. and Geller, I., Effect of noreleagnine, a β-carboline derivative on ethanol perference in the rat, Proc. West. Pharmacol. Soc., 19, 336, 1976.

13. Myers, R.D. and Oblinger, M.M., Alcohol drinking in the rat induced by acute intracerebral infusion of two tetrahydroisoquinolines and a β-carboline, Drug and Alcohol Dependence, 2, 469, 1977.

14. Tuomisto, L., Airaksinen, M.M., Peura, P., and Eriksson, C.J.P., Alcohol drinking in the rat: Increases following intracerebroventricular treatment with tetrahydro-β-carbolines. Pharmacol. Biochem. Behav., 17, 831, 1982.

15. Myers, R.D., Garrison, J.L., and Critcher, E.C., Degradation characteristics of two tetrahydroisoquinolines at room and body temperatures: HPLC determination with electrochemical detection, J. Liquid Chromatography, 6, 343, 1983.

16. Rommelspacher, H., Honecker, H., Barbey, M. and Meinke, B., 6-hydroxy-tetrahydronorharmane (6-hydroxy-tetrahydro-β-carboline), a new active metabolite of indole-alkylamines in man and rat, Naunyn-Schmiedeberg's Arch. Pharmacol., 310, 35, 1979.

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BOLL WEEVIL: DETERMINATION OF ECDYSTEROIDS AND JUVENILE HORMONES WITH HIGH PRESSURE LIQUID CHROMATOGRAPHY

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ABSTRACT

High pressure liquid chromatographic methods were developed to separate 20-hydroxyecdysone and juvenile hormone I in the pupal and adult stages of the boll weevil. Minimum detectable levels were 4 ng for 20-hydroxyecdysone and 4 ng for juvenile hormone. No juvenile hormones were detected in male boll weevils, whereas titers were readily determined in female boll weevils. The ecdysteroid, 20-hydroxyecdysone was present in pupae (not sexed), in adult males from 3 to 6 days of age, and in adult females from days 2 through 6.

INTRODUCTION

Methods currently available for quantification of ecdysteroids in insects include bioassays (1), chromatography (2), radioimmunoassay (3), and high pressure liquid chromatography (HPLC) (4). Ecdysteroids have been identified from hemolymph and ovaries of insects (5,6) and are involved in an interaction with juvenile hormones (JH) in reproductive cycles of insects. The JHs may regulate the synthesis of the vitellogenic protein present in developing eggs (7) and ecdysteroids may inhibit JH synthesis. A

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highly sensitive and specific assay for JHs is the radioimmunoassay technique (8); another method is gas chromatography-mass spectrometry (9). HPLC has not been utilized extensively for determination of ecdysteroids or JHs because of a lack of sensitivity. We report herein the determination of ecdysteroids in pupae and JHs in adults of the boll weevil, Anthonomus grandis Boheman, with HPLC methodology.

MATERIALS AND METHODS

Ecdysone Titers in Pupae

Larvae, pupae and prepupae were washed off the surface of larval diet trays with warm water at 13 days after egg implantation. Pupal age then was synchronized by separating and removing only prepupae. The prepupae were held in total darkness at 50% RH and 30°C.

Juvenile Hormone and 20-hydroxyecdysone Titers in Adults

Newly emerged adult weevils were obtained from the Gast Rearing Laboratory (10) and held at 50% RH and 30°C in a 16 h light:8 h dark regime. One hundred male and 100 female weevils were sexed and collected each day for assay. They were frozen or assayed on the day of collection.

Apparatus and Chemicals

A high performance liquid chromatography system from Waters Associates, Inc. (Milford, Mass.), model M-6000 A solvent pump, a model 6UK injector coupled to a Waters C_{18} Bondapack (10 µ silica) column (3.9 mm x 300 mm) and a model 440 fixed-wavelength detector @ 254 nm was used for ecdysteroid determinations. All HPLC solvents were obtained from Burdick and Jackson Laboratories (Muskegon, Mich.). A Hewlett-Packard 5985A GC-mass spectrometer was used to confirm peak identities. Standards of ecdysone and 20-hydroxyecdysone (Fig. 1) were obtained from Sigma Chemical Co.

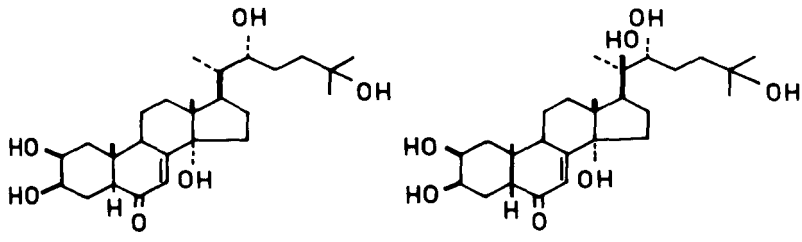


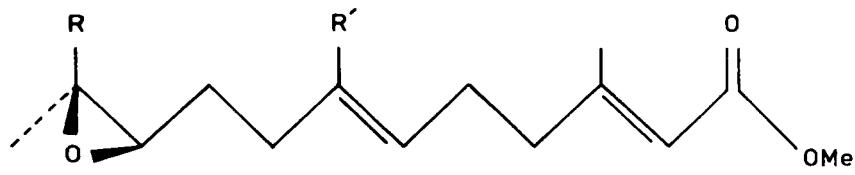
FIGURE 1. The Chemical Structures of Ecdysone and 20-hydroxyecdysone.

(St. Louis, Mo). The juvenile hormones I, II, and III (Fig. 2) were obtained in ampules from Calbiochem (San Diego, CA). An ES Industries (Marlton, NJ) 10 μ MC-18 Chromegabond column (30 cm X 4.6 mm) also was used for ecdysteroids. For JH determinations the HPLC column used was an RCM-100 column system with an 8 mm X 10 cm silica (10 μ).

EXTRACTION PROCEDURES

Extraction of Ecdysone

Assays were conducted at 8 hr intervals on 400-700 weevils with the beginning of the prepupal stage as the starting time (0 hour). At hours 40 to 56 (post prepupal stage) samples were assayed at 4 hour intervals. Weevils were ground in 50 ml acetonitrile with a Willems Polytron homogenizer. Samples were filtered with a Buchner funnel and qualitative filter. The funnel and retained material were rinsed three times with 10 ml acetonitrile, and the filtrate was evaporated to dryness at 60°C under reduced pressure in a rotary evaporator. The sample was taken up in 20 ml of acetonitrile and then was divided into two 10 ml aliquots. One of the aliquots was spiked with 25 ng ecdysone and 25 ng 20-hydroxyecdysone. The two samples were partitioned



JH I: R=R'Et
JH II: R=Et R'=Me
JH III: R=R'=Me

FIGURE 2. The Structure of the Juvenile Hormones.

three times with 20 ml hexane in a separatory funnel to remove nonpolar lipid. The acetonitrile layer was removed and evaporated to dryness at 60°C under reduced pressure in a rotary evaporator. The dry samples were taken up in 1 ml methanol and filtered with a Swinney adaptor through a 0.45 μ filter.

Extraction of Juvenile Hormones

One hundred male or female boll weevils were homogenized in 40 ml acetonitrile. The samples were filtered with a Buchner funnel and the funnel was rinsed with acetonitrile to give a volume of 50 ml. Distilled water (25 ml) was added to make the final volume 75 ml. Juvenile hormones were extracted by partitioning with 25-30 ml pentane in a separatory funnel three times. The pentane fractions were combined and evaporated to dryness under reduced pressure in a rotary evaporator at 30°C. The samples were taken up in 2 ml hexane for injection on the HPLC.

Injections

Ten microliters of the ecdysteroid samples were injected into the chromatograph. The eluting solvent was 18% acetonitrile in water and the flow rate was 2 ml/min. Quantitation was achieved by comparison of peak heights of samples to peak heights of known

quantities of standards. Ecdysone was used as an internal standard.

Collected peaks of 20-hydroxyecdysone were evaporated to dryness and taken up in methanol to be placed on the solid probe of the mass spectrometer for confirmation of peak identity. The spectra of samples were compared to spectra of standards prepared under the same conditions.

One hundred microliters of samples for JH analysis were injected in the HPLC assays. The flow rate was 3 ml/min. and the eluting solvent was 3% tetrahydrofuran in hexane.

Peak areas were obtained and quantitation for JHs and ecdysteroids was made by comparison of sample peak areas to the areas of standards. Hewlett-Packard integrators (3390A) were used to plot peaks and give data reduction. Figure 3 shows chromatograms of standards for JHs and ecdysteroids and of samples.

RESULTS

Ecdysteroids

Figure 4 shows the titers of 20-hydroxyecdysone from 24 through 64 hours after pupation. In the prepupal stages, no 20-hydroxyecdysone was detected. A titer of 20-hydroxyecdysone was found beginning at 24 hours after pupation. The quantity increased to the highest level determined 48 hours after pupation. The 20-hydroxyecdysone was also found in hemolymph at this time. At 48 hours, the quantity of 20-hydroxyecdysone present was 600 $\pm$ 40 ng/g. After 48 hours the quantity dropped rapidly and was not detectable at 64 hours. Ecdysone was not found in any of these samples.

On day 2, the adult female boll weevils produced a peak of 20-hydroxyecdysone (11 ng/weevil). The level of 20-hydroxyecdysone decreased through day 5 (7 ng/weevil) and ended on day 6. The adult male boll weevils on days 3-6 produced small amounts (4 ng/weevil) of 20-hydroxyecdysone (Figure 7).

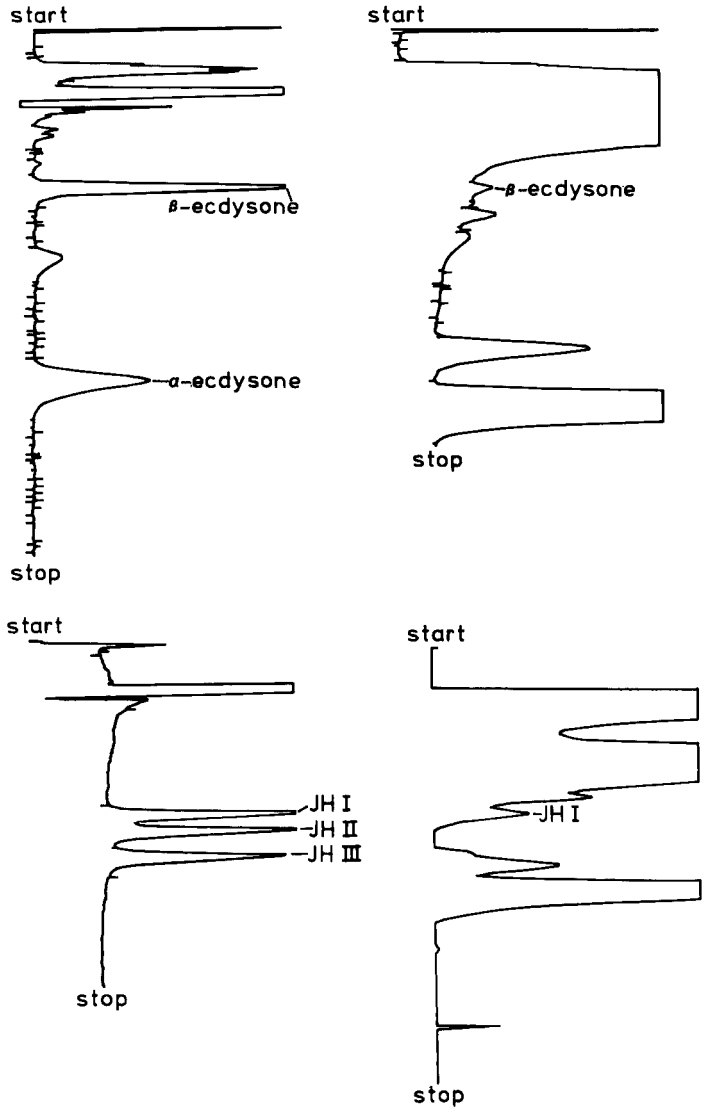


FIGURE 3. Chromatograms of Standards and Samples for Juvenile Hormones and Ecdysteroids.

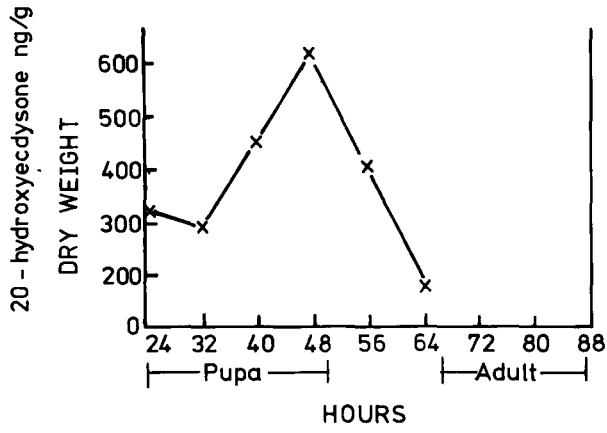


FIGURE 4. Titers of 20-hydroxyecdysone in Boll Weevil Pupae.

The time required to assay one sample for ecdysone (α) and 20-hydroxyecdysone (β) was ca. 20 minutes. The minimum detectable quantity of ecdysone or 20-hydroxyecdysone was 4 ng. Each of the samples tested for recovery averaged 95%.

The standard curve for 20-hydroxyecdysone and ecdysone is in Figure 5. The ratio of area counts obtained from the Hewlett-Packard integrator for concentration of ecdysteroid was linear over a wide range.

Juvenile hormones were not found in male boll weevils in these studies. Figure 6 also shows the pattern of JH I production by the female boll weevil for 10 days. Each point represents 4 replicates. Titers were low until the peak on day 4 (55 ng/weevil) which then decreased until day 9. No JH II or III was found in the samples.

The LDC 214 nm detector had a detection limit of 4 ng for each JH. The assay time on the HPLC was 6 minutes due to the high flow rate and resolution of the RCM-100 silica cartridge system. Figure 6 is the standard curve for the juvenile hormones I, II, and III. The ratio of area counts measured by the Hewlett-Packard

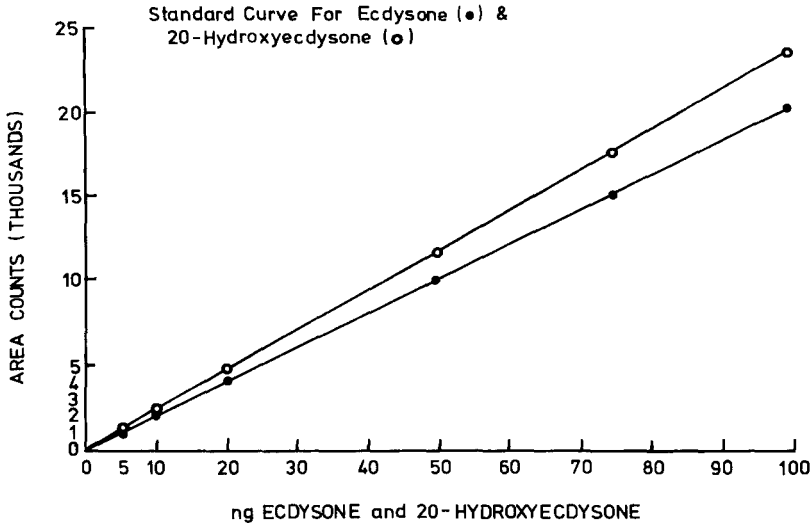


FIGURE 5. Standard Curve for Ecdysone and 20-hydroxyecdysone.

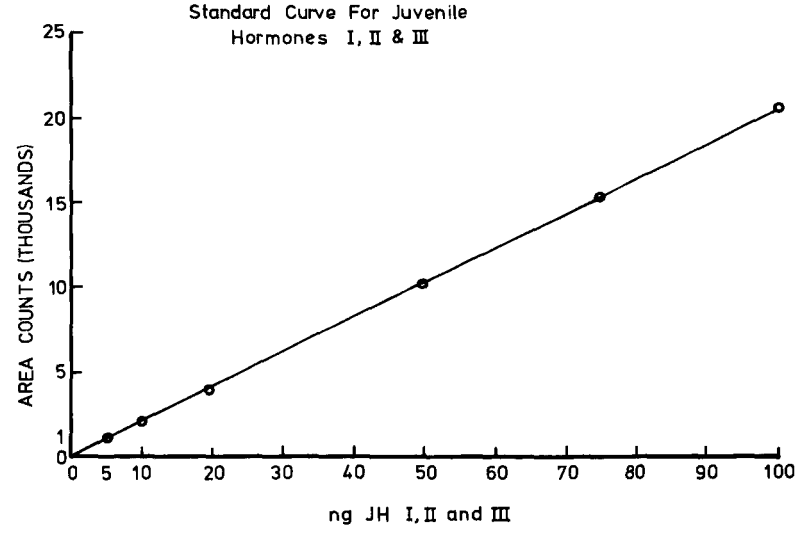


FIGURE 6. Standard Curve for Juvenile Hormones I, II, and III.

integrator to the concentration of JH was linear. All three hormones showed an absorption curve that increased in the UV as the wavelength decreased.

DISCUSSION

To study the hormonal influence on reproduction in the boll weevil, it was necessary to define methodology for definition of the ecdysteroid and juvenile hormones. The extraction methods were compatible for injection and subsequent separation and quantification by the methods developed in this study for these compounds. The sensitivity for the JH was less than GC-election capture technique of Hagenguth and Rembold (11) but this HPLC method was faster with less complications caused by impurities. The RIA method for ecdysteroid quantification is more sensitive but lacks specificity (12).

Our results demonstrate the presence of one ecdysteriod (20-hydroxyecdysone) and JH I were in the boll weevil. The JH found in most Coleopterans is JH III whereas JH I is found primarily in Lepidoptera (13). Henson et al. (14) reported that 20-hydroxyecdysone was found in boll weevil pupae and used a bioassay to estimate 17-35 µg/kg wet weight. Otherwise, little information is available or known concerning the presence or changes in ecdysteroid and JH titers in the boll weevil.

Embryogenesis begins in the pupal stage in this insect and our results indicate that 20-hydroxyecdysone is present in the early prepupal stages (mixed sexes) and decreases below detection levels as the pharate adult stage appears. For days 1 and 2 after adult emergence neither JH I nor 20-hydroxyecdysone was detected but the ecdysteroid, in females, reached a high titer 1 day prior to that of JH I and remained so until day 6. The JH remained at a detectable level throughout day 9 (Fig. 7). Bownes (15) reported that this ecdysteroid must be continuously present in Drosophila for fat body synthesis of yolk polypeptides in females. No JH was

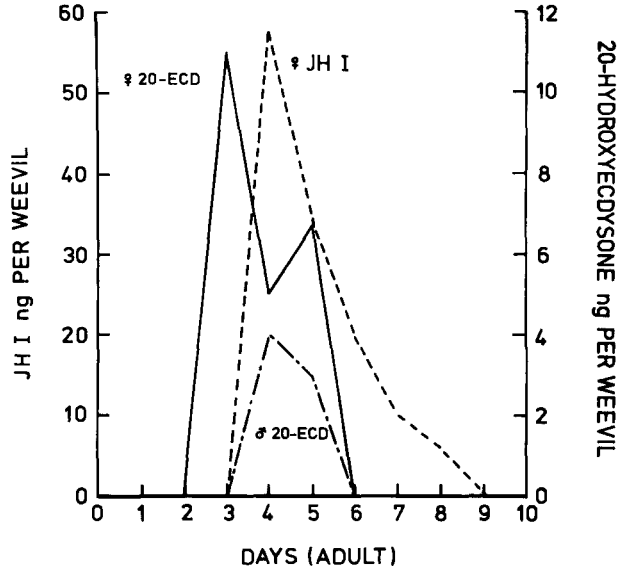


FIGURE 7. 20-hydroxyecdysone and Juvenile Hormone Titers in Adult Male and Female Boll Weevils.

detected in the males and 20-hydroxyecdysone was found in adult males at 3 to 6 days of age. Its role in males is yet unknown.

