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ION CHROMATOGRAPHY ON POLY(CROWN ETHER)-MODIFIED SILICA POSSESSING HIGH AFFINITY FOR SODIUM

M. Nakajima, K. Kimura*, E. Hayata, and T. Shono*

Department of Applied Chemistry, Faculty of Engineering, Osaka University, Yamada-oka, Suita, Osaka 565, Japan

ABSTRACT

Stationary phases which have great affinity for Na^+ were synthesized by incorporating 12-crown-4 polymer on silica gel for liquid chromatography of alkali and alkaline-earth metal ions. The stationary phases interact with Na^+ most strongly of all alkali metal ions as expected, and the retention times on liquid chromatography of alkali metal ions were in the sequence $Li^+<Cs^+<Rb^+<K^+<Na^+$. On the stationary phase, a mixture of Li^+, Na^+, and K^+ can be separated completely by the elution with water/methanol mixture. By the use of spherical type silica gel instead of irregular type one and by effective end-capping of the residual silanol groups, the peak symmetry was improved significantly.

INTRODUCTION

One of the most important analytical applications of crown ether derivatives is chromatographic separation of ionic species. Several studies have been so far appeared regarding stationary phases containing crown ethers for liquid chromatography. Blasius et al. (1-3) have synthesized numerous crown ether resins and reported their applications to stationary phases for liquid chromatography to separate various ionic and organic compounds. Igawa et al. (4) have described an anion-separable stationary phase obtained by coating polyamide-type crown ether resin on

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silica gel. We have also reported some applications of crown ether stationary phases in which poly- and bis(crown ether)s are bonded to silica gel covalently (5,6).

Most of the crown ether stationary phases showed specific chromatographic behaviors which are derived primarily from the ion-complexing abilities of the immobilized crown ethers, possessing great affinity for K^+, Rb^+, or Cs^+. However, very few stationary phase containing a crown ether has specific affinity for Na^+. Therefore, we attempted synthesis of poly(crown ether)-modified silica possessing affinity for Na^+. In this paper we report the synthesis of the stationary phases and the chromatographic separation of alkali and alkaline-earth metal ions on them.

EXPERIMENTAL

Syntheses of stationary phases

The synthetic route and the expected structure of poly(12-crown-4)-modified silica (poly12C4-silica) are illustrated in Fig. 1. In this study both of irregular and spherical type silica gels were employed. The average particle size of the silica gels was 10 μm.

First, the synthesis of poly12C4-silica-I (based on irregular type silica gel) is shown below. To a dry chloroform solution of (3-aminopropyl)triethoxysilane (0.012 mol, 2.66 g) and dry triethylamine (0.012 mol, 1.21 g), a dry chloroform solution of methacryloyl chloride (0.012 mol, 1.1 ml) was added dropwise while stirring and cooling in an ice bath. At room temperature the mixed solution was stirred for 12 h and then refluxed for 2 h in the presence of hydroquinone (6 mg). The solvent was replaced by dry toluene and the triethylamine hydrochloride was filtered off. The toluene solution was placed on 5.90 g of silica gel and the mixture was refluxed while stirring for 4 h. After being filtered off, the resulting vinyl-modified silica-I was washed successively with toluene, chloroform, and methanol, and then dried overnight under vacuum at 80°C. Residual silanol groups of

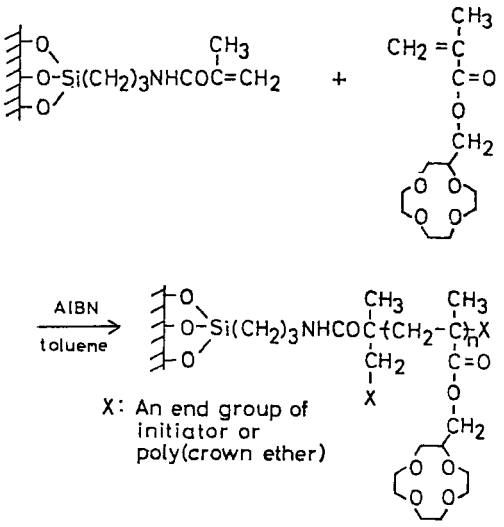

Figure 1. Synthesis andexpected structure of poly(12-crown-4)-modified silica.

the vinyl-modified silica-I were capped in the following way. To a suspension of the modified silica (5.74 g) in dry benzene, a benzene solution of trimethylchlorosilane (0.053 mol, 5.8 g) (TMCS) was added dropwise, and the mixture was allowed to stand for 24 h with occasional stirring. A glass tube containing vinyl-modified silica-I and a toluene solution of (12-crown-4)-methyl methacrylate (7) (6.10 mmol, 1.66 g) and α,α'-azobis-(isobutyronitrile) (0.020 mmol, 33 mg) as the initiator was degassed by freeze-and-thaw method and then sealed. Polymerization was carried out by shaking the sealed tube in an incubator at 70°C for 21 h. After the polymerization, the silica gel was washed successively with toluene, chloroform, and methanol.

Poly12C4-silica-S (based on spherical type silica gel) was synthesized in a similar way to poly12C4-silica-I, but the procedure for end-capping of residual silanol groups of the modified silica was different from that for poly12C4-silica-I.

The vinyl-modified silica-S (4.50 g) was suspended in dry hexane and then a hexane solution of hexamethyldisilazane (0.010 mol, 1.64 g) (HMDS) was added dropwise. The suspension was heated at 75°C for 8 h. The resulting modified-silica was washed sufficiently and then dried.

The crown ether contents of poly12C4-silica-I are 0.62 mmol and 0.72 mmol per gram of dry modified silica, which were determined from weight increase and elemental analysis of carbon, respectively. Similarly, the crown ether contents of poly12C4-silica-S were determined as 0.72 mmom and 0.76 mmom per gram.

Other materials

Alkali and alkaline-earth halides employed here are of analytical grade. Water and methanol used as the mobile phase were purified by distillation, followed by ultrafiltration. The water and methanol were degassed by stirring under vacuum, and mixed eluents of water and methanol were further degassed with an ultrasonic cleaner.

Instrumentation

The chromatograph system employed here consists of commercially available modules, consisting of a pump (Waters Associates 6000A), a sample injector (Waters Associates U6K), and a conductivity detector (LDC C-203). The stationary phases were packed into stainless steel columns (4 mm i.d., 300 mm or 150 mm length) by balanced density slurry technique. These packed columns were incorporated to the chromatograph system.

After the columns were conditioned by elution with a large quantity of water or water/methanol, aliquots of sample (50 µl) containing 0.02-0.04 M salts were injected into the system. Chromatography was performed at room temperature at a flow rate of 1.0 ml/min, unless otherwise specified. The pressure drops ranged from 30 to 100 kg/cm^2 when a column of 300 mm length and a flow rate of 1.0 ml/min were adopted.

RESULTS AND DISCUSSION

Polymers containing 12-crown-4 moiety at the side chain, what we call poly(12-crown-4), exhibit high Na^+ selectivity on complexing alkali metal ions, probably because they form stable 2:1 crown ring to ion complexes with the ion by cooperative action of two adjacent crown ether rings in a polymer chain. Attempts were made to immobilize this type of poly(crown ether)s on silica gel in order to obtain stationary phases for ion chromatography which possess high affinity for Na^+.

Chromatographic behavior of alkali metal salts on poly12C4-silica-I

Chromatography was performed using a column of 300 mm length packed with poly12C4-silica-I. When alkali metal chlorides were chromatographed by the elution with pure water, the retention times of them were not remarkably different. That is to say, Na^+ was slightly retained longer than the other alkali metal ions, which were hardly retained on the modified silica. Generally, in water, which is highly polar solvent, the complexation of crown ether with the ions is weak. When water/methanol (50/50 v/v) was used as the mobile phase, the retention times of the metal ions were increased and marked differences were also observed among them. This stationary phase retained Na^+ most strongly of all alkali metal ions as expected from the complexing ability of the poly(crown ether)s, their retention times being in the sequence $Li^+<Cs^+<Rb^+<K^+<Na^+$. On the chromatography of mixtures of Li^+, Na^+, and K^+ under this elution condition, Na^+ are completely separated from Li^+ and K^+, although baseline separation of Li^+ and K^+ was not attained. Change in the retention time of alkali metal ions were followed with increasing methanol fraction in the eluent (Fig. 2). The retention time of Li^+ was scarecely altered by increasing methanol fraction in the mobile phase, whereas those of Na^+ and K^+ were increased. In the retention times Rb^+ and Cs^+ are close to K^+, even if pure methanol was used as the mobile phase. The elution sequence of them was not changed with any methanol

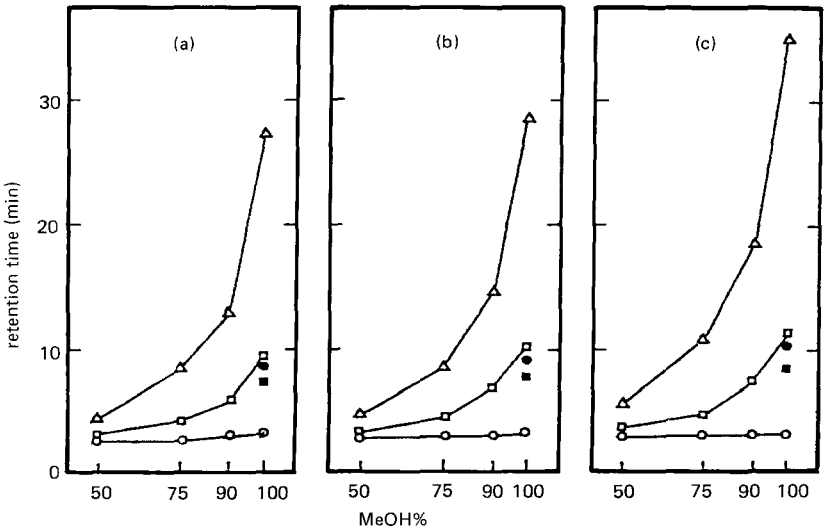

Figure 2. Dependence of the retention time of alkali metal halides upon methanol fraction in eluent. (a) chloride, (b) bromide, (c) iodide; (○) Li, (△) Na, (□) K, (●) Rb, (■) Cs.

fraction in the mobile phase. On the elution with pure methanol, the retention times of chlorides of Li^+, K^+, and Na^+ were 2.7, 9.4, and 27 min, respectively. The retention time difference of them is large enough for their baseline separation, but it takes very long to separate them under this elution condition. In Fig. 3 the separation of Li^+, K^+, and Na^+ is shown under the best elution condition, this is, using water/methanol (25/75 v/v), together with that using water/methanol (50/50 v/v) for comparison. In Fig. 3(a) the separation of Li^+, K^+, and Na^+ was complete, whereas that between Li^+ and K^+ is still insufficient in Fig. 3(b).

It is well known that the ion-complexing abilities of crown ethers were governed by the kind of counter anions as well as of cations. On this stationary phase the retention of metal salts was dependent upon their counter anions, especially, at high

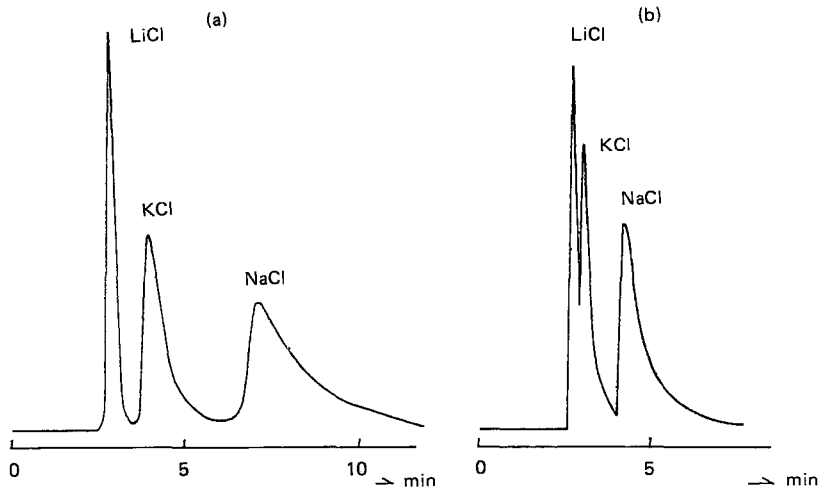

Figure 3. Chromatographic separation of alkali metal chlorides on poly(12-crown-4)-modified silica-I. column length: 300 mm; mobile phase: (a) water/methanol (25/75 v/v); (b) water/methanol (50/50 v/v); sample: LiCl, 0.08 mg; NaCl, 0.12 mg; KCl, 0.15 mg.

methanol fraction in the mobile phase (Fig. 2). The retention of anions accompanying a common cation is increased in the sequence $Cl^- < Br^- < I^-$ except Li^+ which is scarecely retained on the stationary phase even by the elution with pure methanol. However, the differences of retention times among the anions with a common cation were so small that they could not be separated to each other in this chromatography.

Chromatographic behavior of alkali metal salts on poly12C4-silica-S

In order to improve the column efficiency and the peak shapes, spherical type of silica gel was employed instead of irregular type one, and end-capping of residual silanol groups by HMDS was attempted instead of by TMCS.

As described in the experimental section, the stationary phases based on irregular- and spherical-type silica gels are almost the same in the crown ether content. Fig. 4 demonstrates

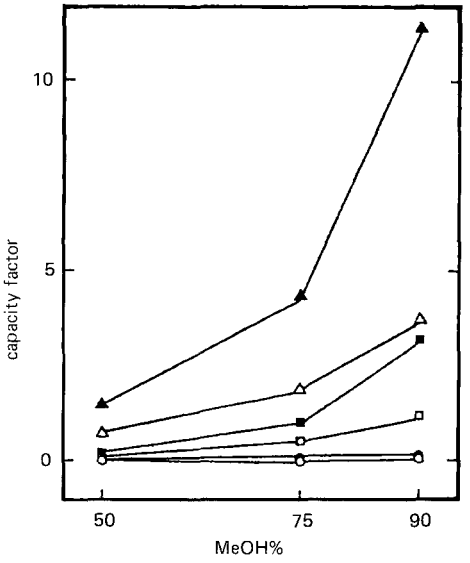

Figure 4. Dependence of capacity factors of alkali metal chlorides upon elution composition on chromatography using the different type of poly(12-crown-4)-modified silicas. (○) LiCl, (△) NaCl, (□) KCl on poly(12-crown-4)-modified silica-I; (●) LiCl, (▲) NaCl, (■) KCl on poly(12-crown-4)-modified silica-S.

the dependence of the capacity factors on the eluent composition of metal ions with respect to both stationary phases. Since Li^+ was hardly retained on either stationary phase, the capacity factors of Li^+ was not changed practically. However, Na^+ and K^+ have larger capacity factors on poly12C4-silica-S than poly12C4-silica-I. One of the reason is that the spherical type silica gel can be packed into a column more densely than irregular one. In other words, the former stationary phase is larger in the contents of crown ether per a column than the latter one, which in turn results in the strong retention of metal ions in a column packed with poly12C4-silica-S column compared with poly12C4-silica-I. By the elution with water/methanol (40/60 v/v), baseline separation of three alkali metal chlorides was attained on poly12C4-silica-S

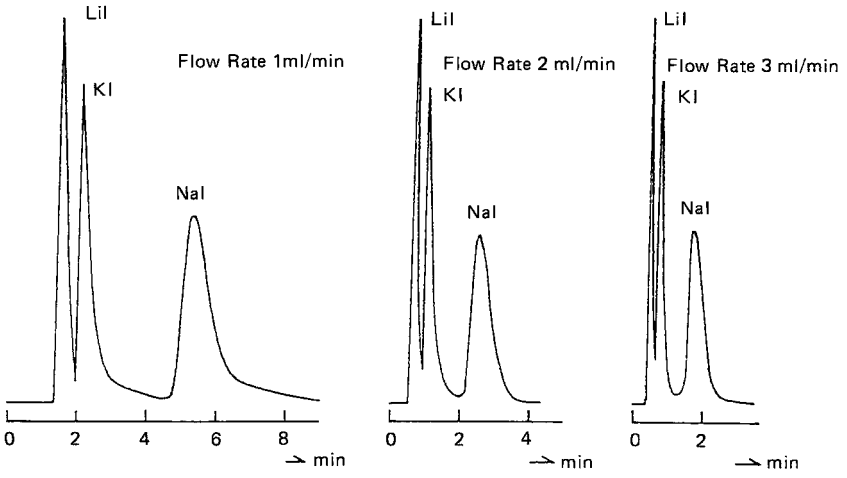

Figure 5. Effect of flow rate upon chromatographic separation of alkali metal iodides on poly(12-crown-4)-modified silica-S. column length: 150 mm; mobile phase: water/methanol (50/50 v/v); sample: LiI, 0.27 mg; NaI, 0.30 mg; KI, 0.33 mg.

whereas this was not the case in polyl2C4-silica-I. Also, intensive tailing of the Na^+ peak which was obseved in polyl2C4-silica-I has disappeared in this case, although slightly taining of K^+ peak still remained. This is thought to be due mainly to the effective end-capping of residual silanol groups on the surface of silica gel by HMDS compared to by TMCS, which was confirmed by the elemental analysis of carbon and the weight increase of the modified silicas on the syntheses.

Although Li^+, Na^+, and K^+ are completely separated on polyl2C4-silica-S under the above-mentioned elution condition, it still takes 9 min to separate them. Some attempts were made to implove the separation time. Generally, separation times are dependent on capacity factor, column length, and flow rate. A column of 150 mm length and flow rates higher than 1 ml/min are tried to attain more speedy chromatographic separation of them. Fig. 5 depicts chromatograms of alkali metal iodides by the elution with water/methanol (50/50 v/v) at a flow rate of 1, 2,

TABLE I

Retention Times of Alkaline-earth Metal Chlorides on Poly-(12-crown-4)-modified Silica-S

	water/methanol (v/v)			
	50/50	40/60	25/75	10/90
$MgCl_2$	2.7	3.0	3.1	5.4
$CaCl_2$	2.8	3.0	3.6	6.9
$SrCl_2$	2.8	3.0	3.9	8.7
$BaCl_2$	3.3	4.9	5.7	10.4

retention time in min.; column length: 300 mm

and 3 ml/min. Even at a high flow rate of 3 ml/min, these metal ions are separated successfully within 2.5 min.

Chromatographic behavior of alkaline-earth metal salts

Crown ethers complex alkaline-earth metal ions as well as alkali metal ions. The poly12C4-silica was tested for its usefulness on chromatographic separation of alkaline-earth metal ions. Alkaline-earth metal ions were eluted in the sequence $Mg^{2+}<Ca^{2+}<Sr^{2+}<Ba^{2+}$, the retention times of them being listed in Table I. The stationary phase in which poly(12-crown-4) is immobilized on silica gel shows large affinity for Ba^{2+}. This retention behavior is very similar to those of the crown ether resins (2,3) and the other poly(crown ether)s-modified silicas reported previously (5,6). When water/methanol (50/50 v/v) was used as the mobile phase, the retention times of Mg^{2+}, Ca^{2+}, and Sr^{2+} are close to each other. Increasing methanol fraction of mobile phase enhanced their retention on the stationary phase, but broadened their peaks concurrently.

ACKNOWLEDGMENTS

The author (K.K.) expresses his grateful thanks to the Foundation Hattori-Hokokai for financial assistance.

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UTILIZATION OF A SYSTEMATIC SOLVENT SELECTION METHOD FOR THE HPLC DETERMINATION OF A TRACE ISOMERIC CONTAMINANT

Jerome E. Haky*, A. Michael Young, Emily A. Domonkos and Robert L. Leeds

Chemistry Department
Warner-Lambert/Parke-Davis Pharmaceutical Research
Ann Arbor, Michigan 48105

ABSTRACT

A systematic solvent selection method is used for the development of HPLC conditions for the separation and detection of trace amounts of threo isomer in samples of the bronchodilating drug procaterol, (±)-(R*,S*)-8-hydroxy-5-[1-hydroxy-2-[(1-methylethyl)amino]butyl]-2(1H)-quinoline (erythro isomer). The method involves the chromatography of a mixture of the two isomers using seven different mixtures of three base solvents as mobile phases and the mapping of resolutions using a computer-generated mathematical model. The optimized mobile phase predicted by the model gives excellent resolution between the two isomers at levels as low as 1% of the threo compound.

INTRODUCTION

Maximizing the HPLC separation of the components of a binary mixture is especially important when the later-eluting material is present in a very small amount. In such cases, loss of detection or quantitation of the minor component can occur when the chromatographic peaks overlap even slightly. Often a trial-and-error

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process is employed for optimization of experimental conditions in such separations. Recently, however, a number of systematic optimization methods have been developed (1-7) which combine fundamental chromatographic principles with existing statistical processes to achieve maximum HPLC resolution.

Of particular utility in optimizing liquid chromatographic separations of complex mixtures is a method developed by Glajch and coworkers (7), based upon the mapping of resolutions between peak pairs using seven different mobile phase mixtures. This "overlapping resolution mapping" (ORM) technique has been used in the development of both normal and reverse phase liquid chromatographic conditions for the separation of a variety of chemical mixtures, including those of substituted naphthalenes (8), isomeric phenols (9) and aflatoxins (10).

Because it has as its basis the optimization of separations between pairs of chromatographic peaks, the ORM method is particularly well-suited for use in the systematic selection of HPLC conditions for maximum separation of a binary mixture. We have explored this potential by applying the ORM method in the development of an optimum solvent system for the separation of the bronchodilating drug procaterol, (±)-(R*,S*)-8-hydroxy-5-[1-hydroxy-2-[(1-methylethyl)amino]butyl]-2(1H)-quinoline (erythro isomer) from its threo isomer, a potential minor contaminant. In this paper, we discuss the details of the optimization of this separation and describe general schemes for the application of the ORM method for similar separations.

EXPERIMENTAL

Materials: All solvents were glass distilled (Burdick and Jackson Laboratories). Samples of procaterol and its threo isomer were synthesized in these laboratories using published procedures (11).

Apparatus: The HPLC system consisted of a Perkin-Elmer Series 4 quaternary solvent delivery system, a Rheodyne injector (20 µl loop) and an octadecylsilane column (Altex Ultrasphere ODS, 5 µm particle size, 250 mm x 4.6 mm). The system was fitted with a Perkin-Elmer Model 85B spectrophotometric detector operated at a fixed wavelength of 254 nm, and a Perkin-Elmer Model ISS-100 chromatographic autosampler. Chromatographic data were recorded and processed on an IBM Model 9000 data system.

Procedure: The optimization procedure consisted of chromatographing 20 µl aliquots of an aqueous solution containing 2 mg/ml of procaterol and 1 mg/ml of its threo isomer, in seven different mobile phases (see Results and Discussion). Final comparisons of optimized and unoptimized conditions were made by chromatographing an aqueous solution of 1 mg/ml of procaterol and 0.01 mg/ml of its threo isomer, using the mobile phases specified in Figure 2. Throughout the entire study, the flow rate of the mobile phase was set at a constant 1 ml/minute.

Equations: Chromatographic capacity factors, k', were calculated by equation I:

$$k' = \frac{t - t_o}{t_o} \qquad (I)$$

where t is the retention time of the component of interest, and t_o is the retention time of an unretained substance, determined by injection of an aqueous solution of sodium nitrite.

Chromatographic resolutions, R, between procaterol and its threo isomer were calculated by equation II:

$$R = \frac{1.18\ \Delta t_r}{W_1 + W_2} \qquad \text{(II)}$$

where Δt_r is the difference in retention times between the two chromatographic peaks and W_1 and W_2 are the widths of the two peaks at their half-heights (12,13).

Predicted acetonitrile-water and tetrahydrofuran-water mixtures of equal strength to the initially-used 30% methanol - 70% water mobile phase (Table I) were calculated using equation III:

$$S_t = \sum_i S_i \Theta_i \qquad \text{(III)}$$

where S_t is the total solvent strength of the initially-used solvent mixture (in this case, 78), S_i is the "solvent strength weighting factor" (water: 0; methanol: 2.6, acetonitrile: 3.2, tetrahydrofuran: 4.5), and Θ_i is the volume fraction of solvent "i" in the mixture (14). For the purpose of these calculations only, the presence of acetic acid in each of the mobile phases was ignored.

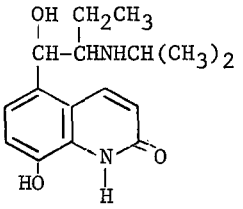
For the quantitative mapping of resolutions (R), data for each of seven mobile phase mixtures was fit to a cubic model (15), based upon equation IV:

$$R = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \qquad \text{(IV)}$$

where the X_i's are the volume fractions of each base solvent (see Table 2) and the B_i's are constants. Equation IV was solved and the resolution map was produced by a BASIC computer program (available on request) written for an Apple II+ computer equipped with an Epson MX-80 dot-matrix printer. This program is based on an earlier FORTRAN program developed by Hare and Brown (16).

RESULTS AND DISCUSSION

The development of an HPLC method for the separation of procaterol (I) from its threo isomer was prompted by the potential for threo isomer contamination in bulk lots of the drug from



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partial inversion of configuration at one of the compound's two chiral centers during its synthesis or storage. While their physical properties are similar, the two isomers differ widely in their bronchodilating activities (11), and thus the quantitation of the amount of threo isomer contamination in samples of procaterol is highly desirable. The two isomers can be distinguished from each other by proton NMR spectroscopy (11,17), but until now no quantitative chromatographic separation has been reported in the literature.

While selecting parameters for a routine HPLC separation of a binary mixture may often be accomplished through methods much simpler than that described here, developing an HPLC system for either trace analysis or preparative separations requires maximization of resolution to reduce peak overlap caused by column overload or any slight tailing of the chromatographic peaks. For trace analysis, this is especially true when the minor component consistently elutes after the main component, which is the case in the present separation. In such situations, a systematic scheme can ensure the development of optimized conditions which may eliminate later uncertainties.

The method described by Glajch and coworkers is designed for the systematic development of the optimum mobile phase for the analysis of a mixture by reverse-phase HPLC (8). Basically, the method consists of four steps:

1. A methanol-water mobile phase, X_1, is chosen which allows for the elution of all components of interest within a given k' range (usually $1 \leq k' \leq 10$).

2. Two other aqueous mobile phases, X_2 and X_3, of different selectivity but equivalent solvent strength (i.e., giving similar k' values) are chosen. The volume fraction of water in each of these iso-eluotropic base-solvents is usually calculated through Snyder's solvent-strength equation III (14) and then refined experimentally if necessary. Generally, these two mobile phases consist of mixtures of acetonitrile and water and tetrahydrofuran and water.

3. The chromatograms of the sample of interest are obtained in each of the three base-solvents as the mobile phase, as well as every possible 1:1 mixture of each base solvent. The chromatogram of the sample is then obtained using a final 1:1:1 ternary mixture of each base solvent.

4. The resolutions between each peak pair in the chromatograms of the sample of interest are either qualitatively or quantitatively compared, and the mixture of base solvents giving the best resolution is deduced and used as the optimized mobile phase.

This method, which is based upon the principles of Snyder's well-known solvent-selectivity triangle (18), seeks to maximize resolution between the components of a mixture by combining the unique selectivities of three base solvents.

Application of the ORM method to the separation of a binary mixture is in many ways simpler than for a multicomponent mixture. Loss of peak identity due to possible inversion of elution order can be eliminated by the development of the method on an unequal mixture of standards, in this case, a 2:1 mixture. Moreover, since only one peak pair is being considered, quantitative mapping and modelling of the resolutions is simplified. For this quantitative mapping, we have written a BASIC computer program, which fits resolutions obtained from the seven solvent mixtures to a cubic model (15), and then predicts resolutions for any other possible mixture. The results are displayed in a triangular

response surface diagram (Figure 1) from which the solvent composition giving the highest resolution may be read (16).

While the general applicability of the quantitative cubic model in the mapping of HPLC resolutions has been demonstrated in several cases (7,8,9,10) its use in the present situation requires a few considerations. Firstly, the sheer number of resolutions needed to be calculated makes it advantageous for these calculations to be carried out by a programmable integrator or data system at the time the data is acquired (19). Since the data system we used (as well as most systems) is capable of determining peak widths at half-heights, we have calculated chromatographic resolutions using equation II, which is based upon this "width at half-height" parameter (12,13). Although such an equation is easily incorporated into most integrators or data systems, the assumption of completely Gaussian chromatographic peaks upon which it is based (13) is not rigorously correct, especially in the present case, where some band broadening and assymetry is expected as the amount of sample injected or the chromatographic scale is increased to look for evidence of a trace contaminant. For this reason, the resolutions calculated by equation II can be considered only in a relative manner in this study. Aside from the inapplicability of the normal assumption of a resolution of 1.5 giving baseline peak separation (13,14), however, this does not limit the applicability or usefulness of the calculated resolutions to a great degree in the optimization process. The

quantitative model is simply used to predict a solvent system giving the maximum resolution, without regard to the magnitude of the actual value obtained.

The composition of the three base-solvent systems used for the present method are displayed in Table 1, along with k' values obtained for the procaterol isomers using these solvents. Each of these solvent systems gave good peak shapes for the two compounds, as well as k' values between 7 and 9. Apparently the addition of 1% acetic acid to the aqueous portion of the base solvents (to improve peak shape) rendered inapplicable the prediction of acetonitrile-water and tetrahydrofuran-water mixtures of equal strength to the methanol-water mixture on the basis of the solvent-strength equation III (14). Both predicted iso-eluotropic solvent mixtures, listed in parentheses in Table 1, were clearly too strong, and needed to be modified somewhat.

Table 2 lists the experimentally-determined resolutions between the procaterol isomers using each of seven mixtures of the three base solvents. Of the seven mixtures used, highest resolution was achieved with the mixture consisting of 50% X_1 and 50% X_2, or 15 parts methanol, 7 parts acetonitrile, and 78 parts aqueous acetic acid. The resolution achieved by mixing the tetrahydrofuran solvent (X_3) with the methanol solvent (X_1) was significantly lower than that obtained with the methanol solvent alone, while mixing the tetrahydrofuran solvent (X_3) with the acetonitrile solvent (X_2) resulted in only slight increases in resolution over that obtained with the acetonitrile solvent alone.

TABLE 1

Base Solvent Compositions and Chromatographic Capacity Factors

Solvent	First Component	Θ_1	Second Component	Θ_2	k'e	k't
X_1	MeOH	.30	HOAc	.70	7.6	8.8
X_2	ACN	.14 (.24)*	HOAc	.86 (.76)*	7.5	8.7
X_3	THF	.03 (.17)*	HOAc	.97 (.83)*	8.0	8.9

MeOH = methanol, ACN = acetonitrile, THF = tetrahydrofuran, HOAc = 1% aqueous acetic acid, Θ_1 = volume fraction of first component, Θ_2 = volume fraction of second component, k'_e = capacity factor of procaterol, k'_t = capacity factor of threo isomer, * Number in parentheses is the volume fraction predicted by equation II to give k' values similar to solvent X_1.

TABLE 2

Experimentally-Determined Chromatographic Resolutions Between Erythro and Threo Isomers

Mixture	$\%X_1$	$\%X_2$	$\%X_3$	Resolution
1	100	0	0	2.41
2	0	100	0	1.77
3	0	0	100	1.87
4	50	50	0	2.66
5	50	0	50	1.29
6	0	50	50	1.90
7	33.3	33.3	33.3	0.98

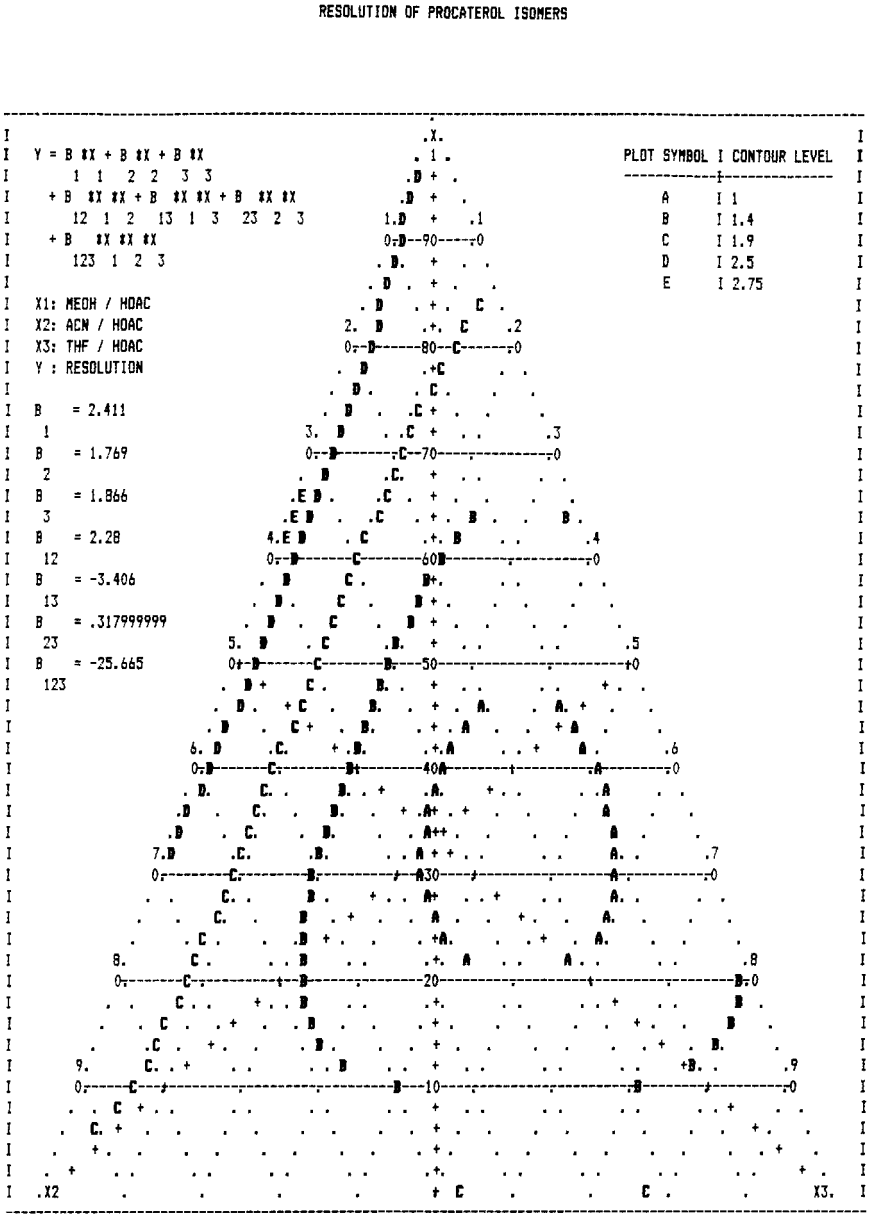

FIGURE 1. Computer-generated resolution map for the separation of procaterol and its threo isomer.

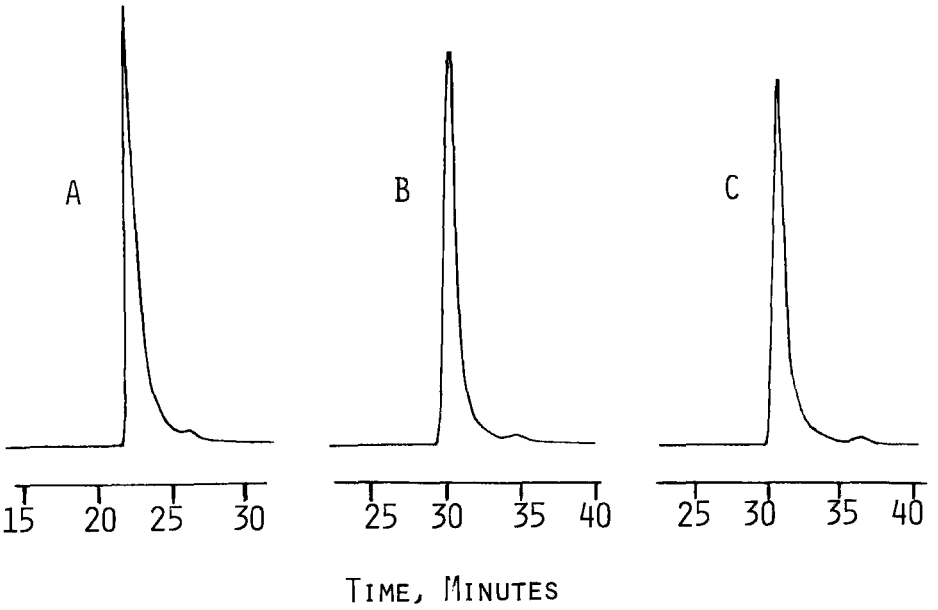

FIGURE 2. HPLC Chromatograms of procaterol containing 1% threo isomer using mobile phases consisting of: A - 33% X_1, 20% X_2 and 47% X_3; B - 100% X_2; C - 63% X_1 and 37% X_2.

