ISSN 0378-4347

VOL. 231 NO. 1 AUGUST 13, 1982

(Biomedical Applications, Vol. 20, No. 1)

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
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Printed in The Netherlands

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(Abstracts/Contents Lists published in Analytical Abstracts, Biomedical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index)

(Continued overleaf)

Electron Capture – Theory and Practice in Chromatography

edited by A. ZLATKIS, *Houston, TX, USA and* C.F. POOLE, *Detroit, MI, USA*

JOURNAL OF CHROMATOGRAPHY LIBRARY – Volume 20

Sept. 1981 xii + 418 pages
Price: US $76.50/ Dfl. 180.00
ISBN 0-444-41954-3

This book provides the first comprehensive coverage of all aspects of the theory, design, operation and applications of the electron capture detector (ECD) from the chromatographer's point of view. In addition, an up-to-date look at the ancillary techniques of selective electron-capture sensitization, atmospheric pressure ionization and plasma chromatography has been included. ECD users will find the solutions to instrumental and technical problems which arise during practice particularly valuable. These have been derived from the experiences of the internationally distinguished team of authors.

Each chapter has been prepared by experts in their field and provides an in-depth coverage of its topic. The basic theory of the mechanisms of electron capture detection is included. Practical sections form the bulk of the book and are devoted to such topics as the construction and operating principles of the detector, including the establishment of instrument design criteria, and the different methods of derivatization. A more personal touch is provided by the inventor of the ECD, J.E. Lovelock, in his review of the development of the technique. Other chapters illustrate the importance of ECD in trace analysis in environmental and biomedical research. A unique feature is the extensive tabulation of all the pertinent data concerning the use of ECD in gas and liquid chromatography.

For those analytical chemists who use chromatography in their research, this book should become a standard text.

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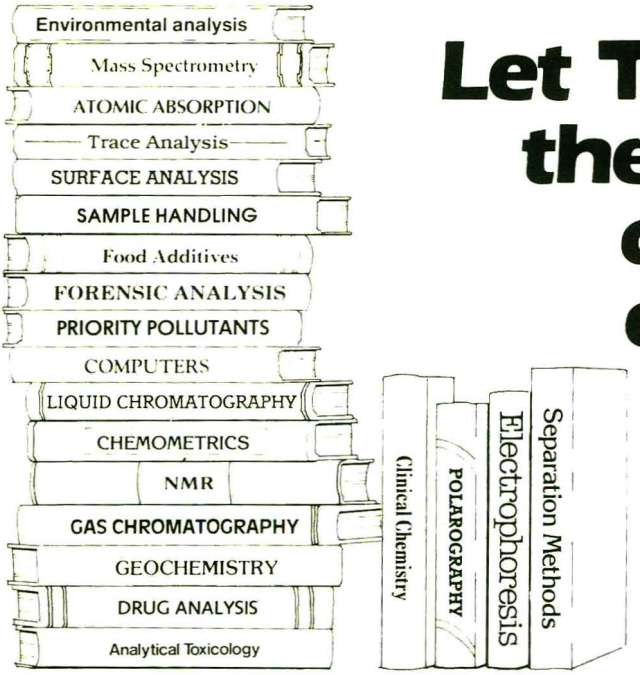

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JOURNAL OF CHROMATOGRAPHY

VOL. 231 (1982)

(Biomedical Applications, Vol. 20)

JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

VOL. 20 1982

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

Printed in The Netherlands

Journal of Chromatography, 231 (1982) 1—12
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1287

ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN SERUM APOLIPOPROTEINS

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(First received November 3rd, 1981; revised manuscript received March 8th, 1982)

SUMMARY

The rapid separation of seven urea-soluble apolipoprotein species from delipidated human serum very low density lipoproteins (VLDL) and high density lipoproteins (HDL) has been achieved by high-performance liquid chromatography on an anion-exchange column of Syn-Chropak AX 300. Effluent chromatographic peaks were detected by absorbance at 280 nm in a flow-through cell. Peaks corresponding to apolipoproteins AI_1, AI_2, AII, CI, CII, $CIII_1$, and $CIII_2$ were identified by amino acid analysis, gel electrophoresis, and isoelectric focusing. Maximum efficient loading of semipreparative columns (250 × 9.0 mm) was established to be ca. 20 mg HDL apolipoprotein. Minimum detectable protein was shown to be ca. 1 μg on an analytical-scale column (300 × 4.5 mm). Chromatographic resolution is comparable to that of conventional DEAE-cellulose column chromatography. The ratio of $apoAI_1$ to $apoAI_2$ was considerably greater in high-performance liquid chromatography, suggesting that the variants seen in conventional chromatography and isoelectric focusing are in part artifactual.

INTRODUCTION

The apolipoproteins of human serum are important determinants of lipoprotein metabolism by virtue of their lipid-binding capacity and transport roles, interparticle exchangeability, enzyme-cofactor roles, and affinity for specific receptors [1, 2]. The human apolipoprotein population includes AI_{1-3}, AII, CI, CII, $CIII_{0-2}$, E_{1-4}, several minor species that are urea-soluble, and apoB, the major apolipoprotein of low density lipoprotein (LDL) and very low density lipoprotein (VLDL), which usually becomes insoluble upon delipidation. The urea-soluble apolipoproteins are present in very different ratios in high density lipoproteins (HDL), in which apoAI and apoAII constitute ca. 90% of the protein mass, than in VLDL, in which apoC's and apoE constitute 40—60% [3, 4].

The continuing elucidation of lipoprotein metabolism, structure, and func-

tion requires both routine isolation of pure apolipoproteins and analytical determination of the apolipoprotein composition of both HDL and VLDL. The advent of SynChropak AX 300, a protein-compatible anion-exchange support [5, 6] for high-performance liquid chromatography (HPLC), provided the opportunity to apply the well documented resolution of anion-exchange chromatography [7, 8] to the separation of the HDL apolipoproteins. We have identified seven of the chromatographic peaks obtained by Tris · HCl gradient elution of a semipreparative (250 × 9 mm) column of SynChropak AX 300 and demonstrated the loading and sensitivity levels that limit anion-exchange chromatography for preparative and analytical applications.

EXPERIMENTAL

Preparation of HDL and apoHDL

HDL (ρ = 1.065—1.210 g/ml) was isolated from the fresh sera of normal males by the sequential flotation ultracentrifugation procedure of Lindgren et al. [9]. VLDL and LDL were removed by centrifugation of a 1.063 g/ml solution for 18 h at 14°C at 103,000 *g* in a Beckman Type 40.3 rotor (Beckman Instruments, Palo Alto, CA, U.S.A.). Total HDL was isolated by centrifugation of a ρ = 1.21 g/ml solution for 48 h under similar conditions. The lipoprotein composition of these fractions was analyzed by gradient polyacrylamide gel electrophoresis on 2.5—27% gradient polyacrylamide gels (Isolab, Akron, OH, U.S.A.). Albumin and LDL contamination was minimal. Salt was removed by dialysis (2 × 100:1, v/v) against nitrogen-saturated double-distilled water. The HDL was delipidated with ethanol—diethyl ether as previously reported [10]. The protein concentration of the apoHDL was determined by the method of Lowry et al. [11].

Isolation of the urea-soluble VLDL apolipoproteins

VLDL (ρ < 1.007 g/ml) of a 12-h fasted Type IV hyperlipidemic male was isolated from the fresh serum by the ultracentrifugal method of Chung et al. [12] using a Beckman VT 50 rotor. The upper 2 ml contained the VLDL, the purity of which was verified by gradient polyacrylamide gel electrophoresis on 2—16% polyacrylamide gels (Pharmacia, Uppsala, Sweden). Delipidation was performed by the tetramethylurea (TMU) extraction method of Kane et al. [13], which precipitates apoB.

Anion-exchange HPLC

Anion-exchange HPLC of apoHDL and apoVLDL was performed with SynChropak AX 300, an oxirane-crosslinked polyethyleneimine-coated 10-μm macroporous spherical silica support. Lots 363, 374 and 403 from SynChrom (Linden, IN, U.S.A.) were used with some variation in results. Both semipreparative (250 × 9 mm) and analytical-scale (300 × 4.5 mm) columns were fabricated from stainless-steel tubing and Whatman high-pressure fittings (Whatman, Clifton, NJ, U.S.A.) and then packed at 400 bar with a slurry of 0.5 g AX 300 support per ml of methanol with a slurry packer (Model CP111, Jones Chromatography, Columbus, OH, U.S.A.). Columns were fitted with 60 × 2 mm guard columns (Whatman) of SynChropak GPC 100, which were replaced

after operating pressures exceeded 140 bars. After 10 min preequilibration with starting buffer, the AX 300 columns were eluted at 1 ml/min with linear gradients of Tris · HCl in 6 *M* urea at pH 7.9 at room temperature (ca. 22°C) and at 40—133 bar, using a pair of Waters 6000A HPLC pumps (Waters Assoc., Milford, MA, U.S.A.). Gradients were programmed with a Waters Model 660 solvent programmer. All injections were made manually with Hamilton syringes (Hamilton, Whittier, CA, U.S.A.) into a Waters U6K injector. Samples for semipreparative columns were injected in 2 ml of 6 *M* urea, 0.01 *M* Tris · HCL at pH 7.9. Samples for analytical columns were stored and injected in nitrogen-bubbled, double-distilled water. Analytical-scale samples preincubated for ca. 20 min at room temperature in 0.01 *M* Tris · HCl, 6 *M* urea at pH 7.9 behaved similarly to those injected in water. Apolipoproteins were detected by absorbance at 280 nm using a Waters Model 440 detector.

Gel electrophoresis and isoelectric focusing

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, and polyacrylamide gel isoelectric focusing (PAGIF) in the pH range of 4—8 on 6% gels containing 6 *M* urea, were performed by methods previously described by Shore et al. [14]. These methods were (with modifications) those of Weber and Osborne (SDS-PAGE) [15] and Wrigley (PAGIF) [16]. PAGIF pH gradients were determined by slicing of replicate gels, followed by overnight aqueous extraction of gel slices, and subsequent determination of the pH of the aqueous extract.

Amino acid analysis

Amino acids were determined as described by Shore et al. [14]. Proteins were hydrolyzed with 4 *N* methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole for 40 h at 110°C in sealed ampoules. Composition of hydrolysates was determined by the method of Liu and Chang [17] with a Beckman Model 121 Amino Acid Analyzer.

RESULTS

SynChropak AX 300 HPLC of HDL apolipoproteins

The HDL apolipoproteins are resolved into at least ten species by anion-exchange HPLC on a SynChropak AX 300 column using an optimized adaptation of the conditions successfully employed for DEAE-cellulose chromatography of apolipoproteins (Fig. 1a). The separation achieved in 180 min by anion-exchange HPLC is similar to that obtained by a 30-h elution of DEAE-cellulose columns. The absorbance profile at 280 nm has a major species at 73 min (peak 1) and a major multiplex between 88 and 100 min with two predominant peaks (peaks 2 and 3). DEAE-cellulose-purified apoAII coelutes with peak 1, apoAI_1 with peak 2 and apoAI_2 with peak 3. The ratio apoAI_1/AII is similar to that observed with DEAE-cellulose chromatography (2.4:1 on DEAE-cellulose, 2.6:1 on AX 300) while the ratio AI_1/AI_2 is substantially higher in anion-exchange HPLC chromatograms (3.0:1 on DEAE-cellulose, 6.4:1 on AX 300) [7]. Repeated chromatography of AX 300-purified apoAII and apoAI_1 produced single peaks with the expected mobilities (data not shown).

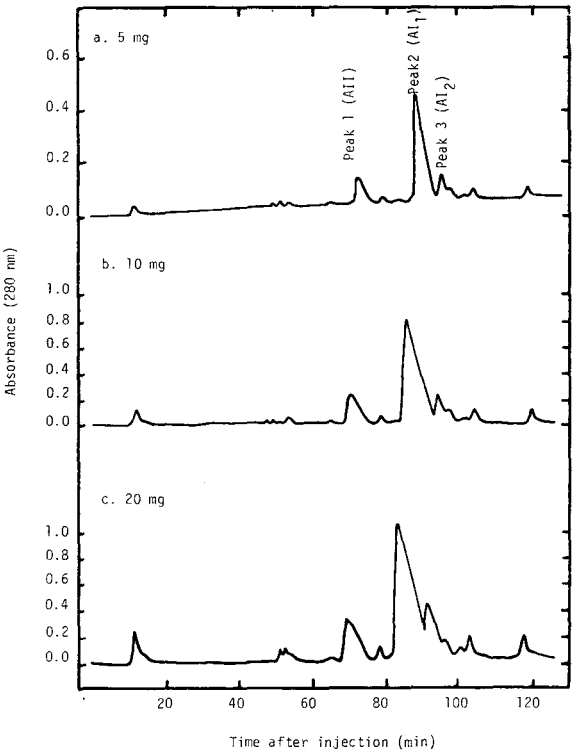

Fig. 1. Semipreparative-scale AX 300 chromatography of HDL apolipoproteins. HDL apolipoproteins were eluted from a 250 × 9 mm column of AX 300 with a linear, 180-min gradient of 0.02—0.20 *M* Tris • HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min. After each run the column was returned to initial conditions and reequilibrated for 10 min. (a) 5-mg, (b) 10-mg, and (c) 20-mg samples of HDL apolipoproteins in 6 *M* urea, 0.01 *M* Tris • HCl, pH 7.9, were each loaded in a single 2-ml injection.

The potential preparative utility of anion-exchange HPLC was attractive enough to justify the determination of loading capacity and the purity of effluent peak material. The chromatograms in Fig. 1a, b, and c were obtained with 5-, 10-, and 20-mg inputs, respectively, of apoHDL on a semipreparative column. Increased loading of AX 300 columns results in shorter elution times for the major species (reduced apparent binding constants and decreased resolution). The AI band broadens substantially as the input load is increased from 5 to 20 mg. Resolution in the region between AI_1 and AII is reduced and the separation between AI_1 and AI_2 is diminished. Input of loads > 20 mg does not appear to be useful.

The purity of the three major peaks obtained by chromatography of 10 mg apoHDL was analyzed by SDS-PAGE, PAGIF, and amino acid analysis of isolated proteins. The composition of a protein from peak 1 (Fig. 1a) corresponds to that derived from the published sequence of apoAII [18], with an average deviation of 5.6%. Hydrolysates from peaks 2 and 3 (Fig. 1a) have compositions that agree with that derived from the sequence of apoAI [19], with average deviations of 4.6% for $apoAI_1$ and 5.0% for $apoAI_2$ (Table I). Apparent molecular weights derived from SDS-PAGE (Fig. 2) for peaks 2 and 3 were

TABLE I

AMINO ACID COMPOSITION OF HDL APOLIPOPROTEINS

Single determination of peak material isolated by HPLC on an AX 300 anion-exchange column. Values are expressed as mol per 10^3 mol of amino acids.

Amino acid	AII peak 1	AII expected [18]	AI_1 obtained, peak 2	AI_2 obtained, peak 3	AI expected [19]
Lys	113	117	88	81	86
His	0	0	18	19	18
Arg	0	0	67	56	65
Asp	44	39	91	89	85
Thr	79	78	36	37	40
Ser	79	78	64	60	60
Glu	208	195	193	193	188
Pro	53	52	43	40	43
Gly	45	39	45	45	42
Ala	63	65	76	78	78
Val	72	78	48	54	55
Met	9	13	10	8	12
Ile	12	13	0	0	0
Leu	107	104	152	162	154
Tyr	53	52	28	28	29
Phe	50	52	23	20	24
Trp	0	0	12	16	16

27,000. Values obtained for peak 1 were 16,000 and 8500, respectively, for samples preincubated with and without mercaptoethanol. These apparent molecular weights also agree well with sequence data. PAGIF analysis of the isolated apoAI isoforms gave apparent isoelectric points of 5.6 for peak 2, and 5.5 for the major constituent of peak 3 (Fig. 3). The apparent isoelectric point of apoAII (peak 1) was 5.0 (data not shown). These values are in good agreement with those derived by thin-layer isoelectric focusing for apoAII, $apoAI_1$, and $apoAI_2$ [20]. The identification of the minor C apolipoproteins of HDL is given in the following section on HPLC of apoVLDL and a mixture of apoHDL and apoVLDL.

PAGIF analyses of $apoAI_1$ fractions consistently produced minor amounts of $apoAI_2$ and $apoAI_3$ that were not apparent upon repeated chromatography. The multiple peaks observed for PAGIF analysis of $apoAI_2$ represent in part the tailing of the $apoAI_1$ peak into the $apoAI_2$ region, and possibly in part, interconversion of the variants. Pure $apoAI_2$ has not been obtained by a single anion-exchange HPLC separation.

In addition to semipreparative usage, anion-exchange HPLC has the potential for quantitative analytical application. Chromatographic separations of 1.5, 15, and 75 μg of apoHDL are displayed in Fig. 4a, b, and c, respectively. With an input of 1.5 μg of apoHDL (ca. 0.9 μg $apoAI_1$ and 0.5 μg apoAII), $apoAI_1$ is clearly detectable but apoAII, whose absorption coefficient at 280 nm is about half that of apoAI, is not detectable (Fig. 4a). Input of 15 μg apoHDL allows reasonable detection of $apoAI_1$, $apoAI_2$, apoAII, and several minor components

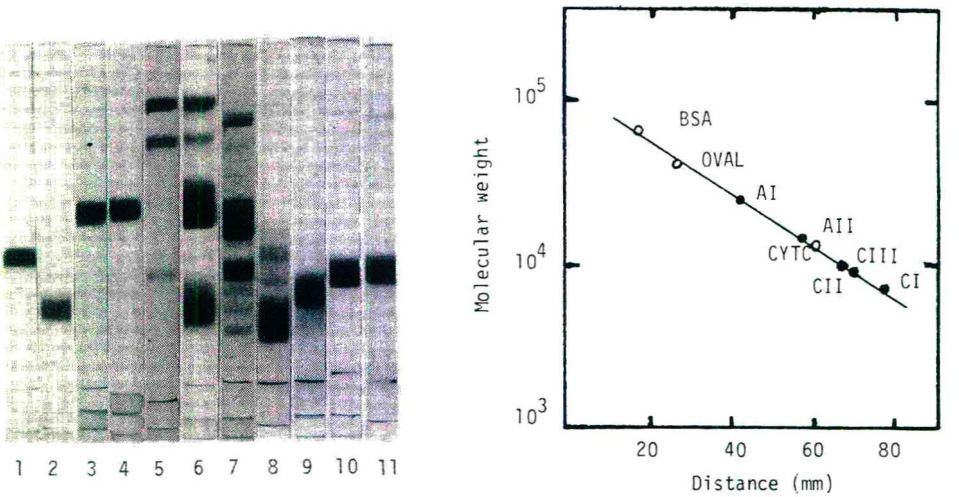

Fig. 2. SDS-PAGE of apolipoproteins. Electrophoresis of apolipoproteins was performed on 10% polyacrylamide gels in 0.2% SDS. Samples (10–100 μg) were run in the following gels: 1, apoAII (peak 1); 2, apoAII (peak 1) + mercaptoethanol; 3, $apoAI_1$ (peak 2); 4, $apoAI_2$ (peak 3); 5, standard mixture of bovine serum albumin (BSA), ovalbumin (OVAL), and cytochrome C (CYTC); 6, HDL apolipoproteins + mercaptoethanol; 7, HDL apolipoproteins; 8, apoCI (peak 1′); 9, apoCII (peak 2′); 10, apoCIII (peak 3′); 11, $apoCIII_2$ (peak 4′).

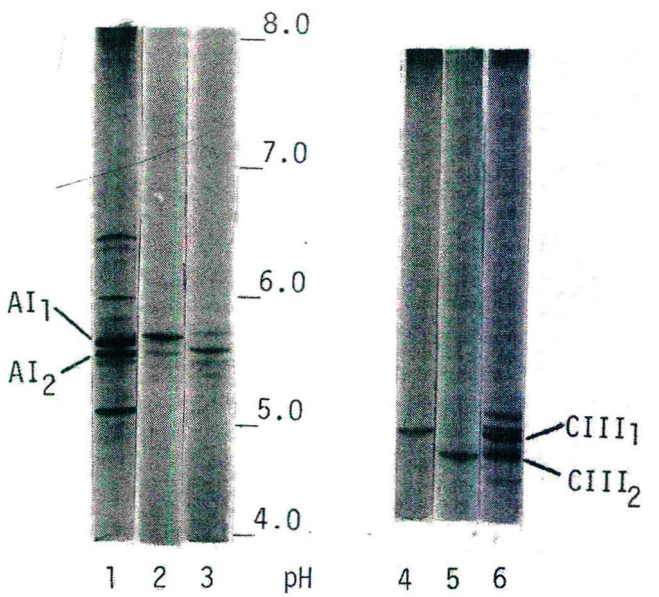

Fig. 3. PAGIF of apolipoproteins. Apolipoproteins were focused in 6% polyacrylamide gels containing 6 *M* urea and 2% pH 4–8 ampholines for 5 h at 400 V. Samples (10–100 μg) were focused in the following gels: 1, HDL apolipoprotein; 2, $apoAI_1$ (peak 2); 3, $apoAI_2$ (peak 3); 4, apoCIII (peak 3′); 5, $apoCIII_2$ (peak 4′); 6, urea-soluble VLDL apolipoproteins.

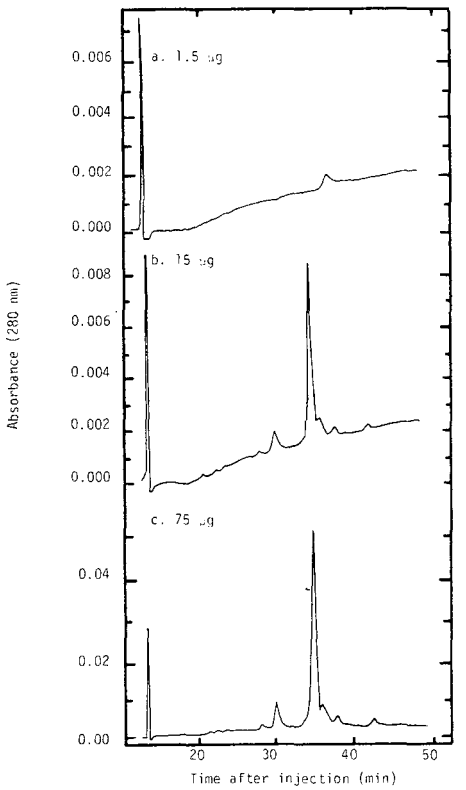

Fig. 4. Analytical-scale AX 300 chromatography of HDL apolipoproteins. Apolipoproteins were eluted from a 300 × 4.5 mm column of AX 300 with a linear 45-min gradient of 0.02–0.15 *M* Tris · HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min. (a) 1.5-µg, (b) 15-µg, and (c) 75-µg samples in double-distilled water were each loaded in a single 100-µl injection.

(Fig. 4b). The chromatography remains similar at 75 µg (Fig. 4c) and analyses of multiple species are possible with 15- to 75-µg inputs. It should be noted that the apoAII peak is contaminated with apoCII when 45-min gradients (Fig. 4) are used. This is not the case when 180-min gradients are employed (Fig. 1).

AX 300 chromatography of apoVLDL and a mixture of apoVLDL and apoHDL

The urea-soluble apolipoprotein moiety of VLDL shares several species in common with the HDL apolipoproteins, but the relative abundances are quite different. The chromatogram of Fig. 5 was obtained by elution of 6 mg of urea-soluble VLDL apolipoprotein from the semipreparative AX 300 column under the conditions employed for separation of the HDL apolipoproteins. The identities of four apoC peaks were assigned and purities estimated on the basis of SDS-PAGE, PAGIF, and amino acid analysis of the collected fractions corresponding to the peaks in the chromatogram. Peak 1′ (Fig. 5) contains apoCI in addition to unidentified contaminants. SDS-PAGE analysis reveals a major band with an apparent molecular weight of 6000 (Fig. 2) and several higher

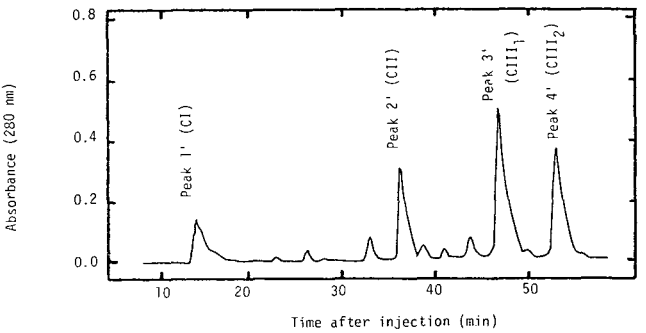

Fig. 5. Semipreparative-scale AX 300 chromatography of urea-soluble VLDL apolipoproteins. Urea-soluble VLDL apolipoprotein (6 mg in 6 *M* urea and 0.001 *M* Tris · HCl, pH 7.9) were eluted from a 250 × 9 mm AX 300 column with a linear 45-min gradient of 0.02—0.15 *M* Tris · HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min.

molecular weight species. The amino acid composition reflects these impurities, differing from sequence-derived composition [21] by ca. 23% per residue (Table II). Peak 2′ (apoCII) has an apparent molecular weight of ca. 9000 (Fig. 2), in agreement with the expected value [22], and differs from sequence-derived composition by 10.4% (Table II). Peaks 3′ and 4′ have amino acid compositions expected for apoCIII [23]. They agree with the expected values within 9.5% and 10.2%, respectively (Table II). The apparent molecular weight by SDS-PAGE is ca. 10,000 (Fig. 2), which is the expected weight for apoCIII's

TABLE II

AMINO ACID COMPOSITION OF VLDL APOLIPOPROTEINS

Single determination of peak material isolated by HPLC on an AX 300 anion-exchange column. Values are expressed as mol per 10^3 mol of amino acids.

Amino acid	CI peak 1	CI expected [21]	CII peak 2′	CII expected [22]	$CIII_1$ peak 3′	$CIII_2$ peak 4′	CIII expected [23]
Lys	115	140	69	76	70	75	76
His	3	0	0	0	15	23	13
Arg	54	58	15	13	24	25	25
Asp	87	88	72	64	94	90	89
Thr	45	53	107	103	55	56	63
Ser	113	88	115	103	131	128	139
Glu	171	105	187	180	138	139	127
Pro	26	17	54	51	29	28	25
Gly	58	17	37	26	44	49	38
Ala	63	53	78	77	124	121	127
Val	44	35	54	38	72	71	76
Met	13	18	16	26	22	20	25
Ile	43	53	7	13	0	0	0
Leu	110	105	104	103	74	70	63
Tyr	8	0	62	51	26	26	25
Phe	48	53	27	26	55	54	50
Trp	16	17	10	13	43	41	25

with carbohydrate moieties [23]. PAGIF analysis of peaks 3′ and 4′ places the major bands at p*I* = 4.8 and 4.6, respectively. These agree fairly well with the thin-layer assessments of isoelectric points of 4.9 and 4.8 for apoCIII$_1$ and apoCIII$_2$ respectively [24]. On the basis of these isoelectric points and the amounts of the species present we have assigned peak 3′ as apoCIII, and peak 4′ as apoCIII$_2$. The amounts of material isolated from the remaining peaks were insufficient for identification.

VLDL apolipoproteins and HDL apolipoproteins were cochromatographed in a mixing experiment (Fig. 6). Comparison of chromatograms of apoVLDL, apoHDL, and a mixture of the two solutions (4:1, v/v) confirms the placement of apoC species on the apoHDL chromatogram. ApoCI and contaminating species run near column volume in all cases. ApoCII, which elutes at ca. 35 min, appears on the trailing edge of the apoAII peak. It is not well resolved in 45-min chromatograms. The apoCIII species that elute at ca. 47 and 50 min co-chromatograph with species eluting at those times from apoHDL. Thus, the shorter run resolves the apoC's. A 120-min run with a shallower gradient is needed to resolve a mixture of apoA's and apoC's.

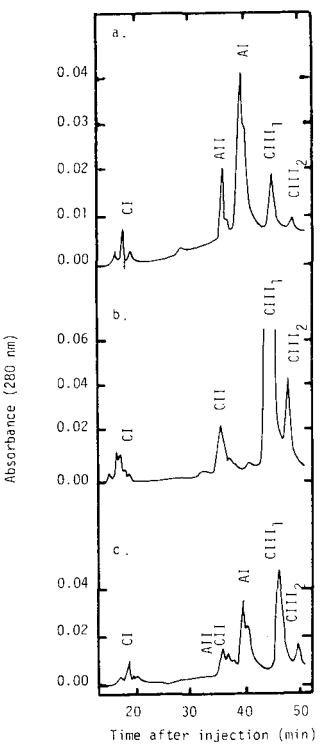

Fig. 6. Semipreparative-scale AX 300 chromatography of urea-soluble VLDL and HDL apolipoproteins. Apolipoproteins were eluted from a 250 × 9 mm AX 300 column with a linear 45-min gradient of 0.02—0.15 *M* Tris · HCl in 6 *M* urea at pH 7.9 and a flow-rate of 1 ml/min. The following samples were run: (a) 250 μg HDL apolipoprotein; (b) 650 μg urea-soluble VLDL apolipoprotein; (c) 130 μg urea-soluble VLDL apolipoprotein + 200 μg HDL apolipoprotein.

DISCUSSION

The preparative isolation and analytical determination of apolipoproteins have been approached by a variety of methods: anion-exchange chromatography [7, 8], gel-exclusion chromatography [25], combinations thereof and thin-layer isoelectric focusing [20, 24] for preparative purposes; urea-PAGE [13], SDS-PAGE [14], PAGIF [26], and a variety of immunological techniques [27] for qualitative and quantitative analysis. The rapid high-resolution methods developed for separation of proteins by HPLC [28, 29] have potential utility for these applications. Separation of apolipoproteins by gel-permeation HPLC with 300 × 22.5 mm columns of TSK-3000 SW (Toyo Soda Manufacturing Co., Tokyo, Japan) has been achieved for < 15 mg inputs of apoHDL in 6 *M* urea [30]. The resolution of purified apoAI into variant forms and the partial separation of a mixture of apoCI, apoCII, and apoCIII (derived from apoVLDL by conventional chromatography) by reversed-phase HPLC on a radially compressed C_{18} microparticulate support (Radial-Pak A, Waters Assoc.) [31] suggests its potential for analytical and possibly preparative work. For the preparative application, the reversibility of the denaturing effects of the organic solvent needs to be established. Apolipoproteins exposed to the denaturing solvents used in ion-exchange and gel-permeation HPLC have been shown to regain enzymatic cofactor activity on dialysis. Anion-exchange HPLC allows resolution of the major urea-soluble apolipoproteins and we have demonstrated the necessary loading capacity and sensitivity to justify development of both analytical and preparative separations of apolipoproteins by this chromatographic technique.

As a preparative method, HPLC conserves time and reagents, and by greatly reducing the exposure to denaturing solvents, reduces the risk of irreversible structural changes in the proteins. The useful loading capacity, with presently available columns, is limited to 10—20 mg. By comparison ca. 30 mg protein can be separated with excellent resolution by DEAE-cellulose chromatography on a 40 × 0.9 cm column. Advances in HPLC with larger-diameter preparative columns and less expensive supports [28, 29] may obviate this limitation in the near future. The alternative is repeated injections of sample, either manually or with an automated HPLC preparative system [32]. The seven apolipoprotein components (AI_1, AI_2, AII, CI, CII, $CIII_1$, and $CIII_2$) separated by anion-exchange HPLC are similar to those isolated by DEAE-cellulose chromatography by SDS-PAGE, PAGIF, and amino acid analysis. The chromatographic profiles are quite similar, with the notable exception that the ratio AI_1/AI_2 is substantially higher for anion-exchange HPLC separations. The number of apoAI variants isolated, and their ratios, appear to be a function of the method of isolation. As many as five variants and as few as two have been reported for ion-exchange separations [33, 34] while four are found with thin-layer isoelectric focusing [20]. Anion-exchange HPLC resolves only two apoAI variants in significant quantity. These results suggest that apoAI variants are generated during long exposure to denaturing conditions. In our hands PAGIF analysis of purified $apoAI_1$ does reveal two additional minor bands while anion-exchange HPLC continues to produce a single species. While shorter exposure to solvent enhances the attractiveness of anion-exchange HPLC, the limitations in resolu-

tion are similar to those for DEAE-cellulose. Purification of some species may require a combination of gel permeation and ion-exchange separations.

The high resolution of apolipoproteins by HPLC indicates the potential for simultaneous qualitative analysis and quantitative determination of the various apolipoproteins in a lipoprotein sample. It offers some advantages over the two basic analytical procedures currently in use. Immunoassays analyze for a single protein with high sensitivity, but the variants of a given apolipoprotein are not distinguished and there are numerous technical constraints [27]. Simultaneous determination with high resolution of apolipoproteins is possible with SDS-PAGE, urea-PAGE, and particularly PAGIF, which resolves the variant forms of apoAI, apoCII, apoCIII, and apoE. The quantitative application of the gel methods has several disadvantages that are obviated by HPLC. These include the uncertainties associated with staining and destaining (which are generally slow and difficult to reproduce), differences in staining intensities of the various apolipoproteins, and the possibility of nonlinear response. In HPLC, peak area is directly related to the amount of protein and its absorption coefficient, which is a characteristic of a given apolipoprotein. HPLC analysis offers the advantages of 1—2 h analysis time and applicability of readily available automated instrumentation, which allows continuous operation. We have demonstrated sufficient resolution and sensitivity for the simultaneous analysis of several apolipoprotein species by anion-exchange HPLC.

Assays for apoAI_1, AI_2, AII, CII, $CIII_1$, and $CIII_2$ are feasible with sufficient input. Differences in peak width and extinction coefficient create some variation in sensitivity. While apoAI can be detected at < 1 μg, AII requires ca. 2 μg for detection (data not shown). Routine analysis of apoHDL will require ca. 50-μg inputs if reproducible analysis of apoC's is to be achieved.

ACKNOWLEDGEMENTS

The investigation was supported by grants HL 23747 and HL 07058 of the U.S. Public Health Service and performed under the auspices of the U.S. Department of Energy by the Lawrence Livermore National Laboratory under contract number W-7405-ENG-48.

We would like to acknowledge the excellent assistance of Marie Laskaris, Joseph Mazrimas, Anthony Silva, and Nancy Shen, and numerous valuable technical discussions with Dr. C.T. Wehr of Varian Associates.

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Journal of Chromatography, 231 (1982) 13—23
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1293

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF HUMAN SERUM LIPOPROTEINS

SELECTIVE DETECTION OF CHOLINE-CONTAINING PHOSPHOLIPIDS BY ENZYMATIC REACTION

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(First received December 29th, 1981; revised manuscript received March 9th, 1982)

SUMMARY

A convenient method for the quantitation of choline-containing phospholipids in each lipoprotein fraction has been developed by combining separation by high-performance liquid chromatography with gel permeation columns and selective detection by enzymatic reaction in the post-column effluent.

The elution patterns monitored by choline-containing phospholipids were compared with those monitored by cholesterol. The elution patterns of choline-containing phospholipids were found to give much more information about the distribution of lipoproteins according to their particle-size differentiation than analyses done by cholesterol.

This choline-containing phospholipid monitoring method not only resolves lipoprotein peaks of the major classes (chylomicron + VLDL, LDL, HDL_2 and HDL_3) quantitatively, but also detects the presence of abnormal lipoproteins containing a large amount of choline-containing phospholipids. We could detect these abnormal lipoproteins using a small amount of whole serum (10—20 μl) from patients with various liver diseases. Our examination of HDL subclasses using this technique showed that the HDL fraction was composed of several subfractions due to their particle-size differentiation.

INTRODUCTION

We have developed a new method for lipoprotein analysis using high-performance liquid chromatography (HPLC) with gel permeation columns (TSK-GEL, Toyo Soda, Tokyo, Japan) which achieves the separation of serum lipoproteins into their major classes due to particle-size differentiation: chylomicron, very low density lipoprotein (VLDL), low density lipoprotein (LDL) and high density lipoproteins (HDL_2 and HDL_3) [1, 2].

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It is well known that the particle size of serum lipoproteins depends on their chemical composition. Especially large-particle-size lipoproteins, such as chylomicrons and VLDL, contain a large amount of triglycerides. On the other hand, phospholipids are an essential component of the serum lipoprotein structure. All lipoproteins, not only normal lipoproteins, (i.e. chylomicrons, VLDL, LDL, HDL_2 and HDL_3), but abnormal lipoproteins which are found in the serum of the patients with dyslipoproteinemia, contain about 18—30% of phospholipids. Some abnormal lipoproteins contain a large amount of phospholipids, more than 60% [3—5]. This means that the detection of triglycerides is very suitable for the analysis of larger-particle-size lipoproteins and that of phospholipids for any lipoprotein, even abnormal ones.

A direct quantitation method for cholesterol in each serum lipoprotein class has been developed using a combination technique consisting of separation by HPLC with gel permeation columns and the selective detection of cholesterol using a commercial enzyme kit [6, 7]. This technique can be applied to the analysis of lipid components other than cholesterol in serum lipoproteins, such as triglycerides and phospholipids. In fact, we have developed a convenient and simple method for the detection and quantitation of triglycerides in serum lipoproteins using a gel permeation column (TSK-GEL, G5000PW) by this technique [8]. Moreover, we have established a quantitation method for choline-containing phospholipids in each lipoprotein class using a commercially available enzyme kit. The study of the optimum conditions for the enzymatic reaction in the flow diagram using a reaction-type high-speed chromatograph is reported in a separate paper [9].

In this paper, we examine the effect of the elongation of the reaction tube on the resolution of lipoproteins using the detection method of choline-containing phospholipids. Elution patterns monitored by choline-containing phospholipids were compared with those obtained by cholesterol monitoring using two different column systems. The size distribution of serum lipoproteins of patients with various liver diseases was examined by monitoring both cholesterol and choline-containing phospholipids.

EXPERIMENTALS

Apparatus

HPLC was carried out using a reaction-type high-speed liquid chromatograph (HLC 805, Toyo Soda) in the same schematic diagram as described in our previous papers [6, 7] except that the enzymatic reaction was performed using Teflon tubes of 0.5 mm or 1.0 mm I.D. with a length varying between 5000 and 30,000 mm in a thermostated water bath (Thermo Mini TM-100, Tokyo Rikakikai, Tokyo, Japan).

Ultracentrifugation for the separation of the standard lipoproteins from human serum was carried out using an RP 55 rotor in an Hitachi 55P-2 ultracentrifuge.

Materials and methods

Samples. Human sera used in these experiments were obtained from normal

men and women or from patients with hyperlipidemia and various liver diseases after 12—16 h of fasting. Chylomicron + VLDL ($d < 1.006$), LDL (d 1.006—1.063), HDL_2 (d 1.063—1.125) and HDL_3 (d 1.125—1.210) were isolated from human serum by the sequential flotation method [10]. After centrifugation (105,000 g, 8°C), lipoproteins in the top layer of the tube were collected using a tube slicer (Model TSU2, Hitachi). The lipoprotein fraction of $d < 1.210$ was prepared by the ultracentrifugation method in the same way as described in our previous paper [1].

Reagents. The concentration of cholesterol and choline-containing phospholipids in the samples applied to HPLC was determined enzymatically using commercial kits: Determiner TC"555" (Kyowa Medex, Tokyo, Japan), for cholesterol, and PL Kit K (Nippon Shoji, Osaka, Japan) for choline-containing phospholipids. The selective detection of choline-containing phospholipids was performed in terms of A_{500} using a commercial enzymatic reagent kit for autoanalyzers (PL Kit K"f", Nippon Shoji). This reagent was given in the form of a premixed lyophilized vial which contained 40 units of phospholipase D (from *Streptomyces* spp.), 130 units of choline oxidase (from *Arthrobacter* spp., EC 1.1.3.17), 110 units of peroxidase (EC 1.11.1.7), and 5.6 mg of 4-aminoantipyrine. For the detection of choline-containing phospholipids we used the enzyme solution which was prepared by dissolving one vial (PL Kit K"f") in 25 ml of a buffer solution for HPLC. This solution contained 44.4 mg of phenol and 93.8 mg of Triton X-100 per 100 ml of 0.05 *M* Tris—HCl buffer (pH 7.8). The details of the examination for the optimum preparation method of this buffer solution are reported in a separate paper [11].

Separation of lipoproteins by HPLC. The separation of lipoproteins was carried out by HPLC with gel permeation columns (TSK-GEL, G5000PW, G4000SW and G3000SW, Toyo Soda). Experimental conditions in this study were as follows. Column: G5000PW, G4000SW+G3000SW, G3000SW+G3000SW+G3000SW (each column 600 mm × 7.5 mm I.D.). Eluent: 0.15 *M* sodium chloride. Flow-rate: 0.50—0.60 ml/min.

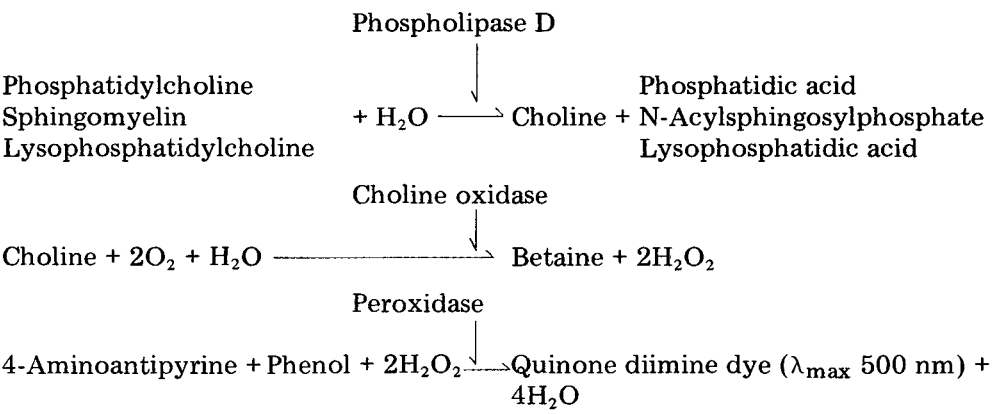
Detection of choline-containing phospholipids and cholesterol. Cholesterol was detected by measuring the A_{550} of the post-column effluent in the same way as described in our previous paper [6, 7].

Choline-containing phospholipids were monitored by the A_{500} of the mixed eluate and enzyme solution (PL Kit K"f") after passage through a reaction tube at constant temperature. Experimental conditions in this study are as follows: temperature of the reaction bath, 39°C; dimensions of the reaction tube, 20,000 mm × 0.5 mm I.D. (Teflon tube); flow-rate of the main path (i.e. the pathway of eluate from the column), 0.50—0.60 ml/min; flow-rate of the enzyme solution, 0.20—0.30 ml/min.

RESULTS AND DISCUSSION

We have already reported the direct quantitation method for cholesterol in each serum lipoprotein by combining separation by HPLC and the selective detection of cholesterol using the enzyme reaction kit [6, 7]. This method can be applied to other lipid components of serum lipoproteins if appropriate reagents for selective detection are obtained.

Takayama et al. [12] have developed a new enzymatic method for quantitation of choline-containing phospholipids. The quantitation of choline-containing phospholipids in a small amount of serum (20 μl) can be carried out in an aqueous system according to the following reaction schemes.



This enzymatic method is specific for the choline-containing phospholipids as shown above. Since these three choline-containing phospholipids comprise about 95% of the phospholipids in serum [13—15], this can be used for the selective detection of phospholipids in serum lipoproteins. For the detection of phospholipids we used a commercial kit (PL Kit K"f") utilizing the reaction schemes as described under Experimental. Using this reagent, choline-containing phospholipids can be detected by the absorbance at 500 nm after passage of the mixed eluate and enzyme solution through the reactor at constant temperature.

The optimum conditions for quantitation of choline-containing phospholipids in the flow diagram were determined as reported in a separate paper [9]. Since a sufficient reaction time, over 4.0 min, can not be given by using a reactor (20,000 mm × 0.25 mm I.D., stainless-steel tube) of the high-speed chemical derivatization chromatograph (HLC 805, Toyo Soda), a reaction tube of larger dimensions than a commerical one must be used.

In order to examine the relation between the dimensions of the reaction tube and the resolution of lipoprotein analysis, the enzymatic reaction was carried out using a Teflon tube (0.5 mm or 1.0 mm I.D.) of various lengths between 5000 and 30,000 mm keeping other experimental conditions optimal. The effect of the dimensions of the reaction tube on peak broadening was examined in the following way with use of the standard lipoprotein fractions LDL and HDL_2. The half band width of these lipoproteins monitored by A_{280} before passage through the reaction tube was compared with that monitored by A_{500} after the enzymatic reaction in the reaction tube. The elution patterns monitored by both A_{280} and A_{500} using reaction tubes of different diameters are presented in Fig. 1. For the reaction tube of 1.0 mm I.D. (Fig. 1c), an increase in band width of A_{500} for both lipoprotein fractions was observed in comparison with that of A_{280}. On the other hand, the increase of that of A_{500} was very small (less than 5%) in the case of the reaction tube of 0.5 mm I.D. (Fig. 1a and b).

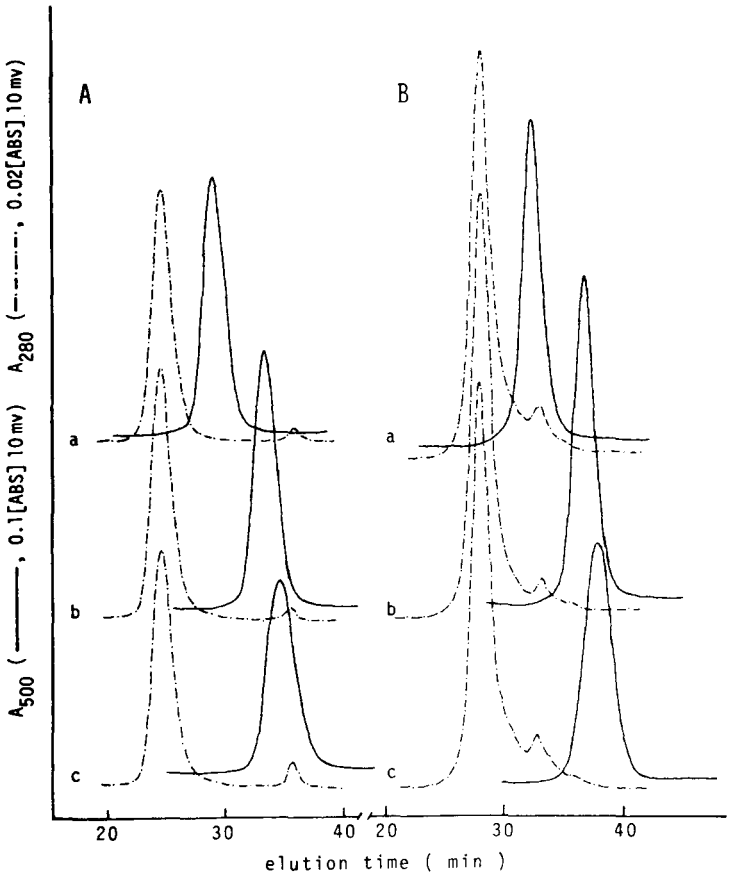


Fig. 1. Elution patterns monitored by A_{280} and A_{500} for standard lipoproteins. Column: G5000PW (600 mm × 7.5 mm I.D.). Eluent: 0.15 *M* sodium chloride. Flow-rate: 0.60 ml/min (main path), 0.30 ml/min (enzyme solution, PL Kit K"f"). Temperature of the reaction bath: 39°C. Dimensions of reaction tube (Teflon): (a) 15,000 mm × 0.5 mm I.D.; (b) 30,000 mm × 0.5 mm I.D.; (c) 11,000 mm × 1.0 mm I.D. Sample: A, LDL (*d* 1.006—1.063) fraction (220.5 mg/dl choline-containing phospholipids; B, HDL_2 (*d* 1.063—1.125) fraction (162.2 mg/dl choline-containing phospholipids). Loaded volume: A, 5 μl; B, 10 μl. Detector: A_{500} (——, 0.1 [ABS] 10 mV) and A_{280} (— · — · —, 0.02 [ABS] 10 mV).

It can be seen from Fig. 1 that the sensitivities for the detection of both LDL and HDL_2 fractions monitored by choline-containing phospholipids are several times higher than those obtained by the A_{280}.

Fig. 2 shows the relation between the half band width ($\omega_{1/2}$, ml) of these lipoprotein fractions monitored by the A_{500} and the length of the reaction tube of 0.5 mm I.D. The half band width of both fractions was constant when the reaction time was over 4.0 min. This reaction time can be obtained by using a reaction tube (0.5 mm I.D.) longer than 15,000 mm. We used a reaction tube of 20,000 mm × 0.5 mm I.D. (Teflon) for the detection of choline-containing phospholipids. Moreover, these examinations indicate that a longer reaction time of over 4.0 min can be obtained by elongation of the reaction tube

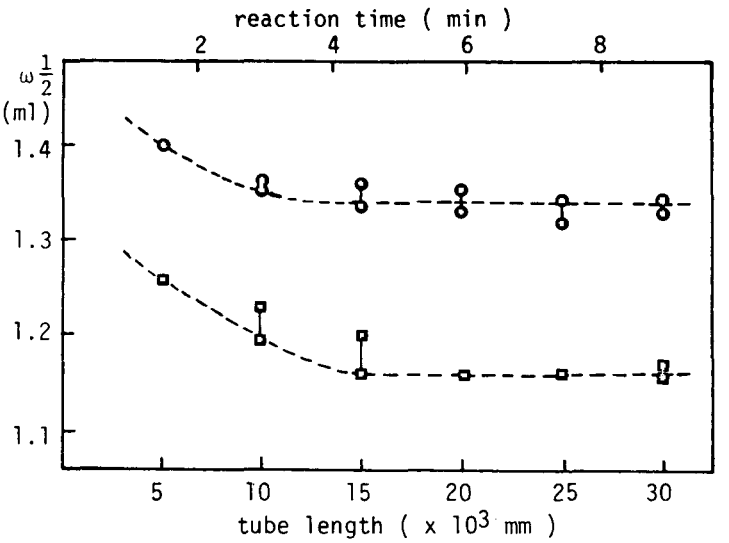


Fig. 2. Relation between half band width monitored by A_{500} and reaction tube length for standard lipoproteins. Sample: LDL (*d* 1.006—1.063) fraction, •; HDL_2 (*d* 1.063—1.125) fraction, ▪. Loaded volume: LDL, 5 μl; HDL_2, 10 μl. HPLC conditions are as in Fig. 1.

without peak broadening, provided the tubing is of appropriate diameter, less than 0.5 mm I.D.

Fig. 3 presents the elution patterns monitored by the A_{500} for the standard lipoprotein fractions (chylomicron+VLDL, LDL, HDL_2 and HDL_3) using the two combined column systems: G4000SW+G3000SW and G3000SW+G3000SW+G3000SW. These standard lipoprotein fractions were prepared from serum of normal males and females by the sequential flotation method [10]. It can be seen from these patterns that the G4000SW+G3000SW system achieves the separation of serum lipoproteins into their major classes: chylomicron+VLDL, LDL, HDL_2 and HDL_3 (Fig. 3A). On the other hand, the G3000SW+G3000SW+G3000SW system (Fig. 3B) was found to be very suitable for the analysis of HDL_2 (*d* 1.063—1.125) and HDL_3 (*d* 1.125—1.210) fractions. The elution patterns of HDL_2 and HDL_3 fractions using this column system suggest the heterogeneity of particle size within these two HDL subclasses.

The elution patterns of whole serum monitored by choline-containing phospholipids were compared between these two column systems (G4000SW+G3000SW and G3000SW+G3000SW+G3000SW) using the same subject. Three examples are shown in Fig. 4. The elution patterns of cholesterol and protein are also presented in the same figure. The arrows in Fig. 4 indicate the elution positions of the major lipoprotein classes and serum albumin. The elution position of each lipoprotein fraction was determined using the standard fractions as shown in Fig. 3.

The elution patterns of whole serum monitored by cholesterol and choline-containing phospholipids using the G4000SW+G3000SW column system gave clear peaks according to the major lipoprotein classes: chylomicron+VLDL, LDL and HDL subclasses. Since the high content of cholesterol in the LDL

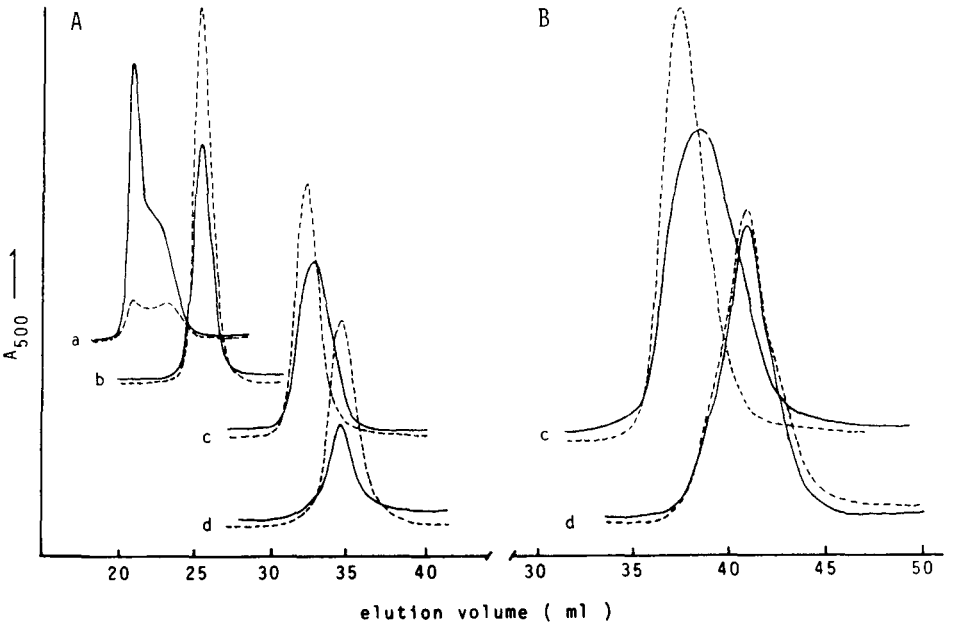


Fig. 3. Elution patterns monitored by A_{500} for standard lipoproteins using combined column systems. Column: A, G4000SW+G3000SW; B, G3000SW+G3000SW+G3000SW (each column size, 600 mm × 7.5 mm I.D.). Eluent: 0.15 *M* sodium chloride. Flow-rate: 0.60 ml/min (main path), 0.30 ml/min (enzyme solution, PL Kit M"f"). Temperature of the reactor (20,000 mm × 0.5 mm I.D. Teflon tube): 39°C. Sample: (a) chylomicron + VLDL fraction ($d < 1.006$); (b) LDL (d 1.006—1.063); (c) HDL_2 (d 1.063—1.125); (d) HDL_3 (d 1.125—1.210) from serum of normal male (——) and normal female (— — —). Loaded volume: 20—150 μl.

fraction interferes with the detection of peaks which appear near the LDL fraction, the resolution of peaks of lipoproteins monitored by choline-containing phospholipids is higher than that monitored by cholesterol.

As previously reported [1], the G3000SW column is suitable for the analysis of small-size lipoproteins such as HDL subclasses. As expected, the G3000SW+G3000SW+G3000SW system gave many peaks and shoulder peaks in the HDL fraction in comparison with the G4000SW+G3000SW system as presented in Fig. 4. Moreover, a shoulder peak of choline containing phospholipids at the elution position of serum albumin is completely separated in the G3000SW+G3000SW+G3000SW system. Elution patterns of the HDL fraction using this column system indicate that there exist many subclasses other than HDL_2 and HDL_3. Heterogeneity of the HDL fraction was examined by peak frequency analysis and rechromatography using this column system, and the results will be reported in a separate paper [16]. Our results for HDL subclasses according to particle-size differentiation using gel permeation columns are consistent with those reported by other investigators who used gradient gel electrophoresis [17] or rate zonal ultracentrifugation [18].

The elution patterns monitored by choline-containing phospholipids were compared between whole serum and its $d < 1.21$ fraction. The two examples

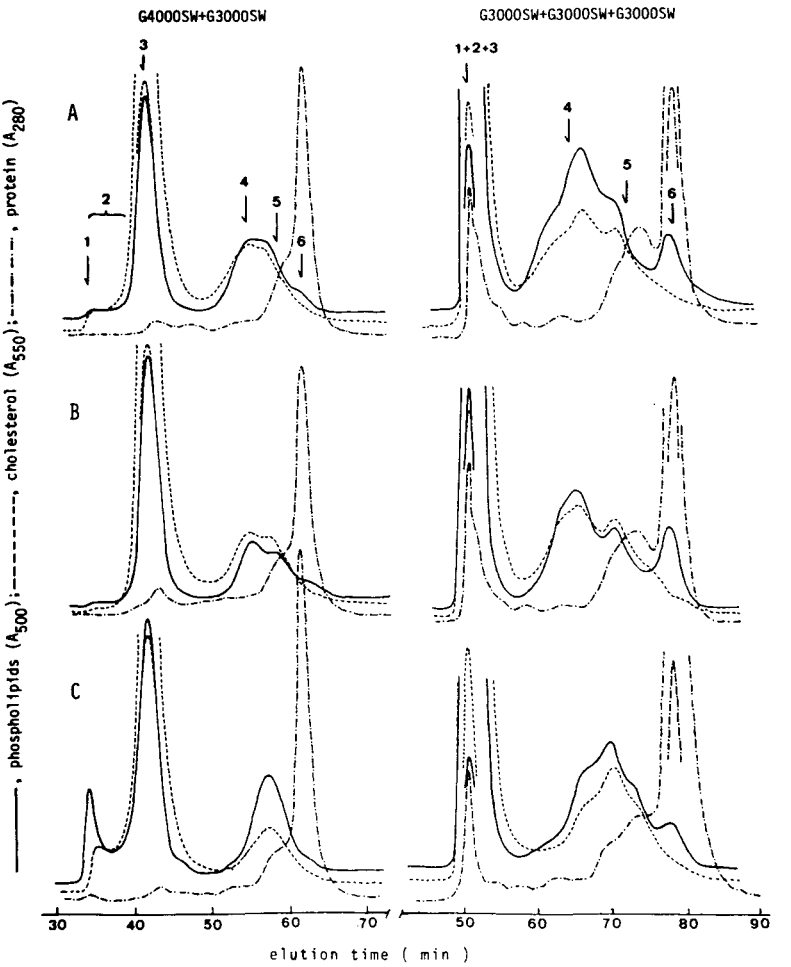


Fig. 4. Elution patterns of choline-containing phospholipids (——), cholesterol (— — —) and protein (— · — · —) for human whole serum using combined column systems. Column: G4000SW+G3000SW, G3000SW+G3000SW+G3000SW. Sample: whole serum of normal male (A), normal female (B) and hyperlipidemia patient (C). Loaded volume: 5 μl (G4000SW+ G3000SW) and 10 μl (G3000SW+G3000SW+G3000SW) for cholesterol and protein monitoring; 10 μl (G4000SW+G3000SW) and 20 or 30 μl (G3000SW+G3000SW+ G3000SW) for choline-containing phospholipids monitoring. HPLC conditions for cholesterol monitoring: eluent, 0.15 *M* sodium chloride; flow-rate of main path, 0.60 ml/min; flow-rate of enzyme solution (TC"555"), 0.20 ml/min; temperature of the reactor (20,000 mm × 0.25 mm I.D. stainless-steel tube), 40°C. HPLC conditions for choline-containing phospholipids monitoring as in Fig. 3. Detector: choline-containing phospholipids (A_{500}, ——), 0.1 [ABS] 5 mV; cholesterol (A_{550}, — — —), 0.1 [ABS] 10 mV; protein (A_{280}, — · — · —), 0.32 [ABS] 5 mV. Elution position: 1, chylomicron; 2, VLDL; 3, LDL; 4, HDL_2; 5, HDL_3; 6, serum albumin.

are shown in Fig. 5. The elution patterns are in fairly good agreement except that peak No. 6 after the HDL_3 fraction disappears in the case of the $d < 1.21$ fraction. This peak might correspond to choline-containing phospholipids in the $d > 1.21$ fraction of serum, because there were considerable amounts of

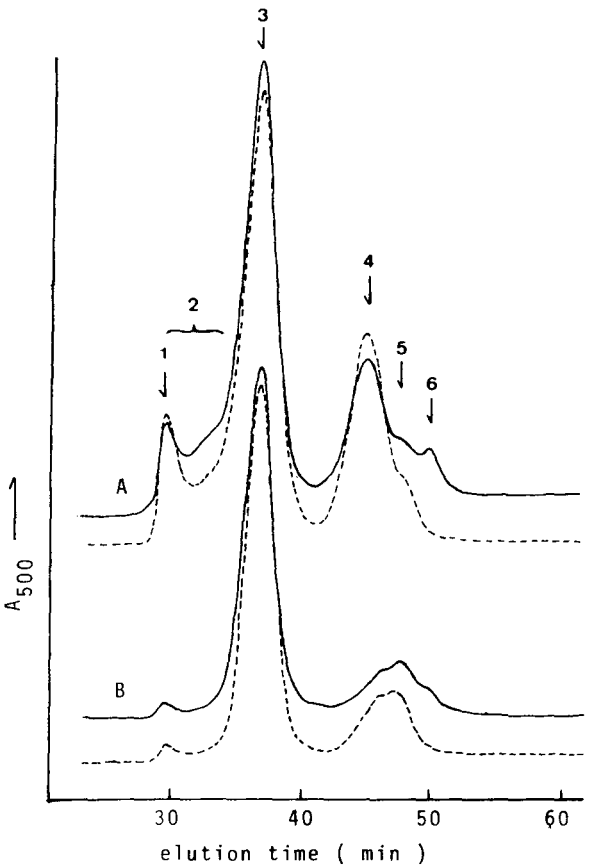


Fig. 5. Elution patterns monitored by A_{500} for whole serum and the $d < 1.21$ fraction. Column: G4000SW. Flow-rate: 0.50 ml/min (main path), 0.20 ml/min (enzyme solution, PL Kit K"f"). Sample: whole serum (——) and the $d < 1.21$ fraction (— — —) of a hyperthyroidemia patient (A) and a hyperlipidemia patient (B). Loaded volume: whole serum, 10 μl; the $d < 1.21$ fraction, 20 μl. Elution position and other HPLC conditions as in Fig. 4.

choline-containing phospholipids in the $d > 1.21$ fraction of serum, 17.6 ± 5 mg/dl ($n = 23$) in our ultracentrifugation examination. Similar results have been reported by other investigators [10, 19—22]. Therefore, peak No. 6 is identified as the choline-containing phospholipids in the $d > 1.21$ fraction which is designated as very high density lipoprotein (VHDL) [21].