The HPLC method developed and the results obtained in this study provide an immediate, rapid, and sensitive system for determining the levels of 20-hydroxyecdysone and JH I in the boll weevil at given times on an immediate basis. These determinations can be performed on whole body tissues, hemolymph, or fractions thereof.

REFERENCES

(1) Staal, G. B. Plants as a source of insect hormones. Proc. K. Ned. Akad. Wet. (C) 70: 409, 1967.

(2) Morgan, E. D. and Poole, C. F. The extraction and determination of ecdysones in arthropods. Adv. Insect. Physiol. 12: 17, 1976.

(3) Hodgetts, R. B., Sage, B. S., and O'Connor, J. D. Ecdysone titers during post embryonic development of Drosophila melanogaster. Develop. Biol. 60: 310, 1977.

(4) Wilson, I. D., Bielby, C. R., Morgan, E. D., and McLean, A. E. M. Comparison of high-pressure liquid chromatography and gas chromatography for analysis of ecdysteriods. J. Chromat. 194: 343, 1980.

(5) Hsiao, T. H. and Hsiao, C. Ecdysteriods in the ovary and the egg of the greater wax moth. J. Insect Physiol. 25: 45, 1979.

(6) Hagedorn, H. M., O'Connor, J. D., Fuchs, M. S., Sage, B., Schlaeger, D. A., and Bohm, M. K. The ovary as a source of α-ecdysone in an adult mosquito. Proc. Nat. Acad. Sci. U.S.A. 72: 3255, 1975.

(7) Wyatt, G. R. and Pan, M. L. Insect plasma proteins. Ann. Rev. Biochem. 47: 779, 1978.

(8) Baehr, J. C., Porcheron, P., Papillon, M., and Dray, F. Haemolymph levels of juvenile hormone, ecdysteroids and protein during the last two larval instars of Locusta migratoria. J. Insect. Physiol. 25: 415, 1979.

(9) Huibregtse-Minderhoud, L., Van den Hondel-Franken, M. A. M., Van der Kerk-van Hool, A. C., Biessels, H. W. A., Salemink, C. A., Van der Horst, D. J., and Beenakkers, A. M. Th. Quantitative determination of the juvenile hormones in the haemolymph of Locusta migratoria during normal development and after implantation of corpora allata. J. Insect Physiol. 26: 627, 1980.

(10) Lindig, O. H., Roberson, J., and Wright, J. E. Evaluation of three larval and adult boll weevil diets. J. Econ. Entomol. 72: 450, 1979.

(11) Hagenguth, H. and Rembold, H. Identification and quantification of insect juvenile hormones as their 10-heptafluorobutyryloxy-11-methoxy derivatives by combination of high performance liquid chromatography and electron capture gas-liquid chromatography. J. Chrom. 170: 175, 1979.

(12) Borst, D. W. and O'Connor, J. D. Arthropod molting hormone: Radioimmune assay. Science. 178: 418, 1972.

(13) Gilbert, L. I., Goodman, W., and Bollenbacher, W. E. Biochemistry of regulatory lipids and sterols in insects.

Biochemistry of Lipids II. Vol. 14 International Review of Biochemistry. (Ed. by T. W. Goodwin) Univ. Park Press, Baltimore. 1977.

(14) Henson, R. D., Thompson, A. C., Gueldner, R. C., and Hedin, P. A. Constituents of the boll weevil. VII. The molting hormone: isolation studies. Annals Entomol. Soc. America 65: 981, 1972.

(15) Bownes, M. The role of 20-hydroxyecdysone in yolk-polypeptide synthesis by male and female fat bodies of Drosophila melanogaster. J. Insect Physiol. 28: 317, 1982.

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The Determination of Iodine in Milk and Milk Chocolate by Anion HPLC

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ABSTRACT

An HPLC method is described for the extraction and analysis of iodine as iodide in milk, cocoa beans and milk chocolate. Prior to analysis samples are finely ground if necessary and combusted in a modified Shoeninger flask. The liberated halide is absorbed in a basic reducing medium which is concentrated and brought to volume with distilled water prior. HPLC analysis used an 8 mm 5μ C_{18} cartridge in a RCM-100 with detection of the iodide ion at 226 nm. The mobile phase consisted of an ion-pairing agent, buffer and acetonitrile. The method is accurate and precise showing reasonable agreement with a National Bureau of Standards spray dried milk sample.

INTRODUCTION

There is much interest in the analysis of iodide in various commodities, especially milk and milk products. With the use of iodophors as disinfectants in the milk industry some data indicates that there is a resulting increase in iodine consumption by the public, leading to a potential increase in thyroid disorders. There is also interest in endogenous iodine levels in various commodities.

Methods employed for the analysis in milk and other food include GC (1) ion-selective electrodes (2) microdistillation (3) and differential pulse polarography (4,5). Other methods proposed include neutron activation analysis (6), which while scientifically stimulating cannot be utilized in any but large laboratories. An AOAC

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Collaborative Study (7) with 7 samples and 8 laboratories using two methods showed a relative standard deviation of 77.9% between labs and 19.1% within labs. In that collaborative study the two methods used were the catalytic effect of iodine on the Ce^{+4}-As^{+3} reaction and neutron activation analysis.

There has been much written about sample preparation for halide determination. A method which has been extremely useful is oxygen flask combustion. This method has seen wide use in the microdetermination of ions (8) and preliminary work on its use in iodine analysis was presented (9) but still required a complicated distillation before a final colorimetric determination step. The method proposed in this paper uses conventional HPLC equipment and a UV detector to accomplish the analysis of iodine in milk, cocoa beans and milk chocolate at lower levels of several hundred ppb. LC has been used for the analysis of various anions but has either used specialty packings (10) conductivity detectors (11) or has been done on relatively pure samples (12,13,14). Others have used various ion pairing agents but were limited to rather theoretical considerations (15,16,17).

EXPERIMENTAL

HPLC Apparatus

The HPLC system used consisted of a Model 6000A Solvent Delivery System (Waters Assoc.), a model 165 Variable Wavelength Detector operated at 226 nm (Beckman Inst.), a model 710B WISP Autoinjector, Model 720 System Controller and Model 730 Data Module (Waters Assoc.). The HPLC Column used was a 8 mm 5μ C_{18} Cartridge for the RCM-100 (Waters Assoc.).

HPLC Mobile Phase

45/25/25 (v/v/v) .0025m hexadecyltrimethylammoniumchloride/.05m Na_2HPO_4 /CH_3CN at pH 6.8 ± 0.1 flowing at 2.5 ml/min.

Iodide Standard

KI (alfa) dissolved in CH_3OH for a final concentration of 0.01 g/µl. Check standard and prepare fresh when degradation occurs. Degradation can be seen by a decrease in peak length for the sample injection and the appearance of a peak in the chromatogram equivalent to the formation of IO_3.

Other Reagents and Supplies

Other equipment used in this study consisted of a 5 liter oxygen combustion flask (9), rubber balloons, and a rotary evaporator. Other reagents were hydrazine monohydrate, KOH and oxygen.

Preparation of Sample

If necessary, grind sample to particle size that allows it to pass through a 25 mesh sieve. For cocoa beans and chocolate weigh 0.5 g into Whatman 541 filter paper. For dry milk, use 0.25 g sample size. Place this into the sample holder of the combustion flask, containing 50 ml of distilled water, 50 µl of hydrazine monohydrate and 3.0 ml of 0.1M KOH and a stirring bar. Attach a rubber balloon to the side neck of the flask to allow expansion of the vapor in the flask. Fill the flask with oxygen, clamp the holder into place and ignite with a 650 watt photography lamp. Allow the sample to burn until the flame is extinguished and place the flask on a stirrer at high speed for at least 20 minutes or until the balloon is totally deflated. Transfer the solution into a 250 ml round bottom flask with three 25 ml portions of distilled water. Place on a rotary evaporate at 85-90°C and rotovap to dryness. Dilute to 2 ml with distilled water for the HPLC determination.

HPLC Determination

Inject 50 to 100 µl of sample extract onto the column. After separation, calculate concentrations of iodide by comparing peak heights with peak heights of iodide standard solutions.

RESULTS AND DISCUSSION

The potassium iodide standard was scanned from 190-700 nm using a Hitachi EPS-3T UV/Vis spectrophotometer. The potassium iodidi exhibited absorbance maximums at 196 and 226 nm. The use of the UV detection was based on earlier studies dealing with the UV spectra of inorganic anions (18). The HPLC standard was injected over a 12 hour period and no decay was evident. The standard was linear from 5 ng to 20 µg with a regression coefficient of 0.98. The 226 nm wavelength was chosen as the one of choice since the baseline was quietier than at 196 nm. Lower limits were 5 ng/inj and 100-150 ppb in cocoa beans. Precision studies were run on standard, cocoa bean and milk with data summarized in Table 1.

An evaluation of the method using KI standard (500 ppb) and filter paper was conducted and 97% recovery was achieved. Whatman 541 filter paper was primarily chosen due to its low ash.

A sample of nonfat dry milk was provided by NBS. The milk was reported to have an iodide concentration, for information use only, of 3.4 µg/g. Using a sample weight of 0.25 g, four analyses were

Table 1

Precision Study

Sample	n	Conc	%Cv
Standard	26	50 ng	2.27
Cocoa bean	3	133.4 ng	8.4
Milk	4	3.8 µg	2.8

Table 2

Cocoa Bean Recovery Studies
n = 2

Amt. Added	Amt. Recovered	% Recovery
+200 ng	174 ng	87
+400 ng	324 ng	81

Sample: Iodide Std

Column: 8mm, 5um C_{18} for RCM

Mobile: 45/25/25 (Buffer/Ion Pairing Agent/Acetonitrile)

Flow Rate: 2.5 ml/min

Detector: Beckman 165 @ 226 nm

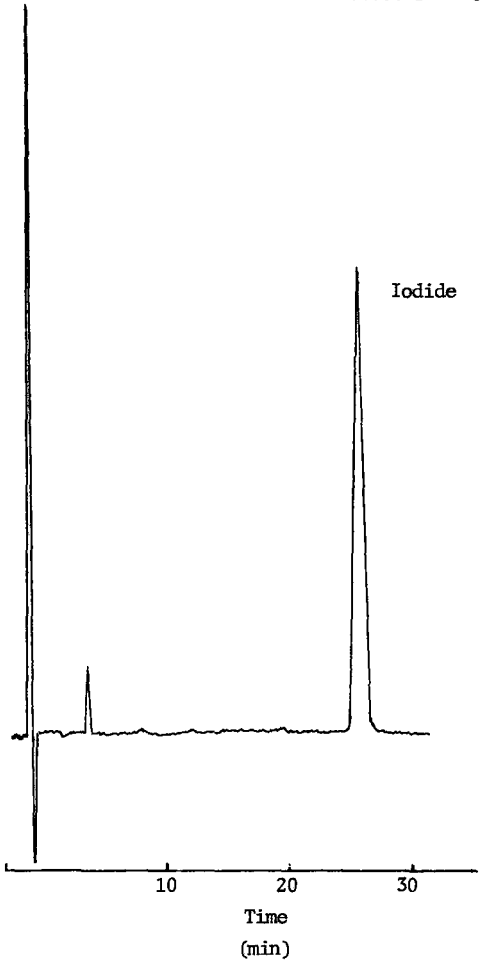


FIGURE 1 Chromatogram of Iodide Std.

Sample: Cocoa Bean

Column: 8mm, 5um C_{18} for RCM

Mobile: 45/25/25 (Buffer/ Ion Pairing Agent/Acetonitrile)

Flow Rate: 2.5 ml/min

Detector: Beckman 165 @ 226 nm

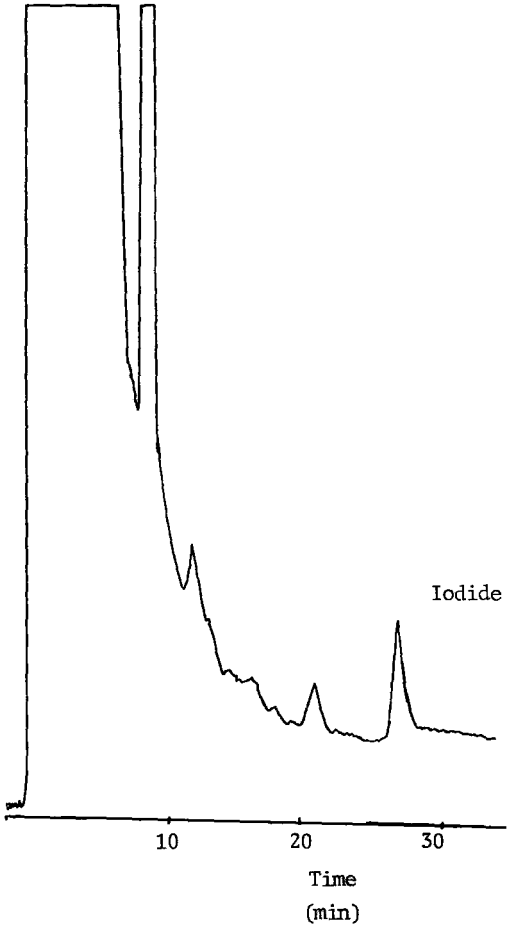


FIGURE 2 Chromatogram of Cocoa Bean Extract

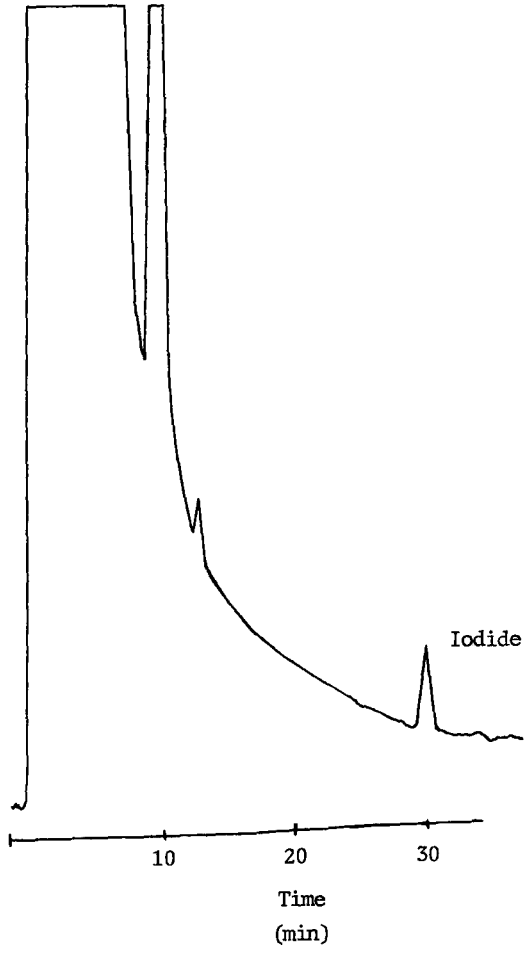
Sample: Milk Chocolate

Column: 8mm, 5um C_{18} for RCM

Mobile: 45/25/25 (Buffer/Ion Pairing Agent/Acetonitrile)

Flow Rate: 2.5 ml/min

Detector: Beckman 165 @ 226 nm



Sample: Nonfat Dry Milk

Column: 8mm, 5um C_{18} for RCM

Mobile: 45/25/25 (Buffer/Ion Pairing Agent/Acetonitrile)

Flow Rate: 2.5 ml/min

Detector: Beckman 165 @ 226 nm

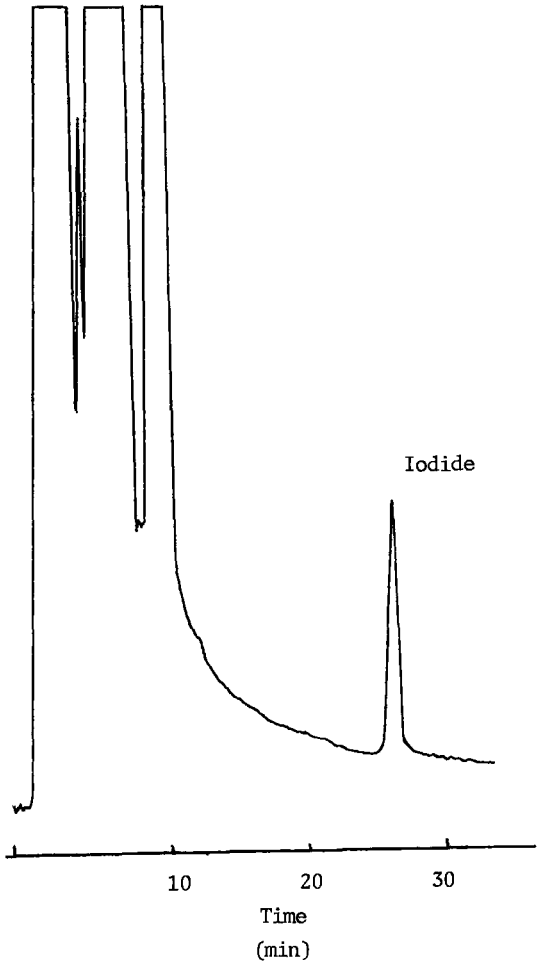


FIGURE 4 Chromatogram of Milk Extract

conducted with an average of 3.8 μg/g iodide. This sample was spiked at a 200 ng level (n = 2) and 95% recovery was achieved.

The method was evaluated on cocoa beans with recovery data summarized in Table 2.

Figures 1, 2, 3, and 4 show chromatograms of iodide standard, nonfat dry milk, cocoa bean and milk chocolate. All iodide peaks were scanned using the Model 165 detector. These scans were compared with scans including max of iodide standard to insure peak identity. The matrix of milk chocolate was analyzed with iodide concentration of 75 ppb for n = 2.

In summary, the method described is an accurate, precise and cost effective alternative for the analysis of iodide. The method proposed does not require the purchase of auxiliary HPLC equipment. It uses HPLC equipment found in many laboratories. The method uses accepted methods of halide determination for sample preparation prior to the final HPLC determination step. It is linear over a wide range with a lower limit of less than 100 ppb. Depending on sample matrix, much lower limits are potentially achievable. Additionally, work is continuing in our laboratory using post column reactions to increase lower limits. If an analyst uses the 190 - 200 nm region for detection of anions, then it is possible to detect fluoride and chloride. In the present mobile phase, these ions elute near void volume. Further work would therefore require adjustments in the mobile phase.

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REFERENCES

1. Bakker, H.J., Gas-Liquid Chromatographic Determination of Total Inorganic Iodide in Milk, JAOAC, 60, 1307, 1977.

2. Altinata, A., Pekin, B. & Ulgu, S., Determination of Iodine using a Kinetic Method with a Iodide Ion-selective Electrode, Analyst, 102, 876, 1977.

3. Joerin, M.M., A Rapid Method for Determining Total Iodine in Bovine Milk, Analyst, 100, 7, 1975.

4. Jolak, W. and Shostak, D., Differential Pulse Polarographic Analysis of Thyroid Hormone: Determination of Iodine, Thyroxine and Liothyronine, J. Pharm. Sci., 68 (3), 338, 1979.

5. Curtis, A.R. and Manning, P., Differential Pulse Polarographic Determination of Total Iodine in Milk, JAOAC, 65 (1), 20, 1982.

6. Allegrini, M., Boyer, K.W., and Tanner, J.T., Neutron Activation Analysis of Total Diet Food Composites for Iodine, JAOAC, 64 (5), 1111, 1981.

7. Heckman, M.C., Analysis of Foods for Iodide:Interlaboratory Study, JAOAC, 62, 1045, 1979.

8. McNally, M.E. and Grob, R.L., Oxygen Flask Combustion Technique, American Laboratory, January 31, 1981.

9. Lento, H.G., and Dogrosa, S.A., A Method for the Determination of Iodine in Foods, Presented at AOAC, Washington, D.C., 1979.

