ISSN 0378-4347

VOL. 224 NO. 1 JUNE 12, 1981
(Biomedical Applications, Vol. 13, No. 1)

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

JOURNAL OF CHROMATOGRAPHY

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications*, which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews*, reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, if possible in three copies. Manuscripts should be submitted to: The Editor of *Journal of Chromatography*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands, or to: The Editor of *Journal of Chromatography, Biomedical Applications*, P.O. Box 681, 1000 AR Amsterdam, The Netherlands. Reviews are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications*. An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography* and the *Biomedical Applications* section can be subscribed to separately.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications, Chromatographic Reviews* and *Cumulative Author and Subject Indexes, Vols. 201–210* and *211–220*) has 24 volumes in 1981. The subscription prices for 1981 are:

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes*) + *Biomed. Appl.* (Vols. 203–226):
Dfl. 3240.00 plus Dfl. 432.00 (postage) (total *ca.* US$ 1708.00)

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes*) only (Vols. 203–220):
Dfl. 2556.00 plus Dfl. 324.00 (postage) (total *ca.* US$ 1339.50).

Biomed. Appl. only (Vols. 221–226):
Dfl. 852.00 plus Dfl. 108.00 (postage) (total *ca.* US$ 446.50).

Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 202) are available at Dfl. 156.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, NY 10164. Tel: (212) 867-9040.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, and Science Citation Index.

See page 3 of cover for Publication Schedule, Information for Authors, and information on the News Section and Advertisements.

Printed in The Netherlands

VOL. 224, NO. 1 JOURNAL OF CHROMATOGRAPHY JUNE 12, 1981

(Biomedical Applications, Vol. 13, No. 1)

CONTENTS

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

(Continued overleaf)

Contents (continued)

JOURNAL OF CHROMATOGRAPHY

VOL. 224 (1981)

(Biomedical Applications, Vol. 13)

JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

VOL. 13 1981

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

Printed in The Netherlands

Journal of Chromatography, 224 (1981) 1—23
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 835

LIPID CLASS AND MOLECULAR SPECIES INTERRELATIONSHIPS AMONG PLASMA LIPOPROTEINS OF NORMOLIPEMIC SUBJECTS

A. KUKSIS*, J.J. MYHER and K. GEHER

Banting and Best Department of Medical Research, University of Toronto, Toronto (Canada)

and

W.C. BRECKENRIDGE, G.J.L. JONES and J.A. LITTLE

Toronto-McMaster Lipid Research Clinic, University of Toronto, Toronto (Canada)

(First received October 1st, 1980; revised manuscript received January 4th, 1981)

SUMMARY

As evidence of lipoprotein interconversion and/or equilibration, a gas—liquid chromatographic (GLC) examination was made of the lipid class and molecular species interrelationships among the major fasting plasma lipoprotein fractions within each of seven male and four female normolipemic subjects subsisting on free choice diets. The lipoprotein fractions were prepared by conventional ultracentrifugation and the lipid class and molecular species composition of the corresponding lipoprotein fractions were determined by GLC of the intact cholesterol and glycerol esters and of ceramides. In general, each lipoprotein fraction possessed a well defined lipid class composition, which was characterized by a dramatically decreasing triacylglycerol and an increasing phospholipid and cholesteryl ester content when progressing from the very low (VLDL), to the low (LDL_2) and high (HDL) density lipoproteins, as already established by conventional analyses. However, the LDL_2 contained about a two times higher proportion of total phospholipids as sphingomyelin than VLDL and HDL. Furthermore, the sphingomyelins of the HDL fraction contained about 30% more of the higher molecular weight species than the sphingomyelins of either VLDL or LDL. Smaller differences were seen in the molecular species composition of the phosphatidylcholines, cholesteryl esters and triacylglycerols among the corresponding fractions of lipoproteins. In general, the lipid class and molecular species distribution is incompatible with the hypothesis which postulates VLDL conversion into LDL and HDL under the influence of lipoprotein lipase and lecithin:cholesterol acyltransferase. The significant differences noted in the lipid class and molecular species distribution suggest that the true transformations of the lipoproteins are much more complex and may also involve cholesteryl ester-triacylglycerol, triacylglycerol and phosphatidylcholine exchanges via appropriate carrier plasma proteins, as well as possible phase separation of lipids during the removal of the excess surface material from the VLDL remnants, as already demonstrated in in vitro experiments. It is concluded that a direct GLC analysis of the neutral and polar lipid components of plasma lipoprotein classes provides important evidence

0378-4347/81/0000—0000/$02.50

of lipoprotein interrelationships which may be utilized to test existing and new hypotheses of lipoprotein interconversion.

INTRODUCTION

Extensive recent work [1,2] has established that the protein moiety of human plasma very low density lipoprotein (VLDL) is a precursor of the protein moiety of low density lipoprotein (LDL). In normal humans, all of the apoprotein B moiety of LDL may be derived from VLDL and all of the VLDL apoprotein B may be converted to LDL-apoprotein B. Detailed analyses of VLDL and LDL particles have revealed that they contain approximately the same number of apoprotein B molecules in single lipoprotein [3,4] implying a direct particle interconversion. The interconversion of VLDL into LDL is mediated by lipoprotein lipase and it results in removal of most of the triacylglycerol, as much as 90% of the phosphatidylcholine and 60% of the sphingomyelin, while the cholesteryl esters are largely retained in the newly formed LDL [3]. Since much of the unhydrolyzed VLDL phosphatidylcholine and sphingomyelin is found in the high density lipoprotein (HDL) density range, Eisenberg [5] has suggested that during the degradation of human VLDL to LDL there may be HDL formed. A similar conclusion had been reached earlier on theoretical grounds by Schumaker and Adams [6], who also proposed that this transformation is mediated by lecithin:cholesterol acyltransferase. Recently several laboratories [7—10] have advanced more or less detailed models for these interconversions, including the above enzymatic pathways. Unlike previous proposals of general cascading transformations of lipoproteins, the latter models are sufficiently specific for analytical testing. The postulated largely self-contained processes [11,12] dictate defined interrelationships among the various components of the precursor and product particles, including the lipids of their polar surfaces and of neutral cores [13,14].

In the following study we have examined the precursor—product relationships among the major plasma lipoproteins by comparing the mass distribution of the lipid classes and molecular species in individual samples of plasma from a significant number of fasting normolipemic subjects. While the data so obtained are consistent with the basic hypothesis of VLDL conversion into LDL and HDL, significant differences are also seen, which suggest a more complex series of transformations than those previously proposed.

MATERIALS AND METHODS

Blood samples were obtained in the fasting state (12—14 h) from eleven healthy normolipemic subjects: seven males, 25—55 years old, and four females, 26—63 years old. The subjects lived at home and subsisted on diets of free choice.

Isolation and characterization of lipoproteins

Lipoproteins were isolated essentially according to the procedure described by Hatch and Lees [15]. Briefly, 5-ml aliquots of plasma were placed in 6.5-

ml cellulose nitrate tubes and overlayered with saline [density (d) = 1.006 g/ml] containing 1% Na_2EDTA. They were centrifuged at 10°C at 100,000 g for 18 h. The supernatant fraction (VLDL) and infranatant fraction were recovered by tube slicing. The infranatant fraction was made up to 5 ml in a volumetric flask at 20°C with saline and then adjusted to d = 1.019 g/ml by the addition of anhydrous solid potassium bromide. The material was placed in a centrifuge tube overlayered with saline adjusted to d = 1.019 g/ml and centrifuged at 100,000 g for 20 h. The supernatant fraction (LDL_1) was not investigated further. The infranatant was reconstituted to 5 ml with saline adjusted to d = 1.019 g/ml. The potassium bromide addition was continued to obtain supernatant fractions of d = 1.063 g/ml (100,000 g, 24 h) (LDL_2) and d = 1.21 g/ml (110,000 g, 48 h) (HDL). A random check of the procedure of density adjustment by picnometry indicated that the adjustments were within 2—3% of the projected value. Each supernatant fraction was washed once under conditions of isolation and collected in a final volume of 2 ml, which was dialyzed against saline. Each fraction was checked for electrophoretic mobility on agarose gel electrophoresis [16]. The apoprotein make-up was assessed by solubility measurements in tetramethylurea [17]. Fractions were also checked by double immunodiffusion against rabbit anti-human albumin, antihuman LDL and HDL. On the basis of agarose gel electrophoresis, the VLDL preparations possessed pre-β mobility with no evidence of chylomicron material or trail to the origin. The LDL_2 preparations showed only proteins of β-mobility. The HDL fraction had α-mobility. Occasionally small amounts of sinking pre-β lipoprotein representing less than 5% of optical density of the total HDL fraction were found. A double immunodiffusion showed the expected cross-precipitation bands only. The protein concentration in each lipoprotein fraction was determined by the method of Lowry et al. [18] using bovine serum albumin as a standard. Preparations of VLDL were extracted with diethyl ether after colour development. In some instances the protein concentration was determined using the modification of the method of Lowry et al. [18] described by Sata et al. [19].

Dephosphorylation and isolation of lipids

Portions of the solutions of the various density fractions (equivalent to 0.1—0.2 ml of plasma) were placed into 18-ml centrifuge tubes with screw caps (PTFE-lined). The contents were diluted with 1 ml of water, and 1 ml of diethyl ether was added followed by 2 ml of a solution (0.1 mg/ml of Tris buffer, pH 7.3) of phospholipase C (α-toxin of *Clostridium welchii*, Sigma, St. Louis, MO, U.S.A.). Finally 1.3 ml of 10% calcium chloride solution were added and the solution mixed. The incubation was performed at 33°C for 2 h with shaking. The enzyme digest was partitioned once with 10 ml chloroform—methanol (2:1) containing 200 μg of tridecanoylglycerol, and the solution centrifuged for 10 min at low speed to break any emulsion. The lower chloroform phase was collected and passed through a Pasteur pipet containing 2 cm length of anhydrous sodium sulfate and the filtrate was evaporated to dryness under nitrogen. To the dry residue were added 75 μl of TRISIL-BSA (Pierce, Rockford, IL, U.S.A.) and the vial sealed with a screw cap. After 30 min at room temperature, the silylation mixture was transferred to a conical sampling

vial and the vial sealed with a clamp-on silicone septum and transferred to a sampling table for gas chromatography.

Gas—liquid chromatography

The quantitative lipid profiles of the various lipoprotein classes were determined by means of an automated gas—liquid chromatography (GLC) system equipped with an automatic liquid injector, programmed heating, cooling and equilibration cycles, and an electronic peak area integrator. The separations were accomplished on a 50 cm × 0.2 cm I.D. stainless-steel column packed with 3% OV-1 (a methyl silicone polymer) on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.), using nitrogen as carrier gas in the temperature range 175—355°C. The peak identification and composition of samples were calculated in relation to the tridecanoylglycerol internal standard using a modification of a commercially available computer program and the results were expressed as mg% and as characteristic molar ratios of lipid classes, as previously described [20,21]. Fatty acid methyl esters of the various lipid classes were prepared by transmethylation with sulfuric acid—methanol and the methyl esters separated, identified and quantitated by GLC on 10% EGSS-X (an ethyleneglycolsuccinate—silicone copolymer), as previously described [22]. The carbon numbers of isolated diacylglycerols [23] and ceramides [24] were determined by GLC as previously described. For this purpose the diacylglycerols and ceramides were first converted into the *tert.*-butyldimethylsilyl ethers. The GLC analyses were performed on a Beckman GC-4 gas chromatograph equipped with a stainless-steel column (50 cm × 0.2 cm I.D.), containing 3% OV-1 packing.

Calculations

In addition to the automated corrections of peak areas for differences in flame ionization response and recovery, the following additional calculations were performed on the peak areas following the completion of the automated GLC. Due to a partial overlap of the higher molecular weight diacylglycerols, ceramides and the lower molecular weight cholesteryl esters in the C_{40}—C_{42} range of carbon numbers the content of phosphatidylcholine of the lipoprotein fractions was calculated from the peak areas of carbon number C_{36}—C_{38} and that of sphingomyelin from the peak areas of carbon number C_{34}, as previously described [25]. Total phosphatidylcholine = total DG × 1.28, where total DG = corrected $(C_{36} + C_{38})/0.81$, and corrected $C_{36} + C_{38} = (C_{36} + C_{38}) - kC_{34}$ ceramide; and where C_{34} ceramide = $C_{34} - 0.051 \times C_{36}$, and $k = 1$ for HDL and VLDL, and 1/2 for LDL. The total sphingomyelin = total ceramide × 1.28, where total ceramide = C_{34} ceramide × 0.758/0.30 for VLDL and LDL, and C_{34} ceramide × 0.758/0.25 for HDL. The factor 0.758 is the ratio of the response factors for ceramide and diacylglycerol TMS ether (0.758 = 0.681/0.898). The factors 0.81 and 0.30 represent the proportion of the measured diacylglycerol and ceramide species of plasma phosphatidylcholine and sphingomyelin as derived from analyses of large plasma pools. The multiplication factor 1.28 converts the ceramides and diacylglycerols into the corresponding phosphorylcholine derivatives.

The core radii of the lipoprotein particles were calculated on the basis of

the surface to volume ratio of a sphere as previously described [24,26]. Surface area = $4\pi r^2 = k[Apl(PL) + Achol\ (C)]$ and volume of core equals $4/3\pi^3 = k[Vtg(TG) + Vce)CE)]$, where k is a proportionality constant dependent on the number of particles per unit lipid mass; *PL*, *C*, *TG*, and *CE* are the mole percentages of phospholipid, free cholesterol, triacylglycerol, and cholesteryl ester, respectively; $Apl = 0.685$ nm^2 and $Achol = 0.391$ nm^2 are the molecular surface areas of phospholipid and free cholesterol, respectively. $Vtg = 1.556$ nm^3 and $Vce = 1.068$ nm^3 are the molecular volumes of triacylglycerol and cholesteryl ester, respectively. The total radii of the particles are obtained by adding the thickness of the surface monolayer (2.0 nm) to the core radii [19]. The number of neutral lipid molecules in the particle cores is calculated by dividing the appropriate proportion of the core volume by the volume of the average cholesteryl ester and triacylglycerol molecules, respectively. Likewise, the number of the polar molecules in the surface shell is calculated by dividing the appropriate proportion of the total area by the area of the average phospholipid and free cholesterol molecule, respectively [27].

RESULTS

Total lipid profiles

The total lipid profiles of the VLDL, LDL_2 and HDL fractions from a normolipemic male subject in the fasting state are shown in Fig. 1. In this elution pattern the various lipid subclasses are represented by their total acyl, acyl plus sterol, or acyl plus sphingosine carbon numbers. Peak 30 represents the internal standard, tridecanoylglycerol, which has been added in equal amounts

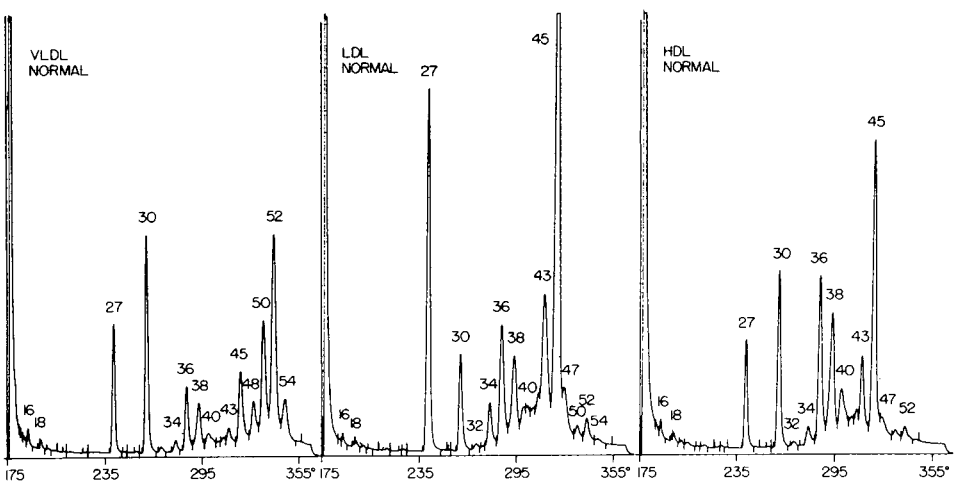


Fig. 1. GLC profiles of total lipids of plasma lipoproteins of fasting normolipemic subjects. Conditions of high-temperature GLC as given in text. Peaks: 16 and 18, trimethylsilyl esters of free fatty acids with 16 and 18 acyl carbons; 27, trimethylsilyl ether of cholesterol; 30, tridecanoylglycerol, internal standard; 34, trimethylsilyl ether of palmitoylsphingosine; 36—42, trimethylsilyl ethers of diacylglycerols of a total number of 34—40 acyl carbons; 43—47, cholesteryl esters of fatty acids with a total number of 16—20 acyl carbons; 48—56, triacylglycerols with a total number of 48—56 acyl carbons. Sample size: 1 μl of approximately 1% solution in silylation mixture. Attenuation: 100 times full sensitivity.

to each of the lipoprotein samples. The relatively small VLDL fraction is characterized by the presence of a high proportion of triacylglycerols (peaks 48—54) and smaller amounts of cholesteryl esters (peaks 43—47), which are resolved according to their carbon numbers, and free cholesterol (peak 27). A carbon number resolution is also seen for the major diacylglycerols (peaks 36—42) derived from the plasma phosphatidylcholines, while only one distinct carbon number (peak 34) is seen for the ceramides derived from sphingomyelin (the other peaks are partially hidden under the diacylglycerol peaks). The LDL_2 lipid profile is characterized by a high proportion of cholesteryl ester and smaller amounts of phospholipid and free cholesterol, and a very small proportion of triacylglycerols. The proportion of the triacylglycerols is reduced still further in the HDL fraction, which contains a high proportion of phospholipid and cholesteryl esters, with smaller amounts of free cholesterol. It should also be noted that the lipid profiles differ in the relative proportions of the peaks for free cholesterol and for cholesteryl esters, and in the ratios of the peaks representing sphingomyelin (peak 34) and phosphatidylcholines (peaks 36—42). Essentially identical elution patterns were recorded for the corresponding

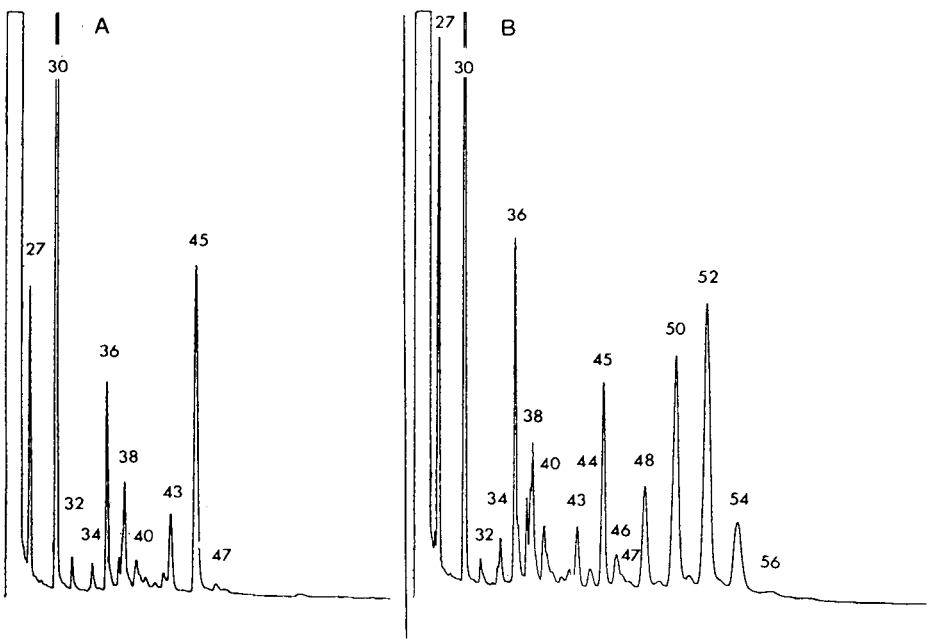


Fig. 2. Capillary GLC profiles of total lipids of plasma lipoproteins of fasting normolipemic subjects. (A) An intermediate density lipoprotein fraction containing negligible amounts of triacylglycerols; (B) VLDL. Peaks 44 and 46 represent triacylglycerols with 44 and 46 acyl carbons; other peaks as in Fig. 1. Capillary GLC conditions: a glass capillary column (5 m × 0.25 mm I.D.) coated with SP-2100 (Supelco, Bellefonte, PA, U.S.A.) and installed in a Hewlett-Packard Model 5880A gas chromatograph equipped with a splitless injector and a hydrogen flame ionization detector. The injector was maintained at 320°C, and the detector at 340°C. The hydrogen carrier gas-flow was maintained with a head pressure of 8 p.s.i. Temperature program: 240°C isothermal for 1 min, followed by temperature programming at 10°C/min to 295°C, then at 3.5°C/min to 340°C. Peak 54 was eluted in 15.80 min at 328°C. Column bleed was minimal and was automatically subtracted via the single column compensation mode of the microprocessor terminal.

lipoprotein classes from other normal subjects on the free choice diets. In VLDL, the low molecular weight triacylglycerols overlap with the cholesteryl esters, as shown by capillary GLC (Fig. 2).

Quantitative composition

The weight percentages of protein and lipid in the VLDL, LDL_2 and HDL fractions isolated from the normal subjects are given in Table I. The values were obtained by summing the estimates for individual lipid classes, calculated from the total lipid profiles, and the estimates for total protein measured independently on aliquots of the lipoprotein solutions. It is seen that the percentage of protein in the VLDL from both sexes averages 9±1%. This corresponds favourably with an average value of 8% reported by Skipski [28]. Eisenberg and Levy [4] have tabulated weight percentages for protein ranging from 4—11% for various subfractions of VLDL depending on particle size. The percentage of protein in the LDL_2 fraction from these subjects averaged 20±1%, which also corresponds well with the average content of protein in this lipoprotein class as tabulated by Skipski [28] and by Lee [29]. The weight percentage of protein in the HDL fraction of these subjects was 50±3%, which could have represented a 1:4 mixture of HDL_2 and HDL_3. Shen et al. [30] have reported 41 and 55% protein for HDL_2, and HDL_3, respectively, of normal human plasma, while Hatch and Lees [15] have estimated the ratio of these two lipoproteins to be about one part HDL_2 and four parts HDL_3. These results would suggest that the normolipemic subjects selected for this study possess the normal lipid:protein ratios for these lipoprotein particles, as well as demonstrate that the GLC quantitation of the lipids corresponded closely with the independently measured protein values. Table I also gives the weight per cent composition of the major lipid classes as measured by GLC. The VLDL is seen to contain 18—22% total phospholipid (phosphatidylcholine plus sphingomyelin), 57—65% triacylglycerol, 11—17% cholesteryl ester and 5—7% free cholesterol. These estimates are in good agreement with the values tabulated for VLDL by Skipski [28]. The total phospholipid content of the LDL_2 fraction is seen to range from 28—34%, the cholesteryl ester content from 46—53% and free cholesterol from 10—12%, with the triacylglycerols accounting for only 6—8% of the total lipid. Again the lipid class proportions are very consistent among the different subjects and well within the range of normal values reported in the literature [28,29]. Likewise, the HDL fraction isolated and analyzed in this study possesses essentially normal composition, with the total phospholipids ranging from 48—56%, the cholesteryl esters from 35—41% and triacylglycerols and free cholesterol from 4—7% of the total lipid. These values again are in excellent agreement with the values reported by Skipski [28].

Lipid class ratios

The molar ratios of the various lipid classes in the major lipoprotein fractions of each normolipemic individual are given in Table II. These ratios are extremely consistent and show only marginal differences between the normolipemic subjects of the two sexes, as well as among different individuals of the same sex. The average ratios for the major lipid classes in the VLDL fraction corre-

TABLE I

OVERALL COMPOSITION OF VLDL, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS

Individual lipid classes estimated as % of total lipid. Each estimate is an average of two or three replicate gas chromatographic analyses.

Chemical component	Males								Females				
	1	2	1690	1765	1767	1689	1858	Average	3	4	18	19	Average
						Weight (%)							
VLDL													
Total protein	10	11	8	8	8	9	9	9±1	10	9	8	9	9±1
Total lipid	90	89	92	92	92	91	91	91±1	90	91	92	91	91±1
PC*	20	18	16	19	18	18	16	18±1	18	19	16	19	18±1
SPH	2	2	2	2	2	2	2	2±0	2	2	2	2	2±0
CE	15	14	11	13	12	14	12	13±1	16	17	12	14	15±2
TG	57	61	65	58	61	59	65	61±3	58	56	65	59	60±4
FC	7	5	5	7	6	7	6	6±1	5	6	5	6	6±1
LDL_2													
Total protein	21	21	20	20	20	20		20±1	19	21	22	19	20±1
Total lipid	79	79	80	80	80	80		80±1	81	79	78	81	80±1
PC	25	25	23	22	23	26		24±1	24	27	25	23	25±2
SPH	7	9	6	6	9	9		8±1	9	7	8	9	8±1
CE	51	46	51	53	49	49		50±2	49	47	48	50	49±1
TG	7	8	8	8	8	6		8±1	6	7	7	7	7±1
FG	10	11	12	11	12	11		11±1	12	11	11	12	12±1
HDL													
Total protein	54	50	57			53	51	53±3	51	56	53	52	53±2
Total lipid	46	50	43			47	49	47±3	49	44	47	48	47±2
PC	44	48	43			43	45	44±3	47	49	45	44	46±2
SPH	5	8	7			5	5	6±1	6	6	5	6	6±0
CE	40	36	39			38	40	39±2	35	35	39	41	38±3
TG	4	7	5			5	4	5±1	6	6	5	4	5±1
FC	7	7	6			9	6	7±1	5	5	6	6	6±1

*PC, phosphatidylcholine; SPH, sphingomyelin; CE, cholesterol ester; TG, triacylglycerol; FC, free cholesterol.

TABLE II

LIPID CLASS RATIOS IN VLDL, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS

Each ratio is an average of two or three estimates from replicate gas chromatographic analyses.

Chemical components	Males								Females				
	1	2	1690	1765	1767	1689	1858	Average	3	4	18	19	Average
						Mole/Mole							
VLDL													
FC/TC*	0.43	0.37	0.44	0.48	0.47	0.44	0.45	0.44	0.34	0.36	0.39	0.40	0.37
FC/PL	0.63	0.47	0.59	0.67	0.64	0.66	0.67	0.62	0.51	0.54	0.57	0.54	0.54
SPH/PC	0.13	0.12	0.14	0.14	0.11	0.14	0.11	0.13	0.13	0.09	0.13	0.12	0.12
EC/TG	0.35	0.30	0.23	0.30	0.25	0.33	0.24	0.29	0.36	0.39	0.25	0.31	0.33
FC/SPH	5.6	4.3	4.8	5.4	6.3	5.3	6.6	5.5	4.3	7.3	5.0	4.6	5.3
FC/PC	0.71	0.52	0.67	0.77	0.71	0.75	0.75	0.70	0.58	0.60	0.63	0.57	0.60
LDL_2													
FC/TC	0.25	0.29	0.28	0.26	0.28	0.27		0.27	0.30	0.28	0.29	0.28	0.29
FC/PL	0.66	0.69	0.80	0.80	0.73	0.62		0.72	0.72	0.67	0.71	0.71	0.70
SPH/PC	0.30	0.37	0.31	0.28	0.38	0.36		0.33	0.30	0.28	0.36	0.28	0.31
EC/TG	10.2	7.3	9.5	6.4	6.5	7.3		7.9	10.1	9.1	9.8	9.5	9.6
FC/SPH	2.8	2.5	3.3	3.6	2.6	2.4		2.9	2.6	2.8	2.7	2.5	2.7
FC/PC	0.84	0.94	1.04	1.02	1.02	0.83		0.95	1.00	0.86	0.97	0.98	0.95
HDL													
FC/TC	0.20	0.23	0.19			0.17	0.22	0.20	0.19	0.20	0.19	0.17	0.19
FC/PL	0.27	0.23	0.23			0.27	0.29	0.26	0.21	0.20	0.23	0.23	0.23
SPH/PC	0.12	0.16	0.18			0.13	0.11	0.14	0.12	0.13	0.12	0.14	0.13
EC/TG	6.26	10.0	7.5			10.2	9.6	8.7	7.5	7.1	7.7	8.4	7.7
FC/SPH	3.3	2.4	1.6			2.5	3.0	2.6	2.0	1.6	1.9	2.0	1.9
FC/PC	0.29	0.25	0.28			0.31	0.34	0.29	0.23	0.21	0.23	0.26	0.23

*TC, total cholesterol; PL, total phospholipid; other abbreviations as in Table I.

spond closely to the values reported in the literature [19,27,31], or to the values that can be calculated from published data [28]. The FC/TC ratio for VLDL averages about 0.4, which is somewhat lower than that reported by Eisenberg [27]. This free cholesterol occurs in a ratio of just less than one molecule cholesterol per two molecules of total phospholipid (0.5—0.6), which has been claimed [32] to be a stable cholesterol—phospholipid complex in artificial liposomes (no sterol—sterol contacts). These VLDL particles possess an extremely consistent SPH/PC ratio (0.12—0.13), the significance of which is not known. Relatively constant is also the ratio of the two neutral ester classes of the VLDL core (EC/TG = 0.29—0.33).

The lipid class ratios of LDL_2 are likewise very constant and again of the order that can be calculated from published data [28,33]. The FC/TC ratio in LDL_2 is significantly lower (0.28) than that in VLDL (0.40) because of the large amounts of cholesteryl ester in the neutral lipid core. The SPH/PC ratio (0.31—0.33) is about two times higher in LDL_2 than in VLDL. The FC/PL ratio in LDL_2 is significantly higher (0.70) than that in VLDL (0.60) and suggests some cholesterol—cholesterol contacts [32].

The HDL particles had the lowest variation in lipid class ratios of the three lipoprotein classes. The average values obtained for HDL in the present study again are in good agreement with those calculated from data reported in the literature [28,34]. The HDL fractions had a slightly lower FC/TC ratio (0.20) than the LDL_2 fractions (0.27), while the FC/PL ratios of HDL (0.23—0.26) were only one half those in VLDL (0.60) and one third those in LDL_2 (0.70). The HDL and VLDL possessed essentially identical SPH/PC ratios, but the sphingomyelins in the two instances were made up of distinctly different molecular species (see below).

Particle size distribution

The calculated particle size distribution for the VLDL, LDL_2 and HDL fractions for each subject is given in Table III. For this calculation the lipid core model was assumed to be valid for all lipoproteins and all free cholesterol, phosphatidylcholine and sphingomyelin were assigned to the polar surface shell, while all the triacylglycerol and cholesteryl ester were assigned to the neutral lipid core of the sphere. The calculated core radii for the VLDL, LDL_2 and HDL averaged 168, 74 and 45 Å, in males respectively. Except for HDL, the equivalent particle radii (core radii plus 20 Å surface shell) are within the reach of the sizes characteristic of normolipemic subjects obtained previously either by calculation from results of conventional analyses or by direct measurement in the electron microscope [30]. Kezdy [35] has reported particle radii of 200 and 95.9 Å for VLDL and LDL, respectively. The core radii of the HDL particles obtained in the present study are about twice that (19 Å) measured by Shen et al. [30] for HDL_3 and about one and a half times that (30 Å) measured by Kezdy [35] for HDL_2. However, the core radii calculated by the present method using the lipid composition of Shen et al. [30] and Kezdy [35] gave values of 39 Å and 50 Å for the HDL_3 and HDL_2 particles, respectively, which is in excellent agreement with the results of this work. The particle size calculated for HDL from the GLC data is overestimated by about 10%. This is due to the failure of the GLC method [25] to account for the

presence of lysophosphatidylcholine and the serine, inositol and ethanolamine phosphatides, which make up a total of 10% of the polar lipids of the HDL surface shell [34]. However, the fact that the calculated particle size of HDL is always larger than the measured size indicates some significant deviation from the ideal spherical model proposed. Furthermore, the triacylglycerol core is known to dissolve about 5 mole % free cholesterol [36], hence the present method of calculation may underestimate the true particle size of the VLDL and LDL_2 by a small percentage because all the free cholesterol in the particle has been assigned to the surface shell. The particle size of the VLDL is seen to vary more than that of the LDL_2 which varies more than that of HDL. These results are consistent with certain previous reports in the literature [30,34], which also have noted a greater individual variability in the VLDL and LDL than in HDL particle size among normolipemic individuals. Table III also includes the calculated masses of the various lipoprotein particles. The masses have been expressed in daltons assuming a molecular weight of 10,000 for the apoprotein moieties [27]. The calculated mass of the HDL particles is about one half that of the LDL_2 particles, which is consistent with the results of other workers [27,29,30].

Surface and core composition

The calculated concentrations of the lipids at the surface and in the core of the VLDL, LDL_2 and HDL particles as the number of molecules are given in Table IV. The average number of molecules calculated for the surface and core of the VLDL and LDL_2 particles is of the order reported by Kezdy [35] for particles of comparable radii. There is, however, considerable individual variation, which reflects the variability in the percent lipid composition of the individual samples pointed out above. The numbers of the various molecules calculated for the surface and the core of the HDL particles greatly exceed the numbers required for the dimensions of these particles measured in the electron microscope or derived from flotation data [30]. However, the estimated number of the molecules in the surface shell and in the core of the HDL particles is consistent with the numbers calculated from the compositional data utilized by Shen et al. [30] and Kezdy [35]. Table IV allows a comparison of the number of molecules of specific lipid classes in the various presumptive precursor and product molecules. Of special interest are the comparisons of the number of cholesteryl ester molecules in the corresponding VLDL and LDL_2 particles. On the basis of the average values it would appear that the LDL_2 particles contain less than one half of the number of cholesteryl ester molecules found in the VLDL, although in specific instances the numbers of these molecules may be identical in the presumed precursor and product particles. In other cases the LDL_2 particles are seen to contain only about a quarter of the number of cholesteryl ester molecules in the precursor VLDL, which suggests cleavage of the VLDL particles during the digestion of the triacylglycerol by lipoprotein lipase. If cleavage of the VLDL particles occurs, the excess cholesteryl ester may be lost as a complex with apoprotein E [37]. In any event, the ratio of sphingomyelin to cholesteryl ester remains the same in both VLDL and LDL_2. A comparable ratio of sphingomyelin and

TABLE III

AVERAGE SIZE DISTRIBUTION OF VLDL, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS

Each value is an average of two or three estimates from replicate gas chromatographic analyses.

Parameter	Males								Females				
	1	2	1690	1765	1767	1689	1858	Average	3	4	18	19	Average
VLDL													
Core radius*	141	177	191	148	162	160	194	168	178	161	205	158	175
Particle weight**	10.4	19.4	23.7	11.7	15.1	14.6	24.9	17.1	19.5	14.9	28.9	14.1	19.3
LDL_2													
Core radius	75	67	78	86	71	66		74	66	64	67	69	67
Particle weight	2.3	1.7	2.6	3.2	2.0	1.7		2.3	1.6	1.6	1.7	1.8	1.7
HDL													
Core radius	45	42	46			42	44	45	41	39	46	45	45
Particle weight	1.0	0.9	1.1			0.9	1.0	1.0	0.9	0.8	1.2	1.0	1.0

*Particle radius (Å) = core radius (Å) plus thickness of outer shell (20 Å).

**Particle weight in daltons $\times 10^{-6}$.

TABLE IV

CALCULATED CONCENTRATION OF LIPIDS AT THE SURFACE AND IN THE CORE OF VLDL, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS (NUMBER OF MOLECULES)

Each value is an average of two to three estimates from replicate gas chromatographic analyses. Abbreviations as in Table I.

Chemical components	Males								Females				
	1	2	1690	1765	1767	1689	1858	Average	3	4	18	19	Average
Surface components													
VLDL													
PC	2376	4021	4348	2500	3143	2959	4466	3402	3917	3316	5127	3134	3873
SPH	316	519	641	374	373	440	531	456	553	287	687	407	483
FC	1721	2150	2996	1963	2296	2268	3435	2158	2310	2026	3344	1830	2377
LDL_2													
PC	579	429	584	709	464	431		532	398	419	419	437	418
SPH	183	167	191	211	191	58		183	161	133	157	184	159
FC	496	410	624	741	485	367		520	403	369	414	442	407
HDL													
PC	284	261	303			242	277	273	244	219	301	288	263
SPH	38	28	42			35	38	36	28	29	41	39	34
FC	87	68	80			102	80	83	56	46	80	77	66
Core components													
VLDL													
TG	6107	12447	16249	7207	9760	8988	17045	11114	11973	8825	19842	8775	12352
CE	2220	3729	3696	2106	2519	2957	4134	3051	4541	3532	4965	2117	3939
LDL_2													
TG	143	134	185	244	139	85		155	94	98	101	116	102
CE	1478	998	1629	2100	1217	1003		1404	970	909	1008	1119	1002
HDL													
TG	24	25	38			25	20	26	29	23	34	20	27
CE	323	259	336			270	312	300	223	194	332	327	277

cholesteryl ester in HDL must be considered a coincidence, because there is no apparent reason for such a constancy.