The computer-generated resolution map for the overall X_1-X_2-X_3 solvent system is shown in Figure 1. As expected from the trends in the experimental data, the computer model predicts that highest resolutions are achieved for the two isomers using mixtures of the methanol and acetonitrile base solvents. The predicted optimum mixture consists of 63% X_1 and 37% X_2, or 18.9% methanol, 5.2% acetonitrile, and 75.9% aqueous 1% acetic acid.

Application of the predicted optimum mobile phase to the determination of trace amounts of threo isomer in procaterol produces consistent and usable results. Figure 2 displays the

chromatograms of a sample of procaterol which is contaminated with 1% threo isomer, using the predicted optimum solvent as well as two other mixtures as mobile phases. While resolution is not complete in any of the chromatograms, owing possibly to column overload, the best resolution is obtained with the predicted optimum solvent (Figure 2C). Also consistent with the computer model is the poor separation of erythro and threo isomers (Figure 2A) with a mobile phase consisting of 9.9% methanol, 2.8% acetonitrile, 1.4% tetrahydrofuran and 85.9% aqueous acetic acid (i.e., 33% X_1, 20% X_2 and 47% X_3). Clearly, the high degree of separation achieved with the use of the predicted optimum solvent reduces overlap to a minimum, and results in a desirable HPLC system for determination of threo isomer contaminant.

CONCLUSIONS

In this study, the systematic solvent selection method developed by Glajch and coworkers was used for the optimization of the mobile phase for the HPLC separation of a drug and a trace contaminant. While three base solvent systems were initially used in the method development, a mixture of two of them was ultimately found to give the best separation. Through the use of this method, the precise composition of the solvent giving the optimum separation was predicted, and one of the three solvents could be confidently eliminated from consideration. Thus, this quantitative, systematic method of solvent selection has been demonstrated to be a highly useful technique for the maximization of

conditions for the HPLC determination of trace contaminants, even when only one such material is known to be present.

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ACTIVE TBC IN BUTADIENE BY HPLC

A.C.Oomens and F.G.Schuurhuis
Analytical Development Department
Dow Chemical (Nederland) bv
Terneuzen, The Netherlands

and

N.E.Skelly
Analytical Laboratories
Dow Chemical Michigan Division
Midland, U.S.A.

ABSTRACT

A high performance liquid chromatographic method has been developed for the determination of active 4-tert-butylcatechol (TBC) in 1,3-butadiene. Following evaporation of the butadiene from an aqueous m-nitrophenol internal standard solution, a 20-microliter aliquot is injected onto a reversed-phase liquid chromatographic column. Recovery of TBC was found to be quantitative over a 17-242 ppm range with a relative standard deviation of 3.8%.

INTRODUCTION

The polymerization inhibitor 4-tert-butylcatechol (TBC) or 4-(1,1-dimethylethyl)-1,2-benzenediol in 1,3-butadiene has been historically determined by colorimetric methods such as ASTM method D1157 (ref 1). In these methods the catechol

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is deliberately oxidized to the colored quinone which is measured by a suitable photometer. This means, however, that these methods are not able to differentiate between the active inhibitor and its oxidized, inactive, form. Therefore, a method that specifically measures the concentration of the active inhibitor would be of considerable importance for industry with respect to butadiene stability during shipping, storage and immediately prior to polymerization. We have developed an analytical method for the determination of the active TBC in liquid butadiene.

MATERIALS

Apparatus :

A modular HPLC has been used throughout this investigation. A Waters M6000A pump or a Spectra-Physics SP8700 pump were applied as a solvent delivery system. Reversed-phase columns included LiChrosorb®RP-18 10 micron or Hypersil ODS packings. A LDC model 1203 UV detector was used with wavelength set at 280 nm. In all cases, a Valco injection valve with a twenty-microliter fixed loop was applied. The unit was interfaced with a Hewlett-Packard 3354 Laboratory Data System.

Reagents :

All reagents were of recognized analytical grades such as TBC (BDH 27520), m-nitrophenol (Baker 1357), methanol (Baker 8045), acetic acid (Baker 6052) and chloroform (Baker 7386). Deionized water from a Milli-Q® system (Millipore Corporation) was used as the extraction liquid. Inhibitor-free butadiene samples in stainless steel containers equipped with an inlet and an outlet valve came directly from our production plant.

Chromatographic conditions :

Column : 250 x 4.6 mm, reversed-phase packing
Eluent : methanol/water/acetic acid, 67/32/1 by volume
Flow rate : 1.5 mL/min.
Detector : 280 nm
Injection : 20 microliter via fixed loop

METHOD

Cool sample container and a 25-mL graduated cylinder to about -20 degC. Measure 25 mL of liquid sample into the graduated glass cylinder. Pour the sample into a 50-mL vial containing 25 mL of internal standard solution (25 mg m-nitrophenol per liter of water) and allow the butadiene to evaporate at room temperature in a fume hood. After the upper organic layer has completely evaporated, close the vial and shake the contents for one minute. Inject the resulting extract into the chromatograph and obtain peak areas for TBC and the internal standard (IS). The ratio of peak areas TBC/IS is used for quantitation. A calibration graph is prepared by analyzing solutions of TBC in water with a concentration range of 0-150 mg/L. These solutions are prepared by adding 0-150 microliter aliquots of a solution containing 2.50 g TBC in 100 mL chloroform to 25-mL aliquots of the internal standard solution.

RESULTS AND DISCUSSION

Figure 1a depicts a typical chromatogram for a sample containing 134 mg TBC per liter liquid butadiene sampled at - 20 degrees C. This corresponds to (134/0.6681=) 200 mg TBC per kg liquid butadiene (ppm by weight).
Linearity of response is excellent as shown in figure 2.

The repeatability of the prescribed analytical method was determined by analyzing an actual sample 10 times, 5 ti-

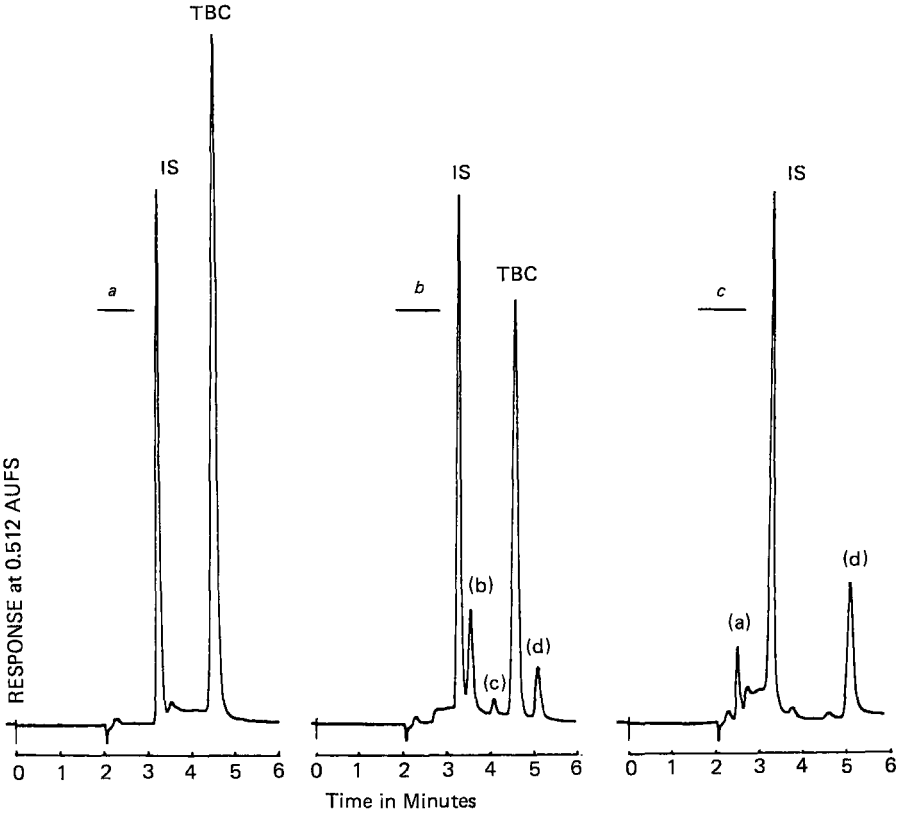


Figure 1: Chromatograms.
1a Normal sample extract (IS = m-nitrophenol),
1b Same solution with addition of NaOH, after 1 minute.
1c Same as 1b, except after 10 minutes.

mes on two successive days. A mean concentration of 95 mg/kg was obtained with a relative standard deviation of 1.9%.

The absolute recoveries of the inhibitor were determined by adding known amounts of TBC from a chloroform solution to inhibitor-free butadiene in stainless steel containers. These spiked samples were then extracted and measured in the normal manner. Recovery was quantitative within the experimental error of the method as shown in Table I.

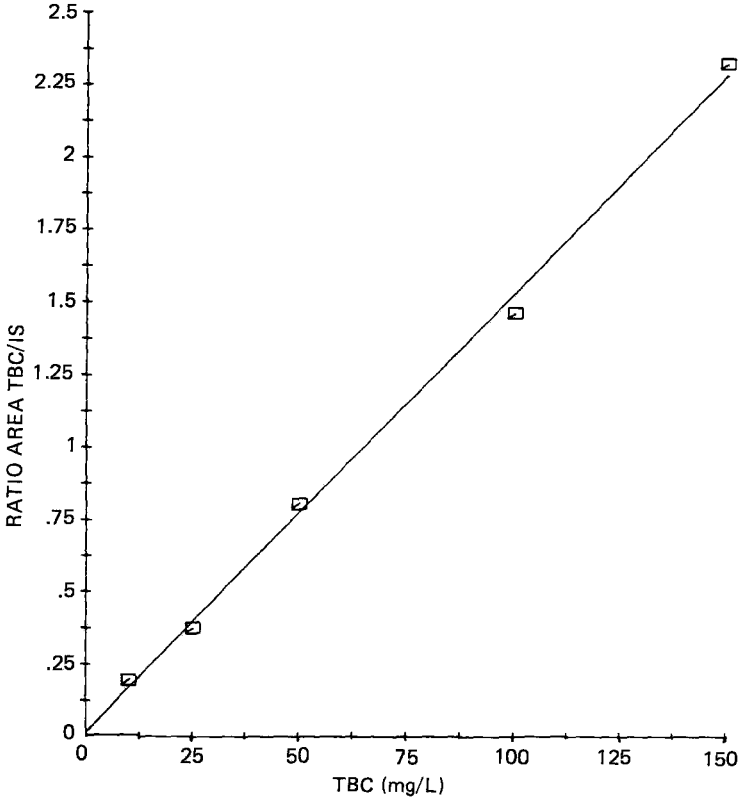


Figure 2: Calibration graph.
Regression data: slope 1.5118E-02, intercept 1.5079E-02, correlation coefficient 0.9987.

TABLE I : Recovery data for TBC added to butadiene.

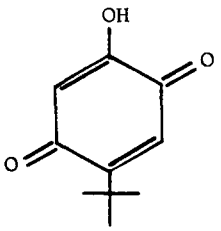
TBC added	TBC found	Recovery
16 mg/kg	17 mg/kg	106 %
40	39	98
79	79	100
159	153	96
238	242	102
Mean recovery		100 %
Relative standard deviation		3.8 %

In order to demonstrate that with this method only the active form of TBC is measured, the following experiment was performed. To 25 mL of an aqueous extract (figure 1a) 200 microliters of sodium hydroxide solution (4N) was added, shaken for 1 minute and immediately chromatographed. Figure 1b shows the decrease of the TBC peak while oxidation products, labeled (b), (c) and (d), are appearing. After another 9 minutes the mixture was injected again with the results vizualized in figure 1c. Now the TBC peak has almost completely disappeared and another reaction product, (a), has appeared.

An experiment was undertaken to isolate and identify the aqueous-caustic oxidation product of TBC. A 25-mg sample of TBC was dissolved in 0.5N sodium hydroxide. Air was bubbled through the solution at a rate of approximately five bubbles per second for ten minutes. The solution was placed in a separatory funnel, acidified with dilute sulfuric acid, and extracted with 5 mL of chloroform. The chloroform extract was washed with an equal volume of water and evaporated under a stream of nitrogen.

Examination of the residue by IR, NMR and MS, together with elemental analysis, proved the oxidation product to be:

2-tert-butyl-5-hydroxy-p-benzoquinone



Injection of this compound into the liquid chromatograph under the conditions used for the TBC analysis gave a peak that had an identical retention time as peak (d) in figure 1c. However, since this peak was generated by alkaline oxidation of TBC, in the butadiene it may be different than oxidation products that result from normal storage of the product. Pilar et al (ref 2) made an electron paramagnetic resonance (EPR) study on the mechanism of the autoxidation of 4-tert-butylcatechol and showed 2-tert-butyl-5-hydroxy-p-benzoquinone to be the final product. The mechanism was in agreement with that of Stone and Waters (ref 3).

Since the mobile phase in the described procedure is acidic, there was some concern as to the durability of the HPLC packing. Using a pneumatically-operated injection valve, a solution of inhibitor with internal standard was automatically injected 36 times a day for 6 weeks, over 1500 injections in all. There was no loss in column efficiency over the period of the test.

It is concluded that a reliable and an accurate analytical method has been developed that enables the specific measurement of active TBC in liquid butadiene.

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DETERMINATION OF ISOCYANIC ACID IN AIR BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

J. E. McClure
P. L. Stehman
American Cyanamid Company
P. O. Box 60
Stamford, Conn. 06904

ABSTRACT

Isocyanic acid in air is determined quantitatively by collection in a scrubber solution of N-4-nitrobenzyl-N-n-propylamine. The urea derivative, which is formed, is determined by using reverse phase high performance liquid chromatography.

INTRODUCTION

Low levels of airborne isocyanates have been measured by a variety of techniques such as colorimetry (1), gas chromatography (2,3,4) and high performance liquid chromatography (HPLC), (5,6). The colorimetric approach to the detection of isocyanate is limited to aromatic isocyanates, and therefore cannot be used for the determination of isocyanic acid (HNCO). Gas chromatography can be used to determine HNCO in the gas phase (7). However, this approach is not attractive in situations where isocyanic acid must be collected at a remote (e.g., plant) site and measured at a later date because HNCO may decompose before it can be analyzed. The gas chromatographic approach also requires the availability of a standard solution of HNCO for calibration.

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The most attractive approach to the measurement of low levels of HNCO in air is a method in which the HNCO is trapped and stabilized. Such an approach is offered by a method in which HNCO is reacted with a secondary amine to form a stable urea derivative. The urea is subsequently measured by HPLC with UV detection. This approach was described for the measurement of several aromatic isocyanates (5,6). This paper describes the use of a derivatization - high performance liquid chromatographic method for the measurement of HNCO in air at concentrations over a range from 0.05 to 25 ppm (v/v). The effect of humidity has also been studied. The preparation of a stable urea derivative for instrument calibration is also described.

MATERIALS

Apparatus

A Spectra-Physics SP-8000B High Performance Liquid Chromatograph with SP-8310 UV/Visible Detector (254 nm) and a Micromeritics Autoinjector with a 100-µL sample loop was used. The column was a 4.6-mm ID x 250 mm stainless-steel column packed with Partisil-5 ODS, 5 micron (Whatman Cat. No. 4220-104). Scrubbers (30-mL capacity) were obtained from Ace Glass (Part No. 7530-05). A Mine Safety Appliances Pump, Model S, was used for sample collection.

Reagents

The nitro reagent (N-4-nitrobenzyl-N-n-propylamine) was obtained as the hydrochloride salt from Aldrich (Catalog No. 22,191-0). Sodium hydroxide, sodium sulfate, toluene, triethylamine, HPLC grade acetonitrile, Type 3A Molecular Sieves, and phosphoric acid were Baker Reagent Grade materials.

Milli-Q purified (Millipore) water was used. Isocyanic acid solutions were made by dilution from a concentrated (17%) solution of isocyanic acid in toluene (U.S. Pat #4,364,913, American Cyanamid Co., December 1982).

METHODS

Preparation of Nitro Reagent

Approximately 240 mg of the nitro reagent hydrochloride were dissolved in 20 mL of distilled water. 25 mL of 1N NaOH were added to precipitate the nitro reagent which was then extracted with 50 mL of toluene. The toluene extraction was dried with ~10 g Na_2SO_4. The toluene was removed by evaporation and the free base was diluted to 500 mL with HPLC grade acetonitrile which had been dried overnight with ~25 g of molecular sieves. The nitro reagent should be stored in the dark and used no more than three weeks.

Preparation of N-4-nitrobenzyl-N-n-propylurea

Dissolve 5.0 g of N-4-nitrobenzyl-N-n-propylamine hydrochloride (Aldrich Chemical #22191-0, 98%) in 50 mL of water in a 250 mL separatory funnel and add 25 mL of 5% NaOH. The free base will separate as a yellow oil. Add 50 mL toluene and agitate gently until the oil dissolves. Draw off the aqueous layer and extract it again with 50 mL of toluene. Combine the toluene layers which contain the free base and filter off the insoluble white solid through Whatman #40 filter paper. Collect the toluene filtrate in a 4-ounce bottle containing 10 g of anhydrous Na_2SO_4. Cap the bottle, shake gently and allow to stand overnight. The theoretical amount of free nitroamine in the toluene solution is 4.13 g. This amount of amine will react theoretically with 0.91 g isocyanic acid. Decant the clear yellow-colored toluene solution into a 250-mL separatory funnel. Add slowly a measured volume of a toluene solution of isocyanic acid (16 mL of solution containing 7.7% HNCO was used which is equivalent to about 1.07 g HNCO). The solution becomes turbid and slightly warm during addition of the isocyanic acid solution, but after mixing, it changes to a clear yellow. Allow the solution to stand for an hour and then extract it twice with 50 mL of aqueous 0.1 N NaOH, once with 50 mL of 0.1N HCl and once with 50 mL H_2O. During the extractions a small amount

of waxy white solid will precipitate out. The solids were kept with the toluene layer. Dilute the toluene layer to 200 mL with toluene and warm gently. The waxy solid will dissolve to give a clear solution. Cool the toluene solution in an ice bath for 1 hour. White crystals will form on the walls of the separatory funnel. Pour off the toluene and dissolve the crystals in the separatory funnel in 60 mL warm toluene. Transfer the solution to a 150-mL beaker and cool in an ice bath. Crystals will form on the walls and bottom of the beaker. Pour off the toluene and dry the crystals in a vacuum desiccator over Drierite. Remove the dried crystals from the beaker and pulverize them with a mortar and pestle. The yield is 1.56 g (31%) and the product has a melting point of 92-95°C.

The following C, H, N values were obtained for the product:

	C	H	N
Found	56.59	6.33	17.64
Theory	55.68	5.95	17.71

The proposed structure of the material was confirmed by proton NMR spectrometry.

Preparation of N-4-Nitrobenzyl-N-n-Propylurea Standard Solution

Approximately 15 mg (to the nearest 0.05 mg) of the urea derivative standard were weighed into a 100 mL volumetric flask and diluted to volume with HPLC grade acetonitrile (15 mg of urea derivative/100 mL corresponds to 27.2 μg/mL HNCO). The standard solution should be stored in the dark and used within two weeks.

Preparation of Mobile Phase

Ten mL of triethylamine were added to 990 mL of Milli-Q purified water. The pH was adjusted to 3.0 with phosphoric acid. 700 mL of this solution were mixed with 300 mL of acetonitrile, filtered through a 0.3 micron pore size glass fiber filter and degassed by sonication before use.

Collection and Treatment of Air Samples Which Contains HNCO

20 mL of ~2 x 10^{-3} M nitro reagent were added to each of two scrubbers. The two scrubbers were then connected in series. A calibrated air pump was connected to the scrubber train and air was drawn through the system at a rate of 0.5 L/min. The scrubber should be wrapped with aluminum foil in order to minimize exposure of the solution to light. Recommended sampling times are shown in Table 1. A blank should be collected from an HNCO-free atmosphere for the same period of time that the sample was collected.

The scrubbers were then removed from the system and the contents of the second scrubber was discarded. The second scrubber was used to prevent small amounts of HNCO from getting into the pump. The first scrubber traps most of the HNCO (see Table 3). The solution from the first scrubber was treated as described in Table 2. After treatment, the solution is ready for measurement by HPLC.

Chromatographic Measurements

The column was equilibrated with the mobile phase at 40°C (ambient conditions can be used) until a steady baseline was achieved. A flow rate of 1.5 mL per minute was used. Linearity of response of the HPLC system was demonstrated by injection of

TABLE 1

Recommended Air Sampling Times

Air Conc of HNCO	Air Conc. of HNCO	Collection time at 0.5 L/min.	Liters Collected	µg HNCO expected
50 ppm	≈0.1 g/m^3	4 min.	2	200
5 ppm	10 mg/m^3	20 min.	10	100
0.5 ppm	1 mg/m^3	1 hour	30	30
50 ppb	0.1 mg/m^3	1 hour	30	3
	Blank	4 min.- 1 hr.	2-30	---

TABLE 2

Treatment of Scrubber Samples for Chromatographic Measurement

HNCO Air Conc.		Scrubber Solution Treatment	µg HNCO expected in 5 mL flask
50 ppm	≈0.1 g/m^3	Dilute to 25 mL with acetonitrile, add 1 mL (from 25-mL flask) and 1 mL acetonitrile to a 5-mL flask, dilute to mark with the aqueous component of the eluting solvent.	8
5 ppm	10 mg/m^3	Dilute to 25 mL with acetonitrile, dilute 2 mL (from the 25-mL flask) to 5 mL with the aqueous component of the eluting solvent.	8
0.5 ppm	1 mg/m^3	Evaporate to ~2 mL, dilute to 5 mL with the aqueous component of the eluting solvent.	6
50 ppb	0.1 mg/m^3	Evaporate to dryness, dissolve in 0.5 mL acetonitrile, dilute to 5 mL with eluting solvent.	3
Blank	Blank	Evaporate to dryness, dissolve in 0.5 mL acetonitrile, dilute to 5 mL with eluting solvent.	<0.5

solutions which contained 3, 6 and 9 µg of HNCO moved (as the urea derivative) in 5 mL of the eluting solvent. These values correspond to 16.5, 33.1, and 49.6 µg of urea derivative/5 mL. The urea derivative elutes at approximately 8.5 minutes. A linearity plot (with additional concentrations) is shown in Figure 1. Typical chromatograms for the standard and sample are shown in Figures 2 and 3, respectively. The sample measurement was carried out by injecting a sample and then a standard which gave a peak with

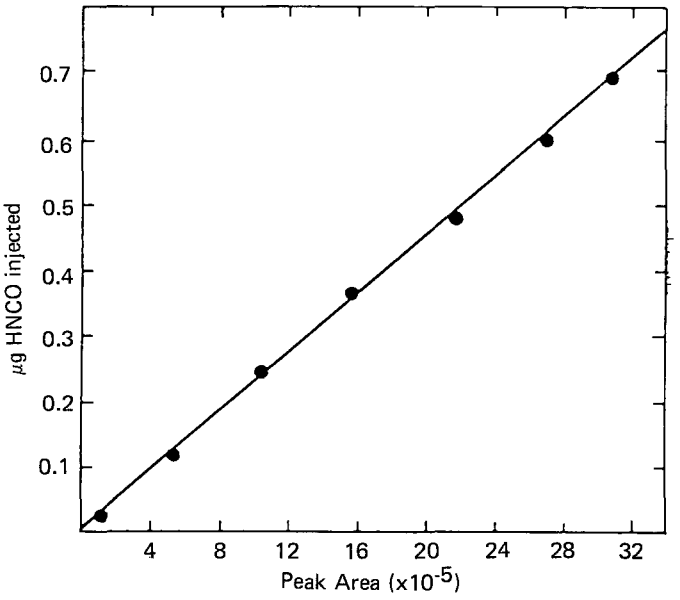

FIGURE 1. Micrograms of isocyanic acid vs peak area

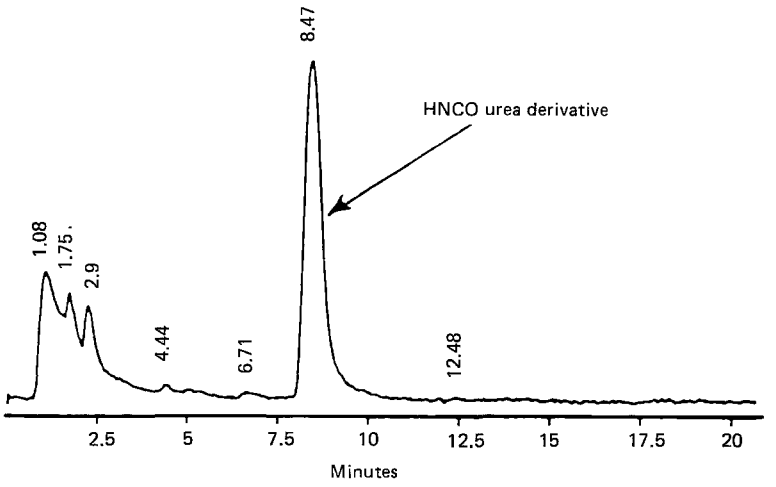

FIGURE 2. Chromatogram of standard urea derivative. 13.6 μg (HNCO basis) in 4 mL. 1 mL diluted to 10 mL. 100 μL (0.0340 μg) injected.

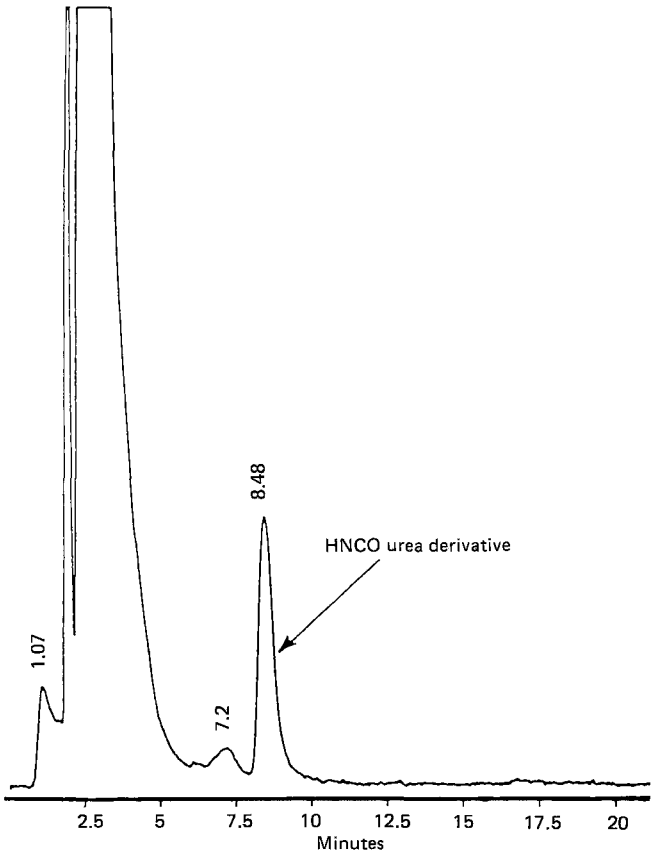

FIGURE 3 Chromatogram of a sample. Total residue dissolved in 4 mL. 1 mL diluted to 10 mL, 100 µL injected, 0.0289 µg found (as HNCO) or 11.6 µg HNCO found in sample.

approximately the same area as the sample. A blank was measured in a similar manner.

Calculations

Case 1: total sample (after complete or partial evaporation of acetonitrile) is in a 5-mL flask. The standard is in a 5-mL flask. (Spl refers to the scrubber solution).

$$\mu g\ HNCO\ spl = \left[\frac{\mu g\ std\ (in\ 5\ mL)}{peak\ area\ std} \times peak\ area\ spl\right] - \mu g\ blank$$

Case 2: the scrubber solution has been diluted to 25 mL. An aliquot of the scrubber solution (from the 25-mL flask) is diluted to 5 mL (see Table II). The standard is in a 5-mL flask.

$$\mu g\ HNCO\ spl = \left[\frac{\mu g\ std\ (in\ 5\ mL)}{peak\ area\ std} \times peak\ area\ spl \times \frac{25}{A}\right] - \mu g\ blank$$

where A is the aliquot volume in mL.

Blank

Blank and standard are in 5-mL flasks

$$\mu g\ \text{"HNCO"}\ blank = \frac{\mu g\ std}{peak\ area\ std} \times peak\ area\ blank$$

Concentration in Actual Atmospheres Tested

$$ppm\ HNCO\ (v/v) = \frac{A(62.36)\ (273 + °C)}{43\ (V)\ (Torr)}$$

where A = μg HNCO collected in trap (from Case 1 or Case 2)
62.36 = Gas constant
°C = Collection temperature
43 = mol. wt. of HNCO
V = Total volume of air (liters) sampled
Torr = Barometric pressure

RESULTS AND DISCUSSION

Recovery Values

The efficiency of the nitro reagent scrubber system for trapping HNCO was determined by drawing known atmospheres of HNCO through the scrubber for a fixed time. Known air concentrations of HNCO were generated by continuous (syringe) injection of a toluene solution of HNCO into a stream of air which was being pulled through the scrubber system. A Sage pump was used to drive the syringe at a constant rate. The pump-syringe system was cali-

TABLE 3

Recovery Data

Air Conc. of HNCO (v/v)	Collection time at 0.5 L/min.	Collection Volume, Liters	Relative Humidity	µg HNCO Expected	µg HNCO found		Recovery (%)
					First Scrubber	Second Scrubber	
24 ppm	8 min.	4		194.2	160	2.4	82
12 ppm	15 min.	7.5		182	147		81
4.65 ppm	30 min.	15		121	114	1.7	94
3.37 ppm	30 min.	15	16%	87.5	92.8	0.16	106
427 ppb	1 hour	30	60%	22.2	18.7	0.96	84
407 ppb	1 hour	30	90%	21.0	21.3	0.66	101
397 ppb	1 hour	30	60%	20.65	14.5	0.36	70
387 ppb	1.5 hour	45	60%	30.7	32.4	0.8	106
350 ppb	1 hour	30	16%	18.2	15.6	0.0	86
338 ppb	1 hour	30		17.6	20.5	0.0	116
295 ppb	1 hour	30	13%	15.4	12.6	0.54	82
152 ppb	1 hour	30		7.9	7.1	0.0	90
121 ppb	1 hour	30		6.28	7.6	0.05	121
92 ppb	1 hour	30		4.8	3.22	0.0	67
90 ppb	1 hour	30		4.65	5.59	0.1	120
77 ppb	1 hour	30		3.98	4.64	0.0	116
77 ppb	1 hour	30		4.00	4.84	0.28	121
70 ppb	1 hour	30	30%	3.88	3.42	0.20	88
68 ppb	1 hour	30	60%	3.67	3.46		94
55 ppb	1 hour	30	42%	2.88	2.71	0.0	94

brated by weighing liquid delivered in one hour. Recovery values were calculated from:

$$\% \text{ Recovery} = \frac{\mu g \text{ HNCO found in first scrubber}}{\mu g \text{ HNCO/hour (syringe-delivery rate)} \times \text{collection time (hours)}} \times 100$$

Data shown in Table 3 indicate that breakthrough of HNCO into the second scrubber is negligible.

Recovery values for gas phase HNCO over a range from 55 ppb to 24 ppm are shown in Table 3. No loss of HNCO was observed when humidified air was used. The overall recovery including all the data shown is 96%. Recovery values are based on the contents of the first scrubber. Only two values were observed at or below 70%.

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DETERMINATION OF BITREX, QUASSIA POWDER AND SUCROSE OCTAACETATE NEXT TO DIETHYL PHTHALATE AND CAMPHOR IN SPECIALLY DENATURED ALCOHOLS BY LIQUID CHROMATOGRAPHY.

Jan Kovar and Mireille Loyer
Laboratory and Scientific Services Directorate,
Revenue Canada, Customs and Excise,
Ottawa, Ontario
K1A 0L5 (Canada)

ABSTRACT

A HPLC method for determination of the bitter principles, bitrex, quassia and sucrose octaacetate, next to other ingredients in Specially Denatured Alcohol formulations is described. The method is based on evaporation of a sample, extraction of the residue with hexane and analysis of the extracted residue on a Cyano-type column with acetonitrile-water used as the eluent. Baseline separation of compounds and satisfactory quantitation has been achieved. Samples without pretreatment can occasionally be analysed with slight modifications of the procedure.

INTRODUCTION

While the bitter substances, Bitrex, (Benzyldiethyl(2,6-xylylcarbamoylmethyl)ammonium benzoate) Quassia powder and Sucrose Octaacetate (SOA), used in several formulations of specially denatured alcohols can be, in exceptional circumstances, identified and to some extent

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quantitated by UV or IR, the spectroscopic determinations generally fail, when other strongly absorbing compounds, such as diethyl phthalate, are present. It was therefore necessary to develop a technique for separating the compounds of interest from the contaminants prior to determination. Liquid chromatography seemed to be such a technique.

Two LC methods have been described in the literature for separation and determination of Bitrex from a sometimes complex matrix of compounds (1,2); there are none, to our knowledge, for the other two denaturants. One of the methods, using a reversed phase type of a column and acetonitrile-water mixture as mobile phase seemed to have promise also for the other compounds and was therefore selected to be adapted for the general application in determining all three bitter principles simultaneously.

The low concentrations of the denaturants used in the allowed (3) formulations (see Table 1) suggested that some sort of preconcentration step might be necessary and therefore a concentration procedure was investigated. The high proportion of possibly interfering compounds called for their removal prior to LC and an extraction procedure was also desirable; the distribution properties of the main ingredients were therefore studied.

EXPERIMENTAL

Apparatus and Chromatography Conditions:

The analyses were performed using a Waters Associates Liquid Chromatography system, consisting of:
Model 6000A Solvent Delivery System
Model U6K Injector
Model SF770 Spectroflow Monitor (Schoeffel Instruments Corp.)
Model 95-8290 Honeywell Dual Pen Flat Bed Recorder.

TABLE 1

Regulation (3) Formulas Containing Bitrex and/or Quassia Powder and/or Surose Octaacetate, with Concentrations Expressed in mg/L of Sample.

Formula	Ethanol % vol	DEP*	Camphor	SOA*	Quassia	Bitrex
1 B a	95	--	--	--	140	--
1 B b	95	--	--	980	--	--
1 B c	95	--	--	420	--	7
1 F a	93	22400	400	--	110	7
1 F b	93	22400	400	210	--	--
1 S	94	11200	--	--	--	14

*DEP Diethyl Phthalate;
*SOA Sucrose Octaacetate

Column: μBondapak-CN (Waters Associates), 3.9 mm ID, 300 mm length, Stainless steel, P/N 41515, particle size 10 μm.
Hamilton Syringe, 10 μL.
Mobile Phase: Acetonitrile, (Caledon) 30% by volume, in water (distilled, for LC) (Baker Analyzed) unless otherwise indicated. The solution was degassed prior to use by sparging for 3-5 min, using pure helium.
Flow rate: 1 mL/min. (Operating range 600 - 1200 psi.) UV: 210 nm, range 0 - 0.4 A, unless otherwise indicated.
Chart speed: 8mm/min.
Rentention times of reference compounds are summarized in Table 2, for 21°C.

TABLE 2

Rentenion times: (min, and standard deviation; number of determinations in brackets)

Acetonitrile %	60	50	40	30	30, in 01-M NaCl
Bitrex	2.0±0.1 (10)	1.79±0.02 (5)	1.86±0.05 (5)	1.90±0.05 (13)	2.4±0.2 (18)
Quassia (main peaks)	3.0±0.1 (9)	3.45±0.07 (2)	4.1 (2) 4.6 (2)	5.27±0.05 (9) 6.10±0.05 (9)	5.2±0.1 6.05±0.07
SOA *	3.53±0.07 (8)	4.69±0.06 (4)	7.1 (2)	13.2±0.4 (13)	12.8±0.1
DEP *	3.74±0.07 (15)	4.76±0.05 (10)	6.6 (2)	9.8±0.1 (12)	9.7±0.2
Camphor	3.63±0.08 (6)			7.2±0.3 (2)	
Ethanol	2.1 - 3.2	2.1 - 3.2		2.5 - 3.6	2.4 - 3.6
Front peak	1.8	1.8	1.8	1.75	2.11±0.03 (20)

* SOA Sucrose Octaacetate; DEP Diethyl Phthalate

Impurities and Contaminants (Retention Times are for 30% Acetonitrile as Mobile Phase):

When samples containing large amounts of DEP were injected, signals at 1.7 - 1.9 min. were sometimes observed , ("Front Peak") possibly due to the presence of small amounts of phthalic acid in the phthalate. Peaks in the same range of retention time appeared upon injection of SOA alone, likely due to free sucrose, and were also present in samples of Quassia. Even if relatively

weak, the signals might be confused with genuine Bitrex peaks from which they are only marginally resolved, and enhance the apparent Bitrex concentration.
Sample chromatograms are attached as Fig.1.