10. Pohl, C.A. and Johnson, E.L., Ion Chromatography - The State-of-the-Art, J. Chrom. Sci., 18, No. 9, 442, 1980.

11. Molnar, I., Knaver, H. and Wilk, D., High Performance Liquid Chromatography of Ions, J. Chrom., 201, 225, 1980.

12. Reeve, R.N., Determination of Inorganic Main Group Anions by High Performance Liquid Chromatography, J. Chrom., 177, 393, 1977.

13. de Kleijn, J.P., Simple Sensitive and Simultaneous Determination of Some Selected Inorganic Anions by High Performance Liquid Chromatography, Analyst, 107, 223, 1982.

14. Cortes, H.J., High Performance Liquid Chromatography of Inorganic & Organic Anions Using Ultraviolet Detection & an Amino Column, J. Chrom., 234, 517, 1982.

15. Hansen, S.H., Helboe, P., Thomsen, M., and Lund, U., High Performance Liquid Chromatography on Dynamically Modified Silica. II. Modification of Various Silica Packings with Cetyltrimethylammonium Bromide, J. Chrom., 210, 453, 1981.

16. Sorel, R.H.A., Hulshoff, A. and Wiersema, S., Reversed-Phase High Performance Liquid Chromatography with Cetrimide Containing Eluents, J. Liq. Chrom., 4 (11), 1961, 1981.

17. Bidlingmeyer, B.A., Separation of Ionic Compounds by Reversed-Phase Liquid Chromatography: An Update of Ion-Pairing Techniques, J. Chr. Sci., Vol. 18, 525 1980.

18. Buck, R.P., Singhadeja, S. and Rogers, L.B., Ultraviolet Absorption Spectra of Some Inorganic Ions in Aqueous Solution, Anal. Chem., 26 (7), 1240, 1954.

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SIMULTANEOUS DETERMINATION OF HEAVY METALS IN WATER BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AFTER SOLVENT EXTRACTION OF HEAVY METALS AS HEXAMETHYLENEDITHIOCARBAMATO CHELATES

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ABSTRACT

A simple, rapid, precise and accurate method for the simultaneous determination of ppb levels of cadmium(II), nickel(II), cobalt(II), copper(II), bismuth(III) and mercury(II) in water was developed. The six heavy metals described above in 50 ml of water sample were quantitatively extracted into 1.0 ml of chloroform by shaking for 15 min as their hexamethylenedithiocarbamato chelates (HMDC chelates), then separated by reversed-phase high performance liquid chromatography (HPLC) and the eluted metal chelates were monitored at 260 nm. The six HMDC-metal chelates were successfully separated on a 5 μm ODS column (4.6× 150 mm, Cosmosil 5 C_{18}), using methyl alcohol-water-diethyl ether-buffer solution (NH_4Cl-NH_3, pH 7.5)-10^{-3}M hexamethyleneammonium hexamethylenedithiocarbamate (HMA-HMDC)=82:9:3:3:3 as the eluent. The linear working curves for the six metals were obtained in the concentration range of 0.3-2000 ng/ml (ppb). Detection limits were 45-600 pg as metals. The recovery and the precision of the proposed method were examined for the composite sample containing the above metals and sixteen foreign ions, and these results were 99.2-101.5 % and 0.5-1.2 %, respectively. The proposed method was applied to the determination of the above six metals in river water and satisfactory results were obtained, compared with those by the flameless atomic absorption (FL-AAS) method.

INTRODUCTION

In recent years, HPLC has become of interest for the simple determination method for metals. HPLC method does not require

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expensive apparatus and skillfulness, and enables simultaneous determination of metals as comparable to FL-AAS or AAS method. N-substituted dithiocarbamates were frequently used as the chelating agent (1-7). Smith et al. (1) reported a simple method for the determination of nickel, cobalt, ion (III) and copper by HPLC using diethyldithiocarbamate (DDTC) as chelating agent. Their method required no pretreatment of sample water, but the detection limits and the reproducibility were not satisfactory (31 ppb and 11.6 %, respectively). Edward-Inatimi et al. (3) also reported HPLC analysis of metal ions after solvent extraction as DDTC chelates. But satisfactory detection limits and reproducibility were not obtained (50 ppb and 4 %, respectively). Young-Tung et al. reported sensitive HPLC method using n-butyl-2-naphthylmethyldithiocarbamate. This method, however, required time-consuming pretreatment (4 h) and large sample size (1000 ml) to analyze ppb levels of metals. Cassidy et al. (8-11) reported determination of ng/ml and pg/ml levels of metals in water or alloy by trace enrichment and HPLC. Their method employed gradient elution and postcolumn reaction. A gradient elution system is essentially inadequate for a routine analysis, because in general the reproducibility is not satisfactory for precise and accurate determination, and then the regeneration of column (time-consuming) and relatively expensive apparatuses are required.

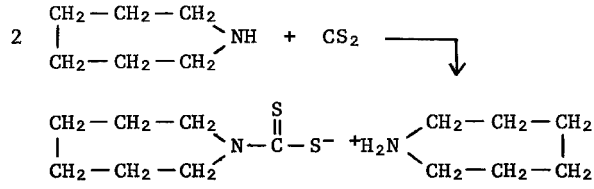
We have reported a simple and rapid method for the simultaneous determination of lead(II), nickel(II), cobalt(II), copper (II), mercury(II) and bismuth(III) in water after solvent extraction as their pyrrolidinedithiocarbamato chelates (APDC chelates) in the concentration range of 4-1000 ppb (6) and applied also to the simultaneous determination of lead(II), nickel(II) and copper (II) in Orchard Leaves (NBS, SRM 1571) (7).

In this paper, we describe a more sensitive method compared with the previous work (6) by using HMA-HMDC as chelating agent. The presented method was possible to determination cadmium (II) which could not determined by previous work (6).

MATERIALS

Reagents

All chemicals used were of analitycal grade purity unless otherwise stated. HMA-HMDC was prepared easily by the method described by Busev et al. (12), and recrystallized twice from methyl alcohol-diethyl ether (2:1). The final crystal was identified by elemental analysis and mass spectrometry. The symthetic method was summarised in the synthetic reaction (eq. 1). The crystal was preserved in glass bottles and stored at -15°C in a refrigerator. The HMA-HMDC solution of 10^{-2} M (for extraction) and 10^{-3} M (for eluent) were adjusted to pH 9.0 by addition of ammonium chloride-ammonia water buffer solution and preserved in polyethylene bottles and stored a refrigerator (5°C). The standard metal solutions were prepared as follows : each of the high-purity metals, such as cadmium, nickel, cobalt, copper and zinc, was dissolved in dilute super special grade hydrochloric or nitric acid, and the other metal solutions were prepared from the metal salts by dissolution with distilled water or slightly acidic or alkaline solution. Each stock solution was made up to contain the metal at a concentration of 1000 ppm. Each 50 ppm standard solution prepared by dilution of the stock solution was acidified to pH 1. A mixed standard solution (for preparation of working curves etc.) containing the six heavy metals (Cd:0.3 ppm, Ni:1.0 ppm, Co:0.5 ppm, Cu:1.0 ppm, Bi:1.0 ppm, Hg:2.0 ppm) were also adjusted to pH 1 with hydrochloric acid. One molar ammonium citrate, dibasic solution was prepared as follows : a 226 g (1 mol) of ammonium citrate, dibasic was dissolved with ca. 900 ml of distilled water then adjusted pH to 9.3 and added water up to 1000 ml. This solution was shaked with 0.01 % diphenylthiocarbazone-chloroform solution for three times in order to remove heavy metals. Two molar ammonium chloride-ammonia water buffer solution was prepared as follows : a 107 g (2 mol) of ammonium chloride was dissolved with ca. 900 ml of distilled water then adjusted to pH 9.0 with ammonia water and



EQUATION 1 Synthesis of Hexamethyleneammonium Hexamethylenedithiocarbamate (HMA-HMDC)

diluted with water up to 1000 ml. The heavy metals in the buffer solution used for the preparation of HMA-HMDC solution were also removed in the manner described above. Chloroform used was purified by distillation before use. The chromatographic solvents were prepared as follows : each commercial reagent-grade methyl alcohol and diethyl ether was distilled and filtered through a membrane filter (Toyo Roshi, TM-2P, 0.45 μ) ; distilled and deionized water was also filtered a membrane filter (Toyo Roshi, TM-2, 0.45 μ). Hydrochloric, nitric acid and ammonia water were super special grade commercial materials and used without further purification.

Apparatus

A liquid chromatograph consisting of a Model 6000 A pump (Waters Assoc.), a U6K universal injector (Waters Assoc.) and UVIDEC-100-III ultraviolet spectrophotometer (Japan Spectroscopic Co., Tokyo, Japan) was used. A 4.6×150 mm reversed-phase column (Cosmosil 5 C_{18} packed column, 5 μm, Nakarai Chemicals, Kyoto, Japan) was employed. This column was cooled at 10.0 ± 0.1°C by a Taiyo Scientific Industrial Co. Ltd. Model 150 L coolpipe and a Model Ace-80 thermominder in all runs. A Hitachi Model 200-20 spectrophotometer was employed for the measurment of UV spectra of HMDC-metal chelates. A Yamato Model SA-31 auto shaker was used for extraction of HMDC chelates and operated at a rate of ca. 250

r.p.m. in all experiments. FL-AAS analysis was performed by Nippon Jarrell Ash (Kyoto, Japan) Model AA-8500 atomic absorption, flame emission spectrometer equipped with Model FLA-100 flameless atomizer and Model MC-100 microprocessor concentration readout system.

METHODS

HPLC System

In general, dithiocarbamates and their metal chelates were thermodynamically unstable. The eluent, therefore, contained trace amount of HMA-HMDC, and the resevoir was cooled in an ice bath, and the column was adjusted to 20 ± 0.1°C. The bubbles which appeared in the connecting tube between a pump and a eluent reservoir by changing of eluent temperature (0°C → ca.20°C) were trapped with a glass tube (6 mm i.d. × 30 mm). Schematic diagram of the chromatographic system is shown in Fig. 1.

HPLC Separation of HMDC-Metal Chelates

A normal-phase (silica gel) and a reversed-phase (ODS) columns were examined as the stational phase, and verious solvents, such as n-hexane, cyclohexane, chloroform, iso-propylalcohol, methyl alcohol, acetonitrile, water, ethyl acetate, diethyl ether, ethyl alcohol etc., and their mixture were examined as the eluent.

Addition of 1.0×10^{-3} M HMA-HMDC to the eluent was needed for the reproducible chromatograms. In order to decide the optimum concentration of HMA-HMDC, the measurements of peak heights of HMDC chelates were carried out at verious HMA-HMDC concentrations.

The effect of injection volume of chloroform extract on the HPLC separation was also investigated.

Ultraviolet spectra of HMDC-metal chelates were measured to decide the detection wavelength.

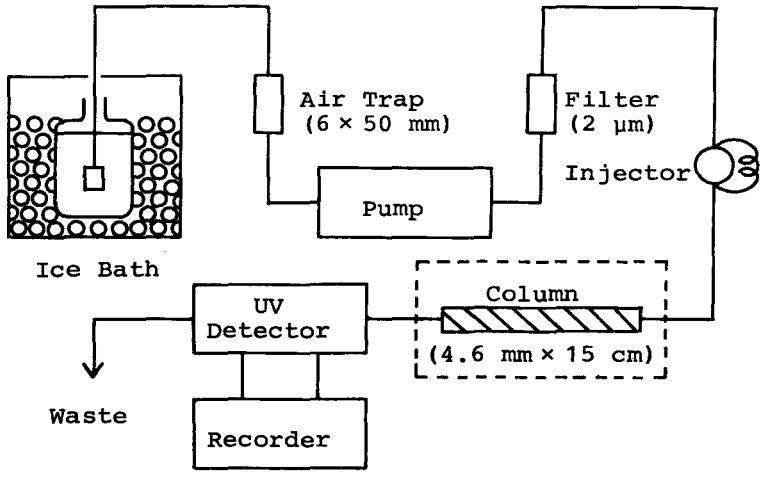


FIGURE 1 Schematic Diagram of the HPLC System.

Extraction Conditions

Optimum amount of HMA-HMDC and shaking time required for simultaneous quantitative extraction of the six heavy metals were investigated. The peak heights were measured at verious amount of HMA-HMDC solution and shaking time. The condition of extraction pH and the amount of buffer and masking agent solution for ion(III) were based on the previous work (6).

Working Curves

The working curves for the determination of six metals were prepared from 50 ml of standard solution containing the six metals, according to the recommended analytical procedure.

Recovery of the Metal Ions from Composite Sample

The effect of foreign ions coexisted on the determination of the six metals were investigated. The composite sample coexisting

sixteen foreign ions was analysed by using the proposed method. The recovery and coefficient of variation for the determination of cadmium(II), nickel(II), cobalt(II), copper(II), bismuth(III) and mercury(II) were investigated.

Determination of Trace Amount of Metals in River Water

The proposed method was applied to the determination of metals in river water samples. The samples were taken from Tamatani, Gôtani and Kakehashi river (Komatsu, Japan), and they were adjusted to pH 1 with hydrochloric acid in situ, then they were stored in polyethylene bottles. After the samples were filtered through membrane filters (Toyo Roshi TM-2, 0.45 μ), the analysis were carried out for the filtrates by the proposed HPLC method and the FL-AAS method.

RESULTS AND DISCUSSION

HPLC Separation of HMDC-Metal Chelates

When reversed-phase column (cosmosil 5 C_{18}) and a mixed solution of methyl alcohol-water-diethyl ether-1.0×10^{-3} M HMA-HMDC were used, Cd(II)-, Ni(II)-, Co(II)-, Cu(II)-, Bi(III)- and Hg(II)-HMDC chelates were sufficiently separated. Addition of a buffer solution (NH_4Cl-NH_3, pH 7.5) to the eluent provided reproducible chromatograms. Decomposition rate of HMA-HMDC and HMDC chelates in the eluent are slow at a high pH, but the ODS columns can not use at more than pH 8. Thus the eluent was adjusted to pH 7.5. The peak heights of the chelates were reproducible in the HMA-HMDC concentration range of $2-6\times10^{-5}$ M.

When 5 μl or above chloroform extract was injected into the column, the width of each peak became broad, therefore 3.0 μl of chloroform extract was used in the HPLC procedure. A typical chromatogram and the optimum separation conditions were shown in Fig.2.

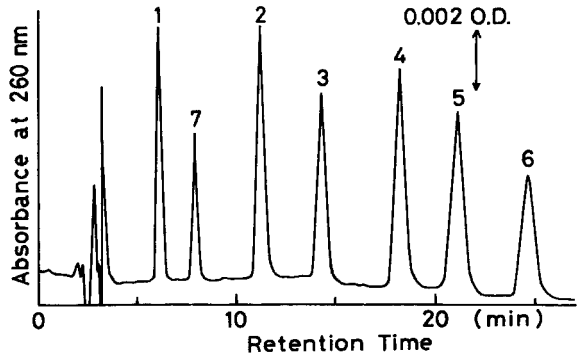


FIGURE 2 Typical Chromatogram of Standard HMDC Chelates

1 : Cd(2.3 ng), 2 : Ni(7.5 ng), 3 : Co(3.8 ng)
4 : Cu(7.5 ng), 5 : Bi(7.5 ng), 6 : Hg(15 ng)
7 : decomposition product of HMA-HMDC
HPLC conditions
Column : Cosmosil 5 C_{18}, 4.6 mm i.d. × 15 cm(10±0.1°C)
Eluent : methyl alcohol/water/diethyl ether/10^{-3} M HMA-HMDC/buffer solution(pH 7.5) = 82/9/3/3/3
Flow Rate : 0.8 ml/min, Injection Volume : 3.0 µl
Eluent reservoir was cooled in an ice bath.

Extraction Conditions

When 0.8 - 4.0 ml of 0.010 M HMA-HMDC was added, reproducible peak heights were obtained over the metal concentration range of 10 - 2000 ppb. The shaking time was investigated in the presence of 3.0 ml of 0.010 M HMA-HMDC over the range of 1 - 30 min. A part of the results was summarized in Fig. 3. The six heavy metals were quantitatively extracted by shaking for 10 - 20 min in the metal concentration range of 10 - 2000 ppb in the presence of 3.0 ml of 0.010 M HMA-HMDC. In the recommended procedure, 3.0 ml of 0.010 M HMA-HMDC and 15 min for shaking time were employed.

Recommended Analytical Procedure

From the studies described above, the following procedure is recommended for the simultaneous determination of cadmium(II),

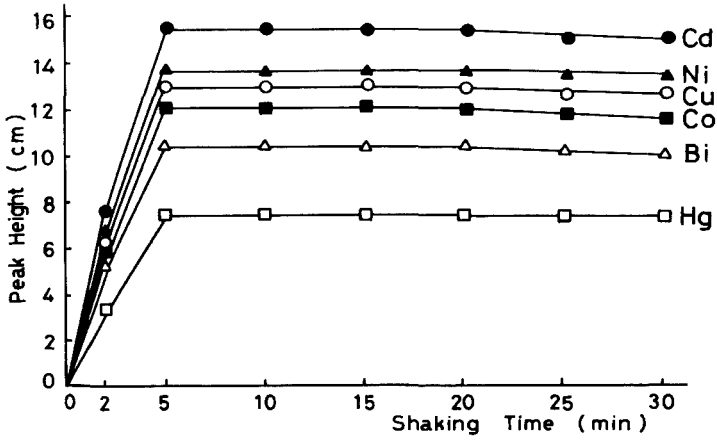


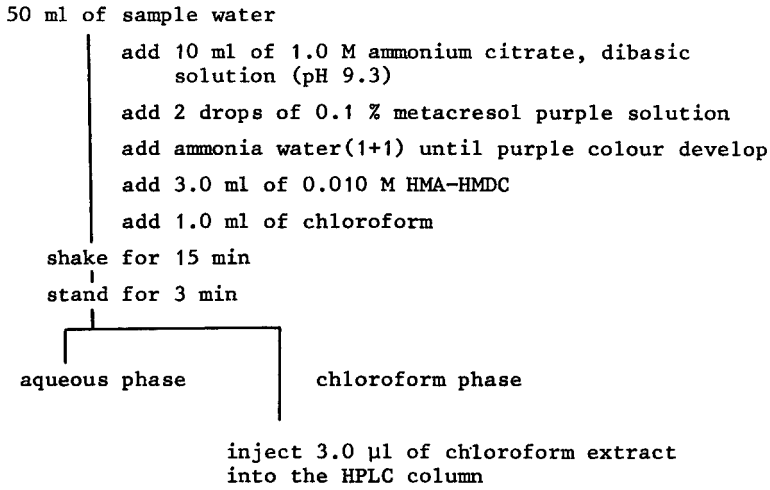
FIGURE 3 Effect of Shaking Time on the Extraction

Metal Concentrations
Cd : 30 ppb, Co : 50 ppb, Hg : 200 ppb, Ni,Cu,Bi : 100 ppb (detector range : 0.04 O.D.)
Extraction was carried out in the manner shown in Scheme 1.
HPLC conditions were same as shown in fig.2.

nickel(II), cobalt(II), copper(II), bismuth(III) and mercury(II) in water (Scheme 1) : a 50 ml of sample water containig 0.015 - 100 μg of each metal is put into a separatory funnel. Then the six heavy metals are extracted according to the recommended analytical procedure as shown in Scheme 1. A 3.0 μl portion of the chloroform extract was injected into the column (Cosmosil 5 C_{18} , 4.6 mm × 15 cm) which adjusted to 10.0 ± 0.1°C. The eluent (methyl alcohol - water - diethyl ether - buffer solution(pH 7.5) - 1.0×10^{-3} M HMA-HMDC = 82:9:3:3:3) cooled in an ice bath was flowed at a rate of 0.8 ml/min and the eluted HMDC-metal chelates were detected at 260 nm. The each amount of metal could be determined by measuring the peak height or the peak area.