Distribution of molecular species

The distribution of the carbon numbers of the cholesteryl esters, triacylglycerols and phosphatidylcholines in the various lipoprotein fractions from the individual normolipemic subjects are given in Table V. It is seen that the variations among the individual subjects are of about the same order as those among the average values of different lipoprotein fractions. Furthermore, the carbon number distribution of the cholesteryl esters is about the same in all

TABLE V

DISTRIBUTION OF MOLECULAR SPECIES OF LIPIDS IN VLDL, LDL_2, AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS

Each value is an average of two to three estimates from replicate gas chromatographic analyses. Values given are percentages of lipid class.

Carbon numbers*	Males								Females				
	1	2	1690	1765	1767	1689	1858	Average	3	4	18	19	Average
VLDL													
36	35	33	33	37	36	36	30	34±2	34	36	40	35	36±2
38	36	40	41	40	39	41	45	40±3	40	37	40	38	39±2
40	29	27	26	23	25	23	25	25±2	26	27	20	27	25±3
43**	20	18	27	21	22	22	16	21±3	17	18	17	20	18±1
45**	80	82	73	79	78	78	84	79±8	83	82	83	80	82±1
46	ND***	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
48	4	5	8	6	7	8	8	7±2	6	9	5	5	6±2
50	19	19	18	19	19	19	19	19±0	25	24	21	17	22±3
52	55	51	40	52	50	48	47	49±5	48	47	48	52	49±2
54	20	22	30	20	22	23	22	23±3	18	17	24	23	21±3
56	2	3	4	3	2	2	4	3±1	2	3	2	3	3±1
LDL_2													
36	38	37	31	39	37	38		37±3	36	38	37	44	39±4
38	39	37	35	38	36	35		37±2	38	37	35	42	38±3
40	23	26	24	23	27	27		25±2	26	25	28	14	23±6
43	19	17	19	19	26	20		20±3	21	32	21	20	21±1
45	72	72	68	74	67	74		71±3	69	68	67	70	69±1
47	9	11	13	7	7	6		9±3	10	9	12	10	10±1
50	26	24	23	23	29	25		25±2	31	33	27	27	30±3
52	49	46	48	50	47	50		48±2	46	45	46	50	47±2
54	25	30	29	27	24	25		27±2	23	22	27	23	24±2
HDL													
36	37	36	32			35	32	34±2	34	38	36		36±2
38	40	41	41			39	40	40±1	41	39	40		40±1
40	23	23	27			26	28	25±2	25	23	24		24±1
43	19	18	16			22	16	18±3	21	24	19		21±3
45	69	72	71			68	72	70±2	66	61	71		66±5
47	12	10	13			10	12	11±1	13	15	10		13±3
50	29	14	28			29	23	25±6	20	25	30		25±5
52	53	61	49			53	45	52±6	60	55	57		57±3
54	18	25	33			18	32	25±7	20	20	13		18±4

*Carbon numbers 43—47 represent the cholesterol esters, with fatty acids of 16—20 acyl carbons; carbon numbers 36—40 represent diacylphosphatidylcholines with a total of 36—40 acyl carbons; carbon numbers 48—56 represent triacylglycerols with a total of 48—56 acyl carbons.

**Due to overlap with C_{46} triacylglycerol C_{47} cholesteryl ester has been omitted. However, analysis of fatty acid methyl esters (Table VI) indicates that C_{47} is $\leqslant 6\%$.

***ND, not determined.

three lipoprotein classes for any one subject. Likewise, closely similar carbon numbers are recorded for the triacylglycerols of all three lipoprotein classes for any one subject. The triacylglycerols of the VLDL were present in the highest amounts and provided the most detailed profiles of their structure. However, the LDL_2 triacylglycerols possessed a closely similar distribution for carbon numbers, when present in sufficient amounts for accurate analysis, as did the HDL fraction, which contained only minute quantities triacylglycerols. A simple determination of the carbon numbers, however, may not be adequate to demonstrate homogeneous populations of species of neither cholesteryl esters nor triacylglycerols for these lipoprotein fractions, because

TABLE VI

FATTY ACID COMPOSITION (MOLE %) OF CHOLESTERYL ESTERS OF VLDL, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS

Each value is an average of two replicate gas chromatographic analyses. Fatty acids identified by carbon number:double bond number.

Fatty acids	Males				
	1924	1690	1689	1858	Average
VLDL					
14:0	0.2		—	0.1	0.1±0.1
16:0	9.9		11.9	7.7	9.8±2.1
16:1	1.7		3.6	1.9	2.4±1.0
18:0	1.0		0.9	1.5	1.1±0.3
18:1	26.9		29.0	19.1	25.0±5.2
18:2	54.2		51.0	63.5	56.2±6.5
18:3/20:1	0.3		2.0	0.5	0.9±0.9
20:3	0.6		—	0.8	0.7±0.1
20:4	5.3		1.5	4.7	3.8±2.0
LDL_2					
14:0	0.6	0.3	0.4	0.4	0.4±0.1
16:0	14.3	11.0	11.8	10.7	12.0±1.6
16:1	2.2	2.3	2.3	1.6	2.1±0.3
18:0	1.0	0.9	0.6	0.9	0.9±0.2
18:1	21.7	20.5	20.1	14.3	19.2±3.3
18:2	53.2	59.2	60.2	67.2	60.0±5.7
18:3/20:1	0.5	0.5	0.4	0.2	0.4±0.1
20:3	0.6	0.8	0.3	0.4	0.5±0.2
20:4	5.9	4.2	3.8	4.3	4.6±0.9
HDL					
14:0	0.5	0.3	0.4	0.4	0.4±0.1
16:0	11.8	11.3	10.4	9.7	10.8±0.9
16:1	2.1	2.5	2.1	1.7	2.1±0.3
18:0	0.7	1.1	0.6	0.8	0.8±0.2
18:1	21.2	19.9	19.6	13.4	18.5±3.5
18:2	55.2	58.9	61.4	68.1	60.9±5.4
18:3/20:1	0.5	0.6	0.5	0.4	0.5±0.1
20:3	0.6	0.7	0.4	0.5	0.6±0.1
20:4	7.3	4.8	4.7	5.0	5.5±1.2

the saturated and the various unsaturated C_{18} fatty acid esters overlap and therefore may hide significant differences in the fatty acid composition of the esters. The true fatty acid compositions of the cholesteryl esters of VLDL, LDL_2 and HDL fractions from the same subjects are given in Table VI. It is seen that the cholesteryl esters of VLDL contain more oleic and less linoleic acid than do the cholesteryl esters of HDL, with those of LDL_2 possessing intermediate compositions. These results are in agreement with the data of Goodman and Shiratori [38] and of Morrisett et al. [39]. The latter observations are consistent with the relatively constant proportion of the C_{47} cholesteryl ester peak in the steryl ester fraction of all three lipoproteins. The fatty acid compositions of the triacylglycerols of the different lipoprotein classes of the same subjects are given in Table VII. It is seen that all lipoproteins contain comparable amounts of palmitic and linoleic acid in contrast

TABLE VII

FATTY ACID COMPOSITION (MOLE %) OF TRIACYLGLYCEROLS OF VLDL, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS

Each value is an average of two replicate gas chromatographic analyses. Fatty acids identified by carbon number:double bond number.

Fatty acids	Males				
	1924	1690	1689	1858	Average
VLDL					
14:0	1.9	1.6	2.0	2.8	2.1±0.5
16:0	28.2	26.7	31.3	26.2	28.1±2.3
16:1	4.0	4.4	4.7	4.6	4.4±0.3
18:0	5.1	5.5	2.8	2.0	3.8±1.7
18:1	42.7	41.0	41.4	32.6	39.4±4.6
18:2	16.2	18.1	16.1	29.7	20.0±6.5
18:3/20:1	1.1	1.8	1.2	1.4	1.3±0.3
20:3/20:4	0.9	0.9	0.5	0.8	0.8±0.2
LDL_2					
14:0	1.5	1.5	1.4	2.5	1.7±0.5
16:0	25.3	29.9	26.6	26.9	27.2±2.0
16:1	3.8	4.2	4.0	4.2	4.1±0.2
18:0	4.4	4.3	3.7	5.0	4.4±0.5
18:1	47.5	43.1	45.7	35.1	42.9±5.5
18:2	15.2	13.5	16.2	24.5	18.0±4.4
18:3/20:1	0.7	1.1	1.0	0.7	0.9±0.2
20:3/20:4	1.3	1.3	1.5	1.1	1.3±0.2
HDL					
14:0	1.7	1.7	1.7	2.8	2.0±0.5
16:0	24.9	28.0	26.6	25.6	26.3±1.3
16:1	4.2	4.8	4.3	4.6	4.5±0.3
18:0	4.4	4.3	2.9	2.5	3.5±1.0
18:1	46.4	45.4	45.1	36.8	43.4±4.4
18:2	16.0	13.9	17.2	25.4	18.1±0.1
18:3/20:1	0.8	0.9	1.1	0.8	0.9±0.1
20:3/20:4	1.6	1.2	1.1	1.6	1.4±0.3

to previous reports [38—40], where more palmitic and less linoleic acid were found in VLDL than in either LDL_2 or HDL.

Also the carbon number distributions for the diacylglycerol moieties of the phosphatidylcholines of the different lipoprotein classes when isolated from the same subjects were closely similar (Table V). The major carbon

TABLE VIII

CARBON NUMBER DISTRIBUTION (MOLE %) OF CERAMIDES OF VLDL, LDL_2 AND HDL PARTICLES OF FASTING PLASMA OF NORMOLIPEMIC SUBJECTS

Each value is an average of two replicate gas chromatographic analyses.

Carbon number*	Males				
	1924	1690	1689	1858	Average
VLDL					
32	1.6		3.1	2.2	2.3±0.8
33	1.1		1.9	1.2	1.4±0.4
34	37.9		39.1	35.2	37.4±2.0
35	1.6		1.6	1.7	1.6±0.1
36	9.4		8.3	9.4	9.0±0.6
37	0.6		0.7	1.1	0.8±0.3
38	3.7		3.7	4.2	3.9±0.3
39	0.9		1.6	1.8	1.4±0.5
40	9.4		11.1	13.0	11.2±1.8
41	4.5		5.5	5.3	5.1±0.5
42	29.4		23.5	25.0	26.0±3.1
LDL_2					
32	2.5	3.1	4.3	2.7	3.2±0.8
33	1.6	1.8	2.3	1.6	1.8±0.3
34	35.1	31.5	32.2	34.1	33.5±1.5
35	2.0	1.3	1.5	1.2	1.5±0.4
36	8.6	7.5	7.3	6.7	7.5±0.8
37	0.6	0.5	0.9	0.6	0.7±0.2
38	4.6	6.0	5.4	5.0	5.3±0.6
39	1.2	2.0	2.1	1.8	1.8±0.4
40	12.4	16.1	14.7	15.9	14.8±1.7
41	4.8	5.6	6.2	5.5	5.5±0.6
42	26.6	24.5	22.0	25.0	24.5±1.9
HDL					
32	1.7	2.7	3.5	1.8	2.4±0.8
33	0.9	1.4	1.6	1.0	1.2±0.3
34	24.3	24.5	26.1	23.0	24.4±1.3
35	0.6	0.9	1.1	0.8	0.9±0.2
36	6.9	6.5	6.8	5.8	6.5±0.5
37	0.1	0.5	0.5	0.4	0.4±0.2
38	4.2	6.4	5.4	5.6	5.4±0.9
39	0.9	1.9	2.1	1.8	1.7±0.5
40	15.3	19.0	17.7	20.5	18.1±2.2
41	6.3	5.5	6.2	6.0	6.0±0.4
42	38.7	30.5	29.0	33.2	32.9±4.3

*Total number of carbons in the fatty acid and the nitrogenous base of the ceramide.

numbers were 36—40 in about the same proportions in all fractions. We have shown elsewhere [41] that the individual molecular species of the phosphatidylcholines are very nearly identical in the different lipoproteins. It was noted, however, that the arachidonoyl species were present in a slightly higher proportion in the HDL_3 fractions, when compared to the corresponding LDL and VLDL fractions from the same subjects.

In agreement with previous findings [24] marked differences, however, were seen in the composition of the molecular species of the sphingomyelins of the different lipoprotein classes when isolated from the same subjects. Table VIII shows the differences in the carbon number composition of the ceramides of the VLDL, LDL_2 and HDL fractions from several subjects. It is seen that the HDL fraction contains a significantly higher proportion of the longer chain species ($C_{24:0}$ and $C_{24:1}$ amides of sphingosine) and a lower proportion of the shorter chain species ($C_{16:0}$ amides of sphingosine) than the VLDL and LDL_2 fractions, which differ little from each other. Detailed analyses of the molecular species have revealed minor differences in the proportions of the nitrogenous bases [24]. For the present purposes it is sufficient to note that the HDL fraction is either not directly derived from the VLDL or it has been extensively diluted by HDL from a different origin.

DISCUSSION

Validity of analytical measurements

The accuracy and precision of the GLC measurements of cholesterol and triacylglycerol [21] as well as of phospholipids [25] have been documented in previous publications and are further indicated in the present work by the overall consistency of the data and a good agreement between the GLC results and the results of conventional measurements reported in the literature [28]. It may therefore be concluded that the lipid class compositions of the various lipoproteins are essentially correct. Also the detailed accounts of the molecular species of the phosphatidylcholines and sphingomyelins necessary for evaluating the precursor—product relationships among the plasma lipoproteins must be essentially correct. The general results obtained in the present comparisons correspond closely to those made earlier on similar preparations of lipoproteins, which had led to comparable conclusions [31,33,40]. Likewise, the differences demonstrated in the C_{18} fatty acid compositions in a limited number of subjects among the plasma lipoprotein cholesteryl esters must be correct, as they correspond to similar observations made in those earlier studies where comparisons were made among samples obtained from the same individuals [39]. The identity implied among the triacylglycerol populations of the lipoprotein classes on the basis of the carbon number distribution and fatty acid composition, however, must be further confirmed by stereospecific analyses of the triacylglycerol structure. We are currently in the process of completing such comparisons and the results will be published elsewhere [42]. The data obtained to date suggest that the triacylglycerols of the VLDL and LDL_2 possess closely similar diacylglycerol moieties and therefore similar enantiomeric structures. The enantiomeric nature of the triacylglycerols of HDL has not been assessed. Since marked differences have been noted

between the enantiomeric structures of the triacylglycerols of chylomicrons and VLDL [42] there remains a need for a more detailed assessment of the equilibration of the triacylglycerols among the plasma lipoprotein classes. Nevertheless, the detailed knowledge of the steryl ester and the choline-containing phospholipid composition representing the neutral lipid core and the surface material of the lipid particle, respectively, is sufficient for at least a partial assessment of the hypothesis of Eisenberg et al. [9].

Furthermore, the lipid class composition provides characteristic lipid class ratios from which the particle sizes can be calculated assuming a lipid core model for all lipoproteins [43]. The calculated values are in the range of the particle sizes derived for these lipoproteins from direct measurements [29,30].

Validity of hypothesis

If a direct precursor—product relationship exists among the plasma VLDL, LDL and HDL lipoproteins, as postulated by Eisenberg et al. [9], it would be anticipated that certain similarities would be found in the mass composition of the lipids between the corresponding lipoprotein fractions. This should be especially so if these cascade-like transformations of lipoproteins took place largely in the plasma compartment with only the concentration of the starting materials and the end-products being modified by the tissues, as claimed on the basis of in vitro studies [11,44]. The interconversion hypothesis predicts that during lipoprotein lipase mediated triacylglycerol hydrolysis, VLDL particles become progressively smaller, change their protein and lipid composition and through a stage of formation of intermediate particles (IDL) (d = 1.006) are converted to plasma LDL. Kinetic studies carried out in normal human subjects have substantiated the scheme and have demonstrated that within experimental error all of the major structural apoprotein of VLDL (apo B) is converted to LDL, and that all of the LDL-apo B originated from VLDL [1,2]. During the process both core and surface components of the VLDL are being affected, resulting in a differential recovery in the LDL end-products. In vitro studies have suggested [11,45] that the cholesteryl ester remains with the residual VLDL triacylglycerols (and apo B) even when 70—80% of the triacylglycerols are hydrolyzed; thereafter they are quantitatively recovered within the particles of the LDL range. In the absence of any known mechanism of synthesis of cholesteryl esters in the VLDL particles, it would be anticipated that both VLDL and LDL_2 would have identical cholesteryl ester compositions. An examination of the carbon number distribution revealed only minor differences, while an assessment of the component fatty acids indicated a significantly higher unsaturation of the LDL_2 cholesteryl esters. In fact, the cholesteryl esters of the LDL_2 fraction resembled more those of the HDL than those of the VLDL. Such a differential distribution of the cholesteryl esters suggests that in addition to a direct transfer of the cholesteryl esters from VLDL to LDL_2 other processes must take place to account for the observed mass distribution. Chajek and Fielding [46] have recently isolated from human plasma a cholesteryl ester transfer protein, which stoichiometrically exchanges one molecule of HDL cholesteryl ester for one molecule of LDL triacylglycerol. Although the significance of this transfer is not known, it could possibly account for an increased unsaturation of LDL_2 cholesteryl esters and the presence

of triacylglycerols in HDL, especially if the HDL cholesteryl esters, which are derived via the lecithin:cholesterol acyltransferase [7] were more unsaturated than those of VLDL or LDL_2. It is also possible that at least a partial equilibration of the cholesteryl esters of LDL_2 and HDL could have taken place by fusion of some HDL particles with either VLDL or LDL_2, as postulated by Anderson et al. [47], Grow and Fried [48] and Tall and Small [10]. On the basis of the similarity in the fatty acid composition it is unlikely that the triacylglycerols of VLDL, LDL_2 and HDL would differ significantly in the composition of their molecular species. An absence of triacylglycerol differences would be consistent with a limited preferential hydrolysis of the unsaturated triacylglycerols by lipoprotein lipase [49]. The residual triacylglycerols of VLDL found in LDL would be therefore anticipated to be similar to those of the original VLDL. Furthermore, the triacylglycerols of HDL could have been derived from LDL via exchange for cholesteryl esters, as postulated by Chajek and Fielding [46], without any change in the composition of their molecular species. No change in molecualr species of the triacylglycerols would also be expected if any triacylglycerols of HDL were eventually returned to LDL_2 and/or VLDL by fusion with any unstabilized HDL particles, as postulated by Tall and Small [10]. Finally, the recently characterized triacylglycerol exchange protein of Barter et al. [50] could also have contributed to the equilibration of the triacylglycerols among all three lipoproteins. Nevertheless, on account of the differences in the cholesteryl ester composition and in cholesteryl ester/triacylglycerol ratios, even the composition of the neutral lipid cores of the VLDL, LDL_2 and HDL particles cannot be fully accounted for by a simple precursor–product relationship, as postulated by Eisenberg et al. [9] and Tall and Small [10], and that plausible additional mechanisms must be invoked to establish the experimentally observed mass distributions.

According to the hypothesis, the LDL particles formed from VLDL retain only sufficient surface material to provide a monolayer coating the entire surface of the sphere, the rest of the surface material being partly hydrolyzed by phospholipase A_2 [51] and partly shed possibly in the form of LPX-like particles [52]. In this process the apo B peptide is retained exclusively on the LDL particles, while the LPX-like aggregates acquire apo A_1 from preformed HDL. The present results indicate that the surface material retained on the LDL is either subject to a preferential loss of phosphatidylcholine due to hydrolysis by phospholipases or there has been a preferential retention of sphingomyelin on the LDL surface during the shedding of the excess surface material in the form of LPX. It is possible that a partial phase separation has taken place during the formation of the LPX-like aggregates, which could have excluded much of the sphingomyelin from the more organized liposomal structure. Phase separation of phosphatidylcholine and sphingomyelin is known to take place under certain experimental conditions [53], but it is not known to what extent, if any, it may take place under physiological conditions. On the basis of physicochemical experiments, Calhoun and Shipley [54] have suggested that a likely candidate for inducing lateral phase separations is a long chain fatty acid ($C_{24:0}$ or $C_{24:1}$) ceramide linked to phosphorylcholine. Should such a phase separation take place during the LPX formation, it could provide a plausible explanation for an eventual enrichment of sphingomyelin in the

LDL_2 and of the longer chain sphingomyelins in the HDL end-product of the excess VLDL surface material. Alternative explanations for the relative enrichment of sphingomyelin in the LDL fraction may be advanced on the basis of preferential binding of this phospholipid by apo B, or a preferential binding of phosphatidylcholine by the apo A_1 transferred to the intermediate LPX from HDL. The available information would suggest that apoprotein A_1 must interact with apoprotein A_2 for a significant phospholipid binding [55,56], but it is not known whether or not any distinction is made between the two types of choline phospholipids. There is not sufficient long chain sphingomyelin in either the liver or intestinal sources of nascent HDL to account for the observed distortion of the molecular species of the ceramides, although the red blood cells could have contributed some of it [57]. In any event, a direct precursor—product relationship, as previously put forward [9,10], cannot be immediately reconciled with the differences in the composition of the molecular species of the sphingomyelins in the VLDL and HDL fractions, and with the differences in the sphingomyelin/phosphatidylcholine ratios in the VLDL, LDL_2 and HDL fractions. Additional mechanisms or alternative series of transformations must be postulated in order to account for the characteristic lipid mass distribution.

The formation of HDL from VLDL is believed to be completed via the action of lecithin:cholesterol acyltransferase, which catalyzes the esterification of free cholesterol by phosphatidylcholine in the LPX monolayer in the presence of apo A_1 resulting in a transfer of the cholesteryl ester into the interior of the phospholipid bilayer [9,10]. According to Tall and Small [10] either the LPX or the discoidal lamellar aggregates derived from it via apo A_1 serve as substrates for lecithin:cholesterol acyltransferase. The lysophosphatidylcholine is relased into the medium where it is presumably bound by albumin. With a gradual depletion of the free cholesterol and phosphatidylcholine the surface area of the aggregate gradually shrinks and the interior is filled with cholesteryl esters resulting in a spherical particle of a diameter characteristic of the apo A_1 containing lipoproteins [34]. According to this scheme the fatty acid composition of the HDL cholesteryl esters should resemble that of the sn-2-position of the HDL phosphatidylcholines. In fact, the cholesteryl esters of the HDL are somewhat more saturated than would have been anticipated from the fatty acid composition of the component phosphatidylcholine. Perhaps during the transfer of the cholesteryl esters from HDL to LDL via the cholesteryl ester transfer protein the more unsaturated species are preferentially removed [46]. It was already noted, however, that the reciprocal transfer of triacylglycerols from LDL to HDL could not have involved a preferential transfer of the more unsaturated triacylglycerols. According to the hypothesis, the HDL should have been essentially free of triacylglycerols. The analytical results, however, demonstrate that triacylglycerols can be reproducibly recovered as a consistent and persistent component of the ultracentrifugally isolated HDL fraction.

CONCLUSIONS

The present evaluation of the plasma lipoprotein mass composition of the lipid classes and molecular species from the same individuals reveals a great

similarity, which may have been achieved either by a non-specific equilibration of the components or by an interconversion of the lipid particles, or both. A direct conversion of VLDL into LDL, via lipoprotein lipase, and of the excess surface material of the VLDL into HDL, via lecithin:cholesterol acyltransferase, however, would appear unlikely in view of the discrepancies in the lipid class and molecular species composition of the phospholipids. If the hypothesis of the lipoprotein interconversion is correct, the various plasma lipoproteins must have undergone such other transformations as those brought about by lysophosphatidylcholine transacylase, phospholipase A_2, cholesteryl ester-triacylglycerol, triacylglycerol, and phospholipid exchange protein, as well as by such physicochemical phenomena as lateral phase separation and preferential binding of phospholipids by specific apoproteins. It is possible that these phenomena have been especially active in the equilibration of the lipids of the major fasting plasma lipoproteins. It would therefore be of interest to perform a comparable examination of the lipid class and molecular species composition on the plasma lipoproteins in the postabsorptive state of normal subjects, as well as of fasting and postabsorptive patients with hyperlipoproteinemia. Ultimately the validity of the lipoprotein interconversion hypothesis should be examined by means of specifically labelled lipid molecules over appropriate periods of time in order to establish any precursor–product relationships of individual lipoprotein classes, as well as the time course of non-specific equilibration of lipid classes and molecular species. Hopefully, the sensitive methods of detailed lipid analysis described here along with measurements of stable or radio-isotope distribution will be adequate for this task.

ACKNOWLEDGEMENTS

These studies were supported by funds from the Ontario Heart Foundation, the Medical Research Council of Canada, and the Heart and Lung Institute, NIH-NHLI-72-917, Bethesda, MD, U.S.A.

REFERENCES

1 G. Sigurdsson, A. Nicoll and B. Lewis, J. Clin. Invest., 56 (1975) 1481.
2 M.F. Reardon, N.H. Fidge and P.J. Nestel, J. Clin. Invest., 61 (1978) 850.
3 S. Eisenberg, D.W. Billheimer, F.T. Lindgren and R.I. Levy, Biochim. Biophys. Acta, 326 (1973) 361.
4 S. Eisenberg and R.I. Levy, Advan. Lipid Res., 13 (1975) 1.
5 S. Eisenberg, Atherosclerosis Rev., 1 (1976) 23.
6 V.N. Schumaker and G.H. Adams, J. Theor. Biol., 26 (1970) 89.
7 J.A. Glomset, Progr. Biochem. Pharmacol., 15 (1979) 41.
8 J.R. Patsch, A.M. Gotto, Jr., T. Olivecrona and S. Eisenberg, Proc. Nat. Acad. Sci. U.S., 75 (1978) 4519.
9 S. Eisenberg, T. Chajek and R. Deckelbaum, Pharmacol. Res. Commun., 10 (1978) 729.
10 A.R. Tall and D.M. Small, N. Engl. J. Med., 299 (1978) 1232.
11 R.J. Deckelbaum, S. Eisenberg, M. Fainaru, Y. Barckholz and T. Olivecrona, J. Biol. Chem., 254 (1979) 6079.
12 T.M. Forte, R.M. Kraus, F.T. Lindgren and A.V. Nichols, Proc. Nat. Acad. Sci. U.S., 76 (1979) 5934.
13 T. Chajek and S. Eisenberg, J. Clin. Invest., 61 (1978) 1654.
14 A.R. Tall, P.H.R. Green, R.M. Glickman and J.W. Riley, J. Clin. Invest., 64 (1979) 977.
15 F.T. Hatch and R.S. Lees, Advan. Lipid Res., 6 (1968) 1.
16 G.F. Maguire and W.C. Breckenridge, Clin. Biochem., 8 (1975) 161.

17 J.P. Kane, Anal. Biochem., 63 (1973) 350.
18 O.H. Lowry, N.J. Rosebrough, A.K. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
19 T. Sata, R.J. Havel and A.L. Jones, J. Lipid Res., 13 (1972) 757.
20 A. Kuksis, J.J. Myher, L. Marai and K. Geher, J. Chromatogr. Sci., 13 (1975) 423.
21 A. Kuksis, J.J. Myher, K. Geher, A.G.D. Hoffman, W.C. Breckenridge, G.J.L. Jones and J.A. Little, J. Chromatogr., 146 (1978) 393.
22 A. Kuksis, Fette Seifen Anstrichm., 73 (1971) 130.
23 J.J. Myher, A. Kuksis, L. Marai and S.K.F. Yeung, Anal. Chem., 50 (1978) 557.
24 A. Kuksis, J.J. Myher, W.C. Breckenridge and J.A. Little, in K. Lippel (Editor), Report of the High Density Lipoprotein Methodology Workshop, NIH Publication No. 79-1661, Bethesda, MD, 1979, pp. 142—163.
25 A. Kuksis, J.J. Myher, K. Geher, N.A. Shaikh, W.C. Breckenridge, G.J.L. Jones and J.A. Little, J. Chromatogr., 182 (1980) 1.
26 A. Kuksis, W.C. Breckenridge, J.J. Myher and G. Kakis, Can. J. Biochem., 56 (1978) 630.
27 S. Eisenberg, in H. Greten (Editor), Lipoprotein Metabolism, Springer-Verlag, New York, 1976, pp. 32—43.
28 V.P. Skipski, in G.J. Nelson (Editor), Blood Lipids and Lipoproteins, Wiley-Interscience, New York, 1972, pp. 471—583.
29 D.M. Lee, in C.E. Day and R.S. Levy (Editors), Low Density Lipoproteins, Plenum Press, New York, 1976, pp. 1—74.
30 B.W. Shen, A.M. Scanu and F.J. Kezdy, Proc. Nat. Acad. Sci. U.S., 74 (1977) 837.
31 R.J. Deckelbaum, A.R. Tall and D.M. Small, J. Lipid Res., 18 (1977) 164.
32 D.M. Engelman and J.E. Rothman, J. Biol. Chem., 247 (1972) 3694.
33 R.J. Deckelbaum, G.G. Shipley and D.M. Small, J. Biol. Chem., 252 (1977) 744.
34 C. Edelstein, F.J. Kezdy, A.M. Scanu and B.W. Shen, J. Lipid Res., 20 (1979) 143.
35 F.J. Kezdy, in H. Peeters (Editor), The Lipoprotein Molecule, Plenum Press, New York, 1978, pp. 83—90.
36 R.J. Jandacek, M.R. Webb and F.H. Mattson, J. Lipid Res., 18 (1977) 203.
37 D. Rubinstein, S.P. Tam and L. Dory, Circulation, 60, Suppl. 2 (1979) 274.
38 D.S. Goodman and T. Shiratori, J. Lipid Res., 5 (1964) 307.
39 J.D. Morrisett, H.J. Pawnall, R.L. Jackson, R. Segura, A.M. Gotto, Jr. and O.D. Taunton, in W.H. Kunau and R.T. Holman (Editors), Polyunsaturated Fatty Acids, American Oil Chemists' Society, Champaign, IL, 1977, pp. 139—161.
40 T.G. Redgrave and L.A. Carlson, J. Lipid Res., 20 (1979) 217.
41 J.J. Myher, A. Kuksis, J. Shepherd, C.J. Packard, J.D. Morrisett, O.D. Taunton and A.M. Gotto, Biochim. Biophys. Acta, submitted for publication.
42 J.J. Myher, A. Kuksis, W.C. Breckenridge and J.A. Little, J. Amer. Oil Chem. Soc., 57, No. 2 (1980) Abstr. No. 413.
43 H. Schneider, R.S. Morrod, J.R. Colain and N.H. Tattrie, Chem. Phys. Lipids, 10 (1973) 328.
44 S. Eisenberg, Progr. Biochem. Pharmacol., 15 (1979) 139.
45 S. Eisenberg and T. Olivecrona, J. Lipid Res., 20 (1979) 614.
46 T. Chajek and C.J. Fielding, Proc. Nat. Acad. Sci. U.S., 75 (1978) 3445.
47 D.W. Anderson, A.V. Nichols, T.M. Forte and F.T. Lindgren, Biochim. Biophys. Acta, 493 (1977) 55.
48 T.E. Grow and M. Fried, J. Biol. Chem., 253 (1978) 8034.
49 N. Morley and A. Kuksis, Biochim. Biophys. Acta, 487 (1977) 332.
50 P.F. Barter, T.M. Gooden and O.V. Rajaram, Arteriosclerosis, 33 (1979) 165.
51 R.O. Scow and T. Egelrud, Biochim. Biophys. Acta, 431 (1976) 538.
52 A.R. Tall, P. Green, E. Abreu and R. Glickman, Circulation, 58, Suppl. 2 (1978) II-15.
53 S.H. Untracht and G.G. Shipley, J. Biol. Chem., 252 (1977) 4449.
54 W.I. Calhoun and G.G. Shipley, Biochemistry, 18 (1979) 1717.
55 G. Assman and H.B. Brewer, Jr., Proc. Nat. Acad. Sci. U.S., 71 (1974) 989.
56 J.D. Morrisett, R.L. Jackson and A.M. Gotto, Jr., Biochim. Biophys. Acta, 472 (1977) 93.
57 G.J. Nelson, in G.J. Nelson (Editor), Blood Lipids and Lipoproteins, Wiley-Interscience, New York, 1972, pp. 317—386.

Journal of Chromatography, 224 (1981) 25—32
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 843

DETERMINATION OF LEVAMISOLE IN PLASMA AND ANIMAL TISSUES BY GAS CHROMATOGRAPHY WITH THERMIONIC SPECIFIC DETECTION

R. WOESTENBORGHS*, L. MICHIELSEN and J. HEYKANTS

Department of Drug Metabolism and Pharmacokinetics, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse (Belgium)

(First received November 12th, 1980; revised manuscript received January 13th, 1981)

SUMMARY

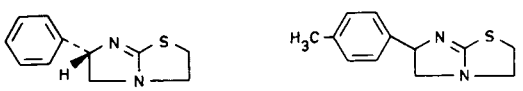
A rapid and sensitive method has been developed for the determination of the anthelmintic levamisole in plasma and tissues from man and animals. The procedure involves the extraction of the drug and its internal standard from the biological material at alkaline pH, back-extraction into sulphuric acid and re-extraction into the organic phase (heptane—isoamyl alcohol). Several extraction steps can be omitted, however, whenever the gas chromatographic background permits and some operations can be simplified using Clin Elut™ extraction tubes.

The analyses were carried out by gas chromatography using a nitrogen-selective thermionic specific detector. The detection limit was 5 ng, contained in 1 ml of plasma or in 1 g of the various tissues, and recoveries were sufficiently high (79—86%).

The method was applied to human plasma samples in a comprehensive bioavailability study of levamisole in healthy volunteers, and to plasma and tissues in a residue trial in cattle. The effect of the blood collection technique on the plasma levels was also studied and pointed to decreased plasma concentrations when Vacutainer® tubes were used.

INTRODUCTION

Levamisole, (*S*)-(−)-2,3,5,6-tetrahydro-6-phenylimidazo[2,1b]thiazole (I, Fig. 1), is widely used as a potent broad-spectrum anthelmintic agent [1—3].



I II

Fig. 1. Chemical structures of levamisole hydrochloride (I) and the internal standard (II).

0378-4347/81/0000—0000/$02.50

More recently it has also been shown to possess a marked influence on the immunological responses of animals and man, particularly in those individuals whose immune defences have for some reason become impaired [4, 5].

A gas—liquid chromatographic (GLC) method was described for determining levamisole residues in milk using an alkali flame ionization detector and external standard calibration [6]. Animal tissue concentrations have been determined by a polarographic method [7].

The present paper describes a rapid and reliable method for the determination of levamisole in plasma and animal tissues by GLC using a thermionic specific detector. The method was used to obtain more detailed information about the pharmacokinetics of the drug in man and animals.

EXPERIMENTAL

Reagents

Levamisole hydrochloride (R 12 564) and the internal standard (R 8493) or (+)-2,3,5,6-tetrahydro-6-(4-methylphenyl)imidazo[2,1b]thiazole hydrochloride (II, Fig. 1) were originally synthesized in our research laboratories and were of analytical grade. Spectrophotometric grade *n*-heptane and methanol were used (Uvasol, E. Merck, Darmstadt, G.F.R.); all other solvents and chemicals were of analytical grade. The Clin Elut^{TM} tubes were of analytical purity (CE 1003, Analytichem International, Lawndale, CA, U.S.A.).

Standard solutions

Using the hydrochloride salts of I and II, stock solutions were prepared corresponding to 1 mg of the free base per ml of methanol. The levamisole stock solution was diluted to concentrations ranging from 0.05 to 30 μg/ml of methanol.

To spike the samples with the internal standard, the stock solution of II was further diluted to 5 μg/ml for the plasma samples and to 1 μg/ml for the various tissue samples.

Extraction procedure

Plasma. Plasma samples (1 ml) were pipetted into 15-ml glass centrifuge tubes and spiked with 0.5 μg of the internal standard. The solution was made alkaline with 2 ml of 1 *M* sodium hydroxide and 4 ml of a heptane—isoamyl alcohol mixture (95:5, v/v) were added. The tubes were carefully rotated for 10 min (35 rpm, Cenco rotary mixer) and then centrifuged (5 min, 1000 *g*). The upper organic layer was transferred to a second centrifuge tube and the plasma was extracted again with 4 ml of the heptane—isoamyl alcohol mixture. The combined organic layers were then extracted with 3 ml of 0.05 *M* sulphuric acid and removed after centrifugation. The remaining acidic phase was made alkaline with 0.15 ml of concentrated ammonia (pH 10) and extracted twice with 2-ml aliquots of the heptane—isoamyl alcohol. The combined organic layers were finally evaporated to dryness under a stream of nitrogen in a water bath at 60°C.

Tissue samples. Aliquots of 1 g of the various tissues (muscle, liver, subcutaneous fat and kidney), ground by means of a Waring commercial blender,

were weighed into 15-ml glass centrifuge tubes and 0.1 μg of the internal standard was added. After the addition of 2 ml of 1 *M* sodium hydroxide, the samples were further homogenized with an Ultra-turrax TP 18/10 homogenizer and extracted as described above.