The elution patterns of cholesterol and choline-containing phospholipids were examined for individual human serum samples (10—20 μl) from normal and pathological subjects using the G4000SW+G3000SW system. This system is the best combination for analysis of all major lipoprotein classes. A few examples obtained for patients with various liver diseases are shown in Fig. 6, where one example of a normal female is also shown as reference. Elution positions of major lipoproteins and serum albumin are shown in the figure in the same way as in Fig. 4. Elution patterns of liver diseases are different from those of normal subjects: disappearance of major lipoprotein classes and/or

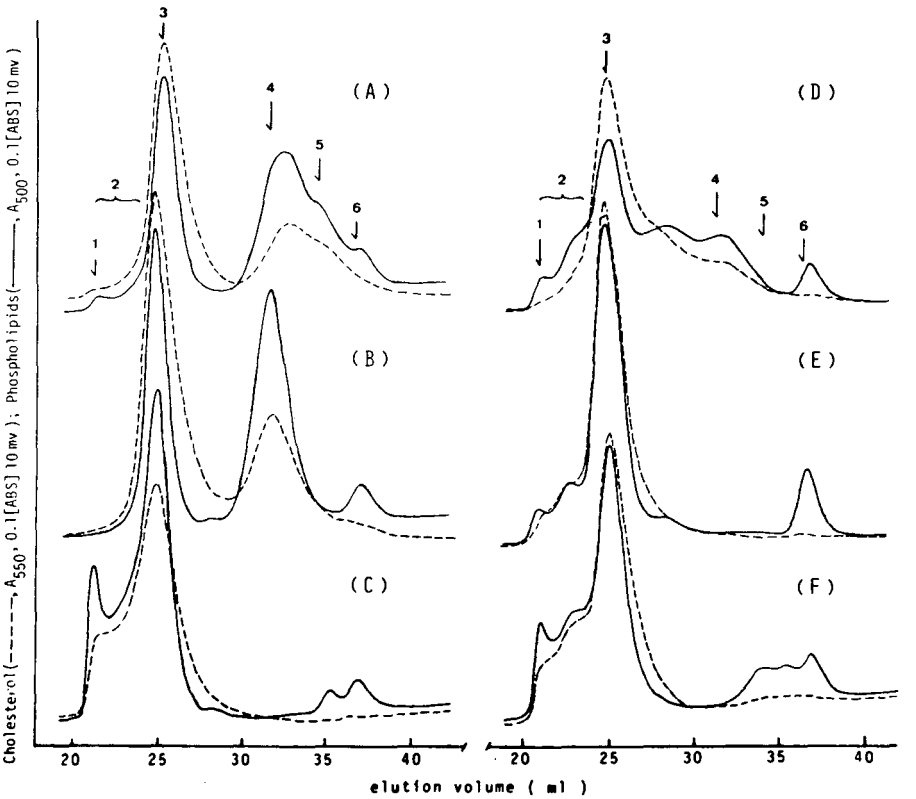


Fig. 6. Elution patterns of choline-containing phospholipids (——) and cholesterol (— — —) for whole serum from patients with various liver diseases. Column: G4000SW+ G3000SW. Sample: whole serum of normal female (A), and from patients with liver cirrhosis (B), acute hepatitis (C), primary biliary cirrhosis (D), intrahepatic cholestasis (E) and drug-induced liver injury (F). Loaded volume: 20 μl for choline-containing phospholipids monitoring, 10 μl for cholesterol monitoring. Detector: choline-containing phospholipids (A_{500}, ——) 0.1 [ABS] 10 mV, cholesterol (A_{550}, — — —) 0.1 [ABS] 10 mV. Elution positions and other HPLC conditions as in Fig. 4.

appearance of peaks other than major classes. It is well known that the liver plays a key role in the synthesis and secretion of lipoproteins and is involved in the uptake of intermediate and end-products of serum lipoprotein metabolism. Therefore, it is not surprising that lipoprotein abnormalities are observed in the elution patterns of liver diseases by our method as a result of hepatic injury. Since these abnormal lipoproteins contain a larger amount of choline-containing phospholipids than normal lipoproteins, the detection of these peaks could be successfully performed by choline-containing phospholipid monitoring as shown in Fig. 6.

Our method for lipoprotein analysis combining the two methods — separation by HPLC using gel permeation columns and selective detection of cholesterol and choline-containing phospholipids by enzymatic reaction — will make progress rapid in the study of lipoprotein metabolism and diagnosis of the various diseases.

We are now examining the characterization of HDL subclasses by combining this HPLC method and the electron-microscopic observations.

ACKNOWLEDGEMENTS

The authors gratefully appreciate the serum gifts by Dr. H. Itakura and Dr. T. Kodama of Tokyo University Hospital and Dr. C. Naito of Tokyo Teishin Hospital. This work was supported by a research grant from the Nissan Science Foundation.

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Journal of Chromatography, 231 (1982) 25—39
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1289

SIMPLE AND FAST SOLVENT EXTRACTION SYSTEM FOR SELECTIVE AND QUANTITATIVE ISOLATION OF ADRENALINE, NORADRENALINE AND DOPAMINE FROM PLASMA AND URINE

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(First received December 15th, 1981; revised manuscript received March 2nd, 1982)

SUMMARY

A very simple solvent extraction system for the selective and quantitative isolation of adrenaline, noradrenaline and dopamine from plasma and urine is described. The extraction system makes use of the complex formation, in alkaline medium, between diphenylborate and the diol group in the catecholamines in combination with ion-pair formation. The influence of various parameters on the distribution coefficient was investigated by analysis of the liquid phases by high-performance liquid chromatography with electrochemical detection. From these results the optimal extraction conditions can be selected. With hexane + 1% *n*-octanol containing 0.25% (w/v) of tetraoctylammonium bromide as extraction solvent, the catecholamines can be quantitatively isolated from plasma and urine at pH 8.6 in the presence of 0.1% (w/v) of diphenylborate. For urine the recovery was 101.5 ± 1.9% for adrenaline, 100.6 ± 2.0% for noradrenaline and 99.9 ± 1.5% for dopamine. For plasma the recoveries were, respectively, 101.8 ± 3.3%, 100.5 ± 2.6% and 92.9 ± 3.5%. The recovery of dihydroxybenzylamine, included in the study as internal standard, was determined to be 96.3 ± 1.6% for urine and 89.9 ± 2.7% for plasma. The applicability of the developed extraction system as clean-up and concentration step for the analysis of catecholamines in plasma and urine by high-performance liquid chromatography with electrochemical detection is demonstrated.

INTRODUCTION

Various analytical methods, such as fluorimetry [1, 2], gas chromatography [3, 4] and in particular liquid chromatography [5—8], are used for the analysis of catecholamines in body fluids and tissues. With all these methods an extensive clean-up procedure, sometimes followed by a concentration step, is inevitable in order to obtain accurate and precise data. Various separation methods have been used as clean-up and concentration step: solvent extrac-

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tion [9, 10], adsorption on alumina [11, 12] and hydrophobized materials [13, 14], ion exchange [8, 15, 16] and isolation on boric acid gels [8, 17, 18]. The adsorption on alumina is by far the most frequently used method because of its unique selectivity towards catechol groups in alkaline medium by which rather clean extracts can be obtained. In this respect the recently developed boric acid gel, which shows a similar selective behaviour towards catechol groups as alumina, becomes increasingly popular, in particular because sometimes problems arise with the reproducibility of the activity of Al_2O_3, which can differ from batch to batch and requires extensive pretreatment before use. The chromatographic methods, although very useful, are rather time consuming and can not be easily automated, which is a great disadvantage when many samples have to be analyzed.

From the point of view of simplicity, reproducibility and automation, isolation by means of solvent extraction offers many possibilities [19]. However, until now the results of the isolation of catecholamines by means of solvent extraction have been mainly restricted to brain tissues [9, 20]. For that purpose *n*-butanol was found to be a suitable solvent for the extraction of catecholamines from acidified solution [20]. However, for plasma and urine samples far too many endogenous substances, interfering seriously with the analysis of catecholamines, are co-extracted with *n*-butanol. An improvement was obtained when the catecholamines were extracted as ion-pairs with diethylhexylphosphoric acid as pairing anion [21, 22]. However, also under these conditions the co-extraction of endogenous substances is still significant.

It is common knowledge that the co-extraction of endogenous (and exogenous) substances always present in body fluids will be suppressed when less polar extraction solvents can be used. In the present paper the results are reported of an investigation to isolate catecholamines from plasma and urine samples by solvent extraction with high recovery and very low co-extraction of endogenous substances, using the complex formation in alkaline medium between diphenylborate and catechol groups. The selectivity and the recovery of the isolation of catecholamines from aqueous, plasma and urine samples was determined using high-performance liquid chromatography (HPLC) with electrochemical detection.

EXPERIMENTAL

Apparatus

The HPLC system used to analyze the extracts consisted of a reciprocating pump (Orlita, Giessen, G.F.R.) equipped with a home-made flow-through bourdon-type manometer and pulse dampener (custom), a coulometric detector (Kipp Analytica, Emmen, The Netherlands) with a potential setting of +0.6 V vs. Ag/AgCl, and a high-pressure sampling valve (type 7010; Rheodyne, Berkeley, CA, U.S.A.) equipped with a 500-μl sample loop. The columns used were of 316 stainless steel and of dimensions of 150 × 4.6 mm (Fast LC-8, Technicon, Tarrytown, NY, U.S.A.).

Chemicals

All chemicals were of analytical grade and used without any further pre-

treatment. The catecholamines were obtained from Sigma (St. Louis, MO, U.S.A.), diphenylborate–ethanolamine was from Aldrich (Milwaukee, WI, U.S.A.), the quaternary amines from BDH (Poole, Great Britain) and octanol from Riedel de Haen (Seelze-Hannover, G.F.R.).

PROCEDURES

Chromatography

After equilibration of the phases, the concentrations of the catecholamines in the aqueous and organic phase (after back-extraction) were determined by means of HPLC with electrochemical detection. For this purpose use was made of a dynamic cation-exchange system as described previously [23]. The column packing was a C_8 bonded silica (Fast LC-8) and the mobile phase was composed of a water–methanol mixture (75:25, v/v) containing 0.05 *M* sodium acetate, 0.15 *M* acetic acid, 0.01% (w/v) of sodium dodecyl sulfate (SDS), 0.01% (w/v) sodium chloride and 0.01% (w/v) EDTA. Depending on the solute concentration, the injection volume ranged between 20 and 450 μl. In order to decrease the background current in detection, the pump, manometer, capillaries and injection port were pumped through, once before use, with 200 ml of 15% nitric acid.

Extraction

The distribution coefficient (D_i) of the catecholamines, defined as the ratio of the total catecholamine concentrations in the organic and aqueous phase, was determined by mixing 5 ml of the organic phase with 5 ml of the aqueous phase. The pairing cations were dissolved in the organic phase, except for the inorganic cations, which were dissolved in the aqueous phase. Diphenylborate–ethanolamine complex (DPBEA) was usually dissolved in the aqueous phase.

The pH of the aqueous phase was adjusted with an NH_4Cl–NH_4OH buffer, except in the case where the influence of pH on D_i was investigated, when an $(NH_4)_2HPO_4$–NH_4OH buffer was used. Known concentrations of the catecholamines (ca. 10 nmol/ml) in the aqueous phase were prepared from a stock solution of the solutes in 0.05 *M* phosphoric acid, stored at 4°C.

The two phases were shaken by hand for 2 min and if necessary centrifuged (1200 *g*) to improve phase separation. The concentrations of the solutes in the organic phase were determined by back-extraction into an aqueous phase; 1 ml of the organic phase was mixed with 1 ml of 0.05 *M* trichloroacetic acid (TCA) and 20–450 μl of the aqueous phase were injected into the HPLC system.

For relatively small D_i values the concentrations in the aqueous phase could be measured directly by HPLC, after adjustment of the pH to 4 with sulphuric acid, by injecting a maximum 20 μl of the aqueous phase. Larger injection volumes were not possible because of serious disturbance of the chromatographic system due to the high salt concentration. In such cases the solutes in the aqueous phase had to be extracted into an organic phase and back-extracted into a small volume (concentration step) of 0.05 *M* TCA before injection into the HPLC system.

Urine and plasma samples

Urine. To 0.5 ml of urine (preserved with 0.025 *M* HCl) 100 µl of a solution of the internal standard (equivalent to 35 ng) dihydroxybenzylamine (DBHA) + 1 ml of 2.0 *M* NH_4Cl—NH_4OH (pH 8.5) buffer containing 0.2% (w/v) of DPBEA and 0.5% (w/v) of EDTA are added. After the addition of 4 ml of *n*-heptane containing 1% (v/v) *n*-octanol and 0.25% (w/v) of tetraoctylammonium bromide (TOABr) the mixture is shaken by hand for 2 min and then centrifuged at 1200 *g*. To 3 ml of the organic phase 2 ml of *n*-octanol and 0.4 ml of 0.08 *M* acetic acid are added and the mixture is shaken by hand for 2 min, followed by centrifugation (5 min); 100 µl of the aqueous phase are injected into the HPLC system.

Plasma. To 2 ml of plasma 100 µl of a solution of DHBA (equivalent to 700 pg) + 1 ml of 2 *M* NH_4OH—NH_4Cl buffer (pH 8.5) containing 0.2% (w/v) DPBEA and 0.5% (w/v) of EDTA are added. After the addition of 5 ml of *n*-heptane + 1% *n*-octanol containing 0.25% (w/v) TOABr, the sample is shaken by hand for 2 min and centrifuged at 1200 *g* for 5 min. Then 4 ml of the organic phase are transferred to a conical tube and mixed with 2 ml of *n*-octanol and 250 µl of 0.08 *M* acetic acid, shaken by hand for 2 min, then centrifuged; 200 µl of the aqueous phase are injected into the HPLC system.

RESULTS AND DISCUSSION

For the isolation of catecholamines by solvent extraction use was made of the complex formation between borate and catechol (diol) groups in alkaline medium [11] and of ion-pair formation. The applicability of this complex formation for the selective isolation of catecholamines from body fluids has been demonstrated in the last years with the so-called boric acid gels [17, 18]. Although the exact reaction scheme has not been elucidated, our opinion is that the extraction proceeds via the scheme given in Fig. 1.

In the present study diphenylborate was used because of its hydrophobic substituents which favour the extraction of borate—diol complexes into organic solvents. Diphenylborate is commercially available as the diphenyl-

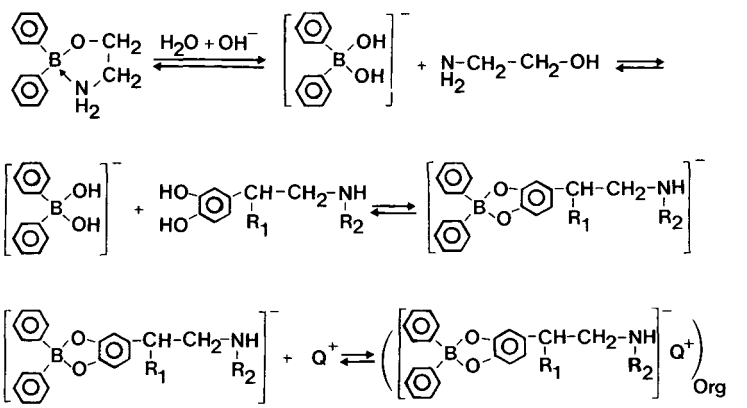

Fig. 1. Tentative equilibrium reactions involved in the extraction of catecholamines using diphenylborate as complexing agent and ion-pair formation.

borate—ethanolamine complex. This complex dissociates in aqueous alkaline medium into a negatively charged diphenylborate and ethanolamine. The diphenylborate forms a negatively charged stable complex with the catecholamines via the diol group, which cannot be easily extracted into an organic solvent. In order to realize this an ion-pair has to be formed with a cation Q^+ (pairing ion) added to the aqueous or organic phase [24]. According to this reaction scheme the distribution coefficient (D_i) will be influenced by the pH of the aqueous phase, the diphenylborate and pairing ion (Q^+) concentration, on the type of pairing ion and on the composition of the organic solvent.

In order to verify the reaction scheme and to determine the optimal conditions for the extraction of catecholamines from plasma and urine samples, the D_i values of these solutes were measured under a variety of conditions. As organic solvents chloroform, *n*-octanol and mixtures of these solvents with *n*-heptane or hexane were chosen because these are found to be useful in ion-pair extraction [24].

Besides the catecholamines dihydroxybenzylamine (DHBA) was also included in the study because of its possible use as an internal standard.

Effect of pH of the aqueous phase

The influence of pH on the distribution coefficient, using NH_4^+ as the Q^+ and *n*-octanol as extraction solvent, is given in Fig. 2. It shows that the D_i increases significantly with increasing pH of the aqueous phase. This can be ex-

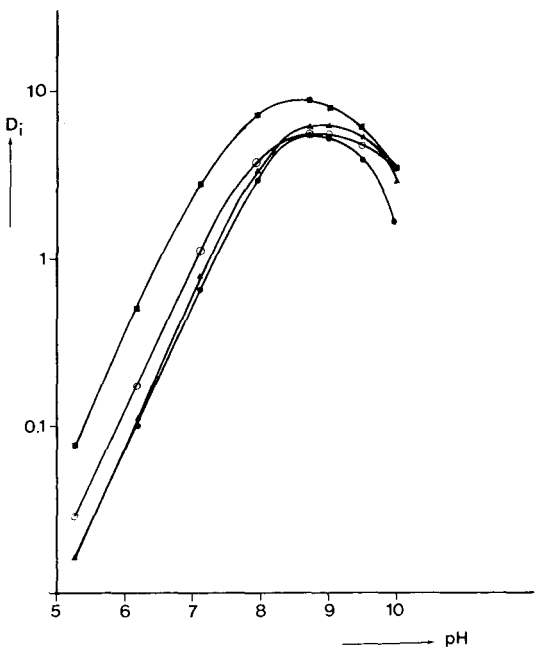

Fig. 2. Effect of the pH of the aqueous phase on the distribution coefficient D_i with NH_4^+ as pairing ion (Q^+) and *n*-octanol as extractant. (▲) Adrenaline; (●) noradrenaline; (■) dihydroxybenzylamine; (○) dopamine.

pected as at low pH the diphenylborate—ethanolamine complex is quite stable [25]. Therefore, the catecholamines can only be transferred to the organic phase as ion pairs with the inorganic anions (HPO_4^{2-}) present in the aqueous phase, which was shown before to be rather ineffective [24]. With increasing pH the diphenylborate—ethanolamine complex starts to dissociate and the negatively charged diphenylborate will be formed and reacts with the diol group of the catecholamines, thus promoting the extractability of the catecholamines (i.e. by masking the hydroxy groups) as a complex ion pair (with Q^+ as pairing ion) into the organic phase. The D_i values reach a maximum at pH 8—9 and then decrease again. This latter effect can be attributed to dissociation of the hydroxy groups of the catecholamines at high pH. Also losses due to oxidation are observed in this region. It should be noted that usually decomposition, due to oxidation, of the catecholamines already starts to occur at about pH 7. However, we found that the decomposition is almost negligible up to pH 9 when diphenylborate is present.

From Fig. 2 it can be concluded that the optimal pH of the aqueous phase must be in the range 8—9, which is in agreement with earlier reports [26]. A similar behaviour of D_i as function of the pH is observed when octanol is replaced by other solvents such as chloroform.

Effect of the type of pairing ion (Q^+)

According to the assumed reaction scheme the diphenylborate—catecholamine complex will be extracted into the organic phase as an ion pair with a

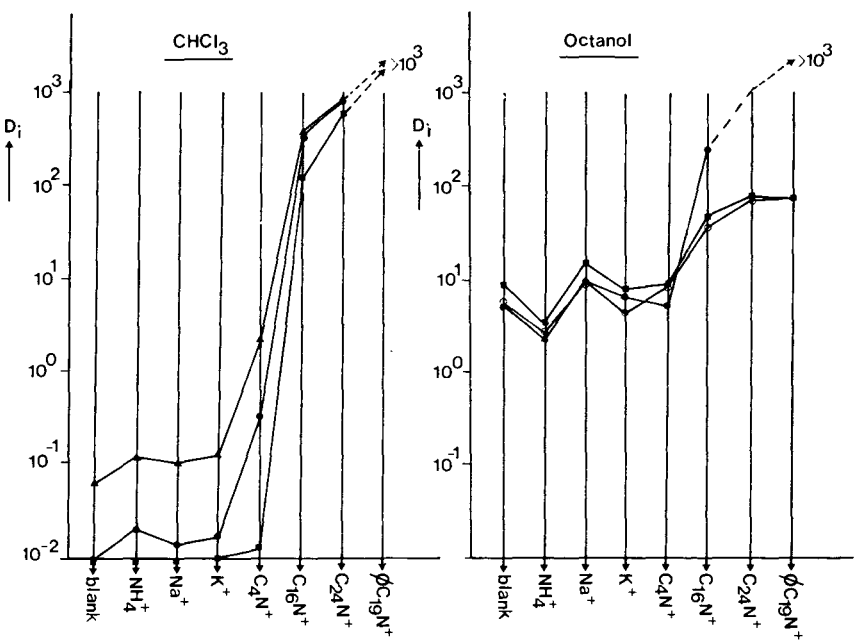

Fig. 3. Effect of the type of pairing ion (Q^+) on the distribution coefficient D_i of catecholamines with chloroform and *n*-octanol as extraction solvents. C_4N^+ = tetramethylammonium chloride; $C_{16}N^+$ = tetrabutylammonium hydrogen sulfate; $C_{24}N^+$ = tetrahexylammonium bromide; $\phi C_{19}N^+$ = benzyldimethyl *n*-hexadecylammonium chloride.

cation Q^+. It is known from ion-pair extraction studies that the extractability of ion pairs is also dependent on the type of pairing ion [24]. Usually a pairing ion with a large hydrophobic moiety enhances the extractability.

In order to investigate this for the present ion-pair extraction system, the distribution coefficient was measured with various types of inorganic and organic cations with chloroform and *n*-octanol as extraction solvents. The results of these measurements are shown in Fig. 3. It shows that the type of cation significantly influences the value of D_i. Further, some significant differences between chloroform and *n*-octanol can be noticed. With the inorganic cations and with tetramethylammonium, very small D_i values (0.01—1) were found with chloroform and significantly larger values (2—10) with *n*-octanol. This can be attributed to the better solvation ability of octanol compared to chloroform [24]. In agreement with previous findings the extraction (D_i value) inproves significantly for all solutes when using more hydrophobic quaternary amines. With chloroform the D_i value of all solutes increases with increasing hydrophobic part of the quaternary amine, reaching D_i values $> 10^3$ with benzyldimethyl *n*-hexadecylammonium chloride. With octanol, however, the D_i values of adrenaline and noradrenaline also reach values $> 10^3$ with benzyldimethyl *n*-hexadecylammonium, but for dopamine and dihydroxybenzylamine significantly smaller D_i values (ca. 70) were found. This might be caused by side-reactions in one or both phases [24].

The results as given in Fig. 3 show that, with hydrophobic quaternary amines as pairing ion, the catecholamines can be almost quantitatively isolated from an aqueous phase with *n*-octanol or even better with chloroform as the extractant.

It must be noted that perchlorate ions, often used for deproteinization of plasma samples, must be absent in the aqueous phase because these anions form extremely stable ion pairs with hydrophobic quaternary amines and thus block the ion-pair formation of the diphenylborate—catecholamine complex with these quaternary amines.

Effect of the amount of chloroform and octanol added to hexane

It is common knowledge that the co-extraction of interfering substances from, for instance, plasma and urine samples is the lowest with very non-polar extraction solvents. Although chloroform and octanol are promising extraction liquids, it would be an advantage if these solvents could be replaced by a less polar solvent or could be used mixed with a non-polar solvent. In order to investigate this, the distribution coefficient was measured with pure hexane and mixtures of hexane—chloroform and hexane—*n*-octanol. The results of these measurements are given in Fig. 4. Pure hexane could not be used as an extraction solvent because a precipitate of unknown identity was formed. By analysis of both phases it was found that the catecholamines adsorb strongly on this precipitate. This precipitate does not occur when about 3% (v/v) of chloroform and 0.5% of octanol is added to hexane. It should be noted that much lower concentrations of chloride as used here lead to an increase of this precipitate problem.

Fig. 4 shows the effect of the amount of chloroform and octanol present in hexane on the distribution coefficient. With chloroform the log D_i values of

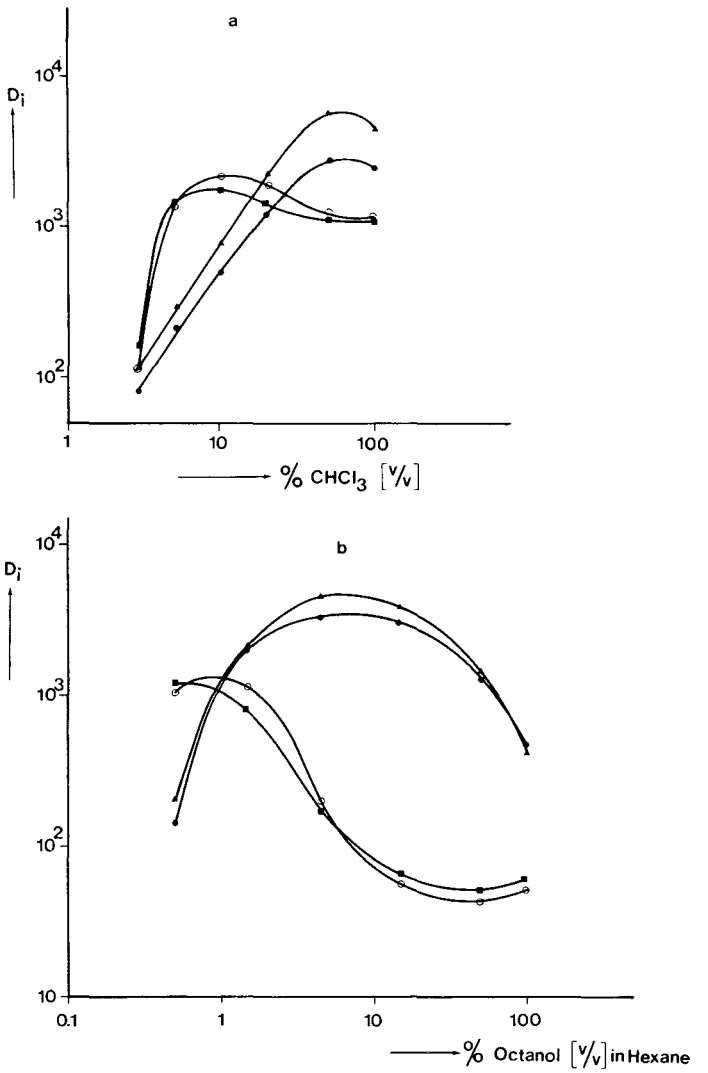

Fig. 4. Effect of the amount of chloroform (a) and octanol (b) in hexane on the distribution coefficient of catecholamines with tetraoctylammonium as pairing ion. (▲) Adrenaline; (●) nonadrenaline; (■) dihydroxybenzylamine; (○) dopamine.

adrenaline and noradrenaline increase almost linearly with increasing chloroform content up to 50% of chloroform and then decrease again towards 100% of chloroform. Dopamine and dihydroxybenzylamine show a significantly different dependence. The D_i values increase with increasing chloroform content and pass through a maximum at about 10% (v/v) of chloroform. With octanol the distribution coefficients of adrenaline and noradrenaline increase with increasing octanol content, pass through a maximum value at about 4% of octanol and then sharply drop again. For dopamine and dihydroxybenzylamine a maximum D_i value is found at about 1% of octanol and also for these solutes the D_i value decreases sharply with increasing octanol content.

From Fig. 4 it can be seen that with about 20% of chloroform or 1% of octanol for all solvents a D_i value $> 10^3$, large enough for a quantitative isolation, can be obtained. With respect to co-extraction of interfering substances, 1% of octanol is preferable.

Effect of the diphenylborate concentration

The influence of the diphenylborate concentration on the distribution coef-

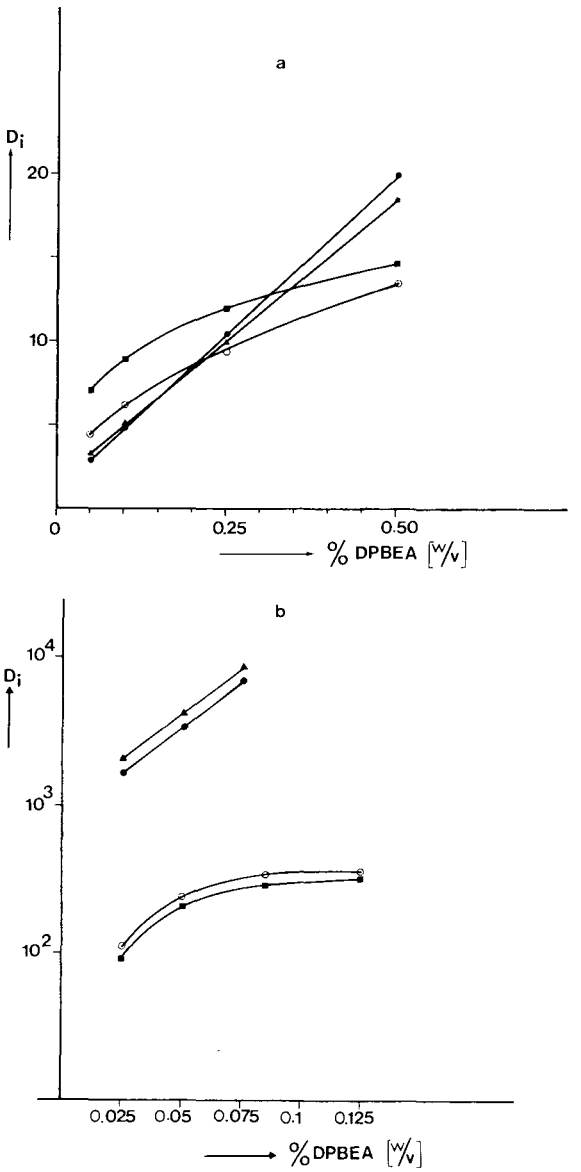

Fig. 5. Effect of the diphenylborate concentration on the distribution coefficient of catecholamines with NH_4^+ (a) and tetraoctylammonium (b) as pairing ion. (▲) Adrenaline; (●) noradrenaline; (■) dihydroxybenzylamine; (○) dopamine.

ficient was investigated with NH_4^+ as the pairing ion and octanol as the extractant, and with tetraoctylammonium as Q^+ and hexane + 2.5% (v/v) octanol as the extraction solvent. The results of these measurements are given in Fig. 5. In both extraction systems the D_i values of adrenaline and noradrenaline increase steeply with increasing diphenylborate concentration as expected on the basis of the assumed reaction scheme given in Fig. 1. With tetraoctylammonium as the Q^+ and diphenylborate concentrations $> 0.075\%$, the D_i values of adrenaline and noradrenaline could no longer be accurately determined because of the extremely low catecholamine concentration in the aqueous phase after phase equilibration (i.e. the D_i values of these two solutes became extremely large). The D_i values of dopamine and dihydroxybenzylamine also increase with increasing diphenylborate concentration (although less steeply than those of adrenaline and noradrenaline) with NH_4^+ as pairing ion and octanol as extractant. However, with tetraoctylammonium the D_i value first increases and then levels off at higher diphenylborate concentrations. This deviating behaviour must be attributed to side-reactions occurring in one or both liquid phases and indicates the imperfectness of the assumed reaction scheme.

From Fig. 5 it can be seen that the diphenylborate concentration should be about 0.1%, which is less than half of the solubility of diphenylborate in water.

Effect of the pairing-ion concentration

The influence of the Q^+ concentration on the distribution coefficient was investigated with tetraoctylammonium as pairing ion and octanol as extractant. The results are shown in Fig. 6. The D_i value of all solutes increases steeply with increasing tetraoctylammonium concentration in agreement with findings in ion-pair extraction systems and in accordance with Fig. 1. A Q^+ concentration as large as possible seems to be of advantage. However, when using hexane

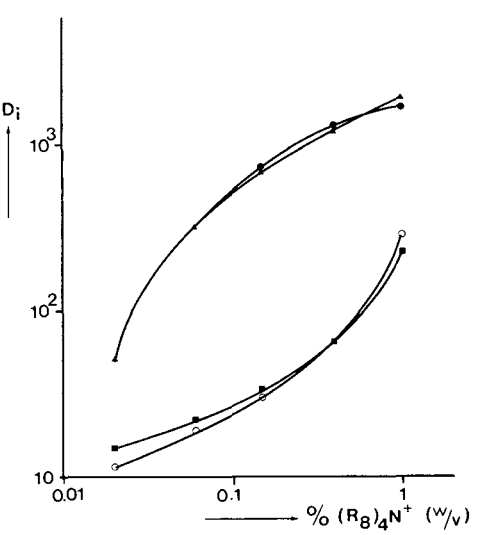

Fig. 6. Effect of the pairing ion concentration [$(R_8)_4N^+$ = tetraoctylammonium] on the distribution coefficient of catecholamines. Extraction solvent: *n*-octanol. (▲) Adrenaline; (●) noradrenaline; (■) dihydroxybenzylamine; (○) dopamine.

+ 1% octanol as extractant, the D_i values are much larger than with pure octanol and become more or less constant (D_i values 10^3 to $5 \cdot 10^3$) at 0.3—0.4% (w/v) of tetraoctylammonium. From the point of view of co-extraction of anions via ion-pair formation with tetraoctylammonium, this concentration seems to be a good choice.

Selection of the extraction conditions

The results as reflected in Figs. 2—6 can be used to determine optimal extraction conditions for the catecholamines. From the point of view of extraction efficiency and selectivity of extraction, a mixture of hexane + 1% of octanol as extractant and 0.1% (w/v) of diphenylborate and 0.2—0.3% of tetraoctylammonium and a pH of the aqueous phase of 8.6 appears to be a good compromise. Under these conditions plasma and urine samples were extracted as described under Procedures and the extracts were analyzed by HPLC using electrochemical detection. It can be noticed that hexane + 20% of chloroform is also favourable as an extractant. However, unfortunately chloroform generates a baseline disturbance at or near the position of dopamine in the chromatogram, at least with the chromatographic and detection system used in this study.

Fig. 7 shows chromatograms of extracts of a standard mixture, a plasma sample and a urine sample. The chromatogram of the plasma sample was recorded at the most sensitive setting of the electrochemical detector (25 nA full-scale). As can be seen the background is almost free of interfering substances, allowing the determination of adrenaline, noradrenaline and dopamine. Adrenaline and noradrenaline can be determined in plasma of normal persons and abnormal levels can easily be detected. However, the concentration of dopamine in plasma of normal persons is usually too low to be detected with the present system. If one wants to determine dopamine, the mobile phase composition has to be changed, by variation of the methanol content, in such a way that the capacity factor k' of dopamine is as small as possible accounting for the disturbance of the chromatogram near the unretained position. Under these conditions adrenaline and noradrenaline can not be determined as they disappear in the front peak.

The chromatogram of an extract of urine is shown in Fig. 7b. Also here the background is excellent and allows a precise determination of the three major catecholamines.

From Figs. 2—6 it can also be seen that dopamine and dihydroxybenzylamine behave significantly differently compared to adrenaline and noradrenaline. Because of this, dihydroxybenzylamine is not the ideal internal standard for adrenaline and noradrenaline. In this respect N-ethylnoradrenaline would probably be a better internal standard for these two solutes.

Quantitative aspects of the extraction

The recovery and reproducibility of the developed extraction method was tested with plasma and urine samples of healthy volunteers. The recovery was determined by spiking the plasma and urine samples with known amounts of the catecholamines (usually ten times the endogenous concentration) and

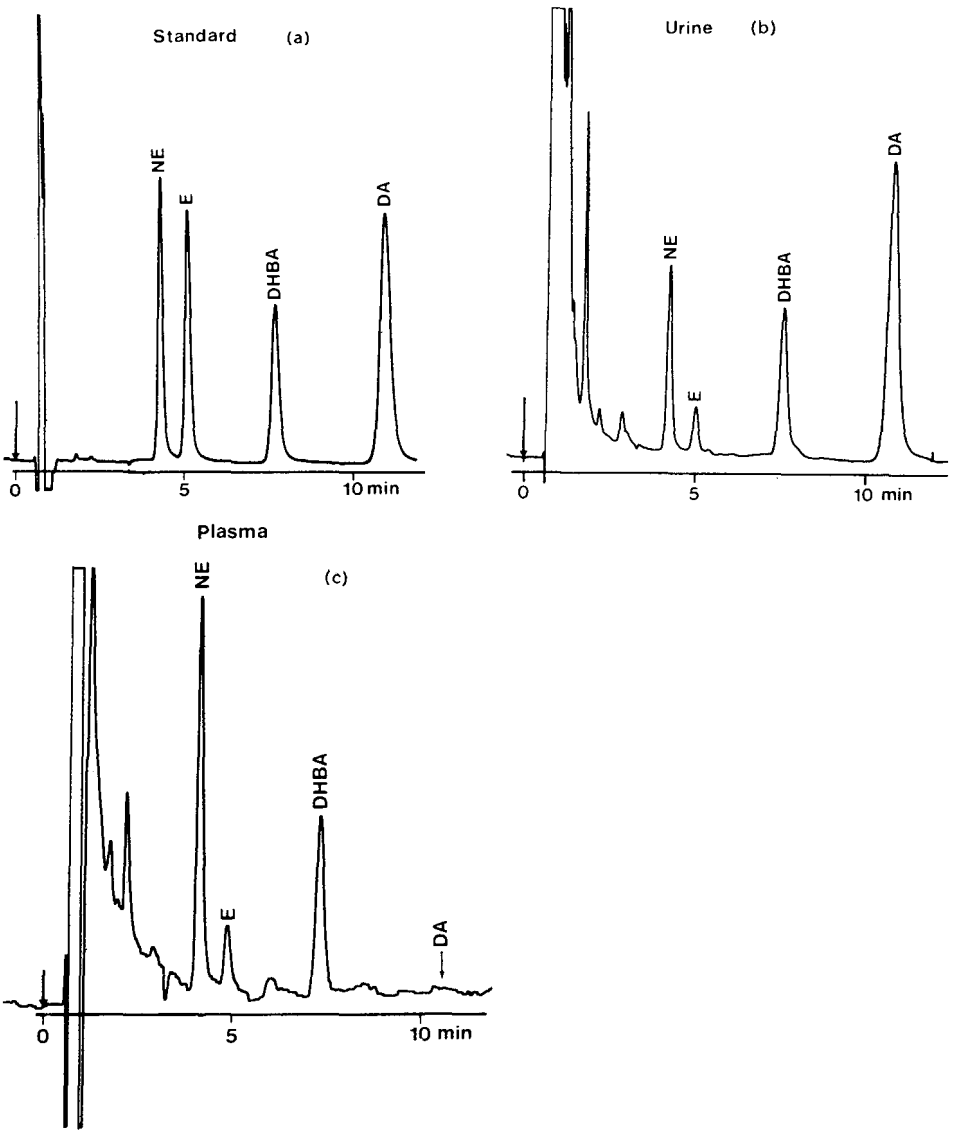

Fig. 7. Chromatograms of extracts of a standard solution (a), urine (b) and of a plasma sample (c) obtained with solvent extraction and HPLC with electrochemical detection. (a) containing 70 ng of noradrenaline (NE), 76 ng of adrenaline (E), 60 ng of dihydroxybenzylamine (DHBA), and 127 ng of dopamine (DA) per ml; (b) containing 56 ng of Ne, 16 ng of E, 60 ng of DHBA, 190 ng of DA per ml; (c) containing 515 pg of NE, 125 pg of E, 600 pg of DHBA per ml.

extracting these samples as described under Procedures, followed by analysis of the extracts by HPLC. The concentrations found were corrected for endogenous concentrations determined in parallel experiments with the non-spiked samples. The results of these measurements are given in Table I. As can be seen from the table, the three catecholamines are completely recovered from urine. However, the recovery of dihydroxybenzylamine is somewhat

TABLE I

RECOVERY OF CATECHOLAMINES* FROM PLASMA AND URINE BY SOLVENT EXTRACTION

	Amount added (ng)	Recovery (%)
Urine (0.5 ml) (n = 7)	NE = 210	100.6 ± 2.0
	E = 230	101.5 ± 1.9
	DHBA = 170	96.3 ± 1.6
	DA = 380	99.9 ± 1.5
Plasma (2 ml) (n = 7)	NE = 7.0	100.5 ± 2.6
	E = 7.0	101.8 ± 3.3
	DHBA = 6.0	89.9 ± 2.7
	DA = 12.0	92.9 ± 3.5

*NE = noradrenaline; E = adrenaline; DHBA = dihydroxybenzylamine; DA = dopamine.

TABLE II

REPRODUCIBILITY OF THE SOLVENT EXTRACTION HPLC METHOD FOR THE DETERMINATION OF CATECHOLAMINES* IN PLASMA AND URINE OF A HEALTHY VOLUNTEER (UNSPIKED SAMPLES)

	Urine (ng/ml) (n = 5)	Plasma (pg/ml) (n = 5)
NE	56.6 ± 1.5	515 ± 17
E	15.7 ± 0.55	125 ± 6
DA	191.4 ± 6.9	—

*Abbreviations as in Table I.

lower (96 ± 1.6%), which raises doubts concerning the use of this solute as an internal standard.

From plasma adrenaline and noradrenaline are completely recovered. The recoveries of dopamine and again dihydroxybenzylamine were found to be significantly lower: 93% and 90%, respectively. An explanation for this has not yet been found. Deproteinization before extraction may improve the recovery, but this was not investigated.

The reproducibility of the method was tested by extraction of a series of non-spiked plasma and urine samples of a healthy volunteer. The catecholamine levels, including the standard deviation, in plasma and urine of this volunteer are given in Table II. As can be seen from this table, the reproducibility of the method is excellent, as is reflected in the standard deviation which ranges between 3 and 4%. The catecholamine levels determined agree with those reported in the literature, except for adrenaline in plasma, which is somewhat higher. However, this might be attributed to the way in which the blood sample was taken, as this might significantly influence the actual concentration level of adrenaline in the plasma.

CONCLUSIONS

The developed solvent extraction system, based on the complex formation in alkaline medium between diphenylborate and catechol groups and ion-pair formation, was found to be extremely suitable for the selective and quantitative isolation of adrenaline, noradrenaline and dopamine from plasma and urine. From the point of view of simplicity, recovery and reproducibility, this solvent extraction system has definite advantages compared to the chromatographic isolation techniques. Further, the simple solvent extraction system lends itself to automation. Preliminary experiments in this direction show that urine samples can be automatically handled with a Technicon Fast LC system. A report on this subject is in preparation.

When combining the solvent extraction with HPLC and electrochemical detection, it is possible to determine the catecholamines in plasma and urine of healthy persons and to detect increased catecholamine levels in patients. However, the described isolation method might be also of great value as a clean-up step for other analytical methods such as fluorimetry, radioimmunoassay, or radioenzymatic methods.

Future work in our laboratory will be devoted to the application of the present extraction principle for the isolation of other compounds containing diol groups and to apply diphenylborate, dynamically coated on various packings, for on-column isolation and/or as a chromatographic HPLC system.

ACKNOWLEDGEMENTS

This work was carried out partly under a Technicon Grant. Thanks are due to Mr. Eric Endert for valuable advice and technical assistance.

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Journal of Chromatography, 231 (1982) 41—51
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1297

ROUTINE DETERMINATION OF PLASMA CATECHOLAMINES USING REVERSED-PHASE, ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received October 20th, 1981; revised manuscript received March 15th, 1982)

SUMMARY

A procedure is described for the determination of plasma catecholamines using reversed-phase, ion-pair high-performance liquid chromatography coupled with electrochemical detection. Optimisation of chromatographic conditions with respect to detector performance and adherence to procedures and precautions described, render the method applicable to both neurochemical research and routine clinical analysis. The limit of quantitative detection of the method was found to be approximately 30 pg per injection for individual catecholamines. A single chromatographic run, providing adequate resolution of each component, could be completed in approximately 12 min.

INTRODUCTION

High-performance liquid chromatography (HPLC) coupled with electrochemical detection (HPLC—ElCD) is a technique which has found increasing application in neurochemical research. Interest in the technique has centred on the advantages it possesses for the analysis of biogenic amines and their metabolites. Recently two state-of-art reviews of HPLC—ElCD and its applications in neurochemical research have appeared in the literature [1, 2].

Catecholamines are one of the groups of compounds amenable to analysis by HPLC—ElCD. The relatively simple procedure of sample preparation, ease of oxidation of the catecholamines to their corresponding quinones, sensitivity of electrochemical detection and the specificity of chromatographic separation means that HPLC—ElCD has distinct practical advantages over existing methods of determination where chemical modification of the catecholamine structure prior to analysis is required. Examples of established methods of catechol-

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amine analysis include gas—liquid chromatography with electron-capture detection [3, 4], gas chromatography—mass spectrometry [5—7], HPLC with ultraviolet detection [8] or fluorescence detection [9, 10], other fluorescence methods [11, 12] and radioenzymatic assay [13—15]. The low concentration of catecholamines in plasma demands that the analytical method has a high degree of both sensitivity and selectivity. Until the advent of HPLC—ElCD, radioenzymatic assay had proved to be the most effective method applicable to subjects where relatively small blood samples (< 5 ml) were available. This technique, however, suffers from the complexity of the sample preparation procedure and is both time consuming and expensive.

The first report of the application of HPLC—ElCD to the analysis of catecholamines was made by Kissinger et al. [16]. The use of the technique for the determination of plasma catecholamines was described by Hallman et al. [17] who used cation-exchange chromatography to achieve separation. More recently the use of reversed-phase ion-pair separation techniques has improved the efficiency, sensitivity and versatility of the chromatography of these compounds [18]. The analysis of picogram quantities of catecholamines that are typically encountered in plasma samples, however, poses additional difficulties due to the small currents (ca. 100—800 pA) generated at the electrode surface as a result of solute oxidation; electrical instability, with a consequent decrease in instrument performance often presents a problem to the chromatographer. The present communication describes a reversed-phase, ion-pair HPLC system, designed to overcome these difficulties. It utilises a high efficiency bonded silica analytical column specifically designed for use in the ion-pair mode and chromatographic conditions have been optimised with respect to parameters known to influence HPLC—ElCD performance. The method is shown to be suitable for the routine laboratory determination of catecholamines in plasma.

EXPERIMENTAL

Reagents and standards

Adrenaline bitartrate, noradrenaline bitartrate, dopamine hydrochloride, 3,4-dihydroxybenzylamine hydrobromide and tris(hydroxymethyl)aminomethane (Tris) were supplied by Sigma (London) (Poole, Great Britain). Methanol (HPLC grade) and sodium octane-1-sulphonate were purchased from Fisons Scientific Apparatus (Loughborough, Great Britain). Aluminium oxide, 70—230 mesh, activity grade 1, neutral washed, was supplied by E. Merck (Darmstadt, G.F.R.).

The aluminium oxide was activated by the method of Anton and Sayre [19] and stored at 37°C until required. The internal standard, 3,4-dihydroxybenzylamine, was prepared as a 0.1 μM solution in 0.1 M perchloric acid (containing 400 μM sodium metabisulphite). Stock solutions of other catecholamines were similarly prepared to the required concentration. The 0.5 M Tris—HCl buffer, pH 8.6, and a diluted solution used in the alumina washing procedure for the isolation of catecholamines, were prepared as described by Adams [20].

Equipment

The liquid chromatograph comprised an Altex 100A pump (Altex Scientific,

Berkeley, CA, U.S.A.), a Rheodyne Model 7125 injection valve fitted with a 20-μl sample loop (Rheodyne, Berkeley, CA, U.S.A.) and a 25 cm × 4.6 mm I.D. stainless-steel analytical column packed with 5 μm diameter Ultrasphere IP particles (Altex Scientific). The analytical column was fitted with a 5 cm × 4.6 mm I.D. precolumn packed with 30—38 μm diameter Co-Pell ODS (Whatman Lab. Sales, Maidstone, Great Britain). The detection system was a Model LC-4 amperometric detector fitted with a TL-5 glassy carbon electrode assembly (Bioanalytical Systems, West Lafayette, IN, U.S.A.). All chromatographic components were supplied by Anachem (Luton, Great Britain).

Chromatography

The mobile phase consisted of 780 ml of the acetate—citrate buffer (pH 5.2), described by Keller et al. [21], 220 ml of methanol, and sodium octane-1-sulphonate (5 m*M* final concentration). Solvent was pre-filtered through a 0.5-μm (pore diameter) Fluoropore membrane filter (Millipore, London, Great Britain) and degassed prior to use. Water used for mobile phase preparation was glass distilled and then deionised. The mobile phase flow-rate was 1.2 ml/min, and the electrode potential was set at +0.6 V vs. the Ag/AgCl reference electrode.

Optimisation of chromatographic performance

The use of the electrochemical detector to determine picomole and femtomole quantities of oxidisable (or reducable) material may be associated with increased levels of detector noise resulting in a reduction of chromatographic performance. A glassy carbon electrode cell provided improved overall performance as compared with a packed carbon paste electrode. Signal-to-noise ratio was enhanced, the detector sensitivity was stable and the electrode surface was unaffected by the passage of air bubbles occasionally formed in the mobile phase. Any surface contaminants could be washed from the electrode using water or methanol. Individual electrode cells have been found to possess a working life of greater than one year, whereas carbon-paste cells required repacking after 2—3 weeks' use.

Previous reports of catecholamine determination by HPLC—ElCD have described the use of the electrochemical detector at a number of oxidation potentials [17, 22—24]. We investigated the response of the detection system to catecholamine oxidation at values of electrode potential over the range +0.35 V to +0.85 V vs. the Ag/AgCl reference electrode in the mobile phase described. It was found that although a maximum detector response was obtained at +0.8 V (Fig. 1), an electrode potential of +0.6 V provided a response with sufficient sensitivity for the determination of picogram quantities of catecholamines, but with minimum interference from solvent effects and electrical noise.

The pH and ionic strength of the mobile phase have both been shown to affect the response of the electrochemical detector to catecholamine oxidation [18]. Optimum response of the electrode was obtained in the pH range 5.0—6.0 and ionic strength (of phosphate) of 0.07 *M* [18]. The mobile phase was composed accordingly with a pH of 5.2 and a total ionic strength of approximately 0.1 *M*.

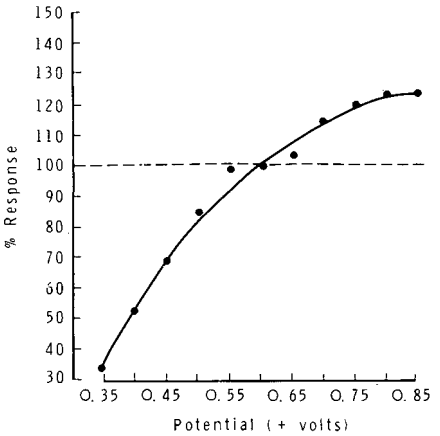


Fig. 1. Response of the electrochemical detector to catecholamine oxidation at a number of electrode potentials in the chromatographic system described. A range of electrode potentials was selected (vs. the Ag/AgCl reference electrode) and the total detector response obtained from the oxidation of individual catecholamines at each potential is represented. The broken horizontal line represents detector response at the chosen optimum oxidation potential of +0.6 V.

Long-term exposure of bonded silica columns to ion-pairing agents may significantly reduce column life [25]. In order to preserve optimum column performance, the chromatograph, when out of use (e.g. overnight), was maintained in a solvent flow of pure water of 0.2 ml/min, and with the electrode activated. The system could be quickly restored to operating conditions by equilibration of the column with mobile phase (15 column volumes approximately) and preparation of the system with two injections of a catecholamine standard mixture solution (total preparation time 1 h). Maintaining the system in this manner also enhanced detector stability.

Collection and storage of blood for analysis.

The stability on storage of blood samples collected for catecholamine determination was investigated in order that a protocol for routine use in a hospital laboratory, ward or clinic might be devised. Petersson et al. [26] recently reported that storage of untreated blood samples at room temperature for several hours did not result in any loss of plasma noradrenaline or adrenaline. We decided, however, to observe certain basic precautions regarding storage of samples for analysis. Blood samples (10 ml) were normally collected into lithium-heparin tubes containing 200 μl of 0.1 *M* sodium metabisulphite and stored immediately at 4°C. Under these conditions, plasma catecholamines were found to be stable for at least 24 h, the maximum time that samples would normally be stored under such conditions. The plasma was then removed and stored at −20° C until analysis.

Plasma for use in the preparation of calibration standards was obtained from a plasma pool. Aliquots of this plasma were treated with increasing amounts of catecholamines and taken through the extraction procedure. Calibration curves for individual catecholamines were constructed from the data obtained.

Isolation of catecholamines from plasma

A 2-ml sample of deproteinised plasma (protein denatured by the storage of plasma in the frozen state was separated by centrifugation at 800 *g* at 4° C), contained in a 15 ml capacity glass conical centrifuge tube, was treated with 200 μl of the internal standard solution. Then 400 μl of 0.5 *M* Tris—HCl, pH 8.6, followed by 20 mg of activated alumina, were added and the contents of the tube shaken gently for 15 min on a spiral mixer (Denley, Billingshurst, Great Britain). Following centrifugation at 600 *g* for 2 min, the supernatant was removed and the alumina washed three times with a buffer preparation [20], centrifuging each time as above. The catecholamines were eluted from the alumina into 50 μl of 0.6 *M* perchloric acid (containing 400 μM sodium metabisulphite). Following centrifugation at 800 *g* for 3 min, 20 μl of the supernatant were injected onto the chromatograph.

RESULTS

Resolution and sensitivity of the chromatographic system were determined daily by the injection of a 20-μl aliquot of a catecholamine reference solution. A typical chromatogram obtained from analysis of this standard mixture is shown in Fig. 2a. A complete separation of individual components of the

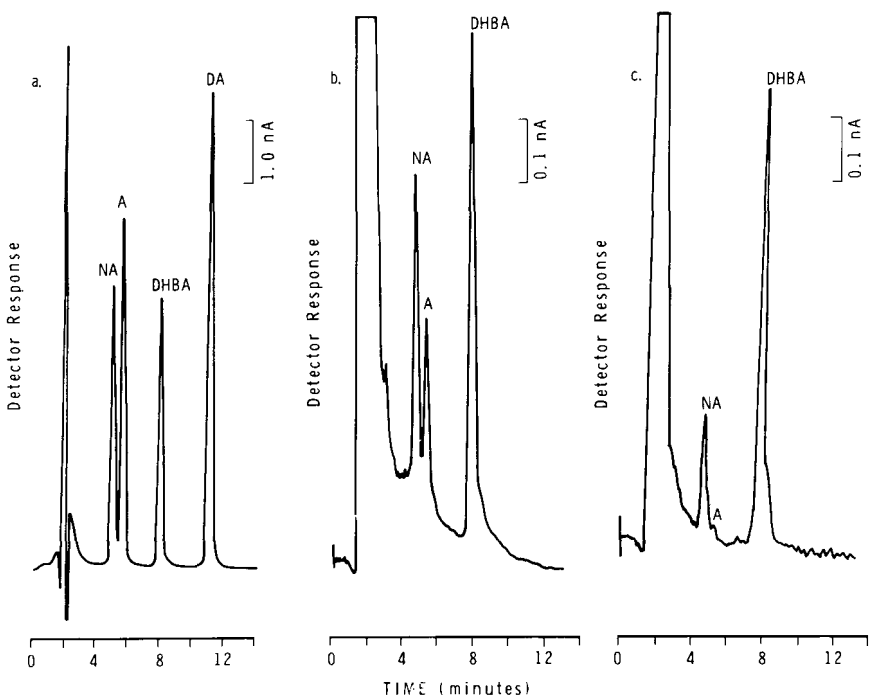


Fig. 2. Chromatograms of HPLC assay of plasma catecholamines. (a) Standard mixture containing 7 ng each of noradrenaline (NA) and adrenaline (A), 6.6 ng of 3,4-dihydroxybenzylamine (DHBA), 9.5 ng of dopamine (DA) injected; (b) plasma extract from a patient on admission to hospital following a road traffic accident; NA = 3.2 pmol/ml, A = 1.6 pmol/ml; (c) plasma extract from the same patient after 24 h treatment; NA = 1.6 pmol/ml, A = < 0.2 pmol/ml.

TABLE I

CHROMATOGRAPHIC PARAMETERS OF THE HPLC SYSTEM FOR THE SEPARATION OF CATECHOLAMINES

Compound	Retention time (min)	Capacity ratio (k')	Resolution factor (R_s)			
			NA	A	DHBA	DA
NA	4.84	1.69	—	1.2	5.3	8.6
A	5.38	1.99	1.2	—	8.1	8.1
DHBA	7.75	3.31	5.3	4.5	—	5.2
DA	10.92	5.07	8.6	8.1	5.2	—

mixture was obtained and a total sample running time of approximately 12 min recorded. Values of column capacity ratios (k') and resolution factors (R_s) are shown in Table I.

The linearity of both the extraction procedure and detector response (determined from peak area) was verified for each catecholamine over the anticipated range of assay. The former was investigated by assaying pooled plasma to which known amounts of noradrenaline (NA), adrenaline (A) and dopamine (DA) had been added and determining the peak area ratios (sample vs. internal standard), obtained for each compound. Calibration curves were constructed for each compound (Fig. 3); in each case a linear relationship between catecholamine concentration and peak area ratio was observed over the concentration ranges studied. The equations for the calibration curves obtained were as follows:

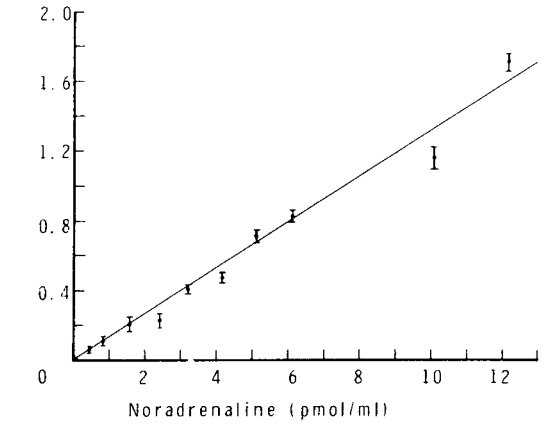
NA: $y = 0.12x - 0.012; r = 0.990$
A: $y = 0.20x - 0.009; r = 0.995$
DA: $y = 0.14x - 0.006; r = 0.998$

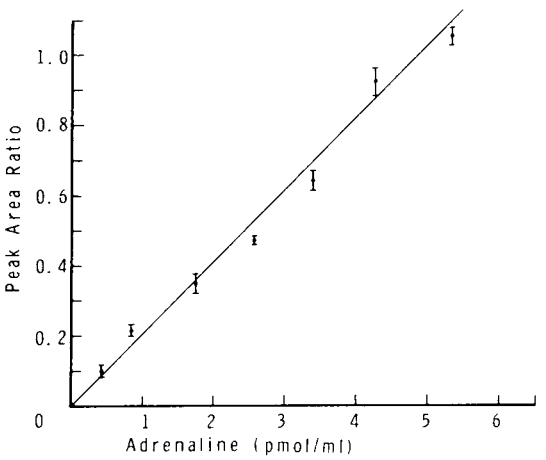
Each point on the calibration curve was established from the mean of five determinations.

The endogenous catecholamine concentrations of the pooled plasma were determined to be NA, 1.5 pmol/ml; A, none detected; DA, none detected.

The linearity of detector response was confirmed by the injection of known amounts of catecholamine standards directly onto the chromatograph. Response for each compound was found to be linear over the range investigated (NA, 0—8.4 ng, $r = 1.0$; A, 0—3.5 ng, $r = 1.0$; DA, 0—4.0 ng, $r = 1.0$). For the routine application described, the amplifier was operated at a sensitivity of either 1.0 nA/V or 2.0 nA/V full scale deflection (f.s.d.). At the former level of sensitivity a noise level of ± 1.0% f.s.d. was observed, which enabled a quantitative detection limit for each catecholamine of about 30 pg per injection to be achieved. This is similar to detection limits reported by other authors [17, 27, 28].

The precision of the extraction procedure and chromatography was evaluated by processing aliquots of pooled plasma containing known amounts of NA, A and DA. Values of inter- and intra-assay are shown in Table II. The recovery from the extraction procedure was determined by comparing the yields from a series of extractions of plasma containing known amounts of





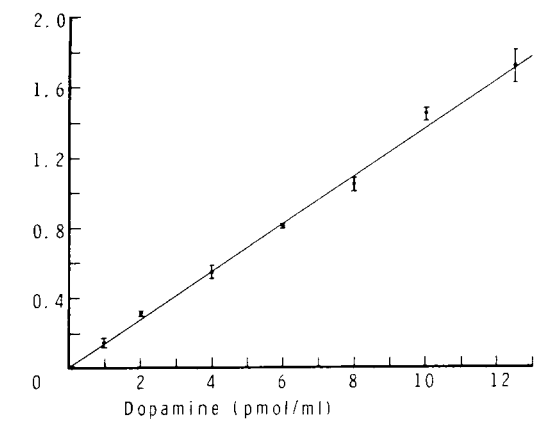


Fig. 3. Calibration curves for the determination of noradrenaline (NA), adrenaline (A) and dopamine (DA) in plasma by the assay procedure described.

TABLE II

INTER- AND INTRA-ASSAY PRECISION FOR THE HPLC DETERMINATION OF PLASMA CATECHOLAMINES

	NA			A			DA		
	Concn. added (pmol/ml)	*n*	C.V. (%)	Concn. added (pmol/ml)	*n*	C.V. (%)	Concn. added (pmol/ml)	*n*	C.V. (%)
Inter-day	0.73	5	10.2	0.5	5	9.2	0.9	5	13.1
	1.45	5	9.7	1.5	5	2.6	1.8	5	10.0
	11.6	5	4.4	6.0	5	5.4	14.4	5	4.7
Intra-day	0.73	9	12.1	0.5	9	7.7	0.9	9	13.7
	1.45	9	11.9	1.5	9	6.9	1.8	9	14.1
	11.6	8	5.8	6.0	8	6.3	14.4	8	5.5

TABLE III

PLASMA NORADRENALINE AND ADRENALINE LEVELS IN ACCIDENT CASUALTIES OF VARYING INJURY SEVERITY

Blood samples were taken from casualties as soon as possible following admission to hospital (Day 0). Further samples were taken at 24-h intervals following admission (Day 1, 2, etc.). Values = mean ± S.D.

Day	Admitted ward		Admitted intensive therapy	
	NA (pmol/ml)	A (pmol/ml)	NA (pmol/ml)	A (pmol/ml)
0	2.96 ± 1.4	1.37 ± 1.2	11.98 ± 7.8	7.52 ± 5.2
	n = 56		*n* = 10	
1	2.77 ± 1.7	0.58 ± 0.49	6.0 ± 3.4	0.82 ± 0.36
	n = 52		*n* = 7	
2	2.98 ± 2.1	0.45 ± 0.6	5.1 ± 4.2	0.96 ± 1.03
	n = 48		*n* = 7	
3	2.67 ± 1.5	0.35 ± 0.28	4.1 ± 3.2	0.60 ± 0.23
	n = 42		*n* = 8	

Discharged following treatment (Day 0; *n* = 15), NA = 2.75 ± 0.8 pmol/ml, A = 0.59 ± 0.38 pmol/ml.

catecholamines with standard solutions. Individual recoveries of 48.7% (NA), 50% (A), 47.5% [3,4-dihydroxybenzylamine (DHBA)], 41.2% (DA) were calculated, representing an overall recovery of 47%. This was lower than has been reported by other workers [17, 27, 29]. However, as was shown in Fig. 3, the procedure was linear over the assay ranges required for each catecholamine.

The present method has been applied to routine clinical analysis and a number of research studies. One such study is an investigation of the report that plasma catecholamines are elevated in response to trauma following injury.

Unless the injuries are life-threatening, a return to normal levels is seen within 72 h [30]. Figs. 2b and c are chromatograms obtained from the analysis of plasma samples of a 59-year-old male injured in a road traffic accident. Blood samples were taken on admission to hospital and following 24 h treatment. Plasma NA and A were found to be 3.2 pmol/ml and 1.6 pmol/ml respectively on admission (Fig. 2b), but following 24 h treatment (Fig. 2c) had fallen to 1.6 pmol/ml and 0.2 pmol/ml (limit of quantitation). Control levels for plasma NA and A of 1.4 ± 0.7 pmol/ml and 0.19 ± 0.08 pmol/ml respectively were calculated from data compilations from two sources [27, 31].

Analysis of the data obtained from 81 casualties has suggested that elevation of plasma NA and A following injury is related to the severity of injury sustained. Table III shows mean plasma noradrenaline and adrenaline levels from casualties whose injury severity has been assessed by the degree of medical treatment required. Patients were grouped into those who could be treated and discharged the same day, those who required admission to a routine surgical ward and those requiring intensive therapy. These increases in plasma catecholamines also correlated with the numerical evaluation of injury severity. Validation of these observations and assessment of their clinical relevance is currently being undertaken. However, the requirement for a reliable, sensitive method for the determination of plasma catecholamines in this and other biomedical applications is of paramount importance.