Old samples originally containing SOA exhibited, in addition to the expected peaks at 13.0 and 1.8 min., signals at 3.4, 5.6 and 8.0 min; concurrtenly the intensity of the main peak was much lower than expected to the point of total disappearance.
These peaks do not interfere with the determination of the other compounds.

Calibration:

Primary standard solutions were prepared by weighing, acurately, about 8 mg Bitrex, 100 mg Sucrose Octaacetate and 60 mg Quassia into separate 10.00 ml volumetric flasks and filling each flask up to the mark with mobile phase (30% Acetonitrile in water).

Note: The amount of SOA is close to the solubility limit under these conditions; e.g., 450 mg did not dissolve in 10 ml of this solvent. Secondary solutions of Bitrex and of Quassia were prepared by diluting to one fifth, one tenth and one twentieth of the original concentration;
Secondary solutions of SOA were prepared by diluting to one half and one fifth of the primary, using the same mobile phase as solvent.

10 µL of each solution were injected in succession, and run under the conditions specified above except that the Range (UV) of 1.0 was used for primary standard of Bitrex, and the Range of 0.1 for solutions a,b and c of Quassia; all other solutions were run at the standard Range of 0.4 A. The heights of peaks corresponding to the compounds analyzed were measured (see Table of retention times) in mm, vertically, from top of the peak to

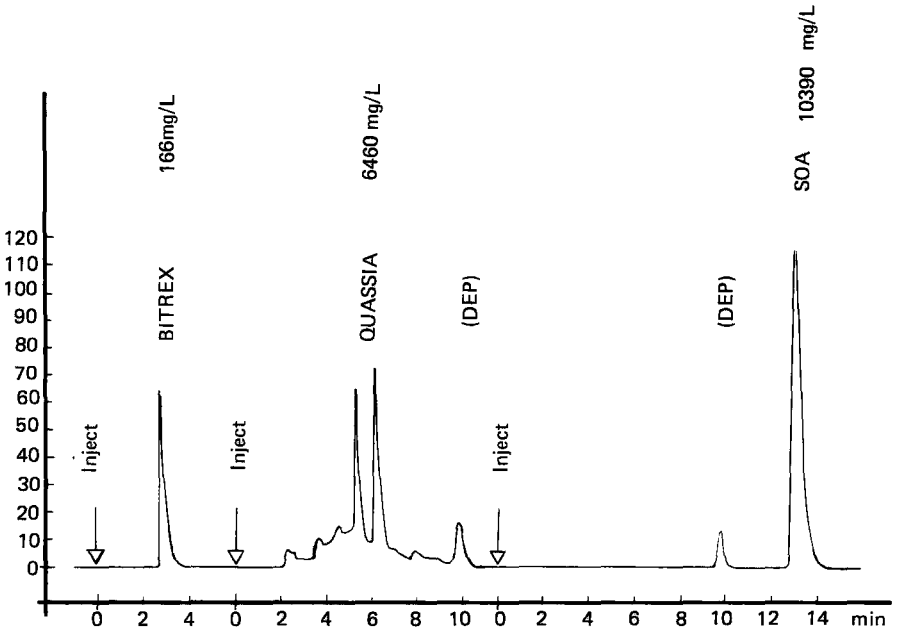

FIGURE 1

Chromatograms of Bitrex, Quassia and Sucrose Octaacetate, 10 µL, 30 % Acetonitrile in 0.01 M NaCl, detector range 0.4

the base line determined as connection between the two adjacent valleys. The heights obtained in runs at Ranges other than 0.4 were recalculated to standard 0.4 range.

Response factors were calculated by linear regression "through origin" (4,5,6) according to the equation:

$$\text{Concentration (mg/L)} = B \times \text{height (mm)}$$

The estimate of the standard error (S) is given as

$$S = \left(\Sigma \ (\delta y)^2 \ / \ (n-1)\right)^{\frac{1}{2}} \ \text{(mg/L)}$$

The response factors are summarized in Table 3.

TABLE 3

Response Factors for Converting Peak Heights (mm) to Concentrations (mg/L)

Compound (Concentration)	30% Acetonitrile/water			30% Acetonitrile/0.01 M NaCl		
	Factor B	Std. error S	Correl. coeff	Factor B	Std. error S	Correl. coeff
Bitrex (40 - 840 mg/L)	0.98	19	0.9991	1.60	5	0.9999
Quassia, at 5.3 m	85	66	0.989	98	18	0.999
at 6.1 m (300-6500 mg/L)	80	38	0.996	80	17	0.999
SOA (2000-20400 mg/L)	90	279	0.997	89	303	0.999

Relative standard deviation of these factors (B) is in the range of 2 - 10%. Corresponding factors for diethyl phthalate and for Camphor are 1.1 and 28 , resp., as determined from single concentration runs.

The range of relative standard error, expressed as the percentage of the standard error from the concentration limits used, is 0.6 to 48% for Bitrex, 0.3 to 22% for Quassia and 3 to 15% for Sucrose Octaacetate, the higher values corresponding to the lower ends of the concentrations.

Sample Preparation:

A/ By evaporation - dissolution.

Suitable for samples containing one or more of the bitter principles only, such as formulas 1-B. (Table 1). The sample as received (10.0 mL) was pipetted into a round glass dish (80 x 45)

and evaporated to dryness from the top of a boiling water bath (about 20 min); the residue was transferred quantitatively into a 3.5 mL vial using 1-2 mL of acetone (SOA or Bitrex suspected) or methanol (Quassia suspected) and the solvent evaporated in a stream of nitrogen with slight heating (up to 40°); the residue was dissolved in 1000 µL of mobile phase (30% or more Acetonitrile in water), 10 µL of the solution injected, and the peaks identified by retention times. The concentrations of the compounds found in the sample were calculated using peak heights and the factors established in calibration.

The concentration in the original sample is taken as one tenth of the concentration so determined.

B/ By evaporation - extraction:

Suitable for samples containing diethyl phthalate, camphor and possibly other contaminants, such as in formulas 1-F and 1-S. (Table 1). The sample was evaporated and the residue transferred to a 7 mL vial as under (A) above; the residue was dissolved by shaking thoroughly for 60 seconds with 1000 µL of 30% Acetonitrile in water mobile phase and 5.0 mL hexanes (UV grade), left stand to separate, and a portion of the bottom layer used for injection into LC. Concentrations of detected bitter compounds were calculated as under (A).

While camphor was virtually removed from the mixture in this procedure, a strong signal corresponding to diethyl phthalate was still present in the chromatogram; this signal is well resolved from, and does not interfere with, the signals of compounds of interest.

C/ Direct injection:

Suitable for clean samples without contaminants; alternative to procedure (A). 20 µL sample as received (only filtered, if necessary) was injected and run at Range 0.1 A. The peak heights were measured and standardised and concentration calculated using

response Factors. The concentration so determined is the concentration in the sample. When procedure (C) was used with samples containing large amounts of phthalates or similar contaminatns, the huge peak of the phthalate (9 - 12 min R.T.), and the minor components usually contained in the phthalates used, tended to interfere with some of the peaks to be measured and made the peak identification difficult; further, the contaminants tended to stay partly behind in the LC system and cluttered the subsequent runs; extensive cleaning of the column and injector was usually required in such a case.

Estimate of the Distribution Coefficient of Diethyl Phthalate.

A known amount of diethyl phthalate was shaken with 1000 µL mobile phase and 5.0 mL hexanes, the mixture left standing to separate and the bottom layer analysed by LC for diethyl phthalate as described above. The results are summarized in Table 4.

As shown, a single extraction of 1000 µL solution of the evaporation residue in mobile phase of not more than 30% acetonitrile with 5 ml hexane removes the phthalate sufficiently for subsequent LC analysis of the watery phase.

Test Runs.

Several samples were prepared by weighing to simulate working solutions obtained by procedure A and precedure B. Samples of denatured spirits were also tested following procedure C. The results are summarized in Table 5.

RESULTS AND DISCUSSION

When inorganic salts are used as constituents in the mobile phase, as suggested in the literature (1,2) a lengthy system

TABLE 4

Distribution Coefficients for Diethyl Phthalate between Mobile Phase and Hexane, at 21° C

Mobile phase: (% acetonitrile)	60	50	25
Distribution coefficient, K	0.75	1.9	19

cleaning is required daily after each series of runs. To save time, most of the experiments were run without the addition of salts; no apparent deterioration of peak shapes or significant changes in retention times were observed when compared to results of experiments using sodium chloride.

The ratio 60/40 for the acetonitrile/water system provided very good separation of the three bitter substances from one another. Sharp, strong singletts were obtained for Bitrex and SOA and a multiplet for Quassia powder as corresponds to the complex nature of the latter. The total analysis time of less than 4 minutes was very appealing also. (Table 2.) However, very little or no separation was observed for SOA and diethyl phthalate, which precludes the use of this system even if most of the phthalate is removed in advance by extraction. Gradual increase of the proportion of water in the mobile system shifted the retention time of SOA sufficiently away from the signal of the phthalate, until a satisfactory separation was achieved at a ratio of 30/70, with the analysis time being still in the acceptable range of below 15 min. At the same time, the resolution between all the other compounds, including camphor and, possibly, ethanol, has improved also. The response (Peak height corresponding to unit concentration) that necessarily decreases with longer retention times due to peak broadening, remained sufficiently high for the ten-fold concentrations, and,

TABLE 5

Chromatography of Test Samples

Sample #	Composition mg/L	Procedure	30% Acetonitrile/water		30% Acetonitrile/1 M NaCl	
			Found, mg/L	% of expected	Found, mg/L	% of expected
a) Compounds dissolved in mobile phase and analysed						
1	SOA: 328 DEP: 56	A	324 ---	99 --		
2	BIT: 8.3 SOA: 520	A			7.5, 7.7 506, 525	90,93 98,101
b) Compounds dissolved in mobile phase, extracted with hexane and analysed						
3	BIT: 8.3 QUAS: 129 DEP: 22400	B			9.3, 10.2 108, 104	112,123 84,81
4	SOA: 208 DEP: 22400	B	122	59	116, 116	56,56
5	BIT: 16.6 DEP: 11200	B			16.5, 19.7	99,119
c) Compounds dissolved in ethyl alcohol (95%)						
6	QUAS: 142.5	A C	148 140	104 99	150, 150	105,105
7	BIT: 7.1	C	6.8	95	5.0, 5.4	70,76

Abbreviations in the table:
BIT Bitrex
SOA Sucrose Octaacetate
QUAS Quassia powder
DEP Diethyl Phthalate

as was shown later, remained sufficient even for concentrations of the bitter compounds in samples as received (Regulation formulas), when the sensitivity setting was properly adjusted.

Addition of salt (NaCl) to the mobile phase (0.01 M) seemed to improve the separation of Bitrex from the front peak, but this advantage proved to be temporary. Repeating the injection of an appropriate mixture after about ten injections of other compounds gave a chromatogram with the first two signals strongly merged, similar to signals obtained with the mobile phase without salt. The resolution power of the column was restored after thorough washing (30 min) with water and Acetonitrile (10 min), but again only for a short time. At the same time the slight increase in retention time of Bitrex brought the peak in close proximity to the ethanol signal, so that this system could not be used for Bitrex determination in alcohols as received, if such an approach were chosen. The salt did not have any significant effect on the position, strength or resolution of signals of all the other compounds investigated. The more practical salt-free phase of 30 % acetonitrile in water is therefore recommended for routine use. When equivocal results regarding Bitrex concentration are obtained, the 0.1 M NaCl containing mixture could be used, but only after thorough conditioning of the chromtographic system.

The linearity of the responses in the ranges defined by the concentration limits of the calibration solutions calculated by linear regression and expressed as correlation coefficients, is satisfactory: 0.999 for Bitrex, 0.998 for Quassia (at 6.1 min) and 0.997 for SOA. While Bitrex and SOA give a single peak under the conditions described and there is no ambiguity as to choosing the analytical reference, Quassia powder invariably gives a multiplet of at least six discernible peaks, two of which are prominent and almost of equal intensity (5.3 and 6.1 min); either one of these two can be used for height (and concentration) measurement, when the proper corresponding factor is used.

Several samples of Quassia powder of very different provenance were analysed using the described procedure; there was no apparent variation between the chromatograms representing the different samples.

There was an opportunity to analyse samples containing SOA (Formulas 1 B type c, and 1 F, type b) that were kept in the laboratory in a closed bottle for 4 - 5 years. Invariably, the content of SOA in these solutions decreased below 50 % of the declared concentration-some had virtually a zero content -, with the concommitant emergence of new, not identified peaks, none of them interfering with the recognised signals described in Table 2. They are most likely due to solvolysis products of SOA, such as glucose tetraacetate, fructose tetraacetate, lower acetates of one of the sugars (sucrose, glucose, fructose) and possibly sucrose itself (ret. time for sucrose was found to coincide with the "front peak", i.e. 1.75 min). While this finding is not detrimental to the procedure, it raises doubts about the long term efficacy of Sucrose Octaacetate as a denaturant.

For reasons of chromatographic practicality - such as working at intermediate sensitivity with resonable baseline stability and signal strengh - it was considered advisable to increase the concentration of the sample about ten times as compared to samples as received; this would at the same time allow for removal of the bulk of ethanol, whose signal just might interfere with the closely neighbouring peaks of Bitrex on one side and Quassia on the other. Such a preconcentration is easily achieved by evaporating exactly 10 mL of sample and dissolving the residue in exactly 1 mL of a non-interfering solvent, such as the mobile phase itself, as described in the Experimental.

Clear solutions were obtained. The multiple transfer of the material, however, was reflected in incomplete recovery.

In view of the concentration ratios of diethyl phthalate to bitter substances in the appropriate formulas (Table 1) it was

deemed advisable - and was born out in experiments - to remove the bulk of the phthalate, and camphor prior to LC analysis, in spite of the good separation of the compounds in question. To assess the suitability of likely solvent systems for the extraction procedure, distribution coefficients were determined by analysing the water layers for residues of the phthalate. The coefficients were found to increase dramatically when water content in the system mobile phase/hexane was increased.

The concentration of SOA is decreased in this process to about 60 % of the original, Quassia to about 80 %, whereas Bitrex concentrations seem not to be affected.

The preconcentration procedure discussed above lends itself directly to the extraction (see Experimental).

The results obtained by chromatography of the concentrates and correspondingly prepared standard mixtures, suggested that the signals are sufficiently strong to be detected in samples as received, if the injection volume is doubled and the sensitivity increased about four times. Experiments have borne this out, at least for simple formulas such as the 1 B types. The lack of sample pretreatment, however, leads to appearance of huge "ghost" peaks in several chromatograms after injections of more complex samples, such as formulas 1 F, samples of questionable purity and older samples. For these reasons, and also for the possibilty of losing the Bitrex signal in the closely following strong ethanol peak, this alternative procedure cannot be recommended for routine use; it might prove useful for quick and simple checks on occasion, particularly for Quassia or SOA analyses in the absence of large amounts of contaminants.

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HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ALPHA-DIFLUOROMETHYLORNITHINE IN RODENT FEED

Florence J. Reavis and John J. Contario
MERRELL DOW RESEARCH INSTITUTE
Cincinnati, Ohio 45215

ABSTRACT

A high performance liquid chromatographic method has been developed for the assay of alpha-difluoromethylornithine, an inhibitor of cell growth, in rodent feed at levels from 0.5 to 50 mg/ml. The drug is leached from the feed with water, and a portion of the aqueous solution is passed through a cation exchange resin to remove some feed components which are simultaneously leached. The drug is removed from the resin column and derivatized with 1-fluoro-2,4-dinitrobenzene to enhance its ultraviolet absorptivity. The derivative is isolated from the reaction mixture and chromatographed on a 25-cm LiChrosorb RP-18 column equipped with a guard column, using a mobile phase of pH 4.8 acetate buffer:tetrahydrofuran (80:20) and photometric detection at 360 nm. The method is also applicable for the assay of feed preparations of other amino acids or similar non-UV absorbing materials which react with 1-fluro-2,4-dinitrobenzene to give dinitrophenyl derivatives.

INTRODUCTION

Alpha-difluoromethylornithine (I) is an enzyme activated irreversible inhibitor of ornithine decarboxylase, the first enzyme in the polyamine biosynthetic pathway.(1) Inhibition of this enzyme by (I) has demonstrated significant antiproliferative effects in vitro and in vivo against a number of tumors and

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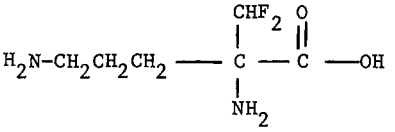
parasitic protozoa. (1,2) The compound is currently undergoing clinical studies in the United States and in Europe.



(I)

The purpose of this work was to provide an analytical procedure to insure adequacy of mixing and stability with time of the drug in feed as required for the toxicological testing. Although an amino acid analytical system had been developed for (I) (3), it was necessary to devise a new analytical procedure for this assay since neither an amino acid analyzer nor a fluorometric detector were available in this laboratory.

Reversed phase liquid chromatography has been used successfully in the past, in this laboratory and by others (4-9), to assay for drugs in feed, and was considered the method of choice for this assay. The following difficulties were encountered in the development of a liquid chromatographic method. Alpha-difluoromethylornithine has the characteristically low UV absorbance of aliphatic amino acids which initially necessitated the detection of the drug at 210 nm. However, at low levels of drug in feed, a large sample was required in order to obtain a measurable amount of drug. Feeds such as Purina Rodent Chow

contain analogous amino acids such as lysine, arginine, etc., at concentrations of over ten times that of the drug in low dosage samples. As a result of this, large feed samples produced high background and interferences from co-leached feed components. These artifacts created difficulties in the ultimate quantitation of the drug despite an additional clean-up procedure.

In order to make UV detection easier, pre-column derivatization with a highly UV absorbant chromophore was explored. Since the detectability of the drug would be increased, a smaller feed sample could be used and lower background absorbance from co-leached feed components would be obtained as a result of detection at a higher wavelength. The reaction of 1-fluoro-2,4-dinitrobenzene with amino acids is known to result in a highly absorbant dinitrophenyl derivative. (10-12) The formation of this derivative of (I) was chosen because the reaction is a fast and simple one, requiring only the mixing of the amino acid and reagent in basic solution to form the derivative, and because it allowed the use of the ubiquitous ultraviolet detector. The reaction of (I) with 1-fluoro-2,4-dinitrobenzene was found to result in the formation of a dinitrophenyl derivative which exhibited the characteristic UV spectrum of dinitrophenyl amino acids, displaying a strong absorption band at 360 nm and a weaker band at 260 nm. A sample preparation procedure was developed employing the dinitrophenyl derivative which would allow the accurate measurement of low levels of (I) in feed using UV detection at 360 nm.

MATERIALS

Chemicals

Glacial acetic acid, hydrochloric acid, methanol, sodium acetate, and ethyl ether, all reagent grade, were purchased from J. T. Baker, Inc. (Phillipsburg, N.J.). Ammonium hydroxide and sodium bicarbonate, both reagent grade, were purchased from Matheson, Coleman and Bell (Norwood, OH.). Absolute ethanol, USP grade was purchased from Aaper Alcohol and Chemical Co. (Louisville, KY.). Tetrahydrofuran, HPLC grade, was purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, MI.). The 1-fluro-2,4-dinitrobenzene, 98%, was purchased from Aldrich Chemical Co. (Milwaukee, WI.). The cation exchange resin was analytical grade, AG 50-W-X2, 100-200 mesh, hydrogen form, purchased from Bio-Rad Laboratories (Richmond, CA.). The feed used was a constant-nutrient rodent feed formulation, Certified Rodent Chow #5002, from Ralston Purina Co. (St. Louis, MO.).

Apparatus

The high performance liquid chromatographic system consisted of an Altex, model 110 pump, Altex Scientific, Inc. (Berkeley, CA.), a Vari-Chrom variable wavelength detector, Varian Associates, Inc. (Palo Alto, CA.), operated at 360 nm, a Rheodyne Model 70-10 injector, Rheodyne, Inc. (Berkeley, CA.), and a recorder, model 252A, from Linear Instruments Corp. (Irvine, CA.).

METHODS

Chromatographic Conditions

The chromatography was carried out at ambient temperature on a reversed phase C_{18} column, Lichrosorb RP-18, 25 cm x 4.6 mm (ID), E. Merck (Darmstadt, Germany). The analytical column was attached to a guard column containing C_{18} packing, Bondapak C_{18}/Corasil, 37-50 microns, Waters Associates, Inc., (Milford, MA). The mobile phase consisted of pH 4.8 acetate buffer: tetrahydrofuran (80:20). The flow rate was 2 ml per minute.

Preparation of the Ion-Exchange Column

The cation exchange column was prepared to contain an appropriate volume of resin in the ammonium form corresponding to various levels of drug in feed as listed in Table I. A small pledget of glass wool was inserted in the column. The appropriate volume of resin (hydrogen form) was added as a slurry in water, allowed to settle, then capped with another pledget of glass wool. The resin was treated with 3M methanolic ammonia until the column effluent was basic using Hydrion pH paper, then washed with water until the effluent was neutral.

Preparation of the Sample Solution

A 2-g sample of feed was used for samples having a concentration of 0.5 mg of drug per g of feed. A 1-g sample of feed was used for samples having a concentration greater than 0.5 mg of drug per g of feed. The accurately weighed feed sample was

TABLE 1

Variation of Ion-Exchange Resin Volume and Aliquot Volume With Sample Drug Concentrations

Alpha-difluoro-methylornithine (mg per g)	Resin bed volume (ml)	Aliquot of extract transferred to resin (ml)	Dilution factor (F)
0.5	4	50	0.040
1.0	4	50	0.040
2.0	4	50	0.080
2.5	4	40	0.100
4.0	2	25	0.160
5.0	2	20	0.200
10.0	2	10	0.400
20.0	2	5	0.800
25.0	2	4	1.00
40.0	2	3	1.33
50.0	2	2	2.00

leached with 250.0 ml of water. The large particulate matter was allowed to settle and a portion of the remaining mixture was centrifuged. An aliquot of the supernatant solution containing 0.4 mg of the drug was transferred to the ion-exchange resin column. Table 1 gives examples of aliquots used for various levels of drug in feed. The sample was drained into the resin bed and the column was washed with 10 ml of water, discarding the eluate. The drug was eluted from the column with 25 ml of 3M methanolic ammonia and the eluate evaporated to dryness. The residue was dissolved in 1 ml of 2% sodium bicarbonate and mixed with 2 ml of 1-fluoro-2,4-dinitrobenzene reagent, prepared by mixing 0.25 ml of 1-fluoro-2,4-dinitrobenzene with 10 ml of absolute ethanol. The reaction mixture was protected from light and shaken for at least 30 minutes.

The mixture was treated with 2 ml of water and extracted twice with 5 ml of ether, discarding the extracts. The aqueous phase was acidified with 6M hydrochloric acid and extracted twice with 2 ml of ether, discarding the extracts. The remaining aqueous solution was quantitatively transferred to a 10-ml volumetric flask, and diluted to volume with mobile phase.

Preparation of the Standard Solution

Approximately 25 mg of a standard sample was accurately weighed into a 25-ml volumetric flask. The sample was dissolved in, and diluted to volume with 2% sodium bicarbonate solution. A 1.0-ml aliquot of this solution was reacted with 2 ml of 1-fluoro-2,4-dinitrobenzene in the dark, with shaking for at least 30 minutes. The reaction mixture was extracted as described under "Preparation of the Sample Solution". The remaining aqueous solution was quantitatively transferred to a 25-ml volumetric flask, and diluted to volume with mobile phase. This solution was used for comparison with samples containing more than 1 mg of drug per g of feed. For samples containing 1 mg of drug per g of feed or less, a 5.0 ml aliquot of the solution was diluted to 10 ml before use.

Chromatographic Procedure

A stable baseline was established with the chromatograph operating under the conditions described. The detector was operated at 0.05 AUFS for samples containing 1 mg of drug per g of feed or less, and at 0.1 AUFS for samples containing more than 1

mg of drug per g of feed. A 20-µl aliquot of the standard solution was injected and its chromatogram recorded. Similarly, a 20-µl aliquot of the sample solution was injected and its chromatogram recorded. Figure 1 illustrates the chromatograms obtained when this method was used to assay a sample prepared to contain 4 mg of drug per g of feed.

Calculations

For each injection, the area of the peak for the dinitrophenyl derivative of (I) was determined, and the assay was calculated as follows:

$$\frac{Au}{As} \times \frac{Ws}{Wu} \times F = \text{mg of (I) per g of feed}$$

where: Au = area of the dinitrophenyl derivative of (I) in the sample chromatogram.

As = area of the dinitrophenyl derivative of (I) in the standard chromatogram.

Ws = weight of the standard in mg.

Wu = weight of the sample in g.

F = dilution factor (obtained from Table 1).

RESULTS AND DISCUSSION

The major difficulty encountered in the method development was the low UV absorbance of (I). Detection had to be accomplished at 210 nm, which monitored the drug via its end absorption. Because a great number of compounds exhibit some

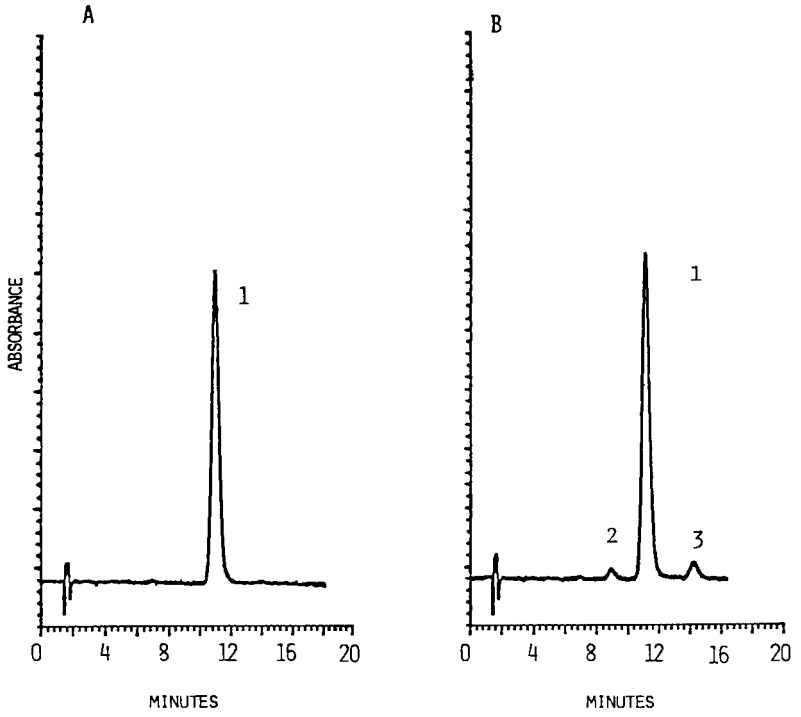


FIGURE 1 - A - Typical chromatogram of the dinitrophenyl derivative of alpha-difluoromethylornithine from a standard solution. B - Typical chromatogram of the dinitrophenyl derivative of alpha-difluoromethyl-ornithine from a feed extract. Key: (1) dinitrophenyl derivative of alpha-difluoromethyl-ornithine leached from feed; (2) dinitrophenyl derivative of arginine leached from feed; (3) dinitrophenyl derivative of unknown leached from feed.

absorbance at 210 nm, the background absorbance from other co-leached feed components increased as the size of the feed sample increased, making quantitation impossible for low levels of drug in feed.

A clean-up procedure was developed in which an ion-exchange resin was used to separate other feed components from (I). As

indicated in Table I, the resin bed volumes were increased when the concentration of drug in feed was low enough to require the treatment of more than 25-ml of feed extract. This was necessary to avoid loss of (I) from an overloaded resin column. Although this procedure improved the quality of the chromatograms, the background absorbance remained high especially at low levels of drug in feed.

In order to overcome this obstacle, a sample preparation procedure was developed which would allow the accurate measurement of the derivatized drug in feed at a detection wavelength of 360 nm. By preparing the dinitrophenyl derivative of (I) leached from feed, a small amount of this normally low UV absorber could be easily detected. In addition to allowing the use of a smaller sample size, less background was obtained as a result of detection at 360 nm and the detector response was increased 140 times when compared to that which was previously obtained for underivatized (I) at 210 nm. The feed itself contains several other amino acids which could also react with the derivatizing reagent. Because of their structural composition, the derivatives of most of these are soluble in ether after acidification of the aqueous reaction mixture and are therefore eliminated with the ether extraction. Those which may remain in the aqueous phase are arginine, lysine, histidine, and threonine. Arginine has been identified as one of these amino acids which is co-leached from the feed, derivatized, and may subsequently appear in the sample chromatogram at a retention time of about 8 minutes. Another peak in the sample chromatogram at about 14.5 minutes is unidentified, however it has

been shown not to be any of the other amino acids listed as present in the feed.

The structure proof of the derivative was accomplished through elemental analysis and proton magnetic resonance spectroscopy. The elemental analysis was in agreement with the formula for a mono-derivative. The spectroscopy confirmed this, and indicated that the dinitrophenyl chromophore was attached to the terminal amino group. The lack of derivatization at the alpha amino group, under the conditions of the reaction, may be due to steric hindrance.

The optimum reaction conditions were determined by varying the reaction time and temperature. A reaction time of thirty minutes at room temperature was found to be optimum, although reaction times up through 2 hours gave similar results. At times of 5 hours and longer, the derivative level began to drop off.

The linearity of detector response was determined for peak areas *vs* column loading from 0.2 to 1.0 μg. The detector response was found to be linear over the range. The 20-μl injection used in the method contains 0.8 μg or 0.4 μg of the dinitrophenyl derivative depending on the level of drug in feed of the sample being assayed.

To determine the accuracy and precision of the method, five synthetic samples were prepared at the 0.5, 5, and 50 mg/g levels by adding known amounts of drug to blank feed samples and assaying by the method. Table 2 lists the recoveries obtained from synthetic samples. As can be noted from the table, recovery

TABLE 2

Recoveries of Alpha-difluoromethylornithine from Synthetic Samples at Three Levels (% Recovery)

0.5 mg/g	5 mg/g	50 mg/g
93.5	95.9	100.0
93.8	93.5	98.4
97.5	94.4	96.1
90.9	96.7	98.3
85.6	98.0	96.2
x = 92.1%	x = 95.7%	x = 97.8%
s = ± 4.3%	s = ± 1.7%	s = ± 1.6%

decreases as the amount of drug in the sample decreases. This phenomenon is not observed when blank feed is leached, and the resulting solution spiked with drug. The decrease in recovery is believed to be caused by some type of irreversible binding of a small amount of (I) to the feed matrix itself.

CONCLUSIONS

A pre-column derivatizaion procedure has been developed for the precise assay of (I), a low UV absorbing aliphatic amino acid. By reacting with 1-fluoro-2,4-dinitrobenzene, a highly UV absorbing dinitrophenyl chromophore was attached, allowing detection and accurate measurement of low levels of the drug in feed. The reaction is simple and fast, and can easily be adapted to the assay of other non-UV absorbing compounds which may be formulated in feed at low levels.

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DERIVATIZATION, SEPARATION AND DIRECT QUANTIFICATION OF MONOHYDROXY-EICOSATETRAENOIC ACIDS USING REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Paul K. Williamson and Robert B. Zurier
Rheumatology Section, Department of Medicine
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania 19104

ABSTRACT

A new method for the separation and quantification of monohydroxy-eicosatetraenoic acids has been developed. The 5-, 12-, and 15-hydroxylated acids were esterified with p-(9-anthroyloxy) phenacyl bromide and subjected to reversed-phase high-performance liquid chromatography. 12-HETE and 15-HETE were readily separated. 5-HETE eluted in 2 peaks, the second co-eluting with 15-HETE. Sensitivity of the assay was 2.81 nM for both the 12- and 15-hydroxylated acids, and 6.25 nM for the 5-hydroxylated acid. This technique lends itself to analysis of biological samples.

INTRODUCTION

Monohydroxy-eicosatetraenoic acids are naturally occurring lipoxygenase-derived products of arachidonic acid. Those with hydroxyl groups at the 5, 12 and 15 carbon positions (5-HETE, 12-HETE and 15-HETE) appear to be individually important in a variety of biological processes, including platelet aggregation and inflammation. Therefore their separation and quantification is important to an understanding of a variety of biological responses.

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Bioassay of these compounds would be tedious and lack specificity. Because these compounds absorb ultraviolet (UV) light of wavelength 234 nm, they may be separated by high performance liquid chromatography (HPLC) and directly quantified by UV absorption. However, maximum sensitivity with such methods is approximately 16 nM (1,2). HPLC has been used with radioimmunoassay (RIA) to combine the best method of separation with the most sensitive means of detection (3-5). Problems with this approach include a lack of antibodies to a variety of HETEs, and a lack of specificity of existing antibodies. In addition, the technique is laborious in that RIA must be performed on multiple eluents from each chromatographic run.

The present work describes a simple and rapid HPLC method for separation and quantification of 5-, 12- and 15-HETE with a sensitivity of 2.8 nM for 12- and 15-HETE and 6.2 nM for 5-HETE.

MATERIALS AND METHODS

Chemicals

5-, 12- and 15-HETE were purchased in crystalline form from Seragen, Inc., Boston Massachusetts. p-(9-anthroyloxy) phenacyl bromide (panacyl bromide, PAB) was a gift from Dr. Walter Morozowich, The Upjohn Company, Kalamazoo, Michigan. Water, acetonitrile, ethanol, acetic acid, triethylamine (TEA) and tetrahydrofuran (THF) were all HPLC-grade (Fisher Scientific, King of Prussia, PA).

Instrumentation

HPLC (Waters Associates, Milford, MA) was performed with the following components: two Model 6000-A Solvent Delivery Systems, a Model 660 Solvent Programmer, a U-6K Injector, a Z Module Radial

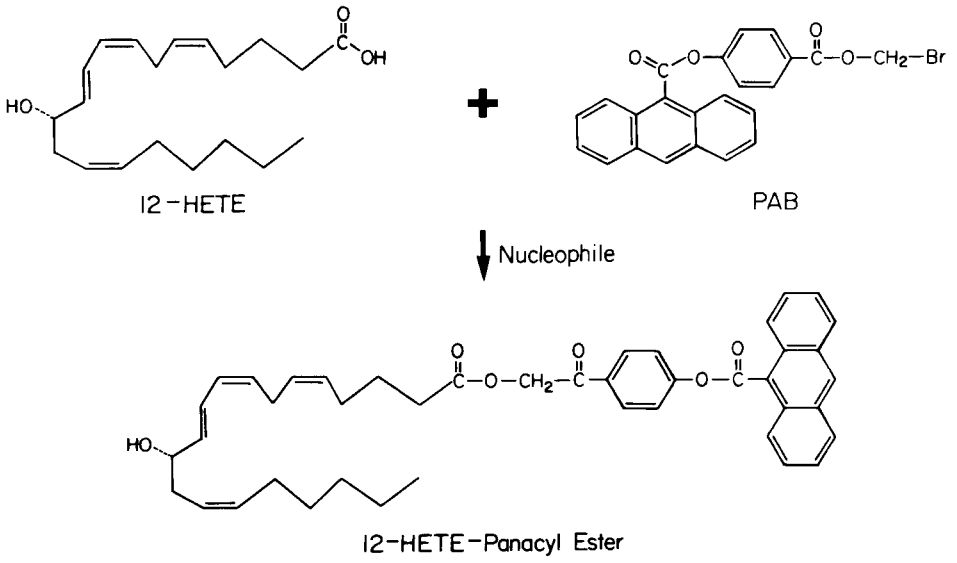

Figure 1.

Esterification reaction of 12-HETE with PAB.

Compression Separation System, a Model 441 Fixed wavelength Ultraviolet Absorbance Detector equipped with a mercury lamp and a 254 nm filter, and a Model 7000 Data Module. Separations were performed on a 5 μ particle-diameter octadecylsilyl (ODS) column (Waters Associates, Milford, MA).