Use of the disposable extraction columns

Plasma samples of 1 ml, made alkaline and spiked with the internal standard as described above, were poured into the Clin Elut™ columns. After 2 min, 4 ml of the extraction solvent were added and the organic phase was allowed to drip into 5-ml test tubes. This step was repeated then with one more 2-ml aliquot of extraction solvent and the combined eluents were evaporated to dryness.

Calibration procedure

Using the levamisole standard solutions, samples of blank control plasma (1 ml) were spiked with levamisole at concentrations ranging from 0.01 to 3 μg/ml, and with the internal standard at a fixed concentration of 0.5 μg/ml.

The calibration tissue samples (rat muscle, 1 g) corresponded to 0.005—1 μg/g and the amount of internal standard was 0.1 μg per sample. These samples were then taken through the extraction procedures described previously.

Apparatus

All the chromatographic analyses were performed on a Varian Model 3700 gas chromatograph equipped with a Varian Model 8000 automatic sample injector and a thermionic specific detector containing an electrically heated ceramic-alkali bead. The glass column (200 × 0.2 cm) was packed with 3% OV-17 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The column temperature was 255°C and the injector and detector temperatures were 290°C and 340°C, respectively. Nitrogen was used as a carrier gas at a flow-rate of 30 ml/min. The detector was operated at a bias voltage of —4 V and the bead heating current was adjusted at 3 A, corresponding to a bead temperature of about 800°C. To optimize the detector for the specific detection of nitrogen compounds, the detector bead was in the path of a gas stream comprising hydrogen and air at flow-rates of 4.5 and 175 ml/min, respectively.

Area integrations, calculations and plotting of the chromatograms as well as the control of the autosampler functions were carried out by a Spectra-Physics Model 4000 data system.

Gas chromatography

The extraction residues from plasma samples were reconstituted with 100 μl of methanol by vigorous vortexing, centrifuged for a few seconds, transferred to 200-μl microvolume vials and placed in the autosampler. Sample volumes of 2 μl were then automatically injected into the gas chromatograph. Tissue extraction residues were dissolved in 50 μl of methanol and aliquots of 2 μl were manually injected.

Calculations

Results were calculated by determining the peak area ratios of levamisole related to the internal standard, and comparing these ratios with the standard curves obtained after analysis of the calibration samples.

RESULTS

The recovery of the extraction procedure, obtained after the analysis of 1 μg of levamisole added to 1 ml of control plasma or to 1 g of blank tissue, amounted to 86 ± 4% (mean ± S.D., n = 5) for the plasma extraction procedure using Clin Elut™ columns, and to 79 ± 5% (mean ± S.D., n = 5) for the tissue extraction procedure; the detection limits were 10 ng/ml of plasma and 5 ng/g of tissue. The precision was calculated as the coefficient of variation (C.V.) of the peak area ratios obtained after ten identical injections of one sample (plasma, 100 ng of levamisole per ml) and was 2.4%. The reproducibility was determined after 10 analyses of the same sample (plasma, 100 ng of levamisole per ml) resulting in a day-to-day variation of 8.9 (C.V., %). The retention times of levamisole and the internal standard were 3.4 and 4.4 min, respectively.

The concentration ranges for levamisole and the internal standard as well as the correlation coefficient and the mathematical expression of the standard curves for levamisole in both plasma and animal tissue are summarized in Table I.

TABLE I

CONCENTRATION RANGES AND MATHEMATICAL EXPRESSIONS OF THE LEVAMISOLE STANDARD CURVES

Biological material	Internal standard (ng/sample)	Levamisole range (ng/sample)	Regression equation $y = ax + b$*		Correlation coefficient	
			a	b	r	n
Plasma (1 ml)	500	10—3000	2.33	—0.01	0.9993	5
Animal tissue (1 g)	100	5—1000	13.23	—0.13	0.9998	5

*y = peak area ratio (levamisole/internal standard); x = ng of levamisole per sample.

DISCUSSION

Initially, a rapid and sensitive method was sought for the determination of levamisole in plasma samples from clinical and bioavailability studies in man.

In most plasma samples, obtained after blood collection in polyethylene tubes containing heparin as the anticoagulant, the gas chromatographic background permitted the omission of the back-extraction steps described in the Experimental section. The procedure could even be further simplified using the Clin Elut™ disposable extraction columns, reducing time-consuming

shaking, centrifuging and phase-transfer steps. In addition, the use of the autosampler allowed the analysis of the samples overnight.

Problems appeared, however, with the extraction of plasma samples whenever Vacutainer® tubes (Model 3200 QS, Becton-Dickinson & Co., Rutherford, NJ, U.S.A.) were used to collect the blood specimens. Interfering peaks were observed and this resulted in the necessity of back-extraction with sulphuric acid and re-extraction into the organic extraction solvent after alkalinization. These purification steps too could be simplified using a second Clin Elut™ tube. However, in comparison with the procedure using single tubes,

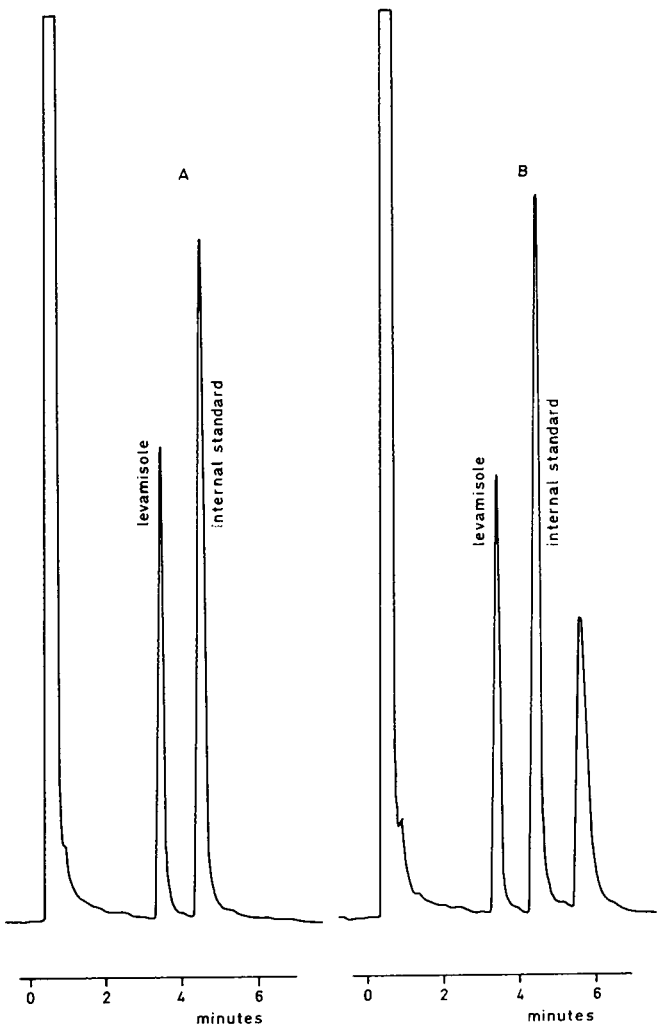


Fig. 2. Chromatograms of plasma extracts from a volunteer, 2 h after oral administration of levamisole, (A) after blood collection in a polyethylene tube, and (B) after collection in a Vacutainer® tube. The extractions were carried out using Clin Elut™ colums. Blood collection in the Vacutainer® tube required the use of an additional purification step using a second Clin Elut™ column. Chromatographic conditions were as indicated in the text.

the total recovery for both I and II declined about 5%, and non-interfering extraneous peaks were still observed, as can be seen in Fig. 2, which shows chromatograms from extracts of the same blood sample collected in a polyethylene tube containing heparin and in a Vacutainer® tube containing EDTA as the anticoagulant. An even more important draw-back of the use of Vacutainer® tubes was the resulting decrease of levamisole plasma concentrations in comparison to other blood collection techniques. Table II shows the levamisole plasma levels in two volunteers after oral administration of 100 mg of the drug. Blood samples were collected from the antecubital vein with Plastipak-BD syringes and transferred to Vacutainer® tubes with rubber stoppers and also to polyethylene tubes with polyethylene stoppers. All samples were then carefully rotated for 5 min (10 rpm); the plasma was then separated after centrifugation and both series of plasma samples were analyzed as described in the Experimental section. In all measurable plasma samples, exposure to the lubricated rubber stoppers lowered the levamisole concentration and resulted in a relative loss of 19.9 ± 8.3% (mean ± S.D.), indicating a highly significant difference ($p < 0.001$ by a paired t-test). A similar phenomenon has previously been reported for propranolol [8], lidocaine [9] and tricyclic antidepressants [10], in which cases it was attributed to the displacement of the drugs from the plasma proteins by stopper constituents and diffusion thereafter into the erythrocytes [11]. The use of Vacutainer® tubes should therefore be avoided whenever levamisole plasma levels are to be determined.

TABLE II

LEVAMISOLE PLASMA LEVELS IN TWO VOLUNTEERS AFTER ORAL ADMINISTRATION OF 100 mg OF THE DRUG

Values are expressed in μg/ml of plasma.

Time after dosage	Subject 1		Subject 2	
(h)	A*	B**	A*	B**
0	⩽0.010	⩽0.010	⩽0.010	⩽0.010
1	0.019	0.015	⩽0.010	⩽0.010
2	0.307	0.249	0.126	0.107
4	0.411	0.261	0.246	0.198
6	0.164	0.133	0.095	0.086

*Blood collected in 10-ml polyethylene tubes with polyethylene stoppers.
**Blood collected in Vacutainer® tubes.

The autosampler can not be used if low tissue residues of I must be detected, since the smaller volumes of methanol used to reconstitute the residues would be consumed in flushing the injection system. If the reconstituted residues are injected by hand, 5 ng of levamisole could be easily detected in 1 g of tissue as well as in 1-ml plasma samples.

The application of the method to the assay of several hundred plasma and tissue specimens demonstrated its suitability; interferences were not

observed and the gas chromatographic column proved to be extremely stable under the conditions used.

APPLICATIONS

The method described has been used in a bioavailability and dose proportionality study of levamisole in human volunteers. Plasma levels from a male volunteer receiving an oral dose of 150 mg of levamisole in three 50-mg tablets are shown in Fig. 3.

The method has also proved to be valuable in tissue residue trials in cattle after topical and oral dosage of the drug. Residues were measured in muscle, liver, subcutaneous fat and kidney at several times after administration.

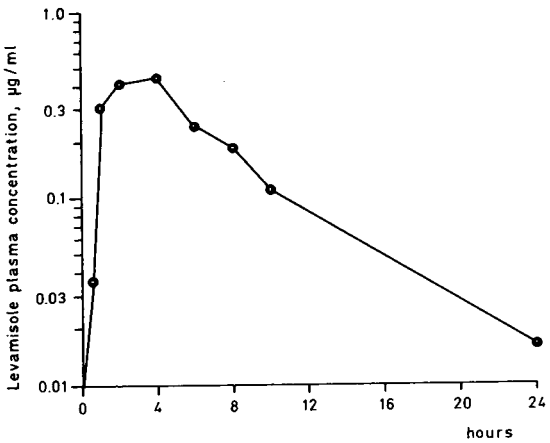


Fig. 3. Plasma concentration profile of levamisole in a human subject following oral administration of 150 mg of the drug in a tablet formulation.

ACKNOWLEDGEMENT

The authors wish to thank Mrs. B. Wouters, Mr. J. Van Mierlo and Mr. D. Ashton for the preparation of the manuscript.

REFERENCES

1 A.H.M. Raeymakers, F.T.N. Allewijn, J. Vandenberk, P.J.A. Demoen, T.T.T. Van Offenwert and P.A.J. Janssen, J. Med. Chem., 9 (1966) 545.
2 M. Moens, J. Dom, W.E. Burke, S. Schlossberg and V. Schuermans, Amer. J. Trop. Med. Hyg., 27 (1978) 897.
3 F. Gatti, F. Krubwa, J. Vandepitte and D. Thienpont, Ann. Soc. Belg. Med. Trop., 52 (1972) 19.
4 P.A.J. Janssen, Fortschr. Arzneimittelforsch., 20 (1977) 347.
5 J. Symoens, J. De Cree, W.F.M. Van Bever and P.A.J. Janssen, in M.E. Goldberg (Editor), Pharmacological and Biochemical Properties of Drug Substances, Vol. 2, American Pharmaceutical Association, Washington, DC, 1979, p. 407.

6 J.E. Smith, N.R. Pasarella and J.C. Wyckoff, J. Ass. Offic. Anal. Chem., 59 (1976) 954.
7 A. Holbrook and B. Scales, Anal. Biochem., 18 (1967) 46.
8 R.H. Cotham and D. Shand, Clin. Pharmacol. Ther., 18 (1975) 535.
9 W.W. Stargel, C.R. Coe, P.A. Routledge and D.G. Smith, Clin. Chem., 25 (1979) 617.
10 J.J. Thoma, P.B. Bondo and C.M. Kozak, Ther. Drug Monitor., 1 (1980) 335.
11 K.M. Piafsky, Clin. Pharmacokin., 5 (1980) 246.

Journal of Chromatography, 224 (1981) 33—41
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 845

DETERMINATION OF POLYTHIAZIDE AND PRAZOSIN IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

J. DOKLADALOVA*, S.J. COCO, P.R. LEMKE, G.T. QUERCIA and J.J. KORST

Quality Control, Pfizer Inc., Eastern Point Road, Groton, CT 06340 (U.S.A.)

(First received November 3rd, 1980; revised manuscript received January 20th, 1981)

SUMMARY

A selective high-performance liquid chromatographic method has been developed for the determination of polythiazide in human plasma down to concentrations of 0.5 ng/ml. Polythiazide and an internal standard (epithiazide) are simultaneously extracted from the sample, the extract is purified on a silica micro-column and analyzed on a μBondapak CN column. Chloroform—methanol (97:3) is the eluent, with spectrophotometric detection at 264 nm.

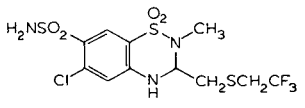
The extraction methodology developed for the analysis of polythiazide in blood plasma allows the simultaneous quantitative determination of prazosin, which is frequently administered together with thiazide diuretics. The precision and accuracy of both the polythiazide and the prazosin assays are excellent and are not seriously affected by the simultaneous presence of both drugs in the plasma. Therefore, determination of polythiazide and prazosin is possible using a single plasma sample.

INTRODUCTION

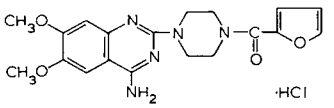
Polythiazide (Renese®) (I) is a diuretic used frequently in the management of hypertension either as a sole drug or in combination with antihypertensive drugs [1]. For instance, prazosin (Minipress®) (II) which is a recognized vasodilating agent [2, 3] can be used for this purpose. Since, under certain circumstances, both drugs are administered concurrently, their simultaneous assay in blood plasma is required for evaluation of bioavailability in clinical studies.

A procedure exhibiting sufficient sensitivity for polythiazide analysis has been published recently [1]. In this procedure, polythiazide is extracted from plasma and hydrolyzed to form trifluoroethylthioacetaldehyde which is quantitated using gas—liquid chromatography with electron-capture detection. Since the procedure does not utilize an internal standard and trifluoroethyl-

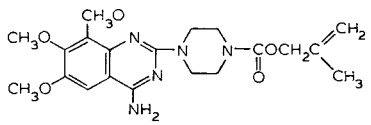
0378-4347/81/0000—0000/$02.50



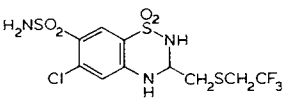
(I) Polythiazide



(II) Prazosin·HCl



(III) ATQ



(IV) Epithiazide

thioacetaldehyde is not available commercially as a primary standard, its practical use requires a high degree of experience.

A relatively simple prazosin determination by high-performance liquid chromatography (HPLC) using fluorescence detection has been described [2, 4]. The procedure involves the 2-methallyl ester of 4-(4-amino-6,7,8-trimethoxy-2-quinazolinyl)-1-piperazine carboxylic acid (ATQ) as an internal standard (III) and provides excellent sensitivity.

Thus, to quantitate both polythiazide and prazosin using a single plasma sample, it was necessary to develop a more practical procedure for polythiazide and couple the method with that for prazosin. The first objective was achieved by development of an HPLC procedure for polythiazide, using epithiazide (IV) as an internal standard, with the eluate monitored by UV detection at 264 nm.

Secondly, an extraction procedure was devised which purified and concentrated both drugs and the internal standards. The extract was then chromatographed on two different HPLC systems, one with UV detection for polythiazide, the other with fluorescence detection for prazosin.

EXPERIMENTAL

Materials and equipment

Polythiazide (I), internal standard epithiazide (IV), prazosin (II) in the form of the hydrochloride salt and internal standard 2-methallyl ester of 4-(4-amino-6,7,8-trimethoxy-2-quinazolinyl)-1-piperazine carboxylic acid, ATQ (III) were supplied by Pfizer (Groton, CT, U.S.A.). Reagents included (1) ethyl acetate, glass distilled; (2) chloroform without preservative; (3) *n*-hexane, glass distilled, all, e.g., from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.); (4) methanol, e.g. Matheson, Coleman and Bell (Cincinnati, OH, U.S.A.); (5) acetonitrile (e.g. Burdick and Jackson); (6) acetic acid, Baker (Phillipsburg, NJ, U.S.A.); (7) silica gel 60, particle size 0.063—0.200 mm, Cat. 7734, E. Merck (Darmstadt, G.F.R.), activated by heating in an oven at 100° overnight; (8) ammonium hydroxide (Baker). A vortex evaporator (Buchler) was used at 40°C. In addition, the reagents included methanol treated with *n*-hexane (9) and *n*-hexane treated with methanol (10). (Reagents 9 and 10 were prepared by transferring 100 ml methanol and 500 ml *n*-hexane to a 1-l

separatory funnel, shaking the mixture and allowing the phases to separate, the bottom layer being methanol treated with *n*-hexane, the upper layer, *n*-hexane treated with methanol.)

All glassware used during the extraction procedure was cleaned with chromic acid, washed with distilled water, rinsed with ammonium hydroxide and air dried.

Chromatography system

The polythiazide and prazosin assays both utilized the same column system and configuration. A pump (Waters 6000A) and variable volume injection valve (Waters U6K) followed by a guard column (Waters PN 84550) containing Bondapak Phenyl/Corasil packing, particle size 37—50 μm (Waters PN 27282) was used preceding the analytical column μBondapak CN, particle size 10 μm, 30 cm × 3.9 mm (Waters PN 84042), all from Waters Assoc. (Milford, MA, U.S.A.).

Polythiazide assay. A UV detector (Schoeffel SF 770) set at 264 nm (range 0.04, time constant 5 sec, recorder 1 mV) was used to monitor the column eluate. The mobile phase, consisting of chloroform—methanol (97:3), was degassed for 3—4 min under vacuum and pumped through the column at a flow-rate of 0.7 ml/min. Injection volume was 25 μl for both standards and samples. Before daily analyses, 1 h was allowed for chromatographic equilibration. Under these conditions, polythiazide eluted at approximately 10 min, epithiazide at 15 min.

Prazosin assay. A fluorescence detector (Schoeffel FS 970) set at an excitation wavelength of 248 nm with a 389 emission filter (range 0.1, time constant 6 sec, recorder 1 mV) was used to monitor the column eluate. The mobile phase, consisting of acetonitrile—distilled water—acetic acid (50:47:3), was degassed for 10 min under vacuum and pumped through the column at a flow-rate of 2.2 ml/min. Injection volume was 3 μl for both standards and samples. Before daily analyses, 1 h was allowed for chromatographic equilibration. Under these conditions, prazosin eluted at approximately 6 min, ATQ at 9 min.

Preparation of standard solutions and calibration curves for polythiazide assay

Prepare methanolic solutions containing (a) 10 μg polythiazide per ml and (b) containing 10 μg eipithiazide per ml.

An external calibration curve can be used, since the extraction efficiencies of polythiazide and epithiazide in the procedure were demonstrated to be identical. Typical recoveries are listed in Table I.

For construction of the external calibration curve, take 1.0 ml (2.0 ml or 5.0 ml) of solution (a) and 10 ml of solution (b), combine and dilute to 100 ml with methanol. The calibration solutions will contain 100 ng (200 ng, 500 ng) polythiazide and 1000 ng epithiazide per ml. Transfer 50 μl of each standard solution into separate 10-ml centrifuge tubes. Under the assay conditions, the amounts correspond to 1 ng (2 ng, 5 ng) polythiazide per ml plasma. Evaporate each solution to dryness using a mild stream of nitrogen. Reconstitute each with 50 μl ethyl acetate, vortex 30 sec and inject 25 μl into the HPLC system. An external standard curve is constructed by plotting the polythiazide—internal

TABLE I

POLYTHIAZIDE DETERMINATION IN BLOOD PLASMA

Polythiazide added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	24	94	8.93
2.0	19	101.5	7.27
5.0	10	93.8	5.69
10.0	10	97.4	4.44
15.0	8	97.9	1.64

standard (epithiazide) peak height ratio vs. ng polythiazide per ml plasma.

For spiking plasma samples with internal standard, accurately prepare a standard solution of epithiazide in methanol containing 1 ng/μl (solution *X*).

Preparation of standard solutions and calibration curves for prazosin assay

Prepare methanolic solutions containing (a′) 10 μg prazosin · HCl per ml and (b′) 1 mg ATQ per ml.

To construct a calibration curve, take 1.0 ml (5.0 ml or 15.0 ml) of solution (a′) and 0.25 ml of solution (b′), combine and dilute to 100 ml with methanol. Transfer 50 μl of each calibration standard solution into separate 50-ml centrifuge tubes. Under the assay conditions, the amounts correspond to 1 ng (5 ng, 15 ng) prazosin·HCl per ml plasma. Evaporate the calibration solutions to dryness using a mild stream of nitrogen, add 5.0 ml human control plasma, mix and proceed as described under Sample preparation. A standard curve is constructed by plotting the prazosin—internal standard peak height ratio vs. ng prazosin·HCl per ml plasma.

For spiking plasma samples with internal standard, accurately prepare a standard solution of ATQ in methanol containing 2.5 ng/μl (solution *Y*).

Since the extraction efficiencies of prazosin and ATQ internal standard are not identical in the procedure, both substances have to be added to blank plasma and carried through the procedure in order to obtain a calibration curve. Typical recoveries are listed in Table II.

TABLE II

PRAZOSIN DETERMINATION IN BLOOD PLASMA

Prazosin added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	12	103	7.24
2.0	14	100	6.62
5.0	14	97	4.97
15.0	10	99	4.59

Extraction of plasma samples

A flow diagram is presented in Fig. 1. Transfer 5.0 ml of a plasma sample to a 50-ml centrifuge tube, add 50 ng epithiazide internal standard (50 μl of solution *X*) and 125 ng ATQ internal standard (50 μl of solution *Y*) and mix well. Extract the plasma sample with 2 × 20 ml *n*-hexane, inverting the centrifuge tube 30 times for each extraction. Centrifuge and remove the *n*-hexane by

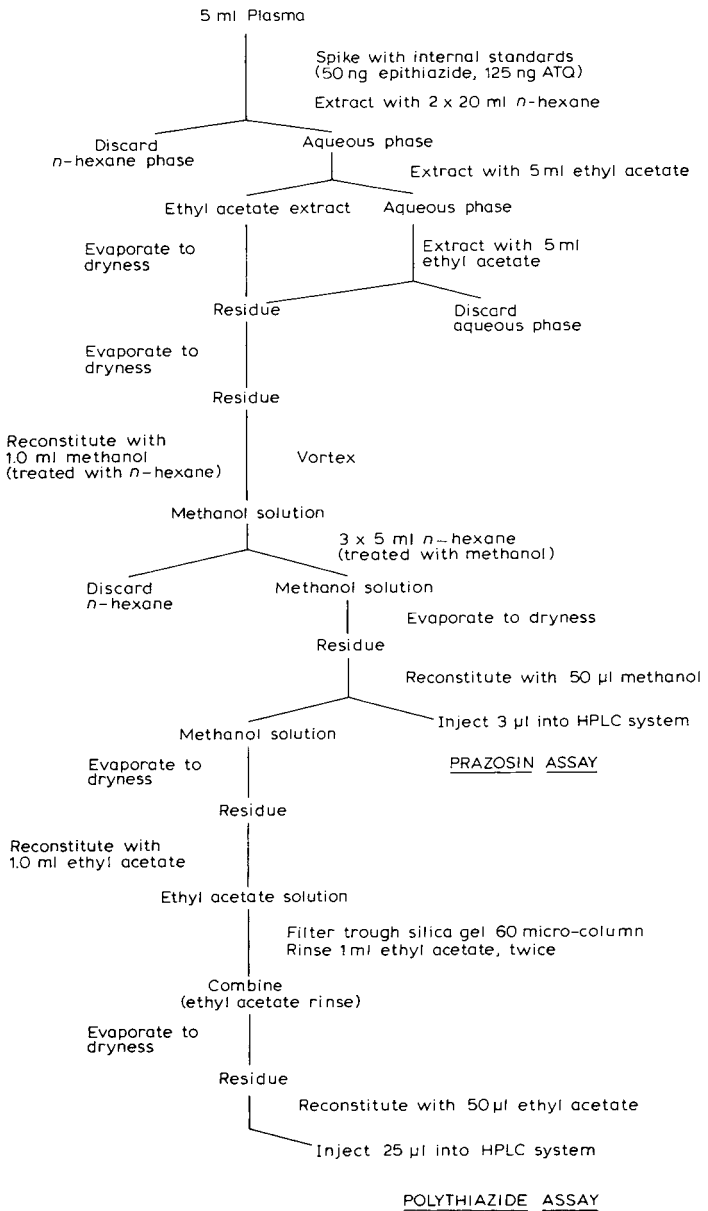


Fig. 1. Schematic diagram for the extraction of polythiazide and prazosin from human plasma.

aspiration. Continue by extracting the plasma sample with 5 ml of ethyl acetate, vortexing 30 sec. Centrifuge the mixture, transfer the ethyl acetate to a 15-ml centrifuge tube, and evaporate the solvent on a vortex evaporator. Repeat the ethyl acetate extraction, combining the solvent with the residue from the first extract. Repeat the evaporation. Reconstitute the residue with 1.0 ml methanol (treated with *n*-hexane). Add 5 ml *n*-hexane (treated with methanol), mix 30 sec, centrifuge. Discard the *n*-hexane layer using a disposable Pasteur pipet. Repeat the extraction with 2 × 5 ml *n*-hexane (treated with methanol). Transfer the methanol to a clean 15-ml centrifuge tube and evaporate the methanol to dryness on a vortex evaporator. The residue (*r*) is used for determining the content of prazosin and polythiazide.

Sample assay for prazosin

Reconstitute the sample residue (*r*) with 50 μl of methanol, mixing 30 sec. Inject 3 μl of the sample solution(s) into the HPLC system for prazosin analysis. Determine the peak heights of prazosin and its internal standard ATQ. Calculate the ratio by dividing peak height of prazosin by peak height of internal standard. Using a calibration curve prepared concurrently with each series of plasma samples, report the content of prazosin in plasma.

Sample assay for polythiazide

Using a vortex evaporator, evaporate to dryness the sample solution(s) remaining after the prazosin assay. Reconstitute the sample residue with 1 ml ethyl acetate, mixing 30 sec. Prepare a micro-column by plugging the tip of a disposable Pasteur pipet (approximately 16 cm long) with glass wool. Transfer 0.5 g of activated silica gel 60 to the column and wet with ethyl acetate. Transfer the ethyl acetate sample solution onto the wet silical gel column with a disposable Pasteur pipet. Collect the eluate in a 15-ml centrifuge tube by applying pressure with a rubber bulb on top of the column. Add an additional 1.0 ml ethyl acetate to the sample tube, mixing 30 sec. Again transfer the ethyl acetate to the silica gel column, collecting the eluate in the 15- ml centrifuge tube. Repeat with an additional 1.0 ml ethyl acetate, the final volume being ca. 3 ml. Rinse the walls of the centrifuge tube with ca. 0.2 ml ethyl acetate. Evaporate the ethyl acetate to dryness on the vortex evaporator and reconstitute the sample residue with 50 μl ethyl acetate, mixing 30 sec. Inject 25 μl of the prepared sample onto the HPLC system for polythiazide analysis. Measure peak heights of polythiazide and its internal standard epithiazide. Calculate ratio by dividing peak height of polythiazide by peak height of internal standard (epithiazide). Using the calibration curve which is constructed concurrently with each series of samples, report the amount of polythiazide present in plasma samples.

RESULTS AND DISCUSSION

An HPLC procedure for the determination of polythiazide in human plasma has been developed. The extraction methodology allows simultaneous determination of prazosin, which is, under certain circumstances, administered together with polythiazide. The procedure was validated in three steps. First,

reference plasma (plasma blank) was spiked with internal standard (epithiazide) and known amounts of polythiazide (corresponding to 1—15 ng polythiazide per ml plasma). The mixture was analyzed according to the procedure described above. A linear calibration curve was obtained. A typical chromatogram is depicted in Fig. 2A. The results summarized in Table I clearly demonstrate the reproducibility and accuracy of the procedure. Second, known amounts of prazosin (corresponding to 1—15 ng prazosin per ml plasma) and internal standard ATQ were added to blank plasma and prazosin content was determined as described. A linear calibration curve was obtained. Fig. 3A shows the separation of prazosin and its internal standard ATQ, while Table II illustrates the accuracy and precision obtained. Amounts as low as 1 ng prazosin per ml plasma and 1 ng polythiazide per ml plasma can be reliably quantified. Finally, polythiazide was determined in blood plasma containing prazosin and ATQ (Table III), and prazosin was determined in blood plasma containing polythiazide and epithiazide (Table IV). No interferences were observed in the chromatographic backgrounds in either procedure. Consequently, polythiazide and prazosin can be analyzed with adequate precision and sensitivity using a single blood plasma sample.

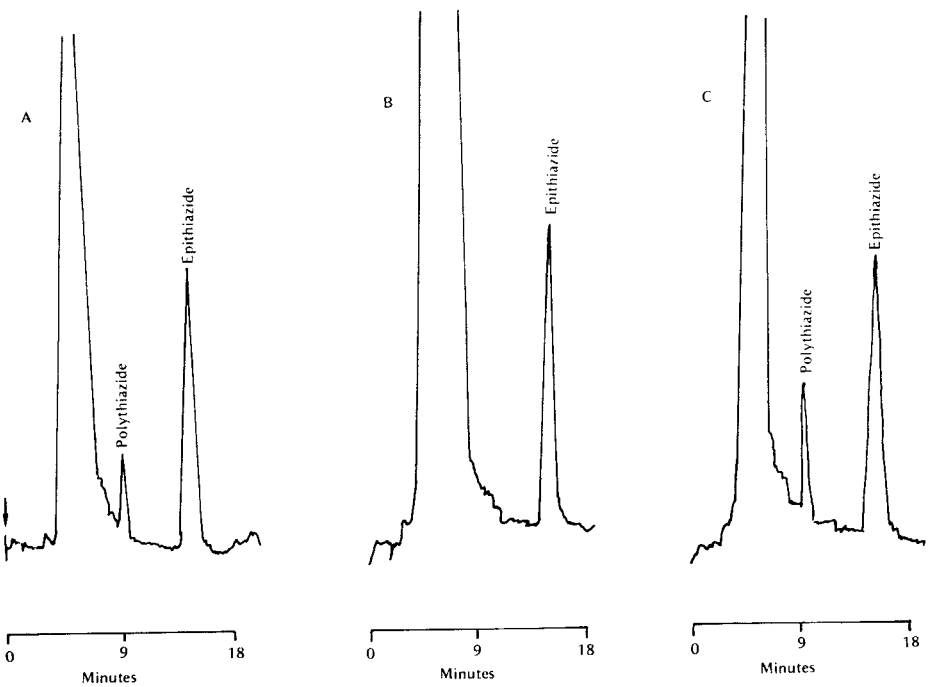


Fig. 2. HPLC chromatograms of (A) the extract from blank plasma spiked with 2 ng polythiazide and 10 ng epithiazide per ml plasma; (B) the extract of plasma sample obtained from a test subject prior to administration of Minizide® spiked with 10 ng epithiazide per ml plasma; (C) the extract of a plasma sample obtained from a test subject 8 h after oral administration of Minizide®.

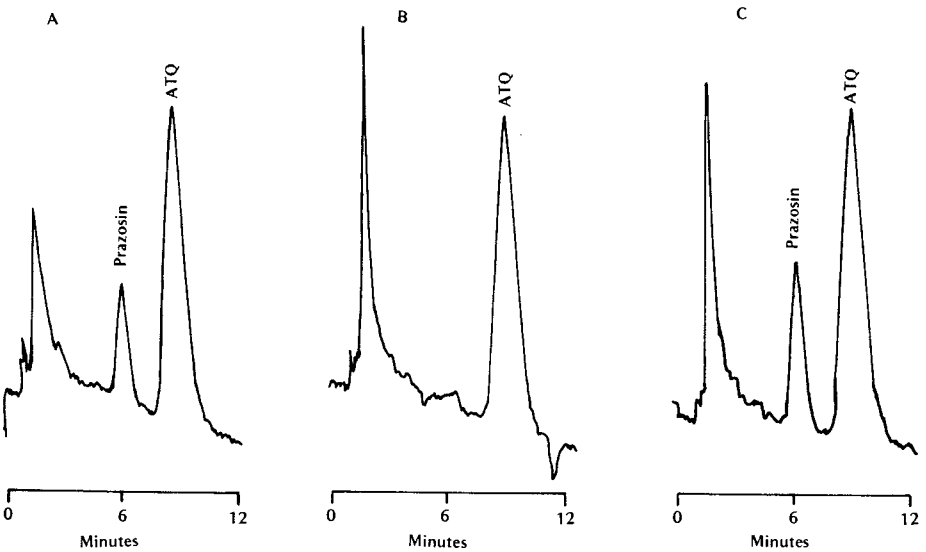


Fig. 3. HPLC chromatograms of (A) the extract from blank plasma spiked with 5 ng prazosin · HCl and 25 ng ATQ per ml plasma; (B) the extract of plasma sample obtained from a test subject prior to administration of Minizide® spiked with 25 ng ATQ per ml plasma; (C) the extract of a plasma sample obtained from a test subject 0.5 h after administration of Minizide®.

TABLE III

POLYTHIAZIDE DETERMINATION IN BLOOD PLASMA IN THE PRESENCE OF PRAZOSIN

Amounts of prazosin added varied from 1 ng per ml plasma to 15 ng per ml plasma at all polythiazide concentration levels studied.

Polythiazide added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	9	94.4	9.32
2.0	9	102.2	6.95
5.0	10	95.8	6.90

TABLE IV

PRAZOSIN DETERMINATION IN BLOOD PLASMA IN THE PRESENCE OF POLYTHIAZIDE

Amounts of polythiazide added varied from 1 ng per ml plasma to 15 ng per ml plasma at all polythiazide concentration levels studied.

Prazosin added (ng/ml)	No. of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
1.0	12	112	6.67
2.0	12	105	4.27
5.0	12	98	3.66

The described methodology was applied to large numbers of blood plasma specimens from a clinical study in which prazosin and polythiazide were administered in the form of the combination drug, Minizide®. Plasma levels for both drugs were determined, with representative chromatograms illustrated in Figs. 2C and 3C.

REFERENCES

1 D.C. Hobbs and T.M. Twomey, Clin. Pharmacol. Ther., 23 (1978) 241.
2 T.M. Twomey and D.C. Hobbs, J. Pharm. Sci., 67 (1978) 1468.
3 A.S. Turner, O.F. Watson and J.E. Brocklehurst, Med. J. Aust., Special Supplement, Aug. 27, (1977) 33.
4 Y.G. Yee, P.C. Rubin and P. Meffin, J. Chromatogr., 172 (1979) 313.

Journal of Chromatography, 224 (1981) 43—49
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 849

QUANTITATIVE DETERMINATION OF NAPROXEN IN PLASMA BY A SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

M. BROQUAIRE*, V. ROVEI* and R. BRAITHWAITE

Department of Clinical Research, Synthélabo—L.E.R.S., 58, rue de la Glacière, 75013 Paris (France)

(First received July 28th, 1980; revised manuscript received January 23rd, 1981)

SUMMARY

A high-performance liquid chromatographic method for the determination of naproxen in plasma is described. The technique is based on the single extraction of the drug from acidified plasma with chloroform using 2-naphthalene acetic acid as internal standard. The chromatographic system consisted of a column packed with Spherisorb ODS (5 μm); the mobile phase was acetonitrile—phosphoric acid (pH 3) (45 : 55, v/v).