DISCUSSION

The HPLC—ElCD system described in this paper has been shown to possess the sensitivity, selectivity and reproducibility required for the determination of picogram quantities of catecholamines from 1—2 ml plasma samples. Improvement in chromatographic performance has been achieved through the use of a high-efficiency bonded silica analytical column specifically designed for use in the ion-pair mode and the problem of electrical instability sometimes encountered in the use of electrochemical detection at high sensitivity was overcome through adherence to the procedures and precautions described above. Column life was extended by eliminating unnecessary exposure of the analytical column to mobile phase containing ion-pairing agent (typical column life ca. 2000 injections).

Previous studies have proved the validity of HPLC—ElCD for the measurement of biogenic amines [28, 32—36]. Comparison of the method with some recent reports on the application of HPLC—ElCD to the determination of plasma catecholamines suggests an improvement in sensitivity of up to four-fold. Jenner et al. [22] required up to 4 ml of plasma for their assay of plasma catecholamines; half of their final eluate was subjected to analysis at a sensitivity of 0.5 nA/V f.s.d. The method of Goldstein et al. [27] was also performed at a similar level of detector response, using half of the total eluate from a 1-ml plasma extraction. In the present assay, basal levels of NA (0.5—1.5 pmol/ml) could be easily determined using 1 ml plasma. However, for basal levels of A (0.1—0.3 pmol/ml), 2 ml were required with a limit of quantitative detection set at 0.2 pmol/ml plasma equivalent. In our experience we have found that the response characteristics of individual glassy-carbon

electrode cells may vary considerably. Caution should therefore be exercised in the selection of electrodes to ensure optimum performance is achieved. However, once installed into an HPLC—ElCD system, their stability of performance renders them more suitable for high-sensitivity work than carbon paste electrodes.

In the traces shown in Figs. 2b and c no chromatographic evidence for the presence of DA was found. The definition of a normal DA level in plasma has been somewhat controversial [36]. Hallman et al. [17] reported a range of resting plasma dopamine levels in healthy volunteers of between < 0.05 and 0.23 pmol/ml, whilst Fenn et al. [37] put the level as high as 0.75 pmol/ml. Since response to stress may not provide significant changes in plasma DA levels [28], normal levels within the lower range quoted above would fall below the limit of detection of dopamine.

HPLC—ElCD possesses the significant practical advantages of speed of sample processing (individual samples may be processed in less than 30 min) and low cost as compared with other methods for the measurement of biogenic amines. In addition to the improvements in technique reported above, these render the present method suitable for research application and routine clinical analysis.

ACKNOWLEDGEMENTS

This work was supported by a grant from the United Kingdom Department of Transport, Transport and Road Research Laboratory, Crowthorne, Berkshire, Great Britain.

We are also grateful to Mr. R.J. Newman, FRCS, of the Nuffield Department of Orthopaedic Surgery, University of Oxford, and to the staff of the Accident Service, John Radcliffe Hospital, Oxford, for their cooperation.

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Journal of Chromatography, 231 (1982) 53—63
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1295

ANALYSES OF NICOTINE AND COTININE IN TISSUES BY CAPILLARY GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(Received January 4th, 1982)

SUMMARY

Selective extraction and chromatographic techniques have been developed to measure low nanogram quantities of nicotine and cotinine in tissues. Analyses were performed by capillary column gas chromatography with a specific nitrogen—phosphorus detector and by gas chromatography—mass spectrometry. With close structural analogues for internal standards, high quantitative accuracy and precision were demonstrated for the range 5—1000 ng per g of tissue. The sensitivity limit was 2—3 ng/g for both compounds. The main advantage of these techniques compared to previously published methods is increased selectivity; the other methods were developed for analysis of biological fluids and are not readily adaptable to more complex biological matrices such as tissue homogenates. With the newly developed techniques, we were able to perform a pharmacokinetic study of nicotine and cotinine in mouse liver following a single intraperitoneal injection of nicotine.

INTRODUCTION

Owing to the widespread use of tobacco products, it is essential to acquire a thorough understanding of the pharmacological properties of nicotine, an important constituent of tobacco smoke [1]. To correlate specific responses with the presence of nicotine in various tissues of intact animals, the concentrations of this compound must be accurately determined. The analytical techniques presently available for the quantitation of nicotine in biological samples have been developed for analyses of blood and urine; the most sensitive and selective of these involve extraction followed by quantitation with gas chromatography utilizing a specific nitrogen—phosphorus detector (GC—NPD) [2—5] or with gas chromatography—mass spectrometry (GC—MS) [6, 7]. Similar techniques have

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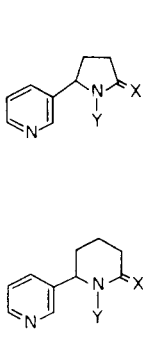

X = H,H
Y = H nornicotine
CH_3 nicotine
CD_3 NIC-d_3
X = O
Y = H norcotinine
CH_3 cotinine
CD_3 COT-d_3

X = H,H
Y = H anabasine
CH_3 MA
X = O
Y = CH_3 OMA

Fig. 1. Structures of compounds cited in the text.

been employed to quantitate cotinine (Fig. 1), a major metabolite of nicotine [5, 8]. Radioimmunoassay methods also have been developed to measure these compounds [9, 10], but because the specific antibodies are not readily available, chromatographic techniques are more generally useful for these analyses than immunochemical techniques. Our attempts to adapt published procedures to the quantitation of nicotine and cotinine at low nanogram levels in liver and brain tissues from mice were unsuccessful because of the simultaneous extraction of compounds which interfere with the analyses.

Tissue homogenates are more complex biological matrices than physiological fluids, requiring analytical methods which combine selective and efficient extractions with selective and sensitive chromatographic techniques. The present report describes the development and application of methods to quantitate low levels of nicotine and cotinine in tissue samples. Extraction schemes were developed to provide maximum recoveries of these compounds with minimum contamination of the extracts by interfering compounds. These methods, together with improved chromatographic and mass spectrometric techniques, allowed reliable quantitative analyses of nicotine and cotinine in tissue homogenates with a demonstrated sensitivity limit of 2—3 ng per g of tissue. The methods were applied to a pharmacokinetic investigation of nicotine and cotinine in mouse liver following a single intraperitoneal injection of nicotine.

EXPERIMENTAL

Reagents and standards

Nicotine (Sigma, St. Louis, MO, U.S.A.) was purified by fractional distillation under reduced pressure and stored at −20°C. A deuterated analogue, 1′-trideuteromethyl-nornicotine (NIC-d_3, Fig. 1), was prepared by the N-alkylation of 50 mg of nornicotine (Dr. N. Castagnoli, University of California, San Francisco, CA, U.S.A.) using sodium hydride and iodomethane-d_3 (99.5 atom % deuterium, Merck, St. Louis, MO, U.S.A.) in a manner which is analogous to a previously published procedure [11]. Methylanabasine (MA) was prepared similarly by the N-methylation of anabasine (Tridom Chemical, Hauppauge, NY, U.S.A.) with methyl iodide. Nicotine was oxidized to cotinine as described [12]. The internal standards 1′-trideuteromethyl-norcotinine (COT-d_3) and 1-methyl-6-(3-pyridyl)-2-piperidone (OMA, Fig. 1) also were prepared by oxida-

tion of NIC-d_3 or MA, respectively, under similar conditions. All synthetic products were purified by preparative thin-layer chromatographic plates (Analtech, Newark, DE, U.S.A.) and a solvent system ethyl acetate—methanol—ammonium hydroxide (85:10:5 for NIC-d_3 and MA; 70:25:5 for cotinine and OMA). The purified compounds were analyzed by GC—MS. Ethyl acetate (reagent grade, J.T. Baker, Phillipsburg, NJ, U.S.A.) was used without further purification, but toluene (reagent grade, J.T. Baker) was redistilled. Ammonium carbonate (HPLC grade, J.T. Baker) was pulverized with a mortar and pestle and washed twice by stirring with ethyl acetate. The solutions 4 *N* sodium hydroxide, 0.2 *N* sodium acetate (at pH 9.5) and 0.1 *N* hydrochloric acid were purified by extraction with ethyl acetate. Water was purified by ion exchange and charcoal filtration (Continental Water Systems, El Paso, TX, U.S.A.) followed by distillation.

Extraction of nicotine

The extraction scheme is shown in Fig. 2. Homogenates of mouse tissues were prepared by adding 4 ml of water to 1 g of liver or a whole brain (average weight about 0.5 g) in a motor-driven PTFE and glass homogenization apparatus. All samples were treated with 300 μl of 4 *N* sodium hydroxide immediately after adding the water to ensure that no further metabolism of nicotine occurred. After homogenization, 250 mg of MA dissolved in 100 μl of ethyl acetate were added as an internal standard for quantitation. The samples were extracted with 6 ml of ethyl acetate by vortexing for 20 sec and additional mechanical mixing for 20 min. The phases were separated by centrifugation at 1500 *g* for 5 min, the organic phase was transferred to a clean tube with a disposable pipet, and the homogenate was reextracted with 2 ml of 0.2 *N* acetate. The combined organic fractions were extracted with 2 ml of 0.2 *N* acetate buffer at pH 4.8 by vortexing for 2 min. The phases were separated by centrifugation and the organic phase was reextracted with 2 ml of the acetate buffer. The combined aqueous phases were saturated with powdered ammonium carbonate and extracted with 3 ml of toluene by vortexing for 3 min. The samples were centrifuged and the aqueous phase was reextracted with 3 ml of toluene. The combined toluene fractions were concentrated to approximately 100 μl by evaporation at 30—40°C under a gentle stream of prepurified nitrogen. The tubes were removed from the evaporation apparatus twice during the concentration procedure and vortexed to redissolve any nicotine which might have adhered to the glass surface. All glassware used in the extraction procedure was either disposable or thoroughly washed with soap and water, soaked in a cleaning solution (Contrad 70, Scientific Products, Houston, TX, U.S.A.) overnight, extensively rinsed with water and dried in an oven at 110°C.

Extraction of cotinine

The procedure developed for the extraction of cotinine from liver, brain and blood samples differed from the procedure for nicotine extractions in a few details. The internal standard added to each homogenate was 150 mg of COT-d_3 dissolved in 100 μl of ethyl acetate. Tissue homogenates were saturated with ammonium carbonate instead of adding sodium hydroxide (step A, Fig. 2). The back-extraction (step E) was performed with 0.1 *N* hydrochloric acid and the

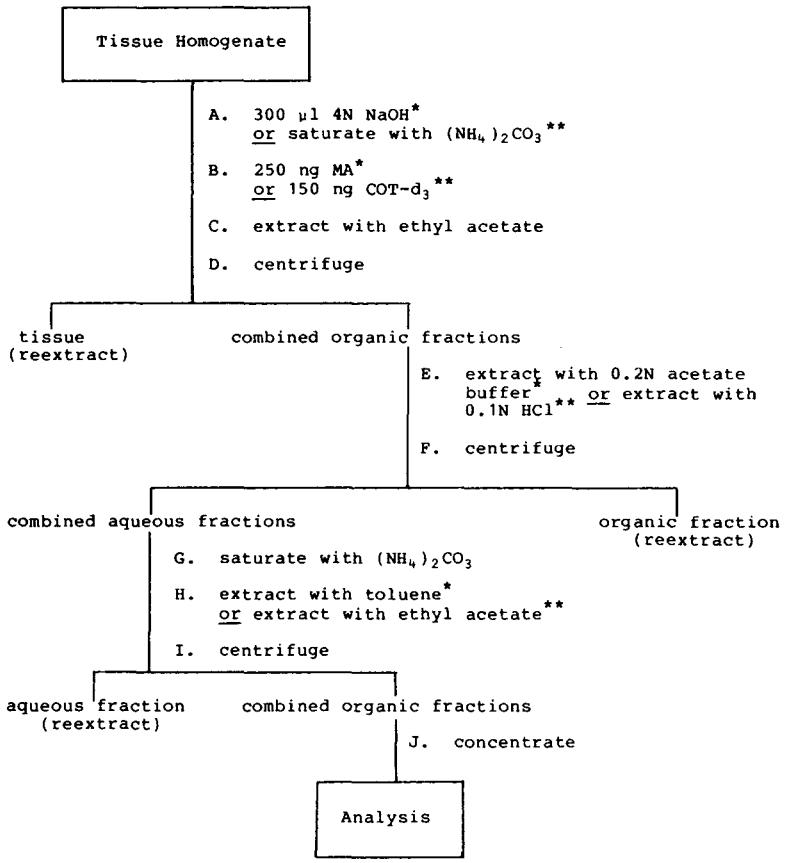

Fig. 2. Scheme for the extractions of nicotine and cotinine from tissue homogenates; (*) denotes procedure for nicotine extraction, (**) denotes procedure for cotinine extraction.

final extraction with ethyl acetate instead of toluene (step H). Finally, the extracts were evaporated to 20—40 µl prior to analysis.

Instrumentation

The gas chromatograph was a Hewlett-Packard Model 5710A equipped with an NPD and a Model 18740B capillary column controller. Analyses were performed on a 12 m × 0.2 mm I.D. fused silica capillary column which had been deactivated with Carbowax 20M and coated with a dimethyl silicone liquid phase (Hewlett-Packard). Peak areas were determined by automatic integration with a Spectra Physics Model 4100 computing integrator. Analyses also were performed with a Hewlett-Packard Model 5984A GC—MS—data system operated in both electron impact (EI) and chemical ionization (CI) modes. A 1.8 m × 2 mm I.D. glass column, packed with 3% OV-22 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.) was interfaced to the mass spectrometer with a glass jet separator (for EI work) or with a direct line (for CI work). Helium carrier gas flow-rates were 30 or 12 ml/min, respectively.

Analyses of nicotine and cotinine

The capillary GC—NPD analyses of nicotine were performed using splitless injection techniques and a fused silica injection port liner. The injection port was maintained at 250°C, the detector at 300°C, and the column was programmed from 80—150°C at 4°C/min. Analyses of cotinine by GC—NPD were performed under identical conditions, except that the column temperature was programmed from 120—200°C at 4°C/min. Determinations of nicotine by GC—MS were carried out with the injection port at 250°C, the column 170°C, the transfer line at 200°C and the ion source at 150°C. The mass spectrometer was operated in the EI mode at 70 eV ionizing energy and with selected ions monitored at m/z 133 (nicotine) and 136 (NIC-d_3) with 100 msec dwell times. Cotinine was determined by GC—MS with the injection port at 250°C, the GC column programmed from 240—270°C at 8°C/min, the transfer line at 250°C and the ion source at 180°C. The mass spectrometer was operated in the CI mode by introducing isobutane as the reagent gas via the direct insertion probe to a source pressure of approximately 1 Torr. Ions were monitored at m/z 177 (cotinine) and 180 (COT-d_3). Peak areas were measured by the standard system software.

Nicotine and cotinine in mouse liver

Male C3H mice (60 days of age) were obtained from the Institute for Behavioral Genetics of the University of Colorado. Nicotine dissolved in normal saline was injected with an intraperitoneal dose of 1 mg/kg of body weight. Control mice were injected with saline only. The animals were killed by cervical dislocation at various times after injection. Livers were removed rapidly, homogenized, and nicotine and cotinine quantitated as described above. Nicotine and cotinine half-lives were calculated from the linear portions of semilogarithmic plots of tissue concentration vs. time as described [13].

RESULTS AND DISCUSSION

Isolation of nicotine from tissue

With ^{14}C-labeled nicotine, it was determined that the precipitation of tissue with a strong acid prior to extraction step C (Fig. 2) resulted in up to 30% losses of nicotine because of tissue adsorption. For this reason, all extractions were performed on whole homogenates. The formation of homogenate—solvent emulsions, a problem with many solvents, did not occur when ethyl acetate was used as the extraction solvent with homogenates maintained at a pH > 10. More than 90% of the ^{14}C-labeled nicotine could be recovered from liver homogenates after two extractions under these conditions. Back-extraction (step E) was necessary to clean up the sample. This was carried out with a weakly acidic buffer so that the extraction would be selective for those compounds which are sufficiently strong bases (such as nicotine, pK_a 7.9 [14]) to be converted to water-soluble conjugate acids. Nicotine was transferred back into an organic solvent (step H) by saturating the aqueous phase with ammonium carbonate and extracting with toluene. The ammonium carbonate served two purposes: to adjust the pH to about 9.2 and to increase the extraction efficiency due to a salting-out effect. Toluene was chosen for this extraction be-

cause the recoveries of nicotine were high, and the simultaneous extraction of more polar compounds which might interfere with the GC analyses was minimized. In addition, the relatively high boiling point of this solvent (111°C) facilitated capillary GC analysis by splitless injection techniques, for which the GC column must be maintained at a sufficiently low temperature during sample injection to produce a chromatographic solvent effect [15]. Unlike chlorinated solvents [16], toluene is compatible with the nitrogen—phosphorus detector, producing a very small response without degrading its performance.

The final concentration step (J) must be performed carefully so that the volume is not reduced to less than 50—100 μl, otherwise substantial losses of nicotine can occur probably due to volatilization. The overall recoveries of nicotine were determined by adding 250 mg to liver homogenates (equivalent to 1 g of liver), extracting nicotine by the methods described, and adding the standard MA to the concentrated samples immediately before GC analysis. The results were compared to the nicotine/MA peak area ratios obtained with standard solutions; recoveries were 81 ± 9% (mean ± S.D., n = 6).

Isolation of cotinine from tissues

Cotinine is more polar and water-soluble than nicotine, thereby requiring more rigorous extraction conditions for its efficient recovery from tissue homogenates. The first extraction step (Fig. 2) was performed after saturating the homogenate with ammonium carbonate to basify the aqueous phase and salt-out cotinine. Because cotinine is a weaker base than nicotine, the back-extraction (step E) was carried out with a strong acid. In the last extraction (step H), ethyl acetate was substituted for toluene, as cotinine was not efficiently extracted by the latter solvent. Due to the lower volatility of cotinine compared to nicotine, the volume of the final extract could be reduced to about 10 μl with no significant loss of the compound. Overall recoveries were determined, as for nicotine, by extracting cotinine from spiked liver homogenates and adding the standard (COT-d_3) to the concentrated sample immediately before analysis. Recoveries were 87 ± 7% (mean ± S.D., n = 5).

GC—NPD analyses of nicotine and cotinine

Previous methods for the GC analyses of nicotine and cotinine were developed with standard packed columns [2—5]. Capillary columns, however, provide higher analytical sensitivity and selectivity because of narrower peak shapes, higher resolution and reproducible retention times. Mouse liver extracts were analyzed by GC—NPD techniques with a dimethyl silicone-coated fused silica capillary column and splitless injection; the chromatograms are shown in Fig. 3. The excellent peak shapes for nicotine and MA are apparent, as well as the highly consistent retention times of these compounds. The inherent GC—NPD sensitivity limit was determined with standard solutions of nicotine to be approximately 50 pg injected on column. Fig. 3, however, shows that extracts of liver homogenates from animals not treated with nicotine contained 1—2 ng of the compound. The identity of this peak was verified by GC—MS methods. The materials used for extraction were highly purified; extractions of water in place of tissue samples demonstrated that most of the background nicotine was from tissue and not from contaminated reagents or solvents. It was, of course,

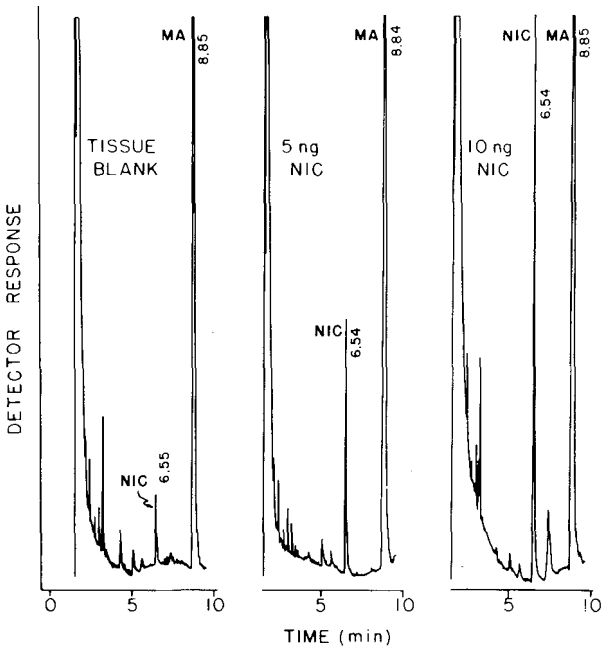

Fig. 3. Analyses of tissue extracts by capillary GC-NPD. Mouse liver homogenates were unspiked or spiked with 5 or 10 ng of nicotine (NIC) along with 250 ng of methylanabasine (MA) and extracted as described in the text.

also necessary to exclude all tobacco smoking from the laboratory and from the animal quarters. When mice were placed in wire cages without bedding, and food and water withdrawn 12 h before sacrificing them, the background levels of nicotine dropped substantially to less than 1 ng per g of liver. Because of this background nicotine in animals housed and fed normally, the effective sensitivity limit for determinations of injected nicotine was 2—3 ng per g of tissue.

The internal standard MA was chosen for GC quantitation because of its similarity to nicotine with regard to pK_a and solubility. A standard calibration curve was constructed with data obtained by analyzing mouse liver homoge-

TABLE I

STANDARD CALIBRATION CURVES FOR THE QUANTITATION OF NICOTINE AND COTININE IN LIVER HOMOGENATES

n = 5 at each concentration.

	Method	No. of concentrations*	Equation of the line	Correlation coefficient
Nicotine	GC—NPD	7	$Y = 0.0054X + 0.0430$	0.999
	GC—MS	7	$Y = 0.0042X + 0.0367$	0.998
Cotinine	GC—MS	8	$Y = 0.0082X + 0.0153$	0.999

*Each homogenate contained 1 g of mouse liver. Concentrations were within the range 5—1000 ng.

TABLE II

ACCURACY AND PRECISION OF THE ASSAYS FOR NICOTINE AND COTININE IN LIVER HOMOGENATES

Mean ± S.D., n = 4 at each concentration.

Amount of nicotine or cotinine added* (ng)	Nicotine determined (ng)	Cotinine determined (ng)
10	10.1 ± 2.1	9.8 ± 2.5
25	24.7 ± 1.3	—
50	50.7 ± 3.7	51.9 ± 3.5
250	252 ± 6	248 ± 8

*Each homogenate contained 1 g of mouse liver.

nates spiked with a quantity of nicotine in the range 5—1000 ng along with 250 ng of MA. The standard curve data are included in Table I. Verification of the extraction and quantitation technique was accomplished by spiking liver homogenates with known quantities of nicotine and analyzing the samples as described. The results (Table II) demonstrate the accuracy and precision of this method.

It is more difficult to achieve narrow, symmetrical chromatographic peaks with cotinine than with nicotine due to the higher polarity and resulting column adsorption of this metabolite. Good peak shape was obtained, however, with a Carbowax-deactivated fused silica column (Fig. 4). A close structural analogue, OMA, was chosen as the internal standard for the GC work with cotinine. Extracts of mouse liver homogenates obtained by the methods dis-

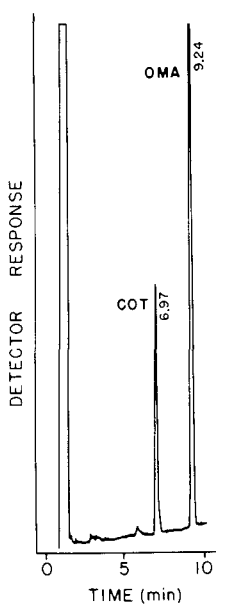

Fig. 4. Analysis of a standard sample of cotinine (COT) and the internal standard, OMA, by capillary GC.

cussed above were found to contain compounds which interfered with the GC—NPD analysis of cotinine at low nanogram levels. Since attempts to produce cleaner extracts resulted in substantially lower recoveries, no further work was carried out to quantitate cotinine by GC—NPD methods. Such techniques could be employed, however, if high sensitivity is not required.

GC—MS analyses of nicotine and cotinine

A method was developed for the GC—MS quantitation of nicotine in tissue extracts. Under EI conditions, the following mass spectral ions are potentially useful for measurement by selected ion monitoring: *m/z* 84 (base peak), *m/z* 133 (relative abundance 40%) and *m/z* 162 (M^+, relative abundance 20%). By analyzing tissue extracts, it was determined that monitoring *m/z* 84 did not provide adequate selectivity and *m/z* 162 did not provide adequate sensitivity for the analyses of low levels of nicotine. Therefore, all GC—MS work was performed by monitoring *m/z* 133 for nicotine and *m/z* 136 for NIC-d_3, the internal standard. The sensitivity limit was equivalent to that obtained by the GC—NPD method, 2—3 ng per g of tissue. The standard calibration data are included in Table I. This method was validated by spiking liver homogenates with known amounts of nicotine and analyzing them as described; the results are included in Table II. The GC—MS method is approximately equivalent to the GC—NPD method with regard to sensitivity, accuracy and precision. Therefore, the GC—MS method provides an alternative quantitative technique to the GC—NPD method for the analysis of nicotine in tissue extracts.

GC—MS analysis of cotinine under EI conditions was not sufficiently selective to quantitate low levels of this metabolite in tissue extracts. By monitoring the M+1 ion for cotinine (at *m/z* 177) under CI conditions with isobutane, however, good sensitivity and selectivity were achieved. The ion current profiles obtained by analyses of liver extracts are shown in Fig. 5. The sensitivity limit is at least 2—3 ng of cotinine per g of tissue, which is equivalent to the sensitivity obtained for nicotine determinations. The GC—MS work was performed with a packed OV-22 column; the use of a capillary column should lead to further improvement in GC—MS sensitivity. A standard calibration curve was

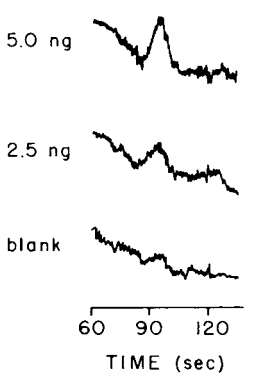

Fig. 5. Ion current chromatograms obtained by monitoring the M+1 ion of cotinine (*m/z* 177) in tissue extracts with chemical ionization GC—MS. Mouse liver homogenates were unspiked or spiked with 2.5 or 5 ng of cotinine and extracted as described in the text.

constructed with data obtained from the analyses of liver homogenates spiked with amounts of cotinine in the range 5—1000 ng per g of tissue. Homogenates also contained 150 mg of the internal standard COT-d_3. The data are summarized in Table I. As with the methods for nicotine quantitation, the extraction and GC—MS techniques for cotinine were validated by spiking tissue homogenates with known amounts of cotinine and performing the analyses. The results (Table II) also demonstrate the reliability of this method.

A pharmacokinetic study of nicotine and cotinine in mouse liver

Mice were injected with a single intraperitoneal dose of 1 mg/kg of nicotine, killed at various intervals after administration and the liver nicotine and cotinine extracted by the methods described. Nicotine was quantitated by GC—NPD and cotinine by GC—MS techniques. The data were used to construct the semilogarithmic plots of concentration vs. time shown in Fig. 6. The disappearance of nicotine was biphasic with the half-life of the elimination phase calculated to be 9.2 min. At 45 min after injection, the levels of nicotine were approaching the lower limit of detection. The maximum tissue levels of cotinine were attained in 5 min and its disappearance was monophasic. Readily detectable quantities were still present in liver at 2 h after injection. The elimination half-life of this metabolite was 27 min.

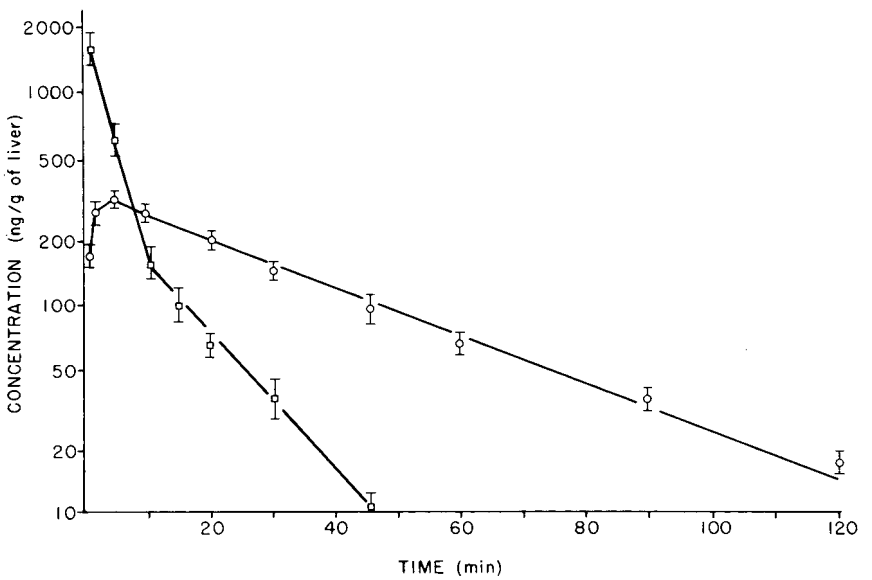

Fig. 6. Concentrations of nicotine (□) and cotinine (○) in livers of mice injected with nicotine vs. time after injection (mean ± S.E., n = 6).

CONCLUSION

Sensitive and selective analytical techniques have been developed for the quantitation of nicotine and cotinine in biological samples. The emphasis of this work was on the accurate measurement of low nanogram amounts of these compounds in tissues rather than in fluids, such as blood and urine [2—8]. Data

were presented to demonstrate that sensitivity for nicotine is limited by background levels of the compound. The internal standards for quantitation are much more similar structurally to nicotine and cotinine than most of those used previously. Nicotine extracts were accurately analyzed by both capillary GC—NPD and GC—MS techniques. Cotinine extracts, however, could only be quantitated by chemical ionization GC—MS methods to achieve the necessary analytical selectivity at low tissue levels of the metabolite. A pharmacokinetic investigation carried out to demonstrate the usefulness of these techniques revealed a very short half-life for nicotine in mouse liver after a single intraperitoneal injection of the compound; the half-life of the metabolite cotinine was three-fold longer. Further studies of the pharmacokinetics of nicotine and cotinine in other tissues and the physiological and environmental factors that affect the disposition of nicotine are in progress.

ACKNOWLEDGEMENT

The authors gratefully acknowledge financial support from the Council for Tobacco Research — U.S.A., grant 1243.

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Journal of Chromatography, 231 (1982) 65—72
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1284

GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF LIDOCAINE IN CAT PLASMA USING MEPIVACAINE AS INTERNAL STANDARD

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(First received November 10th, 1981; revised manuscript received March 1st, 1982)

SUMMARY

A method for the gas—liquid chromatographic determination of lidocaine in cat plasma with mepivacaine as internal standard is described. The investigations demonstrated a high reliability in the method, although the precautions required are relatively few. Under the cited conditions the plasma concentrations determined with the method after lidocaine treatment of cats were proportional to the infusion rates and obeyed a logarithmic normal distribution.

INTRODUCTION

Lidocaine is used systemically in the treatment of cardiac arrhythmias. In order to optimize therapy it is important to know its plasma concentration.

In a recent experimental investigation by Vogt et al. [1] on cats (see Table I), lidocaine plasma concentrations were to be determined by gas—liquid chromatography (GLC) (see also ref. 2)*.

Numerous GLC determinations of lidocaine have already been carried out (see, for example, refs. 3—19 and papers cited therein) using internal and external standards. Because of the expected higher precision an internal standard was used in the present paper.

In most of the works where an internal standard was used, substances with retention times similar to that of lidocaine or in the solvent "tail" region were chosen, both possibly leading to inaccuracies in the results. As mepivacaine does not have these disadvantages, it was used as an internal standard in this

*The cited authors kindly supplied the plasma samples and encouraged work on the present paper.

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paper, as in refs. 15—19, the latter being work using GLC—mass spectrometry. The retention time of mepivacaine was about twice that of lidocaine under the conditions described under Methods (3.35 min for lidocaine).

EXPERIMENTAL

Materials

Lidocaine—HCl (Xylocain®), 20%, and mepivacaine—HCL (Scandicain®), 3%, were from Astra Chemicals (Stockholm, Sweden). Dichloromethane, analytical grade, was from Merck (Darmstadt, G.F.R.).

Methods

Heparinized plasma (0.9 ml) of lidocaine-treated cats (see Table I) was either used at once or stored at —20° C. To the sample, either fresh or thawed out, were added 50 μl of 0.1% mepivacaine—HCl as an internal standard and 50 μl of 10 *M* sodium hydroxide. Then it was agitated twice with 3 ml of dichloromethane for 1 min, each 3-ml fraction being removed as completely as possible after centrifugation. The two dichloromethane fractions were collected in a conical centrifuge tube. The dichloromethane was removed by blowing nitrogen on its surface, occasionally inclining the tube in order to avoid too much deposition of the residue on the wall of the tube. After complete evaporation of the solvent 20 μl of dichloromethane were dispensed onto the bottom of the tube and the latter was agitated carefully for a few seconds. Then 1 μl of the solution was injected directly onto the GLC column. Similar extraction procedures are described in refs. 4 and 15.

The gas chomatograph HP 5730 A was equipped with a flame ionisation detector (FID) and a glass column, 1.83 m × 6.35 mm I.D., containing 3% OV-17 on 80—100 mesh Chromosorb W HP. Both the detector and injector temperatures were 250° C, the column temperature was held constant at 190° C.

The carrier gas was nitrogen at a flow-rate of 30 ml/min; the flame for the FID was generated with air and hydrogen at 240 ml/min and 60 ml/min, respectively.

The lidocaine concentrations were calculated from the ratio of lidocaine/mepivacaine according to a previously established standard curve, as described below.

RESULTS

GLC method

The following points were investigated in order to check the reliability of the method.

Linearity and reproducibility on different days. For the establishment of a standard curve lidocaine—HCl was dissolved in pooled human plasma and the samples were subsequently prepared as described previously. The linearity between peak height and lidocaine—HCl concentration is good (correlation coefficient $r = 0.9998$) and the standard deviation between repetitive experiments on different days is small (corresponding to ± 0.2 μg/ml lidocaine—HCl up to a concentration of ca. 50 μg/ml, $n = 6$ duplicate experiments).

Another standard curve was established in the same way but with half the amount of mepivacaine standard for an investigation on cats with lower infusion rates [2] than in ref. 1.

Recovery and effectivity of extraction. It does not make any difference if cat plasma, human plasma or simply distilled water is used for the preparation of the standard curve samples, as the recovery in terms of the lidocaine/mepivacaine ratio is equal in each case ($p > 0.3$, $n = 4$, lidocaine—HCl = 10 μg/ml). No lidocaine or mepivacaine could be detected in the aqueous phase after extraction.

Precision. Upon examination of the precision for five consecutive determinations in pooled human plasma at lidocaine—HCl concentrations of 5 μg/ml and 10 μg/ml, a coefficient of variation of 1.9% and 1.8%, respectively, was found.

Background peaks. Occasionally small background peaks were found in the lidocaine peak region without any lidocaine treatment of the cats (corresponding to 0.3 ± 0.2 μg/ml lidocaine—HCl in three cats, see Fig. 1). Although the influence on the results was small (compare Fig. 2) a corresponding "blank" value was subtracted. The background did not disappear if additional extraction steps between the organic phase and acid as well as basic media were carried out as described in ref. 5.

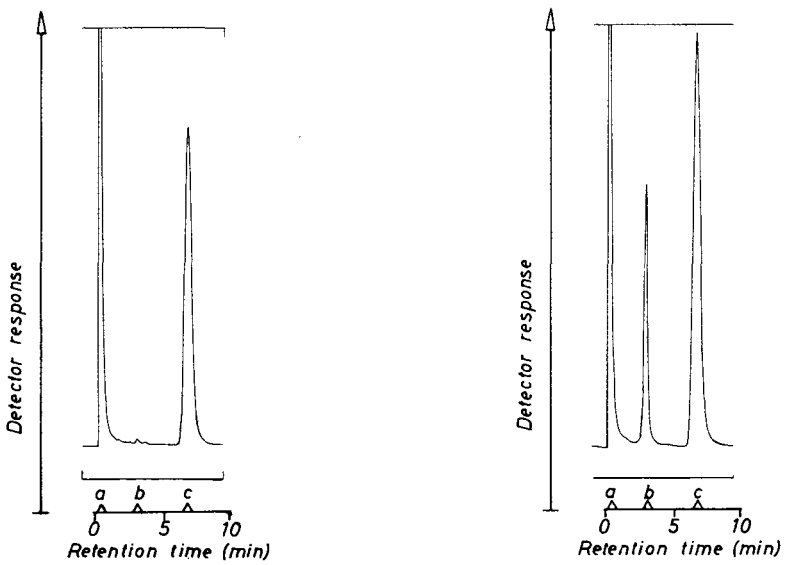


Fig. 1. Typical gas chromatogram of a sample from an untreated cat. a = Solvent peak (methylene chloride); b = endogenous peak with retention time equal to that of lidocaine; c = mepivacaine peak (internal standard).

Fig. 2. Typical gas chromatogram of a sample from a cat at the end of lidocaine—HCl infusion (0.70 mg/kg·min). Same animal as in Fig. 1. a = Solvent peak (methylene chloride); b = lidocaine peak; c = mepivacaine peak (internal standard).

Influence of gas flow-rates. The peak height ratio of lidocaine/mepivacaine was practically independent of the gas flow-rates within ± 50% limits.

Sample stability. Incubation of samples at 25° C and 60° C before and after the alkalinisation with sodium hydroxide showed stability of both lidocaine

and mepivacaine against enzymic and spontaneous hydrolysis. Freezing for one week at −20° C did not alter the results obtained in fresh samples.

Detection limit. If a blank value before lidocaine treatment is available, the detection limit of the method is approximately 0.1 μg/ml, otherwise it is approximately 0.5 μg/ml lidocaine—HCl.

Lidocaine concentrations in cat plasma during and after infusion

The results of the lidocaine determination in cat plasma are summarized in Table I. Apparently a doubling of the infusion rate results in a doubling of the lidocaine concentration. Occasionally small metabolite peaks with retention times less than that of lidocaine partially overlapped with the lidocaine peak obtained 1 h after infusion stopped. The contribution of these peaks to the lidocaine peak height could be neglected.

TABLE I

LIDOCAINE PLASMA CONCENTRATION DURING AND AFTER INTRAVENOUS INFUSION IN CATS

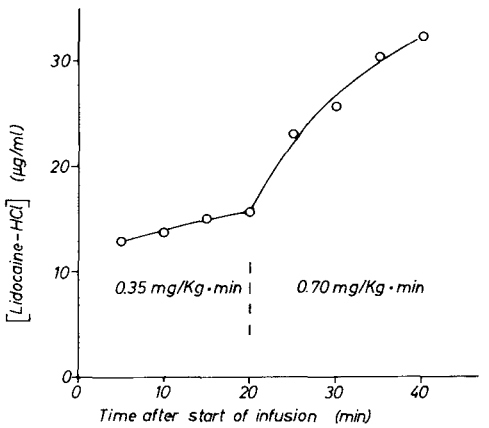
Treatment (see Vogt et al. [1])	Number (n)	Lidocaine—HCl concentration (μg/ml) (mean ± S.D.)	Log (lidocaine—HCl concentration)
Bolus intravenous injection of 2 mg/kg lidocaine—HCl, then 20 min infusion of 0.35 mg/kg·min, finally collection of arterial blood sample	24	10.1 ± 2.8	0.991 ± 0.117
Doubling of the infusion rate: 0.70 mg/kg·min; second sample after further 20 min	20	21.4 ± 6.4	1.312 ± 0.127
Infusion stop and last sample after further 60 min*	11	3.2 ± 1.4**	0.468 ± 0.171

*The peak identification by gas chromatography—mass spectrometry was kindly carried out by Dr. H. Luthe, Medizinische Universitätsklinik Göttingen, Abteilung Klinische Chemie.
**One "outlier" replaced by a new measuring value (see ref. 1).

An arterial blood sample was taken from a cat and then the animal was treated as outlined in Table I (two further samples collected) with the exception that after the second infusion period a third 20-min period with three times the original infusion rate (1.05 mg/kg·min) was included, at the end of which another sample was taken. The dependence of the lidocaine concentration from the infusion rate in this experiment shows a high linear correlation of the two parameters (r = 0.9997).

In another animal several samples were taken at different times during the two infusion periods mentioned in Table I. The lidocaine concentrations during this time are shown in Fig. 3.

Fig. 4 shows the lidocaine concentrations in the elimination period of a third animal on a semi-logarithmic scale. Obviously the elimination occurs in two phases; therefore, the calculations of the pharmacokinetic parameters have



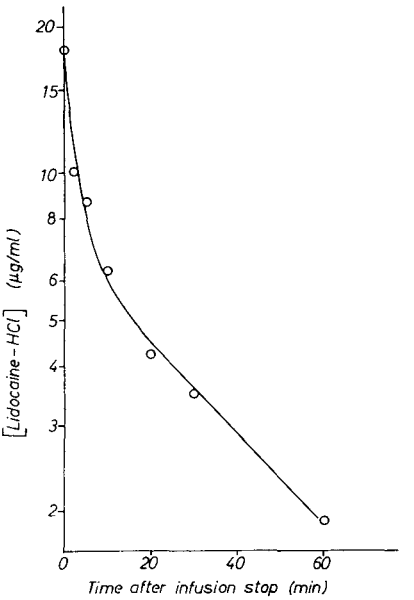


Fig. 3. Lidocaine plasma concentrations during infusion.

Fig. 4. Lidocaine elimination after infusion stop.

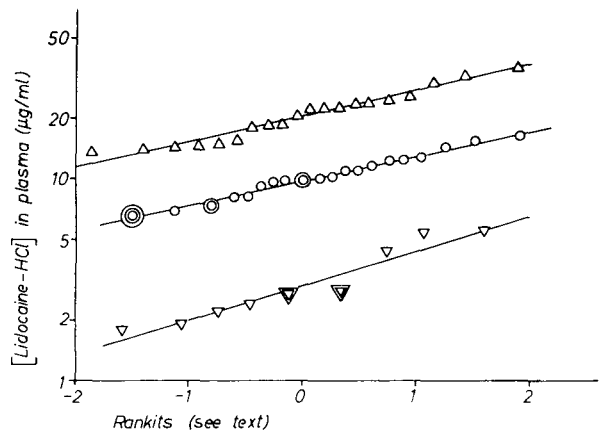


Fig. 5. Test for logarithmic normal distribution of lidocaine concentrations in cats during and after intravenous influsion (see text). △, Infusion of 0.70 mg/kg·min; ○, infusion of 0.35 mg/kg·min; ▽, 1 h after infusion stopped.

been carried out according to a two-compartment model by Gauss—Newton iteration (see refs. 20 and 21).

For the infusion period a one-compartment model gave satisfactory results. The lines drawn in Figs. 3 and 4 were obtained using the calculated pharmacokinetic parameters.

In Fig. 5 the ranked lidocaine concentrations are plotted on a logarithmic scale against the "rankits", i.e. the values theoretically to be expected for a

normal distribution. The rankits [22] were taken from tables [23]. If concentration values were equal, the rankit for the mean of their rank numbers was used.

DISCUSSION

As the peak height of the lidocaine/mepivacaine ratio is proportional to the lidocaine concentration, integration of the peak area is not necessary for the calculation of the concentrations; simple measurement of the height is sufficient.

The high precision and accuracy of the described method follows from the stability of the samples against freezing and storage, completeness of lidocaine and mepivacaine extraction, high linear correlation of the standard curve, low coefficient of variation in consecutive measurements and good reproducibility of the measurements at different days with mepivacaine as an internal standard.

Cyclizine, which has been used, for example, by Zylber-Katz et al. [6] as an internal standard, has a retention time near to that of lidocaine. Although a "to and fro" extraction* between acid and basic media, automatic peak integration and temperature programming was performed by these authors, they found a higher coefficient of variation in consecutive measurements (3.1%) than in the present paper at a similar lidocaine concentration. The possible reasons for this difference are mentioned in the Introduction.

Pape et al. [18] used mepivacaine as internal standard but only a single initial extraction. Their coefficient of variation was about twice that of this paper, suggesting the importance of a double extraction (see below).

Other papers reporting methods with a mepivacaine standard can not be compared with the present one because they used other instrumentation (nitrogen–phosphorus detector [17], gas chromatography–mass spectrometry [19], did not give details concerning the reliability of the method [15], or used material other than plasma [16].

As lidocaine is directly extracted from the plasma without previous protein precipitation, and because of the simplicity of the calculations, the method is relatively easy to carry out.

Due to the small influence of the gas flow-rates on the measured values, the measurements require few precautions and the method can therefore be carried out routinely. The same is true for the extraction. As it is difficult to remove the organic phase completely from the aqueous phase in one step, a double extraction was carried out, which reduced the error possibilities to a minimum. Furthermore, a single extraction may be incomplete and different for lidocaine and mepivacaine [15, 18]. The pipetting of the 50-μl volume of internal standard is essentially the only step to be done with a higher degree of care.

With an appropriate variation the described method should be suitable for mepivacaine determinations with lidocaine as an internal standard as well.

Limitations of the method arise from the low concentrations found during

*The plasma sample is made alkaline and extracted into *n*-hexane, re-extracted into a small volume of an aqueousacid phase, and finally extracted into 50 μl of methylene chloride after alkalinization.

dental anaesthesia. The lidocaine and mepivacaine concentrations in this case are 0.3 μg/ml and 0.4 μg/ml, respectively [13], thus being near to the detection limit of the described method. In lumbar and other forms of regional anaesthesia the maximum lidocaine concentrations were between 1 μg/ml and 4 μg/ml [11, 12], which could still be detected with sufficient reliability by this method. In any case the method is more suitable to analysis after systemic therapeutic treatment or intoxication.

Clearly, the patients must not be treated with mepivacaine at the same time if a high accuracy of the results is desired.

The lidocaine concentrations in the cat fit a logarithmic normal distribution better than a linear one. The elimination half-life of the "slow" phase determined in one animal (32 min) is of the same order of magnitude as found by others [14].

Especially in the high level infusion period (0.70 mg/kg·min) the steady state was not completely reached after 20 min, as at the end of the infusion the plasma concentration curve was not yet parallel to the abscissa and a "fast" elimination compartment could subsequently be observed (Figs. 3 and 4).

ACKNOWLEDGEMENTS

Mrs. D. Volland and Mrs. B. Schilling provided skilful technical assistance in the presented investigation.

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Journal of Chromatography, 231 (1982) 73—82
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1281

IMPROVED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THEOPHYLLINE IN PLASMA AND SALIVA IN THE PRESENCE OF CAFFEINE AND ITS METABOLITES AND COMPARISONS WITH THREE OTHER ASSAYS

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(First received April 29th, 1981; revised manuscript received March 4th, 1982)

SUMMARY

A new ion-pairing reversed-phase high-performance liquid chromatographic assay for theophylline is described which allows the separation of theophylline from 1,7-dimethylxanthine — a metabolite of caffeine which interferes with most theophylline assay procedures. Levels of 1,7-dimethylxanthine equivalent to 3 mg/l theophylline were seen in individuals not taking theophylline but who drank three to four cups of coffee per day. This compound was not seen in individuals abstaining from xanthine-containing foods and beverages.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is a powerful, smooth muscle relaxant used primarily in the treatment of chronic obstructive pulmonary disease where dilation of the bronchi and pulmonary vasculature are warranted [1]. The pharmacological activity of theophylline has been shown to be highly correlated with its concentration in plasma [2]. However, because of the large inter-individual variation in the clearance of the drug, dosages must be individualized in order to optimize therapy [3]. Sensitive and specific assays for theophylline are therefore central to successful therapy with the drug. In this context, older spectrophotometric assays of the drug [4] involving a single or double extraction as the basis for selectivity have been superseded by high-performance liquid chromatographic (HPLC) and immunochemical assays for

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routine clinical determination [5,6]. While interference from other xanthines, e.g. caffeine (1,3,7-trimethylxanthine) and theobromine, have been eliminated with these more sophisticated techniques, a metabolite of caffeine (paraxanthine, 1,7-dimethylxanthine, a chemical isomer of theophylline) has been shown to interfere with theophylline determinations in assays using traditional reversed-phase chromatographic separations [5,7]. Thus the potential for interference with the measurement of theophylline may exist in patients consuming caffeine-containing beverages or foods.

In a previous report [8] we have described an ion-pairing HPLC assay for theophylline and its metabolites in urine which was capable of separating theophylline from paraxanthine. However, this procedure required an ion-pairing liquid—liquid extraction step followed by ion-pairing gradient elution HPLC and was rather complicated and time consuming for the routine application to the measurement of theophylline in plasma and saliva. Consequently, we have made extensive modifications to the above procedure to make it suitable for the routine determination of theophylline in plasma and saliva in the presence of paraxanthine (and other caffeine metabolites) and to assess the magnitude and frequency of this interference. In addition, we report the results of a blind investigation in which plasma samples containing theophylline in the presence and absence of paraxanthine were sent to three other laboratories in order to assess the significance of the interference under the conditions used by each laboratory.

MATERIALS AND METHODS

Apparatus

A Model 8500 high pressure solvent delivery system/automatic sample injector (Varian, Palo Alto, CA, U.S.A.) was used in conjunction with a Hitachi 155-30 variable-wavelength UV detector set at 274 nm (Altex, Berkeley, CA, U.S.A.) and a Hewlett-Packard integrator Model 3380A (Hewlett-Packard, Avondale, PA, U.S.A.). A slurry packed LiChrosorb RP-2 (4.5 cm × 2.0 mm I.D.) precolumn was used in conjunction with an Ultrasphere ODS 5-μm (15 cm × 4.6 mm I.D.) analytical column (Altex) for achieving separation and quantitation.

Reagents and standards

Sources of the xanthine and uric acid derivatives were as follows: caffeine and uric acid (Eastman-Kodak, Rochester, NY, U.S.A.), theophylline and β-hydroxyethyl theophylline (Sigma, St. Louis, MO, U.S.A.), 1-methylxanthine, 3-methylxanthine, 1,7-dimethylxanthine, 1-methyluric acid and 1,3-dimethyluric acid (Adams Chemical, Round Lake, IL, U.S.A.). Tetrabutylammonium hydrogen sulphate was obtained from Aldrich (Milwaukee, WI, U.S.A.) and sodium acetate was of analytical grade. Acetonitrile and methanol were of HPLC grade.

The mobile phase consisted of 0.01 mol/l sodium acetate and 0.005 mol/l tetrabutylammonium hydrogen sulphate with the pH adjusted to 4.75 with either 0.1 mol/l sodium hydroxide or 0.1 mol/l acetic acid. Methanol was added (12.5%, v/v) and the mobile phase was filtered through a Millipore HA

(0.5 μm) solvent filtering system (Millipore, Bedford, MA, U.S.A.).

The internal standard solution was prepared by dissolving 2.5 mg/l β-hydroxyethyltheophylline in acetonitrile. Standard solutions of theophylline were prepared by dissolving theophylline in acetonitrile (100 mg/l). Blank (caffeine-free) plasma and saliva were obtained from a normal volunteer who had abstained from caffeine-containing foods and beverages for at least 72 h.

Procedure

Plasma. Plasma samples (0.5 ml) were transferred to a 7-ml glass tube and the proteins precipitated by the addition of 5 μg of internal standard in 2 ml of acetonitrile and vortexed for 15 sec. After centrifugation (3000 *g*, 10 min) the supernatant was transferred to another 7-ml glass tube and placed in a Buchler vortex evaporator thermostated to 50°C (Fort Lee, NJ, U.S.A.) for approximately 20 min at which time the removal of the acetonitrile was evidenced by an abrupt change in the vacuum chamber pressure. A 20-μl quantity of the remaining aqueous sample was injected directly onto the chromatographic system. Standard curves of theophylline in plasma were prepared by pipetting an appropriate volume of theophylline standard solution into a 7-ml glass tube, evaporation to dryness under a stream of nitrogen, addition of 0.5 ml blank plasma and vortexing thoroughly (15 sec). The samples were then processed as described above.

Saliva. Saliva samples were thoroughly vortexed and approximately 1.5 ml were transferred to a polypropylene Eppendorf microcentrifuge tube and centrifuged (5 min at 15,000 *g*). A 20-μl quantity of the supernatant was injected directly onto the chromatographic system. Standard curves of theophylline in saliva were made by pipetting an appropriate volume of the theophylline standard solution into 7-ml glass tubes, evaporation under a stream of nitrogen, addition of 1.5 ml of caffeine-free saliva and thoroughly vortexing. The samples were then processed as described above.

Chromatographic conditions

A flow-rate of 1.5 ml/min was used at ambient temperature generating a back pressure of approximately 2500 p.s.i. (170 bar). A sample run time of 12 min was selected in order to routinely estimate caffeine levels in outpatient samples. However, the theophylline and internal standard peaks were invariably eluted within 6—7 min.

Comparison with other assays

In order to estimate the frequency with which interference by paraxanthine occurs in routine clinical laboratory theophylline assays, duplicate spiked samples of plasma containing 0, 1, 3, 6, 10 and 18 mg/l theophylline in the presence and absence of 3 mg/l paraxanthine were distributed to three cooperating clinical laboratories routinely assaying theophylline in plasma; one using the EMIT enzyme immunoassay technique and two using HPLC assays, the details of which are as follows.

Laboratory 1. Precipitate 100 μl plasma with 100 μl acetonitrile containing β-hydroxyethyltheophylline (15 mg/l). Centrifuge and inject 20 μl supernatant onto analytical system: mobile phase, 10% acetonitrile in 0.01 mol/l

phosphate buffer, pH 4.5; flow-rate, 2 ml/min; room temperature, Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} column, 30 cm × 3.9 mm I.D.

Laboratory 2. Add 100 μl 0.1 mol/l phosphate buffer, pH 5.0 containing β-hydroxyethyltheophylline (15 mg/l) to 100 μl of plasma. Extract with 200 μl diethyl ether, centrifuge, evaporate supernatant to dryness and make up into 20 μl acetonitrile and inject 10 μl onto analytical system: mobile phase, 10% acetonitrile in 0.01 mol/l phosphate buffer, pH 5.0; flow-rate, 2 ml/min; 50°C; Altex Ultrasphere ODS column 15 cm × 4.6 mm I.D.

Laboratory 3. Used the EMIT enzyme immunoassay analysis technique as described by the manufacturer (Syva, Palo Alto, CA, U.S.A.).

RESULTS

The result of the analysis of a standard solution of theophylline (1,3-MX), β-hydroxyethyltheophylline (IS), caffeine (1,3,7-MX), and 3-methylxanthine (3-MX), 1-methylxanthine (1-MX), paraxanthine (1,7-MX), 1 methyluric acid (1-MU) and 1,3-dimethyluric acid (1,3-MU) is shown in Fig. 1A. The chromatogram from a blank sample of plasma obtained from a volunteer abstaining from caffeine-containing foods and beverages is shown in Fig. 1B. Fig. 1C shows the chromatogram from a patient administered 200 mg aminophylline q.i.d. who had been drinking three to four cups of coffee during the same day. Note the absence of theophylline and other interfering xanthine metabolites in the blank plasma sample but their abundance in significant

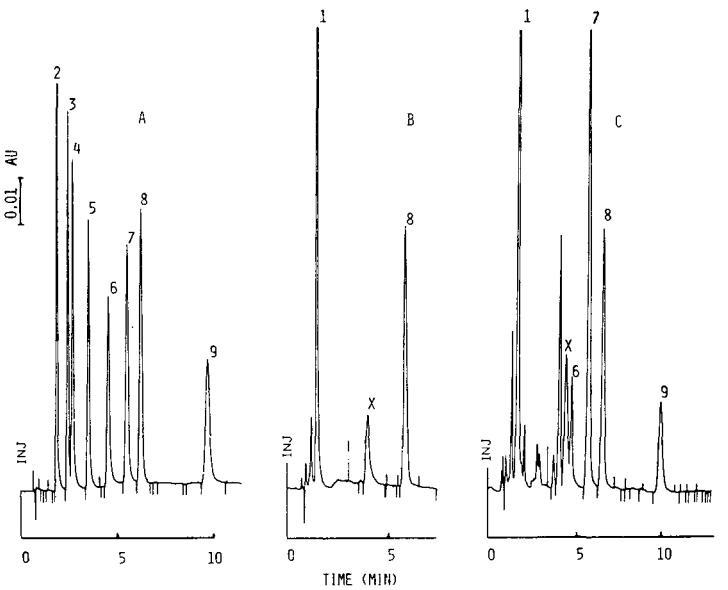

Fig. 1. Chromatograms of: A, standard of methylxanthines and methyluric acids; B, blank plasma; C, plasma from a patient taking theophylline and drinking coffee. Peaks: (1) uric acid, (2) 3-MX, (3) 1-MX, (4) 1-MU, (5) 1,3-MU, (6) 1,7-MX, (7) 1,3-MX (theophylline), (8) IS (β-hydroxyethyltheophylline), (9) caffeine, each at 8.0 mg/l. X is an unknown peak present in all plasma samples and the large peak preceeding X in C was unique to this patient, not corresponding to any of the standards 1—9.

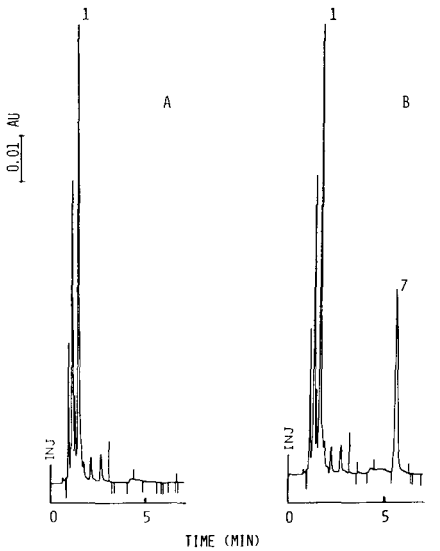

Fig. 2. Chromatograms of: A, blank saliva sample, B, saliva from a patient taking theophylline. Peaks as in Fig. 1.

concentrations in the patient sample. Fig. 2A shows the chromatogram from blank saliva and Fig. 2B, the chromatogram of saliva obtained from a patient administered 400 mg aminophylline q.i.d.

The following drugs were injected onto the chromatographic system at equivalent therapeutic concentrations (where known) and were found not to interfere with either theophylline or the internal standard: propranolol, acebutolol, quinidine, chlorthiazide, hydrochlorthiazide, hydrallazine, acetazolamide, naproxen, ketoprofen, phenobarbital, phenytoin, salicylic acid and procainamide.

Peak area ratio of theophylline to the internal standard was related to plasma theophylline concentration and was found to be linear within the range of 0.5—30.0 mg/l ($r^2 > 0.999$). Table I shows the interday reproducibility coefficient of variation (C.V.) of the theophylline internal standard

TABLE I

REPRODUCIBILITY OF PEAK HEIGHT RATIO VS. CONCENTRATION FOR THEOPHYLLINE IN PLASMA

Within-day C.V. was 1.0% (n = 8).

Concentration (mg/ml)	Mean peak height ratio	Interday C.V. (%)
0	0	—
1	0.1402	2.6
3	0.4244	1.8
6	0.8464	1.1
10	1.4227	0.76
18	2.5390	1.70

TABLE II

REPRODUCIBILITY OF PEAK AREA VS. CONCENTRATION FOR THEOPHYLLINE IN PLASMA

Within-day C.V. was 2.5% (n = 9).

Concentration (mg/ml)	Mean area	Interday C.V.* (%)
9	0	—
1	5.6393	10.25
2	11.2190	8.1
4	22.7000	4.85
8	45.3400	5.06

*See text for explanation of large coefficients of variation.

peak height ratio at several different concentrations (n = 8). The within-day reproducibility (C.V.) of 12 mg/l control sample was 1.0% (n = 5).

Absolute peak area of theophylline was related to salivary concentration and was found to be linear over the range of 0.5—20 mg/l (r^2 = 0.998). Table II shows the inter-day reproducibility (C.V.) of the absolute peak area at several different concentrations (n = 9). The higher C.V. value reflects inter-day variation of the slope of the relationship of peak area vs. theophylline concentration, probably due to minor variations in solvent composition, pH, temperature. The within-day reproducibility (C.V.) of a 4 mg/l control sample was 2.5% (n = 5).

The average plasma concentrations of caffeine and paraxanthine in seven volunteers drinking three to four cups of coffee between the hours of 8 a.m. and noon were 9.0 mg/l and 3.0 mg/l, respectively, while that of theophylline was 0.5 mg/l. Fig. 3 shows a chromatogram from a volunteer (K.T.M.) who

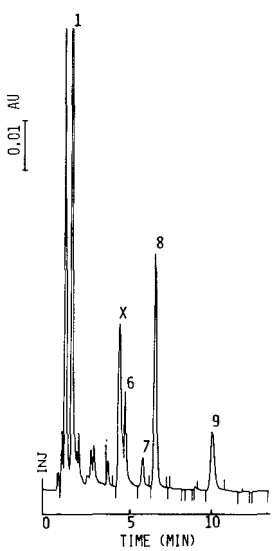

Fig. 3. Chromatogram of plasma from a volunteer drinking coffee. Peaks as in Fig. 1.

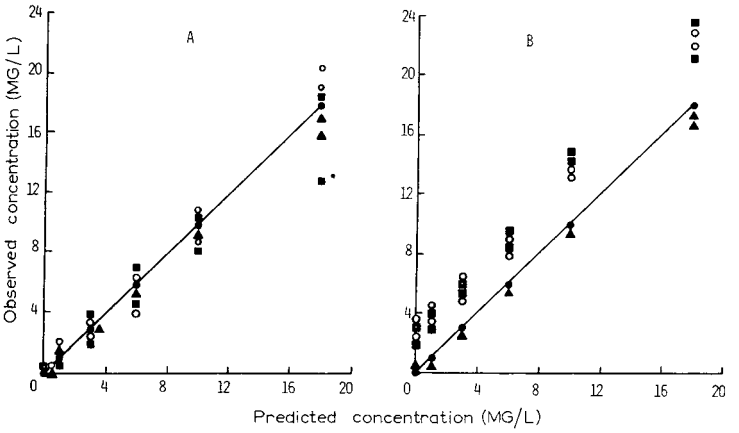

Fig. 4. Relationship between measured and spiked theophylline in plasma for four different assays. A, in the absence of paraxanthine; B, in presence of paraxanthine. The point marked with an asterisk (Laboratory 2) was not included in statistical analyses. •, This assay; ○, Laboratory 1, ■, Laboratory 2; ▲, Laboratory 3.

had drunk four cups of coffee between the hours of 8:00 a.m. and 10:00 a.m. The sample was drawn at 3:00 p.m. Note the presence of caffeine (approx. 6.9 mg/l), theophylline (approx. 0.7 mg/l) and paraxanthine (approx. 2.0 mg/l) indicating metabolic conversion of caffeine to paraxanthine and, to a much lesser extent, to theophylline.

Fig. 4A and Table III show the relationship between the concentration of theophylline reported for the four assays and that with which the samples were actually spiked in the absence of paraxanthine. All assays produced similar results although the variability in the measurements was greater from the three clinical laboratories. The intercepts were not significantly different from zero at the 0.05 level. The variability in the assays as assessed by the residual sums of squares (R.S.S.) and r^2 was much lower in the present HPLC assay than that used by Laboratories 1 and 2. The variability in the EMIT assay used by Laboratory 3 lay between these two extremes. Fig. 4B and Table IV show the relationship between the concentration of theophylline reported for the four assays and the actual spiked concentration in the presence of paraxanthine. The intercept values from Laboratories 1 and 2 were signifi-

TABLE III

SUMMARY OF STATISTICS OF DATA OBTAINED FROM DIFFERENT LABORATORIES FOR THE MEASUREMENT OF THEOPHYLLINE IN THE ABSENCE OF PARAXANTHINE

d.f. = Degrees of freedom.