Working Curves and Detection Limits

The working curves for the determination of the six metals were prepared with 50 ml of standard solution containing these six metals



SCHEME 1 Recommended Extraction Procedure for the Determination of Cd, Ni, Co, Cu, Bi and Hg by HPLC

according to the above procedure. All the working curves obtained showed good straight lines and passed through the origin. They were expressed by least-squares fitting. The regression lines obtained and values of correlation coefficient were summarized in Table 1.

Detection limits of each metal was 45 pg for cadmiun(II), 150 pg for nickel(II), 75 pg for cobalt(II), 150 pg for copper(II), 150 pg for bismuth(III) and 300 pg for mercury(II).

Recovery of Metal Ions from Composite Sample

The effects of presence of other ions on the determination of the six metals were investigated. The results were summarized in Table 2. The limiting value of the foreign ions was taken as that value which caused an error of less than 10 % in the recovery of each at least one metal. Lead(II) and zinc(II) influenced the recovery of nickel only, but almost ions did not have significant influence on the recovery of the six metal ions.

Table 1 Regression Lines of the Working Curves for the Six Heavy Metal Ions

	Metal Ion	Concentration Range (ppb)	Regression Line	Correlation Coefficient
(a)	Cd(II)	30 - 300	y*= 0.50843 x**	0.99985
	Ni(II)	100 - 1000	y = 0.15528 x	0.99967
	Co(II)	50 - 500	y = 0.31130 x	0.99970
	Cu(II)	100 - 1000	y = 0.17996 x	0.99972
	Bi(III)	100 - 1000	y = 0.14309 x	0.99899
	Hg(II)	200 - 2000	y = 0.05077 x	0.99821
(b)	Cd(II)	3 - 30	y = 0.59710 x	0.99948
	Ni(II)	10 - 100	y = 0.15297 x	0.99913
	Co(II)	5 - 50	y = 0.27304 x	0.99927
	Cu(II)	10 - 100	y = 0.15910 x	0.99864
	Bi(III)	10 - 100	y = 0.13045 x	0.99971
	Hg(II)	20 - 200	y = 0.04433 x	0.99959
(c)	Cd(II)	0.3 - 3.0	y = 0.81141 x	0.99917
	Ni(II)	1.0 - 10	y = 0.25629 x	0.99196
	Co(II)	0.5 - 5.0	y = 0.24250 x	0.99923
	Cu(II)	1.0 - 10	y = 0.11839 x	0.99920
	Bi(III)	1.0 - 10	y = 0.09932 x	0.99916
	Hg(II)	2.0 - 20	y = 0.03178 x	0.99839

Detector Range : (a);0.32 O.D., (b);0.04 O.D., (c);0.005 O.D. and all peak heights were calculated at 0.04 O.D.

y* : peak height at 0.04 O.D.(cm)

x** : metal concentration (ppb)

Table 2 Effect of Foreign Ions and Salts on the Determination of the six heavy metals*

Ion	Tolerance Limits	Ion or Salt	Tolerance Limits
Ag(I)	1.0 ppm	Sr(II)	500 ppm
As(III)	100 ppm	Pb(II)	0.05 ppm
Ca(II)	500 ppm	Zn(II)	0.1 ppm
Cr(III)	10 ppm	NaCl	1.0 %
Cr(VI)	500 ppm	KCl	1.0 %
Fe(III)	0.5 ppm	KNO_3	0.5 %
HCO_3^-	100 ppm	$NaNO_2$	0.5 %
Mg(II)	100 ppm	Na_2SO_4	1.0 %
Sn(II)	1.0 ppm	NaH_2PO_4	5.0 %
Sn(IV)	0.1 ppm	$HClO_4$	1.0 %
Mn(II)	5.0 ppm	Na_2SO_3	0.01 %

Concentration of each heavy metal was 15 ppb for Cd(II), 50 ppb for Ni(II), 25 ppb for Co(II), 50 ppb for Cu(II), 50 ppb for Bi(III) and 100 ppb for Hg(II).

Table 3 Recovery of the Six Heavy Metal Ions from Composite Sample in the Presence of Sixteen Foreign Ions or Salts

Ion	Taken (ppb)	Found (ppb)	Recovery (%)	C.V. (%)
Cd(II)	15.9	15.7 ± 0.2	99.4	1.2
Ni(II)	50.0	50.4 ± 0.6	100.8	1.2
Co(II)	25.0	25.2 ± 0.1	100.8	0.5
Cu(II)	50.0	50.7 ± 0.4	101.5	0.7
Bi(III)	50.0	49.6 ± 0.4	99.2	0.9
Hg(II)	100.0	100.9 ± 0.9	100.9	0.9

Coexistent ions(ppm) and salts(%)
Ag(I) 0.1, Ca(II) 50, Cr(III) 0.5, Fe(III) 0.05, Mg(II) 50, As(III) 10, Mn(II) 0.5. Sn(II) 0.1, Sr(II) 10, Zn(II) 0.05, HCO_3^- 10, $HClO_4$ 0.01, KCl 0.1, KNO_3 0.01, NaCl 0.1, $NaNO_2$ 0.01

Table 4 Analytical Results of Metal Ions in River Water Samples

Sample	Method	Concentration ($\bar{x}$ ± s, ppb)*		
		Cd(II)	Co(II)	Cu(II)
Tamatani River	HPLC	2.3 ± 0.0	3.6 ± 0.1	460 ± 4
	FL-AAS	2.0 ± 0.2	< 10	453 ± 62
Gôtani River 1	HPLC	0.9 ± 0.0	1.5 ± 0.1	319 ± 4
	FL-AAS	< 1	< 10	320 ± 23
Gôtani River 2	HPLC	0.4 ± 0.0	1.3 ± 0.1	132 ± 1
	FL-AAS	< 1	< 10	127 ± 12
Kakehashi River	HPLC	0.7 ± 0.1	1.4 ± 0.1	40 ± 1
	FL-AAS	< 1	< 10	44 ± 3

* : average value ± standard deviation, N = 4
FL-AAS conditions
Dry : 25 A, 30 sec, Ash : Cd;30 A, 30 sec, Co,Cu;80 A, 30 sec, Atomize : Cd;150 A, 8 sec, Co,Cu;300 A, 10 sec

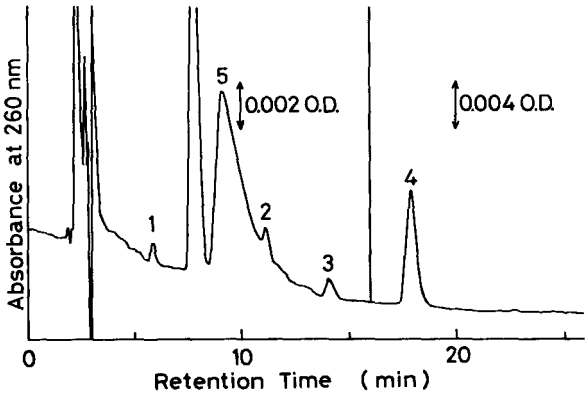


FIGURE 4 Typical Chromatogram Obtained by River Water Samples
1 : Cd(0.7 ppb), 2 : Ni(1.4 ppb), 3 : Co(1.4 ppb),
4 : Cu(40 ppb), 5 : Zn
* : changing of detector range
Other HPLC conditions were same as Fig. 2.

The recovery and the coefficient of variation for the determination of the six metals in the composite sample prepared from the standard solution were investigated by means of the recommended method. The determination of these metals was carried out in the presence of sixteen ions or salts. The results were shown in Table 3. The six metals were recovered more than 99.2 %, and the coefficient of variation was in the range of 0.5 - 1.2 % from six repeated extractions.

Application to the Determination of Trace Amount of Metals in River Water

The proposed method was applied to the determination of trace amount of heavy metals in river water samples. The samples were collected from Tamatani, Gôtani and Kakehashi river (Komatsu,Japan) and they adjusted to pH 1 with hydrochloric acid in situ. After the samples were filtered through membrane filters(Toyo Roshi, TM-2, 0.45 μm), the analysis were carried out for 50 ml aliquots of the

filtrate by the recommended HPLC method. The analytical results were shown in Table 4 and Fig. 4. The metals in the same samples were also determined by FL-AAS method. Bismuth(III) and mercury (II) in river water samples were not detected by both methods. Nickel was only detected in the Kakehashi River(1.4 ± 0.1) by the HPLC method, but the other samples contained less than 1.0 ppb (corresponding to the detection and determination limits of nickel). As can be seen from Table 4, analytical results obtained by the AAS and the proposed method were good agreed.

DISCUSSION

The proposed method permited the simultaneous determination of ppb levels of cadmium(II), nickel(II), cobalt(II), copper(II), bismuth(III) and mercury(II) in water without receiving serious interference from foreign ions. This method is suitable for a routine analysis, because the method is rapid, simple, precise and accurate. In addition, this method does not require so expensive apparatus and special skillfulness, and has extensive concentration range of working curves (by a factor of 1000). Several metal ions whose concentration were very different were simultaneously determined by only changing the detector range without dilution of samples.

ACKNOWLEDGMENT

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REFERENCES

(1) Smith, R.M. and Yankey, L.E., Determination of Metal Ions by Liquid Chromatography Incorporating Dithicarbamates in the Eluent, Analyst, 107, 744, 1982.

(2) Shih, Y.T. and Carr, P.W., Determination of Metals at Trace Levels via Pre-Column Derivatization and Nonpolar Stationary-Phase High Performance Liquid Chromatography with n-Butyl-2-Naphthylmethyldithiocarbamate Complexes, Anal. Chim. Acta., 142, 55, 1982.

(3) Edward-Inatimi, E.B. and Dalziel, J.A., Multi-Element Analysis by Solvent Extraction and HPLC of Metal Chelate Complexes, Anal. Proc. (London), 17, 40, 1980.

(4) Uden, P.C. and Bigley, I.E., High-Pressure Liquid Chromatography of Metal Diethyidithiocarbamates with UV and D.C. Argon-Plasma Emission Spectroscopic Detection, Anal. Chim. Acta, 94, 29, 1977.

(5) Yamazaki, M., Ichinoki, S. and Igarashi, R., Simultaneous Determination of Lead, Zinc and Copper in Water by the Micro High-Speed Liquid Chromatography following the Solvent Extraction of the Metals as Diethyldithiocarbamic Acid-Chelates in Chloroform, Bunseki Kagaku, 30, 40, 1981.

(6) Ichinoki, S. and Yamazaki, M., Simultaneous Determination of Heavy Metals in Water by High Performance Liquid Chromatography following the Solvent Extraction of Heavy Metals as Pyrrolidinedithiocarbamato Chelates into Chloroform, Bunseki Kagaku, 31, E 319, 1982.

(7) Ichinoki, S., Yamazaki, M. and Morita, T.,Simultaneous Determination of Lead, Nickel and Copper in Orchard Leaves by High Performance Liquid Chromatography followed by Solvent Extraction, Bunseki Kagaku, 32, 285, 1983.

(8) Cassidy, R.M., Elchuk, S. and McHugh, J.O., Determination of Metals in Groundwaters by Trace Enrichment and Liquid Chromatography, Anal. Chem., 54, 727, 1982.

(9) Cassidy, R.M. and Elchuk, S., Application of High Performance Liquid Chromatography to the Analysis of Nuclear Materials, J. Liq. Chromatogr., 4, 379, 1981.

(10) Cassidy, R.M. and Elchuk, S., Trace Enrichment Methods for the Metal Ions by High Performance Liquid Chromatography, J., Chromatogr. Sci., 18, 217, 1980.

(11) Cassidy, R.M. and Elchuk, S., Trace Enrichment Methods for the Determination of Metal Ions by High Performance Liquid Chromatography - Part II, J. Chromatogr. Sci., 19, 503, 1981.

(12) Busev, A.I., Byr'ko, V.M., Tereshchenko, A.P., Novikova, N.N., Naidina, V.P. and Terent'ev, P.B., Atomic-Absorption and Spectrographic Determination of Trace of Heavy Metals after Concentrating them by Extraction with Hexamethyleneammonium hexamethylenedithiocarbamate, Zhurnal Analiticheskoi Khimii, 25, 665, 1970.

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HARDWARE FOR MICROPROCESSOR CONTROLLED HPLC: INTERFACING OF AN INTERVAL TIMER AND INTERRUPT CONTROLLER TO THE "S100 BUS SYSTEM"

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ABSTRACT. A hardware interface for the 8253 Interval Timer and the 8259 Interrupt Controller to the standard "S100 Interface Bus System" is described herein. The interface allows the three timers of the 8253 Interval Timer to be synchronized by either an external trigger or an internal trigger issued by the microprocessor. This permits synchronized control of external devices either by the microprocessor, manually, or otherwise externally. Furthermore, two of the three timers are driven by two independent programmable clocks. This enhances their range, in contrast to a fixed clock, while maintaining the lowest possible error. The addition of the 8259 Interrupt Controller further supplements the flexibility of this interface. It permits the synchronization of software along with the external devices and permits the timers to be used in a "stopwatch" fashion by allowing them and the external trigger to initiate an "interrupt".

INTRODUCTION

The advent of "single board" microprocessors such as the "Sym", "Kim" and "Explorer 85" have brought automated HPLC systems within the economic reach of many laborators. These inexpensive programmable controllers are easily coupled to a larger computer in

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a master/slave configuration. The microprocessor, as the slave, is used to control the chromatography apparatus while collecting data for transfer to a master computer for subsequent processing and print-out.

A computer-microprocessor network of this style requires two interfaces, one between the microprocessor and the apparatus to control the system; the other between the microprocessor and the computer to perform data transfer. Fortunately, the major components for various versions of the latter interface are included on most computers and microprocessors. These are usually accessed directly through the "user" or "Peripheral Port". If not, a "Peripheral Port Interface" chip provides a simple, straight-forward interface.

The other interface, between the microprocessor and the chromatography apparatus, is rarely provided and is much more involved. It consists of any number of analog-digital and digital-analog converters, device select decoders and drivers, data registers, timers and controllers to name only a few. However, the literature on such interfaces to a large extent is limited to manufacturer's data books on the individual integrated circuits. The hardware and actual circuitry associated with these chips are somewhat neglected. To fill this gap, it is the purpose of this paper to describe a software programmable interval timer-interrupt controller interface.

The need for such an interface is self-evident. In addition to providing the three independent timers of the 8253 Interval

Timer (I.T.) the interface also provides a means by which an external device (the HPLC apparatus) can communicate with the microprocessor's central processing unit (CPU) by including the 8259 Interrupt Controller (I.C.). Through this controller, devices such as thermostats for constant temperature baths, analog-digital converters for monitoring the output of absorbance monitors and pH meters, pressure sensors in pumping systems, and even fluid level sensors in eluant reservoirs can signal the CPU. The 8259 I.C. is not an "input port" for digital data but rather an interrupt signal source. After receiving an interrupt request signal from a device, the controller issues an interrupt signal to the CPU followed by a subroutine address to which the program jumps. The address and the interrupt priorities are both software programmable.

This, however, is not the only function of the interface. The main intent is to provide the microprocessor with several programmable timers and necessary control and synchronization circuitry for accurately timed and synchronized intervals. Such timed intervals are a must in microprocessor controlled HPLC systems if the potential speed and accuracy of such a system is to be achieved. This is particularly true when making periodic measurements. Even a small error in timing becomes substantial when several thousand measurements are taken. However, this problem is eliminated by this interface. As a consequence, the timers may be programmed in a "free-running" mode without losing the initial accuracy and synchronization. This simplifies

the programming when taking periodic readings of absorbance for integration of peak areas. Furthermore, a provision is provided in the timer control circuitry to enable a timer to be programmed as a "stopwatch", thus allowing the computation of flow rates by the microprocessor and consequently the subsequent computation of retention volumes and times, as well as fraction size.

The remainder of the interface consists of three registers and associated circuitry which are decoded as two output ports and one input port as shown in Diagrams 1a and 1b. The two output ports (timer control register and clock select register) provide software control of all three gates and two of the three independent clocks of the 8253 I.T. The Timer Control Register also provides control of two "External Timed Outputs" and the mode of synchronization (internal or external) of the timers, external devices and software. The third register (Timer Status Register) provides the CPU with the status of the gates and outputs of the timers and the status of the external trigger.

Furthermore, this interface takes full advantage of the microprocessor's reaction time and speed by allowing machine language level programming. This eliminates much of the redundancy in the machine language program generated when using a higher level language such as "FORTRAN" or "BASIC".

MATERIALS

The 8253 Interval Timer and 8259 Interrupt Controller (Intel Corp., Sanat Clara, CA) were purchased from the Digi-Key Corp.,

Thief River Falls, MN. All other integrated circuits used in this interface are of the SN74LS' Series (Texas Instruments, Inc., Dallas, TX). The microprocessor used is the "Explorer 85" single board microprocessor, ASK II terminal version with levels A, B, S, D, and E (Newtronics Research and Development LTD, New Milford, CT). It uses the 8085 CPU and is equipped with a standard "S100 Bus System" and interface card cage. The interface cards (Universal Microcomputer Processor Plugboard, No. 8800V, Vector Electronic Co., Inc.) were obtained from Jameco Electronics, Belmont, CA and are designed for "wire-wrap" assembly. The "Explorer 85" is interfaced to a ASR35 teletype (Teletype Corp., Skokie, IL) with a 20 milliamp loop. All programming is done on the machine language level of the 8085 CPU.

DISCUSSION

Upon initialization of the Explorer 85 Microprocessor, pressing the "Reset Key", a "Reset Signal" is sent out on the Power On and Clear ($\overline{POC}$) line of the "S100 Bus". This in turn causes two events to occur in the 8253 I.T. interface hardware. All output registers are cleared and all flip-flops are reset. This causes all gates to the timers of the 8253 I.T. to go low, which turns off each timer (1); the two programmable clocks are set to 1.0 megahertz and all "external" circuits are disabled. (A low condition of a line or signal is defined as a voltage between 0.45 and 0.0 volts on that line. It is synonymous with a digital logic 0 or false condition.) Consequently, the external trig-

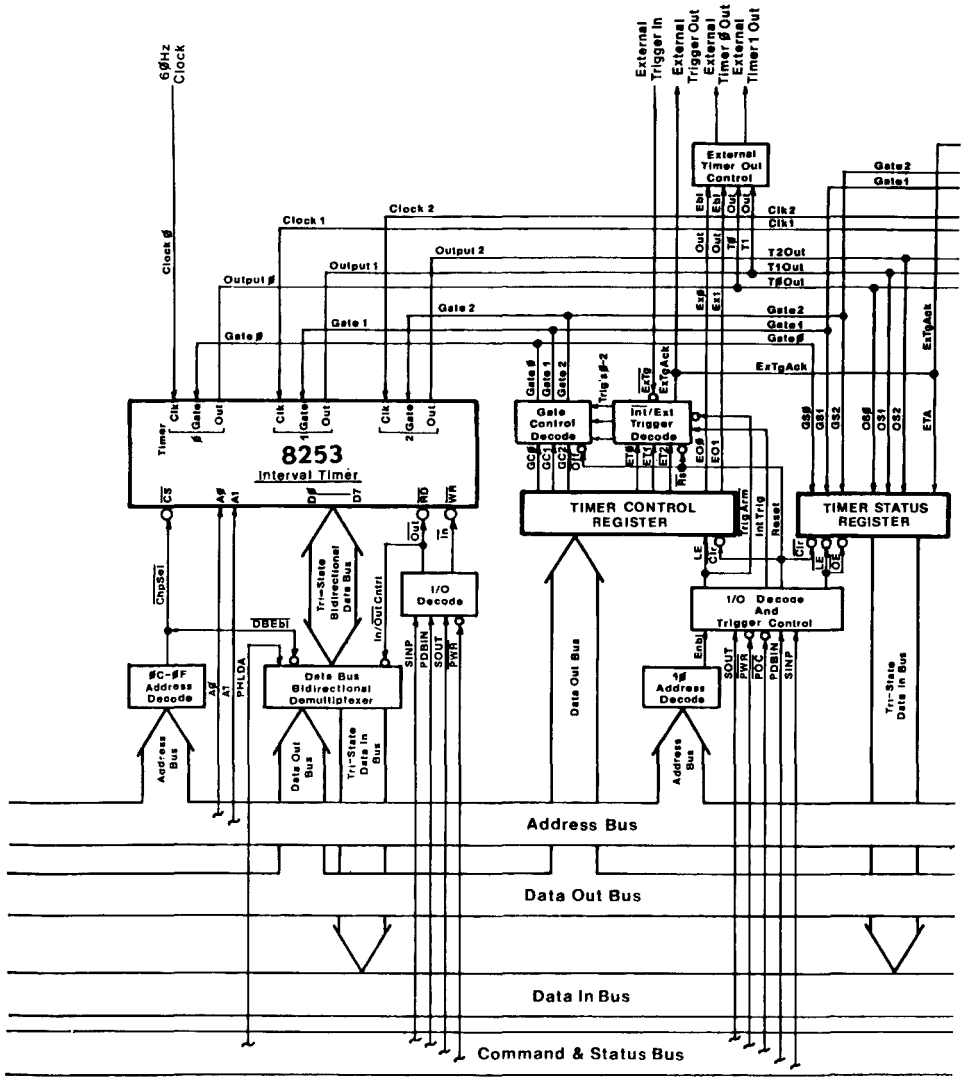

DIAGRAM 1

Microprocessor Controlled HPLC. A schematic view of the flow diagram showing the interfacing of the interval timer, 8253 (Diagram 1a) and the interrupt controller, 8259 (Diagram 1b) to the "S100 Bus System". See the text for details. (Write to the author RPS for a clear diagram and details.)