The method can accurately measure plasma naproxen concentrations down to 1 μg/ml using 100 μl of sample, with no interference from endogenous compounds. The coefficients of variation of the method at 120 μg/ml and 1 μg/ml are 2.8 and 21.6%, respectively, and the calibration curve is linear. The method described is very suitable for routine clinical and pharmacokinetic studies.

INTRODUCTION

Naproxen [(+)-6-methoxy-α-methyl-2-naphthalene acetic acid] is a potent non-steroidal anti-inflammatory, analgesic and antipyretic drug in widespread clinical use in the treatment of rheumatism and osteorarthritis. The drug is normally administered in a daily dose of 250—750 mg [1]. Naproxen is readily absorbed from the gastrointestinal tract and, after a single oral dose of 500 mg, peak plasma drug concentrations are in the order of 100 μg/ml [2]. With the increasing clinical use of naproxen, there is a need for a rapid, sensitive and (because of the large number of drugs which may be concomitantly prescribed) selective assay of naproxen in plasma.

Several methods for the measurement of naproxen in plasma have already

*Present address: Metabolism and Clinical Pharmacokinetics Unit, Delalande Research Centre, Rueil-Malmaison, France.

0378-4347/81/0000—0000/$02.50

been reported. Gas chromatographic methods [3, 4] using derivatization and flame ionization detection are relatively insensitive. Fluorimetric assays [5, 6], although sensitive, are laborious to perform and are not entirely specific. A few high-performance liquid chromatographic (HPLC) methods for the measurement of naproxen have been described but these do not appear to be entirely satisfactory for routine use. The system reported by Slattery and Levy [7] using a CN-bonded phase is relatively insensitive and tedious for routine operation. The method described by Westerlund et al. [8] either lacks specificity when reversed-phase HPLC is used or is tedious to perform and requires a change in the detector attenuation when an ion-pair solvent mixture is used to determine desmethylnaproxen at the same time. Upton et al. [9] proposed a very sensitive reversed-phase method which unfortunately used ketoprofen as the internal standard and is therefore subject to error. The most recently described method of Burgoyne et al. [10], although satisfactory in some aspects, may be criticized for lack of internal standardization and use of a two-stage extraction which is tedious to perform and greatly increases the time taken for each analysis.

In the present study, a new method has been developed using a single-stage extraction of naproxen and internal standard followed by reversed-phase HPLC. The optimization of the mobile phase avoided possible interference with naproxen by some of the most commonly prescribed anti-inflammatory agents. This method is equally as sensitive as previously described fluorimetric [5, 6] or liquid chromatographic techniques [7—10] but because of its simplicity and greater specificity it can easily be applied to routine clinical or pharmacokinetic studies.

MATERIALS

Solvents

Reagent grade chloroform, used for the extraction, analytical grade orthophosphoric acid and LiChrosolv acetonitrile, used for the mobile phase, were all purchased from Merck (Darmstadt, G.F.R.).

Standards

Pure naproxen was extracted from a commercial formulation, Naprosyn®, in the laboratories of Synthélabo—L.E.R.S. Its purity was checked by measuring the melting point and optical activity. The internal standard used in the study (2-naphthalene acetic acid) was purchased from Aldrich Europe (Beerse, Belgium). Standard solutions were prepared by dissolving naproxen and its internal standard in pure acetonitrile.

Equipment

Analyses were carried out on a Micromeritics 7000B liquid chromatograph (Norcross, GA, U.S.A.) equipped with a Micromeritics 730 universal injector and a Micromeritics 785 UV—visible spectrophotometer.

METHODS

Chromatographic analysis

The mobile phase [acetonitrile—aqueous orthophosphoric acid (pH 3) (45: 55, v/v)] was adjusted to a flow-rate of 1.00 ± 0.01 ml/min through a stainless-steel column, 15 cm × 4.6 mm I.D., packed in the laboratory [11] with Spherisorb ODS (5 μm, batch 17/49, Phase Separations, Queensferry, Great Britain). The detector wavelength was set at 230 nm, which corresponds to the highest optical absorption of naproxen dissolved in the mobile phase.

Extraction procedure

The procedure used for the extraction of naproxen in plasma is schematically outlined in Fig. 1. A 100-μl sample of plasma together with 1 ml of KCl solution (1 *M*, pH 2) were introduced into a tapered tube containing 10 μg of the internal standard (10 μl of a 1 μg/μl solution in acetonitrile). This mixture was shaken on a Vortex mixer, then extracted with chloroform (6 ml) on a "rock and roll" shaker for 20 min. The two phases were then separated by centrifugation (1000 *g* for 5 min at 4°C) and the aqueous phase was discarded.

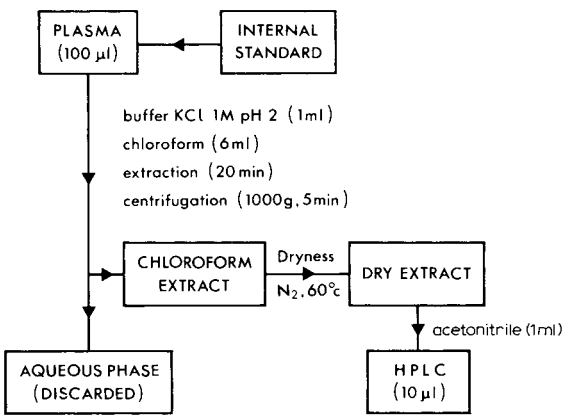


Fig. 1. Extraction scheme for naproxen from plasma.

Then 5 ml of the chloroform extract were transferred into a second tube and evaporated to dryness at 60°C under a gentle stream of nitrogen. The dry extract was then dissolved in 1 ml of pure acetonitrile by agitation on a Vortex mixer; 10 μl of this solution were injected into the column with the detector attenuation set at 0.1 a.u.f.s. or alternatively a 1-μl injection with the detector attenuation at 0.01 a.u.f.s.

RESULTS

Two chromatograms, one obtained from blank plasma and the other from a plasma sample of a patient, are presented in Fig. 2. The peaks corresponding to internal standard and naproxen under the conditions described above were well resolved and no endogenous compound extracted at the same time interfered

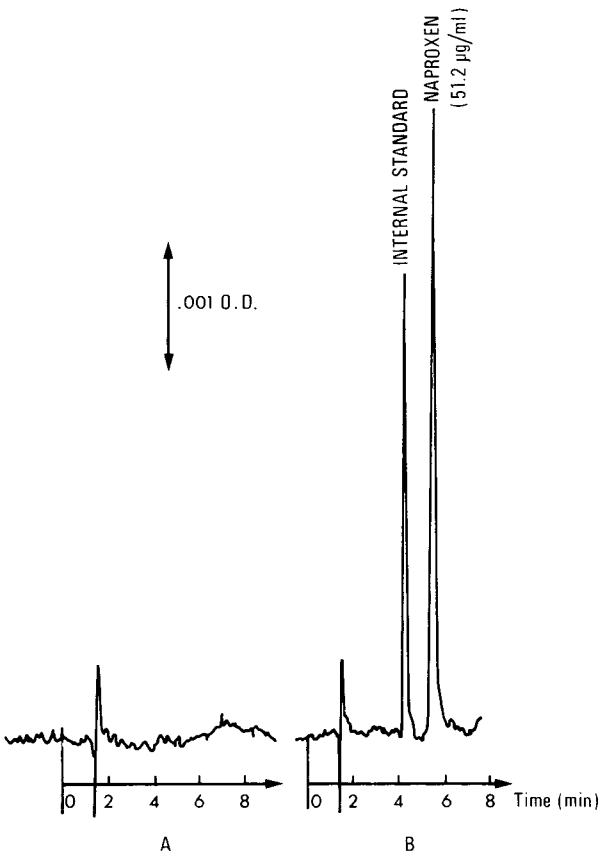


Fig. 2. Typical chromatograms of human plasma extracts for naproxen determination (a) before administration of the dose, (b) 8 h after oral administration of a 500-mg dose (1 µl injected). For chromatographic conditions, see the text.

TABLE I

REPRODUCIBILITY OF THE HPLC METHOD FOR THE MEASUREMENT OF NAPROXEN AT DIFFERENT CONCENTRATIONS IN PLASMA

Spiked concentrations (µg/ml)	Number of observations	Found concentrations (mean values) (µg/ml)	Standard deviation (µg/ml)	Coefficient of variation (%)
1	5	1.3	0.28	21.6
5	5	5.1	0.55	10.9
10	4	10.0	0.99	9.9
20	4	18.8	1.51	8.1
40	3	40.9	0.74	1.9
60	3	60.3	2.50	4.2
80	4	80.1	2.57	3.2
100	5	100.1	2.64	2.7
120	4	119.7	3.27	2.8

with these peaks. The retention times were 4.18 and 5.35 min, respectively, for internal standard and naproxen; with a respective reduced height equivalent to a theoretical plate of 5.8 and 4.7 (mean diameter of the stationary phase was 5.7 μm as determined with a Coulter Counter TA_2 from Coultronics, Margency, France), the interval between each injection was 7 min.

The procedure was quantified by the internal standard method using the peak height ratio method. The response was linear between 1 and 120 μg/ml under the conditions described above, and the day-to-day variation in the slope of the calibration curve was < 3%. The accuracy of the method was determined by analysing spiked plasma samples of naproxen and the results are shown in Table I. The coefficients of variation ranged from 22% for 1 μg/ml to < 3% for the 120 μg/ml plasma drug concentrations (Table I).

DISCUSSION

To extract naproxen from plasma, four different extraction solvents were tried: hexane, toluene, chloroform and diethyl ether. The same conditions described above for chloroform were used. The recoveries of the two compounds (naproxen and its internal standard) were in the following increasing order: hexane < toluene < chloroform = diethyl ether. Chloroform and diethyl ether extracts had almost similar chromatograms but the diethyl ether extracts showed a high and strongly retained peak with a retention time of 40 min. Although obtained at 230 nm, the chromatogram of blank plasma (Fig. 2) extracted with chloroform is considerably cleaner than that presented by Westerlund et al. [8] or Upton et al. [9], and is comparable to that obtained by Burgoyne et al. [10] who used a more specific fluorescence detector and a two-stage extraction.

Different mobile phases were investigated using the same column before making the final selection of the chromatographic conditions: acetonitrile and sodium acetate (0.1 *M*) in different ratios; acetonitrile 30%, KH_2PO_4 (0.01 *M*, pH 6) 70%; acetonitrile 40%, KH_2PO_4 (0.01 *M*, pH 4.6) 60%; acetonitrile and KH_2PO_4 (0.005 *M*, pH 3) in different ratios. The separation and efficiencies were poor with large band tailing peaks when sodium acetate mobile phases were used. Replacing 0.1 *M* sodium acetate with 0.01 *M* KH_2PO_4 at the same pH partly improved the chromatograms. Decreasing the pH or the phosphate concentration of the mobile phase containing KH_2PO_4 increased both the efficiency and selectivity, as well as reducing peak tailing. The same effects were observed by decreasing the concentration of KH_2PO_4 from 0.01 *M* to 0.005 *M* at the same pH. Finally, the chromatograms obtained using pure orthophosphoric acid at a given pH were better than those obtained using a phosphate salt. This suggests that the chromatographic characteristics of naproxen and its internal standard are markedly dependent on the composition of the mobile phase.

The selected mobile phase, acetonitrile—H_3PO_4 (pH 3) (45:55, v/v) (which allowed the system to show more than 37,000 theoretical plates/meter for naproxen on a 15-cm column packed with Spherisorb ODS 5 μm) was similar to that used by Burgoyne et al. [10], namely methanol—acetic acid (0.1 *M*)

(70:30, v/v) at 1.00 ml/min on a 30-cm column (apparently 48,000* theoretical plates/meter for naproxen on μBondapak C_{18}). These two acidic mobile phases were completely different from that used by: (1) Westerlund et al. [8], methanol—phosphate (pH 7) (40:60, v/v) at 1.00 ml/min on a 10-cm column (5500* theoretical plates/meter for naproxen on LiChrosorb RP-8, 5 or 10 μm); (2) Upton et al. [9], acetonitrile—phosphate (pH 7) (8:92, v/v) at 2.00 ml/min on a 40-cm column (3850* theoretical plates/meter for naproxen on Spherisorb ODS 5 μm). The pH, which was the main difference between the four mobile phases, appeared to be one of the most important parameters for obtaining good efficiency and selectivity.

Using the present chromatographic system, it was possible to resolve most of the commonly prescribed anti-inflammatory agents from naproxen (Table II).

TABLE II

CAPACITY FACTORS OF SOME NON-STEROIDAL ANTI-INFLAMMATORY AGENTS

For chromatographic conditions, see the text.

Drug	k'
Paracetamol	0.8
Salicylic acid	1.5
Internal standard	2.5
Alclofenac	3.2
Ketoprofen	3.4
Oxyphenobutazone	3.9
Naproxen	4.1
Sulindac	4.3
Flurbiprofen	5.9
Ibuprofen	7.0
Indomethacin	7.3
Phenylbutazone	8.5
Flufenamic acid	12.1

Only oxyphenbutazone and sulindac were likely to interfere with the measurement of naproxen in plasma. Possible interferences by the metabolites of naproxen in plasma after naproxen administration was considered to be negligible [12]. Because of the simple extraction procedure and the short time of the chromatographic analysis, at least 30 samples can be analysed daily by only one person. If a faster rate of analysis is needed this procedure can be automated. Since the final extract is dissolved in 1 ml of solvent, of which 10 μl are injected, an automatic injector can be used without any modification of the method.

The mean plasma naproxen levels obtained after the administration of a 500-mg single dose (Naprosyn®) clearly demonstrate that the method has sufficient sensitivity for pharmacokinetic studies (Fig. 3). The sensitivity of the method may be greatly increased by reducing the quantity of solvent used in the final pre-injection stage.

*Calculated from the chromatograms shown in the respective papers.

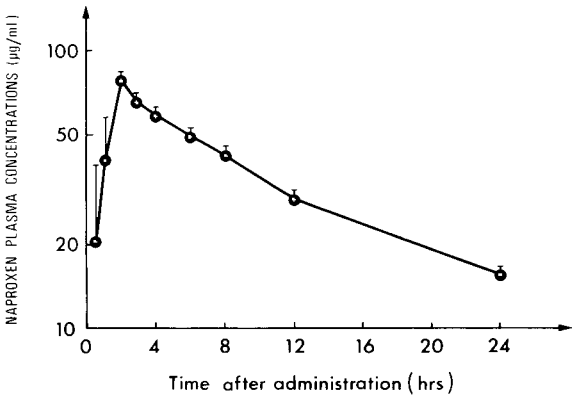


Fig. 3. Mean (± S.E.M.) plasma concentrations of naproxen after administration of a 500-mg dose as Naprosyn® to three adults.

In conclusion, the HPLC method for naproxen in plasma that has been developed, has been demonstrated to be both simpler and more selective than other methods described previously. It is sufficiently sensitive and can serve as a useful tool for pharmacokinetic and clinical studies involving naproxen.

REFERENCES

1 Martindale, The Extra Pharmacopoeia, The Pharmaceutical Press, London, 27th ed., 1977, p. 99.
2 M. Broquaire and V. Rovei, unpublished results.
3 J.P. Desager, M. Vanderbist and C. Harvengt, J. Clin. Pharm., 16 (1976) 189.
4 R. Runkel, M.D. Chaplin, H. Sevelius, E. Ortega and E. Segre, Clin. Pharmacol. Ther., 20 (1976) 269.
5 M. Antilla, J. Pharm. Sci., 66 (1977) 579.
6 H. Held, J. Clin. Chem. Clin. Biochem., 16 (1978) 579.
7 J.T. Slattery and G. Levy, Clin. Biochem., 12 (1979) 100.
8 D. Westerlund, A. Theodorsen and Y. Jaksch, J. Liquid Chromatogr., 2 (1979) 969.
9 R.A. Upton, J.N. Buskin, T.W. Guentert, R.L. Williams and S. Riegelman, J. Chromatogr., 190 (1980) 119.
10 R.F. Burgoyne, P.R. Brown and S.R. Kaplan, J. Liquid Chromatogr., 3 (1980) 101.
11 M. Broquaire, J. Chromatogr., 170 (1979) 43.
12 G. Boost, Arzneim.-Forsch., 25 (1975) 281.

Journal of Chromatography, 224 (1981) 51—58
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 851

SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF 9-(2-HYDROXYETHOXYMETHYL)GUANINE (ACYCLOVIR) IN HUMAN PLASMA AND URINE

G. LAND and A. BYE*

Wellcome Research Laboratories, Beckenham, Kent, BR3 3BS (Great Britain)

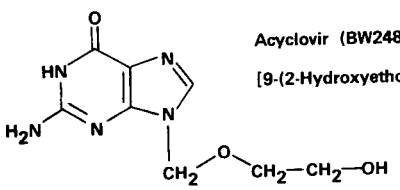
(First received November 11th, 1980; revised manuscript received January 20th, 1981)

SUMMARY

Acyclovir, a new antiviral drug for human use, could not be solvent extracted and was retained poorly by reversed-phase (RP) columns. Drug in urine and plasma (deproteinised) could be chromatographed successfully as an ion-pair (with heptanesulphonic acid) on RP-18 columns. The validated method had the required sensitivity for a wide variety of clinical situations including metabolic and pharmacokinetic studies.

INTRODUCTION

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] is a modified nucleoside which shows strong activity against viruses of the Herpes group [1]. Methods, other than microbiological, for the analysis of acyclovir have been based on radioimmunoassay (RIA) [2] or high-performance liquid chromatography (HPLC) utilising ion-exchange [1, 3] or reversed-phase columns [2]. Following



Acyclovir (BW248U)

[9-(2-Hydroxyethoxymethyl)-guanine]

administration of acyclovir to man it was found that the published HPLC methods lacked selectivity and sensitivity for the analysis of the drug in plasma and urine. Although RIA had the relevant sensitivity we encountered occasional problems in analysing plasma and urine from patients because of possible cross-reactivity with the antiserum. Continued development of RIA

may overcome this problem. As an alternative we looked at ways of improving the HPLC methods.

Selective extraction of acyclovir from plasma or urine was unsuccessful because of its poor lipid solubility. Attempts to couple counter-ions of good lipid solubility followed by extraction also failed. However, reversed-phase ion-pair HPLC met with success and proved superior to either reversed-phase or ion-exchange techniques for the measurement of unchanged drug. This method is reported below.

METHODS

Chemicals and reagents

Acyclovir was obtained from Wellcome Foundation (Dartford, Great Britain); heptanesulphonic acid, sodium salt, HPLC grade from Fisons (Loughborough, Great Britain); and sodium acetate, barium hydroxide and aluminium sulphate, AnalaR grade from BDH (Poole, Great Britain). All aqueous solutions were made up using deionised water (Deioniser type C810, Elgastat, High Wycombe, Great Britain).

Glassware

Centrifuge tubes, 10 ml graduated were purchased from M.S.E. (Crawley, Great Britain), and microvials with PTFE-lined caps from Waters Assoc. (Stockport, Great Britain).

Instrumentation

The chromatography was carried out on a Model SP8000 high-performance liquid chromatograph system using a SP8310 fixed-wavelength (254-nm) detector (Spectra-Physics, St. Albans, Great Britain). An autosampler (WISP-710A, Waters Assoc.) was also used. A refrigerated centrifuge (Mistral 2L, M.S.E.) fitted with an eight-place angle head was used to remove the suspended solids and precipitated proteins from the biological samples.

Determination of acyclovir in plasma

Standard solutions of acyclovir ranging from 0—50 μM were prepared by dilution of an aqueous stock solution (1 mM) with heparinised human plasma. An aliquot of standard or plasma (0.5 ml) was placed in a 10-ml centrifuge tube followed by 200 μl of a 5% w/v aluminium sulphate solution. The tube contents were vigorously mixed (Whirlimixer, Fisons) and a 400-μl aliquot of 0.15 M barium hydroxide was added followed by further mixing. The suspended solids were forced into a sediment by centrifugation (5000 g for 20 min at 10°C). The tubes were covered (Parafilm, American Can Company, Greenwich, CN, U.S.A.) and left to stand overnight at 4°C to aid precipitation. After uncovering, the tubes were again centrifuged (5000 g for 20 min at 10°C) and the clear supernatant was carefully transferred to a microvial which was capped in preparation for injection using the automatic sampler. Standards and samples were analysed in duplicate. The protein precipitation method was based on that of Gyure [4] which was shown to give good recovery of purines. The recovery of acyclovir by this method was shown to be greater than 90%.

Radioimmunoassay of acyclovir in plasma

The method of Quinn et al. [2] was used.

Determination of acyclovir in urine

Standard solutions of acyclovir with concentrations ranging from 0—500 μM were prepared by dilution of aqueous stock solution (1 mM) with filtered human urine. Samples were loaded into the autosampler and injected directly onto the liquid chromatograph. Because of the wide operating range of the urine assay dilution of unknown urine samples was rarely necessary.

HPLC conditions

A 250 mm × 4.6 mm I.D. column packed with 5-μm ODS-silica was used (e.g. Zorbax ODS, 4.6 μm; Du Pont, Hitchin, Great Britain). The mobile phase was an aqueous solution of 0.005 M sodium acetate and 0.0025 M heptanesulphonic acid, sodium salt, filtered before use through grade 50 filter paper (Whatman). The final pH was 6.5. Table I gives the optimum conditions for the HPLC of acyclovir in plasma and urine.

TABLE I

OPTIMUM CONDITIONS FOR HPLC OF ACYCLOVIR IN PLASMA AND URINE

The fixed-wavelength (254-nm) detector was operated at 0.005 a.u.f.s.

	Injection size (μl)	Mobile phase flow-rate (ml/min)	Column temperature (°C)	Minimum run time (min)
Plasma	60	1.2	50	15
Urine	15	1.5	35	35

RESULTS

In plasma extracts acyclovir was well resolved from the void volume and other endogenous peaks (see Fig. 1). No interference was found from the major acyclovir metabolite (9-carboxymethoxymethylguanine), guanine, thioguanine and related purines. In urine a co-eluting endogenous compound was seen in some subjects and made quantitation below 10 μM difficult in these subjects. This effect diminished at higher acyclovir concentrations. A long run time was necessary for urine extracts because of late eluting compounds present in urine under these conditions (see Fig. 2). An increase in the acyclovir peak area, and therefore sensitivity was achieved by larger injections. A plot of peak area (y) obtained at 0.005 a.u.f.s. in μV/sec, against the injection volume of a 10 μM solution of acyclovir (x) gave a linear calibration curve of the form; $y = 1324.8x - 7181$ ($r > 0.99$, $n = 11$ over 11 values of x). It was possible to inject 160 μl of a 10 μM acyclovir solution without affecting peak shape. Shorter run times were possible by using higher column temperatures. However, above 50°C for plasma and 35°C for urine, other endogenous peaks became unresolved from acyclovir making integration of peak areas difficult especially

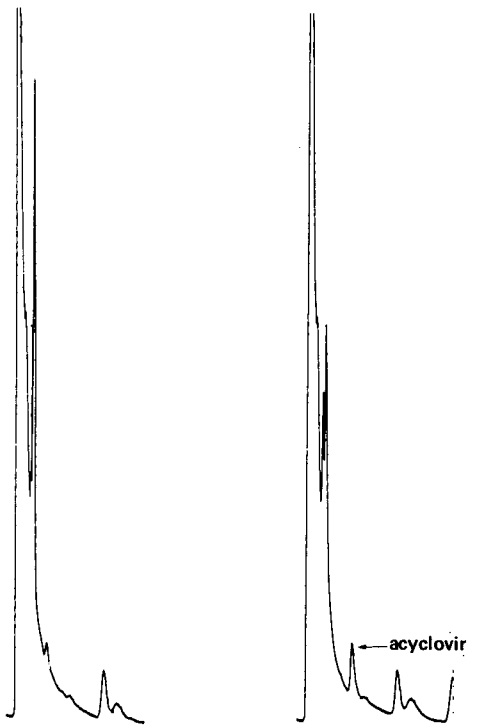


Fig. 1. Typical chromatograms from the plasma of a patient before and after receiving intravenous acyclovir. (a) Blank plasma; (b) plasma containing approximately 7 μM acyclovir.

at low concentrations. Under the described conditions acyclovir had retention times of about 364 and 1360 sec in plasma and urine, respectively.

Plasma validation

The HPLC method showed a linear response in plasma over the range 0—50 μM. A plot of peak height in mm (y) against acyclovir concentration in μM (x) gave a linear calibration curve of the form, $y = 1.9827x + 0.774$ ($r>0.99$, $n = 48$ over 8 values of x). Similar calibration curves were obtained whether peak height or peak area was used on the ordinate. Better precision was apparent at low acyclovir concentrations using peak height (Table II). However, when the percentage standard deviations from the peak height and peak area measurements were averaged, then compared by the t-test modified for inequality of variance, no significant difference was seen at the 5% level. No advantage on precision was obtained after adding an internal standard (oxipurinol). An unkown acyclovir peak height or area from the HPLC chromatogram was quantitated simply by reference to a standard calibration curve.

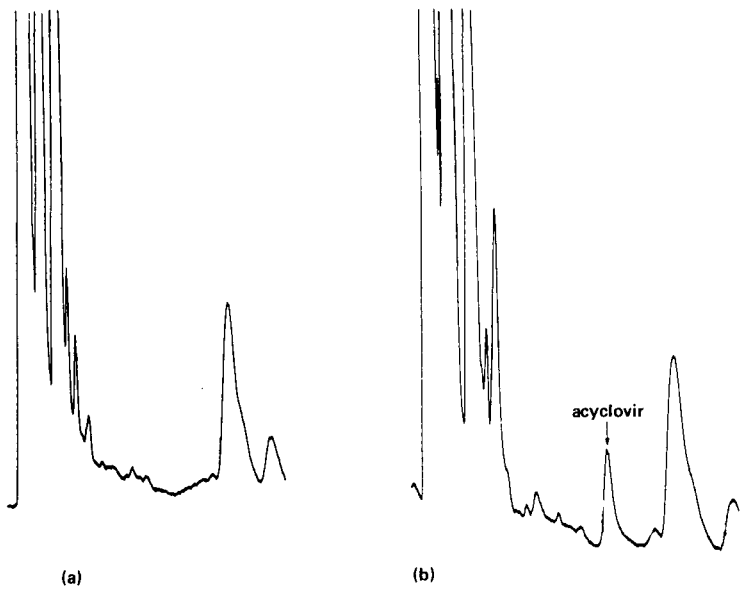


Fig. 2. Typical chromatograms of (a) blank urine and (b) urine containing 20 μM acyclovir.

TABLE II

COMPARISON OF THE ERRORS INVOLVED WHEN PEAK HEIGHT OR PEAK AREA WAS USED IN MEASURING ACYCLOVIR CONCENTRATIONS IN HUMAN PLASMA

Each observation was the mean of six determinations.

Concentration of acyclovir (μM)	Peak height (mm)	S.D. (%)	Peak area (μV/sec)	S.D. (%)
0	0	—	0	—
1	2.25	18.6	19,687	32.1
2	4.58	12.8	40,705	24.6
5	10.75	17.0	283,595	15.0
10	21.5	13.0	464,492	9.2
15	32.4	4.7	680,794	6.9
25	49.2	4.3	972,110	6.4
50	99.75	6.5	2,070,830	5.0

Urine validation

The HPLC method showed a linear response over the range 0—500 μM. However, when undiluted urine from some patients was injected an unknown co-eluting peak was seen on the HPLC chromatogram. This raised the lower reliable detection limit to 10 μM but if the peak was missing, quantitation to 1 μM was possible. A plot of peak height in mm (y) against acyclovir concentration in μM (x) gave a linear calibration curve of the form, $y = 0.15626x + 2.304$ ($r>0.99$, $n = 32$ over 8 values of x). As in the plasma validation no internal standard was needed and peak height or area could be used.

TABLE III

COMPARISON OF HPLC AND RIA FOR THE DETERMINATION OF ACYCLOVIR IN HUMAN PLASMA

In the RIA all samples above 15 μM were diluted 1:10 with the same human plasma as used for the zero so that only the most precise part of the calibration curve (1—10 μM) was used. No dilution was necessary for the HPLC method.

Actual concn. acyclovir (μM)	Concn. acyclovir by HPLC* (μM)			Mean (± S.D.)	Concn. acyclovir by RIA** (μM)			Mean (± S.D.)
0	0,	0,	0,	0	0,	0,	0,	0
	0,	0,	0		0,	0,	0	
2.8	2.605,	1.955,	2.12,	2.65	2.72,	2.52,	2.89,	2.705
	2.995,	3.42,	2.81	(0.55)	2.75,	2.65,	2.70	(0.12)
5.6	4.83,	4.30,	3.825,	4.92	5.03,	5.18,	5.19,	5.35
	4.37,	5.91,	6.26	(0.97)	5.70,	5.63,	5.39	(0.268)
14.0	12.63,	14.44,	13.535,	13.92	13.6,	13.4,	13.4,	13.53
	13.215,	14.29,	15.385	(0.99)	14.4,	13.1,	13.3	(0.45)
25.9	23.455,	23.71,	25.43,	23.90	24.1,	23.9,	25.9,	25.67
	23.49,	25.2,	28.29	(1.23)	28.6,	26.7,	24.8	(1.79)
42.0	38.945,	41.065,	41.24,	42.67	41.63,	41.3,	41.3,	41.37
	45.94,	42.68,	46.125	(2.89)	41.3,	42.5,	40.2	(0.74)
70.0	67.165,	64.29,	70.39,	71.95	69.0,	64.0,	74.0,	70.58
	72.86,	74.445,	82.56	(6.38)	77.0,	69.8,	69.7	(4.47)

*Mean of duplicates.
**Mean of triplicates.

Correlation of HPLC and RIA methods for plasma acyclovir

A set of seven standards was set up in six replicates over the range 0—70 μM acyclovir using plasma which was known not to cross-react in the RIA method. The standards were then analysed blind by both HPLC and RIA. At concentrations greater than 15 μM samples were diluted 1:10 with blank human plasma before RIA so that only the most precise part of the calibration curve was used. On completion the code was broken and the answers were subjected to statistical analysis. A good correlation was obtained ($r>0.99$, $n = 42$; see Table III). At low concentration the RIA had better precision than the HPLC method.

DISCUSSION

Using conventional reversed-phase columns acyclovir was poorly retained and only new, high-efficiency columns had sufficient resolution to allow quantitation. Ion-exchange columns could resolve acyclovir but the poor column efficiency and band broadening effects limited this method to high concentrations of acyclovir. The described ion-pair method combined the advantages of both high resolution and long column life from the previous methods to allow reliable quantitation of acyclovir down to 1 μM in plasma and 10 μM in urine. Greater sensitivity was possible with high-efficiency

columns but the absolute sensitivity was a function of the detector response and the molar extinction coefficient of acyclovir (13,600 at λ_{max} 250 nm). For most situations adequate sensitivity was achieved using the described conditions. The stability and low noise levels of the fixed-wavelength (254-nm) detector outweighed any advantages of using a variable-wavelength detector at λ_{max}. Including automatic injection, 90 samples of plasma or 40 samples of urine can be analysed each day by one technician. Normally a 0.5-ml sample of plasma was needed per determination, however this could be reduced if necessary. Urine can be injected directly into the HPLC so only a few microliters of sample are needed and a result can be available within 35 min from receipt of the sample.

RIA in general has high sensitivity but can suffer from problems of cross-reactivity and non-linear calibration curves. Fortunately the published RIA method currently utilises a fairly specific antibody, the only known major cross-reaction occurring with thioguanine. Minor cross-reactivity does occur but can be overcome either by allowing for the percentage of zero binding of the pre-drug sample or by setting up standards in the pre-drug sample. Where this was not possible the RIA had a tendency to measure apparent acyclovir in some specimens from people who had not yet received the drug. This effect was most noticeable in patients who were immunosuppressed with the drug Azathioprine (Imuran). Although this effect was insignificant at low acyclovir concentration large errors could occur at high concentration by virtue of the logarithmic nature of the calibration curve used. In the RIA, some problems were also experienced in getting a good precipitate after the addition of ammonium sulphate when the original plasma sample contained large amounts of fat. In the described HPLC method no acyclovir was detected in any plasma sample from subjects who had not received the drug, making the method more universally applicable. The HPLC method had a linear calibration curve and no internal standard was needed. Therefore relatively few standards need be run for comparable accuracy with RIA. Also the accurate linear dynamic range of the HPLC method is wider than the described RIA method which obviates the need for preliminary range finding. The HPLC and RIA methods were found to correlate well under optimum conditions and were both useful alternatives. In summary, the HPLC method described has sufficient sensitivity and reliability for the measurement of acyclovir in a wide variety of clinical situations and can also be used for pharmacokinetic and metabolism studies [5, 6].

ACKNOWLEDGEMENTS

We would like to thank Dr. de Miranda and Dr. Quinn for the supply of antibody and tritiated antigen.

REFERENCES

1 H.J. Schaeffer, L. Beauchamp, P. de Miranda, G.B. Elion, D.J. Bauer and P. Collins, Nature (London), 272 (1978) 583.
2 R.P. Quinn, P. de Miranda, S. Good and L. Gerald, Anal. Biochem., 98 (1979) 319.
3 G.B. Elion, P.A. Furman, J.A. Fyfe, P. de Miranda, L. Beauchamp and J.J. Schaeffer, Proc. Nat. Acad. Sci. U.S., 74 (1977) 5716.
4 W.L. Gyure, Anal. Biochem., 50 (1972) 309.

5 D. Brigden, A. Bye, A.S.E. Fowle and H. Rogers, J. Antimicrob. Chemother., 7 (1981) in press.
6 P. de Miranda, R.J. Whitley, M.R. Blum, R.E. Keeney, N. Barton, D.M. Cocchetto, S. Good, G.P. Hemstreet, L.E. Kirk, D.A. Page and G.B. Elion, Clin. Pharmacol. Ther., 26 (1979) 718.

Journal of Chromatography, 224 (1981) 59—66
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 837

ASSAY OF TRIMETHOPRIM, SULFADIAZINE AND ITS N^4-ACETYL METABOLITE IN BIOLOGICAL FLUIDS BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

V. ASCALONE

Pharmacokinetics Laboratory, Department of Clinical Investigation, Prodotti Roche S.p.A., Piazza Durante 11, 20131 Milan (Italy)

(First received September 22nd, 1980; revised manuscript received January 15th, 1981)

SUMMARY

A normal-phase high-performance liquid chromatographic method was developed to determine the concentration of timethoprim, sulfadiazine and its N^4-acetyl derivative in human serum and urine. The unchanged compounds and the metabolite are extracted in organic solvent by a single extraction. The method is accurate and sensitive and suited for pharmacokinetic studies in man.

INTRODUCTION

A combination of trimethoprim (TMP) and sulfadiazine (SDZ) is used as an antibacterial preparation for therapy in humans, following that of trimethoprim and sulfamethoxazole (SMZ), co-trimoxazole. SDZ distributes well into lung tissues, bronchial secretion and saliva [1] thus having a useful chemotherapeutic effect in respiratory tract infections [1]. The proportion of the sulfonamide (TMP/SDZ, ratio 1:3) has been reduced in comparison with co-trimoxazole where TMP/SMZ is in the ratio 1:5.

A number of analytical methods have been employed for TMP determination in biological fluids: microbiological [2], radiochemical [3] or spectrofluorimetric assay (most often used) [4], and thin-layer chromatography (TLC) with densitometry [5].

Recently gas—liquid chromatography (GLC) has been used for the determination of TMP [6] and for TMP, SMZ and the SMZ N^4-acetyl metabolite [7]. Both methods use a nitrogen—phosphorus detector. The most widely used assay for the determination of sulfonamides in biological fluids is a colorimetric assay based on the Bratton—Marshall reaction [8]. Rieder [9] has improved such a method in order to distinguish total sulfonamide (free

0378-4347/81/0000—0000/$02.50

+ N^4-acetyl metabolite) and the "bacteriostatically active fraction" (the free fraction). Other authors describe GLC determination with a flame ionisation detector [10].

Various authors have reported high-performance liquid chromatographic (HPLC) methods for sulfonamide determination in biological liquids [11, 12], for TMP [13], and for determination of both TMP and SMZ [14] by two different chromatographic assays. Recent papers [15, 16] have dealt with direct reversed-phase HPLC for the determination of TMP and SMZ in serum and plasma after protein precipitation, as well as for a mixture of sulfonamides [15], among them SDZ.

A recent paper describes the simultaneous HPLC determination of TMP, SMZ and its N^4-acetyl metabolite [17] after a single extraction from biological fluids. Working in reversed-phase under chromatographic conditions used for TMP and SMZ [17] we obtained a poor separation between SDZ and its metabolite which was not separated from TMP. Using a more polar eluent mixture (15% ethanol and 85% phosphate buffer, pH 4.5) the separation between substances was slightly improved, but TMP, the last to be eluted, showed a broadening tailing peak with a poor response in the UV detector (variation of the pH of the buffer in the range 4—7 did not improve the chromatographic behaviour of the drugs).