	R.S.S.	d.f.	r^2	Slope	Intercept*
This assay	0.0487	10	0.9999	0.9864	—0.03
Laboratory 1	4.9448	10	0.9908	1.0770	—0.32
Laboratory 2	5.8778	9	0.9805	0.9753	0.24
Laboratory 3	1.4906	10	0.9961	0.9081	0.10

*Intercepts not different from zero at $P = 0.05$.

TABLE IV

SUMMARY OF STATISTICS OF DATA OBTAINED FROM DIFFERENT LABORATORIES FOR THE MEASUREMENT OF THEOPHYLLINE IN THE PRESENCE OF PARAXANTHINE

	R.S.S.	d.f.	r^2	Slope	Intercept*
This assay	0.1060	10	0.998	0.9631	−0.00
Laboratory 1	4.6315	10	0.9917	1.0967	2.30
Laboratory 2	4.7778	10	0.9917	1.1167	3.39
Laboratory 3	1.0340	10	0.9975	0.9405	0.03

*Intercepts from Laboratories 2 and 3 were significantly different from zero at $P = 0.05$.

cantly different from zero at the 0.05 level but those from the assay described herein and from Laboratory 3 were not. The variability in the assays, as assessed again by the R.S.S. and r^2 showed that the data from Laboratories 1 and 2 were the most variable. The variability from the described assay herein is the least variable and the variability from the EMIT assay is intermediate. Thus, both of the clinical laboratories using standard reversed-phase HPLC methods failed to distinguish between theophylline and paraxanthine and reported spuriously high values. The reversed-phase ion-pair assay and the EMIT assay, however, are specific for theophylline in these circumstances.

DISCUSSION

The accurate and specific measurement of theophylline in plasma is a necessary part of dosage individualization in patients with obstructive lung diseases. The therapeutic range of theophylline in plasma seems to be between approximately 5 and 20 mg/l although some workers have reported beneficial effects from a concentration as low as 2 mg/l [9]. While the metabolites of theophylline do not appear to accumulate in the plasma of individuals taking theophylline [10], they do appear to be present in the plasma of individuals who consume large amounts of caffeine-containing foods or beverages. This is undoubtedly due to the larger amounts formed from the large quantity of caffeine intake. One caffeine metabolite in particular, paraxanthine, has been shown to interfere with standard reversed-phase HPLC assay [5,7] of theophylline although the magnitude of this interference was not previously known. Our study has shown that the presence of this compound in plasma after drinking a typical number of cups of coffee may result in an apparent plasma theophylline concentration of approximately 3 mg/l although this is expected to be highly variable depending on the individuals, on the nature of caffeine intake, time of sampling, etc. The major difference between the assay described herein and those in which the interference occurs is the presence of the ion-pairing agent tetrabutylammonium which appears to preferentially increase the retention time of theophylline relative to paraxanthine. The reasons for this increase in retention time are not clear. The inclusion of the ion pair in the solvent mixture was originally decided upon in order to increase the retention times of the polar and poorly retained uric acid metabolites of theophylline which, at pH 4.75, would exist in an equilibrium form between the negatively

charged urate ion and the neutral uric acid species. Ion-pair formation between the urate ion and the relatively bulky, non polar tetrabutylammonium ion would therefore increase the retention of these compounds [8]. However, it was also noticed that the retention of theophylline was enhanced to the extent that excellent resolution of theophylline and paraxanthine was attained. Paradoxically, theobromine (the 3,7-dimethylxanthine isomer) had an exceedingly short retention time, appearing between 3-methylxanthine and 1-methylxanthine on the chromatogram. Again, the reason for this unusual chromatographic behavior is unknown.

The ion-pairing system described was exceedingly sensitive to the presence of acetonitrile in the sample. Thus the plasma sample workup by Laboratory 1 resulting in an injection sample of acetonitrile—water (50:50, v/v) produced an unacceptable loss of resolution in the ion-pairing analytical system. The complete or almost complete removal of acetonitrile from the precipitated plasma sample was therefore essential.

The use of a protective precolumn in the system resulted in an analytical column life in excess of 4000 samples although the life of the precolumn was only 500—1000 samples. This may be due to the effect of remaining soluble proteins in the sample or due to the solubility enhancing effect of ion-pairing solvent systems on silicates which may accelerate degeneration of low coverage bonded phase packing materials [11]. Only the exhaustively silylated high coverage bonded phase packing materials have resisted this apparent detrimental effect of the ion-pairing solvent system reported herein. Other packing materials, in particular C_{18} μBondapack (10 μm) and C_{18} Spherisorb (5 μm), resulted in unacceptably short column life as evidenced by a rapid and irreversible loss in retention and resolution.

A comparison of the four theophylline assays is instructive in the light of their intended application. The two clinical laboratory assays utilize slightly shorter sample workup procedures and slightly shorter analysis time although the latter difference is marginal. As the additional step of removing acetonitrile from the sample is necessary in order to perform the assay reported herein, selectivity and accuracy are reduced somewhat by the clinical laboratory procedures in the interests of speed. Although increasing the flow-rate of the ion-pair assay to 2.0 ml/min results in an analysis time of less than 5 min with no loss of resolution between theophylline and the internal standard, this action limited the ability to detect the metabolites of theophylline and caffeine in plasma and, therefore, for routine purposes, was not taken. The EMIT enzyme immunoassay system is apparently specific for theophylline, as would be expected from its immunochemical basis. This assay has been criticized when used in pharmacokinetic studies for its slight, but systematic bias [12], although this was not immediately apparent from the data presented in the present study. However, this is probably not as critical when a single, clinical sample is required as the need for selectivity and accuracy in the concentration ranges typically encountered.

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Journal of Chromatography, 231 (1982) 83—92
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1305

DETERMINATION OF THE ANTICOAGULANT PHENPROCOUMON IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(Received March 1st, 1982)

SUMMARY

The determination of the anticoagulant phenprocoumon in plasma, after acidification and extraction with 1,2-dichloroethane was effected through isocratic high-performance liquid chromatography; a C_{18} reversed-phase column was used as stationary phase using aqueous acetonitrile as eluent and UV detection at 313 nm; *p*-chlorophenprocoumon was used as internal standard.

A high proportion of phenprocoumon in urine is eliminated as the glucuronide and must be hydrolyzed enzymatically before extraction; the same column and detector as for plasma were used, but with gradient elution.

The method was used in the range 0.1—5 mg/l, the sensitivity was 0.1 mg/l for plasma and 0.02 mg/l for urine, the precision was in the range 3—5% and the absence of interference due to other anticoagulants, drugs or endogenous compounds allows the specific determination of phenprocoumon in plasma and urine from patients and volunteers in clinical relevant cases, drug interaction, compliance, toxicological and pharmacokinetic studies.

INTRODUCTION

Phenprocoumon (Fig. 1, 1) [4-hydroxy-3-(1-phenylpropyl)-2H-1-benzopyran-2-one] (Marcumar®, Liquamar®), a member of the 4-hydroxycoumarin-type oral anticoagulants [1, 2] is the most commonly used of its type in many countries. This drug, as well as the congeners warfarin (3) and acenocoumarin (4) are used therapeutically for the prophylaxis and treatment of thromboembolic disorders [2, 3]. Coumarin-type anticoagulants inhibit competitively

*Dedicated to Professor Dr. Dr. h.c.mult. G. Schettler on his 65th birthday.

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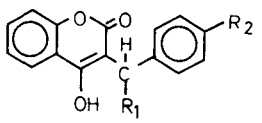

		R_1	R_2
1	Phenprocoumon	$-CH_2.CH_3$	-H
2	p–Chlorophenprocoumon	$-CH_2.CH_3$	-Cl
3	Warfarin	$-CH_2.CO.CH_3$	-H
4	Acenocoumarin	$-CH_2.CO.CH_3$	$-NO_2$

Fig. 1. Structures of 4-hydroxycoumarin anticoagulants.

the last stage of the synthesis of the vitamin K-dependent clotting factors in the liver (carboxylation of glutamic acid to γ-carboxyglutamic acid) [2, 4].

The anticoagulant effect in patients is controlled by the determination of the thromboplastin time [5] or the thrombotest [6] coagulation methods. However, the individual dose of phenprocoumon that leads to effective anticoagulation varies widely with patients [7]. Thus the measurement of phenprocoumon plasma levels and urine excretion rates are indicated particularily during the anticoagulant treatment of patients with oversensitivity or resistance to phenprocoumon, in cases of suspected intoxication or non-compliance and in pharmacokinetic and drug interaction studies [2, 7, 8].

Several methods have been described for the determination of phenprocoumon plasma levels: fluorimetry [9, 10], thin-layer chromatography (TLC) [11—16], gas-liquid chromatography (GLC) [17—22] and high-performance liquid chromatography (HPLC) [16, 23—26]; for the determination of urinary excretion only a GLC method has been reported, after hydrolysis, adsorption and trimethylsilylation, for studies with volunteers after single intravenous injection [19]; however, these methods are inadequate for the analysis of plasma and urine samples from patients, usually under multiple-drug treatment, because they are not specific or sensitive enough, or because they are very time consuming.

In this paper we describe a method for the determination of phenprocoumon in human plasma and urine, involving reversed-phase HPLC after extraction from plasma or enzymatic hydrolysis and extraction from urine, using *p*-chlorophenprocoumon (2) as internal standard.

EXPERIMENTAL

Apparatus

Liquid chromatography was carried out with the following components: Model 6000A pumps, a U6K injector, a Model 660 solvent programmer, a Model 440 UV detector (all from Waters, Königstein, G.F.R.), a BD8 potentiometric recorder (Kipp Analytica, Solingen, G.F.R.), and an Autolab System I computing integrator (Spectra-Physics, Darmstadt, G.F.R.). Centrifugations were run at 2000 *g* at room temperature.

Reagents

All test substances and reagents were of analytical-reagent grade. Acetonitrile (for spectroscopy, Merck, Darmstadt, G.F.R.) and distilled water (B. Braun, Melsungen, G.F.R.) for the mobile phase were used without further purification. Enzymes used for hydrolysis were β-glucuronidase/arylsuphatase from *H.*

pomatia (12 and 60 U/ml) (Merck), β-glucuronidase from *E. coli* (20 U/ml) and arylsulphatase from *H. pomatia* (25 U/ml) (Boehringer Mannheim, Mannheim, G.F.R.). Enzymatic activities were determined using *p*-nitrophenyl-β-D-glucuronide and sulphate as substrates [27]. Phenprocoumon (1) and *p*-chlorophenprocoumon (2) were gifts from Hoffmann-La Roche (Grenzach, G.F.R.) and acenocoumarin (4) and ethyl biscoumacetate from Ciba-Geigy (Wehr, G.F.R.).

Standard solutions

Solutions of 20, 100, 200 and 500 mg/l of phenprocoumon and 500 mg/l of *p*-chlorophenprocoumon (internal standard) in methanol were prepared and kept at 4°C in the dark.

Blood and urine sampling

Venous blood was collected in heparinized tubes, immediately centrifuged, and the plasma kept at −23°C; 24-h urine was stored at the same temperature; samples were thawed and centrifuged before analysis.

Plasma extraction

To 1 ml of plasma contained in a 25-ml centrifuge tube, 10 μl of internal standard solution, 10 μl of methanol, 200 μl of 3 *N* hydrochloric acid and 5 ml of 1,2-dichloroethane were added; the tube was stoppered and shaken for 10 min horizontally at room temperature. After centrifuging for 5 min, 4 ml of the lower phase were transferred into conical centrifuge tubes (15 ml) and evaporated under a stream of nitrogen at 50°C. Before analysis the residue was dissolved in 100 μl of the mobile phase, heated in a water-bath and sonicated. A 20-μl volume of the solution was injected into the chromatograph. A calibration graph was obtained by the same procedure using blank plasma containing 10 μl of the standard solution instead of 10 μl of methanol.

Urine extraction

Enzymatic hydrolysis. A 1-ml volume of urine was treated with 100 μl of acetate buffer (0.1 *M*; pH 4.5) and 10 μl of β-glucuronidase/arylsulphatase and incubated at 37°C for 6 h. Then the mixture was acidified with 200 μl of 3 *N* hydrochloric acid and extracted with 6 ml of 1,2-dichloroethane using the same amount of internal standard and the procedure as for plasma. The corresponding spiked blank urines for the calibration were run in parallel.

Acid hydrolysis. A 1-ml volume of urine was heated with 10 μl of 3 *N* hydrochloric acid at 90°C for 1 h; it was cooled, internal standard was added and the extraction effected as above, omitting the enzymatic hydrolysis step.

Free phenprocoumon. Urine was extracted as described under enzymatic hydrolysis, except that no enzyme or incubation was employed.

Chromatographic conditions

Plasma. The mobile phase was acetonitrile—water—acetic acid (600:400:5), which was filtered (0.45 μm pore size) and degassed before use; the flow-rate was 2.0 ml/min and the pressure 65—100 bar; UV detection was effected at 313 nm at a sensitivity of 0.05 a.u.f.s.; quantitation was effected by area

integration. Calibration graphs were calculated from the linear regression curve from the ratio of the peak area of phenprocoumon to that of the internal standard vs. added phenprocoumon concentration (mg/ml). A reversed-phase C_{18} column was used for the separation (LiChrosorb RP-18 with 10-μm particles, 25 cm × 4 mm I.D.; Hibar RT 250-4; Merck); a guard column (30 × 4 mm I.D.) was filled with Bondapak C_{18} Corasil (35—50-μm particles; Waters) and renewed every few weeks; after finishing a series of analyses the columns were washed with methanol; separations were run at room temperature.

Urine. Urine extracts were analysed using solvent gradient elution with acetonitrile—water—acetic acid (400:600:5) as solvent A and acetonitrile—acetic acid (1000:5) as solvent B; a linear gradient from 0—100% of solvent in 30 min at a total flow-rate of 2.0 ml/min was used. The duration of analysis was 15 min and further 5 min were used for re-equilibration under the initial conditions before the next sample could be injected. Other chromatographic parameters were the same as for plasma.

RESULTS

Plasma

Fig. 2a shows a plasma spiked only with the internal standard; Fig. 2b is from a patient under phenprocoumon treatment and Fig. 2c from a patient with a suspected overdose of phenprocoumon. Retention times were 3.40 and 4.70 min for phenprocoumon and the internal standard, respectively; each sample can be analysed in 5—6 min; peak identity and purity from patient plasma extracts were confirmed by measuring the absorbance ratios for phen-

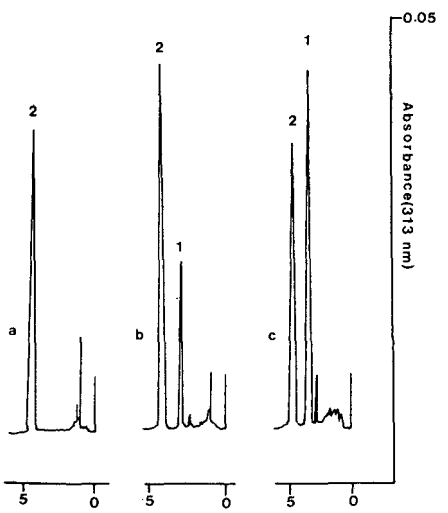

Fig. 2. Chromatograms of plasma extracts: (a) pre-treatment plasma spiked with *p*-chlorophenprocoumon (internal standard; concentration 5 mg/l); (b) extract from a patient under phenprocoumon therapy (phenprocoumon concentration 1.96 mg/l); (c) extract from a patient with suspected phenprocoumon overdosage (concentration 3.8 mg/l). Peaks: 1 = phenprocoumon; 2 = internal standard.

procoumon and internal standards at 280, 254 and 313 nm, which were the same as for the pure compounds. The calculated recoveries for phenprocoumon and *p*-chlorophenprocoumon from plasma were 86.5 ± 3.4% and 88.3 ± 4.7% (n = 5) at a concentration of 5 mg/l; the same values were obtained for aqueous solutions. Plasma analysis were in the range 0.1—5 mg/l with a linear calibration regression line of $y = 0.2188x + 0.0125$, $r = 0.998$; sensitivity 0.1 mg/l.

Urine

Fig. 3a shows the separation of phenprocoumon and internal standard employing the linear gradient elution conditions used for urine analysis (the retention times were phenprocoumon 11.02 min and *p*-chlorophenprocoumon 13.29 min). Fig. 3b shows the chromatogram of a blank urine and Fig. 3c that of a blank urine spiked with 0.2 mg/l of phenprocoumon and 5 mg/l of internal standard. Fig. 4 shows chromatograms of the urine of a patient under chronic phenprocoumon treatment: (a) direct extraction with no hydrolysis, (b) after acid hydrolysis and (c) after enzymatic hydrolysis. The analysis time was 20 min per sample; peaks were identified in a similar way as for plasma. Calculated recoveries at a concentration of 5 mg/l for phenprocoumon and *p*-chlorophenprocoumon were 88.9 ± 2.7% and 89.1 ± 2.2%, respectively (n = 5); analyses were in the range 0.02—5 mg/l with a linear regression line for the calibration of $y = 0.2189x + 0.0189$, $r = 0.998$; sensitivity, 0.02 mg/l. The accuracy and precision for plasma and urine are given in Table I.

Validity

Plasma levels from ten orally anticoagulated patients (dose: 2.33 ± 0.64 mg/day; mean ± standard deviation) showed a mean concentration of 2.10 ± 0.66 mg/l of total phenprocoumon (range 1.54—3.58 mg/l). Urine samples from another group of anticoagulated patients were analysed and the results are shown in Table II; the mean values for the 24-h phenprocoumon excretion were 0.0412 mg for free phenprocoumon and 0.388 mg for total phenprocoumon after enzymatic hydrolysis. The values obtained after hydrolysis with acids were always lower than those obtained with enzymes; the concentraprocoumon after enzymatic hydrolysis. The values obtained after hydrolysis with acids were always lower than those obtained with enzymes; the concentration ratio of enzymatic to acid hydrolysis was 2.19 ± 0.66 (n = 10). The method was also used for the analysis of plasma and urine samples from patients with oversensitivity to anticoagulants, suspected non-compliance or overdose, and for drug interaction studies [2, 8].

Interferences

There were no interferences in the assay from plasma and urine from patients treated with following drugs: allopurinol, atenolol, cimetidine, carbamazepine, digoxin, digitoxin, hydrochlorothiazide, indomethacin, isosorbide dinitrate, β-methyldigoxin, nifedipine, nomifensin, pindolol and triamterene. The following substances were extracted and did not interfere: acenocoumarin, acetaminophen, β-acetyldigoxin, acetylsalicylic acid, ampicillin, atropine, azlocillin, caffeine, cefazolin, cotinine, dihydralazine, ergotamine, ethyl biscoumacetate, furosemide, heparin, heptabarbital,

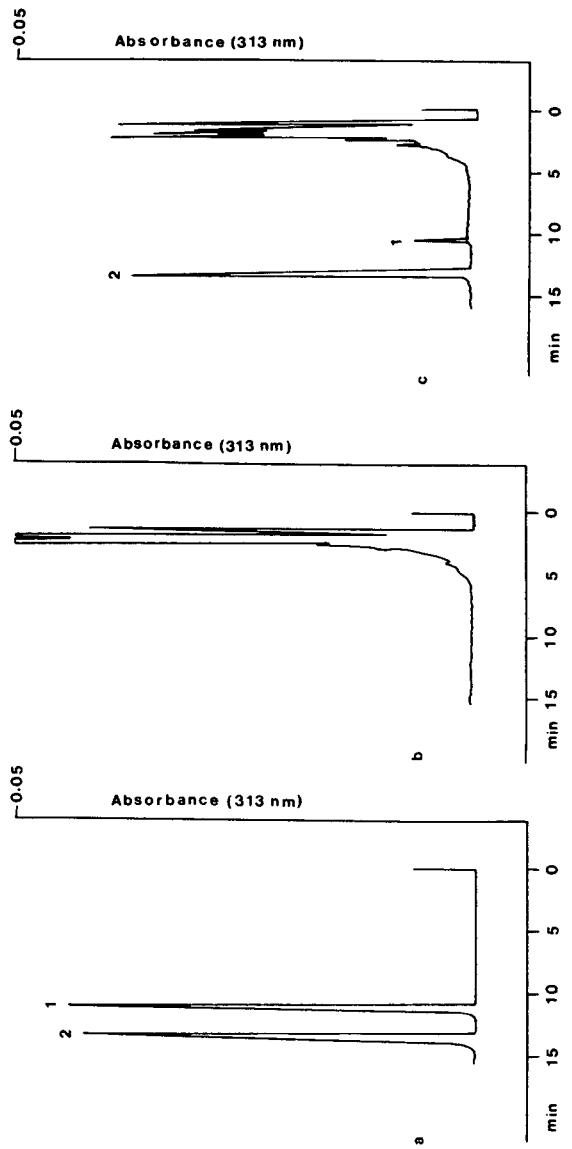

Fig. 3. Chromatograms of urine extracts: (a) separation of pure phenprocoumon and *p*-chlorophenprocoumon; (b) pre-treatment urine with no addition of internal standard; (c) extract from urine spiked with 0.2 mg/l of phenprocoumon and 5 mg/l of internal standard. Peaks as in Fig. 2.

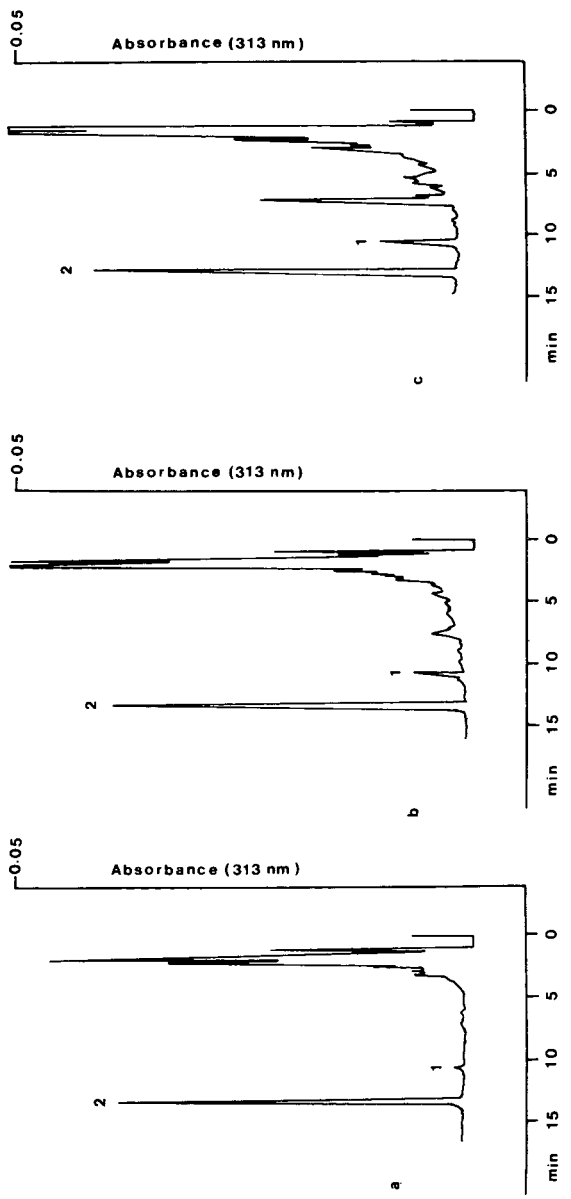

Fig. 4. Chromatograms from the urine of a patient under phenprocoumon treatment: (a) extract with no previous hydrolysis (phenprocoumon concentration 0.02 mg/l); (b) extract after acid hydrolysis (concentration 0.19 mg/l); (c) extract after enzymatic hydrolysis (concentration 0.35 mg/l). In all instances the concentration of the internal standard was 5 mg/l. Peaks as in Fig. 2.

TABLE I

REPRODUCIBILITY OF PLASMA AND URINE ANALYSES FOR PHENPROCOUMON

Parameter	Plasma				Urine		
Concentration given (mg/l)	0.5	1	2	5	0.2	1	5
Concentration found (mg/l)	0.52	1.03	2.03	4.98	0.22	0.96	5.01
Coefficient of variation (%) (n=5)	6.1	5.6	5.0	3.5	2.2	5.3	2.2

TABLE II

PHENPROCCUMON CONCENTRATION IN URINE AND 24-h EXCRETION RATE IN PATIENTS ON PHENPROCOUMON MAINTENANCE THERAPY (2.33 ± 2.82 mg/day; n = 9)

The upper values are mean ± standard deviation; the values in parentheses are the ranges.

Phenprocoumon	Concentration (mg/l)	24-h excretion (mg)
Free	0.0267 ± 0.0245	0.0412 ± 0.0267
	(0—0.07)	(0—0.13)
After enzyme hydrolysis	0.466 ± 0.428	0.388 ± 0.236
	(0.10—1.46)	(0.173—0.936)

hippuric acid, 1-(β-hydroxypropyl)theobromine, isoproterenol, lidocaine, metoprolol, methaqualone, mezlocillin, nicotine, neostigmine bromide, penicillin G, phenacetin, phenobarbital, pindolol, procainamde, propranolol, quinidine, salicylic acid, salicylamide, secobarbital, sulphinpyrazone, theobromine, theophilline, trithiozine, uric acid, vitamin B complex, vitamin C and warfarin.

DISCUSSION

Plasma

Several procedures for the pre-cleaning of phenprocoumon in plasma were tried: precipitation of proteins with solvents [23], prepurification through C-18 cartridges [28] and adsorption [26]; they were found to be non-reproducible or the chromatograms showed interfering peaks; 1,2-dichloroethane extraction in acidic media was found to be the most reproducible method of several tested, and also showed good recoveries and absence of interferences. A wavelength of 313 nm was selected for better selectivity to avoid the detection of substances that would interfere at lower wavelengths. The method measures total phenprocoumon concentration; it has been applied in the cases mentioned above under *Validity* and has been in current use for over a year.

Urine

Only a small fraction (1.8%) of the applied phenprocoumon dose is eliminated unchanged in urine (Table II) and sometimes could not be detected with the described method. In rats, phenprocoumon is metabolized by liver

microsomes through oxidation to the 4'-, 6-, 7- and 8-monohydroxy derivatives [12, 13, 29]; in man, after single intravenous application of phenprocoumon a high proportion is eliminated as the glucuronide [19] (probably conjugated glycosidically to the 4-hydroxy group of phenprocoumon). Prior to extraction, urine was submitted to hydrolysis to liberate phenprocoumon, which was later extracted and quantitated by HPLC. The complex nature of urine samples, due to interfering endogenous compounds, other drugs and metabolites, necessitates the use of solvent gradient elution during the HPLC run. The conditions for hydrolysis described under Experimental were those selected for maximum phenprocoumon after several optimization trials; acid hydrolysis always yielded lower values than enzymatic hydrolysis (see *Validity*), owing in part to the degradation of phenprocoumon at low pH and high temperature; hydrolysis with β-glucuronidase/arylsuphatase gave the same value as with β-glucuronidase alone, confirming the observation that phenprocoumon is not conjugated with sulphates [19].

Excretion of phenprocoumon was studied in 24-h urine samples obtained from hospitalized patients; free phenprocoumon accounts for 1.8% of the applied dose, and after enzymatic hydrolysis 16.6% (Table II); inter-individual differences are present owing to variable dose, different multiple drug treatments and different disorders.

A suspected case of phenprocoumon overdose showed a 24-h elimination of total phenprocoumon (after enzymatic hydrolysis) of 4.6 mg (and a plasma level of 3.80 mg/l; Fig. 2c), significantly higher than the mean and extremes found for patients under normal phenprocoumon therapy, confirming the suspicion of intoxication.

In conclusion, free phenprocoumon or phenprocoumon after acid hydrolysis is not a reliable indicator of urine elimination; values obtained after enzymatic hydrolysis are mor trustworhty in this sense.

For the reasons expressed above the method is simple, rapid, sensitive, specific and reproducible for the quantitation of phenprocoumon in plasma and urine from patients and volunteers in clinical cases, drug interaction, compliance, toxicological and pharmacokinetic studies.

ACKNOWLEDGEMENTS

We thank Miss B. Koroknay and G. Fischer for technical assistance.

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Journal of Chromatography, 231 (1982)93—101
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1283

ROUTINE MONITORING OF CARBAMAZEPINE AND CARBAMAZEPINE-10,11-EPOXIDE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING 10-METHOXYCARBAMAZEPINE AS INTERNAL STANDARD

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(First received October 30th, 1981; revised manuscript received March 4th, 1982)

SUMMARY

Carbamazepine and carbamazepine-10,11-epoxide were separated by high-performance liquid chromatography (HPLC) with acetonitrile—water as mobile phase, and detection was effected by UV absorption at 215 nm with a total retention time of less than 10 min. Plasma samples were extracted with dichloromethane and 4 *M* sodium hydroxide, and 10-methoxycarbamazepine was added as internal standard.

Other commonly used anticonvulsant drugs present in plasma showed no significant interference.

The within-batch coefficient of variation for carbamazepine was 4.9% and carbamazepine-10,11-epoxide 5.9%. Between-batch coefficients of variation were 3.7% and 5.3%, respectively. Mean recovery for carbamazepine was 100.2% and for carbamazepine-10,11-epoxide 100.6%.

This HPLC method was compared with both an enzyme immunoassay procedure (EMIT) and a gas—liquid chromatographic (GLC) method. Correlation coefficient between HPLC/EMIT for carbamazepine was 0.983, HPLC/GLC carbamazepine 0.988 and HPLC/GLC carbamazepine-10,11-epoxide 0.981.

INTRODUCTION

Carbamazepine (Tegretol®, Ciba-Geigy, Basle, Switzerland) is increasingly used in the treatment of epilepsy [1] and is the drug of choice in trigeminal neuralgia [1,2]. Carbamazepine is partly converted in the body to the 10,11-epoxide metabolite which also displays anticonvulsant properties similar to those of the parent compound [3]. The plasma level of carbamazepine-10,11-epoxide is lower than that of the parent drug, the relative percentage ranging from 10—50% [4].

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There is no clear relationship between the daily dose of carbamazepine and its steady state plasma level in epileptic patients receiving long-term therapy with the drug [5,6]. Marked individual variations in the ratio of dose to plasma level [7] make it essential to measure plasma carbamazepine levels in each patient, but no firm data are yet available concerning the importance of measuring the levels of the metabolite in routine practice.

Many different methods have been used for the estimation of carbamazepine including procedures based on UV spectrophotometry [8,9], gas–liquid chromatography (GLC) [10–16], enzyme-multiplied immunoassay technique (EMIT) [17,18] and high-performance liquid chromatography (HPLC), some of which also estimate the epoxide. The subject has been reviewed recently. Nevertheless, there are disadvantages to some of the published methods. In particular the spectrophotometric methods lack specificity and may be subject to interference, and some of the GLC methods yield variable results because of decomposition of carbamazepine on column [19] or during derivatisation [20]. Some of the HPLC procedures employ as internal standards, compounds (or metabolites of drugs) such as nitrazapam [21], lorezapam or N-desmethyldiazapam [22] any of which may be present in the serum of patients in neurological hospitals or clinics, or 10,11-dihydroxycarbamazepine which is the major metabolite of carbamazepine present in urine. Others require a change in wavelength [23] during each run in order to overcome the unfavourable absorption characteristics of the epoxide. The reversed-phase HPLC procedure presented here employs 10-methoxy-carbamazepine as an internal standard, a compound which is not normally administered, nor present as a metabolite in patients' plasma or urine.

EXPERIMENTAL

Materials

Carbamazepine, carbamazepine-10,11-epoxide and 10-methoxycarbamazepine were obtained from Geigy (Horsham, Great Britain). All solvents used were HPLC grade.

Samples

Blood samples were collected from patients on carbamazepine for routine measurement, and plasma was stored at −20°C until required for analysis.

*EMIT** assay

The reagents for enzyme immunoassay of carbamazepine were obtained from Syva (Maidenhead, Great Britain) and were used according to the manufacturer's instructions. Absorption was measured at 340 nm on a Gilford System 3500 Computer Directed Analyser (Gilford Instruments, Teddington, Great Britain) and the results were directly calculated in μmol/l by a CP 5000 EMIT Clinical Processor (Syva).

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* Trade name of Syva.

GLC assay

Plasma containing carbamazepine and its metabolite was analysed by GLC using the method of Chambers [15] with 10-methoxycarbamazepine as internal standard instead of imipramine. The estimation was carried out on a Hewlett-Packard research chromatograph 5750G using an organic nitrogen specific detector (Hewlett-Packard, Winersh, Great Britain).

HPLC assay

Apparatus. The liquid chromatograph used was a Spectra Physics SP 8000 (Spectra Physics, St. Albans, Great Britain) with data processing capability, fitted with an automatic 10-μl Valco loop injector and a Schoeffel S770 variable-wavelength UV absorption detector (Kratos, Manchester, Great Britain). Chromatograms were run at ambient temperature on a column 25 cm × 4.9 mm I.D. packed with LiChrosorb RP-8 10 μm (Hichrom, Reading, Great Britain). A mobile phase (acetonitrile—water, 35:65) flow-rate of 1.8 ml/min at 60 ± 5 bar was used. The column eluate was monitored at 215 nm with sensitivity range of 0.1 a.u.f.s. and chart speed of 0.5 cm/min.

Preparation of standard solutions. The stock solutions of carbamazepine, carbamazepine-10,11-epoxide and 10-methoxycarbamazepine as internal standard were made up in methanol to a concentration of 1 mg/ml. These solutions were kept at 4°C in a sealed container and were stable for several weeks.

Working standards of carbamazepine and carbamazepine-10,11-epoxide were made by further dilution with methanol to give a range of concentrations from 2—16 μg/ml for carbamazepine and 1—8 μg/ml for carbamazepine-10,11-epoxide. The concentration of 10-methoxycarbamazepine in each tube was 4 μg.

Carbamazepine and carbamazepine-10,11-epoxide concentrations were determined by the ratio of the peak areas of each drug to the peak area of internal standard plotted against concentration of the drugs.

Procedure. Plasma or standard (250 μl) to which 10-methoxycarbamazepine (4 μg) had been added as an internal standard was made alkaline with 4 *M* aqueous sodium hydroxide (250 μl); 2 ml of dichloromethane (BDH Chemicals, Poole, Great Britain) were added and the mixture shaken at 120 cycles/min for 5 min. The mixture was then centrifuged at 3 *g* for 5 min and the top (aqueous) layer removed by aspiration. The solvent layer was then carefully transferred to a separate 15-ml conical tube and evaporated to dryness at 40°C under a gentle stream of oxygen-free nitrogen. The sides of each conical tube were washed down with 0.2 ml of acetonitrile (Fisons, Loughborough, Great Britain), which was then evaporated to dryness, the residue dissolved in 100 μl of acetonitrile and 10 μl injected in the chromatograph.

RESULTS

Table I gives the figures for precision, recovery and commercial quality control data.

Specificity

The retention times of carbamazepine and its metabolite and possible inter-

TABLE I

ANALYTICAL PARAMETERS OBTAINED BY THREE PROCEDURES

	HPLC	GLC	EMIT
Carbamazepine (pooled plasma)			
Between-batch precision	31 ± 2.0	32 ± 3.0	36 ± 3.0
Coefficient of variation (%)	3.7 (n=45)	4.2 (n=40)	6.0 (n=35)
Within-batch precision	29 ± 2.0	30 ± 3.0	35 ± 3.0
Coefficient of variation (%)	4.9 (n=20)	5.9 (n=22)	8.9 (n=35)
Carbamazepine-10,11-epoxide (pooled plasma)			
Between-batch precision	7.0 ± 1.0	7.4 ± 2.6	–
Coefficient of variation (%)	5.3 (n=40)	10.7 (n=40)	–
Within-batch precision	6.8 ± 1.0	7.2 ± 2.5	–
Coefficient of variation (%)	5.9 (n=20)	15.6 (n=22)	–
Carbamazepine (spiked plasma)			
Mean of recovery (%)	100.2 (n=20)		
Carbamazepine-10,11-epoxide (spiked plasma)			
Mean of recovery (%)	100.6 (n=20)		
Commercial quality control (Seronorm Pharmaca):			
Carbamazepine			
Recommended value	63.0 μmol/l		
Analytical value	59.07 μmol/l		
Coefficient of variation (%)	2.43 (n=15)		
Carbamazepine-10,11-epoxide			
Recommended value	6.5 μmol/l		
Analytical value	5.9 μmol/l		
Coefficient of variation (%)	3.9 (n=15)		

fering compounds are given in Table II. When these compounds are injected before extraction, it is evident that some of the drugs have very nearly the same retention times as carbamazepine and its derivatives, but after extraction of plasma spiked with (toxic) levels of the compound no peaks are apparent. Fig. 1 shows typical chromatograms of (a) a standard extract and (b) a plasma extract. It can be seen that there is adequate separation between carbamazepine-10,11-epoxide, carbamazepine and the internal standard in both cases.

Precision

Both between-batch and within-batch precision were determined from analyses made on pooled plasma. Patients' plasma samples known by previous analysis to contain carbamazepine were pooled and filtered with thorough mixing, aliquoted into bottles and stored at −20°C until required for analysis. Between-batch precision was determined on this pooled plasma on different days. In all instances the coefficient of variation for HPLC was less than 6%

TABLE II

RETENTION TIMES OF ANTIEPILEPTIC DRUGS AND OTHER POTENTIALLY INTERFERING COMPOUNDS

Compound	Retention time (min)	Retention time after extraction (min)
Carbamazepine	5.9	5.9
Carbamazepine-10,11-epoxide	3.9	3.9
10-Methoxycarbamazepine	7.4	7.4
Phenobarbitone	3.6	No peak
Primidone	3.0	No peak
Phenytoin	6.7	No peak
Ethosuximide	No peak	No peak
Valproic acid	No peak	No peak
Clonazepam	9.3	No peak
Diazepam	18.1	No peak
Desmethyldiazepam	12.4	No peak
Sulthiame	3.5	No peak
Nitrazepam	8.1	No peak
cis-Dihydroxycarbamazepine	2.7	2.6

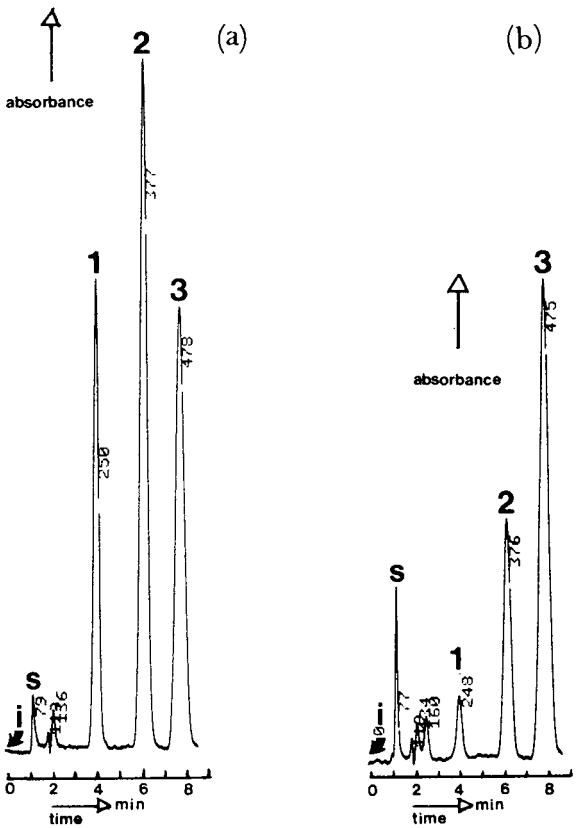

Fig. 1. HPLC chromatograms (a) showing separation of a standard solution and (b) obtained from plasma extract of a patient taking carbamazepine (600 mg/day). Peaks: (1) the metabolite, carbamazepine-10,11-epoxide, (2) carbamazepine and (3) the internal standard, 10-methoxycarbamazepine. (i) Point of injection and (s) solvent front.

which is entirely acceptable for routine measurement of carbamazepine and carbamazepine-10,11-epoxide.

Recovery

Recovery was determined by analysing drug-free plasma samples (spiked with 5 μg/ml carbamazepine and 2 μg/ml carbamazepine-10,11-epoxide) 20 times, giving measured recoveries ranging from 98.8—102.5% for carbamazepine and 97.9—103% for carbamazepine-10,11-epoxide.

Commercial quality control

The commercial quality control serum (Seronorm Pharmaca, BDH) was analysed for carbamazepine and carbamazepine-10,11-epoxide giving coefficients of variation of 2.43% and 3.0%, respectively.

Correlation

Regression analyses of the results obtained between HPLC and GLC (carbamazepine and carbamazepine-10,11-epoxide) and HPLC and EMIT (carbamazepine) are shown in Figs. 2, 3 and 4, which in addition give values for slopes of the regression lines and intercepts. There was good correlation for carbamazepine between HPLC and GLC (r=0.988) and HPLC and EMIT (r=0.983). There was also a good correlation between HPLC and GLC for carbamazepine-10,11-epoxide (r=0.981).

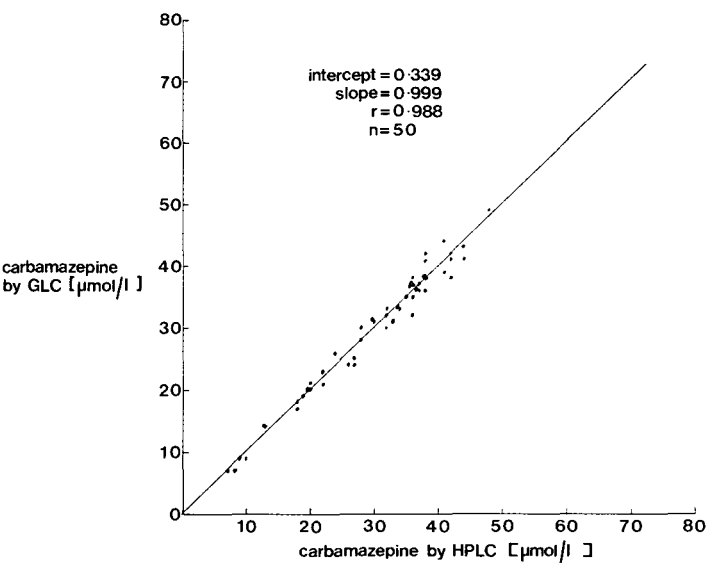

Fig. 2. Correlation of carbamazepine concentration as determined by HPLC and GLC.

Linearity

This HPLC assay is linear over the range of 0—100 μmol/l for carbamazepine and 0—50 μmol/l for carbamazepine-10,11-epoxide. This more than adequately spans the therapeutic range for carbamazepine (12—50 μmol/l).

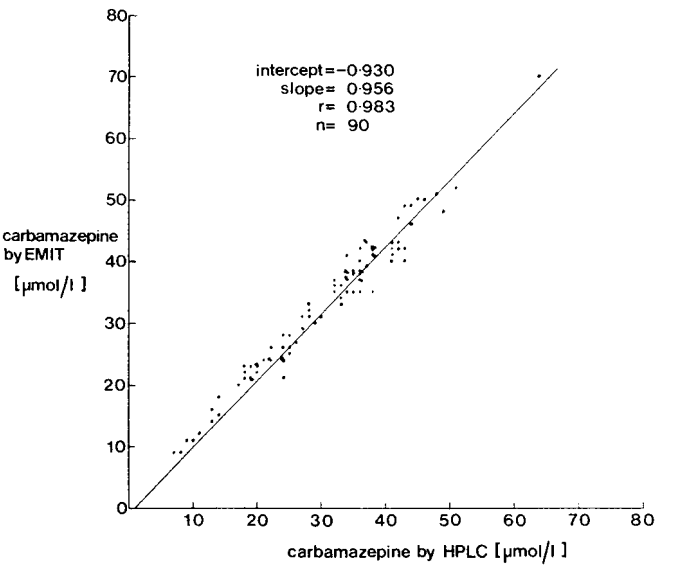

Fig. 3. Correlation of carbamazepine concentration as determined by HPLC and EMIT.

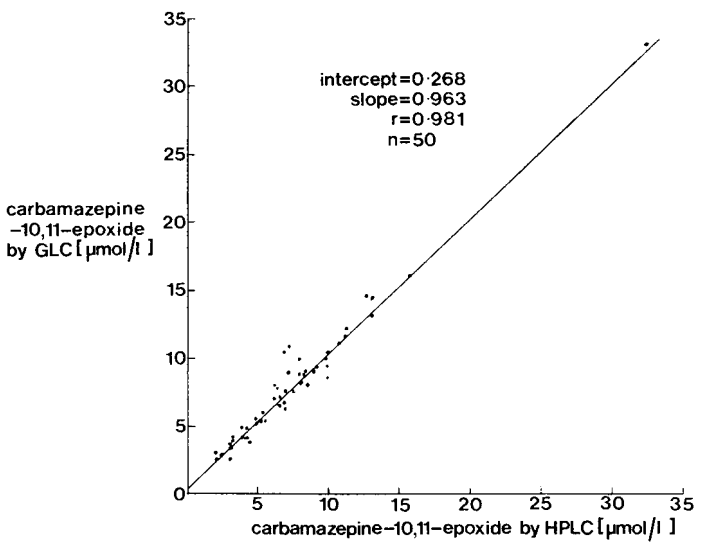

Fig. 4. Correlation of the metabolite carbamazepine-10,11-epoxide concentration as determined by HPLC and GLC.

DISCUSSION

The HPLC system described here for routine monitoring of plasma carbamazepine and its metabolite with use of 10-methoxycarbamazepine as an internal standard has some advantages over published HPLC, GLC and EMIT procedures.

The low UV absorbing properties of the epoxide have been a problem in HPLC analysis but in this HPLC procedure the use of optimum wavelength for

carbamazepine and carbamazepine-10,11-epoxide (215 nm) has contributed significantly to the sensitivity of the method. This factor eliminates the need to change the range of the detector during analysis as is the case for some HPLC procedures [1]. Furthermore, 10-methoxycarbamazepine is a suitable internal standard as it is not present in patients' plasma or urine; the use of lorezapam and N-desmethyldiazepam (a metabolite of diazapam), nitrazepam and imipramine as internal standard may pose problems in neurological hospitals where patients may be taking any one of these compounds together with the carbamazepine.

The high precision of the HPLC procedure shown in Table I as compared with GLC probably reflects better prevailing chromatographic conditions. This may be because the HPLC is carried out at ambient temperature with no possibility of on-column degradation while GLC is carried out at a relatively high column temperature (225°C). By contrast with the above the EMIT procedure is quicker than HPLC but slightly less precise (Table I) and unable to determine the epoxide at present.

No difficulties have been encountered with the HPLC technique in routine use and an interference problem from other drugs has not arisen. Very minor extraneous peaks have been observed around 2 min retention time (Fig. 1) but these peaks are probably caused by impurities from the silicone tubing used for blowing down the extract and do not interfere with the assay.

Sera from a total of 115 patients (dose ranging from 300—1800 mg per day) have been analysed for carbamazepine and its metabolite by this HPLC procedure, and of these one who had symptoms of toxicity was found to have a serum level of epoxide as high as 86.8% of the carbamazepine level (carbamazepine 38 μmol/l and epoxide 33 μmol/l). Another 20 patients with serum carbamazepine levels 50 μmol/l and above had epoxide levels of 12 μmol/l and above. The remaining 95 patients had levels of epoxide below 12 μmol/l and correspondingly serum carbamazepine levels of less than 50 μmol/l.

These results indicate that the upper limit of the therapeutic range for epoxide could be of the order of 12 μmol/l, but it is still necessary to relate the upper limit of epoxide level to clinical toxicity. Further work is necessary in order to clarify this situation and also evaluate interactions between the epoxide and other anticonvulsants co-prescribed with carbamazepine.

CONCLUSION

The HPLC, GLC and EMIT techniques evaluated in this study are all reliable, accurate, simple to perform and linear. The levels of carbamazepine and its metabolite analysed by these methods agree well over a wide range of concentrations. However, the HPLC procedure possesses the advantages of greater precision and sensitivity over the GLC technique examined here, and has the potential for determining carbamazepine and its metabolites in urine. Further it is quicker than GLC (requiring less than 1 h to perform an analysis) and in addition requires smaller plasma samples which is an advantage in paediatric practice. The EMIT technique requires even smaller plasma samples than HPLC and it is more rapid in operation but cannot be adapted at present to analyse carbamazepine-10,11-epoxide in plasma or urine.

ACKNOWLEDGEMENTS

The authors thank Geigy Pharmaceuticals for supplying the carbamazepine, carbamazepine-10,11-epoxide and 10-methoxycarbamazepine used in this study.

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Journal of Chromatography, 231 (1982)103—110
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1286

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF METHOTREXATE, 7-HYDROXYMETHOTREXATE, 4-DEOXY-4-AMINO-N^{10}-METHYLPTEROIC ACID AND SULFAMETHOXAZOLE IN SERUM, URINE AND CEREBROSPINAL FLUID

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(First received November 24th, 1981; revised manuscript received February 26th, 1982)

SUMMARY

An automated high-performance liquid chromatographic system is described for separation and quantitation of the antineoplastic drug methotrexate and metabolites, and the antibiotic sulfamethoxazole in body fluids. The 40-min analysis utilizes a reversed-phase C_{18} column and gradient elution with detection by absorbance of ultraviolet light at 308 nm. The minimum detectable quantities with this assay are: methotrexate 4.4 ng ($9.8 \cdot 10^{-12}$ mole); 4-deoxy-4-amino-N^{10}-methylpteroic acid 11.9 ng ($3.7 \cdot 10^{-11}$ mole); 7-hydroxymethotrexate 30 ng ($6.5 \cdot 10^{-11}$ mole); sulfamethoxazole 125 ng ($4.9 \cdot 10^{-10}$ mole). This analytical method should prove useful for therapeutic monitoring and pharmacokinetic studies of these compounds.

INTRODUCTION

Methotrexate, L-(+)-N-(*p*-[((2,4-diamino-6-pteridinyl)methyl)methylamino]-benzoyl)glutamic acid, a competitive inhibitor of dihydrofolate reductase, belongs to the first class of compounds successfully used to produce remission of leukemia in man. Methotrexate (MTX) is now widely used to treat several human cancers including acute leukemia, osteosarcoma, non-Hodgkin's lymphoma, breast carcinoma and choriocarcinoma.

Renal excretion is the major route of MTX and metabolite elimination. Depending on the dosage and duration of intravenous (i.v.) infusion, from 60—95% of an i.v. dose of MTX may be eliminated unchanged in the urine. More recent pharmacokinetic studies [1,2] have identified substantial amounts of 7-hydroxymethotrexate (7-OH MTX), an oxidative metabolite formed by hepatic aldehyde oxidase [3]. Since 7-OH MTX has 3—5 times lower solubility

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than the parent compound, intratubular crystallization of this metabolite has been proposed as one mechanism of renal damage following MTX administration [3]. MTX may also be metabolized to 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) via a simple amide cleavage by intestinal bacteria [4]. Both of these metabolites are about 100—200-fold less cytotoxic than MTX [4,5].

Because the cytotoxic effects of MTX are due to the inhibition of dihydrofolate reductase and the subsequent lowering of reduced folate pools, the effects of MTX can be negated by administering reduced folates (i.e., leucovorin "rescue"). This has provided a mechanism by which dosage of MTX (1—20 g/m^2) far in excess of conventional dosages (25—50 mg/m^2 without leucovorin) can be given without host toxicity. The rationale of these higher dosages includes overcoming relative resistance due to poor intracellular uptake of MTX and achieving cytotoxic MTX concentrations in tissues where MTX distribution is poor (i.e., central nervous system, testes). However, the administration of high-dose MTX may be severely toxic if adequate leucovorin rescue is not given. A nationwide survey showed approximately a 6% incidence of drug-related deaths after high-dose MTX and leucovorin rescue [6]. This noted mortality rate solidified the need for close clinical and pharmacokinetic monitoring of high-dose MTX and leucovorin rescue.

Several protocols have been described for monitoring high-dose MTX and adjusting leucovorin rescue in patients with delayed MTX elimination [7]. All of these protocols include measurement of MTX serum or plasma concentrations to identify high-risk patients and to guide the dosage modification of leucovorin. Several pharmacokinetic studies have also been conducted to more precisely define the influence of selected clinical features (i.e., pleural effusions, ascites, gastrointestinal obstruction, renal dysfunction, etc.) on the delayed elimination of MTX. However, most studies have not examined the contribution of altered MTX metabolism or perturbations in MTX disposition induced by metabolites of MTX. The absence of such studies is related in part to the lack of an accurate, sensitive and clinically feasible assay for MTX and its two major metabolites in biological fluids. These pharmacokinetic studies are also frequently complicated by the concomitant administration of sulfamethoxazole—trimethoprim, a drug combination which is routinely used as prophylaxis for *Pneumocystis carinii* pneumonia in immunosuppressed patients at high-risk for this potentially fatal infection. The presence of sulfamethoxazole [N′-(5-methyl-3-isoxazoyl)sulfanilamide] in biologic fluids being assayed for MTX and metabolites is potentially important, since this compound coelutes with MTX metabolites on some high-performance liquid chromatographic (HPLC) systems and because sulfamethoxazole may influence the pharmacokinetics of MTX (i.e., protein binding, renal tubular secretion).

Several assay methods for MTX have been reported and include enzyme immunoassay (EMIT) [8,9], radioimmunoassay [10—14], radioenzymatic [13,15] and HPLC assays [16—20]. Of these techniques, only HPLC has the potential to readily measure both parent drug and metabolites. To date, an HPLC assay which provides separation and quantitation of MTX, DAMPA, 7-OH MTX and sulfamethoxazole (SMX) has not been reported.

EXPERIMENTAL

Reagents

Water was distilled and treated with a Milli-Q water purification system. UV grade acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and certified ammonium formate (Fisher, Pittsburgh, PA, U.S.A.) were used as received. Reagent grade trichloroacetic acid was obtained from VWR Scientific (San Francisco, CA, U.S.A.). Ethanol 95% U.S.P. (AAper Alcohol and Chemical Co., Louisville, KY, U.S.A.) was used as received. HPLC solvent A was 5% acetonitrile in 0.01 *M* ammonium formate solution, HPLC solvent B was 20% acetonitrile in 0.01 *M* ammonium formate solution (pH 3.5 for both eluting solvents). HPLC solvents were purged with helium and subjected to vacuum to degas. SMX and aminopterin (AMN) were purchased from Sigma (St. Louis, MO, U.S.A.). DAMPA and 7-OH MTX were gifts from Dr. D. Kabbakoff. DAMPA was also synthesized as described below. MTX, prepared as the sodium salt, NCI No. NSC-740 was supplied by the National Cancer Institute. Structures of the compounds of interest are shown in Fig. 1.

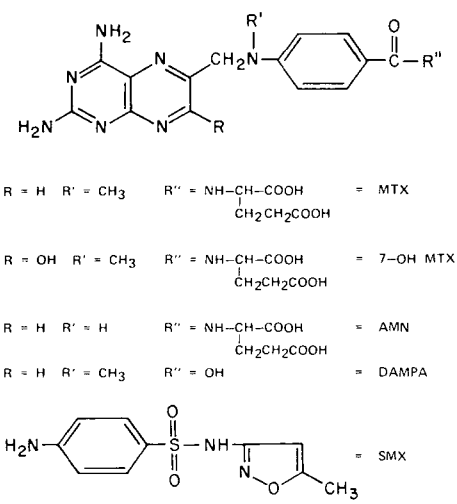


Fig. 1. Chemical structures of methotrexate (MTX), 7-hydroxymethotrexate (7-OH MTX), aminopterin (AMN), 4-deoxy-4-amino-N^{10}-methylpteroic acid (DAMPA) and sulfamethoxazole (SMX).

Synthesis of DAMPA

Amides are cleaved to carboxylic acid salts with aqueous bases, acidification gives the actual acid. To 20 ml 0.05 *N* sodium hydroxide were added 60 mg MTX, and the solution was refluxed overnight. Conversion of MTX to DAMPA was about 50% as determined by UV absorbance with no side products. The solution was neutralized or slightly acidified with hydrochloric acid and rotary evaporated under vacuum. DAMPA was isolated and MTX recovered easily in small amounts by using the HPLC system described.

Samples

Calibration samples were prepared from pooled normal human serum spiked with the compounds of interest and serially diluted. To 0.5 ml of sample were added 5 μl (250 ng) of AMN as internal standard, with vortexing. Proteins were precipitated by adding 75 μl 2 *M* trichloroacetic acid in 95% ethanol and vortexing. Centrifugation at 1500 *g* for 15 min yielded a clear supernatant suitable for HPLC injection. A volume of 250 μl was auto-injected on a 200-μl loop.

Blood, cerebrospinal fluid (CSF), and urine were collected at various times from patients receiving MTX (200 mg/m^2 i.v. bolus, followed by 800 mg/m^2 infused i.v. over 24 h). These patients also received an intrathecal MTX dose of 12 mg/m^2 at the start of the 24-h infusion (time = 0 h). Blood was centrifuged and serum was removed by pipet for analysis. Urine was diluted 1:10 with buffer A. CSF was submitted for analysis as collected. All samples were otherwise treated exactly as the calibration standards.

Instrumentation

HPLC was performed on a prepacked μBondapak 10-μm C_{18} column 30 cm × 3.9 mm I.D. (Waters Assoc., Milford, MA, U.S.A.) protected with a 2.3 cm × 3.9 mm precolumn packed with C_{18} Corasil, 37—50 μm (Waters Assoc.). An M-45 HPLC pump (Waters Assoc.) augmented with a 980A solvent programmer (Tracor Instruments) was used as the solvent delivery system. Gradient elution was utilized for optimum resolution with the following parameters: 3.3% change per min, 20% non-linear convex curve, 25—100% B, flow-rate 2.0 ml/min. Samples were handled by a Model 8055 Autosampler (Varian) fitted with an AH-60 pneumatic injector (Valco Instruments). Column effluent was detected by a Model 1203 UV III monitor (Laboratory Data Control) illuminated with a zinc lamp at a fixed wavelength of 308 nm. A sensitivity of 0.002 a.u.f.s. was used. Detector signal data were processed with an S/P-4100 computing integrator (Spectra Physics). Retention times and areas of peaks calculated by the SP-4100 were stored on tape by a D-980M Cassette Tap Deck (Hitachi) for possible reprocessing.

RESULTS AND DISCUSSION

Of several solvent system—column packing combinations investigated, the system described (see Experimental) proved convenient and satisfactory, achieving baseline resolution for all components of interest. Separation of a standard mixture in pooled serum is shown in Fig. 2, along with a chromatogram from a serum blank. Retention volumes and capacity factors for the compounds of interest and other folates are given in Table I. Leucovorin (5-formyl-tetrahydrofolate) and its metabolite 5-methyl-tetrahydrofolate (5-MeTHF), which may be present in patient samples after leucovorin rescue is begun, are resolved from the compounds of interest.

Quantitative estimation of the amounts of parent drug and/or metabolites was obtained with this system. Calibration curve data with internal standard correction were processed by the computing integrator calculating a linear fit to detector response. The linear correlation coefficients were: MTX 0.9898, DAMPA 0.9575, SMX 0.9965, 7-OH MTX 0.9956. The minimum detectable

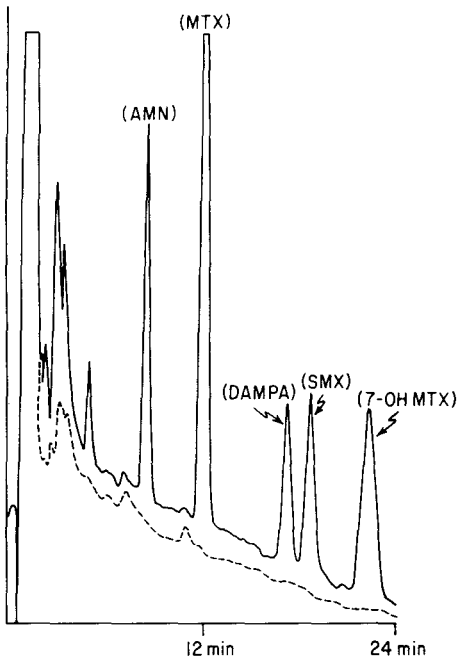


Fig. 2. Chromatogram of blank serum (– – –) and serum spiked with known standards (——). Abbreviations as given in Fig. 1, recorded attenuation 32 mV full scale.

TABLE I

RETENTION VOLUMES AND CAPACITY FACTORS FOR THE COMPOUNDS INVESTIGATED

Compound	Retention volume (ml)	Capacity factor (k')
5-MeTHF	11.3	3.5
AMN	17.0	5.1
Leucovorin	19.3	6.7
MTX	23.3	7.3
Folic acid	24.9	9.0
DAMPA	30.6	9.9
SMX	35.0	11.5
7-OH MTX	43.3	14.5

quantities with this assay were calculated as: MTX 4.4 ng ($9.8 \cdot 10^{-12}$ mole), DAMPA 11.9 ng ($3.7 \cdot 10^{-11}$ mole), 7-OH MTX 30 ng ($6.5 \cdot 10^{-11}$ mole), SMX 125 ng ($4.9 \cdot 10^{-10}$ mole) and found to be in agreement with our experimental values. The technique can thus easily detect and measure quantities of these molecules in the nanogram range. The ultimate usefulness of the method is in detecting the presence of MTX and metabolites in patient body fluids and conducting pharmacokinetic studies of MTX and its metabolites. The minimum detectable concentration of samples prepared as described in the experimental procedure proved to be: MTX $4.8 \cdot 10^{-8}$ *M*, DAMPA $1.8 \cdot 10^{-7}$ *M*, 7-OH MTX $3.2 \cdot 10^{-7}$ *M* and SMX $2.5 \cdot 10^{-6}$ *M*. Obviously, with an added technique of sample

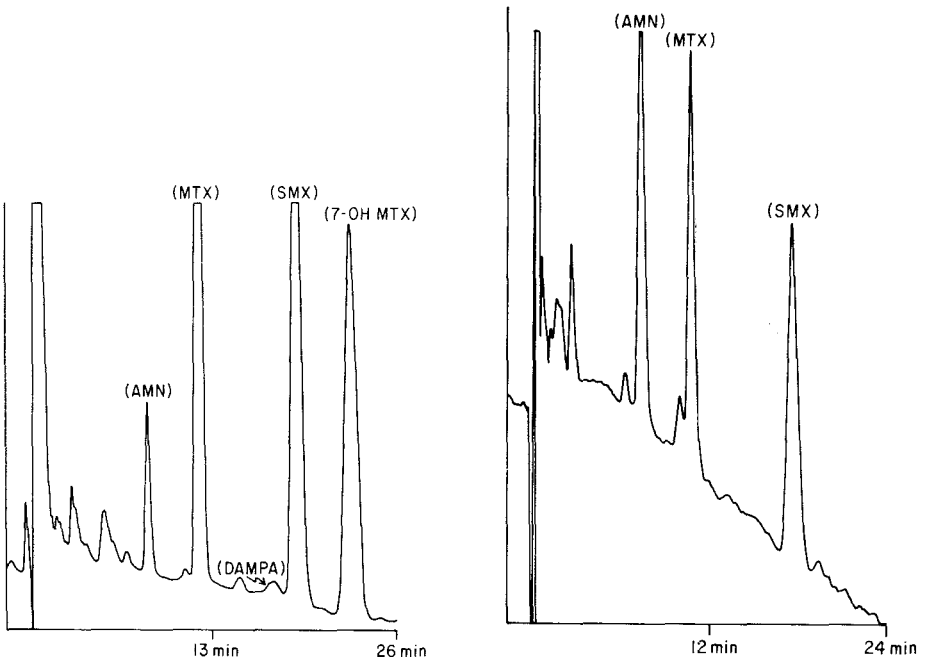


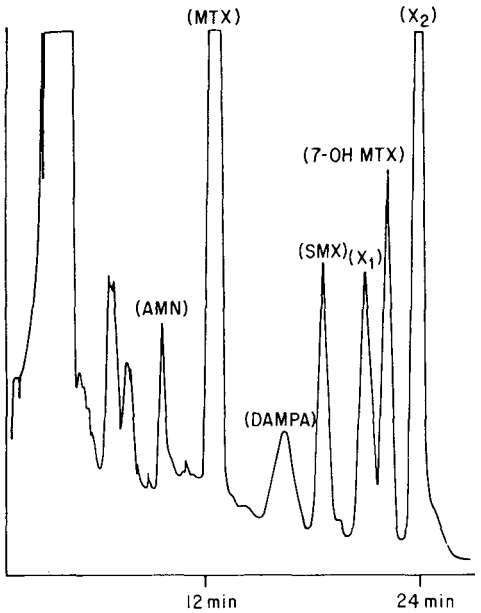
Fig. 3. Chromatogram of a patient serum sample obtained 25 h after starting a 24-h i.v. infusion of MTX (200 mg/m^2 i.v. bolus, 800 mg/m^2 infused over 24 h). SMX, 20 mg/kg had been given orally 6 h prior to collection of this serum sample. Abbreviations as given in Fig. 1, recorded attenuation 64 mV full scale.