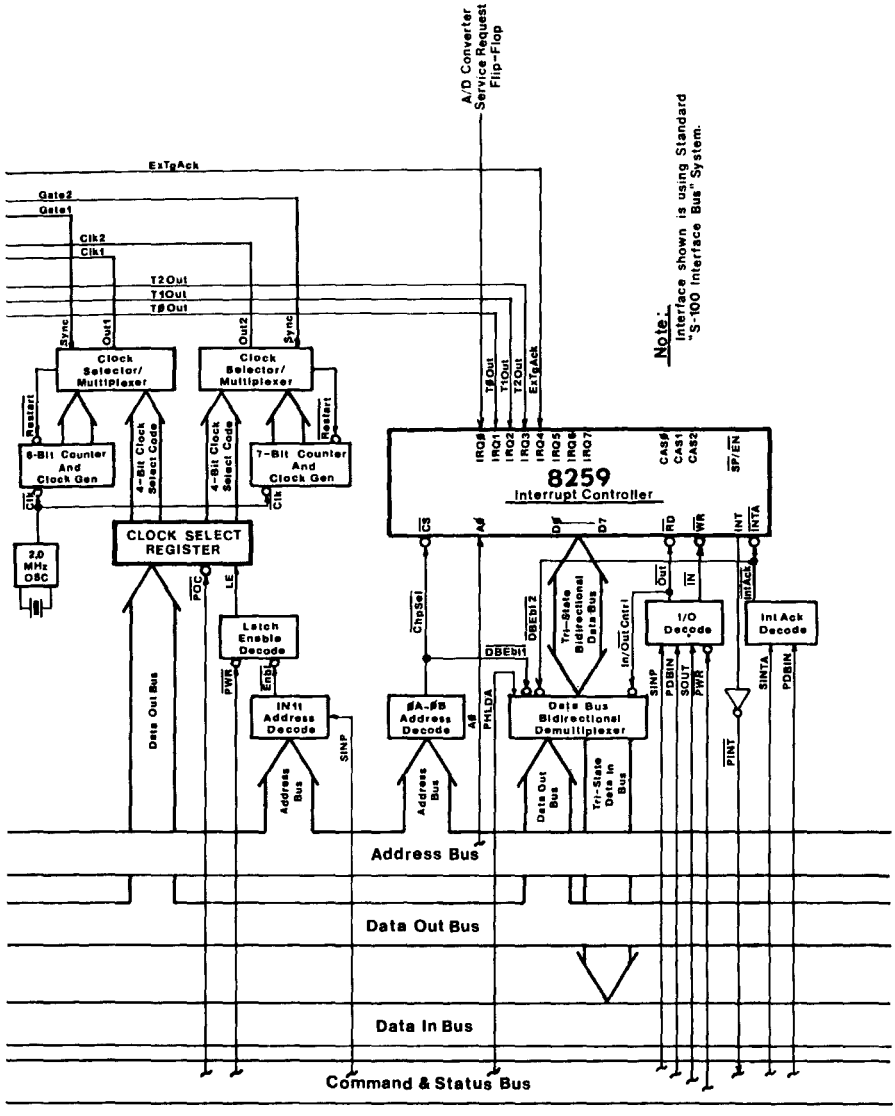

DIAGRAM I (continued)

ger is cleared and disabled and the External-Timer-Outputs are reset low; thus preventing the timers or the external circuitry from causing a system interrupt through the 8259 I.C. or triggering an external device to which they are connected.

The 8253 I.T. and associated registers are now ready to be programmed. The 8253 I.T. is programmed in the usual way (1). Its Mode Control Register and the three counters are "loaded" with "output" instructions. However, the timers do not start since all gates are maintained low.

1. The Timer Control Register.

a. *Control of the gates of the timers.* The gates are controlled by the Timer Control Register as illustrated in Diagram 1a. The outputs of the Timer Control Register are divided into three sets as shown in Figure 1. The first three bits of the register (D_0 to D_2) control the status of the gates to the timers located in the 8253 I.T. After initialization, each bit (D_0 to D_2) is reset low. As a consequence, this inhibits the counters in the timers, in effect, keeping them turned off. To start a particular timer the bit corresponding to that gate is set high. (A high condition of a line or signal is defined as a voltage between 2.4 and 5.0 volts on that line. It is synonymous with a digital logic 1 or true condition.) A specific timer(s) can be turned on or off by setting or resetting the appropriate bit(s) since the state of the bits is independent. In this way the computer can control the running of the timers directly in a synchronous manner.

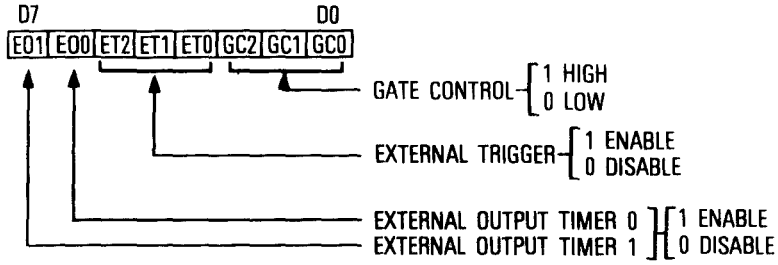

FIGURE 1

The Time Control Register (see Diagram 1a for its relation with other components). The gates and synchronization of the timers are controlled by the register.

b. *Synchronization of the timers.* The synchronization of the timers is achieved by generating a trigger pulse which clocks the data in bits D_0, D_1 and D_2 to the outputs of three "D" filp-flops which are connected to the gates of the timers (see Diagram 1a). The trigger pulse can be generated either internally or externally. This trigger pulse generating source is controlled by the next three bits of the Timer Control Register (D_3 to D_5) (see Figure 1). In the case where the bit (D_3 to D_5) is low, the trigger pulse for the corresponding gate is generated internally at the end of the "output" instruction cycle which loads the Timer Control Register. However, when the bit for the desired gate is set high, the internally generated trigger is inhibited and the external trigger is enabled for that gate. The external trigger can be generated at any time at the operator's discretion by grounding the "External Trigger In" line after setting one or more of the trigger control bits. In addition, after generating

the external trigger, an External Trigger Acknowledge signal is generated which can be used to initiate an interrupt through the 8259 I.C. and thus synchronize software with the External Trigger. Furthermore, this acknowledge signal can be used to control an external device via the "External Trigger Out" line.

c. *Control of external devices by the timers.* The last two bits of this register (D_6 and D_7) are an External Timer Output Enable (see Diagram 1 and Figure 1). The setting of bit D_6 or D_7 (or both) make outputs of Timer 0 and Timer 1, respectively, available to an external device. Consequently, these two timers can be used to control and synchronize an external devide or event such as the timed collection of fractions or the switching of eluant after a prescribed length of time.

2. The Clock Select Register.

a. *Selection of clock frequency.* The second output port is the Clock Select Register (see Diagram 1b). This register is divided into two 4-bit registers as shown in Figure 2. The first four bits (D_0 to D_3) determine the clock frequency for Timer 1 while the second four bits (D_4 to D_9) determine the clock frequency for Timer 2. (Timer 0 has a fixed clock frequency of 60 hertz derived from the AC supply of the power supply.) The actual frequency is selected by the last three bits of each 4-bit register. The clock periods and associated three bit code for each timer are shown in Figure 3. The periods are prime numbers listed in micro-seconds. These prime numbers periods permit a wide variety of

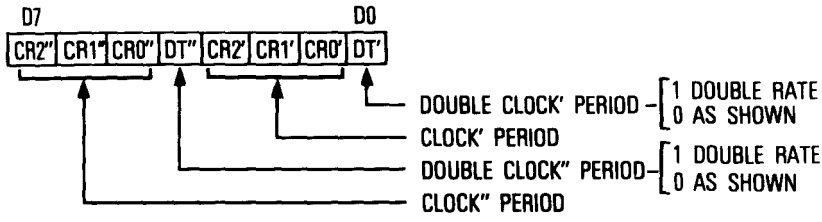

FIGURE 2

The Clock Select Register (see Diagram 1b for its relation with other components). The clock frequency for timers 1 and 2 are determined by the Clock Select Register.

timed intervals with the least redundancy, while the whole number increments simplify selection. The first bit of each 4-bit Clock Select Register, D_1 and D_4, is a "double time" bit. When it is set high, the clock periods of Figure 3 are doubled. This greatly increases the set of intervals which can be accurately timed by adding a large number of even intervals which cannot be timed with the odd prime clock periods.

b. *Clock synchronization.* In addition, the two clock generators contain a "restart" (or reset) provision. The restart is triggered by a "Sync Pulse" generated when the gate for the timer goes high. Consequently, when the gate for Timer 1 or Timer 2 goes high, the timer is started and the corresponding clock is reset to the beginning of the clock cycle. This synchronizes not only the times but also their clocks and eliminates the possibility of starting the timers in the midst of a clock cycle. Thus the total error in any timed interval is reduced to less than one microsecond for both the normal frequency and the "Double Time" frequency.

	CLOCK PERIOD IN MICROSEC.												
	1	3	5	7	11	13	17	19	23	29	31	37	41
CR0'	0	1	0	1	0	1	0	1					
CR1'	0	0	1	1	0	0	1	1					
CR2'	0	0	0	0	1	1	1	1					
CR0"	0			1		0			1	0	1	0	1
CR1"	0			0		1			1	0	0	1	1
CR2"	0			0		0			0	1	1	1	1

FIGURE 3

A tabulation of clock rate codes indicating the clock periods and corresponding three bit code for each timer. The periods are prime numbers listed in microseconds. Setting of the "double time" bit doubles these clock periods.

3. The Timer Status Register.

The third register associated with the 8253 I.T. is the "Rimer Status Register" (see Diagram 1a). This is an input register which allows the microprocessor to "poll" status of timer outputs, gates, and the External Trigger Acknowledge line as shown in Figure 4. The data are latched into this register upon addressing it with an "input" instruction to insure current data. This register is particularly useful in verifying the status of the aforementioned outputs and lines and determining which half of the count a timer is on by polling the status of its output. This is necessary when the timers are used in a "stopwatch" manner (1) to increase the accuracy of the measured time.

4. The 8259 Interrupt Controller.

The 8259 Interrupt Controller (I.C.) is interfaced as shown in Diagram 1b. There are no auxillary registers associated with

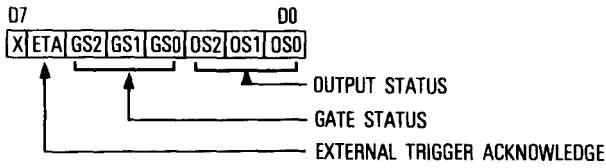

FIGURE 4

The Timer Status Register (see Diagram 1a for its relation with the interval timer, 8253). The microprocessor "polls" status of timer outputs, gates and external trigger acknowledge line.

it. The outputs of Timers 0, 1, and 2; and the external Trigger Acknowledge line are connected directly to the "Interrupt Request" lines (IRQ) 1, 2, 3, and 4, respectively. (IRQ 0 is being used by an A/D Converter in our system.) Consequently, anyone of these lines can initiate an interrupt, provided that the corresponding IRQ is unmasked (2). If this is done, a software subroutine can be synchronized with the timers or external trigger.

The programming of the 8259 I.C. is done with "input" instructions to read its output registers and "output" instructions to load its input registers. This masks and unmasks IRQ lines, determines the memory address vectored to upon receipt of an interrupt request, and determines the mode of operation (2).

The three "Cascade Inputs" (CAS 1, 2, and 3) and the $\overline{SP}/\overline{EN}$ output are not used. They are left available for cascading of three more 8259 Interrupt Controllers with the remaining three IRQ inputs, thus making it possible to add another 24 IRQ lines to the system (2).

ACKNOWLEDGMENTS

The authors thank Drs. W. R. Carper and R. V. Christian, Jr., for discussions and useful suggestions. The research was funded by the Wichita State University.

REFERENCES

1. Component Data Catalog, Intel Corporation, No. 8253/8253-5: Programmable Interval Timer, Santa Clara, CA, Section 8, pages 61-66, (1981).

2. Component Data Catalog, Intel Corporation, No. 8259A/8259A-2/ 8259A-8: Programmable Interrupt Controller, Santa Clara, CA, Section 6, pages 123-136, (1981).

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SOLID PHASE DERIVATIZATIONS IN HPLC: POLYMERIC PERMANGANATE OXIDATIONS OF ALCOHOLS AND ALDEHYDES IN HPLC-SPR

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ABSTRACT

On-line or off-line oxidations of various alcohols, aldehydes, and ketones can now be performed in conjunction with high performance liquid chromatography (HPLC), utilizing a newly developed polymeric permanganate solid phase reactor (SPR). These derivatization reactions are compatible with most reversed phase and normal phase solvents for HPLC separations, and many of these oxidations can be accomplished in real-time, on-line, at or above room temperature. Such HPLC-SPR approaches for chemical modifications and derivatizations of various oxidizable analytes provide a useful and quite practical newer approach for the HPLC-ultraviolet (UV) detection of appropriate analyte species. Difference chromatography, often with improved UV detection, can be used to confirm the suspected presence of a particular oxidizable analyte in a complex sample matrix. All of these solid phase derivatizations utilize conventional, commercially available HPLC instruments and accessories. These HPLC-SPR oxidation methods for chemical derivatization have also been applied to certain real world samples, in order to demonstrate the overall value and applicability of such analytical approaches.

INTRODUCTION (1)

Although a very large number of derivatization approaches have already been described for HPLC applications/utilization, perhaps more than 99% of these have utilized standard, homogeneous type approaches (2-10). Indeed, most conventional homogeneous derivatizations for HPLC are still done off-line, in the pre-injection/column mode, wherein the desired derivative(s) and excess derivatizing reagent(s) are injected together. More recently, automation of

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both pre-column and post-column homogeneous derivatizations, off-line and on-line respectively, have become quite popular and widespread. Nevertheless, it is clear to us and others that homogeneous derivatizations have certain inherent, difficult to avoid/overcome, disadvantages in trace analysis. We have recently summarized many of these more serious disadvantages, and at the same time, we have discussed in depth what we believe are some of the more significant advantages of utilizing solid phase derivatizations in HPLC (2, 3, 11-15). In previous publications, we have described two distinct approaches to performing, on-line, in real-time, pre- or post-column chemical reductions of various aldehydes or ketones (11, 12). In one of these approaches, we used an in-house prepared polymeric borohydride solid phase reactor (SPR), which could then be utilized for the efficient reductions of aldehydes in reversed phase (RP) HPLC separations. In the other publication of ours in this area, we described the preparation and use of a silica supported borohydride reagent for performing similar reductions of aldehydes and ketones, now using normal phase HPLC solvents and separation conditions (11). At the same time, Frei's group in Amsterdam have just described similar approaches in HPLC-SPR, wherein the alumina support commonly used for HPLC separations has been utilized for certain catalytic reactions, pre-column, on-line, in real-time derivatizations (14). Our own interest in HPLC-SPR has now been extended to the development, optimization, and application of a newer polymeric oxidizing SPR for on-line and off-line derivatizations in HPLC.

We describe here the preparation, characterization, evaluation, optimization, and application of a polymeric permanganate resin for performing a wide variety of solid phase oxidations of suitable alcohols (primary/secondary), aldehydes, and some ketones. Such derivatization approaches have been developed for both on-line and off-line applications, with both reversed phase and normal phase HPLC conditions and separations. Percent oxidations have been determined as a function of the derivatization conditions and solvents, and where possible, these have been optimized with regard to time and temperature. At the same time, it has now been possible to evaluate what other classes of organic compounds do not undergo oxidations with this particular SPR. In all of these HPLC-SPR studies, difference chromatography has been utilized, wherein the analyte or sample matrix is first injected onto a dummy SPR plus the analytical column, and then in a separate injection, onto a combination of the oxidizing SPR plus the same analytical column. In both instances, the only change in the two chromatograms thus generated is due to the presence of the oxidizing reagent (permanganate) on the oxidizing SPR, but all other HPLC-SPR operating conditions remain constant. Although UV detection has been emphasized throughout these particular studies, often with enhanced UV detection following SPR oxidations, it is indicated that these approaches are totally amenable and applicable to virtually any known/commercial HPLC detector. This is true, as long as either the starting material and/or the product can be

detected with the particular HPLC detector of choice or availability (2). Indeed, a combination of HPLC detectors could be utilized with HPLC-SPR, and the overall analyte/product specificity could be considerably enhanced over the use of a single such detector.

Finally, these HPLC-SPR oxidation approaches have now been applied to two real world samples, resulting in the partial or complete oxidation of the starting alcohol present. In one such instance, it has been possible to oxidize benzyl alcohol present in a particular hair shampoo, and to observe the formation of the expected aldehyde and carboxylic acid following HPLC-SPR oxidation. In the second instance, riboflavin has been oxidized to a variety of products, perhaps due to the presence of a sugar moiety capable of undergoing oxidations of the various alcohol functionalities simultaneously. It is suggested that a large number of other possibly interesting applications for this particular oxidizing SPR will be developed in the very near future.

EXPERIMENTAL

Reagents and Chemicals

Certain preliminary studies on SPR oxidations were performed with a commercially available sample of chromic acid on Amberlyst A-26 (2.2 meq CrO_3/g reagent), obtained from Alfa Products, Ventron Division, Thiokol Corp. (Danvers, Mass.). This material was used directly in on-line and off-line work with various alcohols, but its oxidizing efficiency was less than adequate or desirable for most HPLC-SPR applications. The polymeric permanganate SPR was prepared using an analytical grade anion exchange resin, AG 1-X8, minus 400 mesh (Bio-Rad Laboratories, Richmond, California). This is a styrene-divinyl benzene based quaternary ammonium (Cl^- form) anion exchange resin which has proven useful in this and previous polymeric SPR studies (12).

HPLC solvents were obtained from Waters Associates, Inc. (Milford, Mass.), as their HPLC grade materials, distilled-in-glass. All such solvents were used as received, with de-gassing and filtering through a 0.45um solvent filtration kit/filter (Millipore Corp., Bedford, Mass.). At times, HPLC grade water was obtained from MCB Manufacturing Chemists, Inc. (Gibbstown, N.J.), as their Omnisolv brand HPLC solvent.