Instead, working in normal phase, under the conditions described in Experimental, a good separation between substances was achieved; TMP eluted first, and gave a high response in the UV detector with a symmetrically shaped peak. In this paper we describe a new method for normal-phase HPLC determination of the drugs and the sulfonamide metabolite in human serum and urine. The organic substances are extracted into ethyl acetate by a single and rapid liquid—liquid extraction at pH 6.8. The assay is performed in the presence of an internal standard, sulfadimethoxine (SDM).

EXPERIMENTAL

Chromatographic system

The determinations were carried out using the following chromatographic system: Altex Model 110 A solvent metering pump, back pressure up to 350 bar (Altex Scientific, Berkeley, CA, U.S.A.); UV—visible (200—850 nm) Kontron Uvicon 725 spectrophotometric detector with a cell volume of 8 μl and path length of 6 mm (Kontron, Zürich, Switzerland) operating at a wavelength of 289 nm with a sensitivity of 0.1 a.u.f.s.; Rheodyne Model 7120 sample injector with a 20-μl loop capacity (Rheodyne, Berkeley, CA, U.S.A.); Hibar® chromatographic column filled with LiChrosorb Si 60 (10 μm), stainless steel, 250 mm × 4 mm I.D. (E. Merck, Darmstadt, G.F.R.); precolumn filled with LiChroprep Si 60 (25—40 μm) (Merck). The detector was coupled through an interface to a chromatographic computer (Sigma 10 data system, Perkin-Elmer, Norwalk, CN, U.S.A.). All calculations were performed according to the "internal standard method".

The eluent was a mixture of dichloromethane—methanol—25% aqueous ammonia (80:19:1). The procedure was carried out at a constant flow-rate of 1.5 ml/min (about 50 bar).

Chromatograms were recorded on a Tarkan 600, W + W recorder, with an input of 10 mV (Kontron).

Under the above conditions the retention times of test substances are as follows: TMP, 2.6 min; SDM (internal standard), 3.7 min; SDZ, 6.7 min; SDZ N^4-acetyl metabolite, approx. 10.4 min (see Fig. 6).

Reagents and solvents

Dichloromethane and methanol (Lichrosolv), ethyl acetate (nanograde), and ammonia solution (25% Suprapur) were all from Merck. Phosphate buffer (pH 6.8, 1 *M*) of USP type prepared with KH_2PO_4 RPE-ACS was from Carlo Erba, Milan, Italy; water was double-distilled on glass. TMP, SDM, SDZ, and the N^4-acetyl SDZ derivative (all approx. 100% purity) were supplied by F. Hoffmann-La Roche, Basle, Switzerland.

Extraction from human serum and urine

A 1-ml volume of human serum is place in a screwcap test tube (for urine 1 ml is diluted with 5 or 10 ml of distilled water, and 1 ml of this solution is used at the start); 0.2 ml of phosphate buffer (1 *M*, pH 6.8) and 6 ml of ethyl acetate are added; the mixture is agitated for 3 min with a Vortex apparatus (Whirlimixer, Fisons, Loughborough, Great Britain) then centrifuged for 5 min at 2400 *g*, and 5 ml of supernatant are carefully collected. The organic extract is evaporated in a thermostated water-bath at 50 ± 1°C under a light stream of pure nitrogen. The residue is redissolved with 200 µl of

TABLE I

RECOVERY OF TMP, SDZ, AND SDZ N^4-ACETYL METABOLITE ADDED TO HUMAN URINE

Results are expressed as the mean of 5 determinations ± standard deviation, from 5 urine samples with the same nominal concentration. The urine was pooled from healthy volunteers who had taken no drugs for at least two weeks.

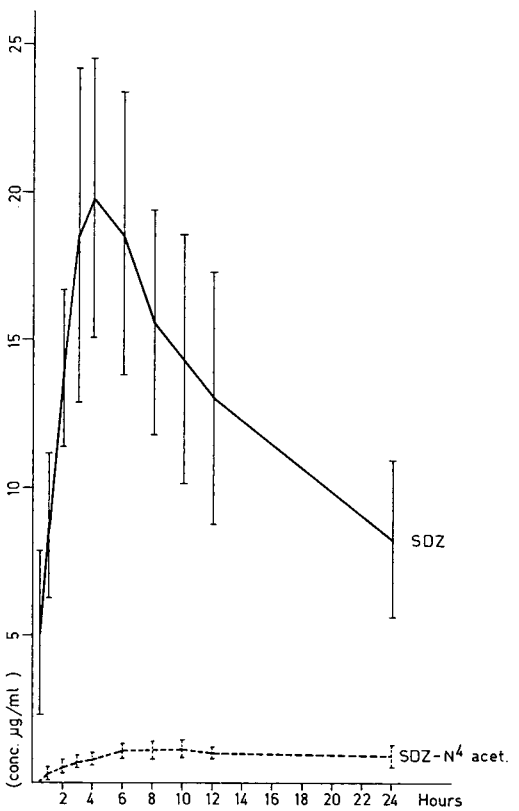
Compound	Quantity added (µg/ml)	Recovery (µg/ml)	Recovery (%)
TMP	45	44.8 ±0.9	99.5 ± 2
	40	39.73± 0.63	99.3 ± 1.6
	35	32.66±0.5	93.3 ± 1.5
	20	18.3 ±0.158	91.5 ± 0.86
SDZ	165	164 ±0.4	99.4 ± 2
	160	155 ±1.0	96.7 ± 0.64
	130	129.3 ±1.5	99.4 ± 1.16
	100	98.94±1.74	98.9 ± 1.75
N^4-acetyl SDZ	60	52.6 ±1.87	87.7 ± 3.5
	55	48.3 ±1.26	87.8 ± 2.6
	45	38.2 ±1.2	85 ± 3.1
	40	35.34±0.54	88.3 ± 1.5

eluent mixture containing internal standard (30 μg/ml). Parallel tests are run with a working standard solution obtained by adding the drugs to blank serum or urine organic extracts and with blank fluid sample.

RESULTS AND DISCUSSION

We have found a linear relationship between concentration of the test substances and the ratio of their peak areas to the internal standard peak area within the following concentration ranges: TMP, 0.03—10 μg/ml; SDZ, 0.1—200 μg/ml; N^4-acetyl SDZ derivative, 0.05—60 μg/ml. (This refers to a 1-ml serum specimen in all cases.) The minimum amounts detectable were about 0.03 μg/ml, 0.1 μg/ml and 0.05 μg/ml for TMP, SDZ and N^4-acetyl SDZ, respectively, working at 0.01 a.u.f.s. under the above conditions.

In chromatography of human serum extract we found a few times a small peak close to that of TMP, on its tail and partly overlapping; in such a case



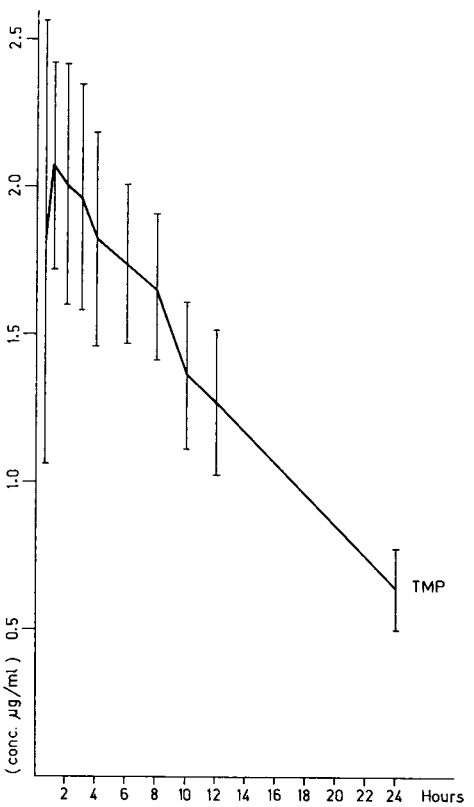


Fig. 1. Serum profile (average of 8 subjects) of free sulfadiazine (SDZ) and its N^4-acetyl metabolite (SDZ N^4-acet.) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

Fig. 2. Serum profile (average of 8 subjects) of trimethoprim (TMP) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

it is necessary in chromatographic calculations to use peak height rather than area.

The column shows no evidence of deterioration after about 1000 injections. Before a period of non-use (for example, a weekend) the column is treated first with 30 ml of dichloromethane—methanol—water (80:19:1), then with 30 ml of dichloromethane—methanol (90:10), 30 ml of dichloromethane and finally with 50 ml of *n*-hexane.

We have performed statistical recovery of drugs from biological fluid blanks to which concentrations simulating therapeutic and subtherapeutic amounts were added. The results are listed in Tables I and II.

TABLE II

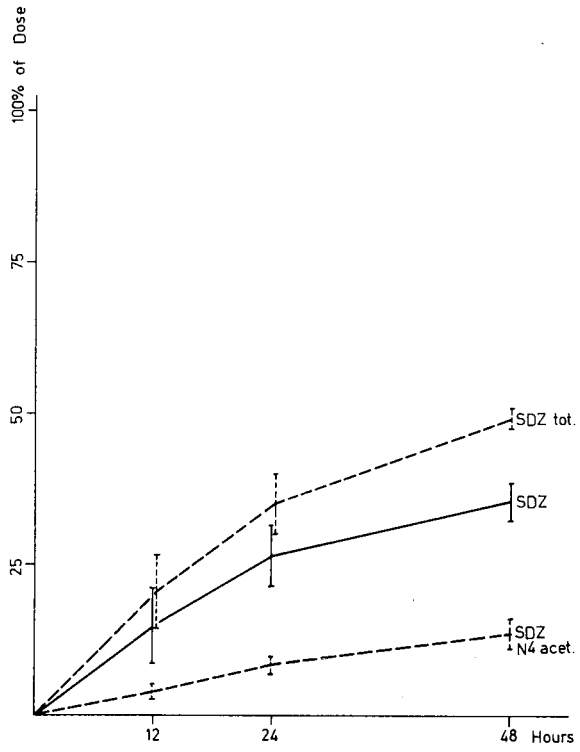
RECOVERY OF TMP, SDZ, AND SDZ N^4-ACETYL METABOLITE ADDED TO HUMAN SERUM

Results are expressed as the mean of 5 determinations ± standard deviation from 5 serum samples with the same nominal concentration. Other values represent the mean of two determinations. Serum was pooled from healthy volunteers who had taken no drugs for at least two weeks.

Compound	Quantity added (μg/ml)	Recovery (μg/ml)	Recovery (%)
TMP	0.2	0.17 ± 0.006	85 ± 3.5
	0.3	0.259 ± 0.008	86.7 ± 3.2
	1.0	0.975 ± 0.012	97.5 ± 1.2
	1.8	1.724	95.8
	2.0	1.850	92.5
	3.0	2.750	91.7
SDZ	3	2.9 ± 0.04	96.3 ± 1.4
	4	4.75 ± 0.07	95 ± 1.5
	10	9.88 ± 0.11	98.8 ± 1.1
	20	20	100
	30	29.5	98.3
N^4-acetyl SDZ	0.2	0.155 ± 0.007	77.5 ± 4.5
	0.5	0.395 ± 0.014	79.0 ± 3.7
	1.0	0.83 ± 0.02	83.0 ± 2.3
	2.0	1.64	82.0
	3.0	2.50	83.3

Clinical application of the method

The behaviour of SDZ and TMP in man can be described by a one-compartment model as also mentioned elsewhere [18]. Pharmacokinetic parameters obtained in a clinical study performed on healthy volunteers agree with those reported by other authors [18, 19]. Figs. 1—4 represent serum and urine excretion profiles of SDZ and TMP in man after oral administration of a solid (tablet) dosage combination.



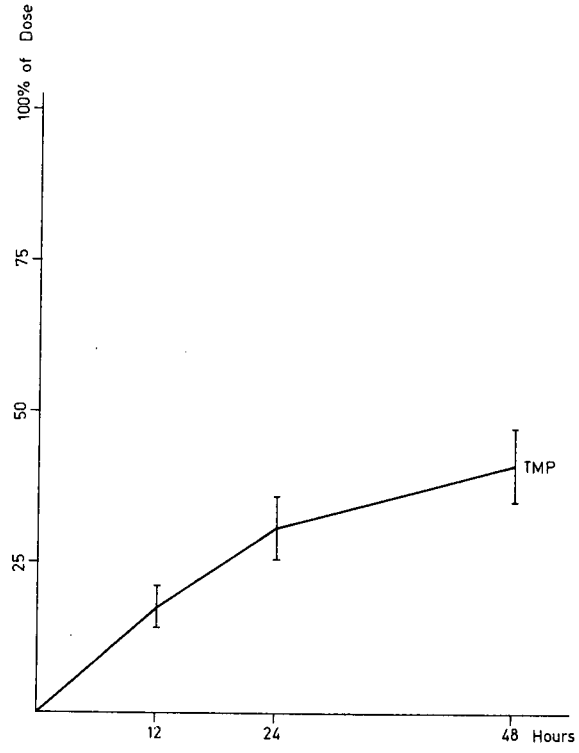
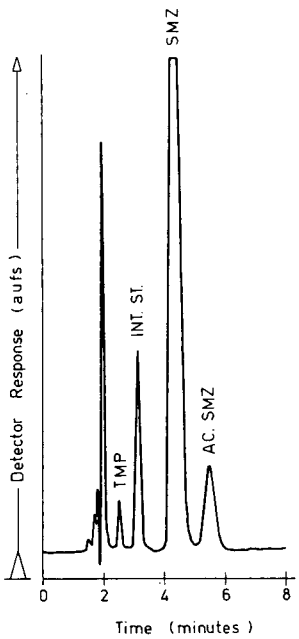


Fig. 3. Cumulative renal excretion profile (in percentage dose) of free sulfadiazine (SDZ) and total sulfadiazine (SDZ + N^4-acetyl metabolite) (average of 8 subjects) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

Fig. 4. Cumulative renal excretion profile (in percentage dose) of trimethoprim (TMP) (average of 8 subjects) after oral administration of two tablets (900 mg SDZ + 300 mg TMP).

The method described here has been successfully applied in the determination of plasma levels of sulfamethoxazole, its N^4-acetyl metabolite and trimethoprim, after oral administration of co-trimoxazole (Fig. 5). It is well suited too for the determination of drugs and metabolite plasma (and urine) levels in man, after administration of a drug combination of tetroxoprim (TXP) and sulfadiazine (Figs. 6 and 7).



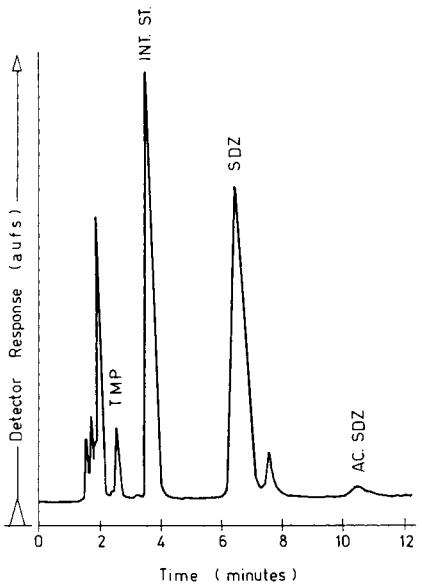


Fig. 5. Chromatogram of human plasma extract from a patient receiving two tablets of co-trimoxazole (Bactrim®; 160 mg TMP + 800 mg SMZ). First administration of a chronic treatment; plasma sample taken at 6 hours.

Fig. 6. Chromatogram of human serum extract from a volunteer receiving two tablets of TMP—SDZ combination (300 mg TMP + 900 mg SDZ; single oral administration, serum sample at 10 hours) (S = 0.1 a.u.f.s.).

Tetroxoprim [2,4-diamino-5-3′,5′-dimethoxy-4′-(β-methoxyethoxy)benzylpyrimidine] is a newly developed, highly active inhibitor of procaryontic dihydrofolate reductase, possessing marked antibacterial activity [20]. Another method has been reported [21] for the determination of TXP and SDZ in biological fluids by reversed-phase HPLC. However, the method requires two different types of liquid—liquid extraction for the drugs and different chromatographic conditions for each drug determination.

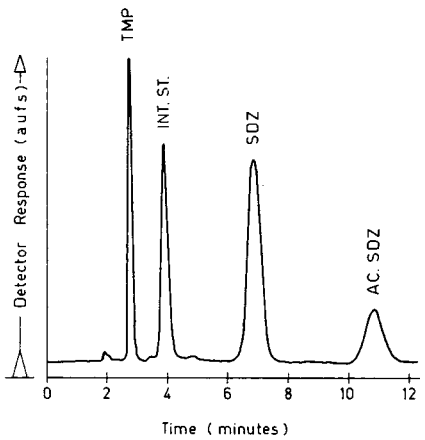


Fig. 7. Chromatogram of human urine extract (1 ml diluted to 10 ml) of a volunteer (same conditions as in Fig. 6). Urine fraction (400 ml) collected from 12 to 24 hours) (S = 0.1 a.u.f.s.).

ACKNOWLEDGEMENTS

The author wishes to thank Mr. G. Cappelletti for technical collaboration and Mrs. C. Marchi for typing the manuscript.

REFERENCES

1 Unpublished data sheet, Hoffmann-La Roche, Basle.
2 S.R.M. Bushby and G.H. Hitchings, Brit. J. Pharmacol., 33 (1968) 72.
3 D.E. Schwartz, W. Vetter and G. Englert, Arzneim.-Forsch., 20 (1970) 1867.
4 D.E. Schwartz, B.A. Koechlin and R.E. Weinfield, Chemotherapy, Suppl. 14 (1969) 22.
5 C.W. Sigel, M.E. Grace, C.A. Nichol and G.H. Hitchings, J. Pharm. Sci., 8 (1974) 1202.
6 G. Land, K. Dean and A. Bye, J. Chromatogr., 146 (1978) 143.
7 V. Ascalone, Boll. Chim. Farm., 117 (1978) 176.
8 A.C. Bratton and E.K. Marshall, J. Biol. Chem., 128 (1939) 537.
9 J. Rieder, Chemotherapy, 17 (1972) 1.
10 E. Röeder and W. Stuthe, Z. Anal. Chem., 271 (1974) 281.
11 K.L. Johnson, D.T. Jeter and R.C. Clairborne, J. Pharm. Sci., 64 (1975) 1657.
12 J.P. Sharma, E.G. Perkins and R.F. Bevill, J. Pharm. Sci., 65 (1976) 1606.
13 R.E. Weinfield and T.C. Macasieb, J. Chromatogr., 164 (1979) 73.
14 A. Bye and M.E. Brown, J. Chromatogr. Sci., 15 (1977) 365.
15 T.B. Vree, Y.A. Hekster, A.M. Baars, J.E. Damsa and E. van der Kleijn, J. Chromatogr., 146 (1978) 103.
16 R.W. Bury and M.L. Mashford, J. Chromatogr., 163 (1979) 114.
17 V. Ascalone, J. High Resolut. Chromatogr. Chromatogr. Commun., 3 (1980) 261.
18 P. Männisto, J. Tuomisto, N.E. Saris and T. Lehtinen, Chemotherapy, 19 (1973) 289.
19 F. Andreasen, L. Elsborg, S. Husted and O. Thomsen, Eur. J. Clin. Pharmacol., 14 (1978) 57.
20 H.S. Aschhoff and H. Vergin, J. Antimicrob. Chemother., 5 (Suppl. B) (1979) 19.
21 H. Vergin and G.B. Bishop, Arzneim.-Forsch., 30 (1980) 317.

Journal of Chromatography, 224 (1981) 67—93
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam -- Printed in The Netherlands

CHROMBIO. 852

SPECIFIC ASSAY FOR RADIOLABELLED DIGOXIN AND ITS KNOWN APOLAR METABOLITES IN BIOLOGICAL FLUIDS. I.

OTTHEINRICH EICHHORST and PETER H. HINDERLING*

Department of Pharmacology, University of Basle, Klingelbergstrasse 70, 4056 Basle (Switzerland)

(First received August 25th, 1980; revised manuscript received January 28th, 1981)

SUMMARY

A specific assay method for radiolabelled digoxin and its known apolar metabolites in plasma, urine and saliva was developed. The assay permits the delineation of the pharmacokinetics of digoxin and its metabolites after single-dose administration of the drug to humans. Column chromatographic and solvent extraction procedures were used for the separation of apolar and polar compounds. Thin-layer chromatography was applied for the individual and specific assessment of digoxin and its apolar metabolites. Apolar and polar standards were used for quantitative assessments of all the procedures used. Accuracy and precision of the assay developed were evaluated in plasma, urine and saliva using biological samples spiked with known amounts of standards and by measuring replicates of biological samples obtained from pharmacokinetic studies with digoxin administration to humans.

INTRODUCTION

A large number of methods have been described for the measurement of digoxin in various biological fluids in humans. These methods can be divided in two classes: biochemical and chromatographic assays. Among the biochemical methods there are immunoassays (radio- and enzyme immunoassay [1,2]), procedures based on competitive protein binding [3], inhibition of red cell ^{98}Rb uptake [4] and Na^+,K^+-ATPase [5]. Double isotope dilution derivative assay [6], gas chromatography [7] alone or in combination with mass spectroscopy [8], high-performance liquid chromatography [9], column chromatography and thin-layer chromatography (TLC) combined with extraction procedures [10,11] or mass spectroscopy [12] are the principal chromatographic methods. The biochemical methods are sensitive but not specific per se; the chromatographic methods are claimed to be specific [13]. Unfortunately, the respective sensitivities of the double isotope dilution derivative assay, the high-

0378-4347/81/0000—0000/$02.50

performance liquid chromatographic and gas chromatographic methods are so low as not to permit pharmacokinetic analyses of digoxin after single-dose administration. Column chromatographic and TLC methods could, however, produce both the necessary specifity and sensitivity if a radiolabelled drug is used. The column chromatographic and TLC procedures which have been reported to separate digoxin from its metabolites while possibly being quantitative do not allow assessment of the efficiency of extraction procedures [10, 11].

There is a definite need for pharmacokinetic investigations with digoxin in humans as a function of dose, formulation and route of administration using specific and sensitive methods of analysis. To our knowledge no published studies have been performed to demonstrate the linearity or otherwise of the kinetics of digoxin or of its metabolites [14]. There is conflicting evidence in the literature regarding the extent of metabolism of digoxin. A total of 80—90% of the amounts excreted in the urine was unchanged digoxin after oral administration of the drug in healthy subjects [15—17]. These results contrast with the results of another study, where equal amounts of digoxin and digoxin metabolites were excreted in the urine [18]. An average of 13% (range 1—47%) of the urinary glycosides was dihydrodigoxin assayed by a gas chromatographic method following multiple doses of the drug [19]. However, less than 1% of the urinary excreted total radioactivity represented dihydrodigoxin (assayed following column chromatography) after single-dose administration of radioactively labelled digoxin [15].

Qualitative and quantitative information on the presence of polar, water-soluble metabolites of digoxin is limited. In a recent investigation on the biotransformation of digoxin the polar metabolites were apparently disregarded [15]. In other studies these radioactive metabolites were determined in aqueous residues of urine following chloroform or ethyl acetate extractions in the first 24 h after oral administration of digoxin [20,21]. The proportions ranged between 16 and 20% (chloroform-extracted residue) and 3—30% (ethyl acetate-extracted residue) of the total urinary excretion of radioactivity [20, 21]. Polar, water-soluble metabolites were defined relative to the solvents used for separation in these studies.

Digoxin is a drug with a narrow therapeutic index with overlapping therapeutic and toxic plasma concentrations [22]. Some of its metabolites have been shown to be pharmacologically active in animals [23—25]. The delineation of the kinetics and the extent and type of metabolism of digoxin is crucial for clinical therapy.

We have now developed a specific and sensitive assay to enable measurement of the parent drug and metabolites in plasma, urine and saliva over adequate time periods after single-dose administration of labelled digoxin. The efficiencies of extraction and separation of all the individual steps have been quantified. Labelled apolar and polar standards have been employed to assess the recoveries. Polar metabolites have been separated relative to a known polar standard. The performance, accuracy and precision of the assay have been determined using biological samples spiked with known amounts of labelled standards and also with biological samples obtained from pharmacokinetic studies with administration of digoxin to humans.

EXPERIMENTAL

Material

The following labelled and unlabelled compounds were used and employed as standards: [12α-^{3}H]digoxin (D*), [12α-^{3}H]digoxigenin bisdigitoxoside (DB*), [12α-^{3}H]digoxigenin monodigitoxoside (DM*), digoxin (D), dihydrodigoxin (DH), digoxigenin bisdigitoxoside (DB), digoxigenin (DG), epidoxigenin (EDG), and digitoxin (DT) were donated by Boehringer, Mannheim, G.F.R.; [12α-^{3}H]digoxin-16′-glucuronide (DGL*), [12α-^{3}H]dihydrodigoxin (DH^*_A), [21,22-^{3}H]digitoxin (DT*), digoxin-16′-glucuronide (DGL) were donated by Beiersdorf, Hamburg, G.F.R.; [21,22-^{3}H]dihydrodigoxin (DH^*_B) was provided by Hoffmann-La Roche, Basle, Switzerland. The compounds with the label in the α-position were synthesized according to the method of Von Wartburg et al. [26] and had the following specific activities: D*, 1460 μCi/mg; DB*, 552 μCi/mg; DM*, 457 μCi/mg; DH*, 12772 μCi/mg; DGL*, 435 μCi/mg. DH^*_B was obtained as described elsewhere [27] and had a specific activity of 539 μCi/mg. DT* was synthesized according to the method of Haberland and Maerten [28] and had a specific activity of 539 μCi/mg.

Different TLC systems were set up for the separation of digoxin and its apolar metabolites (TLC systems A—C) and polar metabolites (TLC systems E and F). TLC system D was used for preliminary clean-up of coeluted biological material. The TLC systems were as follows. System A: chloroform developed on Kieselguhr (F_{254}, 5738, 200 μm, Merck, Darmstadt, G.F.R.) pretreated with ethylene glycol (10%, v/v) in acetone in pre-equilibrated chambers. System B: chloroform developed on Kieselguhr (F_{254}, 5738, Merck) pretreated with formamide (8%, v/v) in acetone in pre-equilibrated chambers. System C: dimethyl malonate—propionic acid (3:1) developed on silica gel ($Silicagel_{60}$, F_{254}, 5642, 250 μm, Merck). System D: chloroform—methanol (9:1) developed on silica gel ($Silicagel_{60}$, F_{254}, 5744, 500 μm, Merck). System E: chloroform—methanol (9:1) developed on silica gel ($Silicagel_{60}$, F_{254}, 5715, 250 μm, Merck) pretreated with ethylene glycol (10%, v/v). System F: methanol—water (1:1) developed on silanized silica gel ($Silicagel_{60}$, silanized, F_{254}, 5747, 250 μm, Merck). All the TLC systems except for system C were developed over the whole length of the plates (20 cm); system C was developed over 15 cm only.

The radiochemical purity of D*, DB*, DM*, DH^*_A, DH^*_B, DT* and DGL* was investigated with systems A, B, C, E and F. The results are listed in Table I. For TLC system A separable contaminants with their percentage of retained radioactivity are given in Table II. TLC system A separated DH and DB optimally from D; however, it could not differentiate between DM, DG and EDG. System B assayed D and all of its known apolar metabolites individually, with the exception of the isomers DG and EDG. The separation of DH from D in the latter system was clearly inferior to that of system A. The only advantage of system C was its selective separation of DM from DG and EDG.

DH has been claimed to be a major apolar digoxin metabolite [12,19]. Since optimum selectivity for DH is achieved in TLC system A, this system was employed in the routine analysis of biological samples obtained in the pharmacokinetic studies with D in humans.

The reproducibility of the separation of D and its apolar metabolites DH,

TABLE I

RADIOCHEMICAL PURITY OF LABELLED STANDARDS OF RECOVERED TOTAL RADIOACTIVITY IN TLC SYSTEMS A, B, C, E AND F

Compound	Percentage purity in TLC system				
	A	B	C	E	F
D*	94.8	94.5	97.8		
DH^*_A	100.0				
DH^*_B	97.5	97.2			
DB*	92.5	94.6	93.0		
DM*	97.8	98.4	95.9		
DT*	100.0			100.0	
DGL*				98.5	94.8

TABLE II

CONTAMINANTS OF LABELLED STANDARDS IN PERCENTAGE OF RECOVERED TOTAL RADIOACTIVITY IN TLC SYSTEM A

Compound	Contaminants			
	D*	DH*	DB*	(DM*+DG*+EDG*)
D*	94.8	1.88	2.39	0.92
DH^*_B	2.49	97.5	0	0
DB*	1.44	0.88	92.5	5.20
DM*	0.67	0	1.49	97.8

DB, DG and EDG was studied with system A. An ethanolic solution containing labelled and unlabelled species of the above compounds was spotted on Kieselguhr plates. After development and visualization the respective R_{DH} values for the different compounds relative to DH (R_{DH} = 1.0) were (n = 6): D, 0.85 ± 0.03; DB, 0.58 ± 0.06; DM, DG and EDG, 0.34 ± 0.03.

All organic solvents were of analytical grade.

A commercially available scintillation fluid (Instagel®, Packard-Becker, Groningen, The Netherlands) was used for the counting of radioactivity.

Instruments

The following were used: commercially available columns (Extrelut®, Fertigsäulen, Merck) for column chromatography, various plates for TLC as indicated above, and liquid scintillation spectrometers (Packard Tri-Carb Nos. 3280 and 3255, Packard Instruments, Downers Grove, IL, U.S.A.) for the measurement of radioactivity.

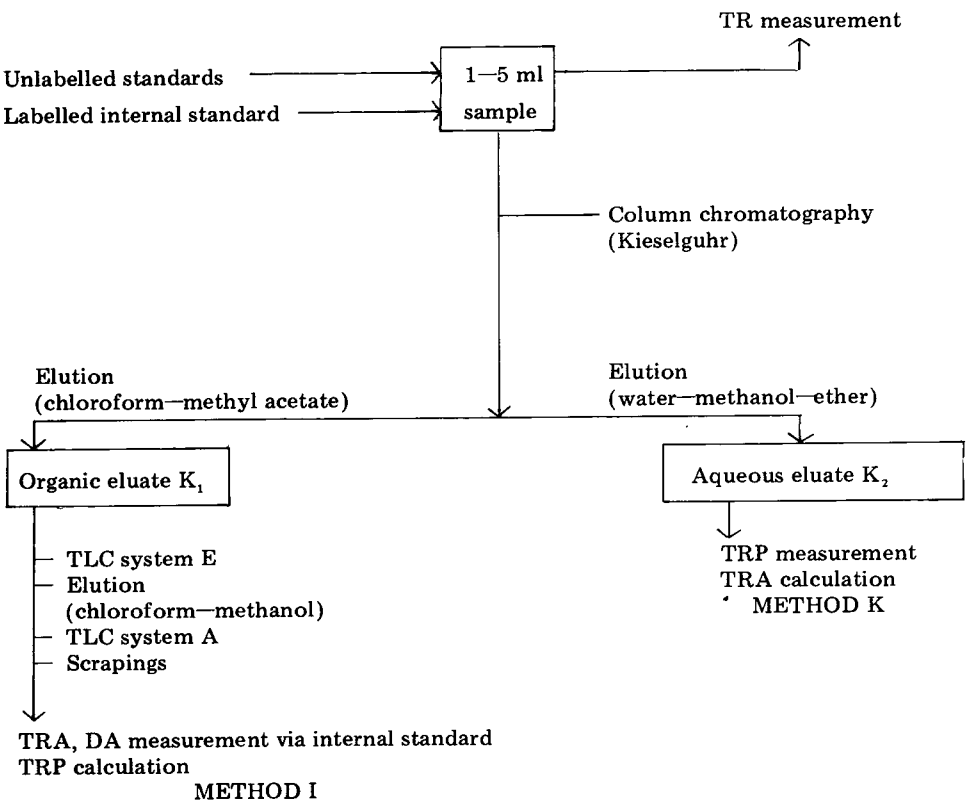
Liquid scintillation counting

Aliquots of biological fluids and their supernatants, eluates, extracts, residues and thin-layer scrapings were transferred into liquid scintillation vials. Eluates and extracts were concentrated to dryness. Then 3.5 ml of water and 10 ml of liquid scintillation fluid were added. After mixing the contents

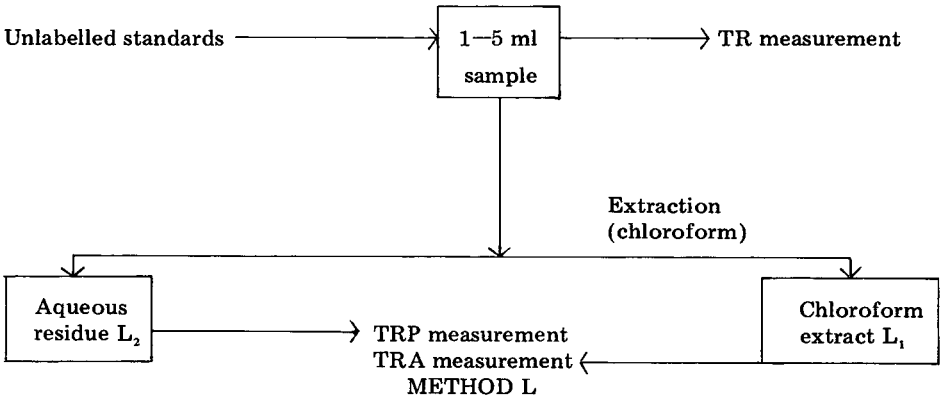
thoroughly, the vials were kept at 37°C for 2 h to enable thorough dissolution with liquid scintillation fluid. Subsequently the vials were kept at 4°C in the dark for several hours prior to counting. The activities measured were corrected for background and quenching using an external standard. Separate quench curves were determined for radioactivity measurements in biological fluids and thin-layer scrapings. Activities of less than twice the background were rejected.

METHODS AND RESULTS

The plus and minus values for mean values in the text refer to the standard deviation (S.D.) of such means; n is the number of experiments performed. The aim of this study was to establish a specific and sensitive assay for (1) labelled D*, (2) its known individual apolar metabolites including DH*, and (3) its (as yet unknown) polar metabolites. The strategy in setting up this assay was to develop three independent methods [methods I, K, and L (Schemes 1 and 2)]: a column chromatographic method (= method K) for the measurement of polar total radioactivity in biological fluids; a solvent extraction method (= method L) for the determination of apolar and polar total radioactivity; a third method (= method I) for the assay of apolar total and individual radioactivity. Method I



Scheme 1. Specific assay for labelled digoxin and metabolites in biological fluids: Flow diagram of methods I and K.



Scheme 2. Specific assay for labelled digoxin and metabolites in biological fluids: Flow diagram of method L.

applied the column chromatographic procedure of method K for separation of apolar and polar total radioactivity followed by specific separation of D, DH, DB and (DM+DG+EDG) by TLC system A. In method I use of DT* as internal standard compensated for losses during the procedure.

Use of unlabelled standards

To ensure constant recoveries, 50 μg unlabelled D, DH, DB, DM, DG, EDG, DGL and DT were routinely added to all the samples prior to processing. Addition of unlabelled standards was also necessary for visualization of the spots on the TLC plates after development. Thus 500 μl of an ethanol—chloroform (1:1) solution containing the standards in a concentration of 100 μg/ml each were placed in glass tubes and subsequently evaporated. Aliquots of the biological samples to be processed were then added.

Measurement of total radioactivity in plasma, urine and saliva

Duplicates of 100—500 μl of plasma, 100 μl of urine and 100—300 μl of pre-centrifuged saliva were assayed and the means used for calculation of the respective concentrations. Saliva samples were centrifuged at 300 *g* for 10 min prior to assay. Radioactivity was subsequently measured in the clear supernatant devoid of mucus. In preliminary experiments fresh saliva was spiked with known amounts of D* and after centrifugation the percentage recovery of the apolar standard, D*, in the supernatant, $r^{s'}_{D*}$, was determined: $r^{s'}_{D*} = 86.6 \pm 3.8\%$ ($n = 4$). Total radioactivity in saliva, TR^s, was then calculated from total radioactivity in saliva supernatant, $TR^{s'}$, on the assumption that the recovery of total radioactivity in saliva supernatant, $r^{s'}_{TR}$, was similar to that of D*, i.e. $r^{s'}_{TR} \approx r^{s'}_{D*}$.

Method I

In this procedure DT* was used as an internal standard, to measure the respective apolar total and individual radioactivities in biological fluids, TRA^{bf} and DA^{*bf} [$= D^{*bf} + DH^{*bf} + (DM^{*}+DG^{*}+EDG^{*})^{bf}$]. Polar total radioactiv-

ity in biological fluids, TRP^{bf}, was a calculated value with method I and was obtained from the difference between the experimentally measured total radioactivity in a sample, TR^{bf}, and its total apolar radioactivity, TRA^{bf}.