Derivatization

Crystalline HETEs were dissolved in absolute ethanol and aliquots were dried under a stream of N_2. PAB was dissolved in a solution of acetonitrile:THF (4:1, v:v). PAB solution (1 ml) and 3 μl of TEA were added to each HETE aliquot. The stoichiometry was adjusted such that the molar ratio of PAB to HETE was at least

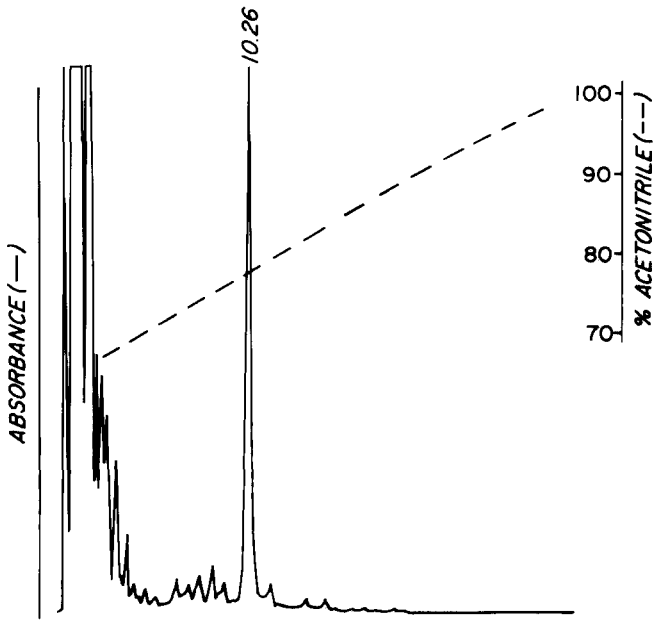

Figure 2.

20 µl of PAB solution injected after incubation at 37°C for 24 hours. Mobile phase is acetonitrile:0.1% acetic acid along a linear gradient from 68% to 98% acetonitrile. Column is a 5µ particle-diameter 5 mm internal diameter ODS flow rate 4.0 ml/min, pressure 2100 psi, 0.050 AUFS.

4:1. Standards were placed in teflon-capped vials and incubated at 37°C for 24 hours, then placed on ice.

Chromatography

Sample volumes were injected into the chromatograph and eluted with a mobile phase of acetonitrile and 0.1% acetic acid employing a variety of gradients. Absorbance as a function of time was recorded and area under peaks were quantified by electronic integration.

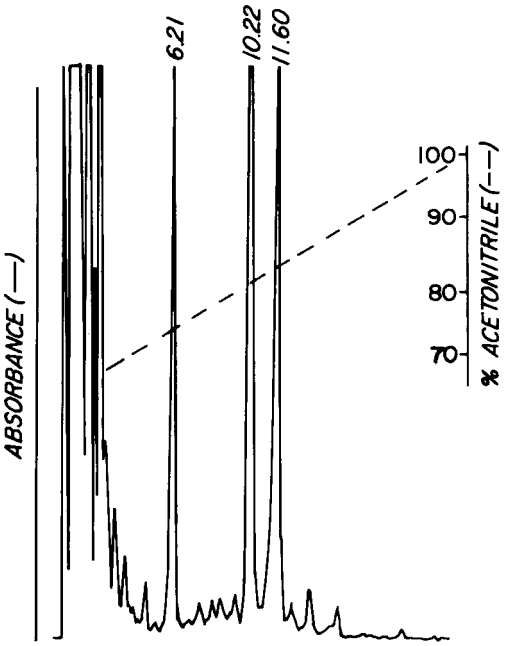

Figure 3.

20 µl of 310 µM derivatized 5-HETE (6.21,11.60). Chromatographic parameters as in Figure 2.

RESULTS AND DISCUSSION

The reaction sequence is depicted in Figure 1. Care should be taken to keep the reaction free of water to prevent hydrolysis back to the acids.

Figure 2 illustrates the chromatogram of the reaction blank (panacyl bromide solution incubated at 37°C overnight). Early peaks and that at 10.26 minutes are due to impurities and breakdown products of panacyl bromide.

Figure 3 shows the chromatogram of the panacyl ester of 5-HETE. Note that this compound elutes in peaks 6.21 and 11.60.

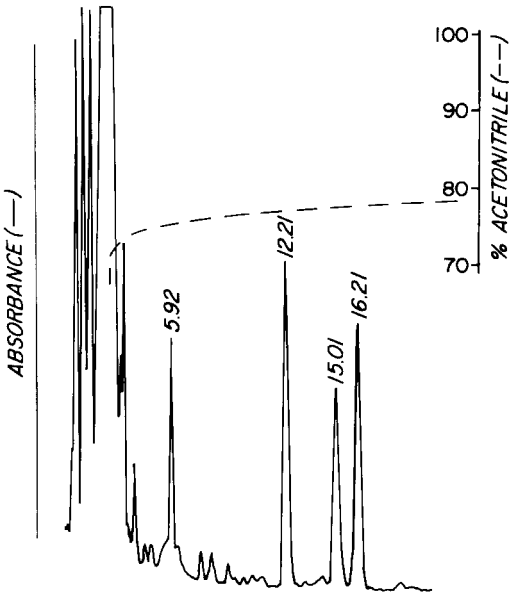

Figure 4.

3.3 µl of 310 µM derivatized 5-, 12-, and 15-HETE. 5.92:5-HETE; 15.01:12-HETE; 16.21:15-HETE. Mobile phase is acetonitrile and 0.1% acetic acid, 68% to 98% of the former over Solvent Programmer gradient #2 (convex) extrapolated to 9 hours. Column is a 5µ particle-diameter 8 mm internal diameter ODS, flow rate 5.0 ml/min, pressure 2100 to 1600 psi, 0.050 AUFS.

This latter peak may represent a 1,5-lactone 5-HETE which is known to occur in acid environment (1).

Figure 4 reveals optimum separation of the panacyl esters (5.92:5-HETE; 15.01:12-HETE; 16.21:15-HETE and presumed 5-HETE lactone). Figure 5 illustrates the maximal sensitivity of 5-HETE (2.91) to be 6.2 nM and Figure 6 the maximal sensitivity of 12-HETE (4.62) and 15-HETE (4.82) to be 2.8 nM.

A plot of area under the peak as a function of concentration of 12-HETE is shown in Figure 7, indicating linearity from 900 pg/ml to at least 10 ng/ml.

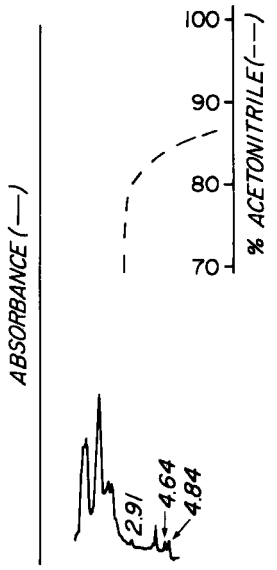

Figure 5.

200 µl of a 6.2 nM solution of 5- (2.91), 12- (4.64), and 15-HETE (4.84). Mobile phase is acetonitrile and 0.1% acetic acid, 68% to 98% of the former over Solvent Programmer curve #2 extrapolated over 20 min. Column as in Figure 2, flow rate 4.0 ml/min, pressure 2100 to 1600 psi, 0.005 AUFS.

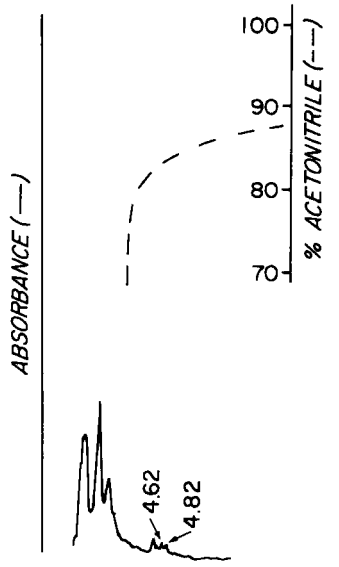

Figure 6.

200 µl of a 2.8 nM solution of 5-HETE, 12-HETE (4.62) and 15-HETE (4.82). Chromatographic conditions as in Figure 5.

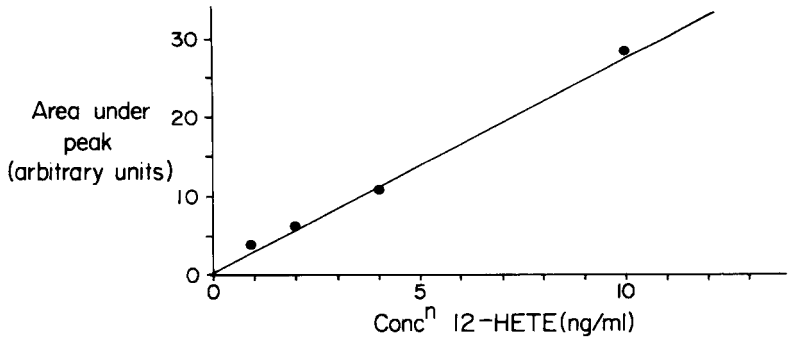

Figure 7.

Concentration curve for the panacyl ester of 12-HETE. Area counts are plotted as a function of concentration 12-HETE.

SUMMARY

A rapid and practical technique is presented for the derivatization of monohydroxy-eicosatetraenoic acids and their separation and quantification using HPLC and UV absorption. A sensitivity of 2.8 nM for 12- and 15-HETE, and 6.25 nM for 5-HETE should allow this technique to be applied to the analysis of biological specimens.

ACKNOWLEDGEMENTS

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HPLC SEPARATION OF TAUTOMERIC COMPOUNDS OF 4-AMINOISOXAZOLYL-1,2-NAPHTHOQUINONES

A.Fernández, M.M. de Bertorello
and M.R.Longhi
Departamento de Farmacia, Facultad de
Ciencias Químicas, U.N.C., Argentina.

ABSTRACT

A sensitive and efficient method for determining a mixture of 4-aminoisoxazolyl-1,2-naphthoquinones isomers was developed using high performance liquid chromatography. The assay is also stability indicating because precursors and isomerization products can be determined simultaneously.The results obtained are in agreement with those obtained by UV spectroscopy.

INTRODUCTION

Several methods have been described for the determination of tautomeric compounds. These include potentiometric[1], nonaqueous titrations and direct spectrometric measurement[2,3].

During the synthesis of new 4-aminoisoxazolyl-1,2-naphthoquinones[4], we verify the formation of a mixture of tautomeric compounds, which were separated by TLC and column chromatography.

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We have also developed a quantitative method for the analysis of tautomeric compounds by ^{1}H-RMN[5] in dimethyl sulfoxide. It was apparent that the values obtained by this method were considerably different from those obtained by column chromatography.

An analytical procedure was then required for their determination in samples arising from the reaction mixture and from the pure form. This procedure had to be accurate and precise, specific in the presence of isomerization products, rapid, simple and stability indicating.

This paper reports the development of a simple, rapid precise and specific HPLC procedure for the determination of N-(3,4-dimethyl-5-isoxazolyl)-1,2-naphthoquinone-4-amino (I); 2-hidroxi-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (II); N-(4-methyl-5-isoxazolyl)1,2-naphthoquinone-4-amino (III) and 2-hidroxi-N-(4-methyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (IV), which were previously synthetized[4] by reaction between the sodium salt of 1,2-naphthoquinone-4-sulfonic acid and the corresponding amino-methylisoxazole.

EXPERIMENTAL

Instrumentation

A constant volume liquid chromatograph from Beckman, consisting of a model 110 A Solvent delivery

TABLE 1

The Optimum Values of the HPLC Parameters

Volume inyected	25 ul
Column	Analytical prepacked 5 micron Silica Gel from Micromeritics
Mobile phase	Methanol-chloroform (90:10)
Flow rate	1,4 ml/min.
Detector	330 nm
Sensitivity	0,02 Amps.
Chart Speed	0,5 cm/min.

system and a Hitachi UV-Vis detector (interfaced with a Altex CR1 A Data System) set at 330 nm, was used.

The optimum values of the established HPLC parameters are shown in Table 1.

UV spectra were carried out in a Beckman DB-G spectrophotometer using 1 cm quarz cells.

Reagents and Materials

Benzene and chloroform were analytical grade and were distilled prior to use. Methanol was treated with 2,4-dinitrophenylhydrazine according to ref.[6].

The isoxazolyl-naphthoquinones were synthesized in these laboratories in the same way as their has been previously reported[4]. 2-Methoxy-N-(3,4-dimethyl-5-isoxazolyl)-1,4-naphthoquinone-4-imine (V) and 2-methoxy-N-(4-methyl-5-isoxazolyl)-1,4-naphthoquinone-

4-imine (VI), were used as internal standard, and they were prepared by reaction between II and IV with diazomethane[4].

Standard and Reference Solutions

The stock solutions of the purified compounds (I-IV) were prepared in distilled water or ethanol by dissolving 15 mg in 100 ml of solvent.

Reference standard solutions were also prepared in water or ethanol, by dissolving compounds V and VI to a concentration similar to that of the standard solutions.

Calibration Curve

A 0,150 mg/ml aqueous or ethanol solution of every compound was diluted with the appropiate solvent to obtain five standard solutions ranging from 2 to 14 ug/ml.

Triplicate 25 ul aliquots of working volume samples and working reference standard solutions were alternately injected into the liquid chromatograph.

Quantification of the samples was carried out by the Altex CR1-A Data System. Calculations were performed using the peak height calibration mode.

Figure 1 shows a standard curve which was obtained by plotting area count (%) vs concentration of II.

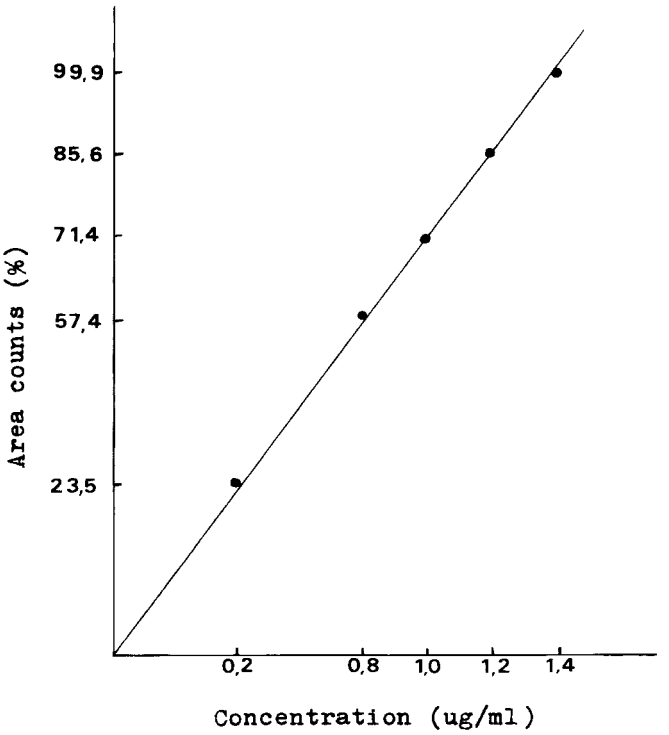


Figure 1: Compound II standard curve. Each point is the average of 3 determination.

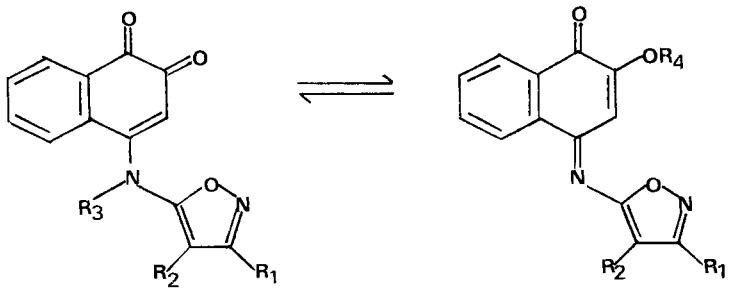
Standard and reference solutions were simultaneously determined by UV spectroscopy[5], using light of 330 nm as the analytical wavelength.

RESULTS AND DISCUSSION

The UV absorption spectra and the chromatographic properties of these drugs have proved to be useful for their separation by HPLC with ultraviolet detection.

TABLE 2

Structure Of 4-Aminoisoxazolyl-1,2-Naphthoquinones



Compound	Subst.R_1	Subst.R_2	Subst.R_3	Subst.R_4
I	CH_3	CH_3	H	---
II	CH_3	CH_3	---	H
III	H	CH_3	H	---
IV	H	CH_3	---	H
V	CH_3	CH_3	---	CH_3
VI	H	CH_3	---	CH_3

The compounds (Table 2), as single entities as well as in combination (Figure 2, 3) were analyzed succesfully using normal silicagel column HPLC.

Two different mobile phases were used: benzene-acetonitrile-chloroform (10:30:10) and chloroform-methanol (10-90). The best results were obtained with the second mobile phase.

Linearity of the method with respect to concentration of compounds I-IV was checked. Peak areas were used to construct calibration lines giving a correlation coefficient of 0,998.

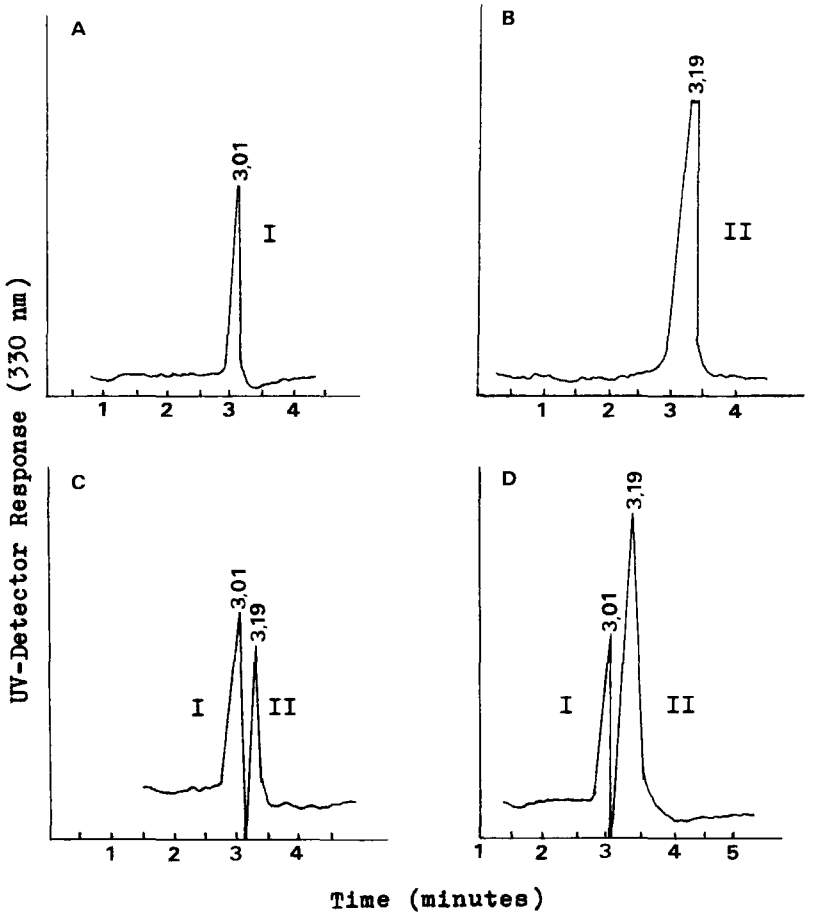


Figure 2: HPLC-UV Chromatograms of standard samples of isoxazolyl-naphthoquinones. A: compound I; B: compound II; C and D mixtrues of I + II, 50:50 and 25:75, respectively.

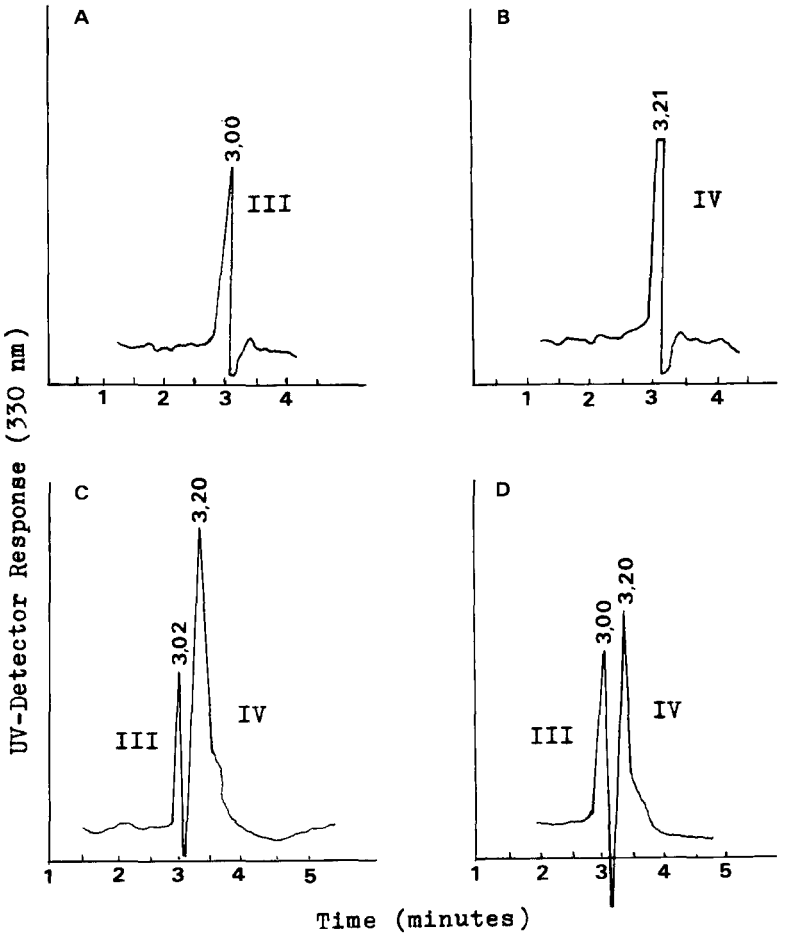


Figure 3: HPLC-UV chromatograms of standard samples of isoxazolyl-naphthoquinones. A: compound III; B: compound IV; C and D: mixtures of III + IV, 20:80; 40:60, respectively.

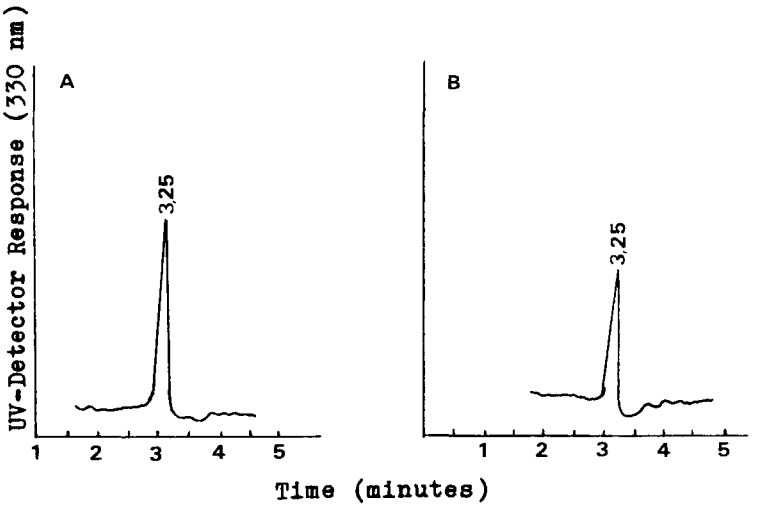


Figure 4: HPLC chromatograms of reference standard; A: compound V and B: compound VI.

Reproducibility of three 25 ul repeated injections of each solution for the compounds II, IV and V was established and the relative Standard deviations of the count/weight ratios were 0,2; 0,4 and 0,5 respectively.

The accuracy of the method was investigated for compounds II and IV using V and VI (Figure 4) as internal standards.

The ratio of peak areas was used to calculate a theoretical concentration. The results were compared with those of the UV assay and are shown in Table 3 and as it can be seen, most of them are in good agreement.

TABLE 3

Recovery Of Isoxazolyl-Naphthoquinones From Ethanolic Solutions By UV And HPLC

Sample	% Recovery		Retention time min.
	UV	HPLC	
I	97,0	100 ± 0,5	3 - 3,01
II	99,9	100 ± 0,2	3,19 - 3,21
I + II	50:50	56,55 ± 43,4	3 - 3,19
I + II	25:75	22 - 77	3,02 - 3,18
III	96	100 ± 0,5	3 - 3,02
IV	89	100 ± 0,4	3,21 - 3,19
III + IV	20:80	17 - 83	3,02 - 3,21
III + IV	40:60	40 - 61	3,02 - 3,21
V	99	100	3,25
VI	99	100	3,25

Stability Studies

The effect of storage on the stability of compounds I-IV in ethanolic solutions and in aqueous acidic and basic solutions was also analyzed.

The results of the isomerization of these compounds at room temperature as measured at 24 hs after their preparation are shown in Table 4.

The isomerization of II and IV in ethanol to give I and III respectively, was undetected over the 24 hs

TABLE 4

Medium Effect On Tautomeric Equilibria Of Isoxazolyl-Naphthoquinones

Sample	% Recovery UV	% Recovery HPLC	Retention Time min.	Figure
I[b]	75-25 K E	70-30	3,03-3,73	7
II[a]	70 K	85-12-2 K E	3,03-3,73-4,02	5
IV[a]	65 K	75-10-1 K E	3,02-3,80-4,02	5
I[c]	---	33-66 K E	3,03-3,19	6
III[c]	---	16-83 K E	2,96-3,19	6

a: aqueous acidic solution; b: Aqueous basic solution; c: ethanolic solution; K: ketoform; E: enolic form.

period. However, their solutions in aqueous acidic medium reveal, after 24 hs, their conversion to the keto form (Figure 5).

The behavior of I and III is different from that of II and IV. Both compounds in ethanolic slutions, after 24 hs, changed into their enolic form (Figure 6).

HPLC analysis of I in aqueous basic medium, indicated that the solution analyzed was a mixture of

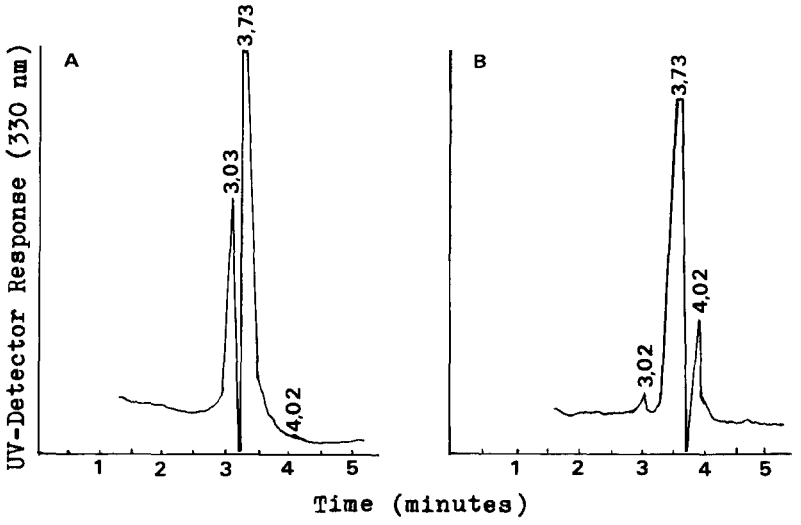


Figure 5: Chromatograms of an aqueous acidic solutions of isoxazolyl-naphthoquinones after 24 hs. A: compound II and B: compound IV.

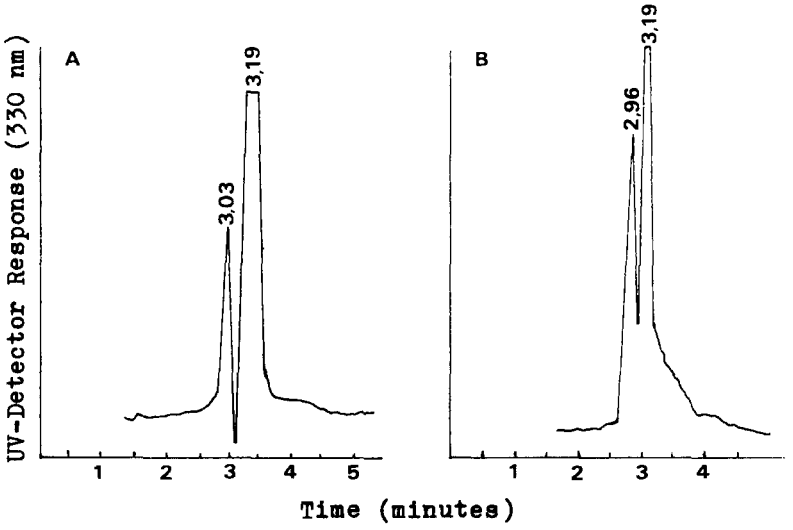


Figure 6: Chromatograms of an ethanolic solutions of isoxazolyl-naphthoquinones after 24 hs. A: compound I; B: compound III.

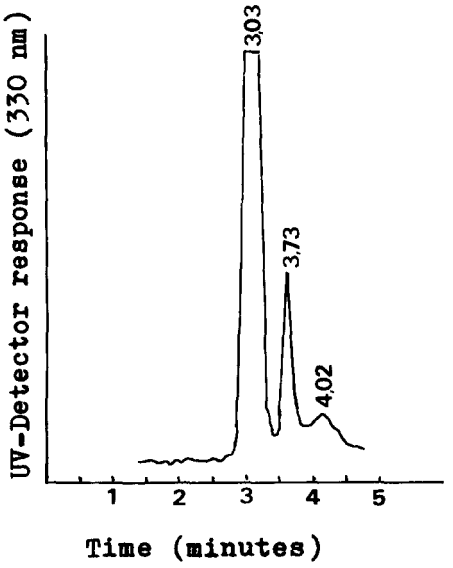


Figure 7: Chromatogram of an aqueous basic solution of I, after 24 hs.

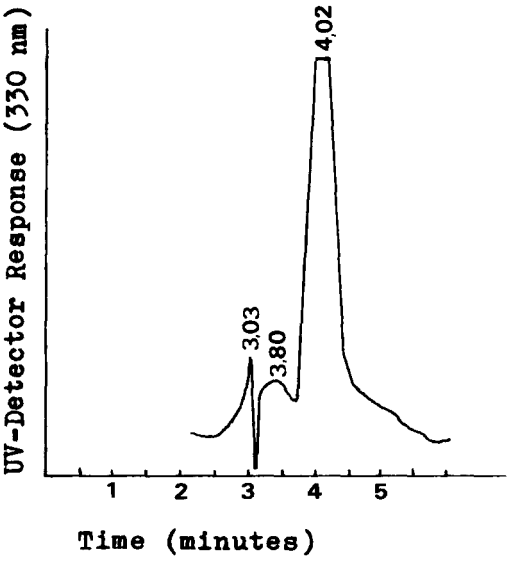


Figure 8: Chromatogram of an aqueous basic solution of III, after 24 hs.

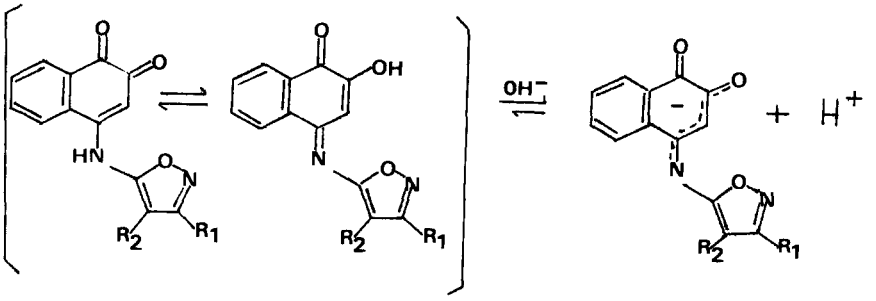
I and a second compound with a larger retention time than that of the enolic form (Figure 7).

Similar results were obtained when III was treated under similar conditions (Figure 8).

According to spectrophotometric data[7], we can assume an enol-enolate equilibrium given by the proton transfer reaction shown in Eq. 1.

These preliminary data on stability studies indicate that in ethanolic solutions the enol forms II and IV are more stable than the ceto forms I and III; whereas in aqueous solutions, at different pH values a ceto-enol equilibrium is established in all the cases.

It is concluded that this method is rapid, precise and exhibits adequate specificity for the analysis of compounds I-IV in samples from different origins as well as it is also useful as a HPLC stability-indicating assay because precursors and



Eq. 1

isomerization products can be determined simultaneously.

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MICRO-SCALE METHOD FOR DETERMINATION OF TOBRAMYCIN IN SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Hiroaki Kubo, Toshio Kinoshita, Yoshie Kobayashi* and Ken Tokunaga*
School of Pharmaceutical Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo, Japan.
*Nihon Waters Ltd., 3, Kioi-cho, Chiyoda-ku, Tokyo, Japan.

ABSTRACT

A rapid, simple, accurate, and micro-scale method for the determination of tobramycin, sisomicin and netilmicin in serum using high-performance liquid chromatography has been developed. The method is sensitive to 0.3 µg/ml using only 20 µl of serum. The serum is deproteinized with methanol containing an internal standard: sisomicin for the tobramycin, netilmicin for the sisomicin, and sisomicin for the netilmicin. After centrifugation, a counter-ion reagent is added to the supernatant, then an aliquot of the solution is injected into the chromatograph. Tobramycin, sisomicin and netilmicin are separated by reversed-phase, ion-pair chromatography and detected by fluorescence using continuous-flow, post-column derivatization with o-phthalaldehyde. For the tobramycin, within-run and day-to-day variation was below 2.5%. Correlation of this method with microbiological assay and homogeneous enzyme immunoassay was good.

INTRODUCTION

Tobramycin is an aminoglycoside antibiotic used for the treatment of serious gram-negative infections. As with other aminoglycoside antibiotics, tobramycin has a narrow therapeutic range and exerts nephro- and oto-toxicity. Therefore, monitoring

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of tobramycin levels in serum is necessary for safe and effective therapy. The advantages and disadvantages of various methods for the determination of tobramycin were reviewed by Maitra et al (1) and Nilsson-Ehle (2). Some of these methods use fluorescence detection with pre- or post-column derivatization of tobramycin. Other methods employ ultraviolet absorption detection with pre-column derivatization of tobramycin. All these methods require time-consuming pretreatment such as solvent or column extraction of tobramycin in serum and require a large volume(50 µl-250 µl) of serum. We have previously reported a micro determination of gentamicin in serum by high-performance liquid chromatography (3). This report describes a modified procedure, using an internal standard, as well as the applicatin of the method to the determination of tobramycin in serum. The values determined by this method were compared with those by a microbiological assay and those by a homogeneous enzyme immunoassay. This method is also applicable for the analysis of sisomicin and netilmicin.

MATERIALS AND METHODS

Chemicals and Reagents

Tobramycin sulfate (manifested potency, 964 µg/mg) was obtained from Shionogi Co. (Osaka, JAPAN). Sisomicin sulfate (646 µg/mg) and netilmicin sulfate (628 µg/mg) were supplied by Essex Nippon K.K. (Shiga, JAPAN). o-Phthalaldehyde was purchased from Nakarai chemical Ltd.(Kyoto, JAPAN); sodium octanesulfonate and disodium 1,2-ethanedisulfonate were from Kanto Chemical Co.(Tokyo, JAPAN). De-ionized and distilled water was used. Methanol used was of liquid chromatographic grade. All other chemicals were of reagent grade.

Counter-ion reagent was prepared to contain 0.2 M disodium 1,2-ethanedisulfonate and 0.01 M sodium octanesulfonate in water, adjusted to pH 2.5 with acetic acid.

Mobile phase was prepared to contain 0.1 M disodium 1,2-ethanedisulfonate and 0.005 M sodium octanesulfonate in a water-methanol mixture (64:36, v/v), adjusted to pH 3.5 with acetic acid.

o-Phthalaldehyde reagent was prepared according to the method of Anhalt and Brown (4).

Methanol solutions of internal standard were prepared by dissolving 0.5 mg of sisomicin sulfate or netilmicin sulfate in 5 µl of 70% perchloric acid and making up to 200 ml with methanol.

Apparatus and Chromatographic Conditions

The chromatographic system consisted of a Solvent Delivery System 6000A, a Universal Injector U6K and a Radial-Pak C_{18} column (10 cm x 8 mm I.D., particle size 10 µm) with radial compression module (RCM-100), all from Waters Assoc.(Milford, Mass, U.S.A.), was used. The flow-rate of mobile phase was maintained at 2.0 ml/min. The o-phthalaldehyde reagent was delivered with a Model 3000 pump (Waters Assoc.) at a flow rate of 0.8 ml/min to the column effluent via a mixing T-piece. A reaction coil consisting of a stainless steel tube (5 m x 0.25 mm I.D.) was placed between the mixing T-piece and a detector. As a detector, a Model S-FL-330 fluorometer(Soma Optics Co., Ltd. Tokyo, JAPAN) equipped with a FL4BLB lamp (energy maximun at 365 nm, excitation), a 440 nm cutoff filter (emission) and a 70 µl quartz flow-cell was used. Chromatography was performed at room temperature.