The various chemicals and reagents utilized here were obtained from a variety of commercial suppliers, of the highest purity available, and were used as received, without further purification. These were obtained as follows: potassium permanganate (Aldrich Chemical Co., Milwaukee, Wisc.); benzoquinone (Aldrich); acetophenone (Aldrich); benzhydrol (Aldrich); benzyl alcohol (Aldrich); trans-cinnamaldehyde (Aldrich); cinnamyl alcohol (Aldrich); benzaldehyde (J.T. Baker Chemical Co., Phillipsburg, N.J.); benzophenone (Fisher Sci. Co., Medford, Mass.); p-nitrobenzaldehyde (Sigma Chemical Co., St. Louis, Mo.); p-nitrobenzyl alcohol (Aldrich); Faberge Hair Shampoo (MediMart Drugs, Boston, Mass.); sec-phenethyl alcohol (Aldrich); salicylaldehyde (Aldrich); hydroquinone

(Aldrich); o-aminobenzyl alcohol (Aldrich); o-aminobenzaldehyde (Aldrich); benzoic acid (Aldrich); riboflavin (Vitamin B_2)(Sigma Chemical Co.). Lanthanum nitrate [$La(NO_3)_3$] was obtained from Matheson, Coleman, and Bell, Inc., (Norwood, Ohio).

The polymeric oxidizing resin (permanganate) was prepared by using the AG 1-X8 resin (5g), potassium permanganate (1.5g), and $La(NO_3)_3$ (0.5g) in 80ml of distilled water. This solution was then stirred for 1hr at room temperature, filtered, and washed extensively with water to remove excess, non-ionically attached MnO_4^-. It is essential to fully remove all excess, physically adsorbed permanganate by water washing/extraction, before this resin is used in any SPR studies/applications. Washing was done in a batch process, until the wash water was totally free of the violet color of MnO_4^-. At that point, the resin could be loaded into an SPR column.

Apparatus

The HPLC system utilized for most of these studies consisted of a Waters U6K syringe loading injection valve (Waters Associates, Inc., Milford, Mass.), a Waters 6000A solvent delivery system/pump, a Waters Model 480 variable wavelength UV-VIS detector, and a Houston Omniscribe, Model 5510 dual pen recorder (Houston Instruments, Inc., Austin, Texas). All HPLC separations were performed with uBondapak C_{18} reversed phase columns, 10um, 30-cm x 3.9-mm i.d. (Waters Assocs.). The dummy SPR column and the solid phase reactor (SPR) column were prepared using glass lined stainless steel tubing, 6-cm x 4.6-mm i.d., from Alltech Associates, Inc. (Deerfield, Ill.). All dummy, solid phase reactor, and analytical column end fittings were zero dead volume (Cambridge Valve & Fitting, Inc., Billerica, Mass.)(Waters Assocs., Inc.). Wherein both the dummy and SPR were both on-line simultaneously, individual injections were switched to either the dummy or SPR via a Rheodyne Model 7000 switching valve (Rheodyne Corp., Cotati, Calif.). The switching valve was located just after the HPLC injection valve and before the dummy and SPR columns, all of which was located just before the analytical column. A schematic diagram of the overall instrumentation arrangement has been published elsewhere (11, 12).

Methods

In all of these studies with the polymeric permanganate resin, standards of all organic compounds being analyzed were injected as solutions in either the mobile phase or neat acetonitrile. Such standard solutions, in known concentrations, were generally injected in 20ul aliquots, first onto a combination of the dummy column plus analytical column, and then onto the polymeric SPR oxidation column and the same analytical column. The dummy column consisted of the commercial anion exchange resin, usually the AG 1-X8, in its original chloride (Cl^-) form. The polymeric permanganate resin in the solid phase reactor and the dummy column were both slurry packed, at pressures of about

2000 psi. During normal operations, both the dummy and SPR columns were stable at pressures of at least 1500-2000 psi. It is probable that these polymeric packings are stable to higher HPLC back pressures, when used in the pre-column mode, but we have not had to determine maximum pressure stabilities. All retention times on both dummy and SPR columns, pre-analytical column mode, were determined by duplicate or triplicate injections of the analyte of interest along with the expected oxidation product(s), the aldehydes or ketones or carboxylic acids, wherever standards for these were known and/or available. In all cases, with various mixtures of mobile phases, the retention times of the alcohols, aldehydes, and ketones on both the dummy and SPR columns agreed very well (±5% or less). Retention times of all compounds were based on the HPLC chromatograms and an external automatic timer started at the point of injection and measured at the point of maximum peak height(s).

In the case of the commercial hair shampoo used for the determination of benzyl alcohol *via* its off-line oxidation, the initial sample of shampoo was first diluted 100-fold in the HPLC mobile phase of 50/50 (v/v) water/acetonitrile (HOH/ACN). The oxidation in this case was performed off-line, at room temperature, in 10 mins, by injecting 100ul of the initially diluted hair shampoo onto a column of the oxidizing resin. After oxidation of the sample, the entire material and oxidation products were eluted from the SPR with an excess of the HPLC mobile phase (10mls). This final, diluted solution was then injected directly onto the HPLC system, with only the analytical column on-line, since the SPR oxidation had taken place off-line here. In the application involving the off-line oxidation of riboflavin, a standard of this vitamin was dissolved in 200ul of 50/50 HOH/ACN, and this solution was then placed onto the oxidizing SPR, off-line, at room temperature, for 10 mins. At the end of this time period, the oxidized products were eluted with 5mls of 50/50 HOH/ACN. An aliquot (20-25ul) of this eluted solution was then injected directly onto the analytical HPLC system, in order to determine remaining riboflavin and its oxidation products.

SPR oxidations of various standard alcohols, aldehydes, or ketones were performed in a number of possible manners, including: 1) off-line, 10 mins or less, at room temperature or 46^{0}C (elevated temperature), followed by elution of the reaction mixture with mobile phase, then HPLC injection; 2) on-line, pre-analytical column mode, room temperature or above, real-time or extended time in SPR, followed by HPLC elution of the oxidized products onto RP analytical column. SPR reactions performed at elevated temperatures were done using a constant temperature water bath, Precision Scientific/GCA Corp. (VWR Scientific, Inc., Boston, Mass.). Although SPR oxidations, in principle, could be performed on-line, real-time, post-column, we have not made use of such approaches in these studies. Previous publications have described and discussed how post-column, on-line SPR derivatizations can be performed, and what sort of qualitative/quantitative results might be expected (2, 11, 12).

Elemental analyses for manganese (Mn) content, and therefore an indication of permanganate loading on the final polymeric SPR, were performed at Galbraith Laboratories, Inc. (Knoxville, Tenn.). Further characterization of the final SPR was done in-house, using a permanganate titration method developed independently. This involved weighing a certain amount of the permanganate resin into a 250ml titration flask with 20ml distilled, deionized water. To this was added an excess of a ferrous sulfate ($FeSO_4$.7HOH, 0.05M) solution, the entire mixture was stirred, with a final pale yellow color developing. The excess ferrous ion (Fe^{+2}) was back titrated with a standard permanganate solution ($KMnO_4$, 0.05M) until the final solution changed color from pale yellow to pale violet. In general, results of these titrations indicated an average percent loading of MnO_4^- on the polymeric SPR of about 9.8% (n=4), which translates into about 100-125mg permanganate in one typical loaded SPR column. A typical permanganate loaded SPR would require about 1.2g of polymeric material to completely load the empty, glass-lined, stainless steel tubing. The elemental analyses for manganese (Mn) performed at Galbraith Labs involved inductively coupled plasma (ICP) emission spectroscopic methods for total Mn. These results, on the same batch of SPR resin used for the above titration determinations, indicated a somewhat higher loading of total Mn/MnO_4^-. This may be due to the titration method measuring only surface available/loaded permanganate, while the elemental analysis, ICP method, measures total Mn, surface and internally loaded. Internally loaded MnO_4^- may not be titratable using the procedure described above.

RESULTS AND DISCUSSION

In general, on-line, real-time, ambient temperature solid phase oxidations/derivatizations are to be preferred over off-line, delayed/stopped-time, elevated temperature approaches with the same polymeric permanganate SPR. That is, on-line approaches permit overall analyses in just two simple injections, one with the dummy column plus analytical column in-line, and the second with the polymeric SPR plus analytical column in-line. The two overall chromatograms obtained *via* these two separate injections of the same sample solution or solution of standards, then leads to the difference chromatography useful for indicating the presence or absence of the suspected analyte capable of undergoing oxidation with this particular SPR under HPLC conditions. Such on-line approaches do not require any sample manipulation prior to SPR oxidation, other than that normally used/required for HPLC injections, and there is a minimal chance of sample loss or contamination. Thus, on-line approaches have always been the desired goal in this and previous HPLC-SPR research and development work (2, 11, 12). However, at times, it has not been possible to utilize on-line, real-time approaches with this type of SPR, and thus it has been necessary to go to off-line, delayed time, above room temperature oxidation conditions, at times. This is a completely feasible

and practical approach, and still possesses some significant advantages over conventional, homogeneous derivatizations now performed off-line, as well. Since polymeric oxidizing reagents can be stored in the SPR column almost indefinitely, they represent a ready source of an efficient oxidizing system for many organic substrates/analytes. This then avoids the necessity of separately preparing the oxidizing solution each time that an oxidation is needed, and in principle, this and other SPRs could be stored in a readily available and usable bank of derivatizing reagents. Because of the relatively high loading of permanganate on this particular oxidizing SPR, often in the range of 100-125mg/SPR, we have found the final oxidizing columns to be stable and usable/practical for extended periods of time, often months. Clearly, they cannot be used on-line or off-line with an HPLC mobile phase or solvent that could itself undergo oxidation, since that would rapidly and inefficiently consume the permanganate loading on the SPR. At the same time, impurities in the HPLC mobile phase, if these are oxidizable, could also quickly consume and expire the SPR. Also, since these polymeric oxidizing resins are derived from an anion exchange support material, the permanganate reagent is only ionically bound to the polymeric support. Thus, wherein inorganic or organic anionic salts are included in the HPLC mobile phase, it is to be expected that these may/will displace the permanganate loading, and that eventually the final SPR will be ineffective as an oxidizing reagent. This is often evident by an inability to stabilize the UV detector, since MnO_4^- has some absorbance at many/most UV-VIS wavelengths of interest in HPLC. Also, the final mobile phase eluent exiting from the SPR and/or detector has a slight violet tinge to it if the permanganate is being released/exchanged from the SPR. Not only can inorganic/organic anions in the mobile phase cause this displacement of the reagent from the SPR, but also certain ionic analytes can have the same effect. Thus, in general, we have not realized much success in the attempted oxidations of ionic or zwitterionic analytes, such as catecholamines, catechols, or phenols. It is not that such compounds cannot be oxidized by permanganate in solution, just that they also will displace the reagent from the SPR into the mobile phase, and then the UV detector becomes unusable and unmanageable for normal HPLC operations and detection of the expected products or analytes.

HPLC-SPR oxidations or any such derivatizations are most useful wherein there is a change in the peak area/peak height of the starting analyte, and a concomitant appearance of one or more expected/known products of such an oxidation reaction. At the same time, the percent disappearance of the starting analyte should closely match the percent appearance of the expected product, in a quantitative sense, in order to truly optimize the selectivity and overall specificity of the analyte identification. However, often qualitative disappearance/appearance results suffice to confirm a suspected analyte, in addition to the conventional use of retention times *vs* a known external standard. In all of this work, we have used changes in both peak areas and/or peak

heights in order to determine percent oxidations of starting material/analyte. It is not always necessary to have 100% oxidations of all analytes, often 50% derivatizations will suffice for confirmation purposes, but such less than 100% results must be highly reproducible in order to be useful and reliable. In using this approach to derivatization on a particular analyte that is not fully oxidized in the sample, it can be most helpful for confirmatory purposes to demonstrate that the analyte in the sample and the external standard of that same analyte are oxidized to the same approximate degree in separate HPLC-SPR experiments. It is also possible that a particular alcohol analyte will undergo an initial oxidation to the aldehyde or ketone, and that in either on-line or off-line approaches, this product will/could then undergo a second stage oxidation to a carboxylic acid. Indeed, we have already seen this type of dual stage oxidation occurring with standards and actual sample matrices. Such multiple oxidations can provide additional confirmation of the nature/structure of the original analyte under investigation *via* HPLC-SPR.

In all of the work that follows, it must be emphasized that the nature of the HPLC mobile phase used must meet at least two criteria, at least for on-line derivatizations. First, it must be compatible with the required oxidation reaction and polymeric reagents, and it must not react or dissolve the reagent present. Second, the mobile phase must be suitable for the desired separations of the starting analyte from its expected/known oxidation products and/or other materials present in the sample matrix/solution. If these two criteria can be met with a single mobile phase composition, then it is quite likely, all other things being equal, that the desired oxidation can be performed on-line, in either real- or extended-time, at room or above temperatures. In certain cases, it may be worthwhile to determine which mobile phase composition may/will provide compatibility with the above suggested requirements for on-line HPLC-SPR work.

To demonstrate the overall approaches utilized here for oxidations in HPLC-SPR, Figure 1 contains two separate chromatograms with analytical column plus reagent SPR on-line. In Figure 1A, a standard solution of only p-nitrobenzyl alcohol has been injected, in real-time, with the SPR maintained at 46^{o}C, on-line. The retention time of the starting alcohol and its known oxidation product, *viz.*, p-nitrobenzaldehyde, were confirmed using a dummy column plus analytical column set-up with separate injections of each standard compound. In Figure 1B, the same HPLC-SPR conditions prevail as in Figure 1A, but now only a standard solution of the oxidation product, p-nitrobenzaldehyde has been injected. Under these conditions, 1B, some of the aldehyde injected has been oxidized further, and this has been demonstrated in a separate set of experiments. However, for purely qualitative purposes, Figure 1B indicates the retention time of the initial oxidation product of the starting alcohol under actual HPLC-SPR conditions. Other HPLC-SPR conditions used here are indicated in Figure 1. Percent oxidations of both alcohol and aldehyde are presented below, Table 1.

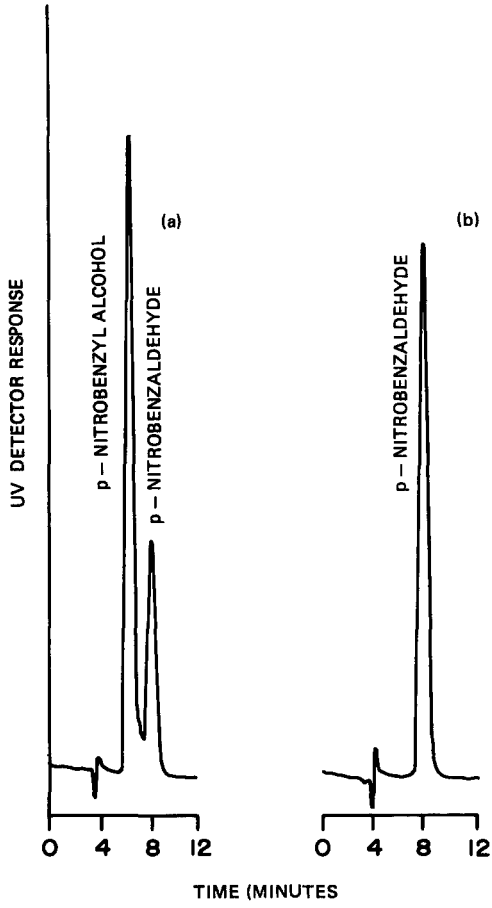

Figure 1. HPLC-SPR chromatograms for the oxidation of p-nitrobenzyl alcohol to p-nitrobenzaldehyde using uBondapak C_{18} analytical column with 50/50 HOH/ACN mobile phase at 0.8 ml/min flow rate: (A) only alcohol was injected with SPR on-line before analytical column, SPR at 46°C; (B) only aldehyde was injected, with HPLC-SPR conditions as in (A).

Whereas p-nitrobenzyl alcohol is only partly/partially oxidized in HPLC-SPR on-line approaches, Figure 1 and Table 1, a similar aromatic alcohol, o-aminobenzyl alcohol, is almost fully oxidized even at room temperature to the expected o-aminobenzaldehyde, Figure 2. Thus, Figure 2 illustrates the exact same HPLC-SPR oxidation of the same starting alcohol, on-line, using the same permanganate SPR, same mobile phase in the HPLC separations, but now varying the temperature of the SPR, pre-column, from room temperature (A) to

TABLE 1.

SUMMARY OF VARIOUS ALCOHOLS/ALDEHYDES STUDIED IN HPLC-SPR WITH POLYMERIC PERMANGANATE PRE-COLUMN ON-LINE IN REAL TIME[a]

COMPOUND NAME	PERCENT OXIDATION[b]
o-AMINOBENZYL ALCOHOL	100%
SALICYLALDEHYDE	100%[c]
p-METHOXYPHENOL	100%[c]
p-METHOXYNAPHTHOL	100%
HYDROQUINONE	42%
BENZYL ALCOHOL	11%
BENZALDEHYDE	15%
p-NITROBENZYL ALCOHOL	52%
p-NITROBENZALDEHYDE	53%

a. Oxidations were performed on-line, in pre-column mode, in real-time, using HPLC-UV with a Waters uBondapak C-18 column with mobile phase of HOH/ACN (50/50) at 0.8 ml/min flow rate, UV at 254nm. Polymeric permanganate SPR operated at 46°C.
b. Percent oxidations determined by changes in peak area or peak heights for dummy vs oxidizing difference chromatograms.
c. In these two analyses, the peak of starting material disappeared, but no new peak appeared for the oxidation product(s). Such products may have remained on ion-exchange support of SPR or not eluted under HPLC mobile phase conditions used here.

46°C (B). In Figure 2A, some of the starting o-aminobenzyl alcohol is still visible at the correct retention time of about 6 mins, but in Figure 2B, with the SPR kept at a higher temperature, there is no remaining alcohol visible, concomitant with an increased peak height for the product aldehyde. The same amounts of alcohol were injected in both (A) and (B), Figure 2, and thus it can be safely assumed that at the higher SPR working temperature used in (B), complete oxidation of the alcohol has now been realized, with an additional observed formation of the known oxidation product, the aldehyde indicated. Clearly, HPLC-SPR oxidations are or can be greatly affected as a function of the temperature of the SPR on-line, in real-time.

We have attempted to demonstrate the linearity of these oxidations over a wide range of concentrations of at least two alcohols injected under HPLC-SPR conditions, as above. Figure 3 illustrates a log-log plot of the amount (ng) of o-aminobenzaldehyde derived/formed from the on-line, real-time, 46°C SPR oxidation of the o-aminobenzyl alcohol already described above. We have here plotted the amount of oxidation product expected *vs* peak height, rather than amount of alcohol injected, since in this example, even at room temperature, all of the starting alcohol is completely oxidized at all levels injected. Thus, it was only possible to utilize amount of product expected *vs* peak heights, in order to demonstrate the linearity of the oxidation reaction over

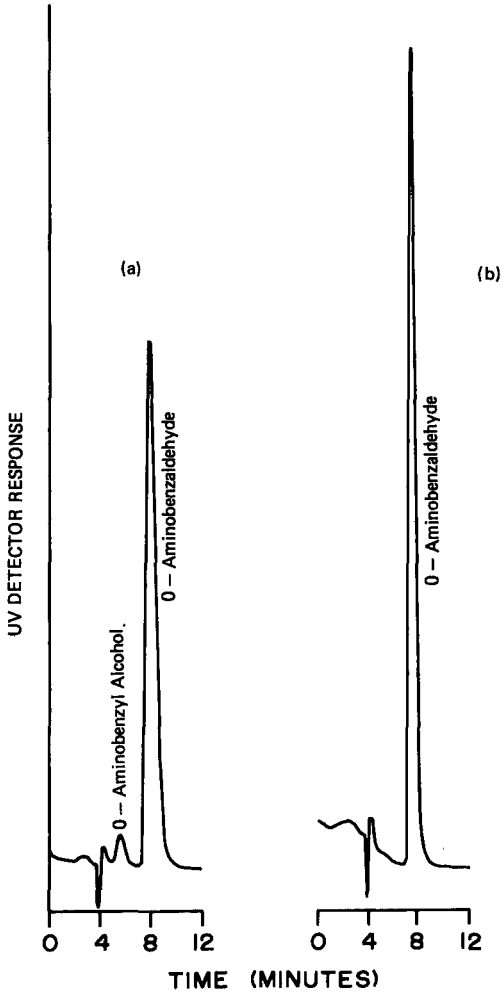

Figure 2. HPLC-SPR chromatograms for the oxidation of o-aminobenzyl alcohol to o-aminobenzaldehyde using analytical column of uBondapak C_{18} with 50/50 HOH/ACN mobile phase at 0.8 ml/min flow rate with SPR on-line, just before analytical column: (A) only alcohol was injected with SPR kept at room temperature; (B) only alcohol was injected with SPR at 46°C throughout.