DT* dissolved in the corresponding biological fluid was added as internal standard to aliquots of 1 or 5 ml of plasma or 1 ml of urine or saliva. DT* radioactivity approximated the inherent total radioactivity of the sample. At the same time an aliquot of DT* as internal standard was transferred to a liquid scintillation vial for measurement of its radioactivity. The biological samples were then diluted with water to total volumes of 20 ml and placed on the Kieselguhr columns according to method K as described below. Eluate 1 containing the apolar D*, DH^*_B, DB*, DM* and DT* was evaporated under vacuum at 40°C and redissolved in 0.5 ml of chloroform—ethanol (1:1). Aliquots of 200—500 µl of this fraction were added in bands of 6 cm on preparative silica gel plates and developed in TLC system D. This was effected by means of pipettes ("Konstriktionspipetten", Becton-Dickinson, Rutherford, NJ, U.S.A.), which had specially prepared polyethylene tubings set over their tips. This first TLC step proved to be necessary for the definitive elimination of coeluted biological material which hindered an effective TLC separation of D and its apolar metabolites (DH, DB, DM, DG, EDG). The silica gel plates of system D were developed as described above.

For reversible visualization of the TLC area containing the glycosides the plates were sprayed with a 1% ethanolic solution of iodine for 30 sec. In preliminary experiments it was shown that the 1% ethanolic solution of iodine did not induce degradation of the standards with loss of the label. Two clearly separated areas were apparent on the plates: one containing the glycosides, the other eluted biological contaminants. The latter part of the plate was cut out and the remaining part was then subjected to an elution procedure for 24 h. Slices (10 cm) of filter paper were used: one end was attached to the upper part of the plate by clamps, the other end reached into a sink containing a solution of chloroform—methanol (1:1). In preliminary experiments the efficiency of this elution procedure was checked at different periods of time. Elution was considered to be complete when spraying with the 1% ethanolic solution of iodine did not reveal any visible spots on the plates. This was accomplished in less than 24 h. The eluates thus obtained were then evaporated under vacuum at 40°C and redissolved in 0.5 ml of chloroform—ethanol (1:1). Aliquots (20—500 µl) of this solution were added to Kieselguhr plates by means of Lambda pipettes (Clay Adams Co., New York, NY, U.S.A.) or added as bands by means of pipettes equipped with specially prepared polyethylene tubings. The plates of system A were then developed as described above. After development the plate areas adjacent to the solvent front were first scraped off. These areas contained the internal standard DT*. When aliquots of 500 µl had been added as bands, rectangular sections (2.5 × 10 cm) were scraped off for radioactivity determination; when aliquots of 20—50 µl had been added as spots, squares of 2.5 × 2.5 cm were scraped off for radioactivity determination. The parts of the plates containing the unlabelled and labelled D, DH, DB, (DM+DG+EDG) and DGL were then heated at 110°C for 5 min and the spots were visualized by spraying the plates with 1% ethanolic iodine solution. Removal of the internal standard DT* from the plates prior to the visualization

procedure proved to be necessary. Preliminary experiments had shown that the DT* employed was thermolabile (contrary to all the other labelled standards used). In other experiments it was demonstrated that the scraped areas contained reproducibly all the DT* present on the plates. In further experiments the relative sizes of the spots of the labelled and unlabelled species of the standard compounds D, DH, DB, (DM+DG+EDG) and DGL were compared. Systematic measurements of the radioactivity from 1-mm scrapings showed that the areas which contained the labelled species exceeded clearly those that contained the visualized fraction of the unlabelled species. The visualized spots and bands were expanded, respectively, to 2 × 2 cm squares and 10 × 2 cm rectangular sections. Such scrapings yielded radioactivity peaks for the individual standard compounds similar to those obtained from the scrapings of 1-mm bands. In the routine procedure the visualized spots and bands were expanded as described and marked accordingly. Prior to scraping the plates were heated again for 2 min at 110°C to eliminate remaining traces of iodine, since iodine was shown to be a potent quencher of radioactivity. The scrapings (2-mm bands) were then individually transferred to liquid scintillation vials and their radioactivity counted. The radioactive distribution pattern was plotted by a computer plotter programmed to give the total radioactivity scraped from the plates, the number of radioactivity peaks, their R_F values and the percentage of radioactivity in each peak.

The accuracy and precision of method I were tested by spiking plasma, urine and saliva with a solution containing known relative radioactivities of D* (59.5%), DH^*_B (6.22%), DB* (7.61%), DM* (6.14%) and DLG* (20.51%). Similar percentage radioactivities of the individual compounds were expected to occur in biological samples after administration of digoxin to humans [20,21]. The internal standard, DT*, had a radioactivity equal to the total radioactivity of the labelled digoxin derivatives. Aliquots of fresh plasma (1 and 5 ml), urine (1 ml) and saliva (1 and 5 ml) were spiked, subsequently assayed by method I as described above and the recoveries for the individual apolar radioactivity, DA^{*bf}_I, total apolar radioactivity, TRA^{bf}_I, and total polar radioactivity, TRP^{bf}_I, determined (see Appendix). Percentage recoveries (accuracies) and deviations (precisions) for DA^{*bf}_I, TRA^{bf}_I and TRP^{bf}_I as obtained by method I in plasma, urine and saliva are listed in Table III.

The percentage recoveries of the experimentally measured DA^{*bf}_I and TRA^{bf}_I, $r^{bf}_{I,DA*}$ and $r^{bf}_{I,TRA}$, were, except for $r^{bf}_{I,DH*}$, in the range 91—113% in all the biological fluids tested (Table III). $r^{p}_{I,DH*}$ and $r^{u}_{I,DH*}$ deviated more and were 55% and 119%, respectively. The percentage recoveries of the calculated TRP^{bf}_I, $r^{bf}_{I,TRP}$, differed more from 100% from those of the experimentally measured TRA^{bf}_I, $r^{bf}_{I,TRA}$ (Table III). It was concluded that method I was most adequate for measuring DA^{*bf}_I and TRA^{bf}_I reliably with reservations regarding DH*. The results obtained for TRP^{bf}_I suggested that these calculated values were less reliable and more biased.

Methods K and L

Methods K and L were set up for a direct measurement of polar total radioactivity in biological fluids, TRP^{bf}. Apolar total radioactivity in biological fluids, TRA^{bf}, was a measured value with method L and a calculated value with

method K. TRA_K^{bf} was obtained from the difference between the experimentally measured total radioactivity in a sample, TR^{bf}, and its polar total radioactivity, TRP_K^{bf}, with method K.

The values for TRP^{bf} and consequently for TRA^{bf} (so obtained) respectively by methods K and L were not expected to be identical, since the separation procedure of the two methods differs.

Individual apolar radioactivity, DA^{*bf}, could only be assessed by methods K and L if the relative radioactivities for D*, DH*, DB* and (DM+DG+EDG)* simultaneously obtained by method I were used.

Method K

Columns (8 × 2.5 cm) were packed with Kieselguhr of 0.5 mm particle diameter (Extrelut®, Fertigsäulen, Merck). Aliquots of 1 ml of urine and saliva and 1—5 ml of plasma were diluted with water to total volumes of 20 ml and then immediately applied on the columns. The first elution was carried out with 40 ml of chloroform—methyl acetate (1:4) (flow-rate, 0.4 ml/min); 25 ml of eluate 1 containing the apolar digoxin and metabolites were obtained and processed as described above (method I). The second elution obtained on passage of 40 ml of water—methanol—diethyl ether (1:1:1) yielded eluate 2 containing the polar metabolites (flow-rate, 0.2 ml/min). Eluate 2 was then concentrated to dryness by evaporation at 40°C under vacuum, redissolved in 3 ml of methanol and transferred to a liquid scintillation vial. The radioactivity was measured after concentrating it to dryness under a stream of air.

In preliminary experiments the separation and elution efficiency of method K was tested with the apolar and polar standards D* and DGL*, respectively. Aliquots of 1, 2, 3 and 5 ml of plasma and 1 ml of urine were spiked with known amounts of D* and DGL* in separate experiments, and the recoveries determined (see Appendix). The percentage recovery of D* in eluate 2 for 1, 2, 3 and 5 ml of plasma was 2.2 ± 0.40% (n = 6) and was volume-independent. The percentage recovery of DGL* for 1, 2, 3 and 5 ml plasma was 83.9%, 81.0%, 69.3% and 56.8%, respectively, and was volume-dependent (two-tailed paired t-test: t (0.05) = 2.02, t_{cal} = 13.64). The percentage recovery of D* and DGL* in eluate 2 of urine, was 2.3% and 89.5%, respectively. These results indicate that separation of apolar and polar total radioactivity in eluate 2 was not complete and that the measured activity represented total radioactivity, $TR^{K_2,bf}$.

The accuracy and precision of method K were tested by spiking aliquots of plasma, urine and saliva with a solution containing known amounts and radioactivities of D*, DH_B^*, DB*, DM* and DGL* as described above (see method I). Samples of fresh plasma (1 and 5 ml), urine (1 ml) and saliva (1 and 5 ml) were spiked with these standards, carried through method K as described above, and the recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} were then determined. DA^{*bf} was assessed by combining data obtained by methods I and K (see Appendix). The percentage recoveries (accuracies) and deviations (precisions) for TRP_K^{bf}, TRA_K^{bf} and $DA^{*bf}_{I/K}$ as obtained by method K in plasma, urine and saliva are listed in Table IV.

The percentage recoveries of the experimentally measured TRP_K^{bf}, $r_{K,TRP}^{bf}$, ranged between 84 and 116% in the three biological fluids studied (Table IV).

TABLE III

DETERMINATION OF PERCENTAGE RECOVERIES OF APOLAR TOTAL AND INDIVIDUAL RADIOACTIVITY AND OF POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES BY METHOD I

Plasma, urine and saliva samples were spiked with known amounts and relative radioactivities of D*, DH_B^*, DB*, DM* and DGL*.

Biological fluid	Volume (ml)	$r^{bf}_{I,TRA}$		$r^{bf}_{I,D*}$		$r^{bf}_{I,DH*}$		$r^{bf}_{I,DB*}$		$r^{bf}_{I,DM*}$		$r^{bf}_{I,TRA}$		n
		Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	
Plasma	1/5	95.4	1.61	99.5	2.08	54.9	1.45	93.5	1.50	99.2	1.29	118.5	6.68	4
Urine	1	112.1	3.91	113.0	3.36	118.7	6.01	103.1	3.98	106.9	7.96	53.3	15.1	4
Saliva	1/5	97.7	1.70	98.2	1.36	103.2	6.34	91.4	2.70	94.9	2.68	109.1	6.58	4

TABLE IV

DETERMINATION OF PERCENTAGE RECOVERIES OF APOLAR AND POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES BY METHOD K AND OF APOLAR INDIVIDUAL RADIOACTIVITY BY METHODS I AND K COMBINED

Plasma, urine and saliva samples were spiked with known amounts and relative radioactivities of D*, DH_B^*, DB*, DM* and DGL*.

Biological fluid	Volume (ml)	$r^{bf}_{K,TRA}$		$r^{bf}_{I/K,D*}$		$r^{bf}_{I/K,DH*}$		$r^{bf}_{I/K,DB*}$		$r^{bf}_{I/K,DM*}$		$r^{bf}_{K,TRP}$		n
		Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	
Plasma	1	96.0	1.06	100.5	1.07	53.8	0.29	92.1	1.03	100.0	1.81	115.6	4.12	2
	5	100.1	0.85	104.3	0.23	59.3	1.00	100.4	2.10	104.3	2.49	99.6	3.31	2
Urine	1	102.5	0.54	103.4	0.88	108.6	2.19	94.3	0.89	97.7	7.58	90.3	2.09	4
Saliva	1	104.2	0.87	104.7	0.87	110.0	5.86	97.4	1.98	101.2	2.99	83.9	3.38	4

The percentage recoveries of the calculated TRA_K^{bf} and $DA^*_{I/K}{}^{bf}$, $r^{bf}_{K,TRA}$ and $r^{bf}_{I/K,DA^*}$ respectively, were even closer to 100% and were (except for $r^{bf}_{I/K,DH^*}$) in the range 92—105% (Table IV). The precision of method K for TRP^{bf} and TRA^{bf} appeared to be superior to that of method I. It was concluded that method K was suitable for assessment of TRP^{bf} and TRA^{bf}.

Method L

A volume of 1 ml of plasma, urine or saliva was diluted with water to a total volume of 5 ml and then extracted three times with chloroform. Plasma was extracted with five times the volume of chloroform and urine and saliva with three times the volume of chloroform. These volume ratios were adequate for the clear separation of the organic and aqueous phases with minimal emulsion. The chloroform extracts were then combined and 1- or 2-ml aliquots were transferred to liquid scintillation vials. The radioactivity was determined after concentration under a stream of air to dryness. Aliquots of 1—3 ml of aqueous residues were transferred to liquid scintillation vials and the radioactivity determined after the remaining chloroform had been eliminated.

In preliminary experiments the extraction efficiency of method L was tested with the apolar and polar standards D* and DGL*, respectively. Aliquots of 1 ml of plasma or urine were spiked with known amounts of D* and DGL* in separate experiments and the respective recoveries determined (see Appendix).

The percentage recoveries were: in plasma for D* (n = 2), in eluate 1 = 98.9, 97.1%, in eluate 2 = 1.1, 2.9%; and for DGL* (n = 2) in eluate 1 = 2.5, 3.3% and eluate 2 = 97.5, 97.1%. The corresponding percentage recovery values for D* in urine were (n = 2), in eluate 1 = 98.9, 98.8%, in eluate 2 = 1.1, 1.2%; and for DGL* (n = 2), in eluate 1 = 2.8, 2.5%, and in eluate 2 = 97.2, 97.5%.

The extraction efficiency of D* and DGL* in saliva with method L was not determined and was assumed to be identical to that in urine.

The accuracy and precision of method L were tested by spiking plasma, urine and saliva with a solution containing known amounts and relative radioactivities of D*, DH^*_B, DB*, DM* and DGL*, as described above (see method I). Samples of fresh plasma (1 and 5 ml), urine (1 ml) and saliva (1 and 5 ml) were spiked with those standards, carried through the procedure of method L as described above, and the recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} were then determined. The extraction efficiency obtained for D* and DGL* in preliminary experiments showed that the measured radioactivities in both the aqueous residue, L_2, and the organic extract, L_1, of a biological sample represented total radioactivity, $TR^{L_2,bf}$ and $TR^{L_1,bf}$, respectively. DA^{*bf} was assessed by combining data obtained by methods I and L (see Appendix).

Percentage recoveries (accuracies) and deviations (precisions) for TRP^{bf}_L and TRA^{bf}_L in the biological samples as obtained by method L in plasma, urine and saliva are listed in Table V. Percentage recoveries for $DA^*_{I/L}{}^{bf}$ are also included.

The respective percentage (accuracies) of the experimentally measured TRP^{bf} and TRA^{bf}, $r^{bf}_{L,TRP}$ and $r^{bf}_{L,TRA}$, were close to 100% and ranged between 99 and 105% in all three biological fluids studied (Table V). The percentage recoveries of DA* (except for DH*) ranged between 92 and 101%. The precision of TRA^{bf} assessments by method L appeared to be similar to that by

TABLE V

DETERMINATION OF PERCENTAGE RECOVERIES OF APOLAR AND POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES BY METHOD L AND OF APOLAR INDIVIDUAL RADIOACTIVITY BY METHODS I AND L COMBINED

Plasma, urine and saliva samples were spiked with known amounts and relative radioactivities of D*, DH^*_B, DB*, DM* and DGL*.

Biological fluid	Volume (ml)	$r^{bf}_{L,TRA}$		$r^{bf}_{I/L,D*}$		$r^{bf}_{I/L,DH*}$		$r^{bf}_{I/L,DB*}$		$r^{bf}_{I/L,DM*}$		$r^{bf}_{L,TRP}$		n
		Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	
Plasma	1	99.5	2.06	98.6	0.48	54.4	1.75	100.2	2.56	100.4	0.70	101.9	7.89	2
Urine	1	100.2	3.07	101.1	0.64	106.1	2.03	92.2	1.23	95.5	4.10	99.2	1.29	2
Saliva	1	98.8	1.30	99.2	0.65	104.3	5.11	92.5	1.06	95.9	2.49	104.8	5.03	2

TABLE VI

PRECISION* OF METHODS I, K AND L ASSESSED FROM REPLICATE MEASUREMENTS OF APOLAR AND POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES OBTAINED FROM A PHARMACOKINETIC STUDY WITH DIGOXIN ADMINISTRATION TO HUMANS

Median, (range), and number of samples with replicate measurements (n) are given.

Biological fluid	TR^{bf}	TRA^{bf}			TRP^{bf}			D^{*bf}	DH^{*bf}	DB^{*bf}	DM^{*bf}**
		I	K	L	I	K	L	I***			
Plasma	1.4 (0.3—12) n=17	2.7 (1.1—16)	4.5 (0.1—52) n=17	17 (0.5—15)	140 (0.1—140)	79 (4.2—140) n=17	13 (0.5—65)	0.5 (0.1—5.4)	15 (1.1—71)	5.2 (0.1—67) n=16	20 (0.1—110)
Urine	2.4 (0.4—10) n=13	2.4 (0.1—6.8) n=13	1.9 (0.1—3.5) n=7	0.6 (0.3—1.1) n=7	66 (0.1—140) n=13	21 (0.1—140) n=7	5.4 (1.7—67) n=7	0.1 (0.1—0.7)	10 (1.3—47)	7.1 (0.1—20) n=13	13 (0.1—50)

*Precision is defined as percentage standard deviation about the mean of replicate measurements.

**DM^{*bf} = $(DM+DG+EDG)^{*bf}$.

***Estimated from replicate measurements of $DA^{*I,bf}/TRA^{I,bf}$.

methods I and K. The precision of TRP^{bf} estimates by method L was closer to that by method K (Table V). It was concluded that method L was adequate for assessments of TRP^{bf} and TRA^{bf}.

Stability and radiolysis

Four plasma and urine aliquots (20 ml) were spiked with known amounts of D*. The D* used had the known percentage impurities [DH* (1.9%), DB* (2.4%), (DM+DG+EDG)* (0.9%)] previously determined with TLC system A. The spiked plasma and urine aliquots were deep-frozen and kept at −20°C for 1—10 months. The aliquots underwent different treatments. The first pair of plasma and urine aliquots was thawed and subsequently refrozen four times at 1, 4, 7 and 10 months after spiking. The second pair of aliquots was thawed and refrozen three times at 1, 4 and 7 months after spiking. The third pair of aliquots was thawed and refrozen twice at 1 and 4 months after spiking, and the fourth pair was thawed after it had been kept frozen for 1 month. After thawing all the aliquots were kept at room temperature for 8 h and samples were then taken. They were processed according to method K.

The percentages of recovered total radioactivity, TR^{bf}, which really represented total apolar radioactivity, TRA^{bf}, were time-independent and constant and were on the average for plasma and urine 97.9 ± 5.1% (n = 10) and 96.2 ± 4.9% (n = 11), respectively. These values agreed with the respective 97.8% and 97.7% which optimally could be expected on the basis of the data of preliminary experiments in which plasma and urine aliquots had been spiked with D* and its recovery had been determined in eluate K_2. Of the recovered TRA^{bf} constantly 95.3 ± 0.46% (n = 10) was D*, 1.4 ± 0.36% (n = 10) was DH*, 2.4 ± 0.15% (n = 10) was DB* and 0.96 ± 0.14% (n = 10) was (DM+DG +EDG)* in plasma. In urine the corresponding figures were: 94.5 ± 0.76% (n = 11) was D*, 2.7 ± 0.20% was DH*, 2.4 ± 0.09% (n = 11) was DB* and 0.82 ± 0.09% was (DM+DG+EDG)*. It was concluded that the tested compounds were stable for at least ten months at −20°C including repeated thawing and exposure to ambient temperature.

Identical studies were performed with DGL* in plasma and urine to investigate a possible deconjugation of this compound. The DGL* used had a known percentage impurity (D* 1.5%, 5.2%) previously determined with TLC systems E and F. TR^{bf} was determined in eluates K_1 and K_2 according to method K (see Scheme 1) except that eluate K_1 was obtained by using 40 ml of methyl acetate instead of chloroform—methyl acetate (1:4). The percentages of recovered TR^{bf} in the experiments with plasma and urine were on the average 102.2 ± 4.5% (n = 10) and 101.7 ± 2.8 (n = 10) respectively. 2.2 ± 0.38% (n = 10) and 2.5 ± 0.79% (n = 10) respectively of TR^{bf} were recovered constantly and time independently in the apolar eluate K_1 with plasma and urine. It was concluded that hydrolysis of DGL* did not occur for at least ten months at −20°C including repeated thawing and exposure to ambient temperature.

Precision of methods I, K and L assessed from replicate measurements of unknown radioactivity in biological samples obtained from pharmacokinetic studies

Replicate determinations of TRA^{bf} and TRP^{bf} in plasma and urine were performed by methods I, K and L. The samples were obtained from two healthy male subjects to which [^{3}H]digoxin had been administered intravenously (0.6 mg to H.R., 1.2 mg to R.F.). The samples chosen covered the whole concentration range of radioactivity found after intravenous dosing of the labelled drug. Unknown DA^{*bf}, TRA^{bf} and TRP^{bf} in biological samples were determined by method I by measuring TR^{bf} and $DA^{*I,bf}$ ($TRA^{I,bf}$). These values were corrected by the individual recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} previously determined with spiked plasma, urine and saliva samples (see Appendix). Unknown TRP^{bf} and TRA^{bf} in biological samples were determined by method K by assaying TR^{bf} and $TR^{K_2,bf}$ (see Appendix). These values were corrected by the individual recoveries of TRA^{bf} and TRP^{bf} previously determined with spiked plasma, urine and saliva samples. Unknown TRP^{bf} and TRA^{bf} in biological samples were determined by method L by measuring $TR^{L_2,bf}$ and $TR^{L_1,bf}$ and considering the previously found individual recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} with spiked plasma, urine and saliva samples (see Appendix). Replicate determinations were also made of $DA^{*I,bf}/TRA^{I,bf}$ by method I and of TR^{bf} by direct measurement.

Precision was defined as percentage standard deviation about the means of replicate measurements. Medians and ranges of precision for TRA^{bf} and TRP^{bf} obtained by the different methods and for TR^{bf} obtained by direct assay are listed in Table VI. The estimated precisions of TR^{bf} and TRA^{bf} determined directly and by methods I, K and L were similarly high in both biological fluids studied (Table VI). The precisions of TRP^{bf} were high for method L only and were significantly lower for methods I and K in plasma and urine. The precisions of $DA^{*I,bf}$ decreased in the order $D^* > DB^* > DH_B^* > DM^*$, and the individual values for the compounds susggested that at least D^{*bf} and DB^{*bf} measurements were sufficiently precise (Table VI). It was concluded that method L displayed the highest overall precision and was the only method in which reproducible estimates of TRP^{bf} in plasma and urine were obtained. Methods I, K and L were considered to be equally precise regarding TRA^{bf} measurements. The comparatively lowest precision of TRP^{bf} obtained by method I was considered to result from the fact that after intravenous administration the percentage of total polar radioactivity was small [$10^2 \times (TRP^{bf}/TR^{bf}) < 5\%$] in both plasma and urine. The value of TRP_I^{bf} was calculated and obtained from the difference of two values of almost equal size ($TR^{bf} \approx TRA^{bf}$). Estimates of TRP^{bf} by method L have a higher precision than those by method K. This could be due to the fact that with method L both TRA^{bf} and TRP^{bf} were measured (and normalized) values, whereas with method K only TRP^{bf} was a measured value, but TRA^{bf} a calculated value. Alternatively, the column chromatographic procedure of method K (and method I) could have produced more variable estimates of TRP than the chloroform extraction procedure of method L.

Correlations of apolar and polar total radioactivity determinations by methods I, K and L in plasma and urine

It appeared to be useful to study the nature of existing relationships between methods I, K and L regarding their respective assessments of TRA^{bf} and TRP^{bf}. Knowledge of the nature of such interassay relationships could give valuable information on the individual characteristics and possible biases of the methods used. Correlations between the different TRA^{bf} values and between the different TRP^{bf} values determined by methods I, K and L in plasma and urine were attempted. The plasma and urine samples for this investigation had been obtained from volunteer R.F. who had received four dosages of [^{3}H]-digoxin on separate occasions: 1.2 mg and 0.6 mg intravenously as well as orally. The correlations were performed with the data sets of each study: separately for TRA^{bf} and TRP^{bf} and separately for these values in plasma and urine. No attempt was made to correlate TRP^{bf} data obtained by the three methods in the intravenous studies. The precision of TRP^{bf} measurements by methods I and K (Table VI) was considered to be too low.

Linear regressions of the type $y = mx + c$ were attempted, where y and x correspond to the TRA^{bf} (or TRP^{bf}) data sets obtained by two different methods and where m and c represent slope and intercept, respectively. A statistical program [29] was used which yielded correlation coefficients, slopes and intercepts. Both variables were assumed to be normally distributed. The means of the variances for TRA^{bf} and TRP^{bf} measurements by methods I, K and L, obtained previously from replicate determination of samples by these methods, were used as variability estimates. Coefficients of correlations were also calculated according to non-parametric statistical procedures [30].

Highly significant linear correlations existed between the TRA^{bf} values (y,x) and between the $TRP^{bf}(y,x)$ values assayed by methods I, K and L in plasma and urine at both dosage levels

$$y_K^{bf} = mx_I^{bf} + c \quad (1)$$

$$y_L^{bf} = mx_I^{bf} + c \quad (2)$$

$$y_L^{bf} = mx_K^{bf} + c \quad (3)$$

The parameters of eqns. 1—3 are given for TRA^{bf} and TRP^{bf}, respectively, in Tables VII and VIII. For TRA^{bf} the slopes of all regressions were close to 1.0 and the intercepts negligible [not withstanding the fact that in some cases slopes and intercepts were statistically significantly different from 1.0 and 0, respectively (Table VII)]. Methods L and K gave practically identical values and small interassay variability in plasma and urine. TRA^{bf} estimates by these methods tended to be higher than those by method I. Regressions between TRP^{bf} values by method L or K and by method I yielded slopes that were clearly smaller than 1.0 and intercepts that were close to zero (Table VIII). TRP^{bf} estimates by method I were thus larger than those by methods K or L. TRP^{bf} values by methods L and K were equivalent in urine, whereas in plasma method K tended to give smaller values than method L.

It was concluded that method I estimates of TRP^{bf} and TRA^{bf} differed from those by methods K and L. This discrepancy was pronounced for the TRP^{bf}

TABLE VII

LINEAR REGRESSIONS OF APOLAR TOTAL RADIOACTIVITY DETERMINATIONS IN PLASMA AND URINE BY METHODS I, K AND L ($TRA^{bf}_{I,K,L}$)

Dose (mg)	Mode of administration	Fluid	n*	Range (dpm/ml)	Methods	m**	S.D.	c***	± S.D.	r§
1.2	Intravenous	Plasma	32	365,120—12,146	K / I	0.997*	0.0059	−23.0	238.7	1.000
					L / I	0.997*	0.0066	130	263.7	0.999
					L / K	0.980*	0.0022	159	88.5	1.000
		Urine	13	127,299—0	K / I	1.058*	0.0091	−2005	2408	1.000
					L / I	1.059*	0.0081	−3432	2154	1.000
					L / K	1.000	0.0023	−1391	655.0	1.000
0.6	Intravenous	Plasma	32	478,530—22,892	K / I	1.076*	0.0110	−377	308.4	0.999
					L / I	1.063*	0.0101	354	284.8	0.999
					L / K	0.987*	0.0034	33.6	102.1	1.000
		Urine	13	116,470—0	K / I	1.027	0.0229	609.5	5010	0.997
					L / I	1.021	0.0231	739.2	5050	0.997
					L / K	0.992*	0.0019	453.7	421.9	1.000
1.2	Oral	Plasma	27	544,744—22,930	K / I	1.235*	0.0163	−366*	78.3	0.998
					L / I	1.201*	0.0165	−342*	79.3	0.990
					L / K	0.971*	0.0087	17.5	49.8	0.990
		Urine	13	16,110—0	K / I	1.127*	0.0268	−5853	5450	0.997
					L / I	1.133*	0.0254	−5985	5177	0.997
					L / K	1.004	0.0162	—332.9	1157	1.000
0.6	Oral	Plasma	27	310,367—20,802	K / I	1.002	0.0107	20.6	50.7	0.999
					L / I	0.969*	0.0091	34.6	43.1	0.999
					L / K	0.967*	0.0043	52.2*	20.4	1.000
		Urine	13	12,510—75	K / I	1.056*	0.0220	−1463	3155	0.998
					L / I	1.059*	0.0219	−1797	3142	0.998
					L / K	1.001	0.0039	−150.8	583.1	1.000

*Number of pairs.
**Slope (*significantly different from 1.0, $p < 0.05$).
***Intercept (*significantly different from zero, $p < 0.05$).
§ Correlation coefficient.

TABLE VIII

LINEAR REGRESSIONS OF POLAR TOTAL RADIOACTIVITY DETERMINATIONS IN PLASMA AND URINE BY METHODS I, K AND L ($TRP^{bf}_{I,K,L}$)

Dose (mg)	Mode of administration	Fluid	n*	Range (dpm/ml)	Methods	m**	±	S.D.	c***	±	S.D.	r§
1.2	Oral	Plasma	26	2153—0	K / I	0.361*		0.050	184		77.6	0.811
					L / I	0.607*		0.080	88.7		116	0.826
					L / K	1.282*		0.044	−11.6		32.2	0.986
		Urine	13	114,410—0	K / I	0.519*		0.064	2602		3144	0.921
			13		L / I	0.528*		0.063	1918		3073	0.927
			13		L / K	0.980*		0.041	89.1		1146	0.990
0.6	Oral	Plasma	27	2273—0	K / I	0.759*		0.047	53.3		30.3	0.954
					L / I	1.075		0.083	99.0		51.6	0.932
					L / K	1.331		0.033	49.6*		17.2	0.993
		Urine	13	39,864—0	K / I	0.756		0.146	−1243		2864	0.817
			13		L / I	0.832		0.171	−2441		3271	0.814
			13		L / K	1.007		0.038	−94.0		531	0.992

*Number of pairs.

**Slope (*significantly different from 1.0, $p < 0.05$).

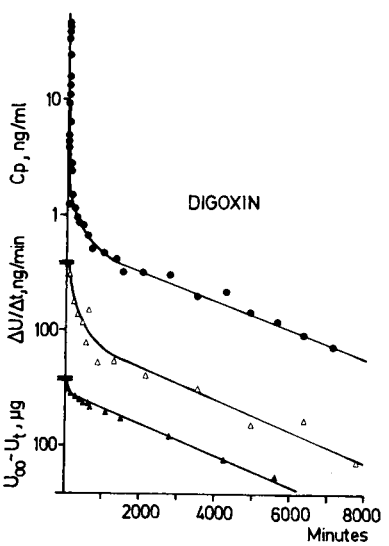
***Intercept (*significantly different from zero, $p < 0.05$).

§ Correlation coefficient.

values and was probably a consequence of the fact that with method I TRP^{bf} was a calculated value, obtained from the difference of two similarly large values, TR^{bf} and TRA^{bf}. The smaller discrepancies between the experimentally measured TRP^{bf} values by methods K and L were most likely due to the use of different separation procedures. It was concluded that method I estimates of TRP^{bf} (and TRA^{bf}) were more biased than those by methods K and L. Method L which, unlike the other two methods, determines both TRA^{bf} and TRP^{bf} experimentally, was regarded as the most adequate assay. In conclusion, method L in combination with method I appears to be the best procedure for the determination of digoxin and its apolar and polar metabolites in biological samples obtained from humans after administration of radiolabelled digoxin.

DISCUSSION

The assay methods presented allow a complete pharmacokinetic analysis of digoxin and its metabolites in biological fluids in humans after single-dose administration of the drug (Figs. 1—6). They provide the necessary specificity and, if radiolabelled drug is used, sensitivity. Parent drug and apolar individual



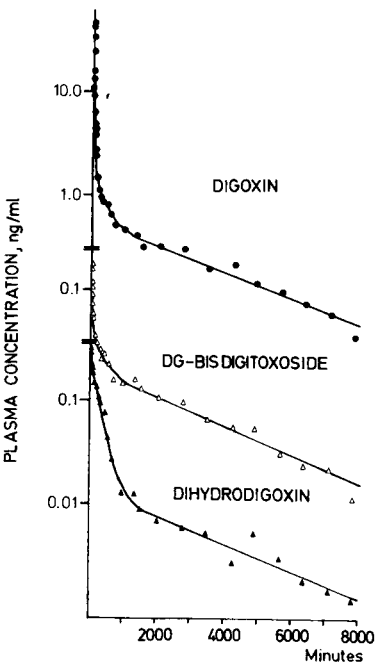


Fig. 1. Typical semilogarithmic plots of plasma concentrations of digoxin, C_p (●), rates of urinary excretion of digoxin, $\Delta U/\Delta t$ (△), and amounts of digoxin yet to be excreted, ($U_\infty - U_t$) (▲), against time for intravenous administration (0.6 mg of [^{3}H]digoxin to H.R.). Data were obtained by method L in combination with method I.

Fig. 2. Typical semilogarithmic plots of plasma concentrations of digoxin (●), digoxigenin bisdigitoxoside (△) and dihydrodigoxin (▲) against time for intravenous administration (0.6 mg of [^{3}H]digoxin to H.R.). Data were determined by method L in combination with method I.

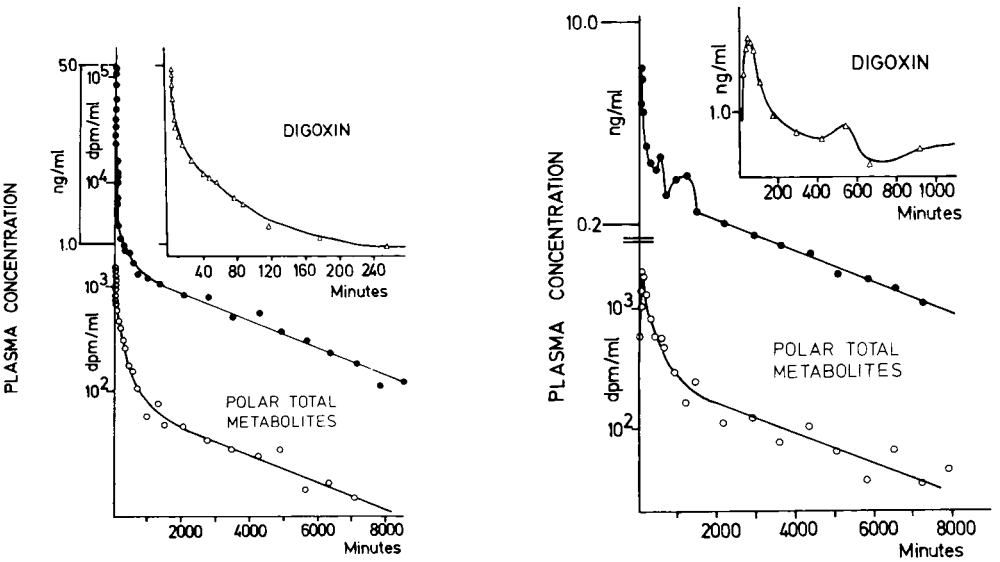


Fig. 3. Typical semilogarithmic plots of plasma concentrations of digoxin (●) and polar total metabolites (○) against time for intravenous administration (0.6 mg of [^{3}H]digoxin to H.R.) (△). Inset: plasma levels of digoxin on an expanded time scale. Data were obtained by method L in combination with method I.

Fig. 4. Typical semilogarithmic plots of plasma concentrations of digoxin (●) and polar total metabolites (○) against time for oral administration (0.6 mg of [^{3}H]digoxin to R.F.) (△). Inset: plasma levels of digoxin on an expanded time scale. Data were determined by method L in combination with method I.

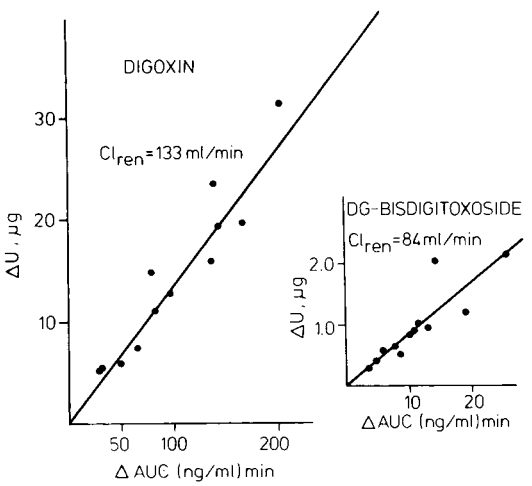


Fig. 5. Typical linear renal clearance plots for digoxin and digoxigenin bisdigitoxoside (inset) after oral administration of 0.6 mg of [^{3}H]digoxin to R.F. The values of the renal clearance, Cl_{ren}, were obtained from the slopes of these plots. Data were assayed by method K in combination with method I.