Fig. 4. Chromatogram of a patient CSF sample obtained 24 h after starting a 24-h i.v. MTX infusion (see Fig. 3 for dosage). An intrathecal dose of MTX (12 mg/m^2) had been given at the start of the MTX infusion and a dose of SMX (20 mg/kg) had been given orally 6 h prior to collection of this CSF sample. Abbreviations as given in Fig. 1, recorded attenuation 16 mV full scale.

preconcentration lower concentrations can be quantitated. For our pharmacokinetic studies, the minimum detectable concentrations achieved were sufficient.

As a measure of system precision, 16 replicate injections ($5 \cdot 10^{-6}$ *M* MTX, $0.4 \cdot 10^{-6}$ *M* DAMPA, $0.7 \cdot 10^{-6}$ *M* 7-OH MTX) were performed under assumed identical conditions. The coefficients of variation (C.V.) for MTX, DAMPA and 7-OH MTX concentrations were 9.7%, 12.7% and 13.7%, respectively.

Sample preparation was fast, simple and reproducible. Total analysis time including gradient return and column re-equilibration was 40 min. Representative chromatograms of patient serum, CSF, and urine are shown in Figs. 3, 4 and 5, respectively. Note from Fig. 4 there were no apparent metabolites of MTX in CSF, and SMX readily crossed the blood—CSF barrier. In these patients, MTX was administered as an initial 200 mg/m^2 i.v. bolus followed by an 800 mg/m^2 i.v. infusion over 24 h. The intrathecal MTX dosage was 12 mg/m^2. SMX, 20 mg/kg body weight, was given orally every 12 h. In Fig. 5, peaks X_1 and X_2 are presently unidentified components which were not seen in all urine samples and do not appear to be due to other drugs being administered to these patients.



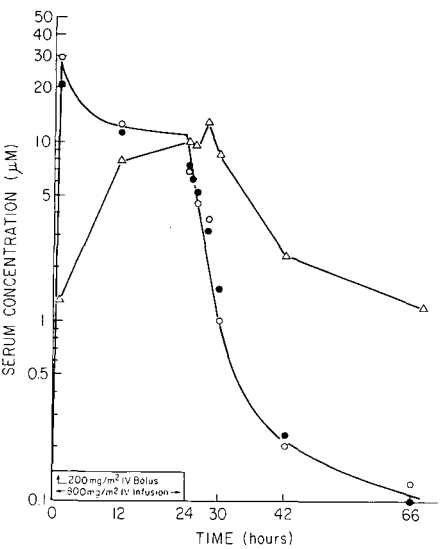


Fig. 5. Chromatogram of a patient urine sample collected 0—48 h after starting a 24-h i.v. MTX infusion (see Fig. 3 for dosage). SMX, 20 mg/kg was also given orally every 12 h during this interval. Abbreviations as given in Fig. 1, recorder attenuation 64 mV full scale.

Fig. 6. Serum concentration—time profiles for MTX (○, HPLC; •, EMIT) and 7-OH MTX (△, HPLC) from a representative patient. DAMPA was not detected in serum.

Serum concentration—time profiles for MTX and 7-OH MTX, obtained from a representative patient, are shown graphically in Fig. 6. DAMPA was generally not detected in serum or CSF, and only in low concentrations in urine. It is possible that the antibacterial effects of SMX may have affected the intestinal bacteria responsible for metabolizing MTX to DAMPA.

CONCLUSION

For the analysis of body fluids, a sensitive HPLC method should be fast, selective and have a wide linear dynamic range. The procedure described represents such a method for quantitating MTX, DAMPA, 7-OH MTX, and SMX. The technique is simple, easily automatable and inexpensive to operate. Measurement of parent drugs and metabolites at times 66 h post infusion can be achieved.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Paul Hutson, Dr. Clinton Stewart, Ms. Cindy Riley and Mr. Delbert Williams for their assistance with this work.

This work was supported in part by Leukemia Program Project Grant CA20180, Solid Tumor Program Project Grant CA23099, Cancer Center (CORE) Grant CA21765, and ALSAC.

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Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1291

DETERMINATION OF TRIPAMIDE AND ITS METABOLITES IN PLASMA, RED BLOOD CELLS AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received November 10th, 1981; revised manuscript received March 10th, 1982)

SUMMARY

A procedure for the determination of tripamide and its hydroxylated metabolites in plasma, red blood cells and urine by reversed-phase high-performance liquid chromatography is described.

The concentrations in red blood cells showed a monophasic decline and the half-life was 9.5 h. The concentration in red blood cells was markedly higher than that in plasma, showing that 95—98% of the drug is present in whole blood, after a dose of tripamide (90 mg) in man. The specificity and sensitivity of this procedure appear to be satisfactory for pharmacokinetic studies.

INTRODUCTION

Tripamide, N-(4-aza-*endo*-tricyclo[5.2.1.$0^{2,6}$]-decan-4-yl)-4-chloro-3-sulfamoylbenzamide, is a newly developed antihypertensive drug [1, 2] for which the clinical effect has also been reported [3—5]. Metabolic studies after administration of [^{14}C]tripamide to rats showed that the compound is extensively incorporated into red blood cells, resulting in low plasma concentration. In vitro experiments also show that the compound is rapidly incorporated into red blood cells of humans. Therefore, the present method is proposed for the measurement of the concentration of tripamide in plasma and red blood cells.

The drug is also rapidly and extensively metabolized by amide hydrolysis and ring hydroxylation. Although the major metabolic pathway is the hydrolysis of the amide of tripamide in rats [6], hydroxylation on the 3 or 8 position of the tricyclodecane ring seems to be an important pathway on the basis of the possible pharmacological significance of the hydroxylated compound.

It has been already reported that sulfonamide-like compounds, such as

hydrochlorothiazide [7], chlorothiazide [8], polythiazide [9], mefruside [10] and furosemide [11], can be analyzed by high-performance liquid chromatography (HPLC). Therefore, this method for the analysis of tripamide and its metabolites in biological fluids was developed. The method which was sensitive enough for the determination of tripamide in kinetic studies was established and preliminary pharmacokinetics of tripamide in healthy volunteers are presented in this paper.

MATERIALS AND METHODS

Reagents

The chemical structures of tripamide, the hydroxylated metabolites and internal standard for the determination of unchanged drug in plasma, red blood cells and urine are shown in Fig. 1. These were prepared in our laboratory as previously reported [12] and two hydroxylated metabolites (3-hydroxy- and 8-hydroxytripamide) were biosynthesized as previously described [6]. Acetic acid (Kanto Chemical Co., Tokyo, Japan) and methanol (Wako Pure Chemical Industry Co., Osaka, Japan) were used.

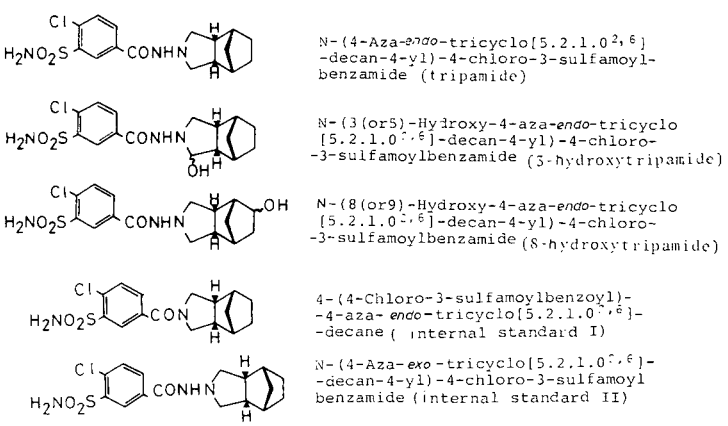

Fig. 1. The chemical structures of tripamide, hydroxylated metabolites, and of internal standards I and II used in the analysis of the blood samples and urine samples, respectively.

Apparatus, column and solvent

An Altex Model 100A high-performance liquid chromatograph, equipped with a variable-wavelength spectrophotometric detector (Jasco Uvidec Model 100-II, Japan Spectroscopic Co.) was used. The chromatographic column, solvent and temperature are shown in Table I.

Human subjects

To three healthy male volunteers (aged 36, 42, and 47 years, weight 62.0, 62.5, and 65.7 kg, respectively), tripamide was administered orally 1 h after a meal at a dose of 90 mg as Normonal tablets (Eisai Co., Tokyo, Japan). Blood samples of 7 ml were collected at scheduled time intervals in heparinized tubes and centrifuged at 1000 *g* for 5 min; plasma was immediately separated from the red blood cells. Remainders (red blood cells) were analyzed as the

TABLE I

CONDITIONS OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

	Compound analysed	
	Tripamide	Hydroxylated tripamide
Column*	Hitachi gel 3011 (1) [Styrene divinylbenzene (SVB)]	Nucleosil C_{18} (2)
	Reversed-phase	Reversed-phase
Particle size	10 μm	10 μm
Dimensions	500 × 4.6 mm	250 × 4.6 mm
Mobile phase	1% aqueous acetic acid—methanol (10:90, v/v)	water—methanol (60:40, v/v)
Flow-rate	0.7 ml/min	0.7 ml/min
Temperature	32°C	40°C
Injector	Rheodyne Model 7120 (injection loop of 100 μl)	
Detection	254 nm	
Pre-column*	Permaphase ODS (50 × 4.6 mm) (3)	

*The packing materials 1, 2 and 3 were purchased from Hitachi Co. (Tokyo, Japan), Macherey-Nagel Co. (Düren, G.F.R.) and DuPont (Wilmington, DE, U.S.A.), respectively.

concentration of tripamide in the red blood cells. Urine samples were collected at the scheduled time intervals.

Extraction from plasma

To 3 ml of plasma placed in a screw-capped tube (50 ml, pyrex, Iwaki Co.), 50 μl of a methanol solution containing internal standard I (625 ng) and 3 ml of distilled water were added. The extraction was made twice using 20 ml of diethyl ether each time by shaking for 15 min and centrifuging for 5 min at 1000 *g*. The combined ether layers were condensed to 5 ml in a screw-capped tube (20 ml) at 45°C in a stream of nitrogen. Two milliliters of 0.05 *M* borate buffer (pH 9) were added and shaken gently for 15 min. After centrifuging for 5 min at 1000 *g*, the ether layer was taken and evaporated to dryness at 45°C in a stream of nitrogen. The residue was dissolved in 0.25 ml of methanol by sonication at 80°C with a Bransonic 12 (Yamato Kagaku). After centrifuging for 5 min at 1000 *g*, a 50-μl aliquot of the solution was injected into the HPLC column.

Extraction from red blood cells

To red blood cells obtained from 7 ml of blood, 0.8 ml (20 μg) or 0.2 ml (5 μg) of methanol solution of internal standard I was added, then 50 ml of distilled water were added for hemolysis. The hemolyzed red blood cell solution was placed in separating funnel (volume 300 ml) and extracted with 100 ml of diethyl ether by shaking by hand vigorously and then with a mechanical shaker gently for 20 min. After standing for 30 min, the ether layer was separated and evaporated in vacuo using a rotary evaporator. The residue was dissolved in 5 ml of methanol and transferred to test tubes. The methanol was evaporated at 45°C in a stream of nitrogen. The residue was dissolved in 0.4 ml

of methanol and a 40-μl aliquot of the solution was injected into the high-performance liquid chromatograph.

Extraction from urine

Ten milliliters of urine placed in a screw-capped tube (50 ml) were adjusted to pH 7 with 1 *N* sodium hydroxide solution, then 20 μl of methanol solution containing 3.3 μg of internal standard II were added. Extraction was made twice using 20 ml of diethyl ether each time by shaking for 15 min and centrifuging for 5 min at 1000 *g*. The combined layers were condensed to 10 ml in a screw-capped tube (20 ml) at 45°C in a stream of nitrogen. Then, 0.5 ml of 1 *N* sodium hydroxide solution was added to the residual solution and shaken for 5 min. After centrifuging for 5 min at 1000 *g*, the ether layer was discarded. To the aqueous solution, 0.2 ml of 6 *N* hydrochloric acid solution and 5 ml of diethyl ether were added and then shaken for 5 min. After centrifuging for 5 min at 1000 *g*, the ether layer was discarded and the water layer was adjusted to pH 7 with 1 *N* sodium hydroxide solution and 1 ml of 0.05 *M* phosphate buffer (pH 7) was added. To the solution 10 ml of diethyl ether were added and shaken for 15 min. After centrifuging for 5 min at 1000 *g*, the ether layer was transferred to a test tube and evaporated at 45°C in a stream of nitrogen. The residues were dissolved in 0.4 ml of methanol and a 40-μl aliquot of the solution was injected onto the column.

Recovery

The addition of 200 ng of tripamide to plasma and red blood cells resulted in overall recoveries of 90% for plasma and 97% for red blood cells. The recovery of tripamide from urine was 80% at a concentration of 2.5 μg/ml and that of 3-hydroxy- and 8-hydroxytripamide was 37.2 ± 0.6% at 0.19 μg/ml and 37.3 ± 2.3% at 0.17 μg/ml, respectively.

RESULTS AND DISCUSSION

Determination of tripamide in plasma and red blood cells

A chromatogram of plasma extract is shown in Fig. 2A. Two peaks, originating from endogenous compounds, are observed at retention times of 18.1 and 19.3 min. Fig. 2B is a chromatogram of extract to which 100 ng of tripamide (retention time 17.2 min) and 125 ng of the internal standard I (22.2 min) were added. They are sufficiently separated from both tripamide and internal standard I and their peaks did not interfere with the peaks of either tripamide or internal standard I.

A standard curve was prepared by adding known amounts of drug to blank plasma samples and determining the peak height ratios (tripamide/internal standard). The tripamide standard curve was linear in the range 16.7—166.7 ng/ml plasma, and extrapolated through the origin.

The sensitivity limit was 16.7 ng/ml.

A similar result was obtained when known concentrations of drug were added to red blood cells. A typical chromatogram of extracted red blood cells is shown in Fig. 3. To facilitate analysis of red blood cells of high concentra-

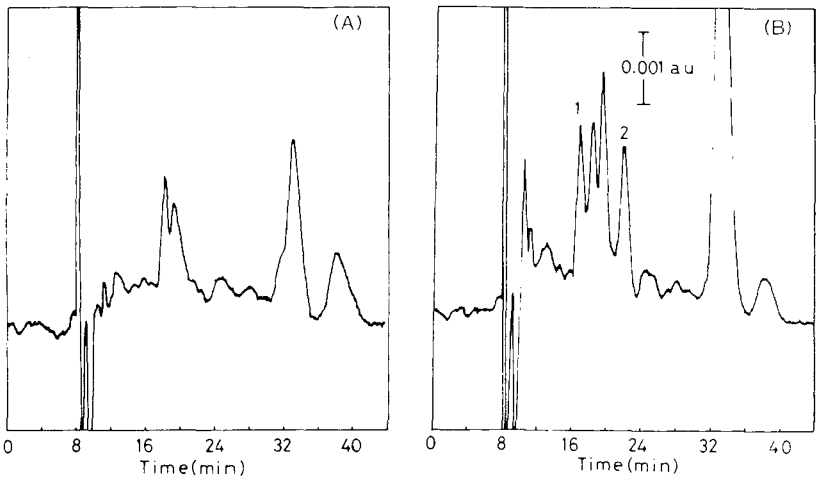

Fig. 2. Chromatograms of (A) plasma extract and (B) plasma extract containing 100 ng of tripamide (1) and 125 ng of internal standard I (2). Three peaks eluting at 18.1, 19.3 and 33.0 min were endogenous plasma constituents. HPLC conditions as in Table I. The detector range for both chromatograms was 0.001 a.u.

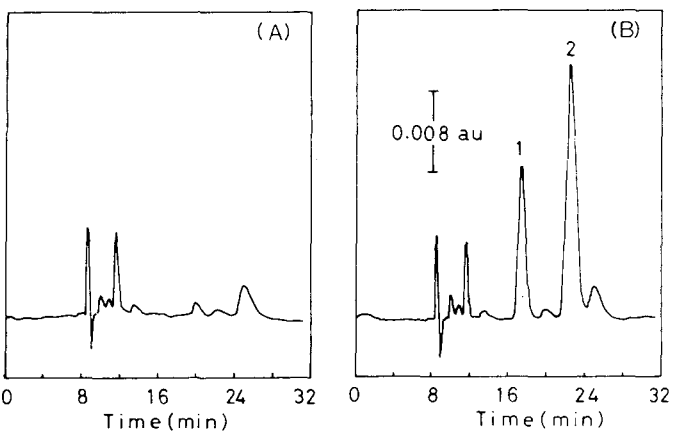

Fig. 3. Chromatograms of (A) red blood cell extract and (B) red blood cell extract containing 1 μg of internal standard and 400 ng of tripamide. HPLC conditions as in Table I. The detector range for both chromatograms was 0.008 a.u.

tion, a high-range standard curve was also prepared by adding known high concentration of the drug and 5 μg of internal standard I to red blood cell samples.

Determination of tripamide and its metabolites in urine

Fig. 4B is a chromatogram of an extracted urine sample to which 200 ng of tripamide and 330 ng of the internal standard II were added. As shown in the blank sample (Fig. 4A), no interfering peak originating from an endogenous compound was found. The calibration curve was obtained by adding known amounts of tripamide to urine samples. The ratio of the peak height of tripamide to that of the internal standard was linear in the range 50—800 ng/ml.

Fig. 5B shows a chromatogram of a urine sample obtained from a volun-

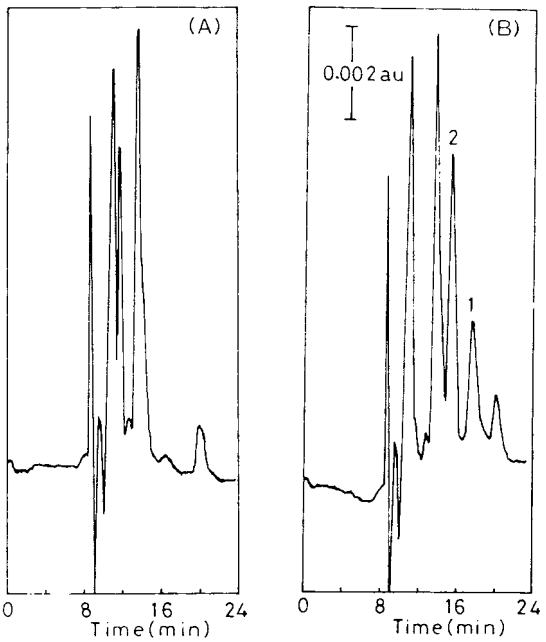

Fig. 4. Chromatograms of (A) blank urine and (B) urine extract containing 200 ng of tripamide (1) and 330 ng of internal standard II (2). Solvent: 1% aqueous acetic acid—methanol (10:90, v/v). Solvent flow-rate: 0.7 ml/min. Column: Hitachi gel 3011, 10-μm particle size, 500 × 4.6 mm. Column temperature: 32°C.

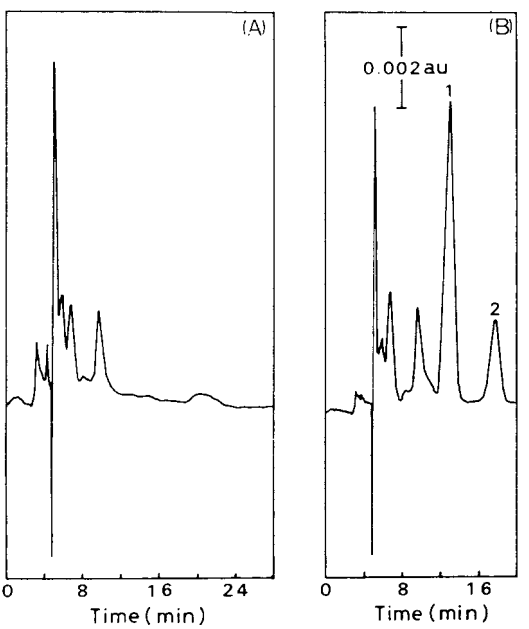

Fig. 5. Chromatograms of (A) blank urine and (B) the hydroxylated metabolites as a single medication in a human urine sample. 1 and 2 on the chromatogram show 8-hydroxy- and 3-hydroxytripamide, respectively. Solvent: water—methanol (60:40, v/v). Solvent flow-rate: 0.7 ml/min. Column: Nucleosil C_{18}, 10-μm particle size, 250 × 4.6 mm. Column temperature: 40°C.

teer after oral administration of tripamide at the dose of 90 mg. As shown in the blank urine sample (Fig. 5A), no interfering peaks were found. Retention times were 12.4 min for 8-hydroxytripamide and 16.8 min for 3-hydroxytripamide.

The calibration curves of 3-hydroxy- and 8-hydroxytripamide were obtained as follows. [^{14}C]Tripamide was incubated with rat liver microsomes under NADPH and O_2, and the two hydroxylated metabolites formed were purified by thin-layer chromatography [6]. Known amounts of 3-hydroxy- and 8-hydroxytripamide, which were calculated on the basis of specific radioactivity of [^{14}C] tripamide, were injected onto the column. The peak area of the compounds was linear in the range from 0.17—1.09 μg per injection for 8-hydroxytripamide and 0.25—1.28 μg for 3-hydroxytripamide.

Preliminary pharmacokinetic study

The analytical method described above has sufficient sensitivity for pharmacokinetic studies in human subjects administered orally 90 mg of tripamide. Table II shows the levels of tripamide in plasma and red blood cells in three volunteers. The plasma concentration reached a maximum of 117.6 ± 17.3 ng/ml at 4 h after administration and could not be detected at 24 h. The red blood cell concentration also attained a peak of 4.98 ± 0.33 μg/ml 4 h after administration.

The time—course of the red blood cell concentration showed a monophasic decline and the half-life was 9.5 h. The concentration of tripamide in red blood cells was markedly higher than that in plasma, showing that 95—98% of tripamide in total blood is present mainly in red blood cells.

Table III shows the renal excretion rate of tripamide in volunteers after oral administration. The unchanged drug excreted in the urine was 0.8 ± 0.1% of the dose. The rate of renal excretion with monophasic phase ($t_{½}$ = 8.9 h) was similar to that of disappearance from red blood cells ($t_{½}$ = 9.5 h). Table IV shows the renal excretion of 3-hydroxy- and 8-hydroxytripamide follow-

TABLE II

CONCENTRATIONS OF TRIPAMIDE IN PLASMA AND RED BLOOD CELLS

Each value is the mean ± S.E. (n = 3). n.d. = not detectable.

Time (h)	Concentration	
	Plasma (ng/ml)	Red blood cells (μg/ml)
1	22.2 ± 2.2	1.94 ± 0.70
2	101.2 ± 17.2	4.18 ± 0.76
3	103.1 ± 6.0	4.81 ± 0.41
4	117.6 ± 17.3	4.98 ± 0.33
6	49.0 ± 16.5	3.90 ± 0.35
8	51.9 ± 20.5	3.35 ± 0.47
24	n.d.	1.09 ± 0.26
48	n.d.	0.21 ± 0.08
72	n.d.	n.d.

TABLE III

RENAL EXCRETION OF TRIPAMIDE IN HUMANS

Subject	Excretion			
	μg in 72 h	%	K (h^{-1})	$t_{½}$ (h)
T. I.	749	0.8	0.063	11.0
A. K.	557	0.6	0.092	7.5
T. K.	813	0.9	0.086	8.1
Mean ± S.E.		0.8 ± 0.1		8.9 ± 1.1

TABLE IV

RENAL EXCRETION OF THE HYDROXYLATED METABOLITES IN HUMANS

Subject	Metabolite	Excretion			
		mg in 72 h	%	Total (%)	3-Hydroxy / 8-Hydroxy
T. I.	3-Hydroxytripamide	1.95	2.2	8.1	0.37
	8-Hydroxytripamide	5.29	5.9		
A. K.	3-Hydroxytripamide	6.21	6.9	11.0	1.68
	8-Hydroxytripamide	3.70	4.1		
T. K.	3-Hydroxytripamide	5.62	6.2	11.8	1.11
	8-Hydroxytripamide	5.05	5.6		

ing administration of tripamide at the dose of 90 mg. The excretion of total hydroxylated metabolites ranged from 8.1 to 11.8%, about ten times that of the unchanged drug. Preliminary pharmacokinetic results in humans as shown in this paper indicate that tripamide is metabolized extensively and excreted as hydroxylated metabolites in urine. None of the hydroxylated metabolites could be detected in the blood. The hydroxylated metabolites do not appear in the blood, since the metabolites formed in the liver are mainly excreted into the bile [6] and the hydroxylated metabolites excreted in the urine seem to be hydroxylated by renal microsomes.

The finding of individual variations in the excretion of 3-hydroxy- and 8-hydroxytripamide may be attributed to inherent variations in its metabolism in the volunteers.

In conclusion, the specificity and sensitivity of this procedure appear to be satisfactory for pharmacokinetic studies on tripamide.

ACKNOWLEDGEMENTS

The authors are deeply indebted to Dr. T. Fujita, Director of the Section of Drug Metabolism and Biopharmaceutics, for valuable discussions.

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Journal of Chromatography, 231 (1982) 121—127
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1299

METHOD FOR THE SIMPLIFIED ANALYSIS OF DEPROTEINIZED PLASMA AND URINARY ISOPROTERENOL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received December 7th, 1981; revised manuscript received March 16th, 1982)

SUMMARY

A simple method for the determination of isoproterenol in urine and plasma by high-performance liquid chromatography coupled with an automated trihydroxyindole method is described. No pre-purification procedures are required. The sensitivity was 0.2 pmol and the average recoveries of isoproterenol added to plasma and urine were 89% and 101%, respectively. The method has been applied successfully to clinical samples.

INTRODUCTION

Isoproterenol (ISP) has been used as a bronchodilator for the treatment of asthma and as an inotropic agent for certain cardiovascular disorders. Although improved β2-stimulators have begun to replace ISP as an anti-asthmatic drug, the inotropic action of ISP is still important for the treatment of low-

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output syndrome in children. However, its β-action causes changes in fatty metabolism, hormonal secretion and arrhythmia [1, 2]. There has been a report of sudden death of asthmatic patients who had been treated with ISP [3].

To find out the relationship between the plasma concentration of ISP and changes in fatty metabolism, hormonal secretion or arrhythmia, it is desirable to examine the blood level of ISP. However, no simple and reliable method for the determination of ISP for clinical application has yet been established.

For the determination of ISP in biogenic materials and for checking the pharmaceutical quality of ISP, several methods, such as fluorimetry [4], gas chromatography [5] and radioisotope methods [6], have been used. However, these methods require complicated procedures for the extraction of ISP from biological specimens. Because of this disadvantage, these methods are unsatisfactory with respect to speed, accuracy and reproducibility.

The purpose of this study was to establish a method for the direct determination of ISP in deproteinized plasma and urine by high-performance liquid chromatography (HPLC) coupled with the trihydroxyindole (THI) method [7, 8].

EXPERIMENTAL

Apparatus

An LC-2 high-performance liquid chromatograph, an RF500LCA spectrofluorimeter, an R-12 recorder from Shimazu Seisakusho (Kyoto, Japan), a KMT-24 autosampler from Kyowa Seimitsu (Osaka, Japan) and a Technicon proportionating pump were used.

Strong cation-exchange columns (Zipax-SCX, particle size 30 μm, 150 cm × 2.1 mm I.D. for the main column, 15 cm × 2.1 mm I.D. for the guard column) from Shimazu Seisakusho were used.

Reagents

ISP was purchased from Sigma (St. Louis, MO, U.S.A.). O-Methylisoproterenol was supplied by Nikken Chemical Industry (Tokyo, Japan). Sodium phosphate (monobasic), hydrochloric acid, ethylenediaminetetraacetic acid (EDTA), potassium hexacyanoferrate(III), perchloric acid, ascorbic acid, sodium bisulphite and sodium hydroxide were of analytical-reagent grade from Wako (Osaka, Japan).

Reagent preparation

All aqueous reagent solutions were prepared with water distilled twice in glass. A stock standard solution of ISP (4.7 m*M* ISP) was prepared by dissolving 1 g of ISP in 1 l of 0.1 *M* hydrochloric acid containing 10 m*M* sodium bisulphite. A working standard solution was prepared by 100-fold dilution of the stock standard solution with 0.1 *M* hydrochloric acid containing 10 m*M* sodium bisulphite. Further 100-fold and 1000-fold dilutions of the working standard solution were made daily with 0.1 *M* hydrochloric acid containing 5 m*M* ascorbic acid as a calibration standard for urinary and

plasma ISP, respectively. As reagents for oxidizing, reducing and intensifying the fluorescence in the THI method, 4 m*M* potassium hexacyanoferrate(III) dissolved in 0.5 *M* phosphate buffer (pH 6.8), 6 m*M* ascorbic acid and 8 *M* sodium hydroxide solution were used. As the mobile phase in HPLC, 0.25 *M* monobasic sodium phosphate solution (pH 4.3) was used.

Sample preparation

Plasma. A 5-ml volume of healthy human blood was collected and immediately transferred into a plastic tube containing 5 mg of solid EDTA, disodium salt. The blood was mixed by gentle inversion of the tube, and then centrifuged at 3000 *g* for 10 min at 4°C. The plasma (2.0 ml) was mixed with 200 μl of 4 *M* perchloric acid and vortex-mixed vigorously for 1 min, then centrifuged at 6000 *g* for 20 min at 4°C. To the deproteinized plasma, 20 μl of the working standard solution of various concentrations were added. The final concentration of ISP in the plasma ranged from 1 to 240 pmol/ml.

Urine. To 10 ml of freshly voided healthy human urine, 100 μl of concentrated hydrochloric acid and then 10 μl of the working standard solution of various concentrations were added. The final concentration of ISP ranged from 20 pmol/ml to 4.8 nmol/ml.

For the assay of plasma and urinary ISP in patients who had been administered ISP after cardiac surgery, samples were treated as mentioned above.

Analytical procedure

A flow diagram of the assay system is shown in Fig. 1. The sample solution was injected automatically into the column at intervals of 30 min (injection volume: 200 μl of deproteinized plasma or 10 μl of urine). The column was eluted at a flow-rate of 1.0 ml/min and the column temperature was

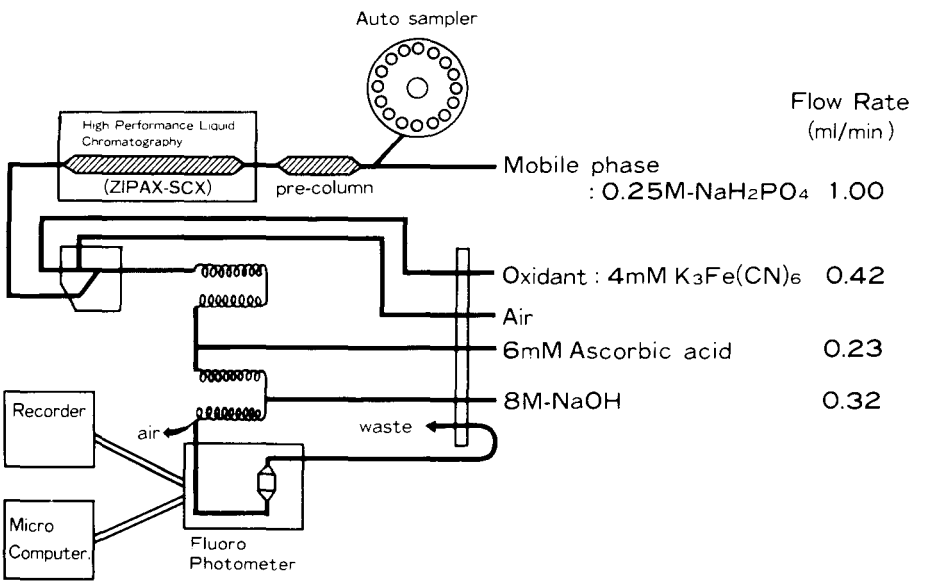

Fig. 1. Analytical flow diagram.

maintained at 40°C. ISP in the column eluate was converted into trihydroxyindole derivatives automatically and the fluorescence intensity was measured with a spectrofluorimeter (excitation wavelength 400 nm, fluorescence wavelength 510 nm) equipped with a recorder. The fluorescence intensity was calculated from the peak height.

RESULTS

Typical chromatograms of the standard solution, a plasma sample and a urine sample are shown in Fig. 2. The retention time of ISP was 24 min. ISP was well separated from interfering substances in urine and deproteinized plasma. The peak of metanephrine appeared at 18 min in the urine sample. The peak of noradrenaline (retention time 10 min) and adrenaline (12 min) were overlapped by unknown interfering substances.

Concerning the metabolites of ISP, O-methylisoproterenol (main intermediate metabolite of ISP) appeared in the chromatogram at 69 min only when a large dose was applied and did not affect the ISP peak. In addition, no substances interfering with the ISP peak were found on application of either urine or deproteinized plasma from patients who had been treated with ISP. Further, we investigated possible interferences in the assay system due to drugs used in intensive care units. These drugs included other catecholamines, α,β-blockers, antibacterial drugs, several vitamins, narcotics and sedatives. None of them interfered with the ISP peak.

Fig. 3 shows the relationship between the injection volume with and without pH adjustment and relative fluorescence intensity (the pH was adjusted to 4.5 with 2 *M* potassium hydroxide solution). A linear correlation was

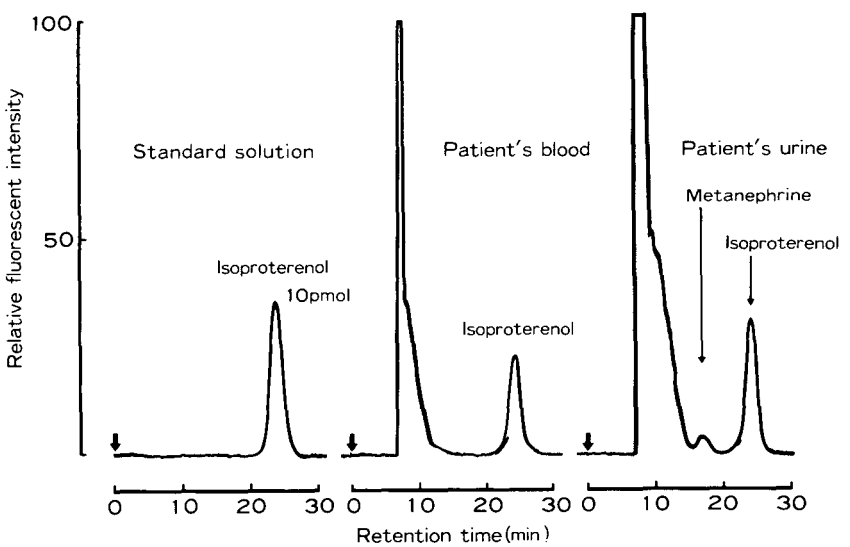

Fig. 2. Chromatography of ISP. Deproteinized plasma and urine samples were taken from a patient who had been administered isoproterenol (734 pmol/kg/min, 20 h) after cardiac surgery. Sample size: 200 μl of deproteinized plasma, 10 μl of urine and 200 μl of standard solution, respectively.

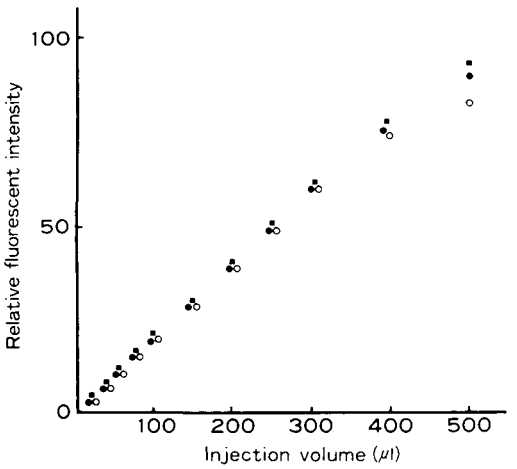

Fig. 3. Correlation between injection volume and fluorescence intensity. Deproteinized plasma containing isoproterenol (50 pmol/ml) with and without pH adjustment was subjected to HPLC. Injection volume ranged from 25 to 500 μl (pH adjustment of deproteinized plasma was effected with 2 *M* potassium hydroxide solution. •, Standard (pH 1.0); ○, deproteinized plasma (pH 0.5); ▪, deproteinized plasma (pH 4.5, adjusted).

observed from 10 to 300 μl without pH adjustment, and up to larger injection volumes with pH adjustment.

There was a linear relationship between relative fluorescence intensity and amount of ISP added from 0.2 to 48 pmol both for urine (10-μl injection) and deproteinized plasma (200-μl injection, without pH adjustment).

An amount of 0.4 pmol of ISP added to deproteinized plasma was detected at a signal-to-noise ratio of 4. Thus, the minimal sensitivity for determination of plasma ISP was 0.2 pmol.

Intra-assay variation was examined by the analysis of five samples of three sorts of doses both in urine and deproteinized plasma. The mean coefficient of variation (C.V.) was 3.2% for deproteinized plasma ISP and 1.2% for urinary ISP (Table I).

TABLE I

INTRA ASSAY AND INTER ASSAY OF DEPROTEINIZED PLASMA AND URINARY ISOPROTERENOL

Assay	Parameter	Deproteinized plasma isoproterenol			Urinary isoproterenol		
Intra-assay	$\bar{X}$ (pmol)	9.4	4.8	1.9	19.5	9.8	4.8
	n	5	5	5	5	5	5
	S.D. (pmol)	± 0.2	± 0.1	±0.1	± 0.1	± 0.1	± 0.1
	C.V. (%)	± 2.1	± 2.1	±5.3	± 0.5	± 1.0	± 2.1
Inter-assay	$\bar{X}$ (pmol)	9.4	1.8		19.3	4.8	
	n	10	10		10	10	
	S.D. (pmol)	± 0.4	± 0.1		± 0.5	± 0.1	
	C.V. (%)	± 4.3	± 5.6		± 2.6	± 2.1	

TABLE II

RECOVERY OF ISOPROTERENOL FROM PLASMA AND URINE

Values of recoveries obtained by adding standard compound to plasma and urine samples.

No.	Plasma isoproterenol			Urinary isoproterenol		
	Added (pmol)	Measured* (pmol)	Recovery (%)	Added (pmol)	Measured* (pmol)	Recovery (%)
1	37.9	34.3	91	37.9	38.9	103
2	18.9	16.6	88	18.9	19.0	101
3	9.5	8.3	87	9.5	9.5	100
4	4.7	4.2	89	4.7	4.8	102
5	2.4	2.1	88	2.4	2.4	100

*Average of duplicate assay.

Inter-assay variation was measured by using samples stored in a refrigerator for 10 days at 4°C. The mean C.V. was 5.0% for deproteinized plasma ISP and 2.4% for urinary ISP (Table I).

When several doses of ISP (2.4—37.9 pmol) were added to both plasma and urine, the recovery of ISP from plasma ranged from 87 to 91% and that from urine from 100 to 103% (Table II).

It was possible to measure the ISP concentration in blood and urine from patients (n=12) who had received ISP (23.7—734.0 pmol/kg/min) after cardiac surgery. The plasma concentration of ISP ranged from 1.3 to 28.7 pmol and that in urine from 41.2 pmol/ml to 1.10 nmol/ml.

DISCUSSION

The retention time of ISP is longer than that of other catecholamines and related substances when they are applied to Zipax-SCX [7]. In addition, we found by the present HPLC method coupled with the THI method that most fluorescent interfering substances in urine or deproteinized plasma appeared in the early phase of the chromatograms and the peak of ISP appeared clearly without any interfering peaks. The peak of O-methylisoproterenol appeared at a later phase of the chromatogram. Therefore, urinary or deproteinized plasma samples could be injected directly. In addition, we did not use an internal standard for calibration, but only used an external standard because of the good recovery and low coefficient of variation.

To measure low concentrations of ISP, a large injection volume was necessary as well as enhancement of fluorescence. As shown in Fig. 3, if the sample pH was adjusted to higher values, the sample volume could be increased. However, in this study, the separation of ISP was performed as simply as possible. Therefore, we curtailed the pH adjustment and 200 μl of deproteinized plasma without pH adjustment was chosen as the sample of blood to be applied. As the concentration of urinary ISP is high [9], 10 μl of urine was enough for the measurement.

Although the sensitivity of the method was adequate for our purpose,

a situation where it is necessary to measure lower concentrations of ISP might arise. To improve the sensitivity, several parameters can readily be altered, for example, increased sample volume and improvement of the detection method. The sample volume will be increased by pH adjustment and by an increase in the capacity factor of the column. The latter will be improved by increasing the theoretical plate number of Zipax-SCX. For an improved detection method, there are more sensitive THI methods [10] and a more sensitive fluorescence method for catecholamines [11]. The final question is how many times a column can be used even if direct injection of the sample is applied. We found we could analyse more than 200 samples with one column and exchanged the pre-column after every 200 samples.

The proposed method requires only deproteinization of plasma as the pre-treatment procedure and, because of its speed, accuracy and reproducibility, the method is suitable for clinical application.

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Journal of Chromatography, 231 (1982) 129—136
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1306

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PRIFINIUM QUATERNARY AMMONIUM ION IN HUMAN SERUM AND URINE

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(Received March 10th, 1982)

SUMMARY

A simple, sensitive method for the determination of the prifinium ion, a quaternary ammonium ion, in human serum and urine is described. The method is based on extraction of the test solution with chloroform in the presence of saturated potassium bromide solution and normal-phase high-performance liquid chromatography using aqueous methanol as the mobile phase at pH 10. To prevent the dissolution of silica from the analytical column, the mobile phase is pre-saturated with silica by using a silica saturation column. Quantitation is possible down to 0.5 ng/ml of prifinium ion using 2 ml of serum and down to 5 ng/ml using a 1 ml of urine. The coefficients of variation of the method are less than 1.3% in both serum and urine. Serum levels and urinary excretion data obtained with this method are given for three healthy volunteers who had received a 60-mg oral dose of prifinium bromide.

INTRODUCTION

Prifinium bromide is a quaternary ammonium compound possessing anticholinergic properties [1], which has been used for some years in the treatment of gastro-enteritis, gastro-duodenal ulcer, irritable colon syndrome, etc. Although the pharmacokinetics of ^{14}C-labelled prifinium bromide in experimental animals have been reported [2], little is known about its pharmacokinetics in man. Generally, anticholinergic ammonium compounds are not well absorbed when given by the oral route [3—9]. Consequently, it was necessary to develop a sensitive assay for measuring prifinium ion in serum and urine after oral administration of the usual clinical dose in man.

Gas—liquid chromatography (GLC) and gas chromatography—mass spectrometry (GC—MS) are commonly used to determine quaternary ammonium ions in biological fluids [10—14]. Because these methods usually include a dealkyla-

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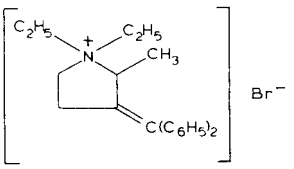

Prifinium bromide

tion or oxidation procedure to produce volatile derivatives, they are time consuming and tedious. Recently, the use of high-performance liquid chromatography (HPLC) for the quantitative determination of quaternary ammonium ions in biological fluds has been shown to have advantages over GLC and GC—MS with respect to speed, simplicity and/or reliability [15, 16]. De Ruyter et al. [15] have reported the use of reversed-phase high-performance ion-pair liquid chromatography for the determination of pyridostigmine, neostigmine and edrophonium in the biological fluids. Assay of ORG NC45 (a myoneural blocking agent) in human plasma using normal-phase HPLC has been reported by Paanakker and Van de Laar [16].

This paper describes a highly sensitive and simple method for the determination of prifinium ion in human serum and urine. The method is based on extraction of the test solution with chloroform in the presence of saturated potassium bromide solution and normal-phase HPLC using aqueous methanol as the mobile phase at pH 10.

EXPERIMENTAL

Reagents and materials

Prifinium bromide (Riabal) was prepared by Fujisawa Pharmaceutical Co. (Osaka, Japan). Methanol and chloroform of UV grade were used. Purified deionized water used for all solutions and mobile phases was prepared with a Millipore Milli-Q water purification system. All other solvents and reagents were of analytical-reagent grade. Sodium hydroxide solution saturated with potassium bromide was prepared from the supernatant after vigorously mixing 100 ml of 0.05 *M* sodium hydroxide with about 70 g of potassium bromide. Blank human serum was obtained from fresh blood of healthy male volunteers. Healthy male volunteers provided blank human urine. Serum and urine samples were stored at −20° C until taken for analysis.

Preparation of standard solution

Diphemanyl methylsulphate was used as an internal standard for the assay. It was dissolved in water and diluted to 1, 2 and 30 μg/ml. Standard solutions of prifinium ion were prepared by dissolving prifinium bromide in water and diluting to appropriate concentrations.

Apparatus

Analysis in serum was made on Waters Assoc. liquid chromatograph equipped with a Model 440 absorbance detector (254 nm fixed wavelength), a Model 6000A pump, a U6K universal injector and a 10-mV recorder. Analysis in urine was made using a Waters Intelligent Sample Processor (WISP) and a Data Module in place of the U6K universal injector and the 10-mV recorder, respectively.

Chromatographic conditions

A pre-packed LiChrosorb Si 60 (particle size 5 μm) analytical column (Umetani Seiki, Osaka, Japan) of 25 cm × 4 mm I.D. and a home-packed LiChrosorb Si 60 (30 μm) guard column of 1 cm × 4 mm I.D. were used. A home-packed LiChrosorb Si 60 (30 μm) pre-column of 5 cm × 2 mm I.D. was placed between the pump and injector (Fig. 1) to prevent the dissolution of silica from the analytical and guard columns at high pH. The mobile phase of 10% 1 *M* ammonium acetate adjusted to pH 10 with 28% ammonia solution in methanol was prepared freshly each day of analysis. The mobile phase was deaerated under vacuum before use. The operating temperature was ambient and the flow-rate was 1.0 ml/min. Retention times with this system were about 8 min for prifinium ion and 11 min for the internal standard.

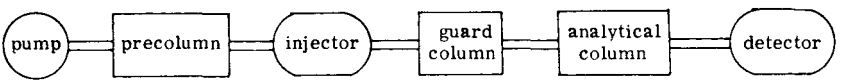

Fig. 1. Pre-column and guard column arrangement.

Extraction from biological fluids

To a glass-stoppered 10-ml centrifuge tube containing 2 ml of a serum sample were added 0.1 ml of water, 0.1 ml of internal standard solution (containing 0.1 μg of diphemanil methylsulphate), 3 ml of sodium hydroxide solution saturated with potassium bromide and 6 ml of chloroform. The mixture was shaken for 10 min and centrifuged at 1900 *g* for 10 min. The aqueous phase was carefully aspirated. A 4-ml volume of the chloroform phase was transferred into a glass-stoppered 10-ml centrifuge tube. The solvent was removed by evaporation in a stream of nitrogen. The residue was dissolved in 100 μl of the HPLC mobile phase and an 80-μl aliquot was injected on to the liquid chromatograph.

To a glass-stoppered 10-ml centrifuge tube containing 1 ml of urine sample were added 0.1 ml of water, 0.1 ml of the appropriate internal standard solution (containing 0.2 or 3 μg of diphemanil methylsulphate), 3 ml of sodium hydroxide solution saturated with potassium bromide and 5 ml of chloroform. The other extraction procedure was carried out in the same way as described for serum. An 8- or 40-μl aliquot of the extract dissolved in the HPLC mobile phase was injected on to the liquid chromatograph.

Quantitation

The procedure was standardized by analysing the blank serum or urine samples to which had been added 0.1 ml of prifinium standard solution instead of 0.1 ml of water as in the extraction procedure. Peak-height ratios and peak-area ratios of prifinium ion to internal standard were used to establish the calibration graph for serum and urine samples, respectively. The calibration graph was fitted to a $y = ax + b$ equation by the least-squares method. The concentrations in the unkown samples were subsequently calculated using the calibration graph.

Clinical study

A clinical study was performed in which three healthy volunteers received an

oral dose of 60 mg of prifinium bromide (Riabal tablet). Serum samples were obtained from blood collected by venipuncture at designated time intervals, and stored at −20° C until taken for analysis. The total urine output was collected at intervals of 0–2, 2–4, 4–6, 6–8 and 8–24 h. The urine volumes were measured, and the aliquots were kept at −20° C prior to analysis.

RESULTS

Separation

Typical chromatograms obtained from the human serum and urine samples are shown in Figs. 2 and 3, respectively. As shown in Figs. 2A and 3A, the background peaks of blank human serum and urine have short retention times and are almost completely separated from those of prifinium ion and internal standard. Figs. 2C and 3C show typical chromatograms of serum and urine samples from a healthy volunteer after oral administration of 60 mg of prifinium bromide. In these chromatograms there are no interferences at the retention times of prifinium ion and the internal standard.

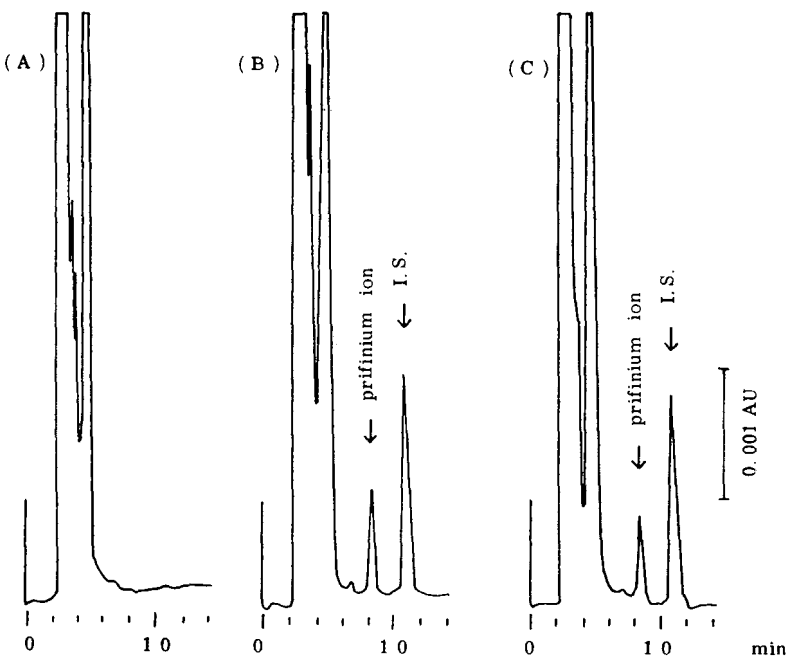

Fig. 2. Chromatograms of (A) blank human serum, (B) serum containing 10 ng/ml of prifinium ion and 50 ng/ml of internal standard and (C) serum from a healthy volunteer after oral administration of 60 mg of prifinium bromide. Conditions: column, 25 cm × 4 mm I.D. LiChrosorb Si 60 (5 μm); mobile phase, 10% 1 *M* ammonium acetate adjusted to pH 10 with ammonium solution in methanol; flow-rate, 1.0 ml/min; detection, UV at 254 nm; injection volume, 80 μl.

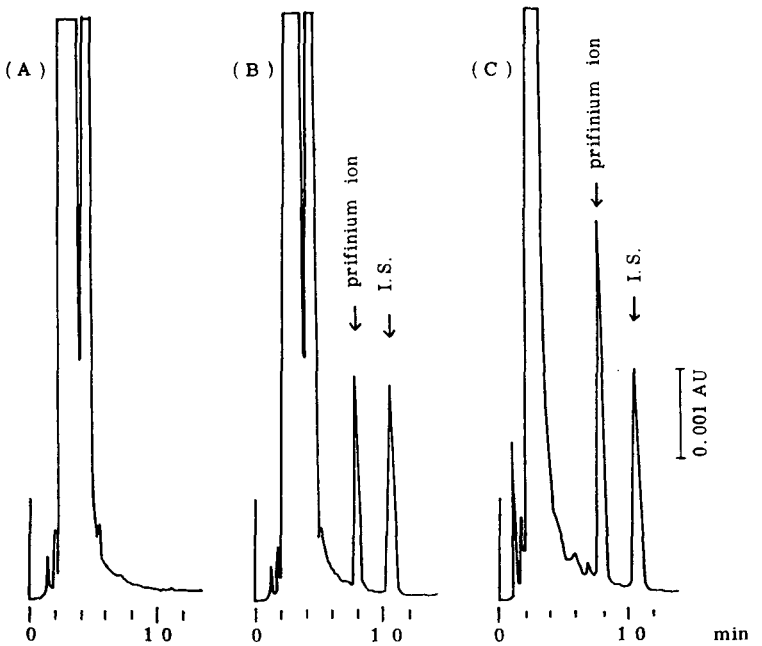

Fig. 3. Chromatograms of (A) blank human urine, (B) urine containing 100 ng/ml of prifinium ion and 200 ng/ml of internal standard and (C) urine from a healthy volunteer after oral administration of 60 mg of prifinium bromide. Conditions as in Fig. 2 except injection volume, 40 μl.

Recovery

Absolute overall recoveries from the samples were estimated by comparing peak heights or peak areas obtained from the injection of known amounts of prifinium ion with peak heights or peak areas obtained from the injection of extracts of samples spiked with prifnium ion. The values obtained from the serum and urine samples were respectively 57.0 ± 1.2% (mean ± S.D.) for prifinium ion concentrations in the range 2.5—50 ng/ml and 72.8 ± 1.5% in the range 25—100 ng/ml. These values, corrected for the ratio of the added solvent volume to the transferred volume, were 85.5% and 91.0% in serum and urine, respectively.

Calibration graph

Typical calibration graphs in human serum and urine are indicated in Table I. All calibration graphs show good linearity in each range. The lower limit of sensi-

TABLE I

TYPICAL CALIBRATION GRAPHS FOR HUMAN SERUM AND URINE

Sample	Concentration range (ng/ml)	Slope	Intercept	Correlation coefficient
Serum	0.5—100	0.04893	0.00169	0.9999
Urine	5 —200	0.007579	—0.00711	0.9998
Urine	50 —2500	0.0005384	—0.00156	0.9996

tivity for serum was 0.5 ng/ml, with a signal-to-noise ratio of 4, when 2 ml of serum was used. In urine, although peaks were obtained at lower concentrations, 5 ng/ml was the lower limit of sensitivity using 1 ml of urine and taking into account the variation of the background peaks in urine.

Reproducibility

The reproducibility was obtained by performing five replicate analyses of spiked serum and urine samples. The results are given in Table II. The coefficients of variation were less than 1.3% in both serum and urine. The actual prifinium ion content measured by HPLC ranged from 97 to 101% in the 15 serum and urine samples analysed. This HPLC method for the analysis of prifinium ion in human serum and urine thus provides good accuracy and precision.

TABLE II

REPRODUCIBILITY OF THE DETERMINATION OF PRIFINIUM ION IN HUMAN SERUM AND URINE

Parameter	Serum	Urine	Urine
Actual prifinium ion concentration (ng/ml)	5.00	50.0	500
Number of analyses	5	5	5
Mean analysed concentration (ng/ml)	4.96	49.7	490
% of actual concentration	99.2	99.4	98.0
Range (ng/ml)	4.91—4.99	48.7—50.3	484—499
S.D. (ng/ml)	0.03	0.62	6.2
Coefficient of variation (%)	0.61	1.2	1.3
Concentration range of calibration graph (ng/ml)	0.5 —100	5 —200	50—2500

Serum levels and urinary excretion in clinical studies

Serum levels of prifinium ion after oral administration of 60 mg of prifinium bromide to healthy volunteers are shown in Fig. 4. The drug reached maximum levels (4.1—14.7 ng/ml) within 3 h after administration of tablets; 0.6—2.1% of the administered dose was excreted as prifinium ion in the 0—24-h urine.

DISCUSSION

Early studies on methods for determining prifinium ion in the biological fluids were carried out by reversed-phase high-performance ion-pair liquid chromatography [15, 17] using heptanesulphonate or laurylsulphate as a counter ion. However, these methods did not separate the background peaks of blank biological fluids from those of prifinium ion. In later studies, normal-phase HPLC with aqueous methanol mobile phases [16, 18] was tested using various salts and pH of the mobile phases. These tests showed that mobile phases at high pH containing ammonium acetate to provide the best conditions for separating the background peaks of blank biological fluids from those of

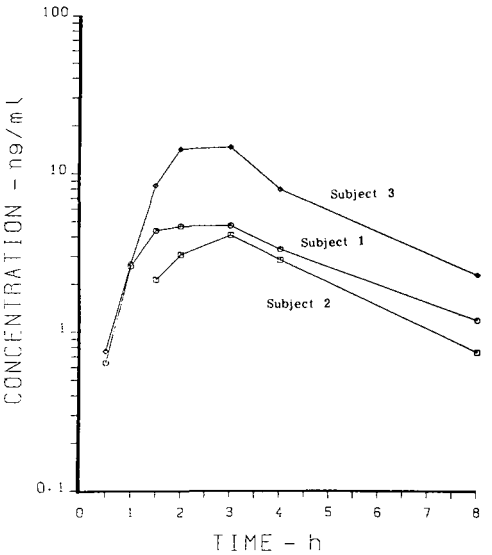

Fig. 4. Serum levels of prifinium ion in healthy volunteers after an oral dose of 60 mg of prifinium bromide.

prifinium ion and the internal standard (Figs. 2 and 3). In this study, a silica gel pre-column was placed between the pump and injector (Fig. 1) to prevent dissolution of silica from the analytical and guard columns at high pH [19—21]. The use of this silica pre-column technique increased the lifetime of the analytical and guard columns. Approximately 200 samples were analysed on one analytical column before a significant decrease in efficiency was observed.

The assay was shown to be sufficiently sensitive to quantify prifinium ion in human serum and urine after oral administration of the usual clinical dose (Fig. 4). A cross-over pharmacokinetic study of prifinium bromide in six healthy volunteers after intravenous and oral administration is in progress. The detailed results and a discussion of the pharmacokinetics of prifinium bromide in man will be published elsewhere.

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Journal of Chromatography, 231 (1982) 137—144
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1300

DETERMINATION OF ELLIPTICINE IN BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received December 9th, 1981; revised manuscript received March 15th, 1982)

SUMMARY

Ellipticine, a plant alkaloid effective against murine leukemias and solid tumors, is presently undergoing toxicological assessment prior to clinical trial. A rapid, sensitive, reversed-phase high-performance liquid chromatographic method employing an internal standard was developed for the detection of ellipticine and its principal metabolite 9-hydroxyellipticine after extraction from biological samples. The method was successfully applied to the quantitation of ellipticine in mouse blood and tissues after intravenous administration of ellipticine and to mouse blood levels of drug after oral administration. Similar success was achieved in determinations of ellipticine and 9-hydroxyellipticine in samples of spiked human blood and plasma. Mouse blood ellipticine levels monitored over 3 h after the intravenous administration of drug demonstrated a biphasic decline with a terminal half-life of 52 min.

INTRODUCTION

Ellipticine (NSC-71795; 5,11-dimethyl-6H-pyrido[4,3-*b*]carbazole) (Fig. 1) is a natural product isolated from *Ochrosia elliptica* and several other species of *Ochrosia* and *Bleekeria vitiensis* [1]. Ellipticine has demonstrated a broad spectrum of antitumor activity in both murine leukemias and solid tumors [1]. Initial toxicological studies in animals found significant hemolysis when the drug was given intravenously [2]. Since the drug is orally active [3] this route of administration would seem to be a reasonable alternative to minimize hemolysis. Ellipticine is currently undergoing toxicological evaluation in animals by the oral route at the National Cancer Institute. Pending a satisfactory completion of these studies, clinical trials will be initiated in man.

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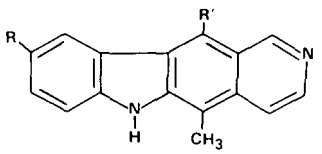


Ellipticine	R = H, R' = CH_3
9-Hydroxyellipticine	R = OH, R' = CH_3
11-Demethylellipticine	R = H, R' = H

Fig. 1. Structures of the ellipticines.

Ellipticine has been determined in various media by several methods. In a biological matrix ellipticine and metabolites have been determined radiochemically after thin-layer chromatographic separation of radiolabeled drug [4]. Also ellipticine equivalents have been measured by ultraviolet spectroscopy after extraction from tissues and fluids [3, 5]. High-performance liquid chromatography (HPLC) has been used in the preparative mode to collect synthetic and in vivo metabolic products of ellipticine [6]. HPLC with fluorescence detection has been employed to separate ellipticine and a number of analogues in non-biological samples [7]. However, we are aware of no reports describing the quantitation of ellipticine and metabolites in tissue and biological fluids by HPLC.

The objective of this work was to develop a simple and reliable reversed-phase HPLC procedure for the determination of ellipticine and its principal metabolite, 9-hydroxyellipticine, in biological materials. Such a method would be valuable in future pharmacological and bioavailability studies of ellipticine.

EXPERIMENTAL

Chromatographic apparatus and conditions

A modular high-performance liquid chromatograph (Model 3500B, Spectra-Physics, Santa Clara, CA, U.S.A.), including a reciprocating piston pump with flow feedback control, delivered mobile phase at a flow-rate of 1.4 ml/min to a stainless-steel column (300 × 4.0 mm) packed with fully porous 10-μm silica particles bonded with a monomolecular layer of octadecylsilane (μBondapak C_{18}, Waters Assoc., Milford, MA, U.S.A.). A stainless-steel guard column (50 × 4.6 mm) filled with 40-μm pellicular C_{18} packing material (Supelco, Bellefonte, PA, U.S.A.) preceded the analytical column. Samples were introduced to the column with a manual injection valve equipped with a 100-μl sample loop (Model CV-6-UHP-a-N60, Valco, Houston, TX, U.S.A.). The eluted compounds were detected with a variable-wavelength ultraviolet detector (Model 770, Spectra-Physics) set at 300 nm and/or a filter fluorometer equipped with a 70-μl flow cell (Fluoro-Microphotometer, American Instrument Co., Silver Spring, MD, U.S.A.), a narrow pass 360-nm filter for excitation and a sharp cut 455-nm emission filter. Detector output signals were

recorded with a variable input strip-chart recorder (Model B5116-1, Omniscribe, Houston Instruments, Austin, TX, U.S.A.).

The mobile phase used when ellipticine alone was quantitated consisted of acetonitrile—0.01 *M* NaH_2PO_4 (36:64, v/v) adjusted to pH 3.5 with 2 *N* phosphoric acid (System A). When ellipticine and 9-hydroxyellipticine were determined simultaneously, the mobile phase consisted of acetonitrile—0.01 *M* NaH_2PO_4 (25:75, v/v) adjusted to pH 3.5 with 2 *N* phosphoric acid (System B). All separations were effected isocratically at ambient temperature.

The compounds were quantitated using an internal standard method. Standard curves constructed from the ratio of peak heights of ellipticine and 9-hydroxyellipticine to the internal standard (I.S.), 11-demethylellipticine, versus concentration were linear ($r > 0.999$) in the range of 20—500 ng/ml.