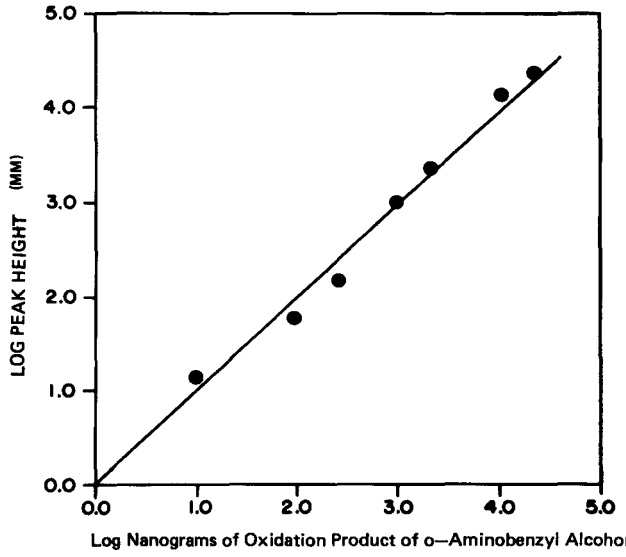

Figure 3. Plot of amount (ng) of expected oxidation product formed as a function of varying amounts of starting alcohol injected onto HPLC-SPR, on-line, real-time, at 46°C, starting alcohol was 100% oxidized at all levels injected onto HPLC-SPR to the expected o-aminobenzaldehyde.

at least 4-5 orders of magnitude of amount (ng) injected via HPLC-SPR. This might have been expected based on the known/demonstrated absolute amount (mass) of permanganate loaded onto a typical SPR oxidizing column (100-125mg/column). The correlation coefficient for linearity of the plot in Figure 3 is 0.995. A similar study is described/summarized in Figure 4, which is another log-log plot of peak heights observed at various levels/amounts (ng) of the oxidation product expected from the HPLC-SPR derivatization of p-nitrobenzyl alcohol. In this instance, the starting alcohol is not fully oxidized at room temperature, Table 1, and thus we have also been able to use peak heights for the alcohol injected at various levels together with peak heights for the oxidation product expected/observed. Again, using either peak heights for the alcohol or aldehyde at various amounts/levels injected, the calibration plot is linear over about 2-3 orders of magnitude. The correlation coefficient of linearity for this plot of Figure 4 is in excess of 0.900. However, in this example, there is observed some degree of non-linearity at the higher ranges/levels of alcohol injected. This is probably due to the fact that this alcohol is incompletely oxidized at lower levels, and thus requires a longer residence time or higher SPR reaction temperature to provide linearity at the higher levels injected. As in all types of derivatizations, it is desirable to utilize

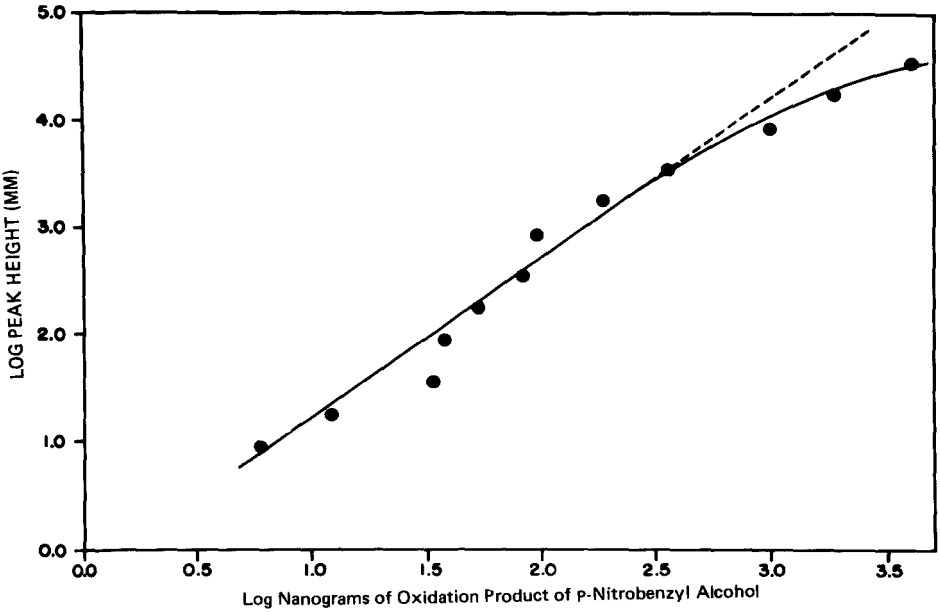

Figure 4. Plot of amount (ng) of expected oxidation product formed, p-nitrobenzaldehyde, as a function of varying amounts of starting alcohol injected onto HPLC-SPR, on-line, real-time, room temperature. Starting alcohol was not 100% oxidized at all levels initially injected in these studies.

ratios of reagent/substrate that will provide for maximum possible conversion/derivatization at all times/levels. Hence, in this particular case, it might be advantageous to inject amounts of the starting alcohol that would fall on the linear portion of the plot in Figure 4.

We have now studied a fairly large number of alcohols and aldehydes in oxidative HPLC-SPR approaches, and these are summarized in Table 1, along with the percent oxidations determined for each starting compound. The percent oxidations were calculated/determined by measuring changes in either peak areas or peak heights, or both, for the same starting material injected onto the dummy column plus analytical column and the SPR plus the same analytical column. In most of these studies, disappearance of the starting material peak was concomitant with appearance of the expected/known oxidation product. However, in two cases, salicylaldehyde and p-methoxyphenol, there was no evidence of any oxidation product formed. This may have been due to retention of the oxidation product on the anion exchange support of the SPR, because on the dummy column, each of these two starting materials were unretained and eluted

TABLE 2.
COMPARISON OF ON-LINE AND OFF-LINE HPLC-SPR OXIDATIONS OF VARIOUS COMPOUNDS USING NORMAL PHASE SOLVENT CONDITIONS IN HPLC FOR OXIDATION/SEPARATION

COMPOUND STUDIED	PERCENT OXIDATION	
	ON-LINE[a]	OFF-LINE[b]
BENZYL ALCOHOL	4.3%	66%
BENZALDEHYDE	3.4%	46%
CINNAMYL ALCOHOL	3.1%	71%
CINNAMALDEHYDE	6.7%	83%

a. HPLC conditions used a uPorasil column (10um) with 5% THF/HEXANE as mobile phase at flow rate of 1.1 ml/min at room temperature, with polymeric permanganate SPR in pre-column mode of operation. Injections made on-line, in real-time, at room temperature, with percent oxidations indicated.
b. Oxidations performed off-line, preparing THF solution of compound studied, placed onto oxidizing column for 10 mins at room temperature, analyte eluted from SPR off-line, injected directly onto HPLC-UV, as above.

in reproducible retention times. Hence, we suspect that it is the products of the SPR oxidation that are being held-up on the polymeric anion exchange support in the SPR only. In Table 1, these analyses were all done with the SPR on-line, real-time, with the SPR maintained at 46°C, with other conditions as indicated. Percent oxidations have varied from a low of 11% for benzyl alcohol to a high of 100% for several of these starting materials/analytes.

Whereas the above studies, Table 1, involved the use of reversed phase solvents, viz., HOH/ACN, it was also important to determine if normal phase organic solvents could be used with this particular oxidizing SPR in HPLC. These results, Table 2, suggest that, at least in the on-line mode, at room temperature, the percent oxidations observed with 5% tetrahydrofuran (THF)/hexane as the mobile phase, were less than adequate or satisfactory for most applications. However, these same oxidations could be adequately performed in the off-line manner, Table 2, using THF alone as the reaction solvent, and then analyzing for the product and starting material by injecting onto an analytical HPLC system, using again 5% THF/HEX as the mobile phase. In the off-line mode of SPR derivatization, the exact same polymeric permanganate material was used for the oxidation reactions, but the oxidation was allowed to proceed for 10 mins at room temperature. The extended period of time that the analyte/alcohol(aldehyde) was allowed to remain in contact with the polymeric SPR was presumably responsible for the increased percent oxidations observed, Table 2. It would therefore appear feasible/possible to utilize this particular SPR in an off-line mode/approach, even with normal phase solvents such as THF for the derivatization step, followed by normal phase HPLC separations/methods.

In all of the above examples of HPLC-SPR with suitable substrates, we have not yet described the possibility of oxidizing secondary alcohols. Table

TABLE 3.
SOLID PHASE OXIDATIONS OF SECONDARY ALCOHOLS WITH HPLC-SPR OFF-LINE OR ON-LINE

COMPOUNDS STUDIED	PERCENT OXIDATION	
	OFF-LINE[a]	ON-LINE[b]
BENZHYDROL	83.8%	30%
sec-PHENETHYL ALCOHOL	83.0%	16%

a. Oxidations performed off-line by preparing acetonitrile solution of analyte (secondary alcohol), injecting this onto polymeric oxidizing SPR, holding for 10 mins at 46°C, eluting and injecting onto HPLC. HPLC conditions used a uBondapak C_{18} column with mobile phase of ACN/HOH (65/35) at 0.9 ml/min flow rate, UV detection at 254nm.
b. On-line oxidations performed at 46°C in real-time, using HPLC-UV conditions as above with ACN/HOH was mobile phase at 0.9 ml/min flow rate.

3 indicates the solid phase oxidations of two such secondary alcohols, again comparing off-line with on-line approaches. And again, it is clear that the on-line derivatizations are less effective/complete overall than the off-line approaches, even when now both approaches use an elevated temperature of 46°C. In each of these two examples of secondary alcohol SPR oxidations, the expected ketone derivatives were observed at the end of each reaction, off-line or on-line, and thus we are fairly certain that these are indeed SPR caused oxidation type reactions of the substrates indicated. Additional experimental conditions for these particular studies are indicated in Table 3.

With regard to specific applications of the use of this oxidizing SPR in HPLC, Figure 5 indicates the analysis of benzyl alcohol present in a commercial hair shampoo. In Figure 5A, the original, diluted shampoo sample has been analyzed *via* reversed phase HPLC, with a peak at the correct retention time for standard benzyl alcohol. However, use of just chromatogram 5A to confirm the presence or amount of benzyl alcohol in this particular sample would be difficult, since there are so many other UV absorbing compounds/peaks present close to the peak of interest. Improved HPLC resolution conditions could be developed in order to further resolve the suspected benzyl alcohol peak from other materials in this sample. However, Figure 5B indicates the considerably simpler overall chromatogram for the same sample injected now onto the permanganate SPR, off-line, with oxidation at room temperature for 10 mins. There is now no longer a peak at the correct retention time for benzyl alcohol, but there are peaks now present for the expected oxidation products, *viz.*, benzaldehyde and benzoic acid. The benzaldehyde is the expected initial oxidation product of the alcohol, while the benzoic acid is the expected/known oxidation product of this aldehyde. The retention times for these two oxidation products were confirmed by injecting separate standards of each compound onto the HPLC system alone.

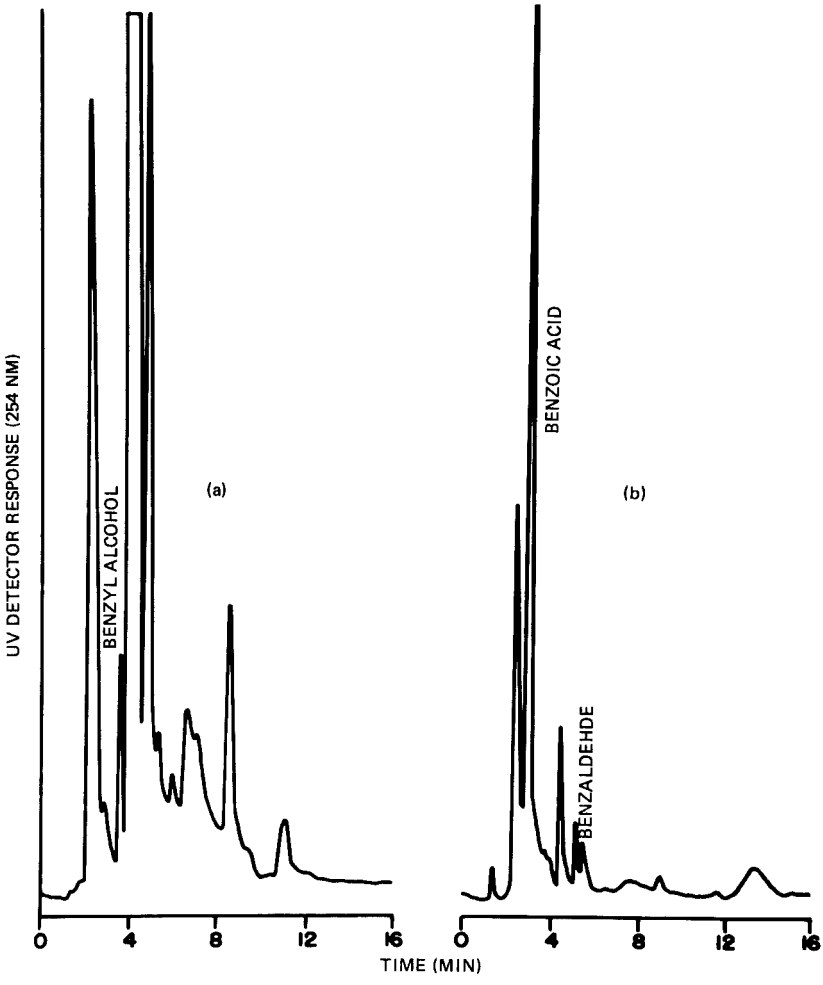

Figure 5. HPLC-UV chromatograms using Waters uBondapak C_{18} analytical column with mobile phase of HOH/ACN (50/50) at a flow rate of 1.0 ml/min, UV at 254nm, for off-line polymeric permanganate oxidation of benzyl alcohol in a commercial hair shampoo: (A) direct analysis of the diluted shampoo sample before oxidation; (B) after off-line oxidation at room temperature for 10 mins, showing disappearance of the alcohol.

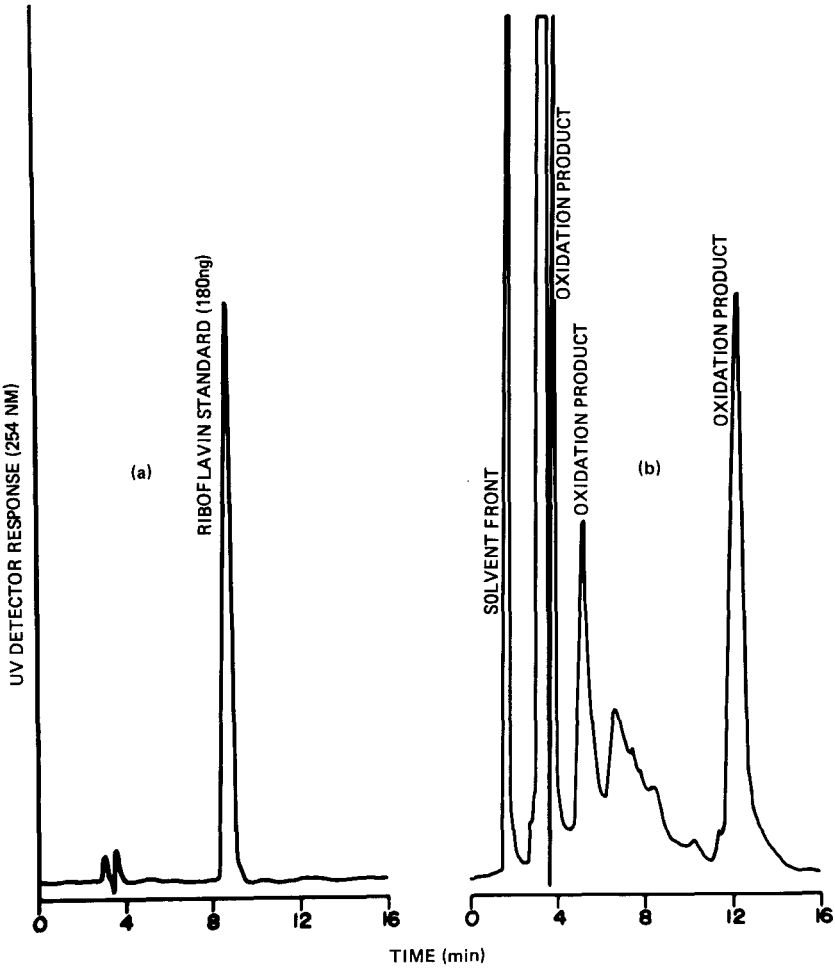

Figure 6. HPLC-UV chromatograms using Waters uBondapak C_{18} analytical column with mobile phase of HOH/ACN (85/15) at flow rate of 1.0 ml/min, UV detection at 254nm, for off-line polymeric permanganate oxidation of riboflavin: (A) standard of riboflavin before SPR oxidation off-line; (B) HPLC analysis of oxidation products of riboflavin oxidized off-line at room temperature for 10 mins in ACN/HOH (50/50).

A second application of HPLC-SPR techniques is indicated in Figure 6, which is the off-line SPR oxidation of a standard of riboflavin, vitamin B_2. This particular substrate was chromatographed before oxidation, Figure 6A, and then following off-line oxidation, Figure 6B, with the specific conditions as indicated. In this case, since riboflavin has a sugar side-chain on the parent molecule, there are several sites for oxidation, and thus several possible oxidation products. Indeed, the chromatogram of Figure 6B indicates the formation of a number of oxidation products, with essentially no starting riboflavin present. Thus, the formation of multiple oxidation products, Figures 5 and 6, provides additional confirmation for the presence of the original analyte, as long as a standard of this compound is available to demonstrate in a separate set of experiments the nature/complexity of the reaction products formed via HPLC-SPR. We have not, as yet, attempted to analyze for riboflavin in an actual vitamin supplement sample or a biological/food sample, but such applications are being planned and should be practical/successful in the future.

CONCLUSIONS

We have now demonstrated the preparation of a polymeric permanganate oxidizing reagent that is useful in various HPLC-SPR derivatizations for alcohols and aldehydes. Although we have not fully delineated what other classes of organic compounds might be suitable for these off-line or on-line reactions, it should be possible to at least oxidize various ketones, based on certain preliminary results. These approaches are compatible with both reversed and normal phase solvents, in either on-line or off-line modes, at room temperature or above. Percent oxidations of various substrates are influenced by a number of factors, including: solvent, analyte structure, residence time within the SPR, temperature of SPR, nature of HPLC mobile phase, and ratio of reagent/substrate injected. It is suggested that these derivatization approaches should find wide acceptance and application in the area of HPLC derivatizations, especially when the ease and convenience of solid phase reactions/reagents becomes better recognized and appreciated. Additional applications of this particular HPLC-SPR approach are now under investigation and development.

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Additional financial support was provided, in part, by a NIH Biomedical Research Support Grant, No. RR07143, Department of Health and Human Services, to Northeastern University.

This is contribution number 169 from the Institute of Chemical Analysis at Northeastern University.