Procedure

Twenty µl serum in a 1.5-ml tapered polypropylene centrifuge tube was vortex-mixed with 100 µl of the methanol solution of internal standard for a few seconds. The tube was then centrifuged at 7,800g in a Model H-25FL centrifuge (Kokusan Co., Tokyo, JAPAN) for 1 min. Then 160 µl of the counter-ion reagent were directly added to the tube, which was vortex-mixed again for a few seconds and centrifuged at 7800g for 1 min. A 200 µl aliquot of this mixture was injected into the chromatograph.

Standard sera spiked with various known amounts of tobramycin (potency, 1.85 - 14.83 μg/ml), sisomicin(potency, 1.66 - 13.33 μg/ml) or netilmicin(potency, 1.63 -13.06 μg/ml) were prepared and treated in an identical fashion to that described above. The peak height ratios between tobramycin and sisomicin as internal standard, sisomicin and netilmicin as internal standard, or netilmicin and sisomicin as internal standard were calculated to construct a calibration curve. Tobramycin in patient sera was determined in duplicate and the results averaged.

Homogeneous enzyme Immunoassay

Homogeneous enzyme immunoassay was performed by using commercially available kits (Emit®-amd, Syva Co., Palo Alto, CA., U.S.A.).

Microbiological Assay

Microbiological assay was performed by a paper disc-diffusion technique, using antibiotic medium No. 5 (Difco Labaratories, Detroit, MI., U.S.A.) and Bacillus subtilis ATCC 6633 as a test organism.

RESULTS

The chromatogram obtained from a standard mixture of tobramycin, sisomicin and netilmicin dissolved in mobile phase is shown in Fig. 1. The retention times for tobramycin, sisomicin and netilmicin are 9.0, 12.5 and 17.0 min, respectively. The antibiotics are well-resolved chromatographically. Figure 2 shows chromatograms obtained from the antibiotics-spiked serum without and with a counter-ion. The chromatogram obtained from the supernatant of methanol-treated serum gave different results from that obtained from the standard mixture of antibiotics. The solution of methanol-treated serum containing the counter-ion reagent resulted in a chromatogram that was virtually identical with the chromatogram obtained from the standard mixture. Figure

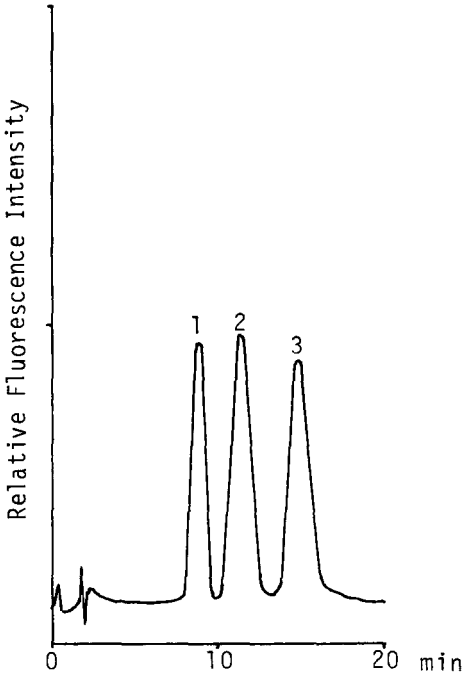

Figure 1. Chromatogram of standard mixture.
1.tobramycin, 2.sisomicin, 3.netilmicin.

3 shows typical chromatograms obtained from tobramycin-free serum that was treated with the methanol and from patient serum containing tobramycin that was treated with the methanol solution of internal standard. The tobramycin-free serum showed no peaks that would interfere with the determination of tobramycin. Linear regression analysis of the calibration curve of tobramycin(1.85 -14.83 μg/ml) yielded the equation, $Y=0.101X - 0.057$($r=1.000$). The peak height ratios(Y) were related to serum tobramycin concentration (X) with high linearity. Those of the calibration curve of sisomicin(1.66- 13.33 μg/ml) and netilmicin(1.63-13.06 μg/ml) were $Y=0.136X + 0.002$($r=0.999$), and $Y=0.100X + 0.003$($r=0.993$), respectively. The limit of detection for these antibiotics is 0.3

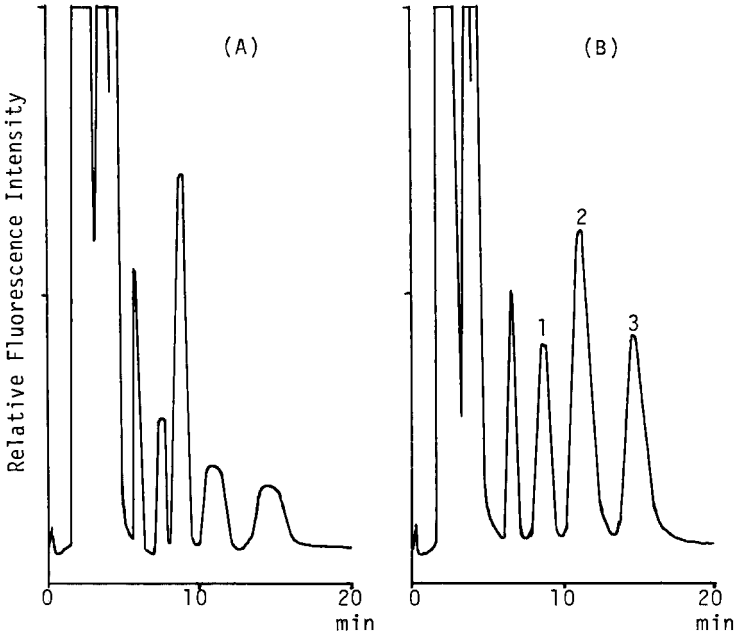

Figure 2. Chromatograms of antibiotics-spiked serum (A):without and (B):with a counter-ion.
1.tobramycin, 2.sisomicin, 3.netilmicin.

μg/ml. This sensitivity is high enough for routine clinical purposes. In order to estimate the analytical recovery, an aqueous solution of tobramycin (5.5 μg/ml) and a tobramycin-added serum (5.5 μg/ml) were analyzed and their peak height ratios compared. The recovery was excellent (97.5-102.5%).
Within-run and day-to-day precision were determined on two serum pools containing tobramycin 3.5 μg/ml and 10.3 μg/ml. As Table 1 shows, the coefficient of within-run variation was less than 2.2% and that of day-to-day variation was less than 2.5%.

The results obtained by the proposed method on patient sera were compared with those by a microbiological assay and those by a homogeneous enzyme immunoassay (Fig. 4). The correlation coefficients were 0.956 and 0.988, respectively. The correlation between the three methods is good.

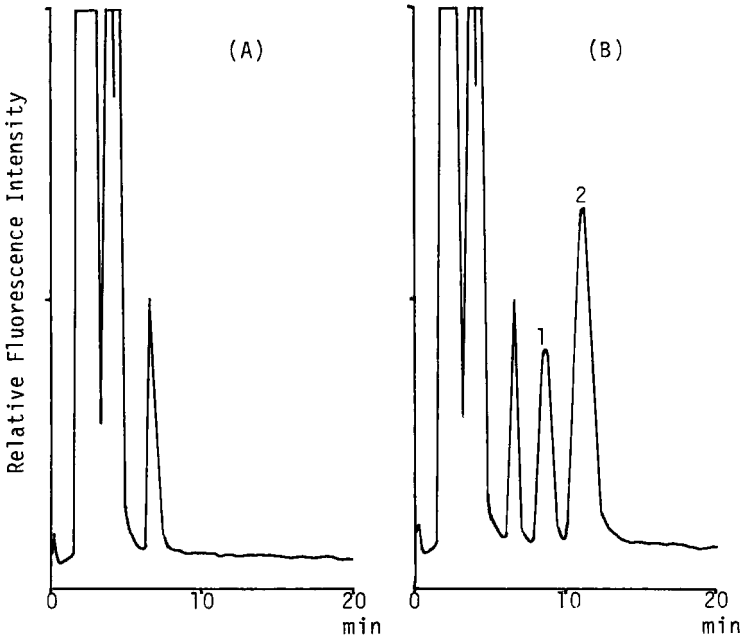

Figure 3. Chromatograms of (A):tobramycin-free serum and (B): tobramycin-containing patient serum.
1.tobramycin, 2.sisomicin(internal standard).

TABLE 1

Coefficient of Variation(%) for Analyses of Tobramycin in Serum

Concentration(μg/ml)	Within-run(n=10) (%)	Day-to-day(n=10) (%)
3.5	2.2	2.5
10.3	2.0	2.3

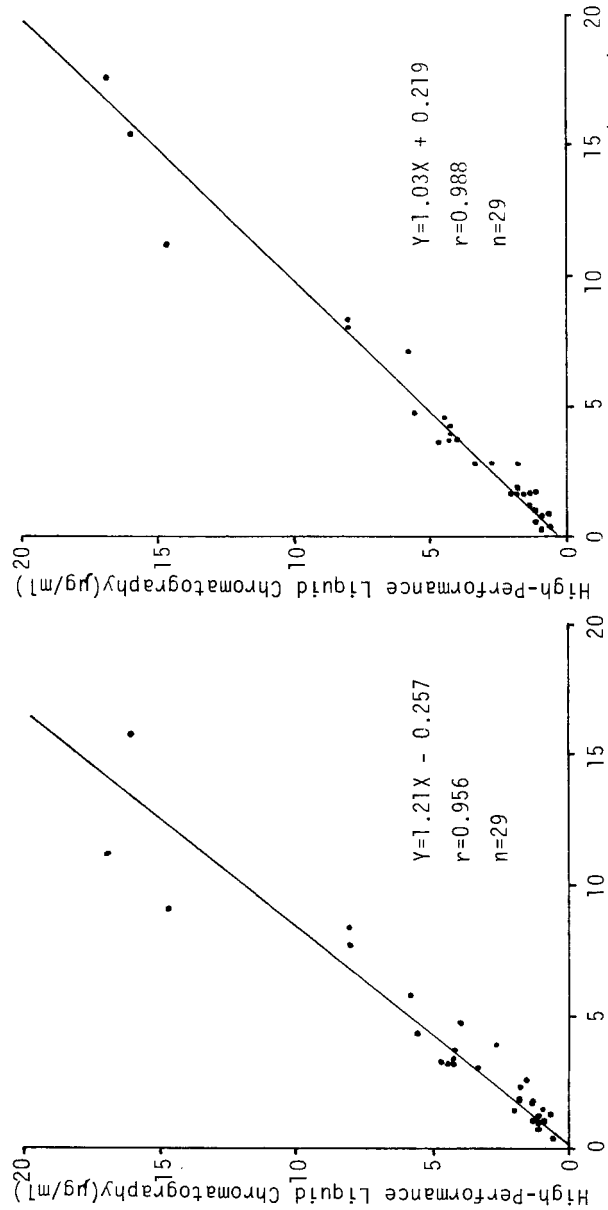

Figure 4. Comparisons with the microbiological assay and with the homogeneous enzyme immunoassay.

DISCUSSION

As we reported previously, precipitation of serum proteins with methanol was essentially complete (3,5). The methanol content in the supernatant of methanol-treated serum is higher than that in the mobile phase solvent and a tobramycin in the supernatant could not form an ion-pair with a counter-ion in the mobile phase during separation on the column. Therefore, it is necessary that the supernatant be diluted with the counter-ion reagent prior to injection into the chromatograph.

Sisomicin sulfate and netilmicin sulfate are very poorly soluble in methanol, but sisomicin perchlorate and netilmicin perchlorate are very soluble in methanol. Therefore, the methanol solutions of internal standard were prepared by dissolving sisomicin sulfate and netilmicin sulfate with perchloric acid and diluting with methanol.

Reversed-phase, ion-pair chromatography was effective in eliminating the interference caused by serum components such as amines, amino acids and small peptides which would form fluorescent products with the o-phthalaldehyde. Tobramycin, sisomicin and netilmicin were separated from these serum components by the use of sodium octanesulfonate. Because tobramycin, sisomicin and netilmicin have five amino groups, their ion-pairs were held more strongly on the reversed-phase column than the ion-pairs of other serum components which have fewer amino groups. By the addition of disodiumn 1,2-ethanedisulfonate, the resolution of tobramycin , sisomicin and netilmicin which have subtle molecular differences could be increased. The retention times for tobramycin, sisomicin, netilmicin and these components could also be shortened.

The method described here simplifies the sample pretreatment greatly by avoiding tedious steps such as solvent or column extraction, and reduces the analysis time significantly. The method is sensitive and accurate. Each analysis requires only 20 µl of serum, and the result can be obtained in less than 15 min for the tobramycin.

This method can be used for pharmacokinetic studies and for routine therapeutic monitoring of pediatric patients.

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ASSAY OF SERUM THIOPENTAL CONCENTRATIONS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

M.T. Parviainen*, A. Harmoinen, G. Baer and H. Jokela

Tampere University Central Hospital
Department of Clinical Chemistry
SF-33520 Tampere 52, Finland

*Present address: Kuopio University Central Hospital
SF-70210 Kuopio 21, Finland

ABSTRACT

A description is given of a specific and simple liquid chromatographic method for the determination of thiopental in protein-free serum. The analysis time per sample is only 4 min, the retention time of thiopental being 2.32 min. No drug interferences were found and the detection limit is about 0.3 mg/l. The day-to-day coefficient of variation is less than 4.3 %, and the within-day variation is less than 4.0 %. The method recovered 98.6 % of thiopental supplemented to a blank serum.

INTRODUCTION

Thiopental (5-ethyl-5(1-methylbutyl)-2-thiobarbiturate) is a short acting i.v. anaesthetic drug, which has also been used for cerebral resuscitation (1). Its use is well established as a single dose

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induction agent, but its pharmacokinetic (2) has not yet been widely studied because of the lack of a simple and sufficiently sensitive assay. Sensitivity is essential when the drug is studied as a single intravenous infusion anaesthetic (3).

Several methods exist for the determination of thiopental. The spectrophotometric method (4) involves three different wavelengths and has a sensitivity of 1.0 mg/l. The method is of limited use because of its non-specificity. Gas-chromatographic procedures (5) require a large sample volume as well as tedious sample preparation steps prior to assay. High-performance liquid chromatography has recently been utilized (6, 7, 8, 9) for the assay of thiopental in serum. The main limitations of these methods are the large sample volumes required (6, 7, 8), the tedious sample preparation (6) needed and the lack of an internal standard (7). Another drawback also is the various and rather complicated solvent systems used as a mobile phase (7, 8, 9).

The purpose of this study was to develop a sensitive and reliable assay for thiopental for use in therapeutic drug monitoring and for pharmacokinetic studies.

MATERIALS AND METHODS

Materials and reagents

The thiopental used in this study was manufactured by Lääketehdas Leiras (Turku, Finland) and the phenolphtalein internal standard came from E. Merck AG (Darmstadt, F.R.G.). The HPLC grade methanol and acetonitrile were purchased from Orion Corp. (Espoo,

Finland). An internal standard was prepared by dissolving 7.5 mg of phenolphtalein in 100 ml of acetonitrile.

Sample preparation

A serum (150 µl) was vortex-mixed with 450 µl of the internal standard solution in a 1 ml Eppendorf polypropylene tube and then centrifuged for 2 min at 12000 rpm using an Eppendorf 5411 centrifuge (Hamburg, F.R.G.) to remove the precipitated serum proteins. After centrifuging, 20 µl of the supernate was used for the analysis.

High-performance liquid chromatography

A Varian 5000 Liquid Chromatograph with variable wavelength UV-100 detector (Walnut Creek, CA, U.S.A.) was used. Sample injection was carried out via a Kontron MSI660 autosampler (Kontron AG, Zürich, Switzerland). A Shimadzu C-R1B Chromatopac computing integrator was used for automatic calculation of the final results.

A MPLC cartridge column system from Brownlee Labs. (Santa Clara, CA, U.S.A.) with a guard column (4.6 mm ID x 3 cm) and an analytical columns (4.6 mm ID x 10 cm) were used for the analysis. Both had Spheri-5 C-18 reverse-phase packing. The mobile phase consisted of 60 % methanol in distilled water.

The flow rate was 2 ml/min. The detector was set to a wavelength of 280 nm.

Calibration

The thiopental standard (20 mg/l, 75.7 µmol/l) was made up in a drug-free serum. The linearity of the

detector response to thiopental was established by using this calibrator from 0 to 250 mg/l. The standard was handled in the same way as the samples.

RESULTS AND DISCUSSION

The separation of phenolphtalein (internal standard, 1.34 min) and thiopental (2.32 min) in a patient serum is shown in Figure 1a. A drug-free serum showed no peaks in the retention times above 1 min (Figure 1b). The phenolphtalein internal standard gave a major peak at 1.34 min and minor impurity peak at 1.75 min, as illustrated in Figure 1c.

Protein in the samples is precipitated with three volumes of acetonitrile, which gives a clear protein-free supernatant (10). This assures a longer life-time of the column than when adding only two volumes of acetonitrile to the serum (9).

The absolute recovery of thiopental from serum was measured by analysing blank serum supplemented with 40 mg/l of the drug. The recovery (n=6) was 98.6 $\pm$ 2.7 % (mean $\pm$ SD), compared with the standard made up in water.

Serum samples containing potentially toxic levels of carbamazepine, clonazepam, digitoxin, disopyramide, ethosuximide, lidocaine, phenobarbitone, phenytoin, primidone, procainamide, propranolol, quinidine, theophylline or valproic acid did not interfere with the assay. Carbamazepine, if present, will elute between phenolphtalein and thiopental with a retention time of 1.94 min, and carbamazepine was the only drug peak seen in this system.

Precision was evaluated in the series by repeated analysis of serum samples containing 20.1, 14.3 and

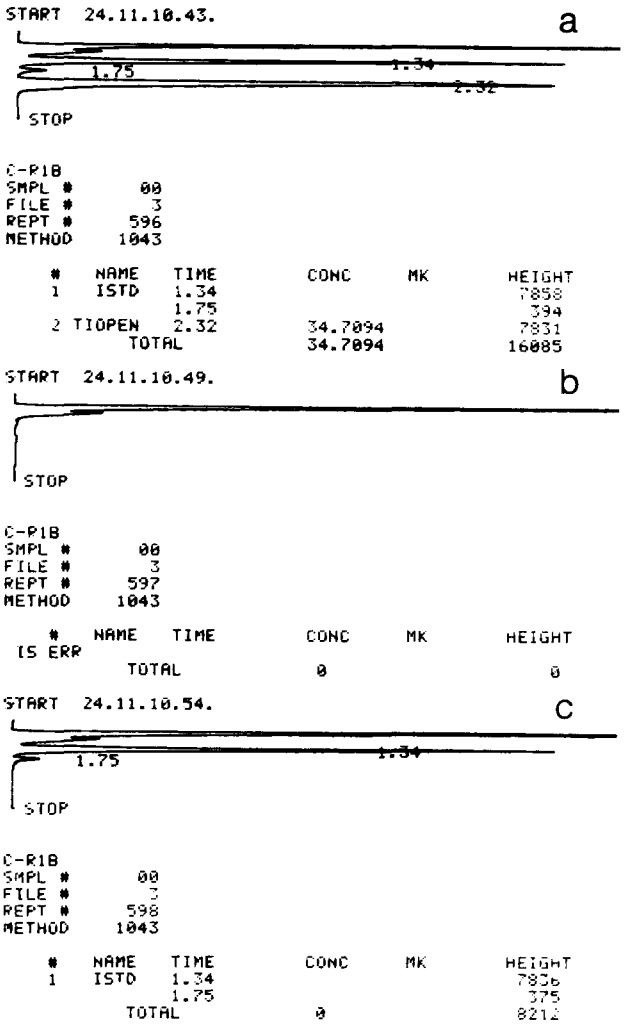

Figure 1. Separation of thiopental in serum. A) A patient serum with 34.7 mg/l of thiopental (retention time 2.32 min) and internal standard phenolphtalein (1.34 min); B) Blank serum; C) Blank serum with internal standard phenolphtalein (1.34 min). Minor peak at 1.75 min is a phenolphtalein impurity.

4.6 mg/l of thiopental. The coefficient of variation was 1.25 % (n=11), 2.40 % (n=15) and 4.0 % (n=15) respectively. The precision achieved from day to day with samples containing 14.1 and 4.5 mg/l thiopental was 2 % (n=10) 4.3 % (n=10) respectively.

We also measured serum thiopental concentrations during and after Fentanyl-complemented (0.01 mg i.v.) thiopental infusion anaesthesia (11). The induction dose was 5 mg/kg and maintenance 12.5 mg/min thiopental. The patients: 10 male and 11 female, aged 50 years (SD 10.4, range 30-64), weight 72.5 kg (SD 16, range 48-120).

The concentrations were measured 10 min, 20 min, 60 min and 24 hours after the induction dose. The concentrations were respectively 12.8 $\pm$ 4, 9.9 $\pm$ 3, 4.4 $\pm$ 2, and 0.4 $\pm$ 0.2 (mean $\pm$ 1 SD).

A detection limit of 0.3 mg/l was achieved using the routine attenuation of the detector (about 0.016 absorbance units of the full scale). This seems to be sufficient for phamacokinetic studies and for the therapeutic monitoring of the drug.

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DETERMINATION OF CINNARIZINE IN WHOLE BLOOD AND PLASMA BY REVERSED PHASE HPLC AND ITS APPLICATION TO A PHARMACOKINETIC STUDY

M. Puttemans*, M. Bogaert*, G. Hoogewijs*,
L. Dryon*, D.L. Massart*, L. Vanhaelst**
*Farmaceutisch Instituut, Vrije Universiteit Brussel;
**Division of Endocrinology, Academic Hospital,
Vrije Universiteit Brussel;
Laarbeeklaan 103, B-1090 Brussels (Belgium)

ABSTRACT

Cinnarizine is determined in whole blood and plasma by reversed phase HPLC on a RP-18 stationary phase. The one-step extraction is performed with a chloroform/hexane (2/3) mixture. A high recovery of 91% and a detection limit of 2 ng/ml are obtained as well as a good precision. The internal standard is meclozine. Pharmacokinetic parameters found are in accordance with data cited in literature.

INTRODUCTION

Cinnarizine, an antihistamine of the diethylenediamine type is widely used in the treatment of cerebral and peripheral vascular insufficiency (1). The plasmalevels in man are situated between 0 and 200 ng/ml. To determine cinnarizine in plasma a sensitive and precise analysis procedure is required.

The methods for the analysis of cinnarizine described in the literature (2-6) exhibit several disadvantages. Dell and Fiedler (2) detected only a metabolite of cinnarizine, benzhydrol, in the urine of rabbits and dogs. The determination consisted of a TLC separation and a U.V. photometry. The determination of cinnarizine itself was not attempted. Morrison et al. (3) described a

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GC method with a low recovery of about 60% and a quite high detection limit of 10 ng/ml. The GC procedure of Woestenborghs et al. (4) is characterized by a high recovery and a low detection limit, but a disadvantage is the time consuming extraction consisting of three successive steps. For the HPLC method of Hundt et al. (5) several criticisms are possible : the recovery of 67% is low, the three-step extraction is tedious, the detection was done at 285 nm while the maximal absorption is situated at 255 nm which means a loss of sensitivity with a factor 6. In the HPLC method of Nitsche et al. (6) a pre-column was used, which is profitable since it extends the life expectancy of the separation column. A disadvantage is the working temperature of 60°C which complicates experimental chromatographic conditions. In addition the selected internal standard has no structural similarity with cinnarizine in contrast with what is desirable for such an analysis. Therefore, it was desirable and necessary to look for an alternative way of analysis of cinnarizine in plasma.

EXPERIMENTAL

Apparatus

Varian LC 5060 equipped with a standard Varian 254 nm U.V. detector and a manual Valvo loop injector (loop size 100 µl). Detection was performed at 0.01 A.U.F.S.

The column was Ultrasphere RP-18 from Altex, packed with 5 µm particles (l = 250 mm, i.d. = 4.6 mm). A pre-column packed with 10 µm Ultrasphere RP-18 (Altex) was also used (l = 30 mm, i.d. = 4.6 mm).

Integrations were performed with a Varian Vista CDS 401, the signal to noise ratio was 2, the WI (= initial peak width) was 10.

pH measurements were done with an Orion Ionalyser 601 and a combined glass electrode.

Extractions were carried out in 30 ml glass centrifuge tubes, sealed with a PTFE-coated screw stop; a mechanical reci-

procating shaker (Orlando Valentini), a heating block Sillitherm® (Pierce), 5 ml glass tubes and high-purity nitrogen were also used.

Chromatography

The mobile phase consisted of a mixture of 9 parts of methanol and 1 part of phosphate buffer (pH = 7.0 and ionic strength 0.1). The eluents were always filtered through a 0.45 µm Millipore filter. The flow rate was 1.5 ml/min. All experiments were carried out at room temperature, a back-pressure of ca. 220 atm was obtained.

Reagents

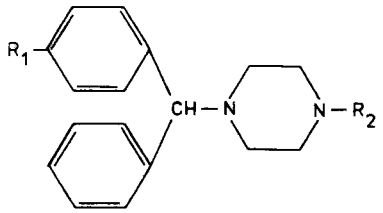
The chemical structure of cinnarizine and potential internal standards are given in Fig. 1. Cinnirazine and clocinizinehydrochloride were obtained from Janssen Pharmaceutica (Beerse, Belgium), buclizinehydrochloride, meclozinehydrochloride and chlorcyclizine were obtained from U.C.B. (Braine l'Alleud, Belgium). Methanol was HPLC-grade and sodiumphosphate, chloroform and hexane were analytical grade from Merck (Darmstadt, G.F.R.).

Stock Solutions

A cinnarizine stock solution was prepared by dissolving 80 mg of cinnarizine in 100 ml of methanol, 1 ml of this solution was then diluted to 500 ml with 0.1 N hydrochloric acid (concentration of the stock solution = 160 ng/100 µl). The stock solution of clocinizinehydrochloride was prepared as described for cinnarazine. The stock solution of meclozinehydrochloride was 4 mg in 100 ml of 0.1 N hydrochloric acid.

Determination of Extraction Recovery

a) from buffered aqueous solutions : 10 ml of the analyte's solution in phosphate buffer pH = 3.0 (1 mg/100 ml for cinnarizine and clocinizine, 35 mg/100 ml for meclozine) was extracted for



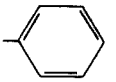
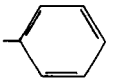
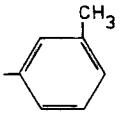
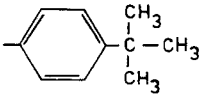
Name	R_1	R_2
Cinnarizine	H	$-CH_2-CH=CH-$ 
Clocinizine	Cl	$-CH_2-CH=CH-$ 
Chlorcyclizine	Cl	$-CH_3$
Meclozine	Cl	
Buclizine	Cl	

Figure 1

Structure of diethylenediamine antihistamines.

30 min with 10 ml of the organic phase. After phase separation, the amount of drug in the aqueous phase was determined by U.V. photometry

b) from biological fluids : to 4 ml of plasma or whole blood, transferred to a glass centrifuge tube, was added 250 µl of cinnarizine or clocinizine stock solution. For meclozine, 500 µl of the stock solution was added. The tube was then vortexed and 10 ml of phosphate buffer (pH = 3.0 and ionic strength 0.4) and 5 ml of organic phase were added. The tubes were sealed and shaken for 30 min on a mechanical shaker at the rate of 90 strokes/min. The tubes were centrifuged for 30 min at 2500 rpm. Three ml of the organic phase was transferred to a 5 ml glass tube, the solvent was evaporated at 40°C under a gentle stream of nitrogen. The residue was redissolved in 200 µl of methanol and 100 µl of this solution were injected in the HPLC system for analysis.

Pharmacokinetic Study

- 10 ml of blood was taken from six healthy volunteers after 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 hours following administration. Blood was collected in heparinized syringes (Sarstedt Monovette®) which were immediately centrifuged, the plasma was separated from the blood cells and was frozen at -20°C until analysis
- calibration : to 4 ml of plasma was added 0.5 ml of internal standard solution and 0, 10, 50, 100, 200, 400 or 500 µl of cinnarizine stock solution. The analysis was then carried out as described under "extraction recovery from biological fluids", starting from : "The tube was then vortexed ...". Chloroform/hexane (2/3) was taken as the organic phase
- analysis of samples : to 4 ml of plasma was added 0.5 ml of internal standard solution. The analysis was then carried out as described under "extraction recovery from biological fluids", starting from : "The tube was then vortexed ...". Chloroform/hexane (2/3) was taken as the organic phase.

Determination of Cinnarizine in Single Capsules

Ten capsules were chosen at random, each capsule was opened and its content was transferred quantitatively to a centrifuge tube. 20 ml of methanol was added to the centrifuge tube which was then sealed, ultrasonicated for 5 min and shaken for 15 min on the mechanical shaker, the tube was centrifuged for 15 min at 2000 rpm, and the clear supernatant liquid was transferred to a 100 ml flask. The extraction was repeated twice with 20 ml fractions of methanol. The combined extracts were diluted to 100 ml with methanol and 1 ml of this solution was then diluted to 50 ml with methanol. 100 µl of this final solution was then injected in the HPLC system for analysis. Calibration was performed with cinnarizine standards which contained 0.5, 1.0, 1.5 and 2.0 mg of the solute in 100 ml of methanol.

RESULTS AND DISCUSSION

Selection of the Chromatographic System

Cinnarizine, which is a lipophilic drug, is nearly insoluble in water and is strongly bound to the RP-18 stationary phase. In order to elute it, a mobile phase with a high methanol content is required. The pH of the mobile phase is important in view of the separation of cinnarizine from matrix constituents. The mixture methanol/phophate buffer pH = 7 (9/1) was revealed to be the most successful of the systems investigated since it permitted the baseline resolution of cinnarizine from endogenic plasma or blood compounds. Furthermore, a detection limit of 0.5 ng was obtained for this system at 0.005 absorbance units full scale, a signal to noise ratio of 2 and a WI value of 10. These latter parameters fix the slope sensitivity of the integrator. Eight replicate injections of 1 ng of cinnarizine yielded a relative standard deviation of 10.3%. Finally, the use of such a high methanol content permitted a flow rate of 1.5 ml/min even with the 5 µm stationary phase. This shortened the analysis time but had no negative effect on column life due to high back-pressures. The performance

TABLE 1
Retention Times of Diethylenediamine Antihistamines on Ultrasphere RP-18 (1 = 25 cm, dp = 5 µm). Mobile Phase : Methanol/Phosphate Buffer pH = 7 (90/10). Flow : 1.5 ml/min.

Name	Retention time (min)
Chlorcyclizine	5.8
Cinnarizine	6.8
Clocinizine	8.9
Meclozine	10.7
Buclizine	17.6

of the column was stable for several months which corresponds to the injection of several hundreds of extracts of biological fluids. The pre-column was changed after ca. 100 injections of extracts. It should be emphasized that this determination required the use of a 5 µm stationary phase in order to get the detection limit down to the required low level and to obtain satisfactory separations.

Selections of the Internal Standard

The internal standard was chosen among the diethylenediamine antihistamines shown in Fig. 1. Their structures, and consequently their U.V. absorption and extraction characteristics are very close to cinnarizine's properties. As can be seen in Table 1, chlorcyclizine elutes before cinnarizine but is incompletely resolved from matrix compounds. On the other hand, buclizine's retention is too high. Two possibilities remain : meclozine and clocinizine, of which clocinizine should be preferred in view of its greater resemblance with cinnarizine.

Selection of the Extraction Scheme

Many papers published in the literature describe the extraction of basic drugs from biological fluids. Such extractions are performed mostly from a basic medium and with various organic solvents. The extraction scheme consists very often of further

purification steps, e.g. a back- and re-extraction. Some procedures for cinnarizine cited in the literature (4-5) use such a methodology. In our laboratory, a standardized extraction procedure for basic drugs has been developped (8). It consists of an ion-pair extraction with n-octylsulphate at pH = 3.0 or HDEHP (=di-(2-ethylhexyl)phosphoric acid) at pH = 5.5 with chloroform as the solvent. It yields pure extracts and also high recoveries. The method is especially interesting for hydrophilic drugs which are very difficult to extract by classical means. Cinnarizine is lipophilic and is extracted quite well to chloroform (3) or tetrachlorocarbon (4) even from acidic solutions. Preliminary experiments carried out on aqueous buffered solutions (pH = 3.0) showed that upon addition of 2.5×10^{-2} M octylsulphate a recovery of 98% to chloroform was obtained, which is equal to the yield obtained with chloroform alone. As both extractions are equally good, the simplest one was chosen for routine application.

As stated by Hundt (5) the pH has no distinct influence on the extraction recovery to chloroform. Experiments were carried out at pH = 3 since at a higher pH higher blanks were obtained. The efficiency of the extraction to chloroform and hexane is given in Table 2. In order to facilitate manipulations (e.g. the transfer of a known amount of organic phase) during the extraction of drugs from blood or plasma, a supernatant organic phase is preferable. A chloroform/hexane mixture (2/3), which has a density less than one, was used by our laboratory for the determination of papaverine in blood (7). The recoveries obtained with this mixture for cinnarizine and the internal standards are nearly quantitative and are also given in Table 2.

Extractions carried out on whole blood and plasma indicate that the chloroform/hexane mixture is a much better extractant than chloroform alone (Table 3a). As shown in Fig. 2, the extract with chloroform/hexane is also purer than with chloroform. Furthermore, it was observed that in most plasma samples analysed, a peak was obtained which interfered with clocinizine. Therefore meclozine was preferred as the internal standard since no interference was observed and because of its higher extraction yield for plasma (Table 3b).

TABLE 2

Extraction Efficiency (%) from 10 ml of Aqueous Buffer Solution pH = 3.0 to 10 ml of Organic phase. N = 3

Substance	Organic Phase		
	Chloroform	Hexane	Chloroform/Hexane (2/3)
Cinnarizine	98.5 ± 0.5	67.0 ± 0.8	96.7 ± 2.1
Clocinizine	98.4 ± 0.1	94.7 ± 0.6	98.0 ± 0.2
Meclozine	94.2 ± 0.2	97.9 ± 0.6	99.6 ± 0.8

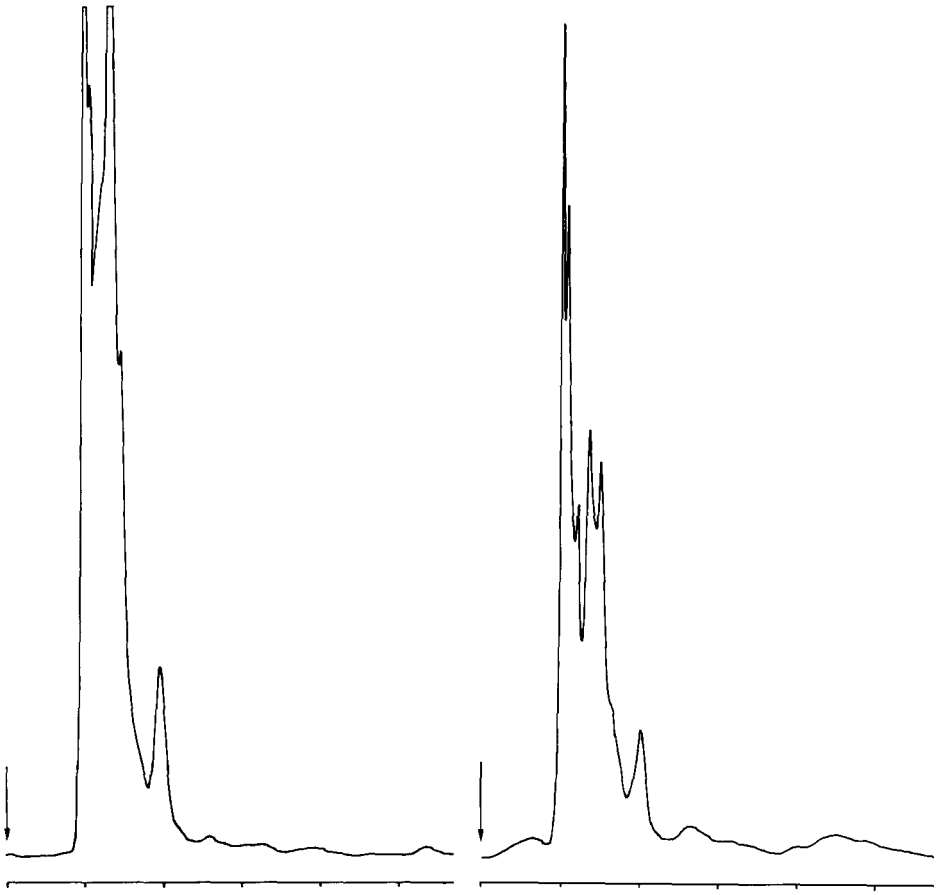


Figure 2

Blank extracts from whole blood.