Reagents

Ellipiticine (NSC-71795), 9-hydroxyellipticine (NSC-210717) and 11-demethylellipticine (NSC-87206) were supplied by the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). Acetonitrile, HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.) and distilled water were filtered through 0.5-μm and 0.8-μm solvent resistant filters respectively (Millipore, Bedford, MA, U.S.A.). All other chemicals were reagent grade and were used as received.

Stock solutions of ellipticine hydrochloride, 9-hydroxyellipticine and 11-demethylellipticine were prepared (100 μg/ml in 0.01 *N* hydrochloric acid) and used as chromatographic standards after appropriate dilutions.

Biological studies

Ellipticine hydrochloride (0.15 mg/ml in 5% Dextrose Injection, U.S.P.) was administered to male BDF_1 mice (20—28 g) at 3.0 mg/kg via a lateral tail vein in a volume equivalent to 2% of body weight. Mice were housed in conventional cages with no access to food or water after drug treatment. Blood was collected (0.3—0.7 ml) with heparinized pasteur pipettes from an orbital sinus cavity at 5, 15, 30, 60, 120 and 180 min after drug administration. Blood was transferred to test tubes and was immediately frozen (—20°C). For tissue distribution studies, mice were sacrificed by cervical dislocation at 4 h after the administration of ellipticine. Liver, spleen, kidney, brain and heart were rapidly excised, rinsed in a cold 0.9% sodium chloride solution, lightly blotted with a lint-free towel, and immediately frozen (—20°C).

Prior to oral administration of drug, the male BDF_1 mice were fasted for 16 h. Ellipticine hydrochloride (1.5 mg/ml in Sterile Water for Injection, U.S.P.) was administered directly into the stomach at 30 mg/kg via oral intubation in a volume equivalent to 2% body weight. Blood was collected 2, 3 or 4 h post administration by the method described above.

Extraction

The extraction of ellipticine, 9-hydroxyellipticine and the internal standard 11-demethylellipticine was accomplished using a modification of a procedure described previously [5]. Blood and tissue samples (ca. 0.5 g) were weighed and subsequently homogenized in the presence of 1.5 ml of 0.05 *M* sodium

phosphate buffer (pH 7.4) containing internal standard using a manual all-glass homogenizer. The homogenates were extracted with water-saturated ethyl acetate (3.0 ml) by vigorous mixing on a mechanical mixer (Vortex Genie Mixer, Scientific Instruments, Bohemia, NY, U.S.A.) for 2 min. After centrifugation at approximately 1300 *g* and 4°C for 30 min (Centra-7R, International Equipment Co., Needham Heights, MA, U.S.A.) a 2.0-ml aliquot of the organic layer was removed and added to 0.5 or 1.0 ml of 0.01 *N* hydrochloric acid. The contents were mixed for 30 sec after which the layers separated upon standing. The aqueous layer was removed and a 100-μl aliquot was injected for HPLC analysis.

Extraction efficiency

Extraction efficiencies for ellipticine, 9-hydroxyellipticine and internal standard were determined by extracting pooled mouse blood spiked with 50—500 ng of the three compounds. Extraction efficiencies from human plasma and blood were determined in a similar manner. The efficiencies of extraction of ellipticine from various murine tissue were determined by extracting homogenates spiked with 100 or 150 ng of drug.

RESULTS AND DISCUSSION

Chromatographic behavior

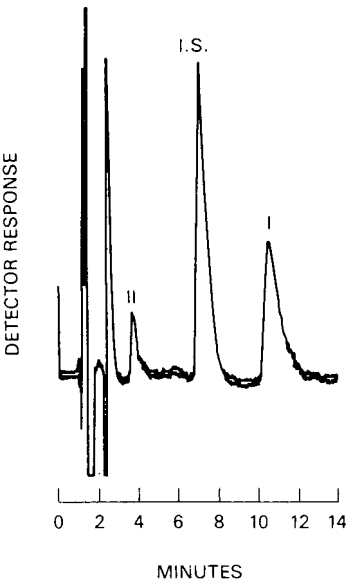
9-Hydroxyellipticine, 11-demethylellipticine and ellipticine eluted from the column in that order in either HPLC system. The retention volumes (V_R), capacity factors (k') and separation factors (α) for the three compounds were calculated using standard methods [8] and are listed in Table I. The time for the chromatographic separation in System B was about 15 min. A typical chromatogram used in the quantitation of ellipticine and 9-hydroxyellipticine is seen in Fig. 2. The slightly lower organic composition of this mobile phase was necessary to ensure adequate resolution of 9-hydroxyellipticine, internal standard, ellipticine and endogenous components from the biological matrix. Since 9-hydroxyellipticine did not fluoresce under the chromatographic conditions employed it was necessary to use a UV detector separately or in series with the fluorescence detector in order to accomplish the assay of 9-hydroxyellipticine and ellipticine simultaneously. Alternatively, it was possible to deter-

TABLE I

RETENTION VOLUMES (V_R), CAPACITY FACTORS (k') AND SEPARATION FACTORS (α) FOR CHROMATOGRAPHIC SYSTEMS A AND B

Component	V_R (ml)		k'		α	
	A	B	A	B	A	B
9-Hydroxyellipticine	—*	5.18	—*	2.70		2.22
11-Demethylellipticine	8.96	9.80	5.40	6.00	1.37	
Ellipticine	11.76	14.70	7.40	9.50		1.58

*9-Hydroxyellipticine was not detected using System A with fluorescence detection.



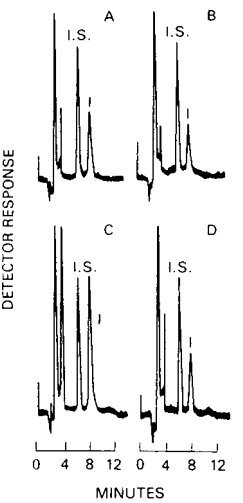


Fig. 2. Typical chromatogram showing the separation of ellipticine (I, 50 ng/ml), 9-hydroxyellipticine (II, 50 ng/ml) and the internal standard, 11-demethylellipticine (I.S., 150 ng/ml) after extraction from mouse blood (System B, UV detection).

Fig. 3. Typical chromatograms used for the quantitation of ellipticine (I) extracted from mouse kidney (A), spleen (B), liver (C), and heart (D), 4 h after the intravenous administration of 3 mg/kg of drug (System A, fluorescence detection).

mine ellipticine alone more rapidly using System A with fluorescence detection (9-hydroxyellipticine does not fluoresce and fewer early eluting endogenous components from the biological samples are encountered with fluorescence detection). The time for a complete chromatographic separation of internal standard and ellipticine in System A was about 10 min. Chromatograms illustrating the determination of ellipticine extracted from mouse blood and various tissues are seen in Fig. 3.

The detection limit for ellipticine using fluorescence detection was 5 ng/ml when a 0.5-ml blood sample was extracted. Using a UV detector the minimum detectable concentrations were 10 ng/ml and 25 ng/ml for ellipticine and 9-hydroxyellipticine respectively when extracted from a 0.5-ml blood or plasma sample.

Various proportions of methanol and 0.01 *M* sodium phosphate buffer (pH 3.5) were also evaluated as potential mobile phases. However, consistently sharper peaks were obtained using either of the two systems (A or B) containing acetonitrile. Variation of the pH in the aqueous component of the mobile phase demonstrated the apparent necessity of forming the protonated species to facilitate the elution of ellipticine from the column. When mobile phases containing water or buffers at >pH 7 were employed, the elution time for ellipticine at comparable flow-rates was more than 1 h. The reported pK_a for ellipticine is 5.8 [9].

TABLE II

EXTRACTION EFFICIENCY FROM BIOLOGICAL SAMPLES

Values are mean % ± S.D.

Drug	Mouse blood	Human blood	Human plasma	Mouse brain	Mouse heart	Mouse liver	Mouse kidney	Mouse spleen
Ellipticine	93.3 ± 4.7 (n = 19)	93.5 ± 5.6 (n = 25)	94.8 ± 3.7 (n = 30)	86.6 ± 7.2 (n = 6)	87.6 ± 3.0 (n = 6)	89.8 ± 3.7 (n = 6)	98.8 ± 3.1 (n = 6)	98.0 ± 6.5 (n = 6)
9-Hydroxy-ellipticine	92.4 ± 7.5 (n = 6)	90.7 ± 4.8 (n = 29)	94.7 ± 5.0 (n = 11)	—	—	—	—	—

Extraction efficiency

The extraction efficiencies for 9-hydroxyellipticine and ellipticine from spiked pooled mouse blood and from human blood and plasma are seen in Table II. Each value represents the mean and standard deviation of at least six separate extractions. The extraction efficiency for the recovery of ellipticine from various murine tissues is also presented in Table II. Each value reported is the mean ± standard deviation of at least six separate extractions. The internal standard was well extracted from all fluids and tissues (mean extraction efficiency overall was 93.0 ± 4.8%) with the exception of mouse liver. Extractions of 11-dimethylellipticine from liver homogenates were extremely variable and unpredictable. Therefore, when mouse liver samples were assayed for ellipticine, the internal standard was incorporated into the acid extract just prior to HPLC analysis.

Biological application

Blood ellipiticine levels in mice after a single intravenous administration at 3.0 mg/kg are seen in Fig. 4. Each plotted value represents the mean concentration obtained from measurements in at least four animals. The decline in blood levels appears to be biphasic. The mean blood concentration of ellipticine 5 min after administration was 696 ± 113 ng/g (mean ± S.E.). The blood levels declined rapidly during the first 60 min with a more gradual decline over the next 2 h. The half-life and rate constant calculated from the terminal phase were 52 min and 0.0132 min^{-1}, respectively. Table III presents representative mouse tissue levels after intravenous administration of drug at 3.0 mg/kg. Ellipticine was still detectable in various tissues 4 h after drug administration.

Blood levels of the 9-hydroxymetabolite of ellipticine have been shown to be relatively low after the intravenous administration of ellipticine [4]. The 9-hydroxymetabolite was not detected after intravenous administration of drug in the present study. However, analysis of blood samples after the oral administration of drug (30.0 mg/kg) showed significant levels of 9-hydroxyellipticine at 2, 3 and 4 h. Two hours post administration blood levels of 9-hydroxyellipticine and ellipticine were 164 ng/g and 590 ng/g respectively. Significant levels of 9-hydroxyellipticine were also detected 3 and 4 h after dosing (99.8 and 55.4 ng/g respectively). These relatively high concentrations of 9-hydroxy-

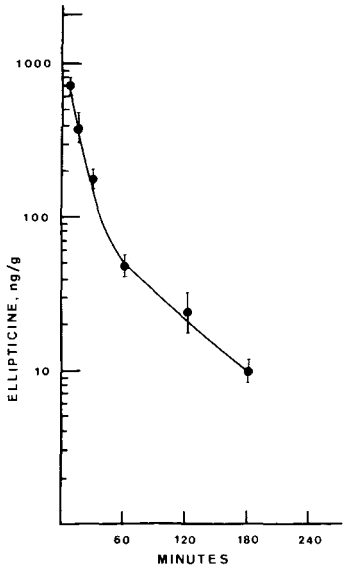


Fig. 4. Blood ellipticine levels in mice after the intravenous administration of drug at 3.0 mg/kg ($n \geqslant 4$).

TABLE III

TISSUE CONCENTRATIONS IN MICE AT 4 h AFTER INTRAVENOUS ADMINISTRATION OF ELLIPTICINE (3.0 mg/kg)

Each value represents the mean (± S.E.) of at least four animals.

Tissue	Concentration* (ng/g)
Brain	72.8 (13.2)
Liver	25.9 (5.6)
Kidney	126.8 (36.1)
Spleen	224.3 (38.8)

*Chromatographic System A, fluorescence detection.

ellipticine may indicate the existence of a significant first-pass metabolism of ellipticine when given orally. Since the drug will be administered orally to humans, this possibility should be given consideration in the design of protocols and pharmacological studies. Since 9-hydroxyellipticine possesses antitumor activity in animal models [10], the blood levels and elimination rates of both ellipticine and 9-hydroxyellipticine should be monitored to understand the proper dosing intervals and subsequent physiological effects.

In summary, a simple HPLC method for the determination of ellipticine and 9-hydroxyellipticine in biological samples was developed. The method has been successfully applied to the assay of mouse blood and tissue samples after the intravenous administration of ellipticine. Additionally, the method (System B) performed well in the determination of ellipticine and 9-hydroxyellipticine

in spiked human blood and plasma. Hopefully, the method should be useful in the assay of human plasma after the oral administration of ellipticine in clinical trials. Since relatively small sample volumes were extracted in this study, it should be possible to increase the sensitivity of the method by extraction of larger plasma samples during clinical studies.

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Journal of Chromatography, 231 (1982) 145—154
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1288

DETERMINATION OF AMIKACIN IN MICROLITRE QUANTITIES OF BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING 1-FLUORO-2,4-DINITROBENZENE DERIVATIZATION*

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(First received December 8th, 1981; revised manuscript received March 5th, 1982)

SUMMARY

Pre-column derivatization of amikacin with 1-fluoro-2,4-dinitrobenzene in 25 μl of guinea pig plasma or human serum produced a stable chromophore which was measured by UV detection after rapid separation on normal-phase or reversed-phase high-performance liquid chromatography systems. The reversed-phase system, selected for routine analysis due to instability of the normal-phase column, consisted of an Ultrasphere-ODS C18 column preceded by a guard column, and used acetonitrile—water (68 : 32) as the mobile phase. A high degree of linearity was found in the range of 2—64 μg/ml with a coefficient of variation averaging less than 5%.

INTRODUCTION

Amikacin is a semisynthetic derivative of kanamycin and has a broad spectrum of activity against aerobic gram-negative bacilli. Because the amikacin molecule has fewer points susceptible to enzymatic attack than have most other aminoglycosides [1] it is often used clinically for infections resistant to gentamicin or tobramycin. Its ototoxicity [2] and nephrotoxicity [3], however, require careful monitoring of blood levels especially in treating life-threatening infections, in patients with impaired renal function or when therapy is of long duration [4].

Amikacin serum or plasma levels are most often determined by a microbiological method [5] but this is time consuming and can be affected by the pres-

*Presented in part at the Pittsburgh Conference, Atlantic City, NJ, U.S.A., March 9—13, 1981.

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ence of other antibiotics. Other techniques which are more specific and rapid include radioenzymatic assay [6], radioimmunoassay [7], gas–liquid chromatography [8] and high-performance liquid chromatography (HPLC) [9]. Due to equipment availability a HPLC technique was the most suitable for our purposes. The published HPLC method for amikacin [9] used fluorescence detection of an *o*-phthalaldehyde derivative, and needed 1 ml of serum for analysis. Our experiments with guinea pigs required frequent sampling of amikacin blood levels from the same animal and therefore a micro-technique was essential.

1-Fluoro-2,4-dinitrobenzene (FDNB) has been used as a reagent for the derivatization of amino function groups for a number of years [10]. Recently, HPLC using FDNB derivatization has been described for neomycin [11], gentamicin [12], sisomicin [12] and fortimicin C [13]. The 2,4-dinitrophenyl (DNP) derivative formed is stable and can be measured using UV detection. This technique appeared suitable for the measurement of amikacin levels since this aminoglycoside has four primary amino functional groups. The ability to measure by UV detection is an advantage since a high-performance liquid chromatograph with a UV detector is rapidly becoming standard equipment in clinical laboratories.

In this report we describe a sensitive micro-technique for the determination of amikacin in plasma which was developed for ototoxicity studies in guinea pigs. Since no interfering peaks are detectable in human serum even in the presence of several other drugs, this technique is readily adaptable for the clinical monitoring of amikacin levels.

MATERIALS AND METHODS

Reagents

Amikacin base was donated by Bristol-Myers Pharmaceutical Group (Candiac, Quebec, Canada). 1-Fluoro-2,4-dinitrobenzene, 98% pure, was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other chemicals were reagent grade and obtained from commercial sources.

Normal-phase chromatographic system

A Varian-Aerograph Series 4100 liquid chromatograph equipped with a positive displacement pump capable of developing a pressure of 34.5 MPa (5000 p.s.i.) was used as the solvent delivery system. Flow-rate was always maintained at 1 ml/min. Samples were injected using a Valco loop (125 μl) injection valve. UV absorbance was monitored at 360 nm with a variable-wavelength UV detector (Varian Vari-Chrom, Model VUV-10) connected to a strip chart recorder (Linear Instruments). Attenuation was set at 0.01, 0.02 or 0.05 a.u.f.s. for a 100-mV full scale deflection. Chromatographic separation was achieved with a 200 × 4.6 mm stainless-steel column packed with 5-μm Spherisorb silica (Phase Separations, Queensferry, Great Britain) using a high-pressure (34.5 MPa) balanced-density slurry technique [14]. The mobile phase consisted of the lower layer of a mixture of chloroform–methanol–glass distilled water–acetic acid (214:35:20:1) passed through a Whatman 2V filter and purged with nitrogen.

Reversed-phase chromatographic system

The solvent pump and UV detection systems were the same as that used in the normal-phase system. Samples were introduced using a Valco loop injection valve (CV-10-UHP$_a$-N60) equipped with a 50-μl loop. A Beckman Instruments (Montreal, Canada) column (Ultrasphere-ODS C18, 250 × 4.6 mm, particle size 5 μm) preceded by a guard column (RP-18, 30 × 4.6 mm, particle size 5 μm, Brownlee Labs., Santa Clara, U.S.A.) was used for chromatographic separation. The mobile phase consisted of acetonitrile—glass-distilled water (68 : 32) passed through a Whatman 2V filter and purged with nitrogen.

Sample preparation

Stock solutions were prepared by adding amikacin base to glass-distilled water to give concentrations ranging from 0.080 to 2.560 mg/ml. Spiked samples were prepared by adding 25 μl of stock solution to 975 μl of guinea pig plasma or human serum to give final concentrations in the range of 2 to 64 μg/ml. A 25-μl aliquot of spiked or blank plasma or serum was placed in a 0.3-ml Reacti-Vial (Pierce, Rockford, IL, U.S.A.) together with 10 μl of 0.1 *M* borate buffer (pH 9.3). After 100 μl of methanol were added to precipitate the plasma proteins, the vials were vortexed, sealed and centrifuged at 2000 *g* for 5 min (IEC Clinical Centrifuge, Damon/IEC, Needham, MA, U.S.A.). A 75-μl aliquot of supernatant was transferred to a 1.0-ml Reacti-Vial and 10 μl of FDNB in methanol (180 mg/ml) were added. The vials were sealed, incubated for 30 min at 80°C in a dry bath (Fisher Scientific, Isotemp Model 145) and cooled to room temperature in a freezer.

Normal-phase chromatography of samples

Chloroform (500 μl) was added to the sample vials which were then vortexed for 1 min and centrifuged at 2000 *g* for 5 min. Aliquots (125 μl) of the chloroform layer were injected onto the column.

Cleanup of samples and reversed-phase chromatography

The contents of the sample vials were evaporated with nitrogen until an oily residue remained and then 6—8 mg of binder-free silica gel (MN-Kieselgel N, Macherey, Nagel and Co., Düren, G.F.R.) were added. After introducing a 600-μl aliquot of diethyl ether the vials were shaken (Burrell Wrist Action Shaker, Burell Corporation, Pittsburgh, PA, U.S.A.) for 5 min, vortexed and then centrifuged for 5 min at 2000 *g*. The diethyl ether was discarded by decanting and the washing procedure repeated once more with 600 μl of diethyl ether, then with 400 μl of 0.2 *M* acetate buffer (pH 4.0). Vortexing without prior shaking was all that was needed for the final two washes. The derivative was then extracted by vortexing the silica gel pellet with 300 μl of mobile phase. After centrifuging for 5 min, 50-μl aliquots of the liquid above the silica gel pellet were injected onto the column.

RESULTS AND DISCUSSION

HPLC separation

The amikacin derivative has a retention time of approximately 6 min in the

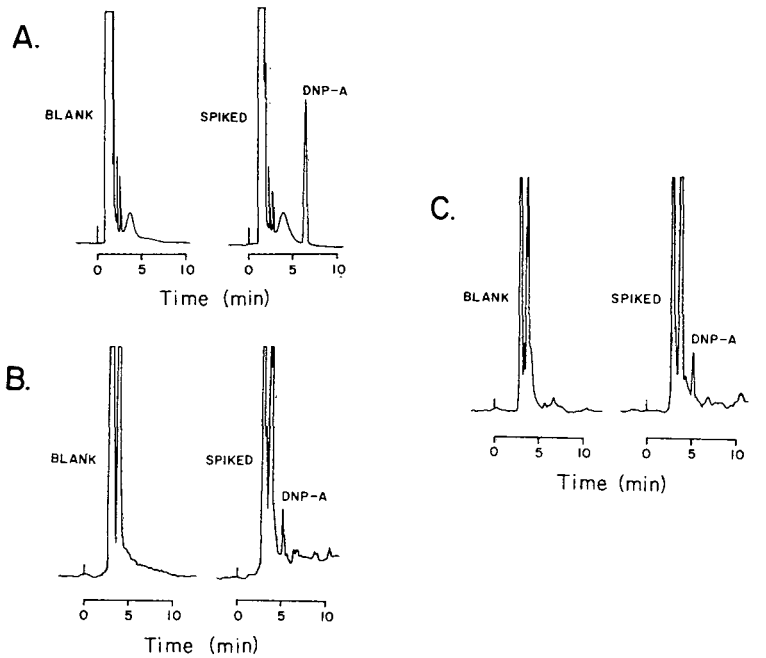

Fig. 1. Typical chromatograms at 0.01 a.u.f.s. of blank and spiked guinea pig plasma (A and B) and human serum (C). The normal-phase HPLC system was used for (A) and the reversed-phase system for (B) and (C). Concentrations of amikacin were 16 (A) and 4 (B and C) μg/ml. DNP-A = DNP-amikacin.

normal-phase system (Fig. 1A). Guinea pig plasma contains a few endogenous peaks which elute in less than 5 min and therefore do not interfere with the analysis. Although the normal-phase system provided adequate sensitivity, it had two major drawbacks. The first was retention time instability from day to day due to extreme sensitivity to the water content of the mobile phase and the long time required to fully equilibrate the column to new batches of mobile phase. The second drawback was that the retention time of the column started to deteriorate after about 100 samples. Since the packing material was found to take on a yellow colour the deterioration of the column could have been due to the presence of unreacted FDNB in the samples. For these reasons, a reversed-phase system was developed together with an extensive cleanup procedure. Guinea pig plasma and human serum chromatograms produced with the reversed-phase system are shown in Fig. 1B and C. Apart from the amikacin derivative peak with a retention time of 5.1 min, all other major peaks occur with the solvent front. FDNB, which has a retention time of 7.0 min is either absent or occurs as a tiny peak. The other minor peaks which follow the DNP-amikacin (DNP-A) peak are eluted by 11 min, and therefore samples can be run about 7 min apart by allowing these peaks to come out in the solvent front of the next sample. These minor peaks probably arise from degradation products of compounds in the solvent front since they do not appear if the sample is chromatographed immediately after the cleanup procedure (e.g., the chromatogram of blank guinea pig plasma in Fig. 1B). The cleanup procedure appears

somehow to catalyze this degradation process since derivatized samples can be stored in the freezer several days prior to extraction without an increase in the size of the peaks following DNP-A. For this reason, chromatograms should be run within 2—3 h of the cleanup procedure, especially for low levels of amikacin. The derivatization procedure however, can be carried out several days in advance without loss of DNP-A since it is stable for at least one week. To date, 150 samples have been injected with no appreciable loss of sensitivity or alteration of retention time. A gradual increase in back pressure occurred with usage but this was eliminated after the guard column was replaced. This reversed-phase system is thus now used routinely for the analysis of amikacin.

Derivatization of amikacin

The optimal conditions for the formation of DNP-amikacin were determined by investigating the effects of pH, FDNB concentration, reaction time and temperature on the derivatization of aqueous amikacin solutions.

A number of buffer systems (acetate, triethanolamine HCl, tris(hydroxymethyl)-aminomethane, bicarbonate, borate) were tested and borate buffer at pH 9.3 was found to give the maximum yield of product. The effect of borate buffer concentration on DNP-amikacin peak height was also examined for

TABLE I

EFFECT OF BORATE BUFFER STRENGTH ON PEAK HEIGHT OF AMIKACIN DERIVATIVE

Concentration of borate buffer aliquot (*M*)	*n*	Peak height* ± S.D. (mm at 0.02 a.u.f.s., normal-phase system)
0.016	3	90.6 ± 3.09
0.032	2	108 ± 6.06
0.064	3	112 ± 0.62
0.128	3	114 ± 3.76

*Plasma samples spiked with amikacin (64 μg/ml) were used.

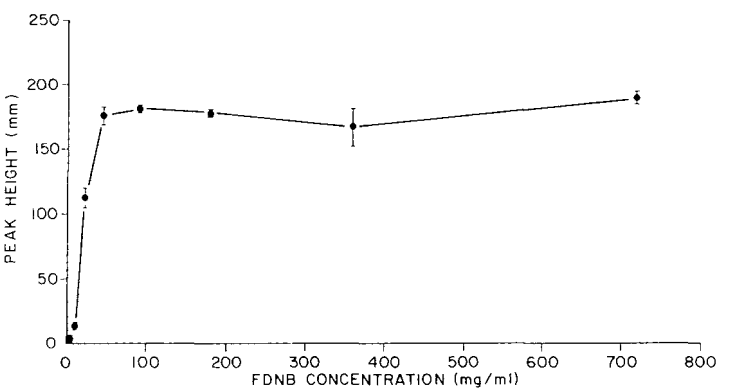

Fig. 2. Peak height (mm at 0.05 a.u.f.s., normal-phase system) of DNP-amikacin as a function of FDNB concentration. Amikacin concentration was 200 μg/ml in guinea pig plasma. Vertical bars are standard deviation. $n = 3$.

guinea pig plasma (Table I). Peak height plateaued above 0.032 *M*. A borate buffer concentration of 0.1 *M* was selected for routine analysis.

The amount of FDNB required to ensure complete derivatization was determined by incubating a high concentration of amikacin (200 μg/ml) in guinea pig plasma with varying FDNB concentrations (Fig. 2). The peak height of the amikacin derivative approached a maximum at a FDNB concentration of 45 mg/ml and leveled off with increasing concentrations. The concentration selected for routine analysis was 180 mg/ml, which gives a molar ratio of 283 for each amikacin primary amino group at a drug concentration of 200 μg/ml.

The effect of reaction time on the peak height of DNP-amikacin was examined at 80°C (Fig. 3). The reaction was judged to be maximal by 10 min but 30 min was selected for routine analysis to ensure stable results. The formation of the amikacin derivative was also examined at 60°C and 100°C. At the lower temperature the reaction was found to proceed too slowly whereas at the higher temperature the problem of solvent evaporation was encountered.

An attempt was made to determine recovery but we were unable to obtain an authentic derivative of high enough purity for this calculation.

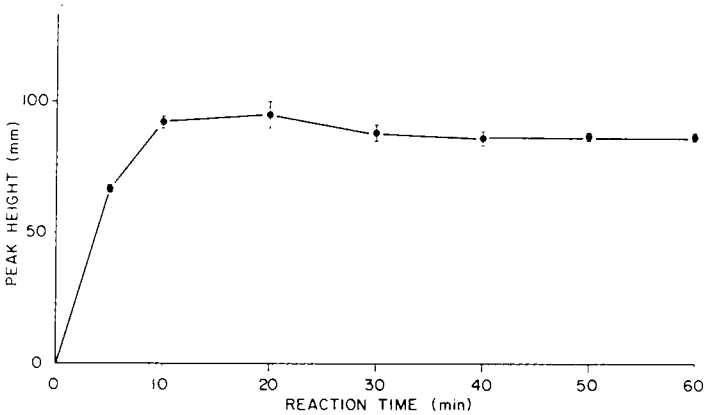

Fig. 3. Peak height (mm at 0.05 a.u.f.s., normal-phase system) of DNP-amikacin as a function of reaction time at 80°C. Amikacin concentration was 100 μg/ml in guinea pig plasma. Vertical bars are standard deviation. $n = 3$.

Cleanup of derivative

Samples injected onto the reversed-phase columns after derivatization without cleanup contained a number of large interfering peaks. Also, the column quickly deteriorated possibly due to unreacted FDNB. Since the amikacin derivative was soluble only in semi-polar solvents such as methanol, partitioning was unsuccessful. Hence silica gel was used to retain the derivative while more and less polar compounds were washed out with suitable solvents. Washing the derivatized sample with diethyl ether reduced unwanted peaks, especially that arising from excess FDNB, without reducing the size of the DNP-amikacin peak. Washing with acetate buffer (pH 4.0) greatly reduced other unwanted peaks, especially the large solvent front peak (arising partly from dinitrophenol). The derivatized amikacin peak was also reduced by about 20% but since this did not appear to affect precision, the reduction in peak size seemed

a fair price to pay for a cleaner chromatogram. The amount of silica gel used in the cleanup process to retain the derivatized amikacin was not critical in the range 5—15 mg per vial. Amounts of silica gel below this range caused a reduction in the size of the amikacin derivative peak. Amounts greater than 10 mg increased the amikacin derivative peak slightly but also greatly increased the size of contaminant peaks. Hence, we used an amount of silica gel in the range of 6—8 mg for routine analysis.

Characterization of derivative

The number of DNP substitutions on the DNP-amikacin derivative was examined by proton magnetic resonance (PMR) spectroscopy (Fig. 4). The PMR spectrum of underivatized amikacin shows two doublets between 5 and 6 ppm arising from the two anomeric carbon protons. The PMR spectrum of the DNP-amikacin derivative shows the presence of aromatic protons of the DNP groups

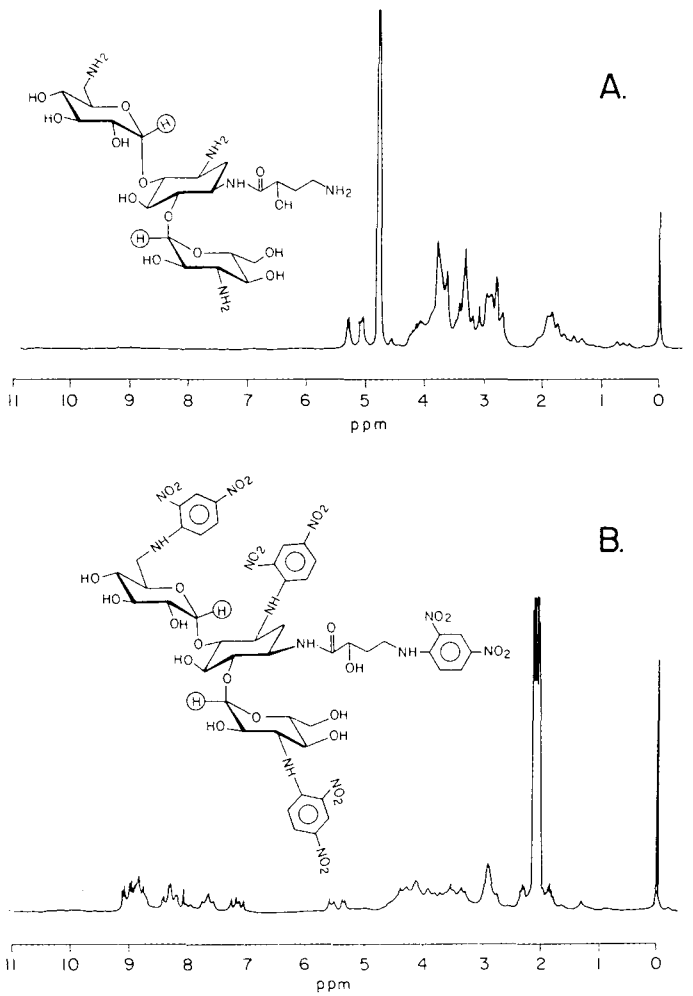

Fig. 4. PMR spectra of underivatized amikacin (A) and DNP-amikacin (B).

between 7 and 9.1 ppm. The ratio of the combined area of the aromatic proton peaks to that of the anomeric proton peaks was 12:2 suggesting that all four primary amino groups of amikacin were substituted. Differing solubility properties required that DNP-amikacin be run in deuterated acetone as opposed to underivatized amikacin which was run in deuterated water.

Quantitation

Spiked plasma samples, each containing one of seven different concentrations of amikacin were analyzed using the reversed-phase technique. The results are summarized in Table II. The standard curve was linear in the range of 2—64 μg/ml for guinea pig plasma and the mean coefficient of variation was less than 5%. Although regression analysis shows the standard curve to possess a high degree of linearity (r = 0.9996), it has been a consistent finding with all the

TABLE II

STANDARD CURVE OF AMIKACIN ADDED TO GUINEA PIG PLASMA

Amikacin added (μg/ml)	No. of samples	Peak height ± S.D. (mm at 0.02 a.u.f.s.)	Coefficient of variation (%)
2	4	5.75 ± 0.57	9.9
4	4	11.5 ± 0.42	3.6
8	4	23.6 ± 0.60	2.6
16	4	49.2 ± 1.14	2.3
32	3	96.9 ± 0.81	0.8
48	4	152.2 ± 2.83	1.9
64	4	204.9 ± 2.34	1.1

Mean coefficient of variation = 3.17%.
Y-intercept = −1.92 (95% confidence limits are −3.10 and −0.73).
Slope = 3.21.
Correlation coefficient = 0.9996.

TABLE III

ACCURACY OF REVERSED-PHASE METHOD USING PEAK HEIGHT COMPARISON WITH 32 μg/ml STANDARD*

T (Theoretical concentration in μg/ml)	E (Estimated concentration from peak height comparison)	Accuracy (%) $\left(\frac{E-T}{T} \times 100\right)$	E' (Estimated concentration using eqn. 1)**	Accuracy (%) $\left(\frac{E'-T}{T} \times 100\right)$
2	1.90	−5.1	2.02	+1.0
4	3.79	−5.3	4.02	+0.4
8	7.80	−2.6	8.19	+2.4
16	16.26	+1.0	16.78	+4.9
48	50.25	+4.7	48.48	+1.0
64	67.66	+5.7	63.15	−1.3

*Data taken from mean peak height values in Table II.
**$(E') = (E) \times$ (C.F.). The value of Z used in eqn. 1 was 0.936.

amikacin standard curves which we have run (with both normal-phase and reversed-phase techniques) that the relationship between peak height and plasma concentration is actually slightly curvilinear. One indication of this is the observation that the regression line passes slightly below the origin (i.e., the 95% confidence limits of the *Y*-intercept do not include zero). The slight curvature is shown more clearly in Table III when estimates of amikacin concentrations are made by comparing sample peak heights with the peak height obtained for a concentration of 32 μg/ml, using the mean data in Table II. Although deviations from theoretical values are less than 6%, concentrations below 32 μg/ml tend to be underestimated whereas those greater than 32 μg/ml tend to be overestimated. The slight curvature may arise from the small amount of plasma protein binding of amikacin [15] which would be proportionately more important at lower concentrations. Accuracy can be improved (Table III) by multiplying the first estimate by the following correction factor (C.F.) which partially compensates for the curvature:

$$\text{C.F.} = (\text{P.H.})_s/[Z(\text{P.H.})_s + (1 - Z)(\text{P.H.})_u] \quad (1)$$

where $(\text{P.H.})_s$ is the mean peak height of 32 μg/ml amikacin standards, $(\text{P.H.})_u$ is the peak height of the unknown sample and Z is an empirical constant whose magnitude is inversely related to the degree of curvature. The value of Z was determined to be 0.936 ± 0.025 (mean ± S.D.) using the best fit data from four separate standard curves.

Specificity

The following drugs were added to human serum at high or higher than clinical concentrations and were not found to produce peaks which would interfere with the reversed-phase technique for amikacin analysis: ampicillin (20 μg/ml), chlorpromazine (500 ng/ml), diazepam (500 ng/ml), digoxin (2 ng/ml), doxycycline (15 μg/ml), gentamicin sulfate (10 μg/ml), sodium heparin (3 I.U./ml), imipramine (1 μg/ml) and phenobarbital (30 μg/ml).

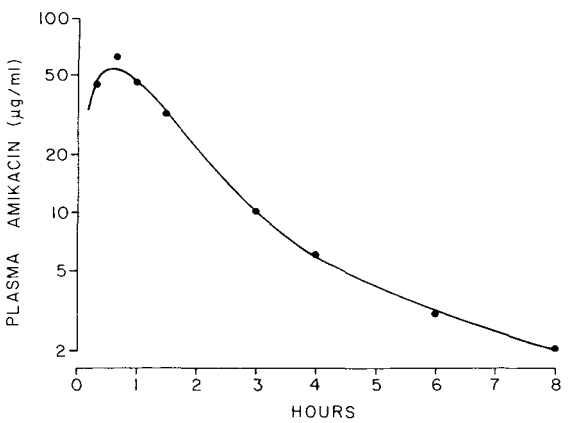

Fig. 5. Amikacin in guinea pig plasma after subcutaneous injection (50 mg/kg). The curve is drawn according to the best fit equation to a two-compartment open model as determined by computer analysis.

Application

The time profile of amikacin in plasma from a guinea pig after a subcutaneous injection of 50 mg/kg is shown in Fig. 5. The data were fitted to a two-compartment open model with a computer program run in a NOVA 800 computer (Data General Corporation, Southboro, MA, U.S.A.). The program used an iterative technique to minimize the squared deviations between the curve and the data values. The equation for the best fit curve was determined to be:

$$C_p = -271.3e^{-2.24t} + 259.9e^{-1.36t} + 11.4e^{-0.22t} \quad (2)$$

where C_p is the plasma amikacin concentration at time t. The β half-life was found to be 3.14 h which is similar to 2.79 h reported for human adults after an intramuscular injection of 500 mg of amikacin [16]. The α half-life for the guinea pig was 0.51 h which compares to 0.25 h found in children [17] and 2.77 h in the human adult study [16].

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Journal of Chromatography, 231 (1982) 155—160
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1282

Note

Flash heater derivatization of unconjugated bile acids using trimethylanilinium hydroxide

Rapid analysis by capillary gas—liquid chromatography*

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(Received October 27th, 1981)

Bile acid methyl ester methyl ethers (BAMME) are stable derivatives which are highly suited to capillary gas—liquid chromatography [1]. In a previous study we described their preparation and characterization, using the reagents sodium methylsulfinylmethanide and methyl iodide [1]. While this procedure has the advantage of quantitatively forming BAMME derivatives in a single step, it is relatively slow, taking several hours for reaction and work-up.

The quaternary base, trimethylanilinium hydroxide (TMAH), has been used in the preparation of alkyl esters of bile acids in a room temperature reaction with alkyl halides [2]. It has also been used for the derivatization of carboxylic acids and amino groups in the vapour phase by pyrolytic methylation. For instance, N,N-dimethyl derivatives of phenobarbitone can be formed in the hot injector port of a gas chromatograph [3]. Other quaternary bases such as tri-

*This work has previously appeared in abstract form in Gastroenterology, 79 (1980) 1003.

0378-4347/82/0000—0000/$02.75

methylammonium hydroxide and (*m*-trifluoromethylphenyl)trimethylammonium hydroxide have been used to form methyl esters of fatty acids in a similar way [3, 4].

In this study we report an on-column derivatization procedure for the quantitative analysis of unconjugated bile acids which uses TMAH for the pyrolytic methylation of both the hydroxyl and carboxylic acid moieties in the vapour phase in the hot injector. Advantage was taken of the splitless injection technique for capillary gas—liquid chromatography which conveniently causes the condensation of the derivatives into a sharply defined starting zone prior to the start of the temperature program sequence.

MATERIALS AND METHODS

Materials

The sources of the bile acids used in this investigation are given in Table I. Each gave a single spot when 30 μg was analyzed by thin-layer chromatography (TLC). 23-Norcholic acid (nor-24-3α-7α,12α-trihydroxy-5β-cholan-23-oic acid), however, gave three spots and was purified by preparative TLC on silica gel G (Rediplates, Fisher Chemical, Norcross, GA, U.S.A.) using hexane—ethyl acetate—acetic acid—propan-2-ol (2:1:1:0.1, v/v) as the mobile phase. 5α-Cholestane and *n*-alkane standards were purchased from Applied Science Labs. (College Park, PA, U.S.A.).

Chromatography grade methanol and dimethylformamide were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Dimethylsulfoxide was distilled over calcium hydride. Methyl iodide and N,N-dimethylaniline were obtained from Fisher Chemical.

Commercial TMAH was purchased from Applied Science Labs. and Pierce (Rockford, IL, U.S.A.). TMAH was also prepared by first reacting methyl iodide and dimethylaniline to form trimethylanilinium iodide. The latter was recrystallised four times from methanol and then was converted to the hydroxide by passing a solution of the compound in dry methanol over a column of AG-1 ion-exchange resin (OH^- form), previously thoroughly equilibrated with methanol. The final concentration was adjusted to 2 *M* by the addition of anhydrous methanol.

Gas chromatography

A Hewlett-Packard 5880 gas chromatograph, equipped with a flame ionization detector and capillary column splitless injector was used. The glass capillary column, 10 m × 0.25 mm, was wall-coated with the liquid phase Poly S-179 (Applied Science Labs.). Preliminary studies were also carried out with the phase SP-2100 coated onto a 12 m× 0.2 mm fused silica capillary column (Hewlett-Packard). The injector port temperature was varied between 200—350°C and the samples were injected at an oven temperature of 60°C. The inlet purge flow of 60 ml/min was interrupted for 18 sec after the moment of injection. The oven temperature was programmed at 30°C/min to 220°C and then at 3.5°C/min to 260°C. The column was then heated to 300°C for 5 min. Hydrogen was used as the carrier gas at an inlet pressure of 15 p.s.i. which gave a flow-rate of 10 ml/min through the column at 60°C and 3 ml/min at 260°C.

TABLE I

KOVÁTS' RETENTION INDICES (KRI) OF BILE ACID METHYL ESTER METHYL ETHER DERIVATIVES

Bile acid*	Source**	KRI
Dinor-5αB-3β-ol	2	36.65
Dinor-B^{5}-3β-ol	2	36.63
Nor-5βB-3α,12α-diol	7	38.26
Nor-5βB-3α,7α,12α,triol	2	39.94
5αB-3β-ol	2	40.19
5βB	2	35.28
5βB-3α-ol	1	39.37
5βB$^{9-11}$-3α-ol	3	38.94
5βB^{11}-3α-ol	3	39.26
B^{5}-3β-ol	2	40.13
5βB$^{9-11}$-3β-ol	3	38.93
5βB^{11}-3β-ol	3	38.43
5βB-3β-ol	3	38.39
5βB-7α-ol	4	36.60
5βB-12α-ol	4	35.93
5βB^{3}-12α-ol	3	36.44
5βB-3α,6α-diol	1	41.94
5βB-3α,7α-diol	1	40.73
5βB-3α,7β-diol	6	41.36
5βB-3β,7α-diol	3	39.49
5βB-3β,7α-diol	3	40.59
5βB-3α,12α-diol	1	39.76
5βB$^{8-14}$-3α,12α-diol	3	40.04
5βB-3α,12β-diol	3	39.71
5βB-3β,12α-diol	3	38.97
5βB-3β,12β-diol	3	38.85
5βB-3α,6α,7α-triol	2	41.75
5βB-3α,6β,7β-triol	2	40.89
5βB-3α,7α,12α-triol	1	41.44
5βB-3α,7α,12β-triol	3	40.91
5βB-3β,7α,12α-triol	5	40.40
5βB-3β,7α,12β-triol	3	39.75

*Abbreviated nomenclature: B = unsubstituted cholan-24-oic acid; the 5(αβ) prefix indicates proton orientation at the C_5 position; the superscript gives the position of a double bond.

**1 = Sigma, St. Louis, MO, U.S.A.
2 = Research Plus Steroids Laboratory, Denville, NJ, U.S.A.
3 = F. Chang, Department of Biochemistry, University of South Alabama, Mobile, AL, U.S.A.
4 = W.H. Elliott, Department of Biochemistry, St. Louis University Medical School, St. Louis, MO, U.S.A.
5 = A.F. Hofmann, Department of Medicine, University of California, San Diego, CA, U.S.A.
6 = P. Szczepanik, Argonne National Laboratory, Argonne, IL, U.S.A.
7 = G. Carlson, Gastroenterology Unit, Mayo Clinic, Rochester, MN, U.S.A.

The flame ionization detector temperature was 300°C. Nitrogen at 30 ml/min was the make-up gas. Calculation of peak areas was performed by the data system which was part of the HP-5880 gas chromatograph.

Retention times of BAMME derivatives were expressed as Kováts' retention indices using *n*-alkanes from C_{32} to C_{44}.

Choice of solvent and injector port temperature

The following solvents for the bile acids were used: methanol, ethyl acetate, dimethylformamide and dimethylsulfoxide. Since the trihydroxy bile acids (with four groups to be methylated) were thought to be the most difficult to derivatize, optimization studies were done with cholic acid. The area of the cholic acid BAMME peak was expressed as a ratio to 5α-cholestane (20 ng injected as internal standard). The injector port temperature was varied from 200—375°C.

Injection technique

Initially, 1 μl of TMAH was withdrawn into a 10-μl glass syringe. A 0.2—1.0 μl aliquot of the sample to be analyzed was then taken up followed by a further 1.0 μl of TMAH. This was performed immediately prior to injection.

RESULTS AND DISCUSSION

The use of different solvents for the bile acids had only a minor effect on the chromatograms. Methanol was adopted for the studies since it gave the smallest solvent front when the Poly S-179 coated capillary column was used, and was a good solvent for TMAH. It is probable that any effects the other solvents might have had were overcome by the preponderance of methanol in the TMAH reagent.

The injector port temperature was of far greater importance. Below 200°C no derivatives, partial or permethylated, were detected. Whilst the mono- and dihydroxy bile acids were converted to the permethylated forms at 250°C, maximum conversion of cholic acid to its permethylated form did not occur until 350°C, similar to results obtained for the methylation of fatty acids [5]. The small amount of partially methylated material was a problem when using the SP-2100 fused silica capillary column, since the very low interaction between the column and the free hydroxyl groups did not cause much change in retention volume. However, on Poly S-179 the difference between the partially methylated and permethylated forms was substantial and thus any incompletely methylated bile acid in no way interfered with the analysis. Although a sandwich injection technique was adopted, equally good data were obtained when injecting with the TMAH in front of or behind the sample. Premixing of TMAH and the sample resulted in no derivative peaks at all.

Application of this method to 32 different bile acid standards produced a single peak for each bile acid in the region of the chromatogram assigned to BAMME derivatives prepared as described previously [1]. Most could be resolved by the temperature program, 60 to 220°C at 30°C/min, 220 to 260°C at 3.5°C/min (Table I). The use of hydrogen as carrier gas enabled rapid analysis, the cycle time for each analysis being 18—20 min.

The method was reproducible and quantitative. The coefficient of variation for a 100-pmol sample injection ranged from 2.5% for chenodeoxycholate to 8.7% for cholate. Compared to authentic BAMME derivatives, derivatization was complete for the monohydroxy bile acids, 70% for dihydroxy bile acids and 50% for trihydroxy bile acids.

It has been claimed that TMAH is unstable. This has not been our experience. No attempt was made to keep the reagent cool, or to rigorously maintain anhydrous conditions. The original batch of reagent has been used for more than 15 months. Unlike commercial TMAH, no smell of dimethylaniline was detected in our reagent.

The use of commercial TMAH resulted in the appearance of contamination peaks in the region where BAMME derivatives were eluted. By contrast, TMAH synthesized in this laboratory was free of this contamination. It should be stressed that the contaminating peaks represent an extremely small percentage of the TMAH reagent and would not interfere necessarily with assays of substances of differing elution volumes to BAMME derivatives.

From comparison of the Kováts' retention indices for combination of bile acid derivatives, the contributions of the functional groups were determined (Table II). It can be seen that the greatest effects were caused by introduction of a hydroxyl group at the 3-position, being greater at 3α (4.02) than at 3β (3.01). Hydroxylation at the 12-position caused quite small changes, again being greater at 12α (0.67) than at 12β (0.31). At the 7-position, the β-orientated group (2.10) had a greater effect than the α-group (1.28). Hydroxylation at the 6-position had a more variable effect; in hyodeoxycholate (3α,6α-dihydroxy-5β-cholan-24-oate) the 6α-group caused a large change (2.56) whereas in hyocholate (3α,6α,7α-trihydroxy-5β-cholan-24-oate) the change was small (1.02). The latter could be due to proximity of the 7α-hydroxy group, thus reducing the effect. This was more so in the case of β-muricholate (3α,6β,7β-trihydroxy-5β-cholan-24-oate) which eluted more rapidly than hyodeoxycholate

TABLE II

EFFECTS OF SUBSTITUENTS ON KOVÁTS' RETENTION INDICES FOR BILE ACID DERIVATIVES

Substituent	Change (mean ± S.E.M., No. of comparisons)
3α-ol	4.02 ± 0.10 (3)
3β-ol	3.01 ± 0.07 (3)
6α-ol	1.02, 2.56
6β-ol	−0.47
7α-ol	1.28 ± 0.09 (7)
7β-ol	1.99, 2.20
12α-ol	0.67 ± 0.11 (4)
12β-ol	0.31 ± 0.06 (4)
Nor	−1.50, −1.50
Dinor	−3.50, −3.54
5α/5β	1.80
B^{3}*	0.51
B^{5}	1.74
B^{9-11}	−0.43, 0.54
B^{11}	−0.12, 0.05

*B^{n} = double bond between carbon atoms n and $n+1$, or as indicated.

or ursodeoxycholate, the 6β-group contributing a small negative effect (0.47). Removal of one or two methylene groups from the side chain caused consistent reduction in the retention index. Removal of two hydrogen atoms to form double bonds had a small and variable effect, the largest being due to the 5,6 double bond at the A/B ring junction. This double bond drastically alters the molecular shape by swinging the A-ring into the plane of the B-, C- and D-rings.

The application of TMAH for the pyrolytic formation of BAMME derivatives provides a simple, rapid and quantitative procedure for the analysis of unconjugated bile acids.

ACKNOWLEDGEMENTS

This study was supported in part by NCI grant No. CA-13148, NIH grant No. AM-25511 and faculty research funds of the University of Alabama in Birmingham Graduate School. D.G.P. is a research fellow of the Arthritis Foundation.

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Journal of Chromatography, 231 (1982) 161—165
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1292

Note

Simultaneous determination of tryptophan, serotonin and 5-hydroxyindoleacetic acid in rat brain by high-performance liquid chromatography using a weak acidic cation-exchange resin*

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(First received November 23rd, 1981; revised manuscript received March 10th, 1982)

Serotonin (5-HT) synthesis and metabolism in brain tissue are significantly influenced by L-tryptophan. For an understanding of the physiological role of serotonin, the simultaneous determination of tryptophan, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in brain tissue is important [1—3]. Recently, high-performance liquid chromatography (HPLC) coupled with fluorescence or amperometric detection has simplified the determination of many indoles

*Preliminary reports of this work have appeared previously [S. Hori, S. Ohtani, K. Ohtani and T. Ito, The Fourth Annual Meeting of the Japan Neuroscience Society, Kyoto, Japan, Abstract; Neurosci. Lett., Suppl. 6 (1981) S.51].

0378-4347/82/0000—0000/$02.75

[4—28]. In this paper, we have improved the quantitative determination of tryptophan, 5-HT and 5-HIAA, using a weak acidic cation-exchange resin.

MATERIALS AND METHODS

The weak acidic cation-exchange resin, Hitachi No. 3011C (10—15 μm, spherical), was from Hitachi (Tokyo, Japan). L-Tryptophan, 5-hydroxytryptophan (5-HTP), serotonin creatinine sulphate (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), L-tyrosine, 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylethylamine (dopamine), norepinephrine and epinephrine were from Nakarai Chemicals (Kyoto, Japan). All other chemicals were of analytical reagent grade and used without further purification.

The liquid chromatograph and detection system consisted of a Hitachi 645A pump, a high-pressure sampling valve (Hitachi) and a Hitachi 3011C weak acidic cation-exchange column (4.6 × 250 mm) with a steel jacket. A Hitachi fluorescence spectrophotometer, Model 650-10S, was used; excitation and emission wavelengths were 280 and 340 nm, respectively.

For the column preparation, the packing material (about 2.5 g of Hitachi 3011C resin) was dispersed in methanol—water (1:1, v/v) and packed into the stainless-steel column under high pressure (less than 150 kg/cm^2) using the slurry packing method.

Rat brain (0.1—0.5 g) was homogenized with 0.1 *M* $HClO_4$ (4:1, v/w) by a Handy sonic homogenizer (Model UR-20P, Tomy Seiko, Tokyo, Japan) [15, 22]. After 5 min centrifugation at 25 000 *g* (4°C), the compounds were determined directly by injecting 20—50 μl of the supernatant into the chromatograph.

RESULTS AND DISCUSSION

Separation of tryptophan, 5-HTP, 5-HT and 5-HIAA

The elution patterns of tryptophan, 5-HTP, 5-HT and 5-HIAA on a column of porous polystyrene—divinylbenzene polymer with —COOH as the active functional group (Hitachi Gel 3011C) are significantly affected by the pH, ionic strength and methanol concentration of the mobile phase [29]. As shown in Fig. 1A, pH 4.0—4.4 was the most convenient range of the mobile phase. Increasing the methanol concentration of the mobile phase resulted in decreased retention times (Fig. 1B); it did not affect the separation sequence or the symmetry of the peaks. Ionic strength has a pronounced effect on the retention times of tryptophan, 5-HTP, 5-HT, and 5-HIAA. With 0.8 *M* citrate buffer, the retention time of 5-HT was markedly reduced (Fig. 1C); with 1.2 *M* citrate buffer, the retention time of tryptophan and 5-HTP was slightly reduced while that of 5-HIAA was slightly increased.

Based on these results, the elution conditions selected were 0.5 *M* citrate—sodium citrate (pH 4) containing 20% methanol; the flow-rate was 0.2 ml/min at 60°C.

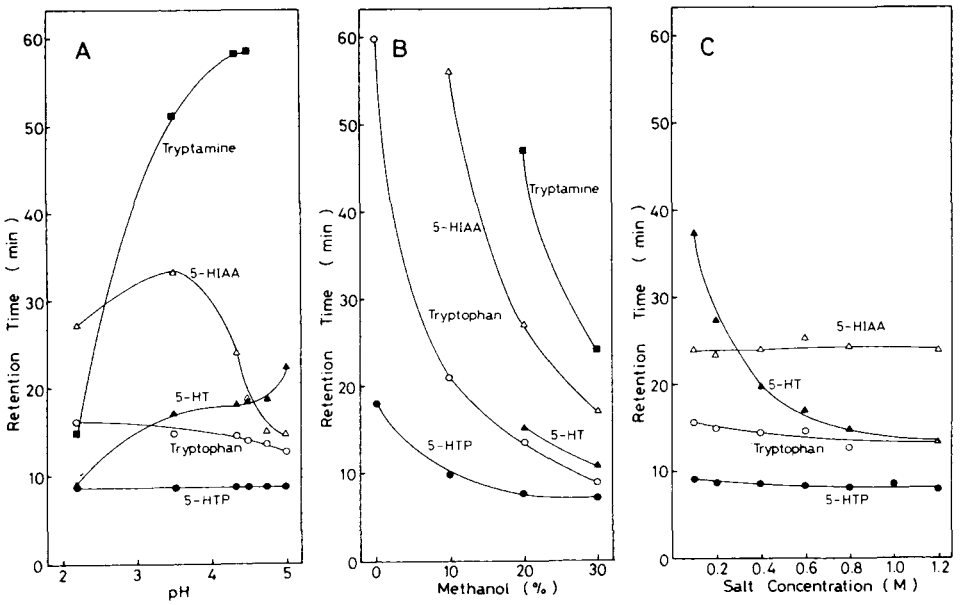

Fig. 1. Retention times of tryptophan, 5-HTP, 5-HT, 5-HIAA and tryptamine. (A) Effect of pH. The mobile phase was 0.5 *M* citrate—sodium citrate containing 20% methanol. (B) Effect of the methanol concentration. The mobile phase was 0.5 *M* citrate—sodium citrate (pH 4). (C) Effect of the salt concentration. The mobile phase was citrate—sodium citrate (pH 4) containing 20% methanol.

Determination of tryptophan, 5-HT and 5-HIAA in rat brain

The standard solution contained 100 pmoles of tryptophan, 5-HTP, 5-HT, 5-HIAA, tyrosine, DOPA, dopamine, norepinephrine, and epinephrine in 1 μl of 0.1 *M* $HClO_4$, and was stored at −80°C in a freezer. Before use, it was diluted to 5, 7.5 and 25 pmoles in 50 μl of 0.1 *M* $HClO_4$. Tyrosine, DOPA, dopamine, norepinephrine, and epinephrine elution preceded other elutions by 10 min; the elution of 5-HTP which, under the conditions used, had a retention time of 8 min, overlapped with dopamine. Tryptophan, 5-HT and 5-HIAA were eluted at 13.0, 19.8 and 25.6 min, respectively (Fig. 2A). Fig. 2B demonstrates the separation of tryptophan, 5-HT and 5-HIAA from rat brain extract. Compounds in the extract did not affect either the separation or the fluorometric detection* of the indoles. The tryptophan, 5-HT and 5-HIAA content of rat cerebral cortex, brain stem and cerebellum is given in Table I. The values obtained with our HPLC method are close to those reported by others [12, 30—33]. The detection limits (signal-to-noise ratio of 2) of tryptophan, 5-HT and 5-HIAA were 0.25, 0.1 and 0.5 pmole, respectively, which were comparable to or even better than the amperometric detection. In conclusion, the simultaneous quantitation of these indoles was simplified by the combination of the improved acidic weak cation-exchange resin and fluorometric detection.

*But if amperometric detection was used, unknown materials were detected near the peaks of the indoles (data not shown).

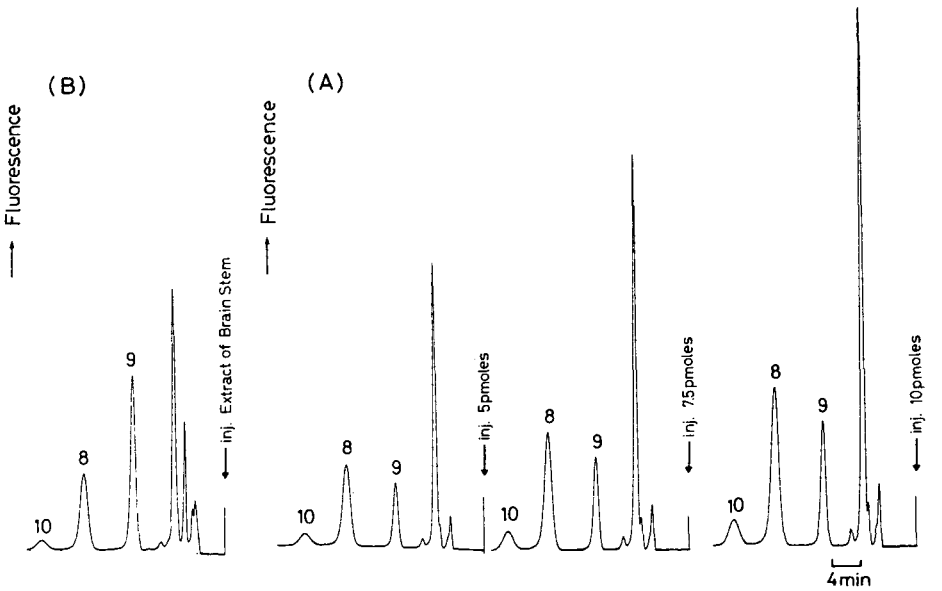

Fig. 2. Separation of tryptophan, 5-HT and 5-HIAA. (A) Separation of the standard solutions. (B) Separation of tryptophan, 5-HT and 5-HIAA in an extract from rat brain stem. Peaks 8, 9 and 10 were identified as tryptophan, 5-HT and 5-HIAA, respectively.

TABLE I

TRYPTOPHAN, 5-HT AND 5-HIAA CONTENT OF RAT BRAIN

Values are nmoles/g wet tissue ± S.D. The number of experiments is given in parentheses.

	Tryptophan	5-HT	5-HIAA
Cerebral cortex	12.8 ± 1.37 (4)	3.51 ± 0.55 (4)	1.95 ± 0.31 (4)
Brain stem	11.5	4.15	2.40
	10.1	3.64	2.15
Cerebellum	13.5	0.45	0.80
	11.6	0.44	0.30

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Journal of Chromatography, 231 (1982) 166—172
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1294

Note

Separation of indole metabolites from urine with an ODS type resin by high-performance liquid chromatography

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(First received January 11th, 1982; revised manuscript received February 22nd, 1982)

The major pathway of degradation of tryptophan in man begins with the cleavage of the indole ring by tryptophan dioxygenase to yield kynurenine, and only a few per cent of administered tryptophan have been reported to be excreted as indole metabolites [1]. In metabolic disorders such as Hartnup disease [2,3], phenylketonuria, etc., large amounts of indole metabolites have been reported to be excreted in the urine [4].

In the present paper, a new ODS type of column (Hitachi No. 3053), which is octadecylsilane-treated silica gel, is introduced for rapid analysis of various indoles in urine by high-performance liquid chromatography (HPLC). Proper extraction of the metabolites from urine in the case of siblings manifesting malabsorption and malelimination of plasma tryptophan may be applicable to detect increased amounts of indole metabolites for the investigation of tryptophan metabolism.

Ether extraction of indole metabolites [5] is recommended to detect all the indole metabolites except for indoleacetic acid, which was efficiently recovered by chloroform [4] prior to phosphate buffer extraction.

MATERIALS AND METHODS

Twenty-four-hour urines were collected from siblings in whom malabsorption of tryptophan from the intestine was observed along with retarded elimination of plasma tryptophan upon oral loading of tryptophan. A tryptophan loading test (100 mg/kg body weight) was conducted on three siblings 3, 4 and 5 years old. In the youngest case, in whom the symptoms were most dominant, niacin was administered for two months at 100 mg a day, then another tryptophan loading test was conducted. Aliquots of the collected urine was treated according to the method of Weissbach et al. [4] to detect urinary indole-3-acetic acid (IAA). This procedure has been reported to extract mainly IAA and indolelactic acid (ILA) without extracting appreciable amounts of other indole metabolites, corresponding to chloroform-extractable indoles. 5-Hydroxyindoleacetic acid (5-HIAA) and other indole metabolites have been reported to be extractable in ether according to Udenfriend et al. [5], corresponding to ether-extractable indoles.

Briefly, for the extraction of indole metabolites, to 4 ml of urine 0.36 ml of concentrated HCl was added and heated for 15 min at 100°C in a stoppered tube to hydrolyze bound metabolites. Then 10 ml of chloroform or ether were added and shaken for 5 min; then 8 ml of the added solvent were transferred to another stoppered tube to which 0.5 ml of 0.5 *M* phosphate buffer (pH 7.0) was added and shaken for 5 min. For ether extraction, NaCl was added to saturation, then 0.4 ml of aqueous phase was pipetted out and 50 μl were applied to the ODS column (Hitachi 3053 gel) in the high-performance liquid chromatograph (Hitachi Model 635A). The column size was 4 × 150 mm, particle size range 4—6 μm, elution was carried out by 1% of 1 *M* sodium acetate, 4% of 1 *M* acetic acid, 10% of 1 *M* Na_2SO_4 and 25% of methanol in water. The flow-rate was 1.0 ml/min; detection was by ultraviolet (UV) light at 280 nm.