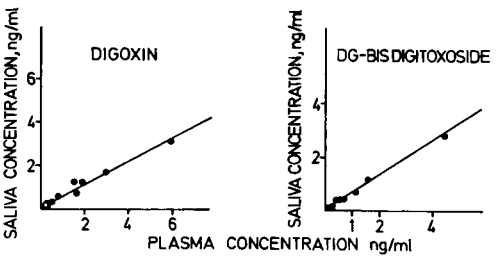


Fig. 6. Typical linear plots of saliva concentrations against plasma concentrations for digoxin and digoxigenin bisdigitoxoside after intravenous administration of 1.2 mg of [^{3}H]digoxin to R.F. Apparent linear correlations existed for digoxin and digoxigenin bisdigitoxoside between the concentrations in saliva and plasma measured at identical times after drug administration.

and total polar metabolites can be followed over time intervals sufficiently large for unambiguous delineations of the pharmacokinetics (Figs. 1—4). The recovery, precision and reproducibility performance of methods I, K and L have been characterized and tested in biological samples spiked with standards and/or obtained from pharmacokinetic studies with digoxin. The critical evaluation of the three methods used showed that the assay performance of method L was best. Method L assayed TRA^{bf} and TRP^{bf} accurately and precisely. Methods K and L determined TRA^{bf} adequately; TRP^{bf} assessments by method K were clearly less precise than those by method L. Method I measured DA^{*bf} specifically and precisely. There was evidence that method I estimates of TRP^{bf} (and of TRA^{bf}) were biased. A combination of $(DA^*/TRA)_I^{bf}$ and TRA_L^{bf} obtained, respectively, by methods I and L in the same biological samples gave optimum (unbiased) data which were suitable for pharmacokinetic analysis (Figs. 1—4). It appeared that the anticipated superior assay characteristics of method L were mainly due to the fact that both TRA^{bf} and TRP^{bf} were experimentally measured values with this method, contrary to those with methods I and K.

APPENDIX

Method I

A. Determination of recoveries in biological samples

The recoveries were obtained in experiments where plasma, urine, and saliva samples were spiked with known amounts of DT*, D*, DH_B^*, DB*, (DM+DG+EDG)* and DGL*.

The percentage recovery of DT* with method I, $r_{DT^*}^{I,bf}$, was obtained from

$$r_{DT^*}^{I,bf} = 10^2 \cdot DT^{*I,bf}/DT_0^{*bf} \qquad (A1)$$

where $DT^{*I,bf}$ represents the measured DT* radioactivity from the scrapings and DT_0^{*bf} the known DT* radioactivity added to a biological sample.

The individual amounts or radioactivities of D_I^{*bf}, DH_I^{*bf}, DB_I^{*bf} and $(DM+DG+EDG)_I^{*bf}$ in a biological sample, DA_I^{*bf}, were obtained from eqn. A2:

$$DA_I^{*bf} = DA^{*I,bf}/r_{DT*}^{I,bf} \quad (A2)$$

where $DA^{*I,bf}$ correspond to the apolar individual radioactivities assignable to D, DH, DB and (DM+DG+EDG) as obtained from the scrapings of the plates from TLC system A. Summation of the individual $DA^{*I,bf}$ values yields $TRA^{I,bf}$:

$$TRA^{I,bf} = \Sigma DA^{*I,bf} \quad (A3)$$

Accordingly the summed individual DA_I^{*bf} values give TRA_I^{*bf}:

$$TRA_I^{bf} = \Sigma DA_I^{*bf} \quad (A4)$$

For the spiked biological samples the percentage recovery of apolar individual radioactivity, $r_{I,DA*}^{bf}$, and the percentage recovery of apolar total radioactivity, $r_{I,TRA}^{bf}$ were obtained from eqns. A5 and A6, respectively

$$r_{I,DA*}^{bf} = 10^2 \cdot DA_I^{*bf}/DA_0^{*bf} \quad (A5)$$

$$r_{I,TRA}^{bf} = 10^2 \cdot TRA_I^{bf}/TRA_0^{bf} \quad (A6)$$

where DA_I^{*bf} and DA_0^{*bf} represent, respectively, measured and known (added) amounts of individual apolar radioactivity and TRA_I^{bf} and TRA_0^{bf} correspond respectively to measured and known (added) apolar total radioactivity.

The percentage recovery of polar total radioactivity (= DGL*), $r_{I,TRP}^{bf}$, for the biological samples tested was obtained from eqn. A7:

$$r_{I,TRP}^{bf} = 10^2 \cdot TRP_I^{bf}/TRP_0^{bf} \quad (A7)$$

where TRP_I^{bf} and TRP_0^{bf} correspond, respectively, to the calculated and known (added) amounts of DGL_0^* radioactivity. The recoveries of DA^{*bf}, TRA^{bf} and of TRP^{bf} obtained by method I are listed in Table III.

B. Determination of unknown concentrations in biological samples

The individual DA_I^{*bf} were obtained by eqn. A8:

$$DA_I^{*bf} = f_I \cdot (10^4 \cdot DA^{*I,bf})/(r_{DT*}^{I,bf} \cdot r_{I,DA*}^{bf}) \quad (A8)$$

where f_I is a correction factor obtained from the experiments which determined the recoveries in samples spiked with the standards (Table III), i.e. $f_I = 10^2/r_{I,DA*}^{bf}$.

TRA_I^{bf} was obtained from eqn. A4 as described above and TRP_I^{bf} was calculated from eqn. A9:

$$TRP_I^{bf} = TR^{bf} - TRA_I^{bf} \quad (A9)$$

Method K

A. Determination of recoveries in biological samples

The recoveries were determined as described above (see method I). The respective percentage recoveries of D* and DGL* in eluate 2 of a biological sample, $r_{D*}^{K_2,bf}$ and $r_{DGL*}^{K_2,bf}$, were determined from

$$r_{D*}^{K_2,bf} = 10^2 \cdot D*^{K_2,bf} / D_0^{*bf} \quad \text{(A10)}$$

$$r_{DGL*}^{K_2,bf} = 10^2 \cdot DGL*^{K_2,bf} / DGL_0^{*bf} \quad \text{(A11)}$$

where $D*^{K_2,bf}$ and $DGL*^{K_2,bf}$, respectively, are the measured radioactivities of D* and DGL* in eluate 2 of a biological sample and D_0^{*bf} and DGL_0^{*bf} are thus the respective known (added) amounts of D* and DGL* in that biological sample. According to the results obtained with the standards D* and DGL*, the measured radioactivity in eluate 2 represents total radioactivity, $TR^{K_2,bf}$, and eqn. A12 holds:

$$TR^{K_2,bf} = TRP^{K_2,bf} + TRA^{K_2,bf} \quad \text{(A12)}$$

where $TRP^{K_2,bf}$ and $TRA^{K_2,bf}$ correspond, respectively, to polar and apolar total radioactivity in eluate 2 of a biological sample.

Since $TRP^{K_2,bf}$ could be estimated from eqn. A13, assuming that D* and DA* behaved comparably throughout the procedures of method K, i.e. $r_{D*}^{K_2,bf} \approx r_{DA*}^{K_2,bf}$, then:

$$TRP^{K_2,bf} = [TR^{K_2,bf} - TR^{bf}(r_{D*}^{K_2,bf}/10^2)] / [1 - (r_{D*}^{K_2,bf}/r_{DGL*}^{K_2,bf})] \quad \text{(A13)}$$

The value of TRP_K^{bf} was thus obtained from eqn. A14:

$$TRP_K^{bf} = 10^2 \cdot TRP^{K_2,bf} / r_{DGL*}^{K_2,bf} \quad \text{(A14)}$$

and TRA^{bf} was calculated subsequently from eqn. A15:

$$TRA_K^{bf} = TR^{bf} - TRP_K^{bf} \quad \text{(A15)}$$

B. Combination of data by methods K and I

Data by methods K and I were combined according to eqn. A16:

$$DA_{I/K}^{*bf} = TRA_K^{bf} \cdot (DA*/TRA)_I^{bf} \quad \text{(A16)}$$

where TRA_K^{bf} represents apolar total radioactivity as obtained by method K and $(DA*/TRA)_I^{bf}$ corresponds to the fractional apolar individual radioactivity as determined by method I.

For the biological samples spiked with known amounts of D*, DH_B^*, DB*, DM* and DGL*, the percentage recoveries of polar and apolar total and individual radioactivity, $r_{K,TRP}^{bf}$, $r_{K,TRA}^{bf}$ and $r_{I/K,DA*}^{bf}$, were thus obtained from eqns. A17—A19:

$$r_{K,TRP}^{bf} = 10^2 \cdot TRP_K^{bf} / TRP_0^{bf} \quad \text{(A17)}$$

$$r^{bf}_{K,TRA} = 10^2 \cdot TRA^{bf}_K / TRA^{bf}_0 \quad (A18)$$

$$r^{bf}_{I/K,DA*} = 10^2 \cdot TRA^{bf}_K \cdot (DA*/TRA)^{bf}_I / DA^{*bf}_0 \quad (A19)$$

where TRP_K, TRA_K and $DA^*_{I/K}$ correspond, respectively, to the measured amounts of polar total radioactivity, apolar total and individual radioactivity by method K, and TRP_0, TRA_0 and DA^*_0 represent the known (added) amounts of these radioactivities.

The recoveries of TRA^{bf} and TRP^{bf} obtained by method K and the recovery of DA^{*bf} received by combining data from methods I and K are listed in Table IV.

C. Determination of unknown concentrations in biological samples

Unknown TRP^{bf} and TRA^{bf} in biological samples were determined by method K by assaying TR^{bf} and $TR^{K_2,bf}$. The value of TRP^{bf}_K was computed from eqn. A20:

$$TRP^{bf}_K = f_K \cdot (10^2 \cdot TR^{K_2,bf} - TR^{bf} \cdot r^{K_2,bf}_{D*}) / (r^{K_2,bf}_{DGL*} - r^{K_2,bf}_{D*}) \quad (A20)$$

The correction factor f_K, was obtained from the experiments in which the recoveries in samples spiked with the standards were determined (Table IV), i.e. $f_K = 10^2 / r^{bf}_{K,TRP}$.

TRA^{bf} was calculated from eqn. A15 as indicated previously. Unknown DA^{*bf} was assessed upon combining the results obtained by methods K and I according to eqn. A16 as outlined above.

Method L

A. Determination of recoveries from biological samples

The recoveries were determined as described above (see method I). The measured radioactivities in both the aqueous residue, L_2, and the organic extract, L_1, of a biological sample represented total radioactivity, $TR^{L_2,bf}$ and $TR^{L_1,bf}$, respectively. They were defined by eqns. A21 and A22:

$$TR^{L_2,bf} = TRP^{L_2,bf} + TRA^{L_2,bf} \quad (A21)$$

$$TR^{L_1,bf} = TRA^{L_1,bf} + TRP^{L_1,bf} \quad (A22)$$

Since $TRP^{L_2,bf}$ can be estimated from eqn. A23, assuming that D* and DA* behaved comparably throughout the procedures of method L, i.e. $r^{L_2,bf}_{D*} \approx r^{L_2,bf}_{DA*}$, then

$$TRP^{L_2,bf} = [TR^{L_2,bf} - TR^{bf} \cdot (r^{L_2,bf}_{D*}/10^2)] / [1 - (r^{L_2,bf}_{D*}/r^{L_2,bf}_{DGL*})] \quad (A23)$$

$TRA^{L_2,bf}$ can be obtained from eqn. A21 after rearrangement. Similarly, $TRA^{L_1,bf}$ can be estimated from eqn. A24:

$$TRA^{L_1,bf} = [TR^{L_1,bf} - TR^{bf} \cdot (r^{L_1,bf}_{DGL*}/10^2)] / [1 - (r^{L_1,bf}_{DGL*}/r^{L_1,bf}_{D*})] \quad (A24)$$

$TRP^{L_1,bf}$ was then obtained from eqn. A22 after rearrangement and TR^{bf} was estimated from eqn. A25:

$$TR_L^{bf} = TR^{L_1,bf} + TR^{L_2,bf} \quad \text{(A25)}$$

The ratio of TR^{bf} (measured directly) to TR_L^{bf} (obtained by method L) yields a correction factor q:

$$q = TR^{bf}/TR_L^{bf} \quad \text{(A26)}$$

TRP_L^{bf} and TRA_L^{bf} were obtained, respectively, from eqns. A27 and 28:

$$TRP_L^{bf} = q \cdot 10^2 \cdot (TRP^{L_2,bf}/r_{DGL*}^{L_2,bf}) \quad \text{(A27)}$$

$$TRA_L^{bf} = q \cdot 10^2 (TRA^{L_1,bf}/r_{D*}^{L_1,bf}) \quad \text{(A28)}$$

B. Combination of data by methods L and I

The value of DA^{*bf} could only be assessed if data from methods L and I were combined according to eqn. A29:

$$DA_{I/L}^{*bf} = TRA_L^{bf} \cdot (DA^*/TRA)_I^{bf} \quad \text{(A29)}$$

where TRA_L^{bf} represents apolar total radioactivity as obtained by method L and $(DA^*/TRA)_I^{bf}$ corresponds to the fractional apolar individual radioactivity as determined by method I.

For the biological samples spiked with known amounts of D*, DH_B^*, DB*, DM* and DGL*, the percentage recoveries of polar total radioactivity, apolar total and individual radioactivity, $r_{L,TRP}^{bf}$, $r_{L,TRA}^{bf}$ and $r_{I/L,DA*}^{bf}$, were obtained from eqns. A30—A32:

$$r_{L,TRP}^{bf} = 10^2 \cdot TRP_L^{bf}/TRP_0^{bf} \quad \text{(A30)}$$

$$r_{L,TRA}^{bf} = 10^2 \cdot TRA_L^{bf}/TRA_0^{bf} \quad \text{(A31)}$$

$$r_{I/L,DA*}^{bf} = 10^2 \cdot TRA_L^{bf} \cdot (DA^*/TRA)_I^{bf} \quad \text{(A32)}$$

where TRP_L^{bf}, TRA_L^{bf} and $DA_{I/L}^{*bf}$ correspond to the measured values of these radioactivities as obtained by method L or I and TRP_0^{bf}, TRA_0^{bf} and DA_0^{*bf} represent the known (added) amounts of the standards in the biological samples.

The recoveries of TRA^{bf} and TRP^{bf} obtained by method L and the recovery of DA^{*bf} received by combining data from methods I and L are listed in Table V.

C. Determination of unknown concentrations in biological samples

Unknown TRA^{bf} and TRP^{bf} in biological samples were determined by method L by measuring TR^{bf}, $TR^{L_2,bf}$ and $TR^{L_1,bf}$. TRP^{bf} and TRA^{bf} could be obtained by eqns. A33 and A34, respectively.

$$TRP_L^{bf} = f_{L_2} \cdot q \cdot (10^2 \cdot TR^{L_2,bf} - TR^{bf} \cdot r_{D*}^{L_2,bf})/(r_{DGL*}^{L_2,bf} - r_{D*}^{L_2,bf}) \quad \text{(A33)}$$

$$TRA_L^{bf} = f_{L_1} \cdot q \cdot (10^2 \cdot TR^{L_1,bf} - TR^{bf} \cdot r_{DGL*}^{L_1,bf}) / (r_{D*}^{L_1,bf} - r_{DGL*}^{L_1,bf}) \quad \text{(A34)}$$

where f_{L_2} and f_{L_1} are correction factors obtained from the experiments which determined the recoveries in samples spiked with the standards (Table V), i.e. $f_{L_2} = 10^2 / r_{L,TRP}^{bf}$ and $f_{L_1} = 10^2 / r_{L,TRA}^{bf}$.

LIST OF SYMBOLS AND ABBREVIATIONS

D	= digoxin (unlabelled)
DH	= dihydrodigoxin
DB	= digoxigenin bisdigitoxoside
DM	= digoxigenin monodigitoxoside
DG	= digoxigenin
EDG	= epidigoxigenin
DT	= digitoxin
DGL	= digoxin-16′-glucuronide
D*	= digoxin (labelled)
TR	= total radioactivity (TR=TRA+TRP)
TRA	= apolar total radioactivity
DA*	= apolar individual radioactivity [DA* = D*, DH*, DB*, (DM+DG+EDG)*]
TRP	= polar total radioactivity
DT*, D*, DGL*, DH*, DB*, DM*, TR*, DA*, TRA*, TRP*	= radioactivity, R
R_0^{bf} (p,u,s)	= known added radioactivity in a biological sample (plasma, urine, saliva)
$r_R^{I,bf}$, ($R^{I,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample spiked with DT* as obtained by method I from TLC scrapings
$r_R^{K_2,bf}$, ($R^{K_2,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample spiked with D* or DGL* as obtained by method K in aqueous eluate, K_2
$r_R^{L_2,bf}$, ($R^{L_2,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample spiked with D* or DGL* as obtained by method L in aqueous residue, L_2
$r_R^{L_1,bf}$, ($R^{L_1,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample, spiked with D* or DGL* as obtained by method L in organic extract L_1
$R_{I,K,L}^{bf}$	= measured radioactivity of a biological sample as obtained by methods I, K or L
$R_{I/K,I/L}^{bf}$	= measured radioactivity of a biological sample as obtained by combining data by methods I and K or I and L

$r^{bf}_{I,K,L,R}$ = percentage recovery of radioactivity in a biological sample as obtained by methods I, K or L

$r^{bf}_{I/K,I/L,R}$ = percentage recovery of radioactivity in a biological sample as obtained by combining data by methods I and K or I and L

ACKNOWLEDGEMENTS

This study was supported in part by a scholarship by the Sandoz Stiftung "zur Förderung der Medizinischen Wissenschaften", Sandoz Ltd., Basel, Switzerland.

We wish to thank Dr. J. Wüersch and Dr. R. Schwob, F. Hoffmann-La Roche Ltd., Basel, for the synthesis of labelled dihydrodigoxin, Professor W. Schaumann, Boehringer Mannheim Ltd., for providing us with labelled and unlabelled digoxin and its apolar derivatives, and Dr. H. Flasch, Beiersdorf Ltd., Hamburg, for the supply of labelled and unlabelled 16′-digoxin glucuronide and dihydrodigoxin.

REFERENCES

1 T.W. Smith, V.P. Butler, Jr. and E. Haber, N. Engl. J. Med., 281 (1969) 1212.
2 A.F. Rosenthal, M.G. Vargas and C.S. Klass, Clin. Chem., 22 (1976) 1899.
3 F.I. Marcus, J.N. Ryan and M.G. Stafford, J. Lab. Clin. Med., 85 (1975) 610.
4 J.M. Lowenstein, Circulation, 31 (1965) 228.
5 G.H. Burnett and R.H. Conklin, J. Lab. Clin. Med., 78 (1971) 779.
6 D.S. Lukas, in O. Storstein (Editor), Symposium on Digitalis, Oslo Glydendal Norsk Forlag, Oslo, 1973, p. 18.
7 E. Watson and S.M. Kalman, J. Chromatogr., 56 (1971) 209.
8 E. Watson, D.R. Clark and S.M. Kalman, J. Pharmacol. Exp. Ther., 184 (1973) 424.
9 M.C. Castle, J. Chromatogr., 115 (1975) 437.
10 M.H. Gault, M. Ahmed, A.L. Symes and J. Vance, Clin. Biochem., 9 (1976) 46.
11 W. Zilly, E. Richter and N. Rietbrock, Clin. Pharmacol. Ther., 17 (1975) 303.
12 H. Greenwood, W. Snedden, R.P. Hayward and J. Landon, Clin. Chim. Acta, 62 (1975) 213.
13 V.P. Butler, Jr., in G. Bodem and H.J. Dengler (Editors), Cardiac Glycosides, International Boehringer Mannheim Symposia, Springer Verlag, Berlin, Heidelberg, New York, 1978, p. 1.
14 P.H. Hinderling, E.R. Carrett and R.C. Wester, J. Pharm. Sci., 66 (1976) 314.
15 M.H. Gault, D. Sudgen, C. Maloney, M. Ahmed and M. Tweedale, Clin. Pharmacol. Ther., 25 (1979) 499.
16 J.E. Doherty, W.H. Perkins and G.K. Mitchell, Arch. Int. Med., 108 (1961) 531.
17 F.I. Marcus, G.J. Kapadia and G. Kapadia, J. Pharmacol. Exp. Ther., 145 (1964) 203.
18 D.S. Lukas, in G. Bodem and H.J. Dengler (Editors), Cardiac Glycosides, International Boehringer Mannheim Symposia, Springer Verlag, Berlin, Heidelberg, New York, 1978, p. 36.
19 D.R. Clark and S.M. Kalman, Drug Metab. Dispos., 2 (1974) 148.
20 A. Dwenger and G. Haberland, Naunyn-Schmiedebergs Arch. Pharmakol., 270 (1971) 102.
21 H.F. Benthe, in G. Bodem and H.J. Dengler (Editors), Cardiac Glycosides, International Boehringer Mannheim Symposia, Springer Verlag, Berlin, Heidelberg, New York, 1978, p. 52.

22 L.B. Sheiner and T.N. Tozer, in K.L. Melmon and H.F. Morelli (Editors), Clinical Pharmacology, MacMillan, New York, Toronto, 2nd ed., 1978, p. 272.
23 K.K. Chen, in W. Wilbrandt (Editor), Proceedings of the First International Pharmacological Meeting, Stockholm, Vol. III, Pergamon Press, New York, 1963, p. 27.
24 G.L. Lage and J.L. Spratt, J. Pharmacol. Exp. Ther., 152 (1966) 501.
25 H. Lüllmann and T. Peters, J. Pharmacol., 14 (1971) 204.
26 A. von Wartburg, F. Kalberer and J. Rutschmann, Biochem. Pharmacol., 14 (1965) 1883.
27 P.H. Hinderling, Agents Actions, 7 (1977) 379.
28 G. Haberland and G. Maerten, Naturwissenschaften, 56 (1969) 516.
29 D.V. Lindley, Suppl. J.R. Statist. Soc., 9 (1947) 218.
30 M.G. Kendall, Biometrika, 30 (1938) 81.

Journal of Chromatography, 224 (1981) 94—98
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 836

Note

Glass capillary quantitative determination of N^{π}-methylhistidine in urine and muscles

E. MUSSINI, L. COTELLESSA, L. COLOMBO, D. CANI, P. SFONDRINI and F. MARCUCCI*

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)

and

F. POY

Dani S.p.A., Via Rovani 10, Monza (Italy)

(Received December 23rd, 1980)

There are a number of methylated amino acid derivatives in muscle, including N^{τ}-methylhistidine (3-methylhistidine) (3-MeHis), N^{π}-methylhistidine (1-methylhistidine) (1-MeHis) [1], the mono- and trimethyllysines [2—4] and N^{G},N^{G}-dimethylarginines [5, 6]. The function of such methylated amino acids is not clearly understood, but it has been proposed that the methyllysines and methylarginines of histones might be involved in the regulation of DNA synthesis and chromatin activity [7]. Furthermore, it has been found that trimethyllysine is the precursor of 4-trimethylaminobutyrate for carnitine biosynthesis [8, 9]. However, neither the function of 3-MeHis residue in actin and myosin, nor the significance of 1-MeHis in the dipeptide anserine has been established. It appears that 1-MeHis is not made by methylation of histidine in proteins.

Accurate measurement of the concentration of methylated amino acids in biological samples is essential because their amount in some instances is very low and no specific assay has been reported. Most workers have determined some methylated amino acids by ion-exchange chromatography and a number of methods have been published specifically for the analysis of methyl amino acids [6, 10—14].

Recently we have developed a method for the isolation of 3-MeHis from biological specimens and its quantitative determination by glass capillary gas chromatography (GC) [15]. This paper describes the application of such a method for the isolation from biological samples and quantitative determination of 1-MeHis.

EXPERIMENTAL

Isolation of 1-MeHis by charcoal column chromatography

The analytical procedure is substantially the same as that used for the isolation of 3-MeHis from biological samples. It can be summarized as follows. Silanized columns (1.5 × 1 cm I.D.) were packed with charcoal—celite (1:1, w/w) (BDH, Poole, Great Britain) suspended in and washed with 1 *N* HCl. Subsequently the columns were washed with 20 ml of water and buffered at pH 5 with 0.33 *M* acetate buffer (5 ml). Biological samples (hydrolyzates of urine and muscles evaporated to dryness under vacuum) were dissolved in 0.33 *M* acetate buffer (pH 5) and an amount corresponding to 0.25 ml of urine and 15 mg of wet muscle was applied to the charcoal—Celite column which was washed first with 20 ml of water, then 5 ml of 80% acetone; 1-MeHis was eluted with 30 ml of dichloromethane—methanol—33% ammonium hydroxide (70:25:5). The eluates were evaporated to dryness under vacuum.

Derivatization of 1-MeHis for gas chromatography

Preparation of the 1-MeHis derivative is a two-step process, initially requiring that the carboxyl group be esterified. Among esterification agents, a mixture of dry acetyl chloride 5% in propanol was chosen; 5 ml of the esterification mixture were added to the residue. Each tube was sealed, mixed and left to react overnight at 90°C in a Reacti—Therm-Heating module (Pierce, Rockford, IL, U.S.A.). Samples were evaporated to dryness under vacuum and then N-acetylated with 150 μl of trifluoroacetic anhydride and 200 μl of dichloromethane for 30 min at room temperature. The 1-MeHis derivative was evaporated to dryness under vacuum and redissolved in dichloromethane solution containing the reference standard (*n*-triacontane 50 μg/ml) before GC analysis. 1-MeHis was completely derivatized (propyl ester, N-trifluoroacetate) as checked by thin-layer chromatography.

Chromatographic conditions

The 1-MeHis derivative gives sharp, symmetrical peaks on common stationary phases such as pretested SE-30, OV-1, OV-17, OV-101 (Applied Science Labs., State College, PA, U.S.A.) in packed columns, but there is no resolution between 1- and 3-MeHis peaks. For this reason we preferred to work with glass capillary columns.

The gas chromatograph was a high-resolution dedicated gas chromatograph 3900B (Dani, Monza, Italy) equipped with a flame ionization detector. The glass capillary column (20 m long, 0.85 mm O.D., 0.30 mm I.D., Duran 50) was prepared according to the barium carbonate procedure described by Grob et al. [16] and given a 0.15 μm thick Pluronic F-68 coating using the static procedure. The split injection mode was used. Temperatures were over 200°C, detector 280°C and injector 300°C. Carrier gas was hydrogen (O_2-free) with a flow-rate of 0.7 ml/min. Splitter flow was 15 ml/min. The practicable sensitivity limit was about 2 μg/ml. The use of an electron-capture detector increased the sensitivity about 100-fold but this detector was not routinely used because rat urine contains a large amount of 1-MeHis. The electron-

capture detector should be very useful for measuring low concentrations of 1-MeHis as, for example, in muscle proteins.

Mass spectrometric assays were performed on an LKB-9000 (Bromma, Sweden) interfaced with a 3% OV-1 packed column.

Quantitation

Quantitation was performed using the method of internal standardization with *n*-triacontane. The calibration curve for 1-MeHis derivative concentrations ranging from 12.5 to 100 ng/μl showed a linear response within this range of concentrations.

Urine creatinine was determined on urine samples using 3,5-dinitrobenzoic acid, according to the instructions supplied with Eurochima Kit (Elvi, Milan, Italy). Protein was determined by the method of Lowry et al. [17].

RESULTS AND DISCUSSION

A typical gas chromatogram of rat urine is illustrated in Fig. 1, where 1-MeHis is present at a greater concentration than 3-MeHis. Recoveries of 1-MeHis from rat urine were 96 ± 2%, the mean ± S.E.M. of four determinations. No significant differences were found between recoveries for different 1-MeHis concentrations (25, 50, 75, 100 ng).

Mass spectrometric analysis confirmed that the 1-MeHis derivative is a

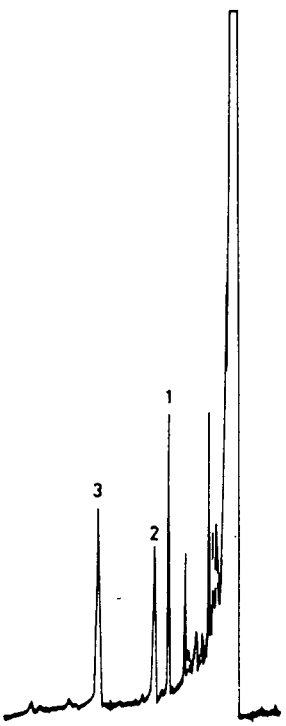


Fig. 1. Gas chromatogram of rat urine sample. 1 = 3-MeHis derivative; 2 = 1-MeHis derivative; 3 = reference standard (*n*-triacontane, 50 μg/ml).

propyl ester, N-trifluoroacetate, as shown in Fig. 2. The basal levels of 1-MeHis and 3-MeHis in rat urine are reported in Table I. Results of preliminary studies of 1-MeHis and 3-MeHis concentrations in rat muscle are reported in Table II.

Chromatographic peaks of biological samples were identified with precision by comparison of the mass spectra of the biological compound and the authentic reference standard, obtained by electron impact.

Critical factors during derivative formation are that reagents must be free of any trace of moisture, and that the preceding reagent must be completely

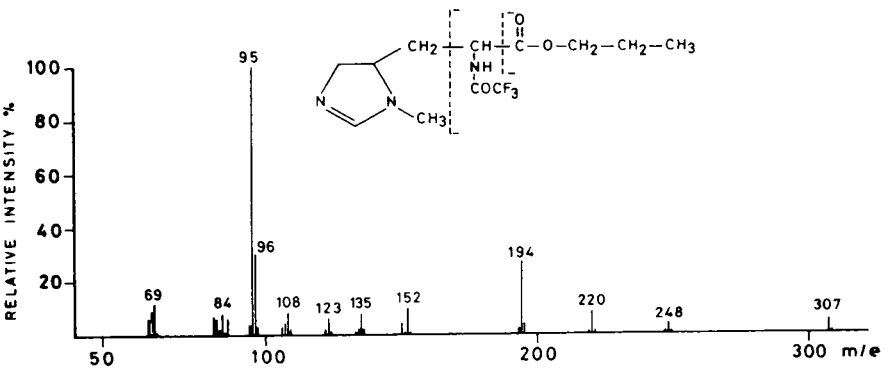


Fig. 2. Mass spectrum of 1-MeHis derivative.

TABLE I

DAILY EXCRETION OF CREATININE, 1-MeHis AND 3-MeHis BY MAN AND BY ADULT MALE RATS

	Creatinine excretion (mmol·24 h^{-1})	1-MeHis excretion (μmol·24 h^{-1})	3-MeHis excretion (μmol·24 h^{-1})
Man*			
C.A.	7.31	958.30	184.80
P.L.	5.34	741.90	151.10
R.M.	3.50	561.08	112.29
R.F.	5.82	920.40	189.17
Mean	5.49 ± 0.95	795.42 ± 99.30	159.34 ± 19.72
Rats**			
1	0.043	2.50	1.64
2	0.057	3.53	2.41
3	0.060	3.15	2.02
4	0.069	3.45	2.31
5	0.079	3.52	2.21
Mean	0.061 ± 0.007	3.03 ± 0.20	2.12 ± 0.15

*Healthy boys (7—12 years), weight 16—25 kg.
**Male CD-COBS rats (Charles River, Calco, Italy), body wt. 250 g.

TABLE II

CONCENTRATIONS OF 1-MeHis AND 3-MeHis IN SOME RAT MUSCLES

Male CD-COBS rats (Charles River), body wt. 250 g, were used. Muscles were pooled from two animals for each determination. Values are expressed as mean ± S.E.M. (n = 4).

Muscle	Protein in wet muscle (mg/g)	Muscle protein (μg/g)	
		1-MeHis	3-MeHis
Palmaris longus	72.96 ± 2	3111 ± 10	886 ± 9
Pectoralis	85.76 ± 3	1762 ± 14	660 ± 10
Gastrocnemius	98.40 ± 5	1730 ± 12	774 ± 8
Heart	103.12 ± 7	543 ± 6	434 ± 6

eliminated by evaporation before the next derivatization step.

The present method, with a sensitivity of 2 μg/ml, appears reliable for measuring 1-MeHis and 2-MeHis in the same sample of urine from animals or men in normal and pathological conditions. The use of an electron-capture detector increases the sensitivity about 100-fold, thus making the method suitable for measuring very low levels of these methylhistidines, in studies of the catabolic rates of muscle proteins, for instance.

Further studies are in progress to measure muscle methylhistidine turnover in animals and men.

ACKNOWLEDGEMENT

The generous financial assistance of "Legato Dino Ferrari" (Maranello, Italy) is gratefully acknowledged.

REFERENCES

1 K.G. Crush, Comp. Biochem. Physiol., 34 (1970) 3.
2 M.F. Hardy, C.I. Harris, J.V. Perry and D. Stone, Biochem. J., 120 (1970) 653.
3 G. Huszar, J. Biol. Chem., 247 (1972) 4057.
4 G. Huszar and Elzinga, J. Biol. Chem., 247 (1972) 745.
5 M. Reporterer and J.L. Corbin, Biochem. Biophys. Res. Commun., 43 (1971) 644.
6 Y. Karimoto, Y. Matsuoka, M. Miyake and H. Konishi, J. Neurochem., 24 (1975) 893.
7 E. Tyihák, B. Szende and K. Lapis, Life Sci., 20 (1977) 385.
8 R.A. Cox and C.L. Hoppel, Biochem. J., 136 (1973) 1083.
9 V. Tanphaichitr and H.P. Broquist, J. Biol. Chem., 248 (1973) 2176.
10 L.N. Haverberg, H.N. Munro and V.R. Young, Biochim. Biophys. Acta, 371 (1974) 226.
11 G.E. Deibler and R.E. Martenson, J. Biol. Chem., 248 (1973) 2387.
12 C.L. Long and J.W. Geiger, Biochem. Med., 12 (1975) 267.
13 R.T. Markiw, Biochem. Med., 13 (1975) 23.
14 C.G. Zarkadas, Can. J. Biochem., 53 (1975) 96.
15 L. Cotellessa, F. Marcucci, D. Cani, P. Sfondrini, L. Colombo, E. Mussini and F. Poy, J. Chromatogr., 221 (1980) 149.
16 K. Grob, G. Grob and K. Grob, Jr., Chromatographia, 10 (1977) 181.
17 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.

Journal of Chromatography, 224 (1981) 99—104
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 844

Note

Liquid chromatographic assay of urinary estriol and electrochemical detection with a battery powered detector

Z.K. SHIHABI*, JUDY SCARO and B.F. THOMAS

Departments of Pathology and Physiology and Pharmacology, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, NC 27103 (U.S.A.)

(First received August 7th, 1980; revised manuscript received January 23rd, 1981)

Urinary estriol represents the final metabolism of estrogens. In pregnancy, estriol levels increase rapidly especially after the 12th week of gestation. Frequent monitoring of this metabolite is utilized in diagnosis of fetal distress in threatened abortions such as toxemia and diabetes.

At the present time, the main methods for estriol assay are colorimetric and fluorimetric [1, 2]. Such methods require lengthy extraction and clean-up steps and they are subject to many interferences [3]. Recently, high-performance liquid chromatography with ultraviolet (UV) detection has been employed for estriol assay [4, 5].

Electrochemical detection has been applied for the detection of many of the urinary metabolites. It is highly sensitive and selective. In this paper we illustrate that estriol can be detected electrochemically with great sensitivity after separation by liquid chromatography. Electrochemical detection of estriol is about 20-fold more sensitive than UV detection. This greater sensitivity allows smaller sizes of sample and solvents to be used, thus speeding up the extraction steps. Column life is greatly increased because of the smaller size of the sample injected.

We used a battery-powered detector in this work. Since the electrochemical detector consumes just minute amounts of electricity, it is well suited to be powered by a battery. The advantages of such a detector are: it would operate free from line noise and would be easier to construct. The detector can be assembled for less than US$75 for parts.