A : with chloroform

B : with chloroform/hexane (2/3)

Chromatographic conditions as in Table 1.

TABLE 3
Extraction Efficiency (%) from 4 ml of Whole Blood or Plasma.
N = 3, *N = 10

	Chloroform	Chloroform/Hexane (2/3)
a) from whole blood :		
Cinnarizine	33.0 ± 1.8	82.0 ± 1.5
Clocinizine	36.3 ± 1.0	85.5 ± 0.9
b) from plasma :		
Cinnarizine	55.2 ± 2.1	90.9 ± 3.3*
Clocinizine	31.8 ± 0.9	60.3 ± 3.5
Meclozine	36.5 ± 1.1	87.0 ± 2.9*

Quality of the Analysis Scheme

As given above, the extraction efficiencies are 91% for cinnarizine and 87% for the internal standard. Calibration curves were constructed by spiking increasing volumes, ranging from 10 to 500 µl, of the cinnarizine stock solutions (160 ng/100 µl) and 500 µl of the meclozine stock solution (4 µg/100 µl) to 4 ml of blank plasma. Calibration curves were constructed by plotting the ratio of the peak areas of cinnarizine to meclozine versus the concentration of cinnarizine (4 - 200 ng/ml plasma). For six curves constructed on different days the following equation was obtained :

$$\text{relative peak area} = 0.0059\ (\pm 0.0005) \times (\text{concentration}) - 0.0308\ (\pm 0.0446)\ (\text{ng/ml plasma} = \text{conc.})$$

the mean correlation coefficient was 0.9991

This indicates that the slope of the calibration curve has a between-day variation of about 8%. All determinations carried out on a single day were always referred to a calibration curve which was also constructed on the day of analysis.

The limit of detection is 2 ng/ml plasma. The precision of the method was determined at two concentration levels, e.g. 20 and 200 ng cinnarizine/ml plasma. The within-day precision is

7.5% at the 20 ng/ml level and 2.9% at the 200 ng/ml level. The between-day precision is 7.7% at the 20 ng/ml level and 5.3% at the 200 ng/ml level. In both cases the number of replicates was 6.

Pharmacokinetic Study

A number of pharmacokinetic parameters were determined for six healthy subjects following oral administration of 75 mg of

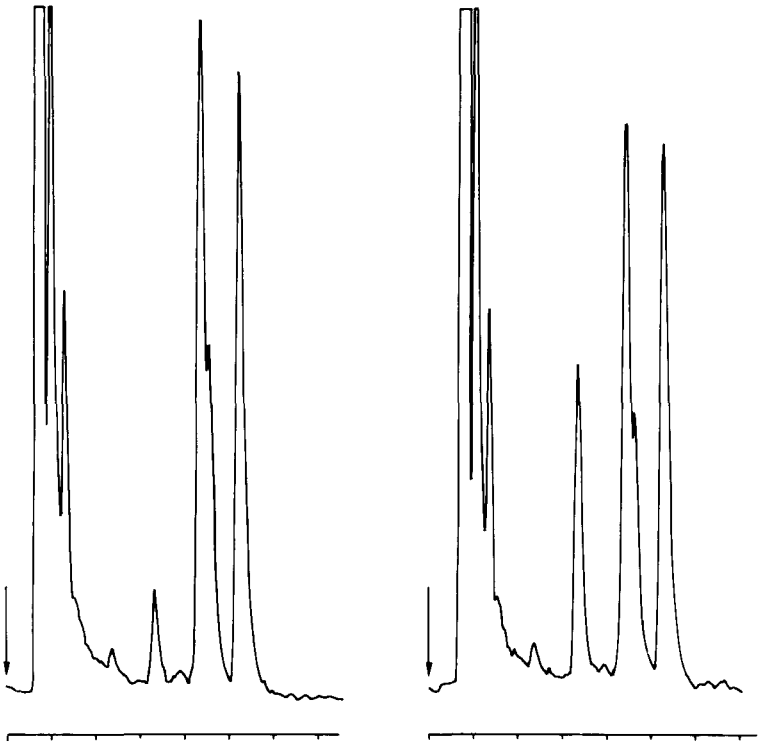


Figure 3

Determination of cinnarizine in plasma samples.

A : 1.5 h after administration

B : 2 h after administration

Chromatographic conditions as in Table 1.

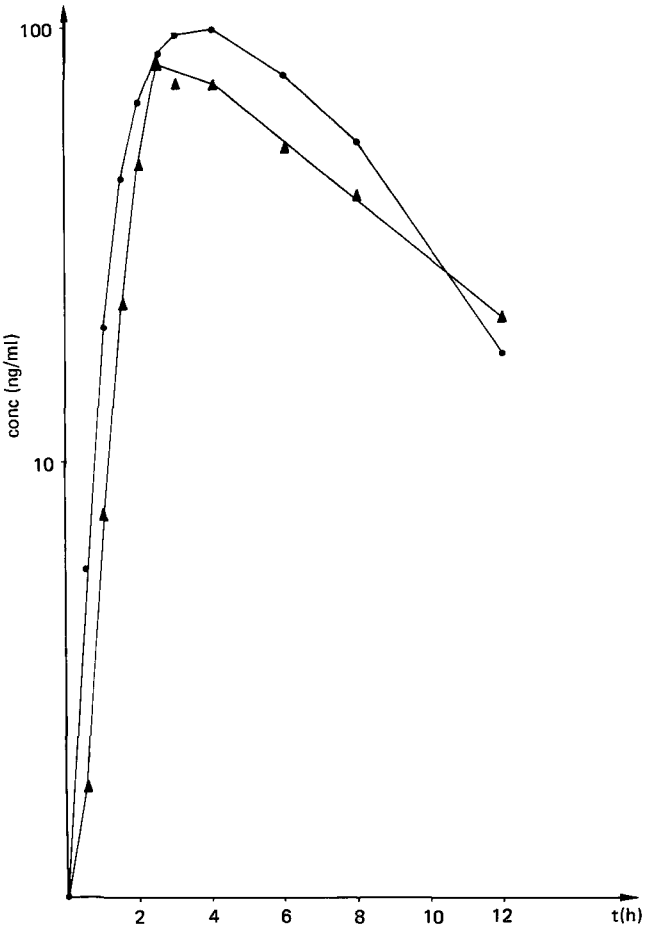


Figure 4

Mean cinnarizine concentration in plasma as a function of time following oral administration of 75 mg of cinnarizine

● : capsules A

▲ : capsules B

Chromatographic conditions as in Table 1.

TABLE 4

Pharmacokinetic parameters : C_{max} = peak concentration, T_{max} = time of peak concentration, T 1/2 = half-life, A.U.C. = area under the curve

	C_{max} (ng/ml)	T_{max} (h)	T 1/2 (h)	A.U.C. (ng x h/ml)
75 mg Cinnarizine				
Capsules A (N=6)	122± 75	3.3±1.6	2.79±1.04	855±431
Capsules B (N=6)	110±133	4.1±2.0	3.24±2.13	637±621
Capsules 75 mg (N=12) (3)	230±130	2.6±1.0	3.43±0.83	1277±440
Tablets 75 mg (N=12) (3)	160±130	3.4±1.2	3.04±1.54	925±603
50 mg Cinnarizine (5)				
Tablets A (N=6)	76± 35	2.3±0.4	4.4 ±1.0	583±180
Tablets B (N=6)	89± 42	2.4±1.1	5.3 ±1.6	721±268

cinnarizine. Two brands of capsules, with similar composition, were compared. Prior to the study the content of single capsules was tested for both brands. The theoretical content being 75 mg, brand A contained 73.9 ± 1.7 mg of cinnarizine, brand B : 73.5 ± 2.0 mg (N=10).

The plasmalevels were determined in samples taken at regular intervals between 0 and 12 hours following oral administration of 75 mg of cinnarizine formulated as capsules. In Figure 3, chromatograms are shown from spiked as well as real plasma samples. The mean plasma concentrations are plotted versus time in Figure 4 for both brands. In Table 4 an overview of the pharmacokinetic parameters obtained from the experimental data are given together with the data from the literature (3) (5). A.U.C. (= area under the curve) values, which are a measure of the bioavailability of a drug, were calculated with the "Estrip" program (9). The data indicate that there is a high biovariability. This is pointed out by our results as well as by the

results found in the literature. The two brands tested out by us do not differ significantly. Considering our data and those found in the literature, one may conclude that after oral absorption of 75 mg of cinnarizine the maximal plasmaconcentration is attained after ca. 3.3 h and the half-life is ca. 3.2 h. The values of the maximal concentration (170 ng/ml) and A.U.C. (980 ng x h/ml) are less certain because of the larger between-brand differences which are observed. Comparison of the data for 75 and 50 mg show that at 50 mg the peak maximum is obtained in a shorter time (ca. 2.3 h) and that the elimination is somewhat slower (t 1/2 = half-life = ca. 4.8 h).

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URINARY URIC ACID DETERMINATION BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

Takeo Iwamoto, Masahiko Yoshiura, and Keiji Iriyama

Division of Biochemistry, Central Research Laboratory,
The Jikei University School of Medicine,
3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105, Japan.

ABSTRACT

Determination of urinary uric acid has been attempted by reversed-phase high-performance liquid chromatography with electrochemical detection. We have found that the electrochemical detection method is suitable for monitoring eluate from reversed-phase column and also that the minimum detectable quantity of uric acid using an electrochemical detector is about 10 pg. Complete separation of uric acid was achieved in about 8 min under the present chromatographic conditions.

INTRODUCTION

Since Kissinger and his coworkers' (1) pioneering work on normal-phase high-performance liquid chromatography (NPHPLC) with electrochemical detection (ECD), the most widely used methods of analysis for catecholamines have been varied in the electrochemical approach. It has been found (2,3) that uric acid (UA) in biological fluids can be determined by NPHPLC-ECD. Clinical laboratory estimations of serum concentrations of UA are currently based on one of two chemical principles: 1) an oxidation-reduction reaction

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in which UA reduces colorless phosphotungstic acid to tungsten blue and measurement at 700 nm (4) and 2) an enzymatic conversion of UA and oxygen by uricase to allantoin and hydrogen peroxide (5). In the latter, there is more heterogeneity of methods due to ancillary linked indicator systems (6). Although the first method is satisfactory for routine analysis, its usefulness is limited because of the presence of other reducing substances such as ascorbic acid (AA) in biological samples. The enzymatic methods proposed until now are sensitive to uricase inhibitors and have poor reproducibility, although the specificity of these procedures are high compared with the phosphotungstic acid method. It has generally been recognized that normal UA levels in human biological fluids (e.g., serum) of different generation must be determined at diagnosis for gout and several other diseases. HPLC may be an obvious candidate as reference method for the determination of UA levels in biological samples. Different separation principles and detectors have been used for the determining UA by aid of HPLC. Ion-exchange (normal-phase) columns have been used, coupled with ultraviolet detection (UVD) (7) or ECD (2,3). Reversed-phase high-performance liquid chromatography (RPHPLC) has been also used with UVD (8) or ECD (9-11). Recently, various types of electrochemical detector of good quality are now commercially available. The inherent higher sensitivity of ECD compared with UVD has been the subject of several publications (9,12). However, there has been no report for the determination of urinary UA by RPHPLC-ECD.

In this article, we describe a simple, rapid, easy, sensitive, accurate and selective method for the determination of UA in human urine samples by RPHPLC-ECD.

MATERIALS AND METHODS

All the experiments were conducted at 25 ± 1°C, unless otherwise stated.

A constant flow pump (Model TWINCLE, Jasco, Tokyo, Japan) allowed a mobile phase to pass through a 25 cm x 4.6 mm I.D. stainless steel column packed with "Fine SIL C_{18}" (Jasco, Tokyo, Japan; particle size, 10 μm) as a reversed-phase adsorbent. Eluate from the column was electrochemically monitored by aid of an electrochemical detector (Model ECP-1, Kotaki, Funabashi, Chiba, Japan) under the potentiostatic condition (+800 mV vs. Ag/AgCl). In addition, eluate from the column was spectrophotometrically monitored by aid of an ultraviolet absorption detector (Model UVIDEC-100-III, Jasco, Tokyo, Japan) set at 254 nm, which was situated between the reversed-phase column and the electrochemical detector, unless otherwise stated. The column temperature was 25 ± 1°C. The mobile phase was 0.2 M phosphase buffer (KH_2PO_4-H_3PO_4, pH 3.0). The flow rate was 2.0 ml/min. For the spectrophotometrical and electrochemical detectors, the limits of detection of UA were in the nanogram and picogram range, respectively, under the present chromatographic conditions.

UA and AA were identified on the basis of retention times by comparison with standards and co-chromatography with standard solutions of UA and AA with various concentrations in different solvent systems.

UA was quantified by comparing the peak height in the respective chromatogram with value from a standard curve. Triplicate injections gave standard deviation of peak heights and retention times of 0.5% and 1%, respectively.

All the chemicals used in this study were the same one as used in our previous reports (13-17). All buffers and aqueous solutions were prepared with glass-distilled deionized water.

An aliquot (0.1 ml) of each human urine sample was diluted with 9.9 ml of the phosphate buffer used as the mobile phase. The diluted urine was passed through a 0.45 μm membrane filter (EKICRODISC 13, Gelman Sci. Jpn. Ltd., Tokyo Japan) to remove the particulate matter (e.g., proteins). The stock solution containing UA(1 mg/ml) was prepared according to the procedure previously

described (16) and was stored at -80°C. Appropriate dilution of the stock solution with the mobile phase was done just before use. 10 μl of each solution containing UA was injected into the chromatographic system for the separation and determination of UA.

RESULTS AND DISCUSSION

Fig. 1 shows a typical chromatogram of the simply deproteinized human urine monitored by the electrochemical detector under the potentiostatic condition (+800 mV vs. Ag/AgCl). As seen here, the UA peak was completely separated as a distinguished one under the present chromatographic conditions. It has generally been recognized that the pretreatment of urine samples previous HPLC analysis is necessary to avoid interferences and extend the column life. Presence of particle materials tends to shorten the column life. One of the most simple pretreatment procedures is the filtration of the liquid sample through a membrane filter to remove the particulate matter. In this way, particles such as proteins are separated from urine sample. Repeated injections of the simply deproteinized human urine samples did not seem to shorten the column life. In addition, interference materials in determining UA were also removed from the human urine samples by the simple filtration procedures, as shown in Fig. 1. The repeated chromatographic examinations revealed that any trace amount of UA was not resultant on the membrane filter, through which the urine sample was passed. Since the sample preparation did not involve any transfer, the value for UA might be nearly absolute, indicative of an endogenous quantity. Content of UA in the human urine, whose chromatogram is shown in Fig. 1, was estimated to be 971 μg/ml.

The peak X component was always appeared in the human urine samples. The component seemed to be chemically very labile in human urine samples, probably forming its degradation product(s) under the present experimental conditions. The Peak X near the so-called

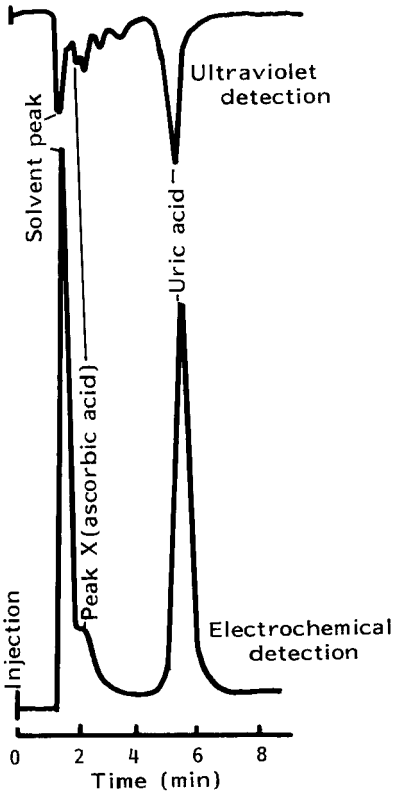

Figure 1. The typical reversed-phase high-performance liquid chromatography, obtained by injecting 10 μl of the simply deproteinized human urine into the RPHPLC-ECD system. Ten microliters of the urine was injected onto the reversed-phase column by using a microsyringe. The eluent was 0.2 M phosphate buffer (KH_2PO_4-H_3PO_4, pH 3.0). The column temperature was 25 ± 1°C. The flow rate was 2.0 ml/min. Eluate from RPHPLC was electrochemically monitored by aid of the electrochemical detector under the potentiostatic condition (+800 mV vs. Ag/AgCl). The sensitivity of the detector was set at 256 nA full scale. At the same time, the eluate was spectrophotometrically monitored by aid of the ultraviolet absorption detector set at 254 nm. The chromatogram is also shown as a reference.

solvent peak was finally identified as AA. Generally, AA is found to be chemically very labile for forming its degradation products, some of which have been found to be electrochemically active (17). Thus, before starting to develop a method for the determination of AA by RPHPLC-ECD, storage conditions for urine samples should be optimized for the determination of AA in biological samples. In the chromatograms of freshly prepared human urine or serum samples,the AA peak was always observed, whereas the AA peak was not observed in the chromatograms of the long-storaged human urine or serum samples. On the basis of the above findings, we are now aiming to develop a method for the determination of AA in biological samples by RPHPLC-ECD. Under the present experimental conditions, the analytical recoveries of AA in biological samples were found to be less than 60%.

As described above, the RPHPLC-ECD method developed in this studyis free from any interferences of the urinary UA determination by urinary reducing substances such as AA. UA standard solutions added to human urine were analyzed with good precision at concentrations comparable to those in physiological samples. The recoveries of UA in different standard solutions added to one hundred different human urine samples prepared on five different days were found to be 99.2 ± 0.5% under the present experimental conditions.

Excellent precision of retention time for UA was always obtained in routine analysis over a six-day period for three hundred different human urine samples, probably due to the fact that the retention time of UA is not affected by the sample matrix. In our laboratory, however, the calibration graphs were daily obtained before and after the UA determination study. Repeated injection gave an average precision (R.S.D) of less than 2%.

CONCLUSION

We have developed a method for the determination of urinary UA by RPHPLC-ECD. The RPHPLC-ECD method has been proven to be

powerful method for the separation and quantitation of urinary UA. It has generally been recognized that the so-called ECD method is more sensitive than the UVD method. However, the higher sensitivity is not crucial in the analysis of urinary UA because of the fact the UVD is adequately sensitive for the determination of urinary UA concentrations (see Fig. 1). Juge and Del-Razo (18) have concluded that the RPHPLC-ECD method developed by them is a powerful method for the determination of urinary UA. At this present stage, no single method is considered to be a standard comparison (reference) method for the estimation of UA in biological samples such as serum and urine. At least, we believe that the RPHPLC-ECD method is also an obvious candidate as a reference method for the determination of UA in urine as well as in any other biological samples.

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USE OF AN OPTIMUM WAVELENGTH TO DETECT GLUTAMIC ACID AND ITS METABOLITES IN HUMAN SERUM BY REVERSED-PHASE HPLC

S.L. Pahuja, J. Albert and T.W. Reid
Department of Ophthalmology and Visual Science
Yale University School of Medicine
333 Cedar Street - BML B219
New Haven, Connecticut 06510

ABSTRACT

Dansylated glutamic acid, glutamine and γ-amino butyric acid (GABA) show maximum absorption at 221 nm. Using this wavelength, the detection limits for dansylated amino acids studied by reversed-phase HPLC are similar to those reported by fluorescence. This technique was used to look for the presence of glutamic acid and its metabolites in human serum. Glutamic acid and glutamine were present in significant amounts and their levels were 2.5 and 6.1 nmoles/ml respetively, while GABA was present in trace amounts, less than 0.3 nmoles/ml.

INTRODUCTION

Dansylated amino acids generally have been detected by absorption at 254 nm. However, as mentioned by Ross (1) in his review article, by using an ultraviolet (UV) wavelength of maximum absorption rather than the arbitrary selection of 254 nm, this technique would be as sensitive as spectrofluorometric detection. Hence, with the availability of variable wavelength UV monitors in conjunction with HPLC equipment, it is possible to utilize the wavelength of maximum absorption to detect dansylated amino acids.

Reversed-phase HPLC may also prove to be a valuable tool in the study of pathological states where abnormalities in amino acid metabolism are involved. Glutamic acid is one such example. Glutamic acid levels have

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been shown to rise in multiple sclerosis (2). GABA, which results from the decarboxylation of glutamic acid, is an inhibitory neurotransmitter in the central nervous system, and has been implicated in schizophrenia, Huntington's disease, Parkinson's disease, and epilepsy (3-6). The biological significance of GABA has been recently reviewed by Holdiness (7). Glutamine, the other major metabolite of glutamic acid, has been shown recently to be of therapeutic value in the treatment of peptic ulcers (8) and is one of the main storage forms of nitrogen in the body.

In view of the significance of these metabolites, we have developed a method for their determination by reversed-phase HPLC with UV absorption at an optimum wavelength.

EXPERIMENTAL

Instrumentation

The HPLC apparatus included a Beckman Model 332 gradient liquid chromatograph, a Beckman 210 sample injection valve with a 20 µl loop, and an LDC UV variable wavelength detector. The chromatographic column was an Altex Ultrasphere reversed-phase ODS-5 (250 x 4.6 mm). The mobile phase was a gradient of 100 mM potassium dihydrogen phosphate pH 2.1 with 0-50% acetonitrile. The flow rate was 1 ml/min at room temperature. A variable wavelength Spectrophotometer (Shimadzu UV-3000) recorder was used to determine the UV absorption spectra of dansylated amino acids. Reagents

Reagents

Dansylated amino acids were obtained from Sigma (St. Louis, MO, USA). Radiolabelled amino acids were obtained from New England Nuclear (Boston, MA, USA). Acetone and acetonitrile were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Potassium dihydrogen phosphate was obtained from Gallard-Schlesinger Chemical Corp. (Carle Place, NY, U.S.A.). Phosphoric acid was obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Dansyl chloride for derivatization was prepared fresh daily by diluting the

stock solution (10% in acetone obtained from Pierce, Rockford, IL, U.S.A.) with acetone to yield a final concentration of 0.25%. Sodium bicarbonate buffer (100 mM, pH 9.5) was passed through a 0.45-μm filter (Millipore).

Dansylation

The amino acids were dansylated as described previously (9). For dansylation of amino acids in human serum, 20 μl of serum was mixed with 80 μl of acetonitrile. The precipitate was removed by centrifugation and supernatant was evaporated under a stream of nitrogen. To the residue was added 50 μl of bicarbonate buffer, pH 9.5, and 100 μl of dansyl chloride solution (0.25% in acetone) in a PTFE lined capped sample vial. After incubation at 70°C for 15 min in a water bath, the vial was removed, cooled in ice for about five min, and 20 μl injected into the column.

RESULTS AND DISCUSSION

Figure 1 shows the UV absorption spectrum of dansylated glutamic acid. It is evident that the maximum absorption is at 221 nm. Absorption at 254 nm which is generally used for detection of dansylated amino acids is not significant. Absorption at 280 nm is more than at 254 nm but it is about one seventh that seen at 221 nm. Other dansylated amino acids showed an identical absorption spectra. The presence of acetonitrile used in the mobile phase does not affect the UV absorption maxima of dansylated amino acids.

Figure 2 shows the separation and detection of dansylated glutamic acid, glutamine and GABA at 221 nm . Thirteen amino acids were tested and found not to interfere with the elution of glutamic acid and its metabolites. The amino acids were arginine, lysine, serine, aspartate, threonine, glycine, alanine, valine, tyrosine, methionine, phenylalanine, isoleucine and leucine. 10–20 picomoloes of each amino acid could be detected easily and is well above background at 0.02 absorption units full scale (AUFS). The reported detection levels for dansylated amino acids by fluorescence are the same (10).

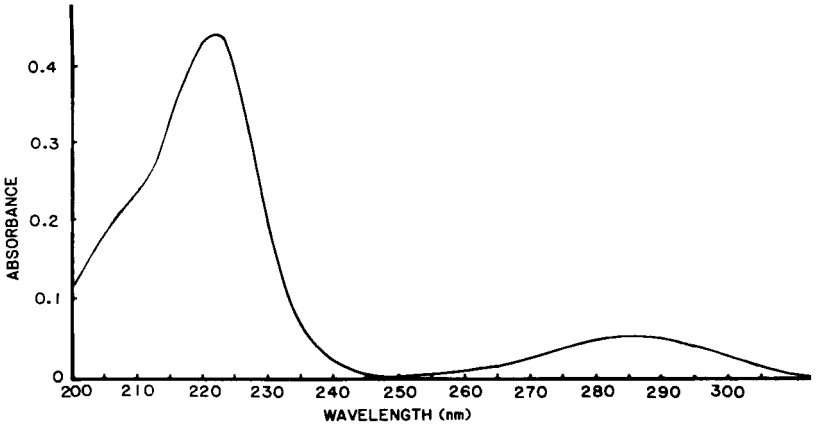

Figure 1. UV absorption spectrum of dansylated glutamic acid. The dansylated amino acid (13 nmoles) was dissolved in 0.1 M phosphate buffer, pH 2.1 and scanned with a chart speed of 2 cm/min at 1.0 AUFS.

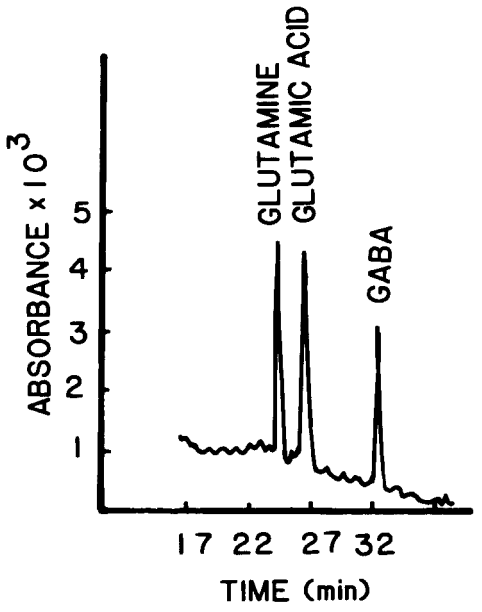

Figure 2. Chromatogram showing the separation of dansylated glutamic acid, glutamine and GABA. The UV detection of dansylated acids (17 pmoles) was at 221 nm (0.02 AUFS). 0.1 M sodium phosphate buffer, pH 2.1 (A) and acetonitrile (B) were used for elution. The composition of the gradient was as follows: 100% A, 3 minμ 0% to 10% B, 5 minμ 10% to 40% B, 30 minμ 50% B,10 minμ 50% to 0% B, 5 min.

In order to see if the procedure would be useful in biological samples, amino acids in serum were dansylated and chromatographed with detection at 221 nm (0.5 AUFS). GABA was present in trace amounts and constituted less than 0.3 nmoles/min, while glutamic acid and glutamine were present at the levels of 2.5 and 6.1 nmoles/ml respectively. The position of these amino acids was confirmed by the use of internal standards. Other amino acids were also present and resolved well from glutamic acid and its metabolites. As studied with radiolabelled amino acids, dansylation of each amino acid was more than 90% and there was no evidence of side product formation (data not shown).

In summary, we have described the usefulness of reversed-phase HPLC coupled with UV absorption at an optimum wavelength for the detection of dansylated amino acids in biological samples. The determination at 221 nm is is at least 20 times more sensitive than determination at 254 nm.

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A FULLY AUTOMATIC METHOD FOR ANALYSIS OF INDIVIDUAL BILE ACIDS IN THE SERUM USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR CLINICAL USE

S. Hasegawa, R. Uenoyama, F. Takeda,
J. Chuma, K. Suzuki, F. Kamiyama*,
K. Yamazaki* and S. Baba.

The Second Department of Internal Medicine,
Kobe University School of Medicine, Kobe, Japan.
*Central Research Laboratory, Sekisui Chemical
Co., Ltd. Osaka, Japan.

ABSTRACT

We have reported highly sensitive methods for the analysis of individual bile acids in the serum using high-performance liquid chromatography coupled with an enzymatic fluorometric system. In this report, a new system equipped with a sample treatment mechanism for chromatographic analysis of serum bile acids is detailed. Most of the protein and other hydrophilic components of the injected serum sample are removed in the pretreatment system, so that only the remaining bile acids are introduced into the chromatographic system and eluted with irrigants containing a coenzyme (NAD) for fluorometric detection. With this method, we are able to simultaneously determine 15 different serum bile acids in an hour without the tedious manual sample processing steps. This system opens up an approach to fully automated analysis of bile acids in the blood.

INTRODUCTION

Bile acids are amphipathic molecules synthesized from cholesterol and conjugated with glycine or taurine in the liver cells. Conjugated bile acids are excreted into the small intestine, where about 30% of them are deconju-

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gated and converted into secondary bile acids by intestinal micro-organisms. Most bile acids are reabsorbed from the terminal ileum by a process of active transport and are returned to the liver via the portal vein. This constitutes the enterohepatic circulation. Disturbances of the enterohepatic circulation can occur secondary to disorders affecting hepatic, biliary, or intestinal function, but may also be of primary relevance in the pathogenesis of clinically important conditions, such as cholelithiasis. Therefore, detailed information on the enterohepatic circulation of bile acids in man is potentially of great clinical importance. For determining serum bile acids in particular, it would be most advantageous to be able to ascertain not only the total concentration, but also the concentrations of each individual bile acid, since these patterns may yield clinical information in hepatobiliary or gastrointestinal diseases. In 1978, we reported a highly sensitive method for the analysis of individual (3α)-hydroxy bile acids in the serum using high-performance liquid chromatography (HPLC) coupled with an enzymatic fluorometric system (1,2); in 1981, we succeeded in combining our original method with a new immobilized enzyme column suitable for use at the alkaline pH that is optimum for (3α)-hydroxysteroid dehydrogenase (3α HSD) enzyme activity (3); and , in 1982, we reported a new column of polymer reversed phase gel exclusively for the analysis of bile acids (4,5). In the present paper, we are presenting a new system for the chromatographic analysis of bile acids.

The system consists of a sample treatment system, a high-performance liquid chromatographic pump with a step-wise gradient programmer, a fractionating column of hydrophilic polymer gel, an immobilized enzyme column and a fluorometer. A serum sample is injected into the sample-treatment-system, through which most of the protein and other hydrophilic components are removed. Then, by a simple valve operation, the remaining bile acids are introduced into the fractionating column and eluted with irrigants containing a coenzyme (NAD). Each fraction of bile acid flows into the immobilized enzyme column where NAD irrigants are transformed into NADH, which is detected fluorometrically. The fractionating step is completed within about 60 minutes. Thus, this system has eliminated the tedious manual sample processing steps and can be controlled fully automatically.

MATERIALS AND METHODS

Materials Used for the Fractionating Column and the Short Column of the Sample Treatment System

As the monomers, tetraethyleneglycol di-acrylate (Sin Nakamura Chem. Co., Ltd. Wakayama, Japan) and tetramethylolmethane tri-acrylate (Sin Nakamura Chem. Co., Ltd. Wakayama, Japan) were prepared. Toluene and benzoyl peroxide were used for the polymerization.

Materials Used for the Immobilized Enzyme Column

Beaded cellulose (Cellulofine GC-200m. Seikagaku Kogyo Co., Ltd. Tokyo, Japan) was adopted as solid support because of its chemical stability at alkaline pH and its superior

mechanical properties. The enzyme was (3α)-hydroxy steroid dehydrogenase extracted from Pseudomonas Testosterony and purified (Sekisui Chem. Co., Ltd. Osaka, Japan)(6).

Eluent Containing a Coenzyme

Triammonium phosphate was dissolved in distilled water to make 0.5% and 1.5% (W/V) triammonium phosphate solution. The 0.5% solution was adjusted to pH 9.1 and the 1.5% solution to pH 9.7 with ammonium hydroxide solution. Each solution was mixed with acetonitrile. Three kinds of eluent were prepared.

The volume ratio (V/V) of Eluent I was 13:87 (acetonitrile: 0.5% triammonium phosphate solution), that of Eluent II was 15:85 (acetonitrile:1.5% solution) and that of Eluent III was 24:76 (acetonitrile:0.5% solution). Each eluent contained 200 mg/l of β-NAD^+ (Sigma Chem. Co., St. Louis, U.S.A.).

Standard Material

Sodium salts of cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) were purchased from Calbiochem-Behring Co. (San Diego, U.S.A.). Sodium salts of glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), glycolithocholic acid (GLCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), taurodeoxycholic acid (TDCA) and taurolithocholic acid (TLCA) were obtained from P-L Biochemicals Inc. (Milwaukee, U.S.A.). Sodium salts of ursodeoxycholic acid (UDCA), glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA) were supplied

gratis from Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan). As an internal standard (I.S.), we used 5β,-pregnan-3α, 17α, 20α-triol (Sigma Chem. Co., St. Louis, U.S.A.).

Instruments

A schematic diagram of the method is shown in Fig. 1. As for the pretreatment system, which is equipped with an injector, we will touch on this later. The high-performance liquid chromatography was carried out on an ALC 204 type system (Waters Associates Instrument, Waltham, U.S.A.). The detector used was a fluorochrome fluorescent detector (Shimadze Seisakusho Ltd. Kyoto, Japan) with an exciting wavelength 350 nm and an emitting wavelength of 460 nm.

Pretreatment System

The sample treatment system consists of a constant flow pump, a short column of polymer reversed phase gel and an injector. The polymer gel is the same copolymer hydrophilic bead packed in the fractionating column mentioned later. The principle of this system is shown schematically in Fig. 2. A serum sample is first injected into the short column, where triammonium phosphate solution (pH 7) from the constant flow pump eliminates most of the protein and other hydrophilic components; then, by a simple valve operation, the remaining bile acids in the short column are introduced into the fractionating column (HPLC column) and are eluted with irrigants containing a coenzyme. This system was devised by the staff of Sekisui Laboratory.

Preparation of the HPLC Column

Copolymer beads were synthesized by a standard suspension

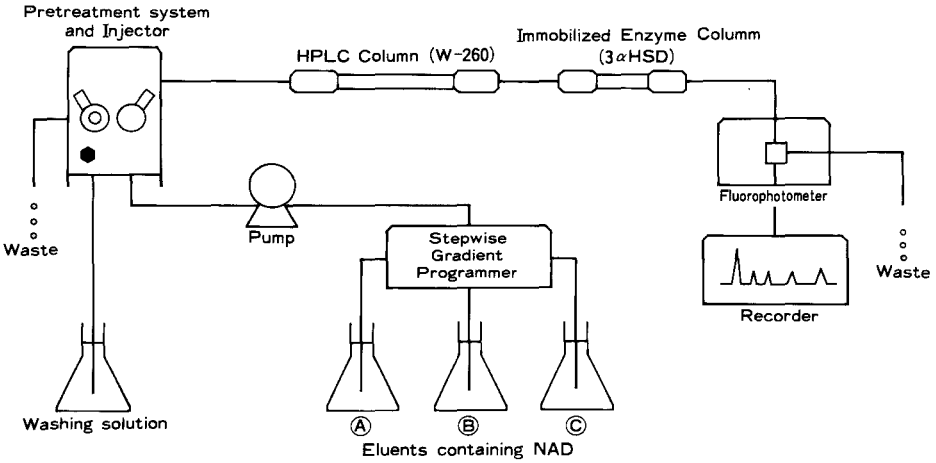

Fig. 1 : Flow diagram of the system

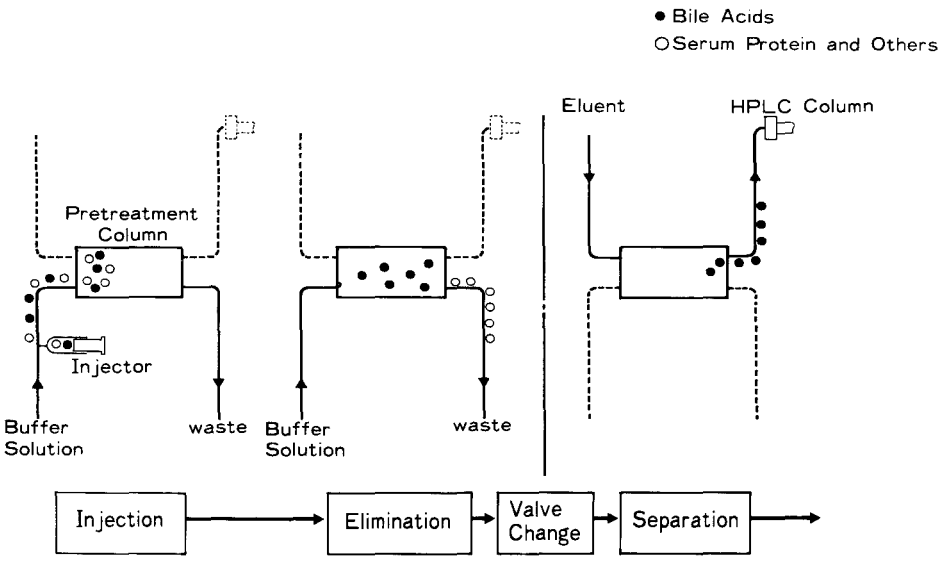

Fig. 2 : Principle of the pretreatment system

polymerization method; the details of the procedure have been mentioned in our recent report (5). The porous beads thus obtained were packed into a stainless steel column (0.6ϕ x 25cm), which was made 20% smaller than that described in our recent report. This was to shorten the retention time of each bile acid.