RESULTS AND DISCUSSION

The elution profiles of various indole metabolites are shown in Fig. 1. Authentic metabolites eluted are sharply differentiated except for tryptamine and ILA, the latter substance being eluted as a broader peak after the tryptamine peak. Indolepyruvic acid (IPA) is also eluted late as a small peak due to low sensitivity to UV light. Both ILA and IPA were applied in amounts five times as high as other metabolites giving lower and broader peaks. Urinary indoles were extracted and analyzed from the youngest siblings before and after tryptophan loading, as shown in Fig. 2. The elution profile of urinary indoles extracted in chloroform is shown in Fig. 2a, while the profile of those extracted in ether is shown in Fig. 2b. Unlike other indole metabolites, IAA is far less extractable from urine by ether than by chloroform, as has been reported by others [4]. In the detection of indole substances derived from tryptophan, chloroform-extractable IAA is considered to be a main metabolite in normal subjects; therefore, in the present study of tryptophan metabolism other than by the kynurenine pathway, the primary choice of extraction procedure may be with chloroform. Two major indole metabolites in urine

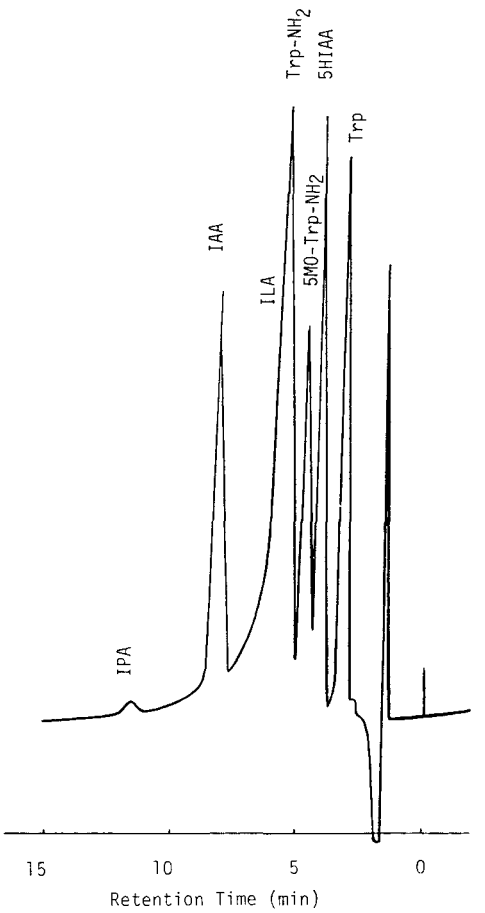

Fig. 1. HPLC elution profiles of indole derivatives. The mixture of authentic derivatives was applied on Hitachi ODS type resin (Hitachi 3053 gel), column size 150 × 4 mm I.D., eluted with 1% of 1 *M* sodium acetate, 4% of 1 *M* acetic acid, 10% of 1 *M* Na_2SO_4, and 25% of methanol in water, detected by UV light at 280 nm; 1 μg of each substance was applied except ILA and IPA (5 μg of each). Trp = tryptophan; Trp-NH_2 = tryptamine; 5MO-Trp-NH_2 = 5-methoxytryptamine.

of three siblings are quantitated by this method as shown in Table I. In these cases, about 3% of orally administered tryptophan is recovered as IAA over basal excretion before loading, while an increasing amount of ILA is observed in the youngest sibling in whom tryptophan absorption from intestine and elimination from plasma were considerably disturbed, and yet no influence of niacin treatment was observed. However, the excreted amount of ILA was lower after niacin treatment, probably because the plasma level of tryptophan did not attain even half the maximal value of the control, which was worse than the previous loading test. On the other hand, ILA was less extractable from older siblings: less than 1% of administered tryptophan was recovered in case 3 in whom almost normal absorption and elimination of tryptophan was observed.

TABLE I

URINARY EXCRETION OF INDOLE ACIDS IN 24-h URINE AFTER TRYPTOPHAN LOADING (100 mg/kg)

Patient	Dose (g)	Indoleacetic acid			Indolelactic acid		
		Pre-load (mg)	Post-load (mg)	Yield (%)	Pre-load (mg)	Post-load (mg)	Yield (%)
Case 1-I	1.5	65.4	109.0	2.9	4.9	110.2	7.0
1-II*	1.5	36.5	106.6	4.6	8.6	59.8	3.4
Case 2	1.8	31.1	83.4	2.9	n.d.**	40.0	2.2
Case 3	2.0	52.0	112.3	3.0	n.d.	15.0	0.8

*After niacin treatment (100 mg/day for two months).
**n.d. = not detectable.

It may be concluded that the metabolic disorder in the present study is the unusual excretion of ILA in the urine at the younger age, rather than of IAA which has been reported to be the main indole metabolite of tryptophan.

Other indole metabolites were extracted in ether and quantitated as shown in Table II. From these analyses, tryptophan itself was not always recovered in larger amounts after tryptophan loading than the preloading basic urinary excretion level, but is recovered as other indole metabolites especially in the form of ILA in this study.

As has been reported, IAA was mostly extractable by chloroform but poorly recovered by ether (cf. Tables I and II), whereas in the case of ILA ether extraction was found to be more efficient: as much as 2—3 times was recovered compared to chloroform extraction.

In these studies, administered tryptophan was constantly recovered as IAA, which was around 3% of loaded tryptophan (Table I), and a fairly large amount of ILA was detected in the urine after tryptophan loading, which is probably due to retarded absorption of tryptophan from the intestine where it is presumably metabolized to indole derivatives by intestinal bacteria rather than absorbed and excreted in the urine without being utilized as a nutrient. Or the metabolic pathway of indoles rather than kynurenine formation may be more active in the present subjects and recovered normal metabolic ability as their age advances which are clinically manifest as retarded physical development accompanying urinary odors of indoles in their younger stage.

It has been reported that in phenylketonuria a large proportion of the chloroform-extractable indoles was ILA, but the highest unusual chloroform-extractable indole is IAA in diabetes, neuromuscular disorders and idiopathic sprue [4].

ILA was excreted in all the cases in the present study, as a basic excretion of indoles besides IAA, and rapid and sensitive analyses of these metabolites by HPLC may be recommended to investigate such metabolic disorders previously detected by thin-layer chromatography or measurement of indoles by colorimetry using xanthydrol [4] or Ehrlich's aldehyde reagent [6].

It is also interesting to note that other indole derivatives such as 5-HIAA, 5-methoxytryptamine or tryptamine, which are metabolic derivatives of 5-hydroxytryptamine, are less influenced by the tryptophan loading test in the present study.

TABLE II

URINARY EXCRETION OF INDOLE METABOLITES IN 24-h URINE BEFORE AND AFTER TRYPTOPHAN LOADING (100 mg/kg)

Patient	Dose (g)		Tryptophan (mg)	5-HIAA (mg)	5-Methoxytryptamine (mg)	Tryptamine (mg)	ILA (mg)	IAA (mg)	IPA (mg)
Case 1-I	1.5	Pre	11.81	1.78	2.42	0.43	1.04	1.1	—
		Post	4.79	3.98	2.11	—	331.20	4.22	—
Case 1-II*	1.5	Pre	3.57	1.61	1.98	0.50	2.44	1.60	1.95
		Post	6.47	3.95	2.79	2.26	88.17	3.40	2.99
Case 3	2.0	Pre	3.40	3.26	3.56	1.31	7.60	2.33	—
		Post	9.66	4.92	3.20	—	27.43	3.82	2.08

*After niacin treatment (100 mg/day for two months).

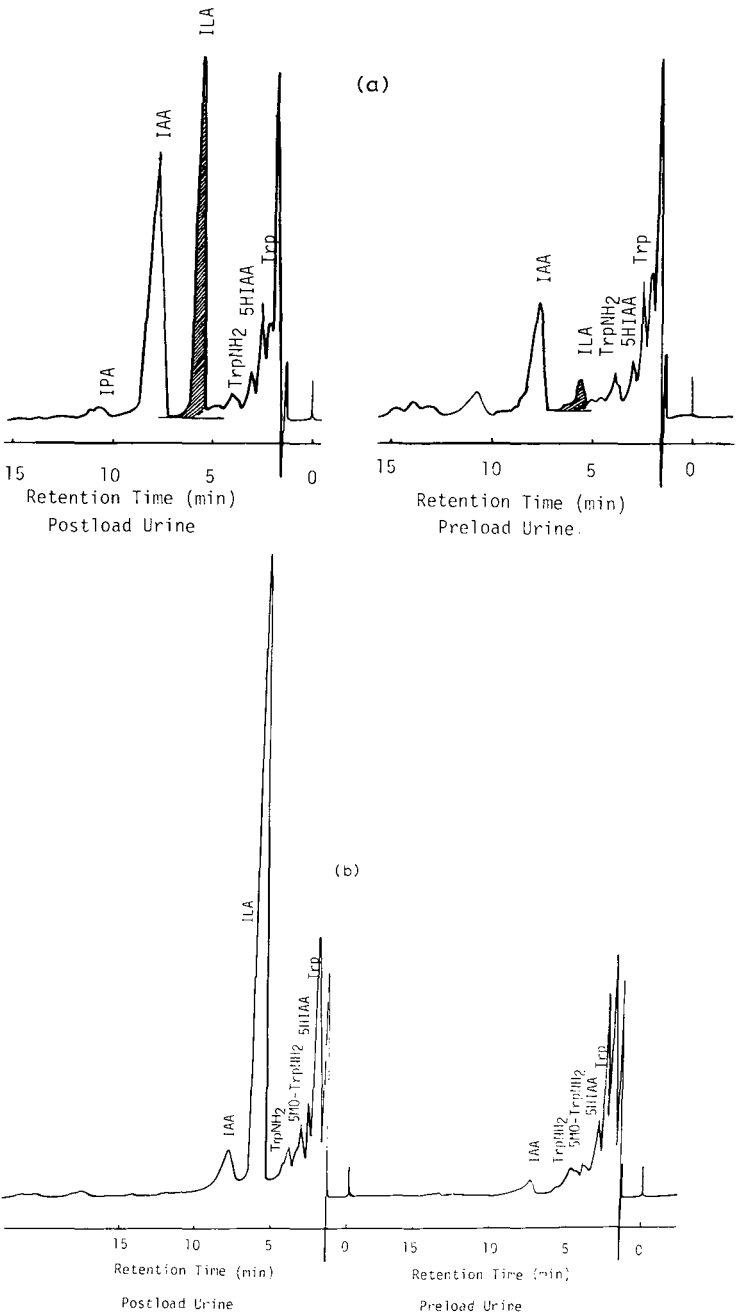

Fig. 2. (a) HPLC analysis of chloroform-extractable indoles in urine. An aliquot of urine was pretreated with chloroform, then extracted in phosphate buffer (see text) and applied on Hitachi 3053 gel, before and after tryptophan loading in a case of malabsorption and excretion of tryptophan. (b) HPLC analysis of ether-extractable indoles in urine. An aliquot of 24-h urine was pretreated with ether, then extracted in phosphate buffer (see text) and applied on Hitachi 3053 gel, before and after tryptophan loading in a case of malabsorption and excretion of tryptophan. For abbreviations see Fig. 1.

ACKNOWLEDGEMENTS

ODS type resin (Hitachi No. 3053 gel) was kindly provided by Nissei Sangyo Co.

Thanks are due to Professors Y. Matsumura and K. Furiya for their support.

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Journal of Chromatography, 231 (1982) 173—177
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1298

Note

Analysis of peptides in tissue and plasma

Use of silicic acid extraction and reversed-phase columns for rapid purification prior to radioimmunoassay

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(Received December 22nd, 1981)

Since the discovery of the endogenous opiate peptides, met- and leu-enkephalin [1] and the endorphins [2, 3], many workers have developed assay systems for the measurement of these compounds in tissue and in plasma. Both radioimmunoassay (RIA) [4—7] and high-performance liquid chromatography (HPLC) [8] or HPLC with bioassay or receptor assay [9, 10] have been used effectively to quantitate these peptides. When large scale studies are undertaken which generate many samples, RIA is the method of choice as hundreds of samples can be processed in a working day. Because antigen—antibody binding is affected by the presence of impurities in the incubation mixture, adequate sample purification prior to RIA is necessary in order to obtain accurate data. Gay and Lahti [11] have recently reported the use of Sep-Pak reversed-phase cartridges for the separation of radioactive and synthetic enkephalins and endorphins. We have developed two simple rapid procedures for sample preparation, one which uses the reversed-phase Sep-Pak cartridges to coelute endogenous met-enkephalin, leu-enkephalin, and β-endorphin from rat brain tissue extracts and the other for plasma which uses silicic acid extraction. The peptides are co-eluted in a single sample for convenience and the procedure is suitable in situations in which the antibodies employed in the RIA are specific for each peptide.

MATERIALS AND METHODS

Brain parts were sonicated in 1 ml 0.2 *N* hydrochloric acid—acetone (25:75)

0378-4347/82/0000—0000/$02.75

containing phenylmethylsulfonyl fluoride, an enzyme inactivator (0.2 mg/ml) and pentachlorophenol (0.02 mg/ml) in Eppendorf plastic tubes. After centrifuging at 12,800 *g* in a Brinkman Eppendorf centrifuge, the supernatant was decanted into a silanized glass 12 × 75 mm test tube, the pellet resuspended in 0.75 ml hydrochloric-acid—acetone, and centrifuged. The supernatants were combined and dried under nitrogen until almost dry. The drying was completed in a vacuum oven. The residue was taken up in 2.0 ml quartz-distilled water and allowed to incubate for 1 h at 4° C.

Sep-Pak reversed-phase C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were used for adsorption of the peptides. These cartridges were used with a Vac-Elut (Analytichem International, Harbor City, CA, U.S.A.) — an evacuated metal box with 10 ports on the top lid. One end of the C_{18} cartridge was inserted into a plastic pipette tip (1—200 μl size, Robbins Scientific Corp., Mountain View, CA, U.S.A.), which in turn went into a port. The other end of the cartridge was attached to a 3-ml plastic disposable syringe. If a Vac-Elut is unavailable, the sample and reagents may be pushed through by means of the syringe plunger. The C_{18} reversed phase was activated before sample application by passing through it 2 ml acetone followed by 5 ml quartz-distilled water. Glass wool was placed in the bottom of the syringe to filter the sample before it reached the C_{18} cartridge. Following sample application, the cartridges were washed twice with 2 ml 4% acetic acid, and met- and leu-enkephalin and β-endorphin were eluted in 1.5 ml 0.2 *N* hydrochloric acid—actone (25:75). The eluate was dried under nitrogen in a vacuum oven and the residue taken up in an appropriate volume of RIA buffer.

The cartridges may be regenerated and used again. After sample elution an additional 3 ml of the hydrochloric acid—acetone was passed through and the cartridges were stored in methanol. Before use, acetone and water were passed through as described above.

Plasma may be applied directly onto the cartridge, but when only β-endorphin-like immunoreactivity was to be measured by RIA, another method that is both rapid and economical was employed. A round disk of filter paper was placed in the bottom of a 3-ml plastic disposable syringe, wetting the paper slightly to keep it in place. Dry silicic acid (Bio-Sil A, 200—400 mesh, Bio-Rad Labs., Richmond, CA, U.S.A.) was added to the 1.5-ml mark using a small plastic dropper bottle and the syringes put directly into the ports of the Vac-Elut. The silicic acid was well wetted with quartz-distilled water. Plasma (1.5 ml) was adjusted to pH 4.0 with 100 μl 1 *N* hydrochloric acid and poured onto the silicic acid. The silicic acid was washed twice with 1.5 ml quartz-distilled water followed by 1.5 ml 1 *N* hydrochloric acid using wash bottles. β-Endorphin was eluted with 2.0 ml 50% acetone. The eluate was taken to dryness under nitrogen and vacuum and the residue reconstituted in RIA buffer. The syringes were discarded after use.

RESULTS AND DISCUSSION

Recovery was monitored by adding iodinated peptide to either brain extracts or plasma and was found to be quite high, 96—98% for all peptides with the exception of iodinated β-endorphin where the recovery was 88%. The S.E.M.

of recoveries for leu- and met-enkephalin and β-endorphin were under 1%. In order to determine whether brain tissue concentration affected recovery, various amounts up to 1 g were extracted with hydrochloric acid—acetone, dried, and passed through the cartridges. The recoveries of the three peptides remained unchanged with the S.E.M. still under 1%. Similarly, cold peptide in concentrations to 1 mg was added to tissue extracts and adsorbed onto the reversed phase with no alteration in recovery. Iodinated β-lipotropin (β-LPH) and dynorphin(1—13) were also adsorbed onto and eluted from these columns with similar results. Approximately 10% of the β-endorphin adsorbed to the silanized glass test tube when the sample was applied to the silicic acid or C_{18}. The β-endorphin was recovered by putting the hydrochloric acid—acetone or 50% acetone into the tube and vortexing well before eluting.

Silicic acid has been used for the extraction of β-endorphin [7] and adrenocorticotrophin [12] from plasma. These procedures involve time consuming end-to-end shaking of the sample with silicic acid, followed by centrifugation and more shaking to elute. Using the column method described above, ten samples can be processed through either column in approximately 5 min with an improved recovery. Table I shows the levels of met- and leu-enkephalin and

TABLE I

PEPTIDE CONCENTRATIONS IN VARIOUS REGIONS OF RAT BRAIN

Five male adult rats were decapitated, their brains removed, and dissected into the areas shown. Protein was determined by the method of Lowry et al. [13]. Data are given in pmol/mg protein ± S.E.M.

Brain area	Met-Enkephalin	Leu-Enkephalin	β-Endorphin
Caudate	12.6 ± 0.95	1.85 ± 0.22	
Hypothalamus	8.21 ± 0.39	2.41 ± 0.56	0.61 ± 0.04
Septum	3.66 ± 0.44	0.62 ± 0.11	0.06 ± 0.01
Brain Stem	2.49 ± 0.08	0.61 ± 0.05	
Mid Brain	2.03 ± 0.09	0.53 ± 0.04	0.03 ± 0.005
Cortex	1.76 ± 0.16	0.25 ± 0.03	
Hippocampus	1.10 ± 0.08	0.20 ± 0.01	
Cerebellum	0.96 ± 0.04	0.12 ± 0.04	

β-endorphin-like immunoreactivity measured by RIA in various regions of rat brain using the reversed-phase C_{18} separation procedure. Using silicic acid extraction, human β-endorphin-like immunoreactivity was equivalent to approximately 6—31 fmol human β-endorphin per ml of plasma. Because the antibody used in the RIA is directed against the C-terminal portion of β-endorphin and crossreacts 100% on a molar basis with β-LPH, these concentrations represent β-LPH as well as β-endorphin-like immunoreactivity.

Serial dilutions of tissue extracts were superimposable on the standard RIA curves of each peptide. A typical met-enkephalin RIA standard curve is shown in Fig. 1 with several dilutions of rat brain caudate, cortex, and midbrain samples.

The silicic acid and reversed-phase column procedures as described are useful for rapid purification of peptides in which the assay will be performed

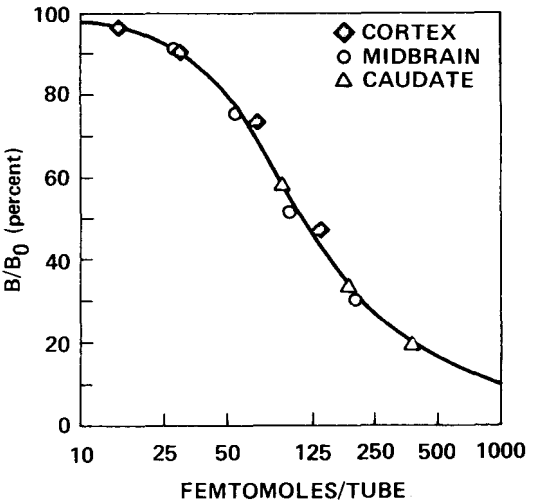

Fig. 1. Comparison of RIA standard curve for met-enkephalin and serial dilutions of various brain region extracts. Procedure as described in text. B/B_0 is binding of ^{125}I-labelled met-enkephalin in the presence of competing peptide compared to binding of ^{125}I-labelled met-enkephalin alone, expressed as percent.

with characterized and specific antibodies. We have demonstrated that the method allows collection from biological samples of various opioid peptides and it can be expected that other opioid and non-opioid peptides are obtained in the collected fraction. Thus, the usefulness of these coelution purification procedures will depend upon the availability of highly specific antibodies. In situations in which such antibodies are not available, or in other situations in which there is reason to believe there may be known or unknown substances which would significantly crossreact with the available antibodies, other more specific isolation procedures will be necessary. Gradient elution using these or related reversed-phase columns may be helpful in such situations. Within the confines of a situation for which it is suited, the described methods are rapid, reproducible, facilitate processing of large numbers of samples, and are easily used for purification of peptides from tissue samples preparatory to specific assay.

ACKNOWLEDGEMENTS

This work supported by NIDA grant DA 02107 and NIMH grant MH 23861. We thank Dr. John Madden for helpful discussions and Sue Poage for her assistance in preparing the manuscript.

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Journal of Chromatography, 231 (1982) 178—182
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1307

Note

Direct isolation of β-endorphin from plasma by column chromatography

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(First received November 17th, 1981; revised manuscript received March 22nd, 1982)

The neuropeptides β-endorphin and β-lipotropin (β-LPH) are secreted in parallel with adrenocorticotrophin (ACTH) into peripheral blood by the pituitary in response to stress and other ACTH-releasing stimuli in the normal human adult [1]. Although β-LPH has no defined biological role, β-endorphin has potent morphine-like activity [2].

The currently available radioimmunoassay (RIA) procedures for measurement of β-endorphin cross-react with β-LPH [1]. To measure the endogenous opiate activity attributable to β-endorphin it is therefore necessary to separate β-LPH from β-endorphin. This paper describes a simplified column chromatography procedure for the isolation of β-endorphin directly from plasma, eliminating the need for an initial extraction procedure [1, 3, 4]. The column chromatography procedure is compared with octadecasilyl-silica (ODS-silica) cartridge extraction [5].

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0378-4347/82/0000—0000/$02.75

MATERIALS AND METHODS

[^{125}I]β-LPH, [^{125}I]β-endorphin and ODS-silica cartridges were obtained commercially from Immuno Nuclear Corp., Stillwater, MN, U.S.A.

Collection of samples

Samples of placental blood (10 ml) were collected in disposable plastic syringes, immediately transferred to tubes containing EDTA as anti-coagulant (Sequestrene) and centrifuged (1000 *g*) at 4°C. Plasma was carefully separated and frozen at −20°C until used for assay.

Chromatography

Plasma (1 ml) was chromatographed directly (without prior extraction) on a 40 × 1 cm Sephadex G-50 (superfine) column (Pharmacia Fine Chemicals, Uppsala, Sweden) with 0.5% bovine serum albumin, fraction V (BSA) in 0.05 *M* borate buffer (pH 8.4). An LKB 2111 Multirac fraction collector (Bromma, Sweden) was utilized and with a slight modification eluates from three columns were collected. A waste volume of 35 min was allowed before collecting fractions at 2.5-min intervals. The column flow-rate (gravity) was 15 ml/h. Each column was primed before β-endorphin isolation by chromatographing a blank plasma sample. Prior to extraction of placental plasma samples, the columns were calibrated by chromatographing a plasma sample containing [^{125}I]β-endorphin. Each fraction (tube) collected was counted for radioactivity (Packard, PGD-Auto-Gamma, Downers Grove, IL, U.S.A.) to identify the endorphin peak. Placental samples were then chromatographed and buffer from the collection tubes corresponding to the β-endorphin fraction was pooled and lyophilized.

Buffer (1 ml 1% BSA in 0.1 *M* borate) spiked with [^{125}I]β-endorphin and [^{125}I]β-LPH was chromatographed on a 40 × 1 cm Sephadex G-75 (superfine) column similar to that described above. Due to the slower flow-rate (4.5 ml/h) a waste volume time of 90 min was allowed before collecting fractions at 7.5-min intervals.

ODS-silica cartridge extraction

Plasma samples (1 ml) were extracted and assayed for β-endorphin according to the procedure of Immuno Nuclear Corp. [5]. Briefly, the plasma was firstly agitated (4°C) with Sepharose anti-β-LPH particles (3 or 24 h) in an attempt to remove β-LPH. Plasma was then acidified and applied to the cartridges. The cartridges were washed with acetic acid (4%) and then β-endorphin was eluted with methanol. The methanol eluate was evaporated to dryness using a gentle flow of compressed air. The resulting residue was reconstituted in 1% BSA—borate buffer prior to RIA.

Radioimmunoassay

Plasma β-endorphin levels were measured using the RIA kit purchased from Immuno Nuclear Corp. The lyophilized samples were reconstituted in distilled water (1 ml) prior to RIA.

RESULTS AND DISCUSSION

[^{125}I]β-Endorphin added to buffer (1 ml 1% BSA in 0.1 *M* borate) was adequately separated from [^{125}I] LPH using Sephadex G-50 (Fig. 1A) but not Sephadex G-75 (Fig. 1B). A higher concentration of [^{125}I]β-LPH than [^{125}I]β-endorphin was used and is depicted in Fig. 1A to confirm there was little carry over of the β-LPH into the β-endorphin peak. This was necessary as β-LPH concentrations in plasma are considerably higher than β-endorphin [1, 6]. Spiked plasma chromatographed slightly slower than the spiked buffer solution (Fig. 1A) probably due to plasma protein constituents. Previous methods for the separation of β-LPH and β-endorphin have involved extraction of plasma (5 ml or greater) with talc [1, 4] and silicic acid [3]. The extracted plasma samples were then chromatographed in Bio-Gel P60 [3] or Sephadex G-50 [1, 4]. Sephadex G-75 has also been used for cerebrospinal fluid [2] and plasma separation [5].

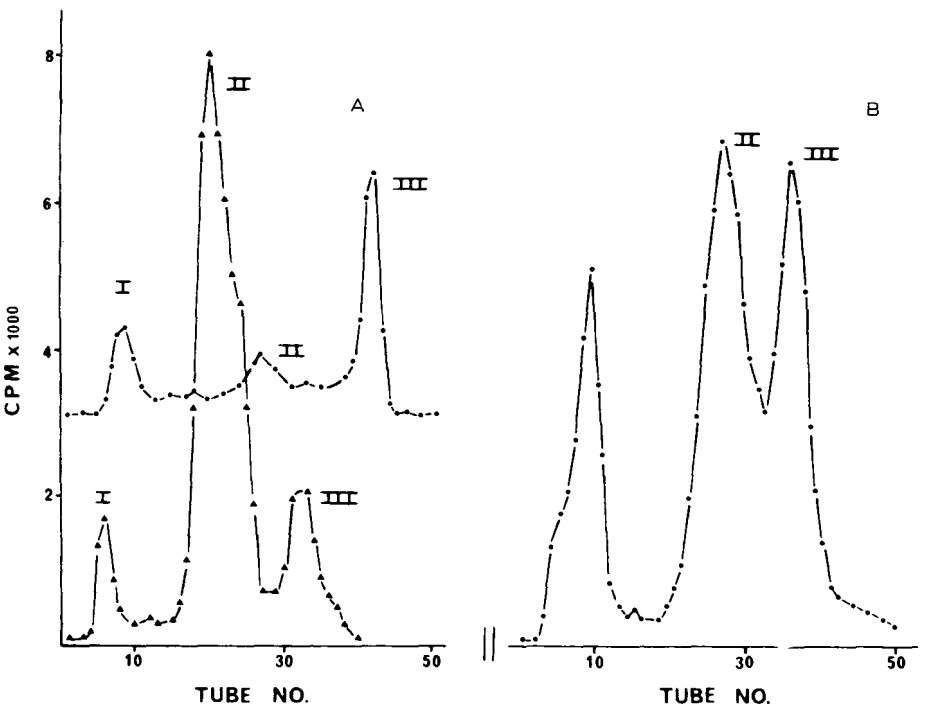

Fig. 1. (A) Elution pattern of a high-molecular-weight immunoreactive substance (I), β-LPH (II) and β-endorphin (III) standards in buffer (▲—▲) and plasma (●—●) from a Sephadex G-50 column. (B) Elution pattern of β-LPH (II) and β-endorphin standards (III) in buffer from a Sephadex G-75 column.

Unlike uniodinated endorphin, [^{125}I]endorphin appears to bind to plasma protein [5]. Peak I (Fig. 1A) may be due to contamination of the ^{125}I-standards with high-molecular-weight immunoreactive material and/or some β-endorphin covalently bound to plasma proteins [3]. We found that if [^{125}I]β-endorphin was added to plasma and left to stand (4°C) for 7 days and then

chromatographed (Sephadex G-50), the separation was lost. The radioactivity was fairly evenly spread over all collected fractions. Wilkes et al. [3] observed that 15% of the radioactivity migrated at the void volume after chromatography of plasma incubated overnight with [^{125}I]β-endorphin. In the present study a freshly prepared [^{125}I]β-endorphin-spiked plasma sample was chromatographed. The buffer eluents corresponding to peak III (Fig. 1A) were pooled, lyophilized and subsequently reconstituted in plasma. The position of the immunoreactive β-endorphin did not alter nor did the radioactivity redistribute on rechromatography of the reconstituted sample. The loss of total radioactivity during lyophilization is negligible. Furthermore, recovery of radioactivity from the column chromatograph was always greater than 94%.

Buffer eluents from tubes 35—44 after placental plasma chromatography were pooled, lyophilized and reconstituted in distilled water for RIA. The mean β-endorphin level of these placental plasma samples chromatographed on Sephadex G-50 was 133 ± 37.7 pg/ml (S.D.) (Table I), a level close to previously reported [6] umbilical arterial concentrations (114 ± 44 pg/ml).

TABLE I

β-ENDORPHIN LEVELS DETECTED BY RIA AFTER (A) COLUMN CHROMATOGRAPHY (SEPHADEX G-50) AND (B) ODS-SILICA CARTRIDGE EXTRACTION

Sample No.	A (pg/ml)	B (pg/ml)
1	165	100
2	103	23
3	122	75
4	180	93
5	95	54
Mean	133 ± 37.7	69 ± 31.3

The use of cartridges for isolation of neuropeptides has recently been described [5, 7]. Rapid elution time has been cited as an advantage. We found ODS-silica extraction does not offer any advantage over Sephadex G-50 column chromatography. Sepharose anti-β-LPH particles did not completely remove β-LPH from spiked plasma samples, even after agitation for 24 h. Loss of radioactivity was observed at various stages of ODS-silica extraction. When acidified, β-LPH-stripped, plasma samples (n = 4) were applied to the cartridges, 22.0 ± 4.5% (S.D.) of the radioactivity was not retained by the cartridge. Sephadex G-50 chromatography of this initial cartridge eluent gave a similar chromatogram to that shown in Fig. 1A, indicating β-endorphin was not completely retained by ODS-silica. A further loss of 14.1 ± 1.3% (S.D.) of the radioactivity occurred after the acetic acid wash and 3.0 ± 0.8% (S.D.) of the radioactivity remained on the cartridge after the final wash with methanol. The mean β-endorphin level of the ODS-silica extracted plasma samples was 69 ± 31.1 pg/ml (S.D.) (Table I). This level is significantly lower than that detected by column chromatography of identical placental plasma samples (133 pg/ml).

In conclusion, a reproducible procedure for the determination of β-endorphin from small quantities of plasma (1 ml) has been developed. This

method is being applied to measurements of plasma and cerebrospinal fluid β-endorphin levels in premature infants. Chromatography of plasma samples without prior extraction or β-LPH stripping minimizes the potential loss of endorphins.

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Journal of Chromatography, 231 (1982) 183—188
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1302

Note

Specific simultaneous assay of hypoxanthine and xanthine in serum by reversed-phase high-performance liquid chromatography using an immobilized xanthine oxidase reactor

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(First received December 30th, 1981; revised manuscript received March 16th, 1982)

The simultaneous determination of hypoxanthine and xanthine in serum and urine has been required for various pharmacological and physiological reasons [1—3]. Several analytical procedures have been developed for these oxypurines at biological levels involving the use of high-performance liquid chromatography (HPLC) [4—7]. However, because many other compounds that absorb at 254 nm are present in serum and urine, good separations by HPLC usually require a long time and accurate quantitation is impossible. It is also difficult to determine these oxypurines simultaneously at 280 nm because of the absence of a strong UV absorption of hypoxanthine at this wavelength.

We first prepared xanthine oxidase bound to controlled pore-glass (CPG) and the properties of the immobilized enzyme have been investigated [8]. In order to simplify the simultaneous determination of hypoxanthine, xanthine and uric acid by HPLC and to provide a means by which they could be quantitated accurately, the performance of a reactor packed with immobilized xanthine oxidase has been also considered theoretically and experimentally [9]; when a post-column reactor packed with immobilized xanthine oxidase is to be employed, the complete conversion of both hypoxanthine and xanthine into uric acid, which can be detected simply and selectively, may be accomplished.

This paper describes a simultaneous assay of hypoxanthine and xanthine in serum from a patient with gout during alloprinol therapy, using an immobilized xanthine oxidase reactor coupled to HPLC.

EXPERIMENTAL

Chemicals

Hypoxanthine, xanthine and alloprinol were purchased from Nakarai Chemicals (Kyoto, Japan) and uric acid from Merck (Darmstadt, G.F.R.). Xanthine oxidase (cows' milk, 0.4 units/mg protein) was purchased from Boehringer-Mannheim-Yamanouchi (Tokyo, Japan), uricase (*Candida utilis*, 3.4 units/mg protein) from Toyobo (Tokyo, Japan) and catalase (bovine liver, 37,000 units/mg protein) from Sigma (St. Louis, MO, U.S.A.). Aminopropyl-CPG (80—120 mesh, 530 Å mean pore diameter) was obtained from Electro-Nucleonics (Fairfield, U.S.A.). All other reagents were of analytical-reagent grade.

Apparatus

The liquid chromatograph was a Model LC-3A (Shimadzu, Kyoto, Japan). Solutes were detected with a Shimadzu Model SPD-2A variable-wavelength UV detector equipped with a Model C-R1A integrator. The pre-column (1.0 × 0.4 cm I.D.) and the analytical column (20 × 0.4 cm I.D.) were prepared with Nucleosil 5 C_{18} (Macherey, Nagel & Co., Düren, G.F.R.) by using a high-pressure slurry packing technique.

Immobilized enzyme reactor

The immobilization procedure was the same as that reported previously [8], in which the enzymes were attached to the aminopropyl-CPG by an intermolecular cross-linking method. The properties and the conversion efficiencies of the immobilized enzymes were determined by the flow-injection method [10]. The pH optima for the immobilized xanthine oxidase, uricase and catalase were 7.5, 8.0 and 8.0, respectively. Acetonitrile contents lower than 5% (v/v) did not degrade the activities of the immobilized enzymes. The immobilized xanthine oxidase and a mixture of the immobilized uricase and the immobilized catalase (mixing ratio 10:1) were packed into stainless-steel tubes (5.0 × 0.21 cm I.D.). Although the long-term stability of each immobilized enzyme was not studied in continuous operation, both enzyme reactors retained their performance without a decrease in activity for more than 7 weeks.

Serum purification

A 0.5-ml portion of serum was mixed with an equal volume of a 6% (v/v) solution of trichloroacetic acid, and the mixture was centrifuged for 15 min at 1500 *g*. Any volume of the supernatant was added to an equal volume of 0.5 *M* tri-*n*-octylamine in 1,1,2-trichlorotrifluoroethane. After mixing and centrifuging, 10 μl of the aqueous solution were injected into the analytical column. Recovery experiments were carried out by adding known amounts of hypoxanthine and xanthine to the control serum. The recoveries of hypoxanthine and xanthine were 93 ± 9.9% (n = 4) and 96 ± 9.2% (n = 4), respectively.

RESULTS AND DISCUSSION

Fig. 1 demonstrates the change in capacity ratios (k') with the pH and the content of acetonitrile in the mobile phase for each solute. By considering the peak broadening by use of immobilized enzyme reactors, 0.01 *M* phosphate buffer (pH 5.5) containing 1% (v/v) acetonitrile was selected for this study.

Fig. 2 illustrates a chromatogram obtained for a serum extract from a patient with gout during alloprinol therapy, which was detected at 254 nm under the conditions described above. It shows that many other UV-absorbing

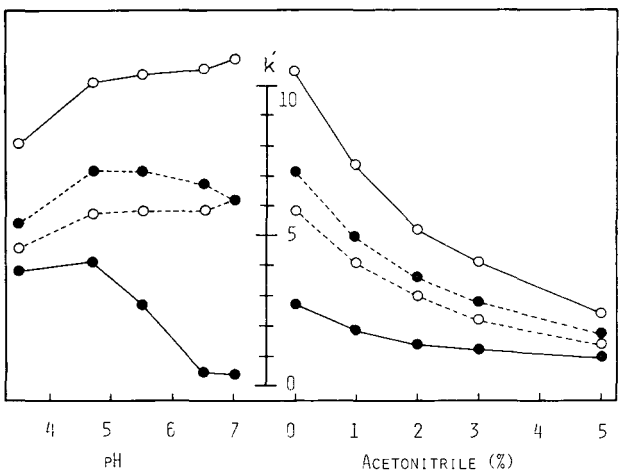

Fig. 1. Effects of pH and acetonitrile content on the capacity ratios (k') of hypoxanthine (- - - - o - - - -), xanthine (- - - - • - - - -), uric acid (—•—) and alloprinol (—○—). Column: Nucleosil 5 C_{18}, 20 × 0.4 cm I.D. Flow-rate: 0.7 ml/min. Detection: 254 nm.

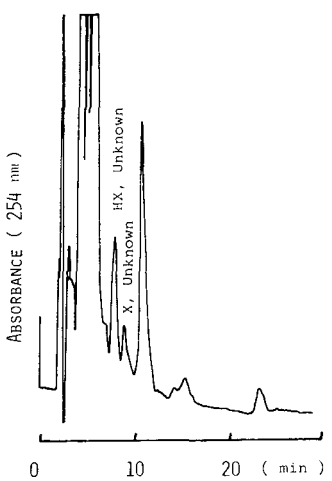

Fig. 2. Chromatogram of serum extract from patient with gout during alloprinol therapy. Injection volume: 10 µl. Column: Nucleosil 5 C_{18}, 20 × 0.4 cm I.D. Eluent: 0.01 *M* phosphate buffer (pH 5.5) containing 1% (v/v) acetonitrile. Flow-rate: 0.7 ml/min. HX = hypoxanthine; X = xanthine.

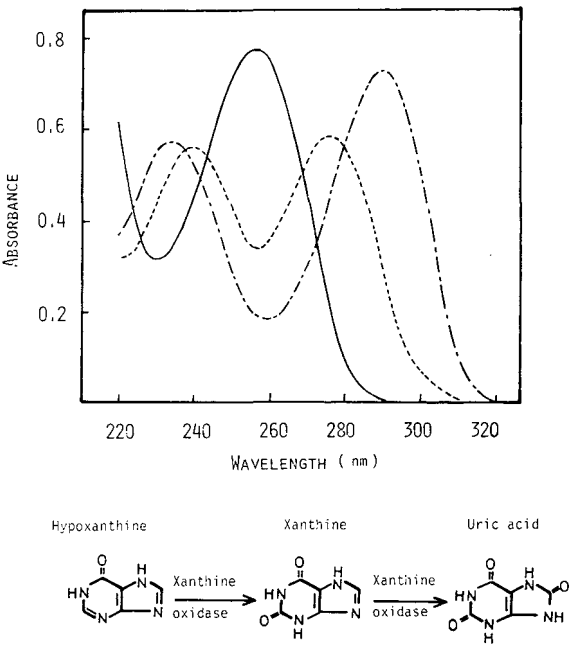

Fig. 3. Absorption spectra of hypoxanthine ($7.5 \cdot 10^{-5}$ *M*) (——), xanthine ($6.6 \cdot 10^{-5}$ *M*) (- - - - -) and uric acid ($6.0 \cdot 10^{-5}$ *M*) (— - —), and oxidative transformation of hypoxanthine and xanthine in uric acid by xanthine oxidase.

compounds interfere with the simultaneous quantitative determination of hypoxanthine and xanthine. On the other hand, when a reactor packed with immobilized xanthine oxidase was coupled to the analytical column, both hypoxanthine and xanthine were oxidized to uric acid and therefore could be detected at 290 nm simultaneously, as shown in Fig. 3. However, in this study it was impossible to determine such oxypurines accurately because of the high concentration of uric acid present in serum. Further, a large excess of uric acid degraded the original activity of the immobilized xanthine oxidase packed in the reactor. Therefore, another reactor packed with a mixture of immobilized uricase and immobilized catalase (mixing ratio 10:1) was utilized in order to remove the interferences of uric acid and its oxidation by-product

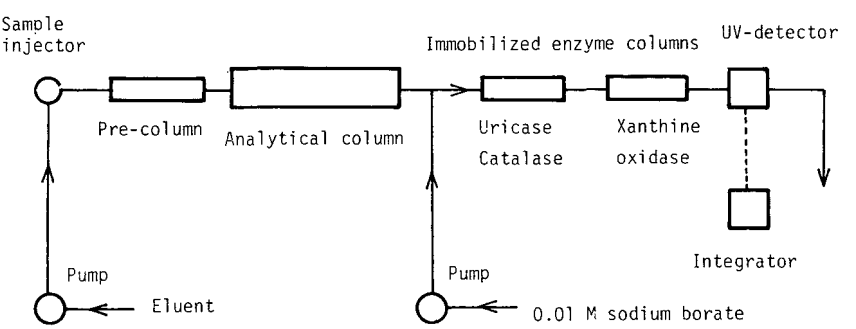

Fig. 4. Flow diagram of HPLC coupled with immobilized enzyme reactors.

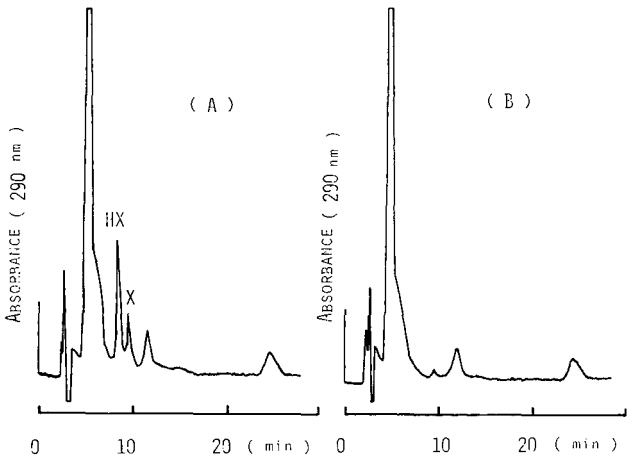

Fig. 5. Chromatograms of the same sample as in Fig. 2, which were obtained (A) with and (B) without the immobilized xanthine oxidase reactor in the system. Flow-rate of 0.01 *M* sodium borate: 0.27 ml/min. Other chromatographic conditions as in Fig. 2. HX = hypoxanthine; X = xanthine.

(hydrogen peroxide), which acts as an inhibitor of the immobilized xanthine oxidase packed in the reactor.

A diagram of the HPLC system coupled with two reactors used in this study is shown in Fig. 4. Fig. 5A illustrates a chromatogram obtained at 290 nm for the same serum sample under identical conditions, in which the effluent from the analytical column was adjusted to pH 7.7 with 0.01 *M* sodium borate and was passed continuously into the enzyme reactors. Fig. 5B shows a chromatogram obtained at the same wavelength, in which only the reactor packed with the immobilized xanthine oxidase had been removed. It shows that both peaks of hypoxanthine and xanthine, as shown in Fig. 5A, were eluted free from other UV-absorbing compounds.

The linearity of calibration graphs of concentration versus peak height was excellent for hypoxanthine, ranging from 0.20 to 1.60 μg/ml, and for xanthine, ranging from 0.19 to 1.52 μg/ml, and both regression coefficients were 0.998. The average coefficients of variation for the normalized peak height over this range of hypoxanthine and xanthine concentrations were

TABLE I

DETERMINATION OF HYPOXANTHINE AND XANTHINE IN SERA FROM PATIENTS WITH GOUT DURING ALLOPRINOL THERAPY

Sample	Hypoxanthine (μg/ml)*	Xanthine (μg/ml)*
1	3.27 ± 0.056	0.74 ± 0.033
2	1.06 ± 0.029	0.81 ± 0.029
3	1.57 ± 0.036	0.72 ± 0.018
4	1.69 ± 0.101	0.46 ± 0.013
5	2.34 ± 0.111	1.21 ± 0.040

*Means ± S.D. (n=4).

2.19 and 1.70%, respectively. The sensitivities of determination for hypoxanthine and xanthine were 68 and 76 ng/ml, respectively. Typical results for the determination of hypoxanthine and xanthine in sera of patients with gout during alloprinol therapy are presented in Table I.

In conclusion, a rapid and relatively simple HPLC method has been developed for the simultaneous identification and quantification of hypoxanthine and xanthine in biological fluids. The procedure can be easily instituted as a routine laboratory procedure in the diagnosis of purine metabolism.

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Journal of Chromatography, 231 (1982) 189—193
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1301

Note

Analytical electrophoretic separation of undelipidated rat plasma apolipoproteins

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Analysis of plasma lipoproteins is most conveniently performed by various methods of acrylamide electrophoresis. Complete resolution of the apolipoproteins (including isomorphic forms) requires two-dimensional electrophoresis [1]. The separation of rat apoproteins by molecular weight is sufficient for many studies. While sodium dodecyl sulphate (SDS) electrophoresis has been used to resolve apoproteins with molecular weights between 15,000 and 80,000 daltons, the apoC proteins remain unresolved [2]. These low molecular weight apoproteins can be resolved by urea—acrylamide electrophoresis [3] or isoelectric focusing [1], methods which do not yield complete resolution of the higher molecular weight apoproteins (in particular, rat apoE and ApoA-IV). The complete spectrum of apolipoproteins can be separated according to molecular weight, provided the samples are first delipidated, by SDS—acrylamide gradient gel electrophoresis [4]. The present paper describes the resolution of rat apoproteins in the molecular weight range from 6000—80,000 daltons using non-gradient SDS—urea polyacrylamide gel electrophoresis without prior delipidation of the lipoprotein samples and illustrates the advantages of this method.

EXPERIMENTAL

Acrylamide, N,N,N,N-tetramethylethylenediamine (TEMED), N,N'-methylene-bisacrylamide, and ammonium persulfate (electrophoresis grade) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Ultrapure urea was purchased from Schwarz-Mann (Orangeburg, NY, U.S.A.), and Ampholines, pH 4—6, were supplied by LKB (Stockholm, Sweden). D,L-Dithiothreitol was purchased from Sigma (St. Louis, MO, U.S.A.). Tetramethylurea was

purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The animals were male rats of the Wistar strain (250—400 g) fed ad libitum standard Purina Rat Chow, Purina Foods (Toronto, Canada). Blood samples were drawn in tubes containing EDTA (1 mg/ml) and plasma was separated by low speed centrifugation (2000 *g*) for 20 min.

Isolation and analysis of rat plasma lipoproteins

Rat plasma lipoproteins were isolated by sequential ultracentrifugation at the following densities: very low density lipoproteins (VLDL), d = 1.006 g/ml; low density lipoproteins (LDL), d = 1.006—1.040 g/ml; and high density lipoproteins (HDL), d = 1.040—1.21 g/ml [5, 6]. Prior to electrophoresis each lipoprotein fraction was dialyzed against 0.15 *M* sodium chloride, 0.04% EDTA, pH 8.6, at 4°C.

The lipoprotein fractions were characterized by lipid and protein analyses. Lipids were quantitated by high temperature gas—liquid chromatography after dephosphorylation and trimethylsilylation, using tridecanoylglycerol as internal standard [7]. Protein was measured according to Markwell et al. [8] using bovine serum albumin (Fraction V, Sigma) as reference standard.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out in a Model DE-102 tube gel apparatus (Hoffer Scientific Instruments, San Francisco, CA, U.S.A.) using a Bio-Rad Model 1420 power supply. The electrophoretic system of Swank and Munkres [9] was used as modified by Butkowski [10]. Twenty-five ml of gel solution was sufficient to make twelve tube gels 90 × 5 mm in 125 × 5 mm glass tubes. Acrylamide gels (15%) were made by mixing the following: 50% acrylamide, 2% bisacrylamide (7.5 ml); 0.8 *M* H_3PO_4, 0.8% SDS, 1.29 *M* tris (hydroxymethyl) aminomethane, pH 6.8 (3.13 ml); urea (9.38 g); and distilled water to 24.75 ml. The above were thoroughly mixed to dissolve the urea, and 1.0% ammonium persulfate (0.25 ml) and TEMED (0.01 ml) were added. The solution was degassed and gels poured and overlayed with distilled water. Polymerization took place within 40 min and gels were used 1—2 h after preparation. After mounting the tubes in the electrophoresis apparatus the upper and lower reservoirs were filled with 0.1% SDS, 0.1 *M* sodium phosphate buffer, pH 7.0 [10, 11]. Electrophoresis was performed at 5 mA per gel constant current for 10—12 h or at 2.5 mA per gel overnight until the tracking dye was 1 cm from the end of each gel. Gels were stained in 9.2% acetic acid, 45.4% methanol, 0.025% Coomassie Brilliant Blue G-250 (filtered before use) for a minimum of 6 h and destained in 7.5% acetic acid, 5.0% methanol. Urea polyacrylamide gel electrophoresis was carried out as described [4]. Isoelectric focusing and two-dimensional gel electrophoresis were performed according to Swaney and Gidez [1]. The pH of the isoelectric focusing gels was determined using a combination pH electrode Model 1885, manufactured by the Markson Scientific Company (Del Mar, CA, U.S.A.).

Samples for electrophoresis were prepared as follows: an aliquot containing 40 μg of protein (25—250 μl of each lipoprotein fraction) was added to an equal volume of glycerol followed by a 0.25 volume of isopropanol. SDS was added to each sample to a final concentration of 2% using a 20% stock solu-

tion. Some samples were reduced by the addition of 0.10 volume of a freshly prepared 400 m*M* dithiothreitol solution as described previously [12]. The samples were heated at 100°C in a boiling water bath for 2 min. Bromophenol Blue (10 μl of a 0.02% solution) was added to each sample as a tracking dye and each sample was layered under the running buffer.

RESULTS AND DISCUSSION

Fig. 1 shows the resolution obtained for the apoproteins of rat HDL with the SDS—urea system. The identity of the apoprotein bands was established by two-dimensional electrophoresis in which isoelectric focusing is used in the first dimension and the SDS-urea system in the second dimension. The p*I* values of the A-I, A-II, A-IV, E, and C-II, C-III-0 and C-III-3 components corresponded to those previously reported [1]. ApoA-II, which is present in low amounts in rat HDL, was detectable in this gel but too faint to reproduce photographically. Fig. 2 compares the separation obtained with the SDS-urea system to that realized with the urea or SDS systems run separately. The urea system resolves the apoC apoproteins; however, the use of tetramethylurea can produce artifactual bands in the apoC region, complicating interpretation of the results (data not shown). In addition, there is considerable overlap of the higher molecular weight apoproteins. Both SDS systems resolve the higher molecular weight apoproteins while only the SDS—urea system resolves the Apo A-II and the C apoproteins (apoC-II, ApoC-III-0 and apoC-III-3) routinely on the basis of apparent molecular weight. It was not possible to positively

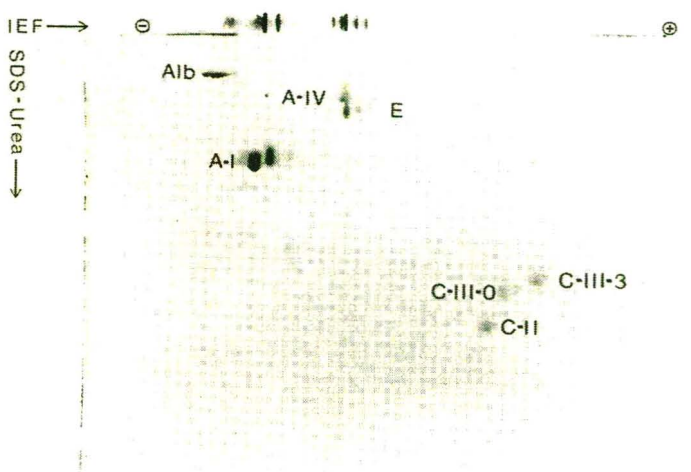

Fig. 1. Resolution of rat HDL apoproteins by SDS—urea gel electrophoresis and identification of components by two-dimensional electrophoresis. Isoelectric focusing was performed in a tube gel (top) using 80 μg of rat plasma HDL. A 1 mm thick slice of the gel was then incubated in 0.2% SDS—phosphate buffer, pH 7.0 (37°C, 1.5 h) and electrophoresis performed in the second dimension using the SDS—urea system. A-IV = apoprotein A-IV (p*I* 5.37—5.60); E = apoprotein E (p*I* 5.37—5.60); A-I = apoprotein A-I (p*I* 5.68—5.81); C-III-3 = apoprotein C-III-3 (p*I* 4.52); C-III-0 = apoprotein C-III-0 (p*I* 4.70); C-II = apoprotein C-II (p*I* 4.80).

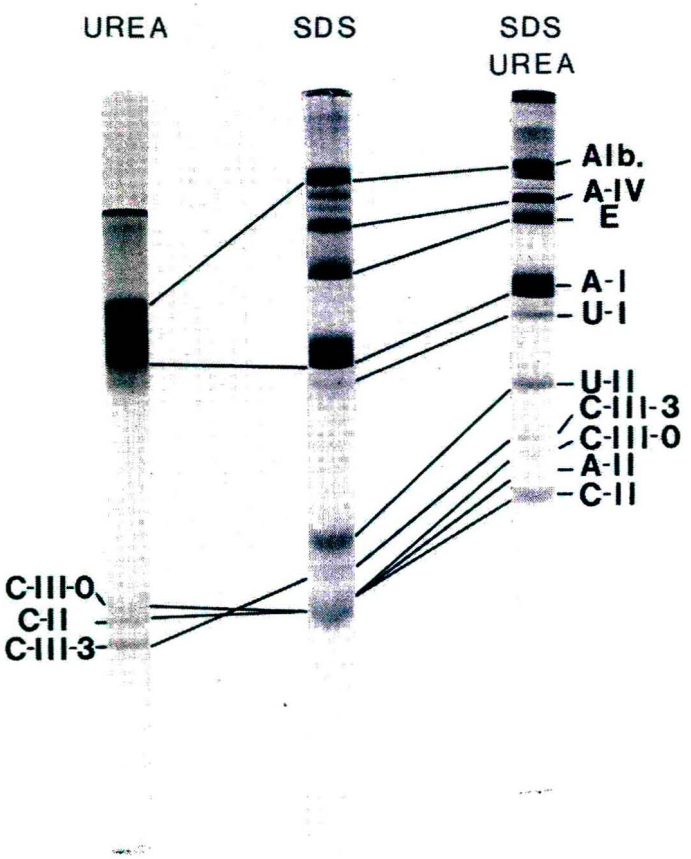

Fig. 2. Comparative resolution of the apoproteins of rat HDL by SDS, urea, and SDS—urea gel electrophoresis. A 40-μg sample of rat plasma HDL protein was applied to each gel. Bands were identified as in Fig. 1. U-I and U-II are unidentified components of rat HDL.

identify apoC-I due to the ambiguity in its position in pH 4—6 isoelectric focusing gels [1]. Under optimal conditions a band apparently corresponding to apoC-I was present below apoC-II, however there was incomplete resolution of apoC-I and apoC-II in most runs. Two additional components referred to as U-I and U-II and not corresponding to previously demonstrated apoproteins, are also resolved in the SDS—urea system. These unknowns were present in several rat HDL fractions run as described or after delipidation with ethanol—diethyl ether [13].

SDS—urea gel electrophoresis was originally proposed for improved separation of low molecular weight peptides because of the increased sieving effect and the improved handling characteristics due to the inclusion of urea in gels of high acrylamide concentration [9]. We have confirmed its suitability for rat apoproteins of low molecular weight. It was noted, however, that the stacking efficiency of the buffer system was inadequate for normal rat VLDL in which the protein concentration is often very low (in the range of 40 μg per 250 μl). The sharpness of the protein bands was considerably improved for sample volumes as large as 400 μl by the use of the buffer combination

described here. However, as previously noted [4], electrophoresis of intact lipoprotein samples caused distortion of the apoprotein bands and resulted in streaks of stainable material along the length of the gel. The addition of glycerol and isopropanol to lipoprotein samples described here eliminates these problems and reproducibly yields distinct aproprotein bands. As well, intact lipoproteins prepared with glycerol—isopropanol—SDS gave the same apoprotein pattern as delipidated lipoproteins treated with SDS alone (data not shown). This method of sample preparation should be compatible with any SDS electrophoretic system. The present system is preferred by the authors due to the inherent ease of pouring these gels relative to gradient acrylamide gels.

In summary, the application of SDS—urea gel electrophoresis to rat plasma lipoproteins results in a resolution superior to that obtained with any previously described conventional system and equals that reported for gradient acrylamide gel electrophoresis. Due to the simplicity of this sytem and the method of sample preparation, it should be advantageous where rapid simultaneous resolution of high and low molecular weight peptides is required.

ACKNOWLEDGEMENTS

These studies were performed with funds from the Ontario Heart Foundation Toronto, Ontario and the Medical Research Council of Canada.

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Journal of Chromatography, 231 (1982) 194—199
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1290

Note

Determination of levorphanol (Levo-Dromoran®) in human plasma by combined gas chromatography—negative ion chemical ionization mass spectrometry

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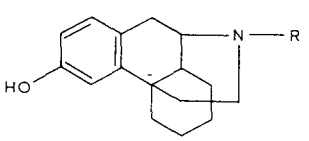
(First received December 30th, 1981; revised manuscript received March 2nd, 1982)

Levorphanol (17-methyl-morphinan-3-ol, Levo-Dromoran®) is a synthetic narcotic analgesic which is more potent than morphine when administered orally, parenterally or subcutaneously [1].



R = CH_3 levorphanol
R = C^2H_3 levorphanol — d_3

Both levorphanol and its metabolite, norlevorphanol, are excreted principally as glucuronide conjugates [2—4]. A recently reported radioimmunoassay (RIA) [5, 6] is the only available assay for measuring plasma concentrations of levorphanol, in spite of the fact that the drug has been marketed for over 25 years. This paper reports a gas chromatography—mass spectrometry (GC—MS) assay for levorphanol which was developed to quantitate plasma concentrations of levorphanol generated following the administration of Levo-Dromoran to man. The assay features the use of a trideuterated analogue of levorphanol (levorphanol-d_3) as the internal standard, conversion of levorphanol to a derivative with electron-capturing properties and ionization of the derivative by methane negative chemical ionization.

EXPERIMENTAL

Gas chromatograph

A Finnigan Model 9500 gas chromatograph is equipped with a glass column (120 cm × 2 cm I.D.) packed with 3% poly S-176 on 80—100 mesh high-performance Chromosorb W (Applied Science Labs., State College, PA, U.S.A.). The column was conditioned overnight at 300°C with a nitrogen flow-rate of 40 ml min^{-1}. Methane (101 kPa) was used in the assay as both GC carrier gas and negative ion reagent gas. The temperatures of the injection block, GC oven and GC—MS transfer line were 290°C, 280°C, and 280°C, respectively. Under these conditions the pentafluorobenzoyl derivative of levorphanol had a retention time of 2 min.

Mass spectrometer

A Finnigan Model 3200 quadrupole mass spectrometer was set to give the maximum response consistent with reasonable ion peak shape and unit resolution. The modification to the instrument to permit the detection of negative ions have been described [7]. The methane ion source pressure was 66.7 Pa. The ion source was operated at an emission of 0.25 mA, an ionizing voltage of 120 V and without any external heating. The voltages on the conversion dynode and electron multiplier were +2.5 and —2.0 kV, respectively. The preamplifier was set to give 10^{-8} A V^{-1}.

Data system

Selected ion monitoring measurements were made using a Finnigan Model 6000 data system with revision I software. A scan of *m/z* 451 and *m/z* 454 was made in 1 sec.

Glassware

Sixteen-ml culture tubes (Pyrex 9825) provided with Teflon®-lined screw caps were used for plasma extractions. Five-ml conical centrifuge tubes (Pyrex 8061) were used for the derivatization procedures and for the final evaporation of the derivatived extract. All tubes, after washing with detergent and water, were treated with Siliclad® (Clay Adam, Parsippany, NJ, U.S.A.) and were finally rinsed with methanol and dichloromethane (Fisher Scientific, Pittsburgh, PA, U.S.A.) just prior to use.

Solvent evaporator

Solvents were removed at 60°C under nitrogen (N-Evap, Organomation Assoc., Worchester, MA, U.S.A.).

Shaker

Extractions were performed by shaking (60 strokes min^{-1}) on a variable-speed reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.).

Centrifuge

A Damon/IEC (Needham, MA, U.S.A.), Model CRU-500 refrigerated centrifuge was operated at 1320 *g* and 10°C.

Scintillation counting

A Packard Tri-Carb Model 3380 scintillation counter was used with an external standard.

Chemicals

Levorphanol tartrate was supplied by Dr. W. Scott, Chemical Research Department, Hoffmann-La Roche, Nutley, NJ, U.S.A. Levorphanol-d_3 hydrochloride was synthesized using the method of Ellison et al. [8]. Tritiated levorphanol was provided by Drs R. Muccino and J. Cupano, Hoffmann-La Roche. Methanol, dichloromethane, chloroform and benzene were supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Pentafluorobenzoyl chloride and 4-dimethylaminopyridine were purchased from PCR Research Chemicals (Gainesville, FL, U.S.A.) and Aldrich (Milwaukee, WI, U.S.A.), respectively.

Solutions

Borate buffer (pH 9) was prepared as previously described [9]. Stock solutions (mg/ml) of levorphanol tartrate and levorphanol-d_3 hydrochloride were prepared in methanol. Aliquots of the stock solution were diluted with methanol to give working solutions containing 100 ng ml^{-1} of levorphanol-d_3 free base and either 0 (solution A), 10 (solution B), 50 (solution C), 100 (solution D), 200 (solution E), or 500 ng ml^{-1} (solution F) of levorphanol free base.

Procedure

Levorphanol-d_3 (10 ng) was added to 1 ml of either the calibration curve plasma samples, i.e., drug-free control plasma, or experimental plasma samples, and the mixture was vortexed briefly. Duplicate calibration curve plasma samples were spiked with 0.1 ml of either solution A (0 ng of levorphanol), B (1 ng of levorphanol), C (5 ng of levorphanol), D (10 ng of levorphanol), E (20 ng of levorphanol), or F (50 ng of levorphanol). One ml of 1 *M* borate buffer (pH 9) was added and the samples were extracted with 7 ml of benzene—dichloromethane (9:1) by shaking for 50 min. The samples were then centrifuged for 10 min and the organic layer was transferred, a portion at a time, to a 5-ml centrifuge tube, followed by evaporation of the solvent. The organic solvents were then evaporated to dryness. The residue was dissolved in 100 μl of chloroform and derivatization was accomplished by adding 20 μl of 1% 4-dimethylaminopyridine in chloroform and 20 μl of pentafluorobenzoyl chloride. After 2 h at room temperature, this solution was washed with 1 ml of borate buffer (pH 9) and was extracted with 2 ml of benzene—dichloromethane (9:1). After centrifugation the organic layer was transferred into a 5-ml centrifuge tube and evaporated to dryness. The residue was dissolved in 50 μl of ethyl acetate and an aliquot of 3—5 μl of the resulting solution was analyzed by GC—MS with the mass spectrometer set to monitor *m/z* 451 and *m/z* 454 in the GC effluent.

At 30 sec after injection the GC divert valve was turned off and 15 sec later the ionizer was turned on and data collection was begun. The ion ratio of *m/z* 451 to *m/z* 454 in an experimental sample was converted to a concentra-

tion of levorphanol using a calibration curve generated from a linear least squares regression analysis of the m/z 451 to m/z 454 ion ratios versus amount added data from the analyses of the calibration curve samples. The slope (m) and intercept (b) values from the least squares analysis were used to calculate the amount (x) of levorphanol in an experimental sample given an ion ratio (R) using the equation $x = (R-b)/m$.

RESULTS AND DISCUSSION

The methane negative chemical ionization mass spectra of the pentafluorobenzoyl derivative of levorphanol and levorphanol-d_3 are shown in Fig. 1. As seen in Fig. 1, the M^- molecular anions dominate the spectra. The pentafluorobenzoyl group appears to confer maximum sensitivity for the analysis of phenols by electron-capture GC [10] and for phenolic morphines and morphinans by GC—MS [11].