REFERENCES

1. Abbreviations used: HPLC = high performance liquid chromatography; SPR = solid phase reactor/reactions; MnO_4^- = permanganate; UV = ultraviolet detection; HOH = water; ACN = acetonitrile; THF = tetrahydrofuran; HEX = n-hexane; mins = minutes; ml/min = milliliters per minute; nm = nanometer.

2. Xie, K-H., Colgan, S., and Krull, I.S., J. Liquid Chromatogr. Reviews, invited contribution, in press (1983).

3. Krull, I.S. and Lankmayr, E.P., American Laboratory, 18 (May, 1982).

4. Lawrence, J.F., Organic Trace Analysis by Liquid Chromatography, Academic Press, Inc., New York, 1981. Chapter 5.

5. Frei, R.W., in Chemical Derivatization in Analytical Chemistry. Volume 1: Chromatography, ed. by Frei, R.W. and Lawrence, J.F., Plenum Press, Inc., New York, 1981. Chapter 4.

6. Frei, R.W. and Lawrence, J.F., eds., Chemical Derivatization in Analytical Chemistry. Volume 2: Separation and Continuous Flow Techniques, Plenum Press, Inc., New York, 1982.

7. Snyder, L.R. and Kirkland, J.J., Introduction to Modern Liquid Chromatography, Second Edition, J. Wiley & Sons, Inc., New York, 1979. Chapter 4.

8. Lawrence, J.F. and Frei, R.W., Chemical Derivatization in Liquid Chromatography, Elsevier Scientific Publ. Co., Amsterdam, 1976.

9. Blau, K. and King, G., eds., Handbook of Derivatives for Chromatography, Heyden & Son, Ltd., London, 1977.

10. Knapp, D.R., Handbook of Analytical Derivatization Reactions, J. Wiley & Sons, Inc., New York, 1979.

11. Krull, I.S., Colgan, S., Xie, K-H., Neue, U., King, R., and Bidlingmeyer, B., J. Liquid Chromatogr., in press (1983).

12. Krull, I.S., Xie, K-H., Colgan, S., Neue, U., Izod, T., King, R., and Bidlingmeyer, B., J. Liquid Chromatogr., in press (1983).

13. Bowers, L.D. and Bostick, W.D., in Chemical Derivatization in Analytical Chemistry. Volume 2: Separations and Continuous Flow Techniques, ed. by Frei, R.W. and Lawrence, J.F., Plenum Press, Inc., New York, 1982. Chapter 3.

14. Nondek, L., Brinkman, U.A.Th., and Frei, R.W., Anal. Chem., in press (1983).

15. Frei, R.W., Chromatographia, 15, 161 (1982).

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(11), 2129-2130 (1983)

BOOK REVIEW

CHROMATOGRAPHY. FUNDAMENTALS & APPLICATIONS OF CHROMATOGRAPHIC AND ELECTROPHORETIC METHODS, E. Heftmann, Editor, in two parts, Elsevier, Amsterdam, 952 pp.

This two-volume set of books represents the fourth edition of a classic reference in the field of chromatography. Many discoveries, changes in ancillary equipment, and broader theoretical understandings have emerged since the appearance of the first edition in 1961. This present edition is a unified collection of chapters written by many experts in the field of chromatography.

Part A contains nine chapters pertaining to the fundamentals and techniques of chromatography and electrophoresis. The first two chapters focus on "survey of chromatography and electrophoresis" and "history of chromatography and electrophoresis." Both chapters have been written by the editor (E. Heftmann) and are concise and well-written as were the corresponding chapters in the previous editions.

Chapter three is the outstanding chapter in this part; theory of chromatography by Horvath and Melander. This chapter is well written; typical of what one would expect of the authors. The reviewer recommends this chapter to all readers, especially those beginning in this very important area of analytical chemistry.

Chapter four unfortunately falls short of the expectations of this reviewer. Column chromatography is an ever expanding realm of chromatography and it is disappointing to find such a meager representation of this technique. The list of references (twenty-four) speaks either for the prejudices of the author or his deliberate omissions as to the vast number of pertinent references available in this area. The reviewer finds it difficult to reason why the classic reference, "Introduction to Modern Liquid Chromatography," by Snyder and Kirkland was missing from the references. The addition of the word LIQUID to the chapter title would assist neophytes to the area of chromatography and give these readers immediate notice that the chapter is concerned with classical liquid column chromatography and HPLC. This chapter would be of little help to the reader wishing current theories and topics in modern liquid chromatography.

Chapter five (planar chromatography) provides an interesting historical discussion of these techniques and sufficient

information to permit the beginner to get started in this area. This is an area of chromatography which is not utilized as much as it should; especially with the present work being done in HPLC.

Chapter six (gas chromatography) overlaps with chapter three and lacks more pertinent information on the present practice of gas chromatography (GC). More discussion should have been given to capillary columns because of the lack of understanding of many new people to the technique of GC on the merits of packed columns versus capillary columns. The section on qualitative and quantitative analysis surely could have been expanded in place of the theoretical discussion.

Chapter seven (ion exchange) is a very readable chapter which summarizes the present state of the technique. The chapter is written with enough theoretical discussion to point out the uniqueness of this technique compared to other chromatographic techniques. The section on ion chromatography is an added plus to this chapter.

Chapters eight and nine (gel chromatography and electrophoresis) are heavy in theoretical discussion and too light in practical applications (i.e., experimental discussions). The practical aspects would have added more usefulnness to these chapters especially in the light of Part B of this two-volume set.

Part B deals with various applications of chromatographic techniques to a variety of sample types. The sample types covered (in order of chapters) are: amino acids and oligopeptides, proteins, lipids, terpenoids, steroids, carbohydrates, pharmaceuticals, antibiotics, nucleic acids, porphyrins, phenolic compounds, pesticides, inorganic compounds, non-hydrocarbon gases, and hydrocarbons. Each of these chapters varies from fair to good in coverage of the particular sample type. They do, however, suffer from the same problem that most multi-authored books (including the reviewer's) have, i.e., the references cannot be more recent than a year of publication. This does not detract from the usefulness of the book. A prejudice of the reviewer would be that a chapter discussing environmental samples, in general, would be an excellent addition to Part B.

Lastly and very important, who should have this set of reference books in their library? Obviously, university and industrial research laboratories should have these on the shelf. It would be very nice if individuals could also have them in their personal libraries. However, the cost of this two-volume set is prohibitive; over 200 dollars (US).

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ANNOUNCEMENT

Federation of Chromatography Discussion Groups

Several chromatography discussion groups met at the 1983 Pittsburgh Conference in Atlantic City and formed the Federation of Chromatography Discussion Groups. This organization was formed to improve communication between the various chromatography discussion groups that currently exist in the United States and to serve as a central focus for those discussion groups in dealing and communicating with similar groups in other countries.

Local chromatography discussion groups will supply information about their activities to the Federation as a clearing house. The consolidated information will then be publicized through the leading chromatography and analytical chemistry journals. The Journal of Liquid Chromatography will publish this information on a regular basis.

Local chromatography discussion groups are encouraged to contact Dr. Joseph L. Glajch, E. I. duPont de Nemours & Co., Experimental Station, 262/315, Wilmington, DE, 19898, USA, Phone: (302)-772-2555 for further information.

JOURNAL OF LIQUID CHROMATOGRPAHY, 6(11), 2133-2136 (1983)

LC NEWS

DISPOSABLE GLASS CHROMATOGRAPHY COLUMN is designed to satisfy a wide range of analytical separations. It consists of a 150 x 8 mm I.D. borosilicate glass column, a 30-50 micron porous polyethylene disc, and a molded polypropylene lower Luer fitting. Kontes, JLC/83/11, P. O. Box 729, Spruce St., Vineland, NJ, 08360, USA.

THE MOLECULAR BIOLOGY CATALOG contains information on DNA/RNA-modifying enzymes, restriction nuclease enzymes, DNA sequencing products, nucleic acids, cloning vectors, oligodeoxynucleotides and other nucleosides and nucleotides of special interest. Included are technique information, references, cloning procedures, cloning vector maps, restriction nuclease assay conditions, and more. Pharmacia P-L Biochemicals, JLC/83/11, 1037 W. McKinley Ave., Milwaukee, WI, 53205, USA.

PHENOLS IN WATER are determined by isocratic mode LC. A large volume of water is injected onto a reversed-phase column, and eluted with a 40:60 acetonitrile/water mobile phase. Detection is at 200 nm down to less than 0.001 ppm. Pye Unicam Ltd., JLC/83/11, York Street, Cambridge CB1 2PX, England.

BIOTECHNOLOGY SERIES highlights a phase in the process of generating and isolating biomolecules through biotechnology. The initial brochure in the series serves as a guide to the use of high-resolution separation techniques in the secondary purification stage of the biotechnology process. LKB Instruments, Inc., JLC/83/11, 9319 Gaither Rd., Gaithersburg, MD, 20877, USA.

POST-COLUMN DERIVATIZATION SYSTEM Provides column temperature control, post-column derivatization reagent flow control for 1 Or 2 reagents, post column reaction temperature control, and sensitive fluorescence or absorbance detection. Kratos Analytical Instruments, JLC/83/11, 170 Williams Dr., Ramsey, NJ, 07446, USA.

HPLC GRADIENT CONTROLLER is a microprocessor-based module designed to provide maximum flexibility for binary and ternary programs. It helps minimize errors by constantly showing run status, all system parameters, and the file name during the run. Tracor, Inc., JLC/83/11, 6500 Tracor Lane, Austin, TX, 78721, USa.

HPLC-GRADE WATER is produced on demand by a system tt employs an organic scavenger resin cartridge combined with absolute 0.45 micron low-extractable membrane filtration to give consistenly pure water from average-quality distilled or deionized feed water. Up to 15 liters of organic-free water is produced by a single cartridge. Millipore Corp., JLC/83/11, Bedford, MA, 01730, USA.

DEDICATED SUGAR ANALYSIS LC is custom-tailored for use by producers and large-scale users of sugar products. It is optimized for rapid analysis of corn, beet, and cane sugars and processing liquors. Waters Associates, Inc., 34 Maple Street, Milford, MA, 01757, USA.

SULFITE IN FOODS is determined without interferences using electrochemical detection at ppm levels. Samples need only be homogenized, diluted, and filtered prior to injection. Dionex Corp., JLC/83/11, 1228 Titan Way, Sunnyvale, CA, 94086, USA.

PROTEIN ANALYSIS LC is described in a recent brochure. Four separation mechanisms are used--ion exchange, gel filtration, reverse phase, and hydroxyapatite fractionation. The methods allow identification and quantitation of components that might not be Omenable to any single mechanism. Bio-Rad Labs, JLC/83/11, 2200 Wright Avenue, Richmond, CA, 94804, USA.

AUTOMATED TLC SAMPLE APPLICATOR consists of a control unit and the actual applicator. The control unit is a Z-80 microprocessor-based device that holds 8 sample application modes, five of which are user programmable. Sample volumes from 100 nL to 20 microL may be applied. Applied Analytical Industries, JLC/83/11, Route 6, Box 55, New Hanover Air Park, Wilmington, NC, 28405, USA.

EC/LC FLOW CELL FOR AMPEROMETRIC DETECTION features negligible dead volume, minimum IR drop, improved detection limit, and better peak shape. IBM Instruments, Inc., JLC/83/11, Orchard Park, Box 332, Danbury, CT, 06810, USA.

THIN-LAYER CELL FOR ELECTROCHEMICAL DETECTION allows placement of the auxilliary electrode both downstream and across from the working electrode. A highly polished stainless steel top extends cell life, permits compatibility with new "high speed" columns, and allows for connection of low dead volume fittings for use with micro columns. Bioanalytical Systems, Inc., JLC/83/11, 1205 Kent Avenue, Purdue Research Park, West Lafayette, IN, 47906, USA.

LIQUID PROCESSING UNIT can be used to feed most analytical instruments. It performs all of the crucial sample pickup, mixing and dispensing operations. A pair of syringes whose plungers are driven by a stepper motor and precision ball lead screws, a mixing chamber, and a hand-held control unit are the principal working parts. A computer program directs all operations. Processing parameters such as time, volume, ratios, and increments are entered thru the control unit and become a part of an individual routine

program. Hamilton Company, JLC83/11, P. O. Box 10030, Reno, NV, 89510, USA.

BINARY GRADIENT HPLC CAPABILITIES include easy setting of flow rate, initial and final solvent composition, and times for equilibration, gradient, and hold steps directly from the front panel. The controller also includes the necessary connections for automated operation with many auto samplers and data handling devices. Perkin-Elmer Corp., JLC83/11, Main Avenue, Mail Station 12, Norwalk, CT, 06856, USA.

PNEUMATIC CLAMPING SYSTEM is for use with HPLC columns and cartridges. No-fault connections ensure consistent results. Columns are added or removed easily by relatively untrained operators without altering analytical integrity. They are useful to 5000 psi. EM Science, Inc., JLC/83/11, 480 Democrat Rd., Gibbstown, NJ, 08027, USA.

PUMP PRIMING VALVE is designed to simplify purging of HPLC pumps when change of solvent is made. When fitted between pump and injector, it permits new solvent to be purged through the pump to waste via a drain tube. Only half a turn is required to open the valve fully. Negretti & Zambra (Aviation), Ltd., JLC/83/11, The Airport, Southampton, SO9 3FR, Hampshire, England.

HPLC FILTER is made of a new fluoropolymer membrane and is housed in solvent resistant polypropylene. It is pressure rated at 75 psi and is available in a 0.45 micron pore size. Gelman Sciences, Inc., JLC/83/11, 600 S. Wagner Rd., Ann Arbor, MI, 48106, USA.

HPLC REAGENTS & COLUMNS are described in a new brochure. Included are buffers, ion pair reagents, derivatizing reagents, hardware, and solvents. Fisher Scientific Co., JLC/83/11, 711 Forbes Ave., Pittsburgh, PA, 15219, USA.

LAB AUTOMATION SYSTEM FOR SAMPLE PREPARATION combines robotics and lab stations to automate procedures. The controller interfaces the operator with the robot. Software is menu-based and uses familiar laboratory terms. Zymark Corp., JLC/83/11, Zymark Center, Hopkinton, MA, 01748, USA.

APPLICATIONS GUIDE deals with sample preparation, highlighting background, principles, and techniques of solid phase extraction. The guide contains over 40 detailed procedures for preparing environmental, pharmaceutical, biological, food, and cosmetic samples, such as priority pollutants, crude oil, trace metals, aflatoxins, steroids, etc. J. T. Baker Chem. Co., JLC/83/11, 222 Red School Lane, Phillipsburg, NJ, 08865, USA.

HPTLC/TLC BIBLIOGRAPHY SERVICE is available free of charge. Publications may be included by mailing to the publisher. Applied Analytical Industries, Inc., JLC/83/11, Route 6, Box 55, Wilmington, NC, 28405, USA.

DETERGENT REMOVING GEL is an affinity chromatographic support that selectively removes detergents from protein solutions, with proteins being recovered in virtually 100% yields. The support can be regenerated for repeated use. Pierce Chemical Co., JLC/83/11 P. O. Box 117, Rockford, IL, 61105, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 6(11), 2137-2140 (1983)

LC CALENDAR

1983

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

SEPTEMBER 22-23: Symposium: "Columns in High Performance Liquid Chromatography," Lady Mitchell Hall, University of Cambridge. Contact: A. G. W. Mulders, Hewlett-Packard, GmbH, Postfach 1280, D-7517 Waldbronn 2, West Germany.

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

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

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

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

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

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

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

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

NOVEMBER 10-11: Electrofocusing and Electrophoresis Workshop, Birmingham, AL, USA. Contact: Workshop Registrar, LKB Instruments, Inc., 9319 Gaither Rd., Gaithersburg, MD, 20877, USA.

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

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

NOVEMBER 22-23: Short Course: "Sample Handling in Liquid Chromatography," sponsored by the Int'l. Assoc. of Environmental and Biological Samples in Chromatography, Palais de Beaulieu, Lausanne, Switzerland. Contact: Dr. A. Donzel, Workshop Office, Case Postale 130, CH-1000 Lausanne 20, Switzerland.

NOVEMBER 24-25: Workshop: "Handling of Environmental and Biological Samples in Chromatography," sponsored by the Int'l. Assoc. of Environmental Anal. Chem., Palais de Beaulieu, Lausanne, Switzerland. Contact: Dr. A. Donzel, Workshop Office, Case Postale 130, CH-1000 Lausanne 20, Switzerland.

NOVEMBER 29-30: Electrofocusing and Electrophoresis Workshop, San Francisco, CA, USA. Contact: Workshop Registrar, LKB Instruments, Inc., 9319 Gaither Road, Gaithersburg, MD, 20877, USA.

DECEMBER 6-7 and 8-9: Electrofocusing and Electrophoresis Workshop, Los Angeles, CA, USA. Contact: Workshop Registrar, LKB Instruments, Inc., 9319 Gaither Road, Gaithersburg, MD, 20877, USA.

1984

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

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

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

JUNE 18-21: Symposium on Liquid Chromatography in the Biological Sciences, Ronneby, Sweden, sponsored by The Swedish Academy of Pharmaceutical Sciences. Contact: Swedish Academy of Pharmaceutical Sciences, P. O. Box 1136, S-111 81 Stockholm, Sweden.

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

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

1985

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

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

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

1986

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

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

1987

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

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

The Journal of Liquid Chromatography will publish announcements of interest to liquid chromatographers in every issue of the Journal. To be listed in the LC Calendar, we will need to know: Name of the meeting or symposium, sponsoring organization, when and where it will be held, and whom to contact for additional details. You are invited to send announcements to Dr. Jack Cazes, Editor, Journal of Liquid Chromatography, P. O. Box 1440-SMS, Fairfield, CT, 06430, USA.

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F. D. Pierce and H. R. Brown
Utah Biomedical Test Laboratory
520 Wakra Way
Salt Lake City, Utah 84108

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

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5. Drawings, graphs, and other numbered figures should be professionally drawn in black India ink (do not use blue ink) on separate sheets of white paper and placed at the end of text. Figures should not be placed within the body of the text. They should be sized to fit within the width and/or height of the type page, including any legend, label, or number associated with them. Photographs should be glossy prints. A typewriter or lettering set should be used for all labels on the figures or photographs;. they may not be hand drawn. Captions for the pictures should be typed single-spaced on a separate sheet, along the full width of the type page, and preceded by the word FIGURE and a number in arabic numerals. All figures and lettering must be of a size to remain legible after a 20% reduction from original size. Figure numbers, name of senior author, and arrow indicating "top" should be written in light blue pencil on the back or typed on a gummed label, which should be attached to the back of the illustration. Indicate approximate placement of the illustrations in the text by a marginal note in light blue pencil.

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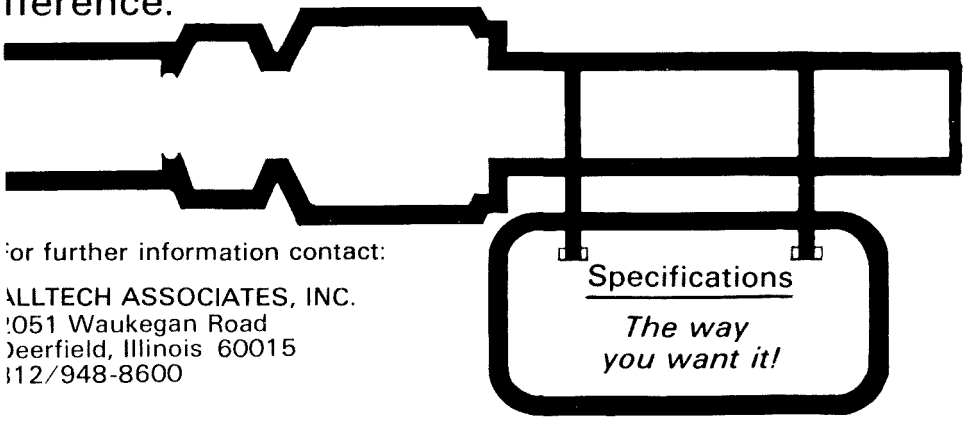

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