Preparation of the Immobilized Enzyme Column

Activation of the beaded cellulose by the addition of CNBr was followed by the coupling reaction with (3α)-hydroxy steroid dehydrogenase. The reactions were performed according to the procedure described by S.C. March et al. (7). The enzyme-bound beads were packed into a stainless steel column (0.4ϕ x 10cm), and this was referred to as the immobilized enzyme column.

Preparation of Serum Material

Human sera were collected from the peripheral veins of healthy volunteers and patients with hepatobiliary diseases after overnight fasting and were frozen (-20°C) until analyzed for bile acids. For the analysis, 5,000 ng of 5β-pregnan-3α, 7α, 20α-triol (dissolved in 10 μl of methanol) were added to 1 ml of serum as I.S.

Measurement Procedure

Twenty-five μl of the samples were applied to the system with a microsyringe (Hamilton Co. Reno, U.S.A.). Eluent was passed through at the rate of 1 ml per minute. In order to shorten the retention time, the mobile phase was changed from Eluent I to Eluent II four minutes after the operation of a valve attached to the pretreatment system

(this corresponds to six minutes after injection). Then Eluent II was replaced by Eluent III 35 minutes later. Each fraction of bile acids flowed into the immobilized enzyme column where the NAD irrigants were transformed into NADH, which was detected fluorometrically. This reaction was carried out at 27°C in a water bath. The chart speed was 2.5 mm per minute. Fig. 3 shows a standard chromatogram. This fractionating step was completed in 68 minutes.

Standard Curves

Five concentrations of a mixture of each of the standard bile acids were prepared 10 μmol/l, 7.5 μmol/l, 5 μmol/l, 2.5 μmol/l and 1.25 μmol/l. These samples were dissolved in distilled water, and I.S. was added as the sera were treated. The standard curves were established by plotting the peak height against the amount of each bile acid.

Recovery Experiments

Recovery experiments were performed by adding a mixture of standard samples to the sera of healthy subjects. This mixture contained 5 μmol/l of each bile acid.

Reproducibility

As retention times, several samples were analyzed 20 times continuously. The %CV of each bile acid's retention times was calculated. As an intra-assay, the same samples were determined five times during a day, and, for interassay, the same samples were determined during another six-day period. The %CV values were calculated from the mean values and SD values.

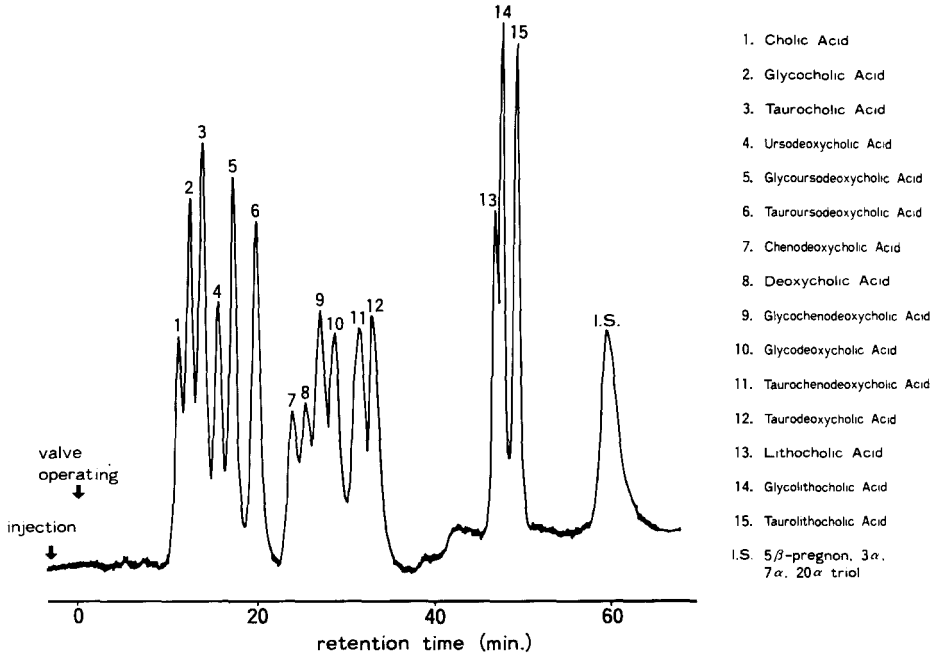

Fig. 3 : Standard chromatograms of individual bile acids (2.5 μmol/l each)

Influences of Bilirubin, Hemoglobin and Lactate Dehydrogenase (LDH)

These tests were performed by adding these substances to the serum of a patient with hepatobiliary disease. The control concentration of bilirubin ranged from 0 to 20 mg/dl, of hemoglobin from 0 to 500 mg/dl and of LDH from 0 to 2500 IU/dl. The concentrations of 15 kinds of bile acids were compared with the sample containing no added substances.

Subjects

The subjects studied are listed in table 4. The control group consisted of asymptomatic, clinically and biochemically normal volunteers, who were not receiving any drugs. The diagnosis of each patient was based on clinical, biochemical and histological findings.

RESULTS

Fig. 3 shows a chromatogram of a mixture of free, glycine- and taurin-conjugates of each of five bile acids. The separation of those bile acids was satisfactorily obtained in the order of CA, GCA, TCA, UDCA, GUDCA, TUDCA, CDCA, DCA, GCDCA, GDCA, TCDCA, TDCA, LCA, GLCA and TLCA. The resolution value of TCDCA and TDCA, in which separation was most difficult, was 0.7. Fig. 4 shows the standard curve of each bile acid. Linear correlations were obtained between the peak height and the amount of each bile acid. Table 1 indicates the reproducibility of each retention time. Each %CV was satisfactory. Table 2 indicates the recovery ratios, reproducibility and minimum detectable range. Satisfactory recovery ratios of between 91.9% and 104.5% were obtained. The intra-assay CV value of each bile acid ranged from 1.6 to 8.7% in replicated determinations and from 2.4 to 9.4 in six replicated determinations for interassay CV. The sensitivity limits of each bile acid by this method were between 0.042 and 0.116 μmol/l. There was no influence of bilirubin ranging from 0 to 20 mg/dl added to human sera as shown in Fig. 5, no

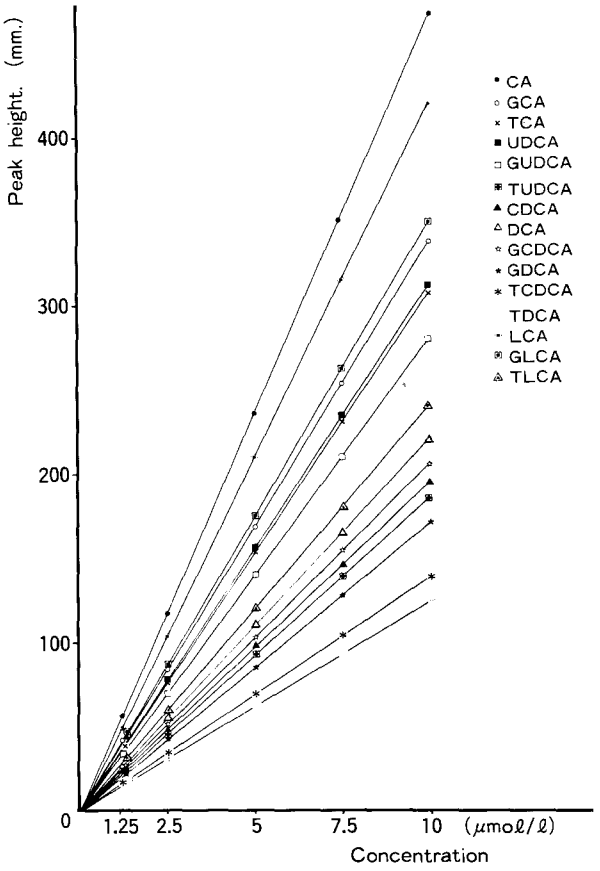

Fig. 4 : Standard curves of individual bile acid

influence of hemoglobin from 0 to 500 mg/dl as shown in Fig. 6 and no influence of LDH from 0 to 2500 IU/dl as shown in Fig. 7. A normal serum analysis by this method is presented in Fig. 8. In addition, an example of serum analysis in a patient with acute hepatitis is shown in Fig. 9. A typical bile acid pattern of acute liver cell

Table 1: Reproducibility of each retention time

Bile Acids	C.V. (%)
CA	0.75
GCA	1.07
TCA	0.13
UDCA	0.13
GUDCA	0.29
TUDCA	0.45
CDCA	0.42
GCDCA	0.68
TCDCA	0.52
DCA	0.72
GDCA	0.50
TDCA	0.65
LCA	0.34
GLCA	0.20
TLCA	0.24

damage was obtained. Normal values analyzed in this system are compared with those from other methods, including our previous HPLC method using 3α-HSD reagent solution in Table 3 (2,5,8,9,10). Table 4 shows mean concentrations of individual bile acids in the sera of six healthy volunteers and 82 patients with hepatobiliary diseases. Characteristic patterns of individual serum bile acids were found in patients with various hepatobiliary diseases.

Table 2: Recovery, reproducibility and minimum detectable range

Bile Acids	Reproducibility of Each Bile Acid Assay		Recovery Rate (%)	Minimum Detectable Range (μmol/ℓ) S/N=2
	Intra asay C.V. (%) n=5	Inter assay C.V. (%) n=6		
CA	2.4	5.3	102.3	0.042
GCA	3.7	8.9	101.5	0.060
TCA	8.7	2.4	104.5	0.071
UDCA	5.3	4.6	99.7	0.065
GUDCA	7.3	8.7	98.8	0.065
TUDCA	1.6	9.4	99.2	0.104
CDCA	8.8	4.9	99.7	0.102
GCDCA	7.6	5.2	100.2	0.097
TCDCA	2.4	8.9	100.2	0.140
DCA	5.3	8.3	101.0	0.092
GDCA	2.9	5.8	101.2	0.116
TDCA	5.7	2.8	100.7	0.162
LCA	2.2	8.7	94.2	0.049
GLCA	2.7	3.7	94.0	0.057
TLCA	5.3	2.8	91.9	0.083

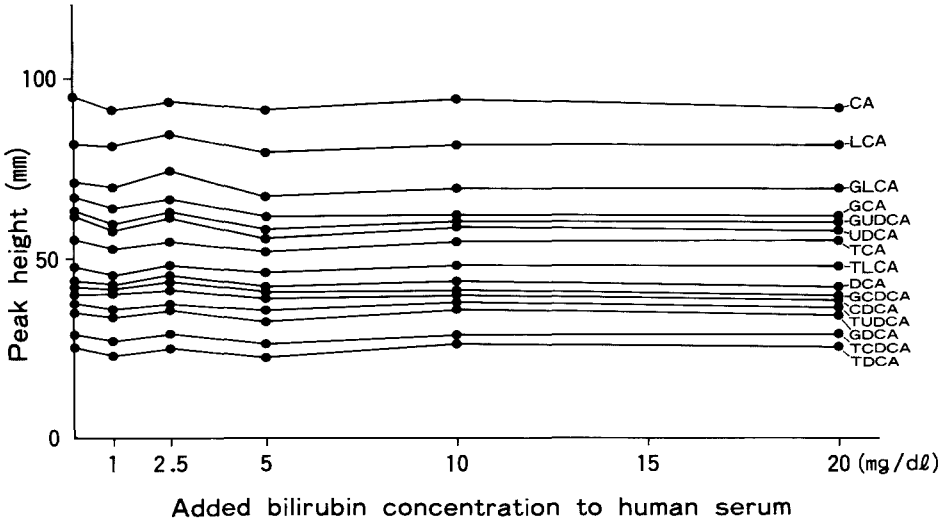

Fig. 5 : Influence of bilirubin

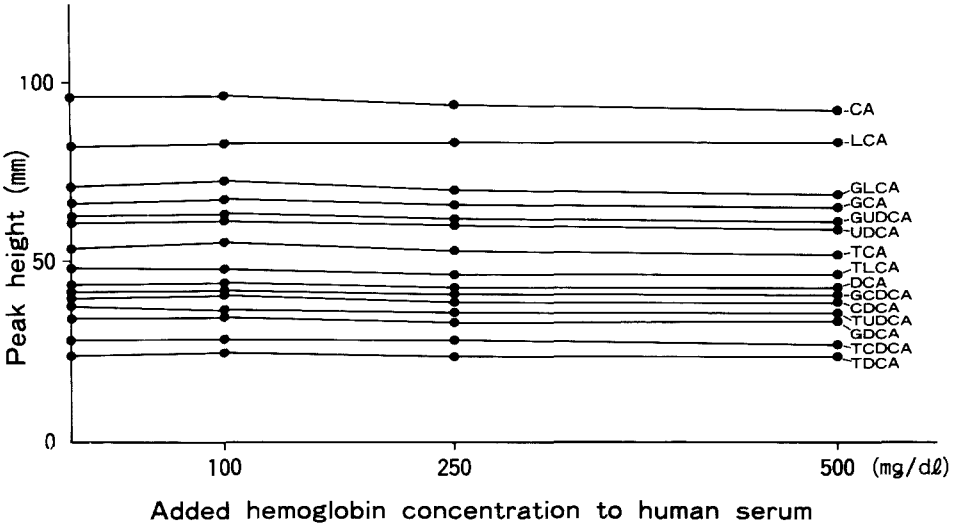

Fig. 6 : Influence of hemoglobin

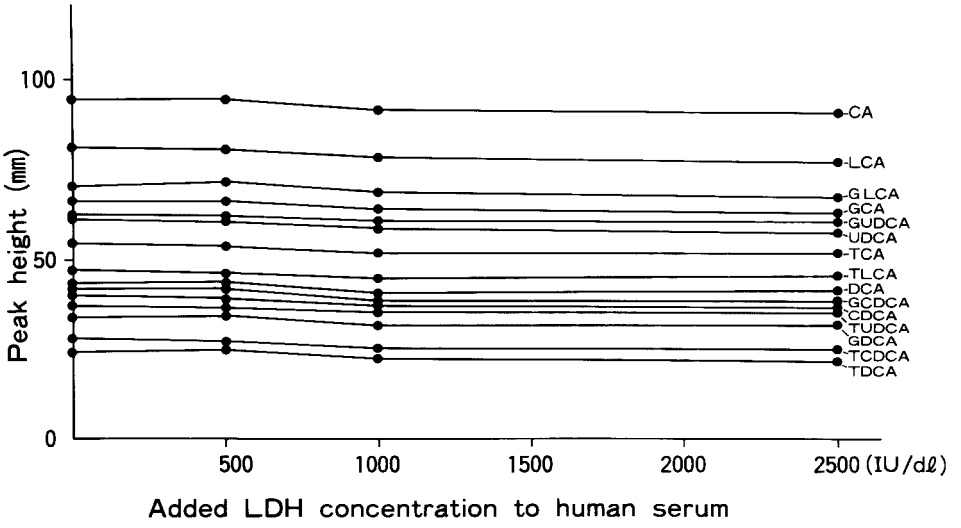

Fig. 7 : Influence of LDH

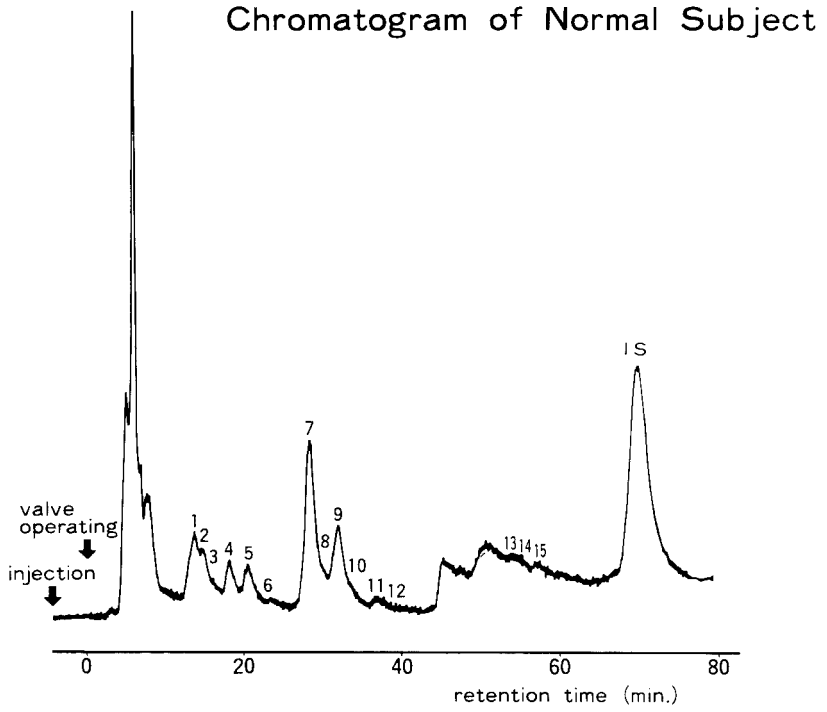

Fig. 8 : A bile acid pattern of normal serum

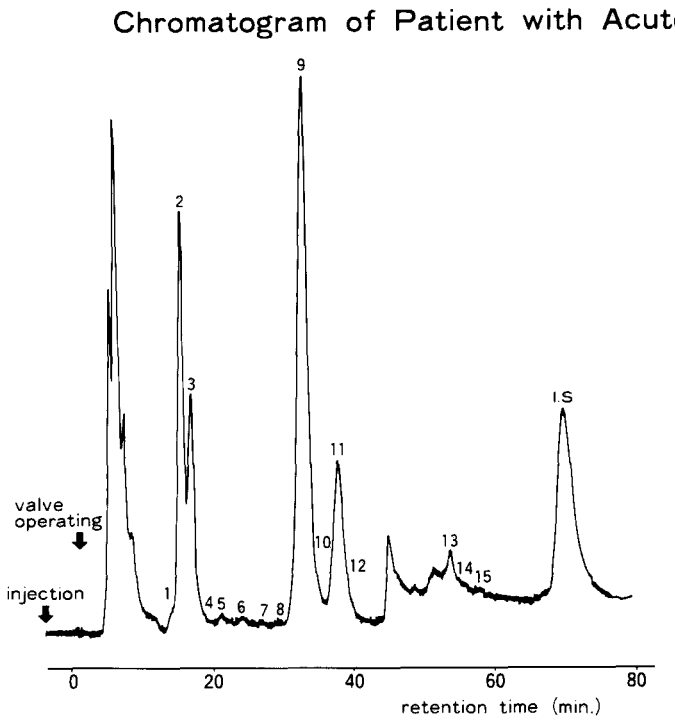

Fig. 9 : A bile acid pattern of serum in a patient with acute hepatitis

The number of peaks on these examples indicates the same substance shown in the standard chromatogram

DISCUSSION

This method allows analysis of 15 kinds of (3α)-hydroxy bile acids in the serum without tedious extraction steps and can be completed within only an hour. The strong points of this method are: firstly, adequate separation of each bile acid with neither chemical modification nor disadvantages

Bile acid μmol/ℓ Author and Method	No.	Total	UDCA group			CA group			CDCA group			DCA group			LCA group		
			UDCA	GUDCA	TUDCA	CA	GCA	TCA	CDCA	GCDCA	TCDCA	DCA	GDCA	TDCA	LCA	GLCA	TLCA
Present Method HPLC (Equipped with pretreatment system)	6	3.32 ±0.37	0.16 ±0.04	0.28 ±0.13	0.14 ±0.06	0.16 ±0.04	0.30 ±0.07	0.12 ±0.03	0.42 ±0.19	0.59 ±0.15	0.27 ±0.06	0.23 ±0.05	0.34 ±0.10	0.27 ±0.06	N.D.	0.04 ±0.01	0.08 ±0.03
Baba (4) HPLC (Using immobilized enzyme)	12	3.90 ±0.85	0.08 ±0.03	0.14 ±0.05	0.07 ±0.02	0.26 ±0.05	0.30 ±0.08	0.21 ±0.03	0.75 ±0.20	0.97 ±0.38	0.45 ±0.13	0.20 ±0.06	0.23 ±0.04	0.23 ±0.05	0.02 ±0.01	0.01 ±0.00	0.01 ±0.00
Baba (2) HPLC (Using enzyme solution)	8	2.88 ±0.74	0.07 ±0.04			0.14 ±0.05	0.16 ±0.07	0.16 ±0.05	0.42 ±0.16	0.47 ±0.05	0.36 ±0.08	0.30 ±0.05	0.46 ±0.26	0.36 ±0.08	N.D.	N.D.	N.D.
Demers (8) RIA	25						0.27 ±0.03			0.20 ±0.03			0.06 ±0.01				
Sandberg (9) GLC	18	0.9 ∿ 6.9		N.D.			0.09∿1.95			0.15∿3.9			0.18∿1.35			N.D.	
Shino (10) GC-MS	4			1.02			0.55			1.81			1.12			0.01	

Table 4: Individual bile acids in the serum of healthy subjects and patients with hepatobiliary diseases. Acute H. = Acute hepatitis. Chronic inact. = Chronic inactive hepatitis. Chronic act. = Chronic active hepatitis. Comp. = compensative stage. I.H.C. = Intrahepatic cholestasis. E.H.C. = Extrahepatic cholestatis

Disease	µmol /ℓ No.	T.B.A	UDCA	CA	CDCA	DCA	LCA	GUDCA	GCA	GCDCA	GDCA	GLCA	TUDCA	TCA	TCDCA	TDCA	TLCA
Normal	6	3.22 ±0.37	0.16 ±0.04	0.16 ±0.04	0.42 ±0.19	0.23 ±0.05	N.D.	0.28 ±0.13	0.30 ±0.07	0.59 ±0.15	0.34 ±0.10	0.04 ±0.01	0.14 ±0.06	0.12 ±0.03	0.27 ±0.06	0.27 ±0.06	0.08 ±0.03
Acute H.	16	216.84 ±34.17	0.10 ±0.06	1.05 ±0.47	0.37 ±0.13	0.16 ±0.07	0.17 ±0.08	1.09 ±0.45	46.02 ±8.70	77.38 ±14.22	2.21 ±1.81	0.41 ±0.15	0.55 ±0.17	37.40 ±6.39	48.25 ±9.89	1.38 ±0.88	0.30 ±0.14
Fatty liver	3	5.70 ±1.65	1.16 ±0.41	0.67 ±0.27	0.22 ±0.13	0.08 ±0.08	0.05 ±0.05	0.23 ±0.06	0.41 ±0.05	1.72 ±0.66	0.42 ±0.28	0.06 ±0.04	0.12 ±0.09	0.16 ±0.11	0.40 ±0.00	N.D.	N.D.
Chronic inact	16	6.51 ±0.93	0.13 ±0.03	0.15 ±0.06	0.31 ±0.09	0.38 ±0.09	0.01 ±0.01	0.26 ±0.05	0.78 ±0.16	1.65 ±0.44	0.64 ±0.17	0.04 ±0.01	0.20 ±0.04	0.44 ±0.11	1.15 ±0.43	0.21 ±0.04	0.10 ±0.06
Chronic act.	9	22.55 ±5.25	0.16 ±0.04	0.98 ±0.34	1.20 ±0.30	1.20 ±0.56	0.05 ±0.02	0.88 ±0.54	3.55 ±1.59	11.84 ±4.56	3.32 ±1.64	0.19 ±0.07	0.30 ±0.11	1.36 ±0.54	4.62 ±1.79	0.51 ±0.30	0.10 ±0.05
Cirrhosis comp.	9	26.36 ±3.86	0.91 ±0.49	0.54 ±0.19	1.22 ±0.31	0.82 ±0.26	0.03 ±0.02	0.24 ±0.05	2.12 ±0.62	7.86 ±2.12	1.43 ±0.56	0.11 ±0.03	0.20 ±0.07	1.94 ±0.51	7.47 ±1.83	1.33 ±0.42	0.14 ±0.09
Cirrhosis non-comp.	12	101.96 ±21.85	0.12 ±0.07	1.00 ±0.43	1.31 ±0.86	0.19 ±0.08	0.03 ±0.01	1.81 ±0.67	16.34 ±2.80	46.51 ±12.36	0.26 ±0.26	0.21 ±0.06	0.48 ±0.19	10.90 ±2.15	24.40 ±7.09	0.53 ±0.28	0.09 ±0.04
I.H.C	8	149.92 ±35.10	0.02 ±0.02	1.77 ±0.30	0.45 ±0.20	0.09 ±0.06	0.23 ±0.14	0.26 ±0.08	32.95 ±8.34	34.29 ±15.36	0.51 ±0.51	0.23 ±0.07	0.30 ±0.10	47.88 ±15.30	29.68 ±7.67	1.09 ±0.90	0.18 ±0.08
E.H.C.	5	377.94 ±182.85	N.D.	0.04 ±0.04	0.09 ±0.09	N.D.	0.03 ±0.02	26.47 ±23.49	73.40 ±33.65	107.88 ±79.41	8.53 ±8.53	0.47 ±0.20	1.73 ±1.35	71.56 ±30.20	79.32 ±50.53	8.15 ±8.15	0.29 ±0.29
Silent Stone	4	6.31 ±2.33	0.25 ±0.21	0.42 ±0.22	0.67 ±0.24	0.29 ±0.15	N.D.	0.29 ±0.17	0.68 ±0.32	2.08 ±0.93	0.47 ±0.27	0.04 ±0.02	0.19 ±0.09	0.11 ±0.04	0.50 ±0.19	0.93 ±0.09	N.D.

in the preparation; secondly, high sensitivity is obtained by high-performance liquid chromatography combined with fluorometric techniques using immobilized enzymes, which enables this measurement to be made in only 25 µl of even healthy human serum. There are several recent approaches to the component analysis of serum bile acids. Gas-liquid chromatography is the standard method. It allows assay of not only nonsulphated but also sulphated bile acids that are not detectable by our method (9). However, gas-liquid chromatography (GLC) suffers from such disadvantages as extraction, nonconjugation, final derivation prior to injection and substantial loss of bile acids during these procedures. All this results in a failure to identify minor components, such as lithocholic acid. Thus, it is inconvenient for routine clinical use. Gas chromatography-mass spectrometry (GC-MS) is the most advantageous of these techniques because no other method allows identification of unknown bile acids (10). However, this technique has the same disadvantages in the pretreatment procedure and is also rather costly. As a result, we must now consider GC-MS as the last resort technique for clinical use.

We can point to radioimmunoassay (RIA) as one of the approaches well suited to routine clinical use, provided the antibody specificity is carefully determined (8). An enzyme immunoassay is an improvement on radioimmunoassay, with respect to safety, because it does not use radioisotopes (11), though it still has many of the same disadvantages as the latter. Several ambitious trials involving high-

performance liquid chromatographic analysis have been reported (12,13,14). However, without exception, they require a tedious extraction step. Some of them have still more complicated procedures, such as a separating step prior to subjecting the bile acids to the high-performance chromatography (14).

In the present method, we made 200 analyses without a significant change in operating characteristics. Thus, this system can be fully automated quite easily. We hope that the ability to analyze individual circulating bile acid species quickly, easily and specifically will become clinically useful in differentiating various kinds of liver disease. We have shown the reproducibility of this method and have defined its practicality in clinical use.

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THE RAPID DETERMINATION OF NEOPTERINE IN HUMAN URINE BY ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Patrick Mura, Claude Tallineau, Daniel Reiss and Alain Piriou

Laboratoire de Biochimie et de Toxicologie
Hôpital Jean Bernard, BP 577 - 86021 POITIERS (FRANCE)

ABSTRACT

An isocratic high-performance liquid chromatography method is described for the determination of Neopterine eliminated in human urine, using a μ-Bondapak C_{18} column (300 x 3.9 mm I.D.) and a strongly polar phosphate buffer (pH 6.2) for elution. This analysis requires only 15 minutes and allows very good reproductibility of retention times. This method is well-suited for automation and routine clinical laboratory in order to quantify human urinary Neopterine in healthy subjects and in subjects with malignant disorders.

INTRODUCTION

Neopterine (2-Amino 4-hydroxy 6-trihydroxypropyl-pteridine) (Fig. 1) is produced from guanosine-5'-triphosphate by proliferating cells (1) or activated T-lymphocytes (2) and eliminated in the urine.

This elimination is increased in several diseases : viral diseases (2), atypical phenylketonuria (3), allograft rejections (2), and in a large number of neoplasic diseases (4) such as haematological neoplasias (5) and genital cancer (6). Furthermore, urinary Neopterine values are in connection with the severity of the disease and a rapid determination offers a great clinical interest.

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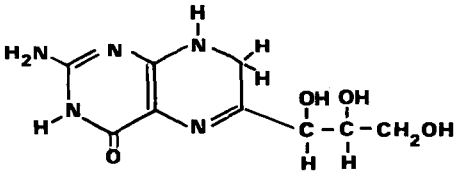

FIGURE 1. Neopterine structure.

The reversed phase liquid-chromatographic methods proposed to date, have used a flow and concentration program by adding acetonitrile (7) or methanol (8) to a phosphate buffer (15 mmol/l). This sophisticated program permits a good separation but does not afford a good reproductibility of retention times. Most of these methods propose a sample pretreatment (7)(9) under dim light which decreases the precision of the measure since pteridines are light sensitive. Niederwieser et al (10) report an automated liquid-chromatographic system for pteridines, thus eliminating sample pretreatment, but their method requires special equipment for automatic switching.

All these methods are complex and we propose here a simple isocratic HPLC method for the determination of urinary Neopterine using a strongly polar phosphate buffer and involving neither sample pretreatment nor special equipment. As an application of the method, variations of Neopterine eliminated according to sex and age were determined.

EXPERIMENTAL

High Performance Liquid Chromatography

The HPLC system used consists of a Beckman 112 solvent delivery system, coupled with a Kontron SFM 23 fluorescence spectrometer. Sample injection is via a Rheodyne 7125 injector fitted with a 50 µl sample loop. A (300 x 3.9 mm I.D.) µ-Bondapak C_{18} column was used for the analysis. The mobile phase was a phosphate buffer pH = 6.20 $\pm$ 0.10 : KH_2PO_4 (7 mmol/l) and $Na_2HPO_4 \cdot 12\ H_2O$

(33 mmol/l), thoroughly degassed before use. The flow rate was 1.5 ml/min. The detector was set at an excitation wavelength of 353 nm and an emission wavenlength of 438 nm.

Neopterine Standard and Reagents

KH_2PO_4 and $Na_2HPO_4 \cdot 12\ H_2O$ are from Merck (Darmstadt ; Germany). Neopterine purchased from Fluka (Buchs ; Switzerland) is dissolved in NaOH 0.5 N for a final concentration of 0.40 µg/ml. This stock solution was stored at -20°C in the dark.

Calibration Curve

The calibration curve (Fig. 2) was obtained from aqueous solutions by diluting the stock solution with NaOH 0.5 N in order to obtain 0.40, 0.20, 0.10 µg/ml of Neopterine and is linear within this range. Each calibration point was run in duplicate.

HPLC Determination

50 µl of the sample thoroughly diluted with NaOH 0.5 N (1:2 v/v) were injected (Fig. 3). The concentration of Neopterine was calculated by comparing peak heights with those of Neopterine standard solutions. The final result was expressed in µmoles of Neopterine per mole of creatinine. Urinary creatinine was quantified by kinetic Jaffé reaction. It is useful to relate urinary Neopterine to creatinine, because of the physiologically variable concentrations of urine.

RESULTS AND DISCUSSION

Many liquid-chromatographic methods for Neopterine have been developed and are considered to be efficient means of determining Neopterine in human urine. Fukushima and Nixon, (1979)(8) proposed an isocratic elution with methanol-water (1:19). According to our experience, acetonitrile or methanol in the mobile phase decreases the quality of the separation : the best separation is obtained using the phosphate buffer alone. Moreover, the absence of a buffer is prejudicial to the reproductibility of the retention time

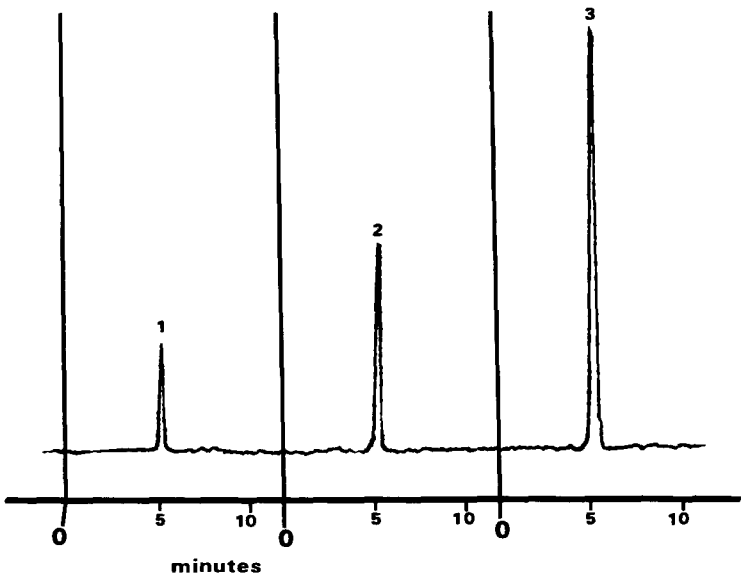

FIGURE 2. Calibration curve
1 : 0.10 µg/ml 2 : 0.20 µg/ml 3 : 0.40 µg/ml

of Neopterine which is very sensitive to changes in pH. Hausen et al (1982) (7) used a flow and concentration program by adding acetonitrile in order to reduce the analysis time. Because of the time required for returning to initial conditions, total analysis time was not really reduced.

The flow-rate of 1.5 ml/mn was found to be suitable to obtain high resolution and to complete the analysis in 15 minutes. Moreover, this constant flow-rate coupled with a high buffering capacity of the mobile phase produces a C.V. of 0.65 % for retention times of Neopterine (n = 20). Within-run precision of the method was evaluated by repeated analyses of a urine sample containing 140 µmoles/mole : the C.V. was 3.2 % (n = 15). We also investigated the influence of pH upon separation : it was optimized at a pH 6.20.

The use of fluorescent detection makes the method both specific and sensitive. Matsubara et al (1984) (3) proposed 338 nm for

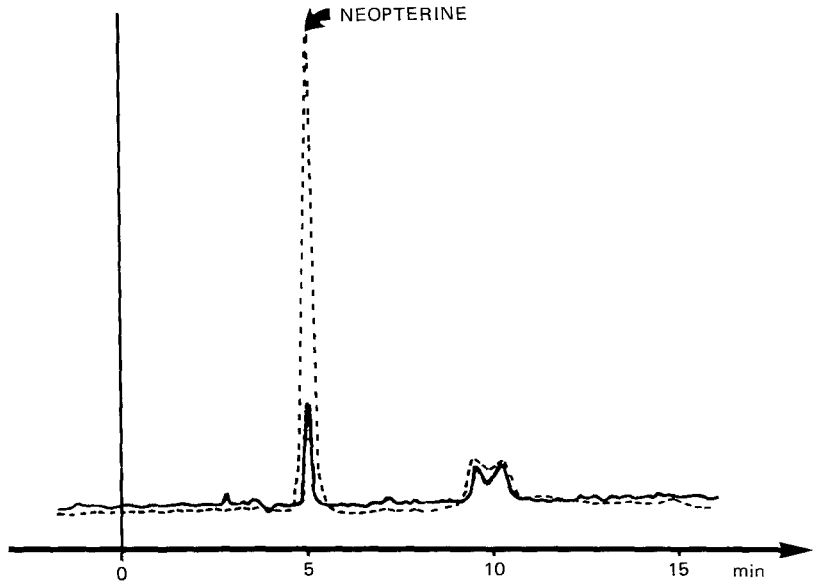

FIGURE 3. Chromatogram of a healthy adult's urine (————) containing 141 µmoles of Neopterine/mole of creatinine and of a patient's urine with ovarian cancer (------) containing 710 µmoles/mole.

excitation and 425 nm for emission. Wachter et al (7) used an excitation wavelength of 438 nm. The comparison of both methods shows that the second is twice as sensitive.