Selected ion current profiles from the analyses of control plasma spiked with known amounts of levorphanol and levorphanol-d_3 and from experimental plasma from a patient receiving Levo-Dromoran® are shown in Fig. 2.

Based on liquid scintillation measurements of the radioactivity extracted from samples containing tritiated levorphanol, the extraction procedure gave recoveries of the drug from plasma of 86—87%. Calibration curves from the assay are linear, i.e. correlation coefficients are greater than 0.99, for concentrations of levorphanol between 1 and 50 ng ml^{-1} using 10 ng of levorphanol-d_3 as internal standard. The y intercept of the calibration curve using

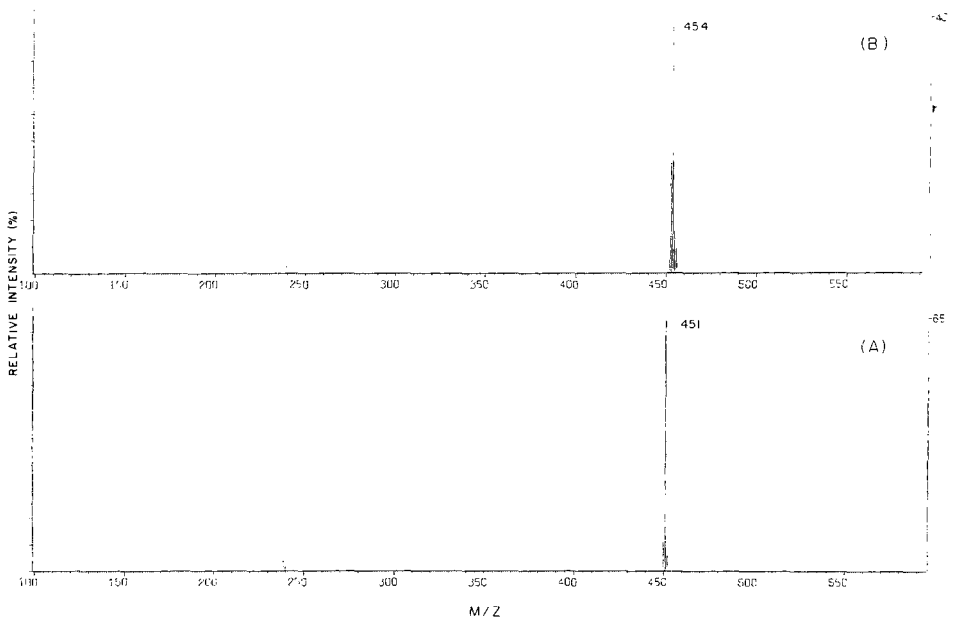


Fig. 1. Methane negative chemical ionization mass spectra of the pentafluorobenzoyl derivatives of (A) levorphanol (mol. wt. = 451) and (B) levorphanol-d_3 (mol. wt. = 454).

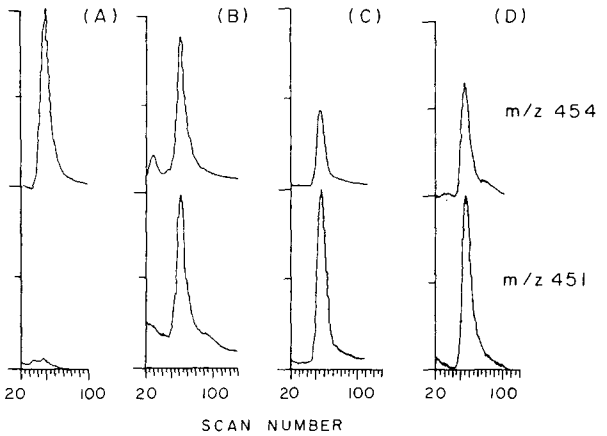


Fig. 2. Selected ion current profiles from the analyses of control plasma (A) and plasma taken from a cancer patient on chronic Levo-Dromoran® therapy just prior to (B), 60 min (C) and 120 min (D) after receiving a 3-mg dose of Levo-Dromoran®. All samples were spiked with 10 ng ml^{-1} of levorphanol-d_3. The concentrations of levorphanol in the samples whose selected ion current profiles are given in (B), (C) and (D) were 10.0, 23.9 and 14.5 ng ml^{-1}, respectively.

TABLE I

CONCENTRATION OF LEVORPHANOL IN PLASMA SAMPLES FROM TEN SUBJECTS ON LEVO-DROMORAN® THERAPY MEASURED BY EITHER THE GC—MS METHOD OR THE RIA PROCEDURE

Sample No.	GC—MS (ng ml^{-1})	RIA (ng ml^{-1}) [5, 6]
1	20	17
2	5	4
3	6	7
4	10	12
5	10	10
6	23	23
7	15	14
8	25	28
9	21	15
10	20	24

10 ng of levorphanol-d_3 as internal standard typically represents less than 0.1 ng of levorphanol. The limit of quantitation is 1 ng ml^{-1}.

The precision of the assay was evaluated by six analyses each of control plasma spiked with either 1, 10 or 50 ng ml^{-1} of levorphanol. The amounts found (± S.D.) were 0.95 ± 0.08, 10.5 ± 0.18 and 50.1 ± 0.17 ng ml^{-1}. These values give the assay a relative standard deviation of 8.2, 1.7 and 0.3% for concentrations of 1, 10 and 50 ng ml^{-1}, respectively.

Comparative analyses of human plasma samples by this GC—MS assay and the published RIA procedure [5, 6] are shown in Table I. A linear regression analysis of the RIA data (y) against the GC—MS data (x) shows them to be

well correlated (correlation coefficient = 0.93). The regression line has a slope of 0.98 and an intercept of only 0.21 ng.

In conclusion, a relatively simple, specific and sensitive assay has been developed to measure levorphanol in human plasma. The assay has confirmed data generated by a published RIA method [5, 6] for levorphanol. The general procedure reported should be useful for the GC—MS analyses of other phenolic morphinans and morphines.

ACKNOWLEDGEMENT

Plasma samples from patients on Levo-Dromoran® therapy were provided by Dr. K. Foley of Memorial Sloan-Kettering Cancer Center and Cornell University Medical College, New York, NY, U.S.A.

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Journal of Chromatography, 231(1982) 200—204
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1303

Note

Decomposition of sulphoxide metabolites of phenothiazine antipsychotics during gas chromatographic analysis

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(Received January 27th, 1982)

During a recent evaluation of a gas chromatographic and gas chromatographic—mass spectrometric (GC—MS) assay for the psychotherapeutic agent chlorpromazine and its major metabolites, it was noted that the sulphoxide metabolite decomposed upon injection into the gas chromatograph to give a small but significant peak with the same retention time as that of the parent drug chlorpromazine. Decomposition of the N-oxide metabolite during GC analysis has been reported [1]. However, information as to the stability of the sulphoxide metabolite in GC analysis is lacking in spite of the fact that there are reports which describe GC procedures for chlorpromazine and its metabolites including the sulphoxide metabolite [2, 3]. Thus it was decided to examine the effect of injection port temperature on the extent of decomposition of phenothiazine sulphoxide metabolites during GC analysis, using both capillary and packed columns with mass spectrometric and nitrogen sensitive detection.

EXPERIMENTAL

All solvents were redistilled in glass before use. The samples of chlorpromazine sulphoxide (CPZSO) (synthesized in our laboratory), trifluorperazine sulphoxide (TFPSO) and prochlorperazine sulphoxide (PCPSO) (provided by courtesy of Rhone Poulenc, Montreal, Canada) were purified by thin-layer chromatography (TLC) (Whatman TLC plates precoated with 40A silica gel, 250 μm) with a solvent system acetone—methanol—diethylamine (100:20:1). The area on the TLC plate corresponding to the sulphoxides (R_F 0.16 for PCPSO, R_F 0.22 for TFPSO and R_F 0.38 for CPZSO) were scraped off and the sulphoxides were redissolved in methanol. The purity of the samples was then confirmed by high-performance liquid chromatography using a Waters M45 pump system and Waters Model 440 UV detector operating at 254 nm wavelength. A 250 mm × 4.6 mm I.D. column packed with 5 μm

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Spherisorb Cyano was used with acetonitrile—0.01 *M* acetate buffer (9:1) as solvent system. Injection was made via a Rheodyne loop Model 7125 fitted with a 500 μl loop.

Gas chromatography with nitrogen selective detection was performed on a Hewlett-Packard 5840 series gas chromatograph fitted with a 1.5 m × 2 mm I.D. glass column packed with Gas-Chrom Q (100—120 mesh) (Applied Science). The injection port was operated at either 250, 270 or 300°C, the column oven was maintained isothermally at 290°C. The helium flow-rate was set at 50 ml/min.

Capillary column GS—MS was performed using a V.G. Analytical 16F single focussing mass spectrometer coupled to a Hewlett-Packard 5700 gas chromatograph via a direct glass capillary coupling to the ion source. A 20 × 0.3 mm I.D. fused silica capillary column coated with methyl silicone OV-1 was used. Injections were made in the split and splitless modes with an injector temperature at 300°C, and with the cold on column injection mode [4]. The column was programmed from 60 to 280°C at 6°C/min in each case. Additional mass spectrometer operating conditions were interface temperature 280°C, ion source 200°C, ion energy 70 eV, emission current 200 μA in the electron impact mode and 500 μA in the chemical ionisation mode using ammonia as reagent gas.

RESULTS AND DISCUSSION

Gas chromatographic analysis of chlorpromazine sulfoxide using a packed column at an injection port temperature of 300°C showed two peaks a and b (Fig. 1A) with a retention time of 1.5 and 4.5 min, respectively. When the injection port temperature was lowered, peak b increased at the expense of peak a. Peak a had the same retention time as authentic chlorpromazine. Both the

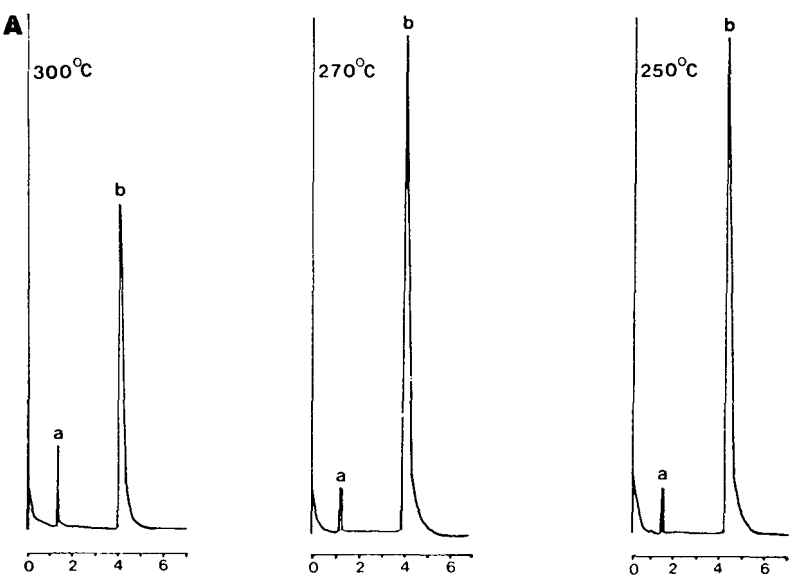

Fig. 1.

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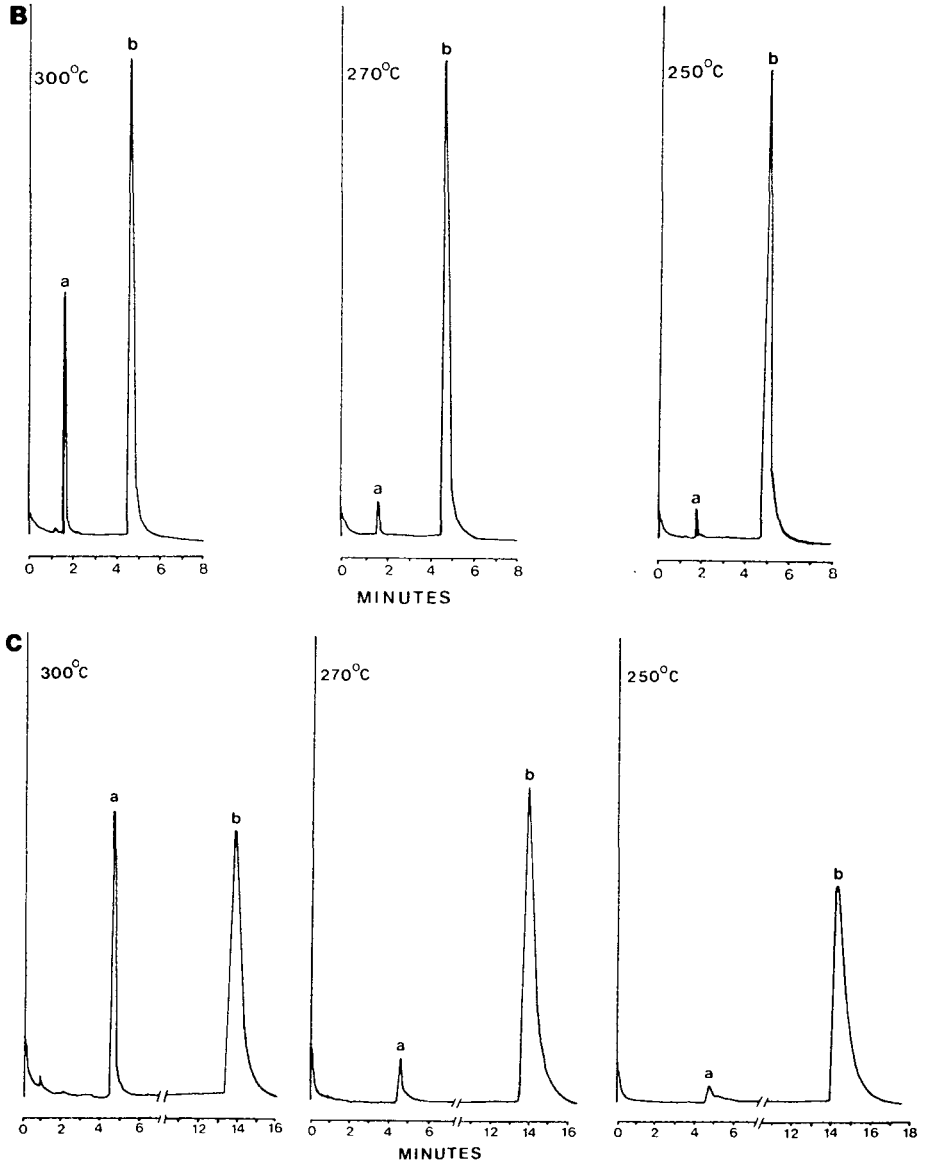

Fig. 1. Typical gas–liquid chromatograms of CPZSO (A), TFPSO (B) and PCPSO (C) at three different injection port temperatures. Peak a is the parent drug and peak b is the respective sulphoxide, in each case.

electron impact (EI) and chemical ionisation (CI) mass spectra of the compound giving rise to peak a were identical to those of authentic chlorpromazine. The EI mass spectrum showed the molecular ion at *m/z* 318/320, the base peak at *m/z* 58 and other diagnostic ions at *m/z* 273/275; 272/274; 233/235; 196, 86 and 85. The mass spectra of the compound giving rise to peak b were consistent with the structure of authentic chlorpromazine sulfoxide. The EI mass spectrum showed a molecular ion cluster at *m/z* 334/336, and other

diagnostic ions at *m/z* 318/320 and *m/z* 317/319. The CI mass spectrum using ammonia reagent gas showed the quasi molecular ion at *m/z* 335/337 $(M+H)^+$. Similar to the GC behavior of CPZSO, decomposition of TFPSO and PCPSO to their respective phenothiazines also occurred in GC (Fig. 1B and C), and was also confirmed by EI and CI MS. The extent of decomposition for these two sulfoxide compounds, however, was more pronounced than in the case of CPZSO.

Results from the injection of the phenothiazine sulfoxides made at three different injection port temperatures using nitrogen selective GC detection, and also capillary column GC—MS using split, splitless and cold on column injection [4] are presented in Table I.

TABLE I

PERCENT DECOMPOSITION OF CPZSO, PCPSO, AND TFPSO IN THE GAS CHROMATOGRAPH AT DIFFERENT INJECTION PORT TEMPERATURES

Each value represents the mean of 3 injections.

Column	Injection port temperature (°C)	Decomposition (%)		
		CPZSO	PCPSO	TFPSO
Packed (1.5 m × 2 mm I.D.) 280°C	300	10	24	20
	270	2	8	2
	250	2	2	2
Capillary (20 m × 0.3 mm I.D.) 60—280°C at 6°C/min	Split 300	8	15	17
	Splitless 300	8	16	18
	Cold on column	0.3	0.5	0.7

It can be easily observed from Fig. 1 and Table I that injection port temperature has a marked effect on the extent of decomposition. In the case of cold on column method of injection, the break down was minimal, although this method of injecting samples is not practical in routine analysis.

Although the decomposition of chloropromazine N-oxide in GC was noted as early as 1964 [1], we are unaware of any reports regarding the decomposition of the sulfoxide metabolite, despite the fact that the injection port temperatures in most reported GC analysis of CPZ and its metabolites were in the range of 275—310°C. The sulfoxide metabolites, although they are thought to be relatively inactive therapeutically [5], may be present in plasma at levels similar to or even exceeding that of the parent drugs [6, 7]. Thus a significant contribution may be made to the concentrations of the parent compounds by thermal breakdown.

To minimize this analytical complication, we would suggest careful optimisation of chromatographic conditions prior to the analysis of phenothiazine sulfoxides. It was found that by maintaining the injection port temperature

between 250—270°C, the decomposition rarely exceeded 2% for the three sulfoxide metabolites tested, and the chromatographic behavior of these metabolites was not changed by the lowering of the injection port temperature within this range.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the Medical Research Council of Canada for the studentship award to P.K.F.Y. and the development grant DG 222 to KKM. Drs. E.M. Hawes, G. McKay and J.K. Cooper of the College of Pharmacy are thanked for helpful suggestions.

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Journal of Chromatography, 231(1982) 205—209
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1285

Note

Ion-exchange high-performance liquid chromatography in drug assay in biological fluids

I. Ethmozin

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(First received December 14th, 1981; revised manuscript received March 1st, 1982)

The reversed-phase sorbents that are most widely used in drug assay in biological fluids by high-performance liquid chromatography (HPLC) have some disadvantages because of their low selectivity. The retention on such sorbents depends mainly on the lipophilic properties of the substances to be analysed. On the other hand, biofluids, blood especially, contain considerable amounts of the various lipid substances, which are extracted, as a rule, with drugs and metabolites. When analysing such extracts on reversed-phase columns one often observes interference to the drug and/or metabolite peaks by endogenous substances. Ion-exchange sorbents are significantly more selective and practically do not retain non-ionogenic substances. This is why these sorbents seem to be highly specific for the assay of the drugs that form ions in solution. Many drugs exhibit such a property.

In this paper the assay method is presented for the new potent antiarrhythmic drug ethmozin (1, Fig. 1), which has been developed in the U.S.S.R. and is at present being studied in the U.S.S.R. and the U.S.A. [1—3]. The advantage of the cation-exchange column compared to the reversed-phase column is demonstrated. The method has a detection limit of about 0.01 μg/ml with a plasma sample volume of 1 ml, and is not time-consuming.

Recently two papers have been published [4, 5] which deal with the HPLC determination of some other phenothiazine drugs in plasma. Both methods use polar bonded-phase columns and require selective detectors: electrochemical detector [4] or fluorescence detector with a post-column photochemical reactor [5].

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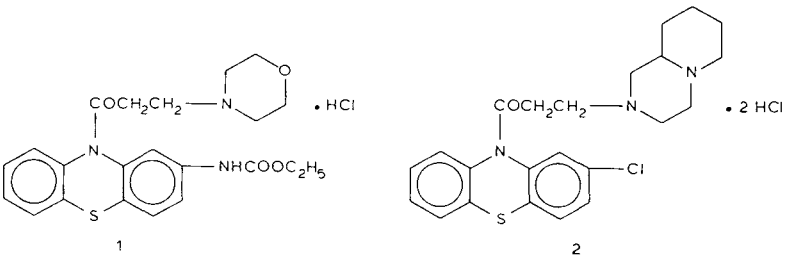

Fig. 1. Structural formulae of ethmozin (1) and nonachlazin (2).

EXPERIMENTAL

Apparatus and columns

The chromatograph used was Altex Model 322 with fixed-wavelength (254 nm) UV detector Altex Model 153 (Altex Scientific, Berkeley, CA, U.S.A.). The recorder was Linear Model 355 (Linear Instruments, Irvine, CA, U.S.A.). Chart speed was 12 cm/h, sensitivity was 10 mV/scale. The columns used were 250 × 3.2 mm packed with Partisil 10-SCX and LiChrosorb RP-18, both 10-μm particle size (Altex Scientific). The precolumns, 40 × 3.2 mm, were packed by us with the same sorbents. The columns were at ambient temperature.

Reagents and solvents

Acetonitrile, dichloromethane, isopropanol and *n*-heptane were analytical grade distilled in glass. Pure diethylamine was distilled twice over sodium hydroxide. The water was double-distilled in glass. Ethmozin and nonachlazin (internal standard) were obtained from the Institute of Pharmacology A.M.S. U.S.S.R. The stock solutions of drug and standard were prepared using double-distilled water and were stored at 4°C. They were stable for at least four months. Two concentrations were prepared: 0.02 and 0.2 μg/ml.

Extraction procedure

To 1 ml of plasma or 0.1 ml of urine in a 20-ml Pyrex tube was added 0.1 ml of standard solution (the concentration depends on the expected ethmozin level) followed by 1 ml of water and 0.1 ml of 0.1 *N* hydrochloric acid. The mixture was gently shaken for 20 min with 10 ml of dichloromethane—isopropanol (10:1). After centrifugation for 5—10 min at 500 *g* the lower layer was filtered into a conical tube and evaporated to dryness at 35°C under a gentle stream of air. To the residue 70 μl of acetonitrile were added followed, after brief mixing, by 70 μl of water. Then 0.5 ml of heptane was added to the tube which was shaken for several minutes to remove lipid particles. Then 100 μl of the lower layer were injected onto the column.

Quantitation

The internal standard method was used with nonachlazin (2, Fig. 1) as standard. For construction of the calibration curves the peak height ratios were plotted against the concentrations of ethmozin added to blank plasma and

urine. The recovery was estimated as the ratio of the ethmozin peak heights after analysis of the extracted drug and standard solution.

RESULTS AND DISCUSSION

A chromatogram of an extract of drug-free plasma on the reversed-phase column is presented in Fig. 2A. The conditions are given in the legend. Chromatogram B is the result of the analysis of the standard ethmozin solution (0.05 μg was injected) under the same conditions. It might be impossible to detect ethmozin in plasma at such a level as 0.1 μg/ml because of interference. Varying the conditions (mobile phase composition, column temperature) does not improve the separation. For the pharmacokinetic study of ethmozin the detection limit must be at least 0.02 μg/ml. Since interfering endogenous substances do not seem to be ion-generating in solution we tried to realize the separation on the cation exchanger Partisil 10-SCX. It has been found that ethmozin is retained on the column with such a sorbent. Drug-free plasma extract gives no peaks that could interfere with ethmozin (Fig. 2C); urine does not give such peaks either. This is why we chose Partisil 10-SCX for ethmozin determination. We tried several buffer systems as mobile phase but the ethmozin peak shape is quite satisfactory with diethylamine only. The organic modifier (acetonitrile) is necessary to shorten the retention time. The complete eluent composition is given in the legend to Fig. 2 (C).

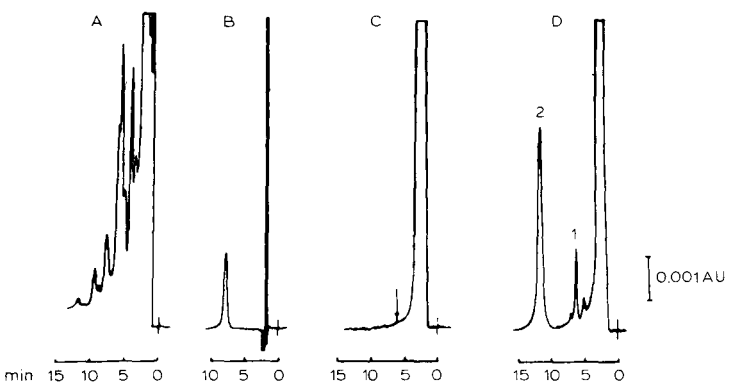

Fig. 2. (A) Chromatogram of a drug-free plasma extract on LiChrosorb RP-18. Eluent: acetonitrile—water—diethylamine (50:50:0.3). Flow-rate: 1 ml/min. Temperature: ambient. (B) Chromatogram of the ethmozin standard solution (sample size 0.05 μg) on LiChrosorb RP-18. Conditions are the same as in (A). (C) Chromatogram of a drug-free plasma extract on Partisil 10-SCX. Eluent: acetonitrile—water—diethylamine—acetic acid (27:73:0.18:0.18). Flow-rate: 1.5 ml/min. Temperature: ambient. The arrow marks the position of the ethmozin peak. (D) Chromatogram of an extract of plasma from a patient who received 300 mg of ethmozin in tablet form. Plasma sample was spiked with 0.5 μg of nonachlazin. Peaks: 1 = ethmozin; 2 = nonachlazin. Conditions are the same as in (C).

Nonachlazin (2, Fig. 1) was chosen as an internal standard since its absorbance spectrum is close to that of ethmozin and nonachlazin can therefore be detected at the same wavelength (254 nm). Its extraction

behaviour is also like ethmozin. The retention times of ethmozin and nonachlazin under the noted conditions are ca. 6 and 11 min, respectively. They depend on the mobile phase composition: the higher the acetonitrile and/or diethylamine—acetic acid content the shorter the rentention time of the drugs.

The calibration curves were plotted in the concentration range 0.02—5 μg/ml for plasma and 0.1—20 μg/ml for urine and were found to be linear (r = 0.97 and 0.98, respectively).

Table I gives data on the within-day and day-to-day accuracy of the ethmozin determination in plasma for four different concentrations. The accuracy of the ethmozin determination in urine was not greatly affected by concentration and was no more than 4% within-day and 5.5% day-to-day.

The ethmozin recovery from plasma was estimated at 0.05 and 2 μg/ml, and was equal to 73 ± 5% (n = 6) and 84 ± 7% (n = 7), respectively. The recovery from urine was not lower than 90%.

TABLE I

WITHIN-DAY AND DAY-TO-DAY ACCURACY OF ETHMOZIN DETERMINATION IN PLASMA

Concentration (μg/ml)	Accuracy (C.V. %, n = 5)	
	Within-day	Day-to-day
0.05	10.5	14.1
0.2	7.1	8.5
0.5	5.2	6.4
2.0	3.8	5.1

TABLE II

DRUGS WHICH DO NOT INTERFERE WITH ETHMOZIN DETERMINATION

Acetylsalicylic acid	Methoprolol
Amitriptyline	Methyldopa
Amobarbital	Mexiletine
Ampicillin	Nitrazepam
Caffeine	Nitroglycerine
Carbamazepine	Oxazepam
Chlorpropamide	Paracetamol
Diazepam	Phenylbutazone
Digoxin	Propranolol
Ephedrine	Quinidine
Furosemide	Sulfamethoxazole
Hydrochlorothiazide	Theophylline
Insulin	Verapamil
Isosorbide dinitrate	Warfarin
Lidocaine	

In Fig. 2D an example of the analysis of a human plasma extract after oral administration of 300 mg of ethmozin is presented. The concentration is found to be 0.087 μg/ml.

Thus the ion-exchange column had in this case obvious advantages over the reversed-phase column because the endogenous lipid substances from plasma, which were retained on the reversed-phase column, were practically not retained on Partisil 10-SCX.

The detection limit of the method is about 0.01 μg/ml and may be somewhat lower if the effluent is monitored at the ethmozin absorbance maximum (269 nm). Table II is a list of drugs which do not interfere in the determination of ethmozin by the method described. The extraction procedure is relatively simple. Up to 30 samples can be worked-up during a day. The residues after extract evaporation can be stored at 4°C for several days. The analysis time of one sample does not exceed 15 min. Provided the precolumn packing is changed regularly, the column can be used for at least two years without any noticeable change in its quality.

With a small variation in the ratio of the mobile phase components the method can be used for the determination of other phenothiazine drugs in biological fluids.

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Journal of Chromatography, 231 (1982) 210—215
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1304

Note

Determination of tolazoline in plasma by high-performance liquid chromatography

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(Received February 24th, 1982)

Tolazoline is an alpha-adrenergic blocking agent which also exhibits dilator action on the peripheral blood vessels. For this reason, it is recommended in the treatment of peripheral vascular disorders such as acrocyanosis and Raynaud's syndrome [1, 2]. Tolazoline has also been shown to be a histaminergic agonist acting at H_2 receptors [3]. Recently, tolazoline has been proposed as a vasodilator for newborns suffering from pulmonary hypertension. As small plasma volumes (< 0.2 ml) are available in these patients, a sensitive method is required for monitoring drug concentrations.

Several methods have been described for measuring tolazoline and other imidazoline derivatives [4—8]. These methods are generally based on spectrophotometry, colorimetry, thin-layer chromatography, gas—liquid chromatography and liquid column chromatography.

The spectrophotometric and colorimetric techniques are often not specific because they cannot differentiate the parent compound from the metabolites or the degradation products. Thin-layer and gas chromatography require several steps for the analysis of these compounds and are therefore time consuming.

Liquid column chromatography appears to be a suitable technique, which allows the analysis of the compound underivatized straight after extraction from biological fluids. We describe in this paper a high-performance liquid chromatographic (HPLC) method suitable for the monitoring of tolazoline plasma concentrations in newborns.

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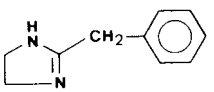
0378-4347/82/0000—0000/$02.75

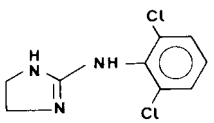
EXPERIMENTAL

Standard and reagents

Tolazoline hydrochloride [4,5-dihydro-2-(phenylmethyl)-1H-imidazole hydrochloride] was supplied by Dr. Monin, Maternité Universitaire of Nancy, France. Clonidine hydrochloride [2-(2,6-dichlorophenylamino)-2-imidazoline hydrochloride], the internal standard, was supplied by Dr. Cavero, Department of Biology, Synthélabo-L.E.R.S., Paris, France. Both compounds were checked for purity by mass spectrometry.



TOLAZOLINE



CLONIDINE

Aqueous solutions of tolazoline (1 mg/ml, 100 μg/ml and 10 μg/ml) and clonidine (100 and 10 μg/ml) were prepared. The final concentrations were calculated as free bases for both compounds. Thes solutions were found to be stable for 3—4 months at 4°C.

Analytical-reagent grade chloroform and acetonitrile (Uvasol) were purchased from Merck (Darmstadt, G.F.R.).

Extraction procedure

A 20-μl volume of an ethanolic solution of clonidine (10 μg/ml) was added to a tapered conical tube together with 0.5 ml of 0.25 *M* potassium hydroxide solution and 0.2 ml of plasma sample. This solution was extracted with chloroform (7 ml) on a shaker (Bioblock) for 20 min.

The sample was centrifuged for 5 min at 1000 *g* and 4°C and the upper aqueous phase was discarded. Approximately 6.5 ml of the chloroform phase was transferred into a second clean tube and evaporated to dryness in a water-bath at 60°C under a gentle stream of nitrogen. The dry residue was dissolved in 870 μl of acetonitrile—0.02 *M* potassium dihydrogen phosphate (KH_2PO_4) (5:97, v/v) and 500 μl of this solution were injected into the chromatograph. The extraction scheme is shown in Fig. 1.

Liquid chromatographic conditions

The LDC Constametric IIG high-performance liquid chromatograph was equipped with a Micromeritics 725 automatic injector fitted with a 500-μl injection loop. The chromatograms were recorded with a Perkin-Elmer Model 56 recorder. The stainless-steel column (15 cm × 4.6 mm I.D.) was packed with Spherisorb ODS 5 μm batch 17/49 (Sopares, Gentilly, France), according to the technique described by Broquaire [9]. The flow-rate of the mobile phase (acetonitrile—0.02 *M* KH_2PO_4, 62.5:37.5, v/v) was adjusted to 1.0 ml/min. The detector wavelength was set at 210 nm.

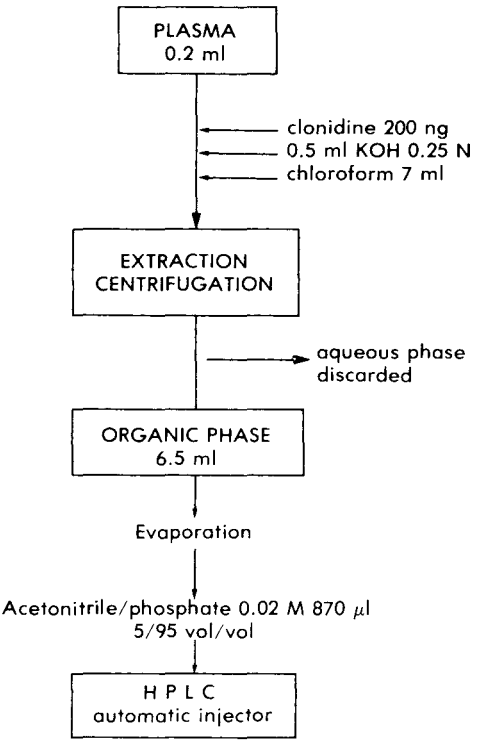


Fig. 1. Scheme for extraction of tolazoline from plasma.

Under these analytical conditions, the retention times were 6.6 and 7.2 min for tolazoline and clonidine, respectively.

RESULTS AND DISCUSSION

First we developed a gas—liquid chromatographic (GLC) method with electron-capture detection. After adding norpethidine (normeperidine) as internal standard, the sample was derivatized with heptafluorobutyric anhydride. This technique was relatively complicated, owing to the difficult acylation of the imidazole nitrogen and the use of norpethidine as internal standard.

Tolazoline absorbs UV light with a maximum at 210 nm, and we therefore decided to develop an HPLC method with clonidine, a structurally related compound, as the internal standard.

The scheme for the extraction of tolazoline from plasma is shown in Fig. 1. The analytical conditions for the extraction of the drug from plasma were established using a spiked human plasma sample containing 5 µg/ml of tolazoline.

A 0.2-ml volume of plasma was acidified with 0.25 *N* hydrochloric acid, made neutral with 0.5 *M* phosphate buffer (pH 7) or alkaline with 0.25 *M* potassium hydroxide solution. Tolazoline was not extracted at acidic pH (ca. 1), poorly extracted at neutral pH and well extracted at alkaline pH (ca. 13).

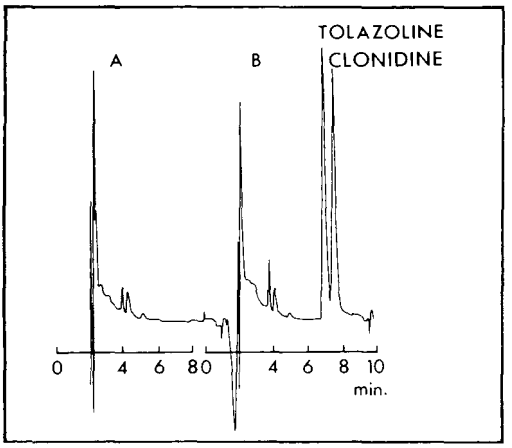


Fig. 2. Chromatograms of plasma extracts. In B, the plasma was spiked with 200 ng of tolazoline and clonidine.

The solvents chloroform, ethyl acetate, freshly distilled diethyl ether and *n*-hexane were tested for optimal extraction. The best solvent was chloroform, which provided after a single extraction at alkaline pH a recovery from the plasma sample of 85 ± 3% (S.E.). Furthermore, the chloroform extract was clean. No endogenous compound present in plasma interfered with tolazoline and clonidine (Fig. 2).

The extraction of tolazoline and clonidine from plasma was completed after 20 min. The UV wavelength selected for the HPLC detector was 210 nm, corresponding to maximum UV absorption for tolazoline. This value gave the best signal-to-noise ratio.

The calibration graph (Table I) was linear for tolazoline plasma concentrations between 0.2 and 20 μg/ml. In these experiments, 200 ng of clonidine were added (20 μl of a 10 μg/ml solution). The minimum sensitivity of detection was 0.2 μg/ml with a coefficient of variation (C.V.) of ± 14.3%. For tolazoline concentrations of 8—20 μg/ml, the coefficient of variation was only 2—3%.

TABLE I

ACCURACY AND REPRODUCIBILITY OF TOLAZOLINE DETERMINATION BY HPLC

Number of determinations: 9.

Added (μg/ml)	Found (μg/ml)	C.V. (%)
0.20	0.21	± 14.3
0.50	0.47	± 8.5
1.00	1.01	± 8.9
4.00	3.96	± 6.3
8.00	8.05	± 2.2
20.00	19.99	± 3.3

Chromatograms obtained from plasma of newborns sampled before and after tolazoline administration are shown in Fig. 2. Gentamicine, cefradine, ampicillin and colistin, drugs that could be associated with tolazoline therapy in newborns, were not co-eluted with tolazoline and the internal standard and do not interfere in the analysis. An example of plasma concentration vs. time curve is displayed in Fig. 3. The newborn received first a loading dose intravenously, followed by an intravenous perfusion to maintain tolazoline plasma levels. At the end of perfusion, the elimination half-life of tolazoline was determined. In this patient, plasma concentrations were stable during perfusion (between 7 and 8 μg/ml), then decreased monoexponentially at the end of perfusion with a half-life of 5.3 h.

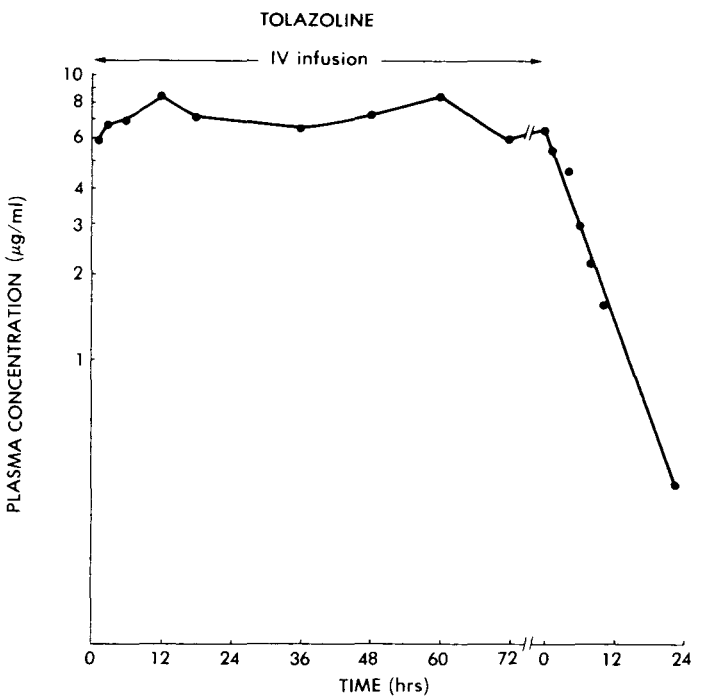


Fig. 3. Plasma concentrations of tolazoline in a 34-week-old newborn (2.38 kg) during and after intravenous infusion. A bolus of 2 mg/kg was injected over a 3-min period, followed by a continuous infusion providing a maintenance dose of 2 mg/kg.

The simplicity of this technique should be emphasized; tolazoline and clonidine can be analysed after a single extraction from alkaline plasma.

The best recovery (85%) was obtained with chloroform, which also provided a clean plasma extract. The reversed-phase conditions allowed the injection of large volumes of the water—acetonitrile mixture, without alteration of the characteristics of the chromatographic separations. Thus, samples could be injected automatically. The chromatographic separation of tolazoline and clonidine was achieved in less than 20 min, and 40—50 samples could be analysed routinely in one day. The described method appears to be suitable for therapeutic plasma monitoring of tolazoline in either newborns or adults.

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Journal of Chromatography, 231 (1982) 216—221
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1308

Note

High-performance liquid chromatographic determination of dipyridamole

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(First received December 23rd, 1981; revised manuscript received March 23rd, 1982)

Although there has been considerable interest in the antithrombotic potential of dipyridamole (DIP) [1—5], studies in different species and in some occasions in the same species, have produced variable results [4, 6—8]. Tyce et al. [9] have suggested that variability in response may be attributed to variability in plasma concentrations. Such intersubject variability in pharmacokinetics was reported by Mahoney et al. [10] who also suggested that the current clinical practice of empirical dosage schedules may be inappropriate. Rajah et al. [11] in man and Buchanan et al. [12] in rabbits demonstrated a positive correlation between plasma concentrations of DIP and effect on platelet behaviour. These data suggest that rational therapy with DIP requires maintenance of plasma concentrations above a certain threshold level. It is therefore necessary that simple and rapid methods be available for the determination of this drug in biological fluids.

Determinations of DIP concentrations have been carried out by spectrophotofluorometric methods [13—16] and by chromatographic [16—19] techniques.

The chromatographic procedures described to date are based either on fluorometric and ultraviolet (UV) detection. While fluorometry offers the advantage of sensitivity, it suffers from the disadvantage of a limited linear dynamic range. For instance, while both Schmid et al. [17] and Wolfram and Bjornsson [18] reported linear concentration—response relationships from 1—400 ng/ml and 1—500 ng/ml, respectively, variable amounts of plasma or blood must be used. It is necessary to calibrate the system for various sample volumes of blood or plasma particularly if whole blood is used [18]. Furthermore, DIP has endogenous fluorescence only at alkaline pH which can cause considerable difficulties with high-performance liquid chromatographic (HPLC)

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analysis due to instability of the support at high pH [17]. This problem can be overcome by the use of ion-pair chromatography wherein the fluorescence can be measured at acidic pH [18]. However, both fluorometric techniques doe not resolve potential problems of a relatively small linear dynamic range which complicates the methods by requiring varying amounts of plasma.

HPLC with UV detection has a wide linear dynamic range, does not require alkaline pH or ion-pair chromatography [19]. However, the reported method [19] does not take advantage of these characteristics. It requires both different volumes and different methods of extraction dependent upon concentrations and finally the method is externally calibrated. We describe an HPLC–UV detection method for DIP which uses uniform volumes of plasma, is sufficiently sensitive to follow the concentration of DIP in man for 48 h and is linear over the range of concentrations found in man at standard therapeutic regimen.

Experimental apparatus

The HPLC system used was a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 liquid chromatograph equipped with a U6K injector and a Model 440 dual-channel filter absorbance detector in conjunction with a Hewlett-Packard (Avondale, PA, U.S.A.) 3380A integrator. The column used was an Altex Ultrasphere 5-μm reversed-phase C18 column (25 cm × 0.46 mm I.D.) purchased from Beckmann (Canada).

Reagents

Dipyridamole was generously supplied by Boehringher Ingelheim (Burlington, Canada). Propranolol (P) was a kind gift from Ayerst Research Labs. (Montreal, Canada). Reagents for the preparation of buffers [tris(hydroxymethyl)aminomethane] (Tris) and both sodium and potassium phosphate salts were purchased from BDH (Toronto, Canada). N,N,N,N-Tetramethylethylene diamine (TMD) was purchased from Matheson Coleman and Bell (East Rutherford, NJ, U.S.A.). Diethyl ether used in the extractions was purchased from Canlab (Toronto, Canada) and was analytical grade. Acetonitrile for HPLC was purchased from Caledon Labs. (Mississauga, Canada). Water for the preparation of all buffers was glass-distilled in-house. Stock solutions of DIP and P, 5 mg/ml in ethanol, were prepared and stored at −20°C in darkness. Under these conditions the solutions were stable for at least nine months.

HPLC conditions

DIP and P were eluted with 33% acetonitrile in 0.02 *M* phosphate buffer containing 0.01 *M* TMD at pH 2.9. The flow-rate was 2.5 ml/min which generated a pressure of approximately 238 bar. The effluent was monitored at 280 nm [16]. Under these conditions both DIP and P could be detected and a baseline separation was readily achieved. The retention time for P was 6.5 min and for DIP it was 7.5 min. Quantitation was by determination of the ratio of the response for DIP to P. Both peak areas and peak heights were taken as a measure of the response of the two compounds.

Standard solutions

A series of serial dilutions from the stock solution of DIP (5 mg/ml) was prepared in ethanol. A 50-μl aliquot of each of these solutions was added to 5 ml of plasma. In this way, a series of concentrations ranging from 2 to 2000 ng/ml was prepared. The blank consisted of the addition of 50 μl of ethanol to 5 ml of plasma. The solutions so prepared were stored at −20°C and were found to be stable for a period of two years.

Sample preparation

To 1 ml of plasma were added 500 ng of P in 50 μl of ethanol. A spatula of sodium chloride was then added and the tube mixed to ensure saturation of the aqueous phase with the salt. One ml of 1 *M* Tris buffer at a pH of 10 was added to the plasma solution and this was further agitated to ensure maximum dissolution of the sodium chloride. Five ml of diethyl ether were added, the mixture was shaken for 5 min and centrifuted at 1500 *g* for 10 min. The clear organic phase was transferred to a second tube containing 3 ml of hexane. The DIP and P were then back-extracted into 50 μl of 0.1 *N* hydrochloric acid. After shaking and centrifugation, the ether layer was removed by aspiration. An aliquot of the aqueous phase or the total phase could be injected for analysis.

RESULTS

HPLC conditions

Factors controlling the elution of DIP from a 5-μm octadecyl silica gel column are the percent acetonitrile in the mobile phase, and the presence of TMD. The results are summarized in Table I.

TABLE I

THE EFFECT OF COMPOSITION OF THE MOBILE PHASE ON RETENTION TIME

Acetonitrile (%)	pH of the aqueous phase	Concentration of TMD (*M*)	Retention time (min)*
30	4	0	—**
30	4	0.01	13.75
35	4	0.01	8
33	2.9	0	10.32
33	2.9	0.01	7.49
33	2.9	0.02	6.82

*Flow-rate = 2.5 ml/min.
**No detectable peak within 15 min of injection.

Extraction procedure

In order to achieve high extraction efficiency from plasma into the relatively small volume of diethyl ether the plasma which was buffered to pH 10 was saturated with sodium chloride. Similarly, in order to facilitate the back-extraction of DIP from diethyl ether into 50 μl of acid, hexane was added to

the ether extract. The overall efficiency of extraction was 87%. This was determined by external calibration method with P acting as an external standard added to the final aqueous extract. In our hands omission of either sodium chloride or hexane resulted in an extraction of only trace amounts of DIP.

Linearity and reproducibility of the method

In the range from 2—2000 ng/ml the calibration curve was linear, following the equation $y = 0.14x + 0.009$ ($n = 20$; $r = 0.998$). At the 100 ng/ml level the relative standard deviation was 3% whereas at the 10 ng/ml level the relative standard deviation was 5% ($n = 5$).

DISCUSSION

The potential utility of monitoring plasma concentrations as an adjunct to therapy with DIP makes it necessary that methods for determining concentration of this drug in biological samples be as selective, simple and rapid as possible. In particular, if such therapeutic monitoring is to become generally used it is important that such techniques be readily established in different laboratories. HPLC using high-resolution columns and UV detection offers the best combination of selectivity and simplicity of instrumentation.

The percentage of acetonitrile in the mobile phase plays a role in determining the elution volume of DIP (Table I) with acidic conditions being a prerequisite [19]. This is expected for a basic drug. However, Table I also shows that TMD has a major effect on elution volume. The purpose of the diamine is to inactivate binding sites that may bind the highly polar nitrogenous base [20]. It is evident from the effect of TMD on elution volume that these binding sites, if not inactivated, can alter the elution volume of a basic analyte.

Recognizing the need for simplicity, conditions were developed which utilized single extraction steps as well as small volumes of solvents and extractants. In order to effect a high efficiency of extraction from buffered plasma we found it necessary to saturate the aqueous phase with sodium chloride. Salting out was also technically useful in that the protein separated out in a very narrow band at the interface between the aqueous solution and the diethyl ether. This permitted a facile transfer of organic phase into the second extraction tube. Similarly, in our hands, the addition of hexane to the diethyl ether prior to back-extraction was necessary in order to have a high extraction efficiency into 50 μl of 0.1 N hydrochloric acid. Again, there was a technical improvement in that the separation of the 50 μl of aqueous from organic phase was free of emulsion. When only diethyl ether was used there was emulsion between the organic phase and the 50 μl of aqueous phase.

The use of an internal standard simplified the entire procedure by eliminating the need for precise transfer of solvent and precise injection volume. Propranolol was used because the drug is readily available and since it is a tertiary amine its extraction characteristics are similar to DIP. Furthermore the differences in structure between analyte and standard permit a separation of these two compounds.

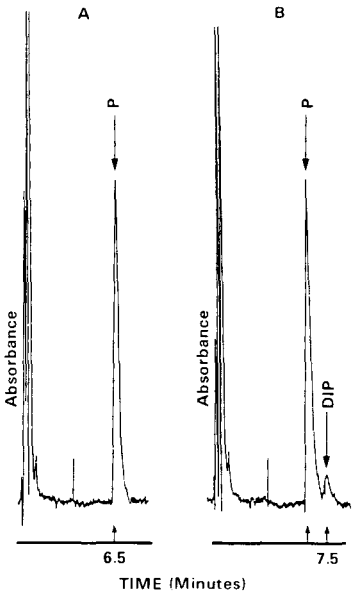

Fig. 1. Plasma prepared from a sample (A) prior to ingestion of DIP; and (B) 48 h after the last dose. The concentration was 10 ng/ml. These results were obtained by injecting a 25-μl aliquot of the 50-μl extract.

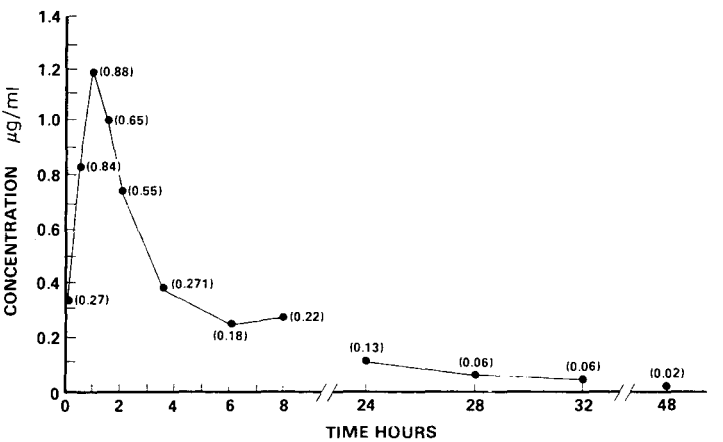

Fig. 2. Plasma concentration profile from ten volunteers receiving 75 mg DIP for 1 week t.i.d. Concentrations shown are averages with standard deviations in brackets. Sample for $T = 0$ was taken at 8:00 a.m. just prior to the last dose.

The extracts of blank plasma so prepared showed no interference to the detection of DIP at low concentrations of DIP (Fig. 1).

This technique is not quite as sensitive as a fluorometric method, but it is not markedly less sensitive. It was found to have sufficient sensitivity to permit a description of the plasma concentration profile over a 48-h period (Fig. 2).

The calibration is linear over the concentration range normally found in

man and uniform volumes can be used. Finally, the method is sufficiently simple and rapid to permit the extraction of 100 samples per day of plasma.

ACKNOWLEDGEMENTS

This work was supported by Boehringer Ingelheim (Canada, Great Britain). We thank Mr. R. Devereaux for his helpful discussions.

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Journal of Chromatography, 231 (1982) 222—223
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1296

Book Review

Liquid chromatography in clinical analysis, edited by P.M. Kabra and L.J. Marton, Humana Press (Wiley), Clifton, NJ, 1981, XVII + 466 pp., price US$ 55.00 (U.S.A.), US$ 65.00 (other countries), ISBN 0-89603-026-1.

The great interest in clinico-chemical applications of liquid column chromatography is reflected in the increasing number of monographs devoted to this topic. Unfortunately, the four books published previously on this theme are only proceedings of international symposia. In this respect the book by Kabra and Marton is different as it originated as an outgrowth of a course organized by the Department of Laboratory Medicine of the University of California.

The book consists of three parts, the first of which is devoted to an Introduction to liquid column chromatography (91 pages), the second to Therapeutic drug monitoring and toxicology (153 pages) and the third to Clinical analysis of endogenous human biochemicals (201 pages).

In the first part there is a short introduction to high-performance liquid chromatography (HPLC), its instrumentation and column technology. In these chapters most attention is paid to column technology which is excellent. A table of the most common sorbents would, however, have been highly welcomed. In the chapter on instrumentation, electrochemical detectors, which play an important role in clinical analysis, are dealt with only very briefly. Post-column reactors are not discussed at all (with the exception of a chapter devoted to proteins). There are some fundamental books missing from the list of references (e.g., Z. Deyl et al., Liquid Column Chromatography, and J.F.K. Huber, Instrumentation for High-Performance Liquid Chromatography).

The section on therapeutic drug monitoring is classified according to the main groups of compounds that receive the most attention today: anticonvulsants, antiarrhythmics, antibiotics, tricyclic antidepressants, antineoplastic drugs and hypnotics. Important groups of compounds that have been omitted include antirheumatics and other analgesics and drugs of abuse. Most chapters contain very useful tabulated surveys of the literature and the text includes many figures. Toxicological screening is presented only very briefly, probably because HPLC cannot yet compete with gas chromatography—mass spectrometry or with thin-layer chromatography as a screening procedure. Another problem is that sample preparation is repeated in every chapter, whereas it would have been better to discuss it as a separate topic in this section.

In the third part there are chapters devoted to tyrosine and tryptophan

metabolites (including catecholamines), steroids, proteins, bilirubin and its carbohydrate conjugates, porphyrins, organic acids, nucleosides, RNA and DNA and polyamines. In this section, omissions include the analysis of amino acids, carbohydrates, lipids and their constituents, and at least a paragraph in the chapter on organic acids should have been included to describe their profiling in body fluids.

In spite of the above imperfections, this book bears the signs of a carefully considered construction and, although several authors contributed to the volume, the style is remarkably uniform. As with any book dealing with a rapidly developing area, although this one has been published fairly quickly, it is unfortunately not completely up-to-date (literature references are mostly up to 1979, and quotations from 1980 are exceptions). As with two other books (edited by Hawk), also in this book the term Liquid Chromatography in the title is incorrect as the book deals only with liquid *column* chromatography (and, for example, planar techniques are not described). At the moment the book represents the most complete publication on the application of liquid column chromatography in clinical analysis and the Editors can be congratulated on their enrichment of this area.

Prague (Czechoslovakia) KAREL MACEK

Vol. 231, No. 1

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

COURSE

BIOCHEMICAL SEPARATION METHODS

The above-mentioned course will be held from March 22–June 7, 1983, at the Uppsala Separation School, Institute of Biochemistry, University of Uppsala, Uppsala, Sweden, and is organized by Professor S. Hjertén and Professor P. Roos.

The course is centered around modern analytical and preparative methods for the separation of cells, virus, proteins and nucleic acids, and their characterization. It consists of lectures and laboratory work dealing with the following methods: moving boundary electrophoresis, free zone electrophoresis, zone electrophoresis in both sieving and non-sieving anticonvection media, isoelectric focusing, displacement electrophoresis (isotachophoresis), molecular sieve chromatography, hydroxyapatite chromatography, hydrophobic interaction chromatography, covalent chromatography, bioaffinity chromatography, gas chromatography, HPLC, counter-current distribution (liquid phase partition), analytical and preparative centrifugation methods (centrifugation in different kinds of density gradients, determination of sedimentation coefficients and of molecular weights), immunodiffusion, immunoelectrophoresis, determination of diffusion coefficients, light scattering, spectrofluorometry, bioluminescence, and radioimmunoassay.

Knowledge of biochemistry and mathematics, corresponding to a basic university degree, is required. Good knowledge of English is necessary. The number of participants is limited to 12, 6 from Sweden and 6 from abroad.

The course fee is US $450. Living expenses to cover food and accommodation in student rooms will be a minimum of US $1600. No fellowships are available through the organizers.

The closing data for applications is January 15, 1983. Application forms can be obtained from: Eva Linder, Secretary, Institute of Biochemistry, University of Uppsala, Box 576, S-751 23 Uppsala, Sweden.

CALENDAR OF FORTHCOMING EVENTS

Sept. 6–9, 1982 Bath, Great Britain	**4th European Symposium on Chemical Structure – Biological Activity: Quantitative Approaches** Contact: Dr. J.C. Dearden, School of Pharmacy, Liverpool Polytechnic, Byrom Street, Liverpool L3 3AF, Great Britain.(Further details published in Vol. 228.)

Sept. 6–9, 1982 Hradec Králové, Czechoslovakia	**8th International Symposium on Biomedical Applications of Chromatography** Contact: Dr. K. Macek, Institute of Physiology, Czechoslovakian Academy of Sciences, Vídeňská 1083, CS-142 20, Prague 4-Krč, Czechoslovakia. (Further details published in Vol. 225, No. 2.)
Sept. 9–10, 1982 Lausanne, Switzerland	**International Meeting on Isolation, Separation and Structural Determination of Natural Products** Contact: Professor K. Hostettmann, Laboratoire de Pharmacognosie et Phytochimie, Ecole de Pharmacie de l'Université de Lausanne, Rue Vuillermet 2, CH-1005 Lausanne, Switzerland.
Sept. 13–17, 1982 London, Great Britain	**14th International Symposium on Chromatography** Contact: The Executive Secretary, Chromatography Discussion Group, Trent Polytechnic, Burton Street, Nottingham, NG1 4BU, Great Britain. (Further details published in Vol. 211, No. 3)
Sept. 19–24, 1982 Singapore, Republic of Singapore	**2nd Asian-Pacific Congress on Clinical Biochemistry** Contact: 2nd Asian-Pacific Congress on Clinical Biochemistry, Singapore Professional Centre, 129B Block 23, Outram Park, Singapore 0316, Republic of Singapore.
Oct. 4–6, 1982 Tarrytown, NY, U.S.A.	**Capillary Chromatography '82 – An International Symposium** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.
Oct. 12–14, 1982 Salzburg, Austria	**DIOXIN 82, 3rd International Symposium – Workshop on Chlorinated Dioxins and Related Compounds** Contact: Dr. E. Merian, Im Kirsgarten 22, CH-4106 Therwil, Switzerland. (Further details published in Vol. 219, No. 3.)
Oct. 18–22, 1982 Columbus, OH, U.S.A.	**Symposium and Workshop on New Spectroscopic Methods for Biomedical Research** Contact: Karen L. Waite, Battelle's Columbus Laboratories, 505 King Avenue, Columbus, OH 43201, U.S.A. Tel.: (614) 424-4179
Oct. 19–20, 1982 Montreux, Switzerland	**Short Course on LC–MS and MS–MS** Contact: Workshop Office, Dr. Alain Donzel, Case postale 130, CH-1000 Lausanne 20, Switzerland. Tel.: 004161/63 27 89 or 004121/33 50 83.
Oct. 21–22, 1982 Montreux, Switzerland	**2nd Workshop on Liquid Chromatography–Mass Spectroscopy (LC–MS) and MS–MS** Contact: Workshop Office, Dr. Alain Donzel, Case Postale 130, CH-1000 Lausanne 20, Switzerland. Tel.: 004161/63 27 89, or 004121/33 50 83. (Further details published in *J. Chromatogr.*, 251 (1982) 225; proceedings to be published in *J. Chromatogr.)*
Dec. 6–8, 1982 Baltimore, MD, U.S.A.	**2nd International Symposium on HPLC of Proteins, Peptides and Polynucleotides** Contact: Shirley E. Schlessinger, Symposium Manager, 2nd International Symposium on HPLC of Proteins, Peptides and Polynucleotides, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011.

Dec. 6–8, 1982 Parsippany, NJ, U.S.A.	**3rd Biennial Symposium on Advances in Thin-Layer Chromatography** Contact: Dr. Touchstone, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A. Tel.: (215) 662-2082. (Further details published in Vol. 235, No.1.)
March 7–12, 1983 Atlantic City, NJ, U.S.A.	**1983 Pittsburgh Conference and Exhibition on Analytical Chemistry and Applied Spectroscopy** Contact: 1983 Pittsburgh Conference, 437 Donald Road, Dept. FP, Pittsburgh, PA 15235, U.S.A.
April 5–8, 1983 Cardiff, Great Britain	**International Symposium in Electroanalysis in Biomedical, Environmental and Industrial Sciences** Contact: Short Courses Section, University of Wales Institute of Science and Technology (UWIST), Cardiff CF1 3NU, Wales, Great Britain.
April 17–23, 1983 Limassol, Cyprus	**1st Cyprus Conference on New Methods in Drug Research** Contact: Conference Secretariat, 1st Cyprus Conference on New Methods in Drug Research, P.O. Box 121, Oxford, Great Britain.
May 2–6, 1983 Baden-Baden, G.F.R.	**VIIth International Symposium on Column Liquid Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrapstrasse 40–42, D-6000 Frankfurt (Main) 90, G.F.R.
May 30–June 3, 1983 Melbourne, Australia	**International Conference on Chromatographic Detectors** Contact: The Secretary, International Conference on Chromatographic Detectors, University of Melbourne, Parkville, Victoria 3052, Australia. (Further details published in Vol. 216.)
June 4–12, 1983 Cologne, G.F.R.	**29th Congress of the International Union of Pure and Applied Chemistry (IUPAC)** Contact: Dr. M. Williams, Executive Secretary, IUPAC, Bank Court Chambers, 2–3 Pound Way, Cowley Centre, Oxford OX4 3YF, Great Britain.
June 7–10, 1983 Brussels, Belgium	**1st International Symposium on Drug Analysis** Contact: Ms. C. Van Kerchove, Secretary, Société Belge des Sciences Pharmaceutiques/Belgisch Genootschap voor Pharmaceutische Wetenschappen, rue Archimedesstraat 11, B-1040 Brussels, Belgium. Tel.: (02) 733 98 20, ext. 33.
June 26–July 1, 1983 Amsterdam, The Netherlands	**23rd Colloquium Spectroscopium Internationale** Contact: Conference Secretariat 23 CSI, c/o Organisatie Bureau Amsterdam BV, Europaplein, 1078 GZ Amsterdam, The Netherlands. Tel.: (020) 44 08 07; Telex: 13499 raico nl.
June 27–July 1, 1983 Gatlinburg, TN, U.S.A.	**3rd Symposium on Separation Science and Technology for Energy Applications** Contact: A.P. Malinauskas, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, TN 37830, U.S.A.
July 17–23, 1983 Edinburgh, Scotland, Great Britain	**SAC '83, International Conference and Exhibition on Analytical Chemistry** Contact: The Royal Society of Chemistry, Analytical Division, Burlington House, London W1V 0BN, Great Britain. Tel.: 01-734-9971. (Further details published in Vol. 214, No. 2.)

Aug. 28–Sept. 2, 1983 Amsterdam, The Netherlands	**9th International Symposium on Microchemical Techniques** Contact: Symposium Secretariat, c/o Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel: (020) 552 3459.
Aug. 29–Sept. 2, 1983 Bratislava, Czechoslovakia	**4th Danube Symposium on Chromatography and 7th International Symposium "Advances and Application of Chromatography in Industry"** Contact: Dr. Ján Remen, The Analytical Section of the Czechoslovak Scientific and Technical Society, Slovnaft, 823 00 Bratislava, Czechoslovakia. (Further details published in Vol. 235, No. 1.)
Oct. 1–5, 1984 Nürnberg, G.F.R.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.
Nov. 22–24, 1984 Barcelona, Spain	**3rd International Congress on Analytical Techniques on Environmental Chemistry** Contact: 3rd International Congress on Analytical Techniques on Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.
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