Under the conditions described, no interference from endogenous compounds in human urine was encountered : pretreatment clean-up sample procedure was therefore unnecessary. By avoiding this procedure, a better sensitivity was obtained and a detection limit of 0.02 µg/ml was observed.

Our experiments over a period of more than 5 months failed to reveal any significant changes in the separation efficiency of the µ-Bondapak C_{18} column. Due to the high buffering capacity of the mobile phase, the injection of this highly basic sample (pH 12) did not significantly shorten the column life.

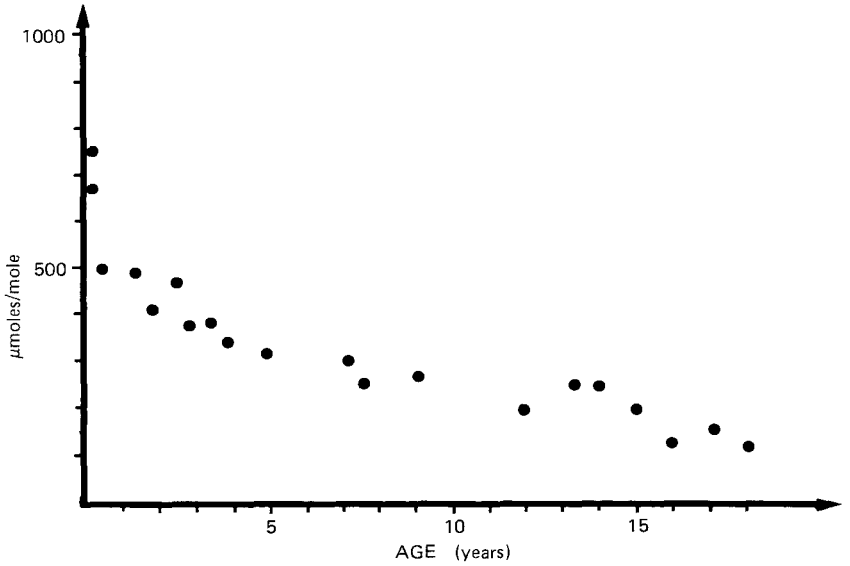

FIGURE 4. Physiological variations of Neopterine according to age.

An isocratic regime in combination with the appropriate phosphate buffer and flow-rate offers an improved analytical method for the assay of urinary Neopterine, which is well-suited for automation and clinical laboratory investigation.

Neopterine Values in Healthy Women and Men

Urinary Neopterine determinations were performed on 20 women and 20 men, all apparently healthy subjects from 30-40 years old. The values found in men were 118 ± 22 µmoles of Neopterine/mole of creatinine and 145 ± 18 µmoles/mole in women. The student t-test gives a t of 4.19, demonstrating that the mean values are significantly different. These results agree with previously published data (11), showing higher values in women than in men.

Neopterine Values in Connection with Age

Urinary Neopterine determination was performed on children of various ages. Fig. 4 shows very high values during the first months

of life and a decrease until the age of eighteen, when they correspond with established adult values.

ACKNOWLEDGEMENTS

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A GPC METHOD FOR ANALYSIS OF LOW MOLECULAR WEIGHT DRUGS

K. Sreenivasan, Prabha D. Nair and K. Rathinam*
Laboratory for Technical Evaluation of Biomaterials
and
*Division of Toxicology
Sree Chitra Tirunal Institute for Medical Sciences and Technology,
Trivandrum - 695 012.

ABSTRACT

Gel permeation chromatography has been shown as a versatile method for the analysis of low molecular weight drugs. Aspirin and its hydrolysis product, salicylic acid, which differ in a molecular weight of 42 units have been efficiently separated by a retention time difference of 3.25 minutes. Qualitative idea of other impurities is also obtained.

INTRODUCTION

Gel permeation chromatography (GPC) separates sample molecules by differences in effective molecular size in solution. This technique has been widely used for the determination of weight average molecular weight M_w molecular weight distribution and dispersity of polymeric materials. GPC is used

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primarily for high molecular weight (> 1000) components and its application in routine drug analysis is limited. We report here the use of GPC for the analysis of low molecular weight drugs. As a representative example we chose to analyse the amount of salicylic acid in commercially available aspirin samples.

Aspirin (acetyl salicylic acid) is widely used for its analgesic, anti-inflammatory and antipyretic effects. Aspirin has been found to interfere with platelet aggregation (1,2) reduce venous thrombosis (3) and is also associated with secondary prevention of myocardial infarction (4,5). Salicylic acid, the major decomposition product of aspirin, irritates the digestive system. The limit of salicylic acid content in aspirin powder is prescribed to be 0.1% by pharmacopoeias (6,7). But the assay for aspirin powder described in them is qualitative. There have been reports on the assay of salicylic acid in aspirin by HPLC (8,9) and GLC (10). However, these methods are time consuming, require chromatographic conditioning and even chemical derivatization for the GLC method (10). Reverse phase methods are not desirable as aspirin is not stable in the mixed

aqueous organic eluents in that form of LC. Gel permeation chromatography has been used to an advantage for the analysis of salicylic acid in aspirin. This method can also be used for other low molecular weight xenobiotics.

EXPERIMENTAL

Apparatus

The chromatographic system was a Waters Associates Model 6000A solvent delivery pump equipped with a U6K injector. A μStyragel column (30x0.78cm I.D. Pore size 100A°) and a model 440 absorbance detector. Chromatograms were obtained in a Houston Instruments Omniscribe recorder and peak absorbance values were directly read from the digital display of the Model 440 system.

Chromatographic Conditions

A flow of 1 ml/min of the mobile phase was used and absorbance monitored at 280nm.

Reagents

Aspirin AR (Sigma Chemicals, USA) and Salicylic acid (Wilson Laboratories, India) were used

for preparing the standard samples. Aspirin was used after recrystallization in acetone. Freshly distilled Dichloromethane Analar (Glaxo Laboratories, India) was used for preparing the solution and as the mobile phase.

Calibration curve

The calibration curve for salicylic acid was prepared by noting the peak absorbance values for salicylic acid solutions at varied concentrations (10 to 100 μg/ml)

Aspirin samples

Standard aspirin sample consisted of a dichloromethane stock solution containing 1.2 mg/ml of standard aspirin.

Other samples were 1-2 mg/ml solution of 6 commercially available aspirin samples in dichloromethane. Samples were injected as soon as they were prepared and the injection volume for all samples was 100 μl.

RESULTS AND DISCUSSION

Under the experimental conditions described above, aspirin and salicylic acid had a retention

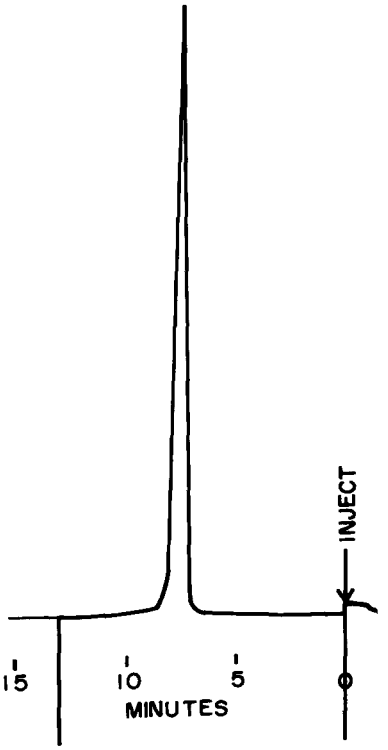

FIGURE 1 - Chromatogram of aspirin (1.2 µg/ml) in the mobile phase.

time of 7.5 ± 0.1 and 10.75 ± 0.1 minutes respectively.

Fig.1 is a chromatogram of standard aspirin (1.2mg/ml) in the mobile phase. Fig.2 is a chromatogram of standard salicylic acid (70 µg/ml) Fig.3 is a chromatogram of one of the commercial samples of aspirin, peak 1 corresponds to aspirin,

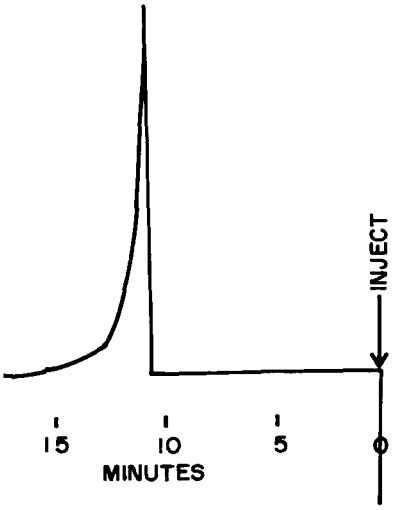

FIGURE 2 - Chromatogram of salicylic acid (70 μg/ml) in the mobile phase.

Peak 2 corresponds to salicylic acid (37.5 μg/ml), other peaks correspond to unidentified impurities. Salicylic acid concentrations in commercial aspirin samples ranged from 3-37.5 μg/ml. The minimum limit of detection for salicylic acid is 0.6 μg.

Adsorption techniques such as normal phase and reverse-phase liquid chromatography require extensive methods development time. However, in GPC analysis time is reduced considerably in additon to the simplicity of operating conditions. Salicylic acid and aspirin differ only in a molecular

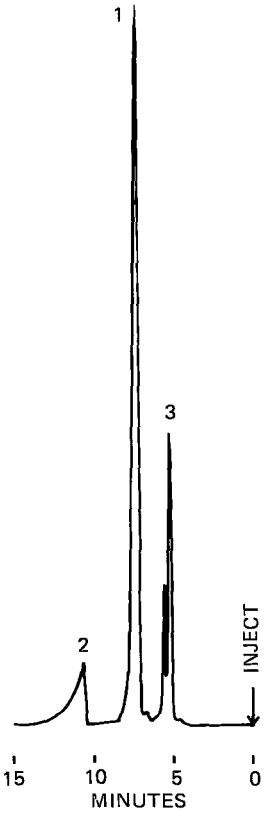

FIGURE 3 - Chromatogram of one commercial aspirin sample. Peak 1 corresponds to pure aspirin. Peak 2 corresponds to salicylic acid (37.5 µg/ml).

weight of 42 units, but are separated by a retension time of 3.25 minutes. Further, a qualitative idea of the nature of other impurities in aspirin can be obtained e.g. Peak 3 in Fig.3 corresponds to an impurity having higher molecular weight than aspirin.

In conclusion, Gel permeation chromatography has clearly been shown as a versatile technique for analysis of low molecular weight drugs.

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ON THE AVAILABILITY OF 2,3,4,6-TETRA-O-ACETYL-β-D-GLUCOPYRANOSYL ISOTHIOCYANATE, A CHIRAL DERIVATIZING AGENT IN HPLC

Joseph Gal
Departments of Medicine and Pharmacology
Division of Clinical Pharmacology
and
Robert C. Murphy
Department of Pharmacology
University of Colorado
School of Medicine
Denver, Colorado 80262

ABSTRACT

During the last few years, the chiral derivatizing agent 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (TAGIT) has been shown to be valuable in the separation of the enantiomers of a variety of compounds by HPLC. The sale of the reagent by its sole commercial supplier was recently discontinued, but its unacetylated analog was made available. The chemical structure of a sample of the unacetylated compound obtained from the supplier was investigated using methane and ammonia chemical ionization mass spectrometry, proton nuclear magnetic resonance, comparison of melting points, and HPLC. By all these criteria the "unacetylated" compound was shown to be, in fact, the acetylated analog, i.e., TAGIT. In view of the value of TAGIT in HPLC, its continued availability, confirmed by the supplier, is welcome.

INTRODUCTION

The high-performance-liquid-chromatographic (HPLC) resolution of enantiomers is receiving considerable attention (1-8). One approach to this problem is derivatization of the enantiomers with a chiral reagent, followed by chromatographic separation of the resulting diastereomers (2,4,6,8). Compound I, 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothio-

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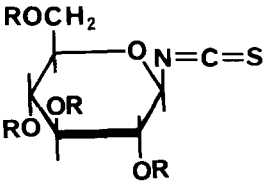
cyanate (TAGIT) was introduced recently as a chiral derivatizing reagent in HPLC (9-14). TAGIT reacts with primary and secondary amino groups rapidly and under mild conditions to give the corresponding thioureas. The reagent has been shown to be highly useful in the HPLC resolution of the enantiomers of a variety of amino acids and their esters (9,10), epinephrine and norepinephrine (11), propranolol and several related β-adrenergic antagonists (12), ephedrine, pseudoephedrine, and norephedrine (13), and several 1-phenyl-2-aminopropanes (amphetamines) (14). TAGIT has been commercially available from Polysciences Inc. (Warrington, PA), but recent attempts by us and by others (15,16) to obtain the reagent from that supplier have been unsuccessful: the sale of the compound was said to have been discontinued. Consistent with this information, the 1984-85 catalog of Polysciences listing the currently available chemicals contains no entry for TAGIT. The catalog does contain, however, an entry for β-D-glucopyranosyl isothiocyanate (catalog no. 8648, structural formula II), i.e., the unacetylated analog of TAGIT. We were interested in exploring this compound as a chiral derivatizing agent alternative to TAGIT, although the stability of II, a polyhydroxy isothiocyanate, appeared doubtful. In this communication we describe the unexpected results obtained.



	R
I	$COCH_3$
II	H

EXPERIMENTAL

Melting points were determined on Fisher-Johns melting point apparatus and are uncorrected.

TAGIT was obtained from Polysciences Inc. before the sale of the compound was discontinued. The m.p. of TAGIT was 114°-116° (lit. (9) m.p. 113-115°). Samples of purported β-D-glucopyranosyl isothiocyanate (II) were also purchased from Polysciences Inc.

Derivatization and Chromatography

The reaction of the chiral reagent with propranolol and the subsequent HPLC analysis were carried out as described previously (12).

Mass Spectrometry

Mass spectrometry was carried out using a VG Micromass 7070H (Altrincham, United Kingdom) instrument in the chemical ionization (CI) mode. Ultra pure methane (Matheson Gas, Caucaln, NJ) and ammonia were used as reagent gases. The reagent gas pressure was adjusted to maximum MH^+ (m/z 107) production for xylene which corresponds to 0.7 to 1 torr in the ion source. The electron beam was 100 eV. Samples were introduced by the solid probe with an ion source temperature of 140°.

Nuclear Magnetic Resonance

The 90-MHz proton nuclear magnetic resonance (PMR) spectra were recorded using a Varian Assoc. (Palo Alto, CA) EM 390 instrument. The compounds were dissolved in deuteriochloroform containing tetramethylsilane (TMS) as internal reference.

RESULTS

The melting point of a sample purchased as II was 113-115°, which is identical to the reported melting point of TAGIT (9). A mixed-melting-point determination with authentic TAGIT also gave 113-115°.

The ammonia CI mass spectrum of purported II is shown in Figure 1. The spectrum was identical to that of a sample of TAGIT. Similarly, the methane CI mass spectrum of the compound thought to be II (Figure 2) was identical to the spectrum of TAGIT.

The 90-MHz PMR spectrum of the two samples were also compared (spectra not shown). Again, the two spectra were identical. In particular, the acetyl methyl protons were present in both spectra, appearing as two singlets at 2.03 and 2.12 ppm downfield from TMS.

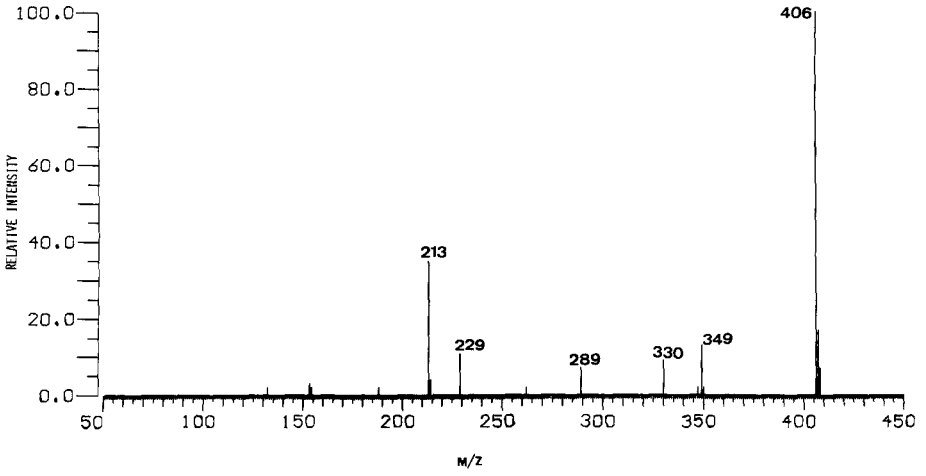


FIGURE 1 Ammonia CI mass spectrum of substance purchased as compound II.

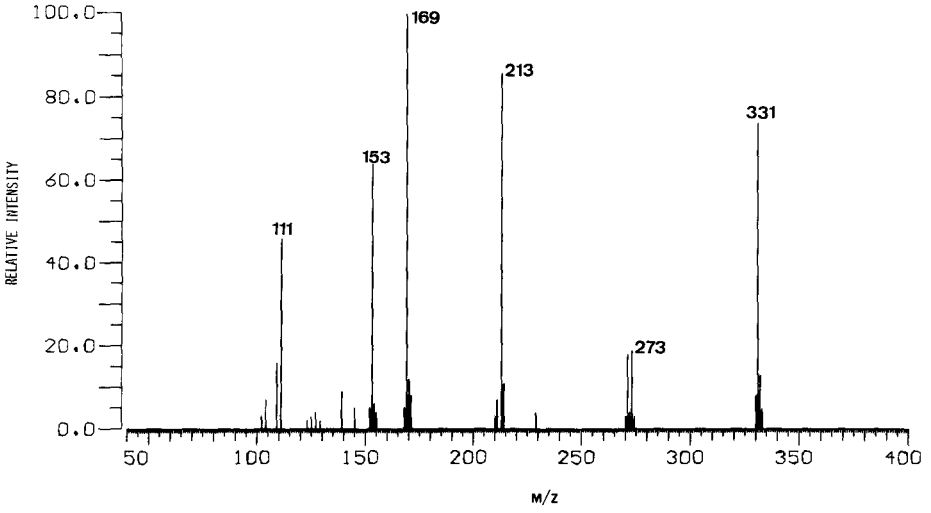


FIGURE 2 Methane CI mass spectrum of substance purchased as compound II.

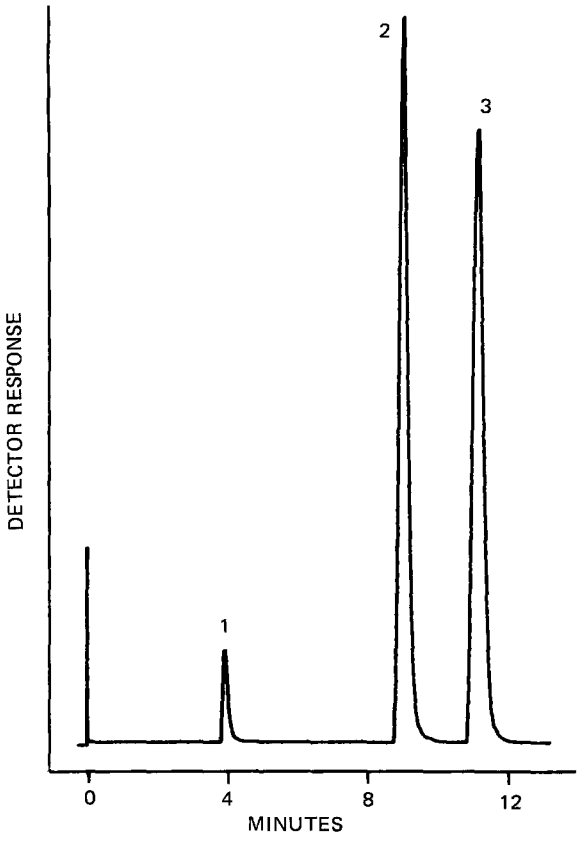


FIGURE 3 Chromatogram of racemic propranolol derivatized with purported II. Peak 1: reagent peak; peak 2: derivative of (-)-propranolol; peak 3: derivative of (+)-propranolol.

Derivatization of (±)-propranolol with purported II and HPLC analysis of the products yielded the chromatogram shown in Figure 3. Derivatization with TAGIT gave a chromatogram indistinguishable from that shown in Figure 3, and essentially identical to chromatograms obtained previously (12). When (R)-(+)-propranolol was derivatized with the reagent obtained as II, only the diastereomer with the longer retention time (peak 3 in Figure 3) was obtained. As expected, this was identical to the results obtained with TAGIT.

DISCUSSION

The substance obtained from the supplier as compound II was analyzed using several analytical techniques. By all criteria the substance was found to have, in fact, structure I and was indistinguishable from TAGIT. For example, the mass spectra of the substance (Figures 1 and 2) were consistent with structure I and were essentially identical to those of TAGIT. There was no molecular ion species observed in the methane CI mass spectrum of TAGIT (Figure 2), which was not unexpected. The major high mass ion at m/z 331 corresponds to a loss of isothiocyanate (58 daltons). The major ions arise from multiple losses of acetate and ketene. The ammonia CI mass spectrum was investigated in view of the known reactivity of TAGIT with amines to form thioureas (Figure 1). In fact the major ion observed corresponded to the ammonia attachment ion at m/z 406 (389+17 daltons). The fragmentation of this ion was greatly reduced, no doubt due to covalent bond formation.

The PMR spectra and the results of the melting-point determinations also confirmed the identity of the substance as TAGIT. Not surprisingly, when the reagent was used to derivatize propranolol, the HPLC chromatogram obtained (Figure 3) was identical to that obtained using authentic TAGIT. Furthermore, derivatization of (+)-propranolol revealed that the enantiomeric purity of the chiral derivatizing reagent was > 99.5% since no peak was detectable for the second diastereomer.

It is clear from the foregoing that the sample of compound no. 8648 in the Polysciences catalog obtained by us is in fact TAGIT. We have reported our findings to Polysciences, and the supplier agrees with our conclusion about the identity of the substance (17). Furthermore, compound II is not available from Polysciences, and it appears to us that its listing was simply an error in identifying TAGIT. We now understand that this error will be corrected and that TAGIT--correctly identified--will again be available (17). In view of the value of TAGIT as a chiral reagent in HPLC (9-14), its continued availability is indeed welcome.

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JOURNAL OF LIQUID CHROMATOGRAPHY, 7(11), 2315-2317 (1984)

LC NEWS

FULLY PROGRAMMABLE UV-VIS DETECTOR is microprocessor controlled. It features variable wavelength from 190-700 nm and .001 AU full scale with less than 2% noise, automation of wavelength, sensitivity, event mark, auto zero, etc. Kratos Analytical Instruments, JLC/84/11, 170 Williams Dr., Ramsey, NJ, 07446, USA.

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TLC STICK is a solid, elongated block of silica gel designed for use in a wide variety of separations. It can be spotted with a maximum of 300mg or 20ml of sample...1,000 to 10,000 times more sample than other related methods. Recovery is achieved by slicing the desired band from the stick and extracting with an appropriate solvent. Alltech Associates, Inc., Applied Science Labs, JLC/84/11, 2051 Waukegan Rd., Waukegan, IL, 60031, USA.

OPTIMIZING HPLC PRECISION BY SKILLFUL INJECTION is a report that can help both the beginner and the experienced chromatographer. Some very practical questions are addressed: "How much sample must be wasted to fill a sample loop?"; "When you are judging the amount of sample being loaded by reading a syringe, how much sample can you inject without impairing accuracy?" Ask for Tech Note No. 5. Rheodyne, Inc., JLC/84/10, P. O. Box 996, Cotati, CA, 94928, USA.

POLYACRYLAMIDE RIGID GEL FOR SEC/GPC may be operated at high pressures and undergoes minimum swelling and shrinkage when transferred between polar eluents. Applications include separations of polysaccharides, polyphenols, and synthetic aqueous polymers. Polymer Laboratories, JLC/84/11, Essex Road, Church Stretton, Shropshire, SY6 6AX, UK.

LC CAPABILITY BOOK contains 56 pages of illustrations and information including "Choosing the System", that helps potential users determine the most appropriate equipment for their needs. Pye Unicam, Ltd., JLC/84/11, York Street, Cambridge CB1 2PX, UK.

CHROMATOGRAPHY AUDIO COURSES are available covering Basic Gas Chromatography; Column Selection in Gas Chromatography; Modern Liquid Chromatography; Modern Liquid Chromatography, Special Topics. American Chemical Society, JLC/84/11, 1155 Sixteenth Street, NW, Washington, DC, 20036, USA.

"NEW FOR OLD" HPLC COLUMNS is a service that includes cleaning out the hardware, changing the frits or meshes, and repacking with new material. Repacked columns are claimed to have the same or higher efficiencies that those of other manufacturers' new ones. Phenomenex, JLC/84/11, 426 Via Corta, Bldg 305, Palos Verdes Estates, CA, 90274, USA.

DUAL PISTON HPLC PUMP includes an electronically controlled motor, two sapphire pistons, two cartridge-type ruby ball check valves, and an ingenious cam arrangement to maintain the flow rate of the mobile phase. The cam achieves the correct precompression and pressure feedback compensates electronically for liquid compressibility. Sonntek, Inc., JLC/84/11, P.O.Box 8731, Woodcliffe Lake, NJ, 07675, USA.

ACETYLCHOLINE ANALYZER utilizes a rapid reverse phase separation of acetylcholine and choline and a post-column enzymatic reactor module to achieve detection limits of 2 pmol or better. Detection is based upon electrochemical oxidation of hydrogen peroxide, released in the enzymatic reaction using a platinum electrode. Bioanalytical Systems, Inc., JLC/84/11, 2701 Kent Avenue, West Lafayette, IN, 47906, USA.

RESOLUTION OF EVEN HIDDEN COMPOUNDS is achieved through use of two user-selected wavelengths to analyze compounds of like or similar

chemical structure. Overlapping peaks are resolved without changing analysis conditions or resorting to exotic and expensive detection methods. Micromeritics Instrument Co., JLC/84/11, 5680 Goshen Springs Road, Norcross, GA, 30093, USA.

JOURNAL OF LIQUID CHROMATOGRAPHY, 7(11), 2319-2322 (1984)

LC CALENDAR

1984

SEPTEMBER 10-14: Advances in Liquid Chromatography, including the 4th Annual American-Eastern European Symposium on LC and the Int'l Symposium on TLC with Special Emphasis on Overpressured Layer Chromatography, sponsored by the Hungarian Academy of Sciences' Chromatography Committee & Biological Research Center and the Hungarian Chemical Society, in Szeged, Hungary. Contact: Dr. H. Kalasz, Dept. of Pharmacology, Semmelweis University of Medicine, P.O.Box 370, H-1445 Budapest, Hungary, or Dr. E. Tyihak, Research Inst. for Plant Protection, P.O.Box 102, H-1525 Budapest, Hungary.

SEPTEMBER 16 - 21: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: D. B. Chase, DuPont Co., Experimental Station 328, Wilmington, DE, 19898, 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.

OCTOBER 8 - 10: ASTM Committee E-19 on Chromatography, St. Louis Sheraton Hotel, St. Louis, MO. Contact: F. M. Rabel, Whatman, Inc., 9 Bridewell Place, Clifton, NJ, 07014, USA.

OCTOBER 24 - 26: Third Workshop/Symposium on LC/MS and MS/MS, Montreux, Switzerland. Contact: R. W. Frei, Dept. of Anal. Chem., Free University, De Boelelaan 1083, NL-1081 HV Amsterdam, The Netherlands.

OCTOBER 28 - NOVEMBER 1: 2nd International Congress on Computers in Science, Washington, DC. Contact: S. R. Heller, EPA, PM-218, Washington, DC, 20460, USA.

NOVEMBER 13 - 16: 23rd Eastern Analytical Symposium, New York Penta Hotel, New York City. Contact: S. D. Klein, Merck & Co., P. O. Box 2000/R801-106, rahway, NJ, 07065, USA.

NOVEMBER 19-24: Expoquimia: International Chemical Forum, Barcelona, Spain. Contact: Expoquimia, Feria de Barcelona, Barcelona, Spain.

DECEMBER 10-12: "TLC/HPTLC-84: Expanding Horizons in TLC," Sheraton-University City, Philadelphia, PA. Contact: J. C. Touchstone, University of Pennsylvania, Dept. OB-GYN, 3400 Spruce eStreet, Philadelphia, PA.

DECEMBER 10-12: Fourth International Symposium on HPLC of Proteins, Peptides and Polynucleotides, Baltimore, MD. Contact: Shirley E. Schlessinger, Symposium Manager, 400 East Randolph, Chicago, IL, USA.

DECEMBER 16-21: International Chemical Congress of Pacific Basin Societies, Honolulu, Hawaii, sponsored by the Chemical Inst. of Canada, Chemical Soc. of Japan, and the American Chem. Soc. Contact: PAC CHEM '84, International Activities Office, American Chem. Soc., 1155 Sixteenth St., NW, Washington, DC, 20036, USA.

1985

FEBRUARY 10-15: Symposium on the Interface Between Theory and Experiment, Canberra, Australia. Contact: Leo Radom, Research School of Chemistry, Australian National University, Canberra, ACT 2601, Australia.

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.

FEBRUARY 25 - MARCH 1: Pittsburgh Conference on Analytical Chemistry & Applied Spectroscopy, New Orleans, LA. Contact: Linda Briggs, 437 Donald Road, Pittsburg, PA, 15235, USA.

MARCH 23-24: Conference on Creativity & Science, Honolulu, Hawaii. Contact: D. DeLuca, Scientists and Humanities Conf., Winward Community College, University of Hawaii, 45-720 Keaahala Rd., Kaneohe, Hawaii, 96744, USA.

APRIL 15 - 17: Second International Symposium on Instrumental TLC, Wurzburg, West Germany. Contact: H. M. Stahr, 1636 College Veterinary Medicine, Iowa State University, Ames, IA, 50011, USA., or Prof. S. Ebel, Institute for Pharmacies, A. M. Hubland, D-8700 Wuerzburg, West Germany.

APRIL 15-18: Materials Research Society Spring Meeting, San Francisco, CA. Contact: Susan Kalso, Xerox Palo Alto Res. Center, 3333 Coyote Hill Road, Palo Alto, CA, 94304, USA.

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

APRIL 29 - MAY 2: Symposium on Analytical Metods in Forensic Chemistry & Toxicology, Miami, Florida; in conjunction with 189th ACS Nat'l Meeting. Contact: Dr. M. H. Ho, Dept. of Chem., University of Alabama, Birmingham, AL, 35294, USA.

MAY 19: Middle Atlantic Regional ACS Meeting, Sponsored by ACS Monmouth County Section. Contact: M. Parker, Dept. of Chem., Monmouth College, West Long Branch, NJ, USA.

JUNE 9-15: ACHEMA 85, Frankfurt, West Germany. Contact: DECHEMA, Organization ACHEMA, P.O.Box 97 01 46, D-6000 Frankfurt, 97, West Germany.

JUNE 24 - 28: 59th Colloid & Surface Science Symposium, Clarkson College of Technology, Potsdam, NY. Contact: J. P. Kratohvil, Institute of Colloid & Surface Science, Clarkson College of Technology, Potsdam, NY, 13676, USA.

JULY 1-5: Ninth International Symposium on Column Liquid Chromatography, sponsored by the Chromatography Discussion Group and by the Royal Society of Chemistry's Chromatography & Electrophoresis Group, Edinburgh, Scotland. Contact: Prof. J. H. Knox, 9th ISCLC Secretariat, 26 Albany Street, Edinburgh, EH1 3QH, Great Britain.

JULY 4: 4th International Flavor Conference, Greece. Contact: C. J. Mussinan, IFF R&D, 1515 Highway 36, Union Beach, NJ, 07735, USA.

SEPTEMBER 1-6: 6th International Symposium on Bioaffinity Chromatography & Related Techniques, Prague, Czechoslovakia. Contact: Dr. J. Turkova, Institute of Organic Chemistry & Biochemistry CSAV, Flemingovo n. 2, CS-166 10 Prague 6, Czechoslovakia.

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

SEPTEMBER 29 - OCTOBER 4: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Marriott Hotel, Philadelphia, PA. Contact: T. Rains, NBS, Center for Analytical Chemistry, Chemistry B-222, Washington, DC, 20234, 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

SEPTEMBER 21-26: XVIth International Symposium on Chromatography, Paris, France. Contact: G.A.M.S., 88,Boulevard Malesherbes, F-75008 Paris, France.

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.

1988

JUNE 5 - 11: 3rd Chemical Congress of the North Americanmn Continent, Toronto, Ont., Canada. Contact: A. T. Winstead, ACS, 1155 Sixteenth St, NW, Washington, DC, 20036, USA.

SEPTEMBER 25 - 30: 196th ACS National Meeting, Los Angeles, CA. Contact: A. T. Winstead, ACS, 1155 Sixteenth 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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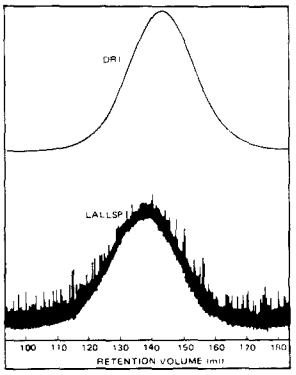

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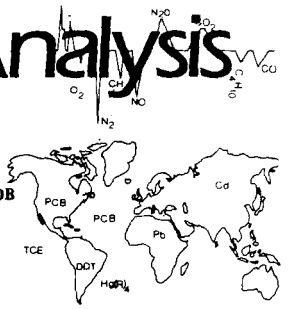

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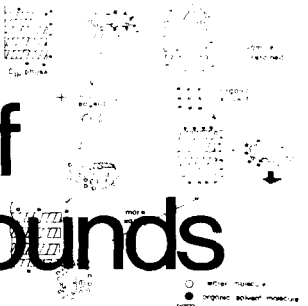

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(Chromatographic Science Series, Volume 26)

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9. Only standard symbols and nomenclature approved by the International Union of Pure and Applied Chemistry should be used.

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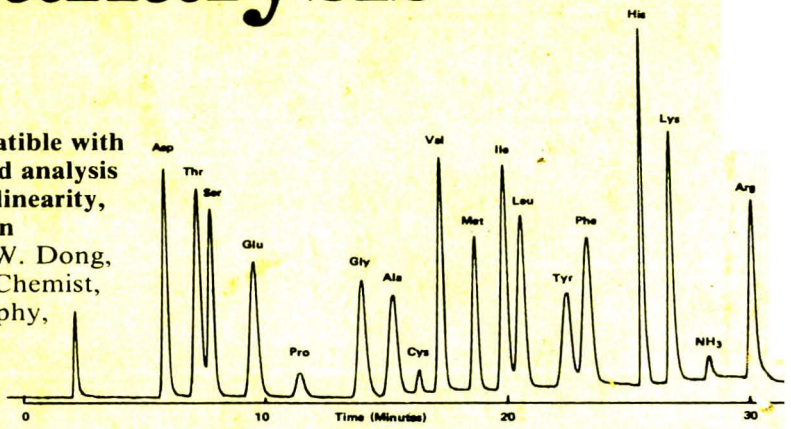
"Buffēlutes are compatible with high-speed amino acid analysis with good precision, linearity, resolution and column lifetime..." Michael W. Dong, Senior Applications Chemist, Liquid Chromatography, Perkin-Elmer.



Your HPLC can resolve amino acids like this with Pierce's new Buffēlute High Performance Buffers and High Speed Ion-Exchange Column. Buffēlutes are sodium based, non-citrate buffers designed for high sensitivity with minimal baseline noise. No other commercial buffers can match the performance of Buffēlutes.

The High Speed Ion-Exchange Column is packed with a special sulfonated polystyrene divinylbenzene copolymer. The column, the Buffēlute buffers and Fluoraldehyde™ Reagent Solution (*o*-phthalaldehyde) are designed to work in combination for the analyst who requires high speed and high resolution of amino acids.

For more information on these products call or write Product Manager Steve Seely. Ask about our **Special Introductory Offer** on these products: 800-435-2960 (Illinois residents call 815-968-0747 collect)

800-8-PIERCE
(direct line to order desk)

27615 Buffēlute Hydrolyzate Buffer Kit
25132 High Speed Ion-Exchange Column
26025 Fluoraldehyde OPA Reagent Solution

PIERCE CHEMICAL COMPANY
P.O. Box 117, Rockford, IL U.S.A. 61105

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