MATERIALS AND METHODS

Equipment

A pump, Model 110A (Altex Scientific, Berkeley, CA, U.S.A.) was used to

0378-4347/81/0000—0000/$02.50

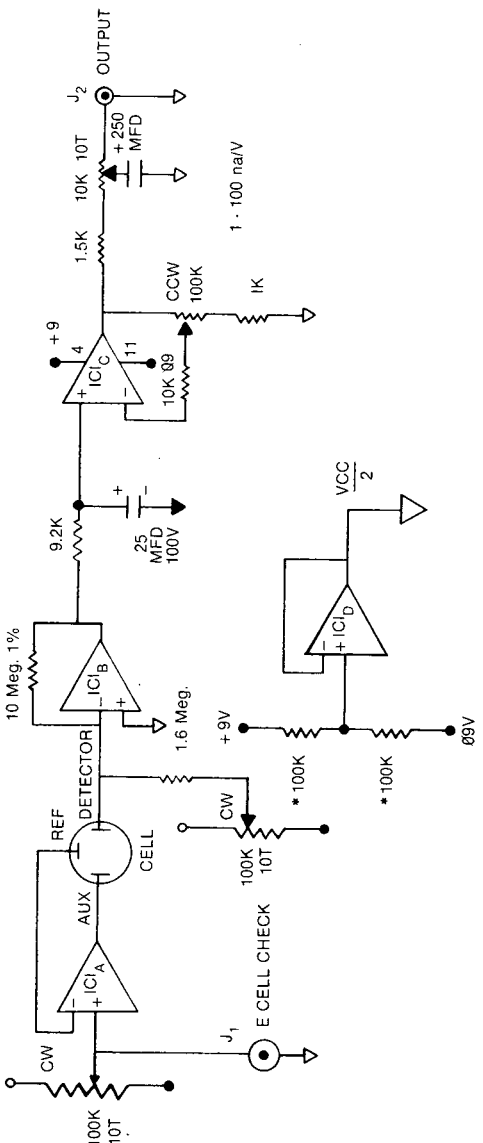


Fig. 1. Schematic diagram of the detector.

deliver the solvent through a 150 × 4.6 mm I.D. column of 5 μm average particle size of C_{18} silica gel (Altex) at a flow-rate of 1.2 ml/min. The samples were introduced through a 30-μl loop injector, Model 7120 (Rheodyne, Berkeley, CA, U.S.A.). The effluent was monitored at 275 nm using a UV detector, Model 785 (Micromeritics Instrument Corp., Norcross, GA, U.S.A.) followed by a thin-layer electrochemical cell (Bioanalytical Systems, West Lafayette, IN, U.S.A.).

Detector

The schematic diagram of the detector is illustrated in Fig. 1. The detector is powered by two 1.4-V mercury batteries and one 9-V alkaline battery. These batteries run for a few months. The detector has a linear range between 1—250 ng of 5-hydroxy-3-indoleacetic acid. The detector responds to as low levels as 0.5 ng of the latter compound.

Reagents

Extraction solvent: 15 ml ethyl acetate and 15 ml methanol were added to 70 ml chloroform.

Pump solvent: 20% acetonitrile in 15 m*M* phosphate buffer, pH 3.5.

Stock estriol standard: 100 mg estriol dissolved in 1000 ml methanol.

Procedure

Urine (100 μl) was buffered with 25 μl of 2 *M* phosphate buffer, pH 6.4 and hydrolyzed with 45 units of glucuronidase (Sigma, St. Louis, MO, U.S.A.) at 60°C for 30 min. After cooling the tubes 500 μl of the extracting solvent were added and the contents were vortex-mixed for 20 sec. The organic layer was separated from the aqueous layer by centrifugation at 9000 *g* for 30 sec. A 50-μl aliquot of the organic layer was removed into the evaporating vial and evaporated at 60°C without air. The contents of the vials were reconstituted with 100 μl of pump solvent and an aliquot of 25 μl was injected on the column.

RESULTS AND DISCUSSION

Estrogens contain a phenolic group, so they are expected to undergo oxidation under an applied potential. The separation and electrochemical detection of the three main estrogens is illustrated in Fig. 2. Estradiol and estrone do not elute from the column using the routine solvent of 20% acetonitrile in 15 m*M* phosphate buffer, pH 3.5 but require increasing the acetonitrile concentration up to 35%. The 16- and 17-epiestriol coelute with estriol; however, the level of epiestriol in urine is about 50-fold less than estriol. The oxidation potential of estriol is illustrated in Fig. 3. For routine use the oxidation potential was set at 0.9 V. Since the oxidation potential depends on many variables [6, 7], it needs to be determined for each instrument. The capacity factor for estriol (Fig. 4) was not affected with pH change.

Representative chromatograms of estriol assay by the present method are illustrated in Fig. 5. The assay is linear by the peak height method between 1—30 mg/l. The average recovery of 10 mg/l standard added to urine is 87%.

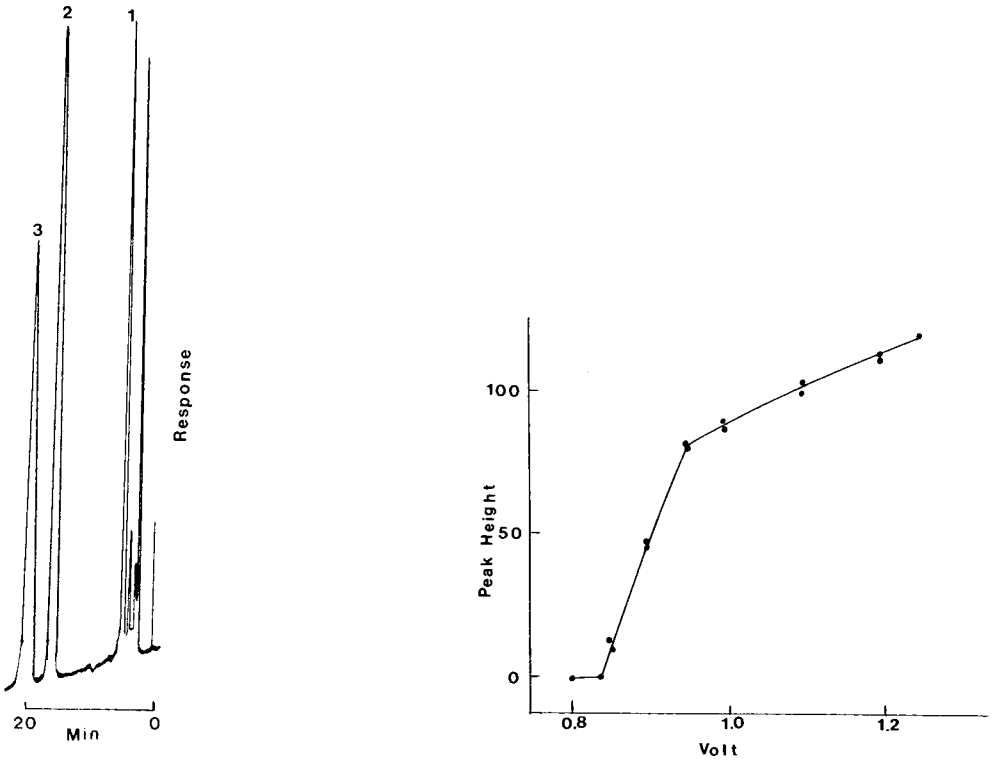


Fig. 2. Separation of estriol (1), estradiol (2) and estrone (3). Eluting solvent: 35% acetonitrile in 15 m*M* phosphate buffer, pH 3.5.

Fig. 3. Oxidation potential of estriol.

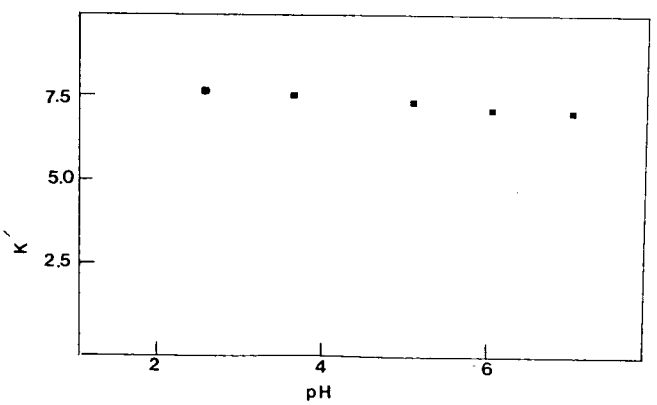


Fig. 4. Capacity factor, k', of estriol against pH.

Two samples with values of 13 and 19 mg/l by gas chromatography gave 12.6 and 16.6 mg/l, respectively, by the present method. Three urine samples from pregnant females — in the trimester — had estriol levels by the present method of 13.6, 14.1 and 17.5 mg/l, while 30 urines from males and non-pregnant

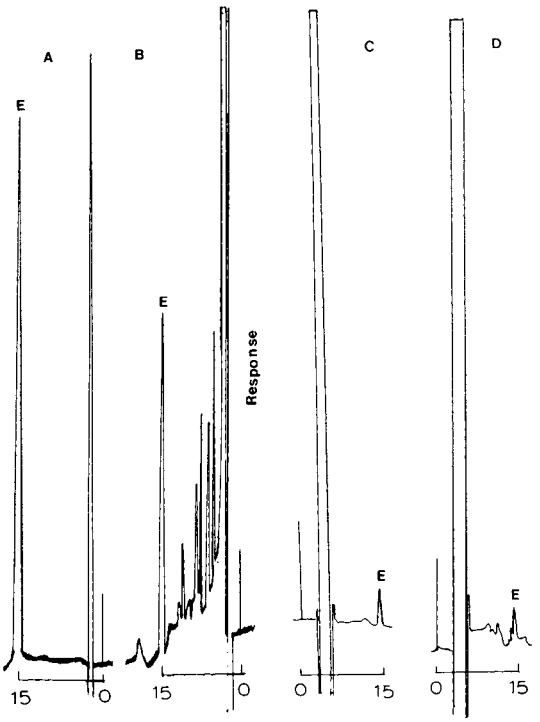


Fig. 5. Chromatograms of (A) estriol (E) standard, 10 mg/l by electrochemical detection; (B) estriol (E) extracted from the urine of pregnant female; electrochemical detection; (C) estriol (E) standard, 10 mg/l; UV detection, 275 nm, 0.010 absorbance; (D) estriol (E) extracted from urine of pregnant female same as (B); UV detection at 275 nm, 0.010 absorbance.

females had undetectable levels. Usually estriol in non-pregnant females is about 100-fold less than in pregnant females. If estriol is to be assayed from non-pregnant females larger urine samples, and more important, further additional clean-up steps are required.

Estriol assay by electrochemical detection is about 20-fold more sensitive than the UV detection at 275 nm (Fig. 5). Estriol concentrations as low as 0.2 mg/l can be assayed by this method. This high sensitivity allows smaller volumes of sample, solvent and enzyme to be used, thus greatly speeding up the extraction step. Column life is greatly extended. The column can be used for over 1000 injections provided that maintenance is carried out as previously described [8]. Although electrochemical detection is more sensitive than UV detection for estriol, the radioimmunochemical methods remain more sensitive than either of them.

The standard deviation for 15 within-run assays by this method is 0.52 with a mean of 11.4 mg/l and a coefficient of variance of 4.5%.

The battery-powered detector is easy to assemble and repair while operating free from line noise. It has similar sensitivity to the electrical one [7, 8]. When it is connected after a UV detector, it will increase the capability of the liquid chromatograph without extra expensive investment.

REFERENCES

1 J.B. Brown, Biochem. J., 60 (1955) 185.
2 J.B. Brown, C. Macnaughtan, M.A. Smith and B. Smyth, J. Endocrinol., 40 (1968) 175.
3 D.S. Young, L.C. Pestaner and V. Gibberman, Clin. Chem., 21 (1975) 294 D.
4 G.R. Gotelli, J.H. Wall, P.M. Kabra and L.J. Marton, Clin. Chem., 24 (1978) 2132.
5 T.H. Maurey and S. Siggia, Anal. Chem., 52 (1980) 881.
6 J.L. Morrisey and Z.K. Shihabi, Clin. Chem., 25 (1979) 2045.
7 Z.K. Shihabi and J. Scaro, Clin. Chem., 26 (1980) 907.
8 D.A. Richards, J. Chromatogr., 175 (1979) 293.

Journal of Chromatography, 224 (1981) 105—110
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 847

Note

Simultaneous determination of histamine and N^{τ}-methylhistamine in human urine and rat brain by high-performance liquid chromatography with fluorescence detection

YASUTO TSURUTA, KAZUYA KOHASHI and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

(First received October 31st, 1980; revised manuscript received January 27th, 1981)

Considerable attention has been given recently to the simultaneous determination of histamine and N^{τ}-methylhistamine [1] in tissues and body fluids. The profile of urinary histamine and N^{τ}-methylhistamine during pregnancy is of interest in connection with the secretion of estrogens and luteinizing hormone [2]. In brain, histamine is catabolized mainly by N-methyltransferase to produce N^{τ}-methylhistamine [3, 4] and has been reported as a neurotransmitter in the central nervous system [5].

Several methods have been reported for the simultaneous assay of the imidazole amines; for example, liquid chromatography after fluorescence derivatization with dansyl chloride [6] and gas chromatography after derivatization with heptafluorobutyrate or N,O-bis(trimethylsilyl)trifluoroacetamide [7].

o-Phthalaldehyde (OPT), which is popular as a fluorescence reagent for amino acids [8] and primary amines [9], reacts rapidly in the presence of 2-mercaptoethanol with histamine and methylhistamines to form fluorescent products. Perini et al. [10] have shown that an amino acid analyzer can be adapted to the determination of histamine and N^{τ}-methylhistamine, and of di- and polyamines by post-column derivatization with OPT.

In the present paper, a simple simultaneous determination of histamine and N^{τ}-methylhistamine by high-performance liquid chromatography (HPLC) (pre-column derivatization with OPT) with fluorescence detection has been developed and is applied to the assay of imidazole amines in human urine and rat brain.

0378-4347/81/0000—0000/$02.50

EXPERIMENTAL

Materials and reagents

Histamine was obtained from Wako (Osaka, Japan), N^{τ}- and N^{π}-methylhistamines were from Calbiochem (La Jolla, CA, U.S.A.) and Cellex-P was from Bio-Rad Labs. (Richmond, CA, U.S.A.). Other chemicals were the same as used in the previous paper [11].

Human urines were obtained from healthy volunteers in our laboratory. Rat brains were obtained from 5—7-week-old male rats (Donryu).

Reaction buffer. The pH of a borate buffer (0.05 *M* sodium tetraborate) was adjusted to 10.0 with 5 *M* sodium hydroxide. A reaction buffer was prepared by mixing 5 ml of the borate buffer, 5 ml of methanol and 0.1 ml of 0.5% (v/v) 2-mercaptoethanol in methanol.

Cellex-P column. Cellex-P (H^+; 0.9 mequiv./g) was purified as described by Kremzner and Wilson [12] with some modifications as follows. Cellex-P (5 g) was washed before use with successive 200-ml portions of 0.1 *M* hydrochloric acid, 1.0 *M* sodium chloride, water, 0.1 *M* sodium bicarbonate, water, 1.0 *M* sodium carbonate, water, 0.1 *M* sodium hydroxide, water, and finally 0.2 *M* sodium phosphate buffer (pH 6.0). Then the Cellex-P was suspended in 0.01 *M* sodium phosphate buffer (pH 6.0) and stored. When required for use, the Cellex-P suspension was poured on to a glass column (150 × 5 mm I.D.) to be 25 mm in height after settling. The column was washed two times with 1.0 ml of the phosphate buffer.

Apparatus

The HPLC system consisted of an Hitachi 635-A liquid chromatograph equipped with a universal injector and an Hitachi 650-10S spectrofluorimeter fitted with a flow-cell unit (cell volume, 18 μl) operating at an emission wavelength of 445 nm and an excitation wavelength of 335 nm. A stainless-steel column (150 × 4 mm I.D.) was packed with LiChrosorb RP-18 (particle size, 5 μm; Japan Merck, Tokyo, Japan) as previously described [11]. An Hitachi-Horiba M-7 pH meter and a Vapor mix S-10 test tube evaporator (Tokyo Rikakikai, Tokyo, Japan) were also used.

Procedure for urine

To urine (1.0 ml) taken in a 5-ml glass stoppered centrifuge tube spiked with N^{π}-methylhistamine (1 nmol) as an internal standard (IS), 5 *M* sodium hydroxide (0.25 ml), *n*-butanol (4.5 ml) and sodium chloride (0.4 g) were added. Histamine and N^{τ}- and N^{π}-methylhistamines were extracted into *n*-butanol with vigorous shaking for 5 min. The butanol layer was separated by centrifugation and a 4-ml portion of the layer was transferred to a glass stoppered test tube containing 0.1 *M* hydrochloric acid (0.5 ml) and benzene (4.0 ml). After 3 min of shaking, the organic layer was discarded.

To the acid layer, 2.5 ml of 0.01 *M* sodium phosphate buffer (pH 6.0) were added. The mixture was adjusted to pH 6.0 with 0.1 *M* sodium hydroxide. The mixture was applied to the Cellex-P column. The column was washed successively with 1.0 ml of the buffer (four times) and 1.0 ml of water. The amines were eluted with 1.2 ml of 0.12 *M* hydrochloric acid. The eluate was adjusted

to pH 8—10 with 0.5 *M* sodium hydroxide and then evaporated to dryness in vacuo at 50°C.

To the residue, 0.2 ml of the reaction buffer and 5 μl of 0.5% OPT in methanol were added and an aliquot (50 μl) of the reaction mixture was applied to the HPLC system. The mobile phase was a mixture of 0.07 *M* disodium hydrogen phosphate (pH 9.45) and methanol (47 : 53, v/v) and the flow-rate was 0.7 ml/min. The column temperature was ambient.

Procedure for rat brain

A portion (100—500 mg) of whole rat brain spiked with N^{π}-methylhistamine (1 nmol, as IS) was homogenized with 4.0 ml of ice-cold 0.4 *M* perchloric acid in a glass homogenizer. The mixture was centrifuged at 1200 *g* for 15 min. The supernatant (4.0 ml) was transferred to a glass stoppered test tube containing 5 *M* sodium hydroxide (0.5 ml), *n*-butanol (10.0 ml) and sodium chloride (1.5 g). The sample was extracted for 5 min with vigorous shaking. The *n*-butanol layer was separated by centrifugation and an 8.0-ml portion of the layer was transferred to a glass stoppered test tube containing 0.1 *M* hydrochloric acid (1.0 ml) and benzene (15.0 ml); the tube was then shaken vigorously for 3 min, followed by centrifugation (1200 *g*, ca. 4 min). After removal of the organic layer, a portion (0.4—0.9 ml) of the acid layer was transferred to a test tube, and then carried through the same procedure as for urine.

RESULTS AND DISCUSSION

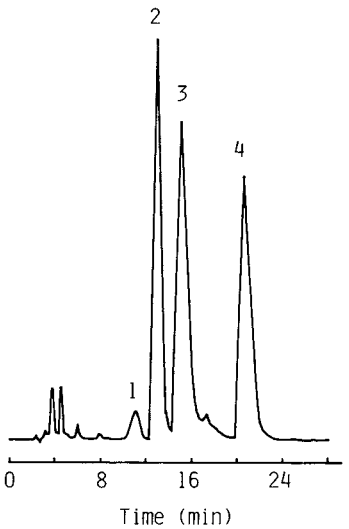
Histamine and N^{τ}- and N^{π}-methylhistamines exhibit fluorescence spectra with an excitation maximum at 335 nm and an emission maximum at 445 nm. The reaction of OPT and 2-mercaptoethanol with these imidazole amines was completed within 30 sec at room temperature and the fluorescent products (OPT derivatives) were stable for at least 60 min under the prescribed conditions [pH 10; solvent, a mixture of methanol and water (50 : 50, v/v)].

The OPT derivatives of histamine, and N^{τ}- and N^{π}-methylhistamines were completely separated with retention times of 13, 21 and 15 min, respectively; the peak at 11 min was due to ammonia occluded as a contaminant and the peaks at 3.5—8.0 and 17.5 min were caused by the reagent blank (Fig. 1).

The methanol content in the mobile phase had an effect on the retention times and the resolution of the OPT derivatives. At a methanol content higher than 60%, the peaks due to ammonia, histamine and N^{π}-methylhistamine closely overlapped, while a lower concentration of methanol (40%) caused a delay in elution with broadening of the peaks.

The pH of the phosphate solution in the mobile phase affected the capacity factors (k') of the OPT derivatives (Fig. 2). The maximum k' values were obtained at pH 7. Above pH 7, the elution pattern of the OPT derivatives remained unchanged, but the derivatives eluted earlier with increasing pH. Since the column packing degenerated irreversibly at pH values higher than 10, a sodium phosphate solution (pH 9.45) was employed to provide adequate resolution.

The concentration of the phosphate in the mobile phase influenced the peak widths of the OPT derivatives. At a phosphate concentration lower than 0.05



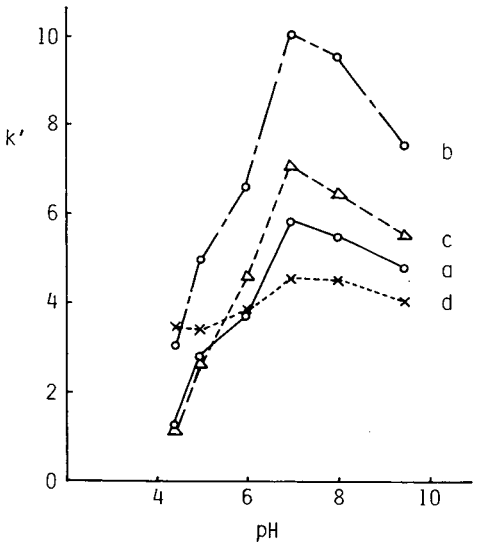


Fig. 1. Chromatogram of the reaction mixture. A mixture of histamine and N^{τ}- and N^{π}-methylhistamines ($1 \cdot 10^{-6}$ *M* each) was treated according to the procedure for urine. Peaks: 1 = ammonia; 2 = histamine; 3 = N^{π}-methylhistamine; 4 = N^{τ}-methylhistamine.

Fig. 2. Effect of pH of the phosphate solution in the mobile phase on the capacity factor (k') of the fluorescent derivatives of (a) histamine, (b) N^{τ}-methylhistamine, (c) N^{π}-methylhistamine and (d) ammonia.

M, the peaks of the derivatives tailed badly, and at concentrations higher than 0.1 *M*, the phosphate separated out in the mobile phase and choked up the HPLC column. Therefore, a concentration of 0.07 *M* was used.

The OPT derivatives of ammonia and some biological amines which elute near to those of the imidazole amines may interfere with the determination of the imidazole amines if present in the sample solution for HPLC. The interfering amines, which were not completely separated by the extraction procedure, could be removed by chromatography on the Cellex-P column. The imidazole amines adsorbed on the column were eluted completely with 0.12 *M* hydrochloric acid. To remove a small amount of contaminating ammonia, the eluate was adjusted to pH 8—10 and evaporated to dryness in vacuo. Care should be taken to avoid contamination with atmospheric ammonia during the assay.

N^{π}-Methylhistamine has not been found in the biological samples, and thus was conveniently used as an internal standard.

The ratios of the peak heights of histamine and N^{τ}-methylhistamine to that of N^{π}-methylhistamine (IS) were plotted against the amounts of each amine. The calibration curves thus obtained were linear up to at least 10 nmol/ml and passed through the origin.

The lower limits of determination (at a signal-to-noise ratio of 2) for histamine and N^{τ}-methylhistamine were 2.0 and 3.8 pmol/ml of urine, and 5.1 and 8.6 pmol/g of rat brain, respectively. Recovery tests were run for urine and rat brain adding known amounts of histamine and N^{τ}-methylhistamine (2 nmol/ml

each for urine and 2.5 nmol/g each for rat brain). Good recoveries (urinary histamine and N^{τ}-methylhistamine, 99 and 102%, respectively; rat brain histamine and N^{τ}-methylhistamine, 94 and 91%, respectively) were obtained.

The precision of the method was examined for histamine and N^{τ}-methylhistamine in urine containing 0.25 and 1.89 nmol/ml, respectively, and in rat brain containing 0.57 and 0.28 nmol/g, respectively. The coefficients of variation were 8.0 and 2.1% for urinary histamine and N^{τ}-methylhistamine (n = 10), respectively, and 7.0 and 10.7% for rat brain histamine and N^{τ}-methylhistamine (n = 4), respectively.

The amounts of histamine and N^{τ}-methylhistamine in the 24-h urines of 18 healthy persons (9 men, 24—48 years; 9 women, 21—23 years) determined by this method are shown in Table I. The concentrations of histamine and N^{τ}-methylhistamine in rat brain determined by this method were 0.62 ± 0.05 and 0.35 ± 0.05 nmol/g (mean ± S.D.), respectively. These values are in good agreement with published data [13—16].

TABLE I

AMOUNTS OF HISTAMINE AND N^{τ}-METHYLHISTAMINE IN 24-h URINES OF NORMAL PERSONS

Age* (years)	Histamine (μmol)	N^{τ}-Methylhistamine (μmol)
48 (m)	0.14	1.54
41 (m)	0.32	2.31
32 (m)	0.21	1.97
31 (m)	0.60	2.30
29 (m)	0.34	3.43
27 (m)	0.33	2.39
24 (m)	0.52	3.52
23 (m)	0.42	2.52
22 (m)	0.34	1.84
23 (f)	0.15	1.35
22 (f)	0.56	4.17
22 (f)	0.54	1.69
22 (f)	0.45	2.00
22 (f)	0.35	2.22
21 (f)	0.33	0.90
21 (f)	1.14	2.46
21 (f)	0.44	2.88
21 (f)	0.98	2.29
Mean ± S.D.	0.45 ± 0.25	2.32 ± 0.78

*m = male; f = female.

This method is sensitive, rapid and simple and should be useful for the simultaneous determination of histamine and N^{τ}-methylhistamine in biological samples.

ACKNOWLEDGEMENT

We thank Daiichi Pure Chemicals Co. Ltd. (Tokyo, Japan) for the generous gift of some reagents.

REFERENCES

1 J.N. Black and C.R. Ganellin, Experientia, 30 (1974) 111.
2 J.P. Green, D.H. Fram and N. Kase, Nature (London), 204 (1964) 1165.
3 W.P. Burkard, K.F. Gey and A. Pletscher, J. Neurochem., 10 (1963) 183.
4 J.C. Schwartz, Ann. Rev. Pharmacol. Toxicol., 17 (1977) 325.
5 J.C. Schwartz, Life Sci., 17 (1975) 503.
6 A. Yamatodani, T. Seki, M. Taneda and H. Wada, J. Chromatogr., 144 (1977) 141.
7 H. Navert, J. Chromatogr., 106 (1975) 218.
8 M. Roth, Anal. Chem., 43 (1971) 880.
9 N. Seiler and L. Demisch, in K. Blau and G.S. King (Editors), Handbook of Derivatives for Chromatography, Heyden & Son, London, Philadelphia, PA, 1978, p. 378.
10 F. Perini, J.B. Sadow and C.V. Hixson, Anal. Biochem., 94 (1979) 431.
11 Y. Tsuruta, K. Kohashi and Y. Ohkura, J. Chromatogr., 146 (1978) 490.
12 L.T. Kremzner and J.B. Wilson, J. Biol. Chem., 238 (1963) 1714.
13 J.A. Oates, E. Marsh and A. Sjoerdsma, Clin. Chim. Acta, 7 (1962) 488.
14 D.H. Fram and J.P. Green, J. Biol. Chem., 240 (1965) 2036.
15 L.T. Kremzner and C.C. Pfeiffer, Biochem. Pharmacol., 15 (1966) 197.
16 L.B. Hough, P.L. Stetson and E.F. Domino, Anal. Biochem., 96 (1979) 56.

Journal of Chromatography, 224 (1981) 111—115
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 850

Note

Enzymatic detection of urinary acidic 3α-hydroxysteroids on thin-layer chromatograms

YOSHIHISA YAMAGUCHI*, CHOZO HAYASHI and KIYOSHI MIYAI

The Central Laboratory for Clinical Investigation, Osaka University Hospital, Fukushima-ku, Osaka (Japan)

(First received December 31st, 1980; revised manuscript received January 27th, 1981)

It is well known that an abnormal metabolism of bile acids occurs in some liver diseases and a number of papers have been published so far [1—3]. Recently, carboxylic acid metabolites of steroids have also been reported by Bradlow and co-workers [4—6], and some aspects of these metabolites have been described. Four carboxylic acid metabolites of cortisol in man have been isolated and identified using [4-^{14}C]cortisol and an Amberlite XAD-2 column.

In this paper the enzymatic detection of these steroids carrying 3α-hydroxy and carboxylic acid groups is described. The principle of the method used [7] is as follows:

$$\text{Acidic 3}\alpha\text{-hydroxysteroid} + \text{NAD}^+ \xrightarrow{3\alpha\text{-HSD}} \text{acidic 3-ketosteroid} + \text{NADH}$$

$$\text{NADH} + \text{2-}p\text{-iodophenyl-3-}p\text{-nitrophenyl-5-phenyltetrazolium chloride} \xrightarrow{\text{diaphorase}} \text{formazan (pink color dye)}$$

MATERIALS AND METHODS

All reagents used were of analytical grade, and 3α-hydroxysteroid dehydrogenase (3α-HSD) and β-NAD were purchased from Nyegaard (Oslo, Norway).

Preparation of the enzyme color development reagent used has been described previously [7]. Briefly, dissolve 6 mg of 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride in 10 ml of 0.2 *M* K_2PO_4 (pH 8.5) containing (per 10 ml) 1 U of 3α-HSD, 50 U of diaphorase and 5 μmol of β-NAD.

0378-4346/81/0000—0000/$02.50

Preparation of acidic and neutral fractions

Urine (15 ml) is pipetted into a 40-ml tube and hydrolysis with β-glucuronidase and solvolysis are performed [7]. The ethyl acetate layer is then washed successively with 3 ml of concentrated sodium carbonate twice and water. After centrifugal separation, the ethyl acetate extract is transferred to a tube and the ethyl acetate evaporated (neutral fraction).

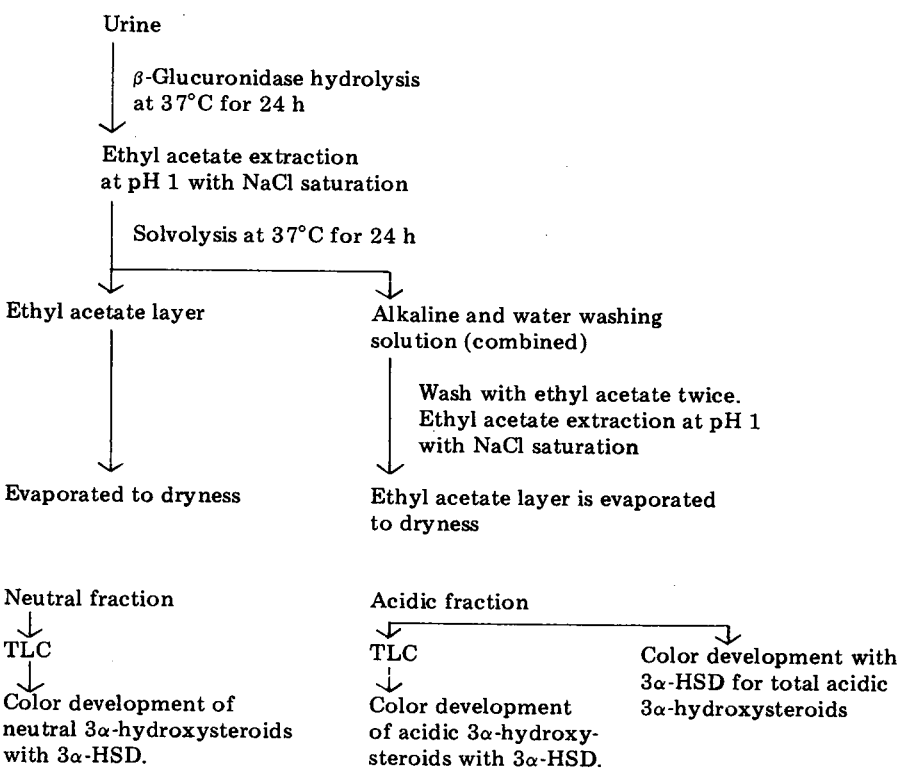
The alkali and water washes of the ethyl acetate extract are combined and the combined solution is acidified to pH 1 and sodium chloride added. Shake the solution with 20 ml of ethyl acetate for 5 min. After centrifuging, transfer 15 ml of the extract to a tube. Evaporate the ethyl acetate (acidic fraction).

Thin-layer chromatography

To the dry residue, a few drops of a chloroform and methanol mixture (1:1, v/v) are added, and the sample is applied to an activated thin-layer plate with marker dye and standards. The thin-layer plate (Kieselgel 60) is developed with a solution of chloroform—methanol (85:15, v/v) for 60 min at 20°C, to a distance of the front from the starting point of about 16 cm.

Color development of neutral 3α-hydroxysteroids and acidic 3α-hydroxysteroids was performed by the method described previously [7].

An outline of the method is shown in Scheme 1.



Scheme 1.

RESULTS

The selectivity of 3α-HSD for steroid molecules is shown in Table I, for which the intensity of the reaction with some steroids has been described in a previous paper [7].

TABLE I

SELECTIVITY OF 3α-HSD FOR SOME STEROIDS AND CHOLIC ACID

Each compound, at 0.1 μmol per tube, was incubated with 2 ml of enzyme solution at 37° C for 20 min.

Compound	Intensity of reaction (absorbance at 500 nm)
Cholic acid	0.555
Etiocholanolone	0.650
Androsterone	0.610
Tetrahydrocortisol Tetrahydrocorticosterone	0.717

Absorption curves of the dye formed by the enzymatic reaction with acidic fractions obtained from various urine samples are shown in Fig. 1.

The excretion pattern of acidic 3α-hydroxysteroids in a sample from a patient with adrenal disease (Cushing's disease) is shown in Fig. 2 together with the excretion pattern of neutral 3α-hydroxysteroids.

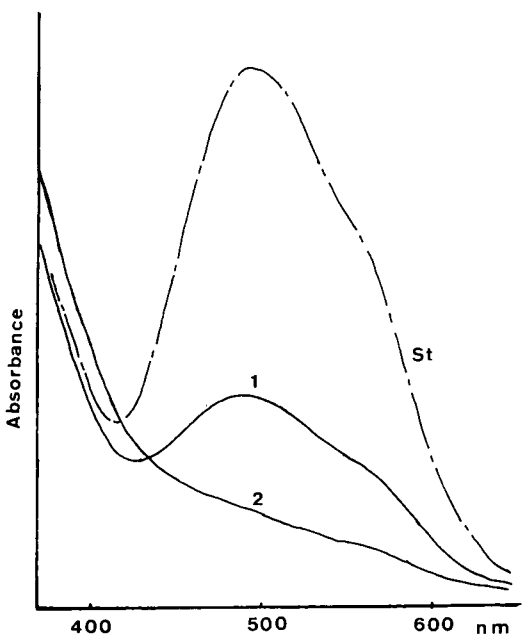


Fig. 1. Absorption curves of dye formed by reaction with the acidic fraction from urine. St = standard; 1 = Cushing's disease; 2 = normal subject.

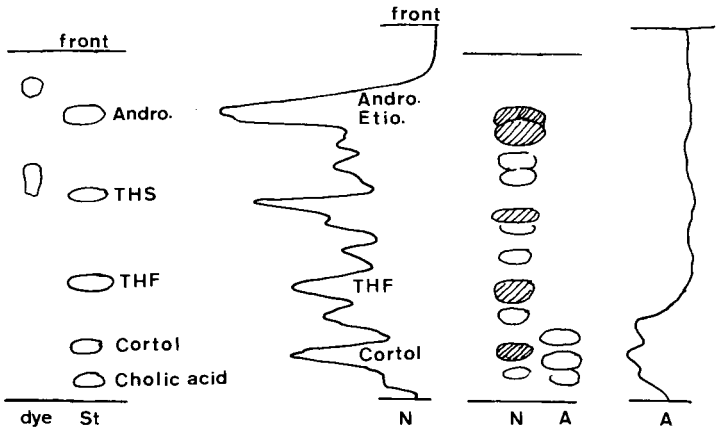


Fig. 2. Detection of acidic 3α-hydroxysteroids and neutral 3α-hydroxysteroids on thin-layer plates using a sample from a patient with adrenal disease. N = neutral fraction; A = acidic fraction.

For determination of total acidic 3α-hydroxysteroids, urine samples from cases of some adrenal and liver diseases and normal subjects (15 cases) were tested. The values obtained were 12.8 mg/day for Cushing's disease, 26.3 and 7.5 mg/day for hepatoma, 12.5 mg/day for some liver function disorder, and not detectable to 4.5 mg/day (N.D., below 0.5 mg/day) in 15 normal subjects. Assay of total acidic 3α-hydroxysteroids was performed by adding 2 ml of enzyme solution for color development to tubes containing the dry residues of the acidic fractions and incubating at 37°C for 30 min. Absorbance was read at 450, 500, and 550 nm and amounts calculated using Allen's formula.

The standard curve for acidic 3α-hydroxysteroids, obtained by submitting cholic acid to the whole procedure was linear for concentrations of cholic acid up to 200 μg.

DISCUSSION

Simple detection and determination of acidic 3α-hydroxysteroids in urine have been demonstrated using a sample from a patient with Cushing's disease. Acidic 3α-hydroxysteroids showed no elevated values in normal subjects by this method. This result seems to be reasonable because increased excretion of acidic steroids has not been reported [6].

This method can be used for detecting urinary bile acids, which are found in some liver diseases, as shown in Fig. 3. Many unidentified steroids and bile acids can easily be seen in an increased amount on chromatograms of neutral and acidic fractions. Use of a different solvent system for development of the thin-layer plates may give more details for diagnosing some metabolic abnormalities.

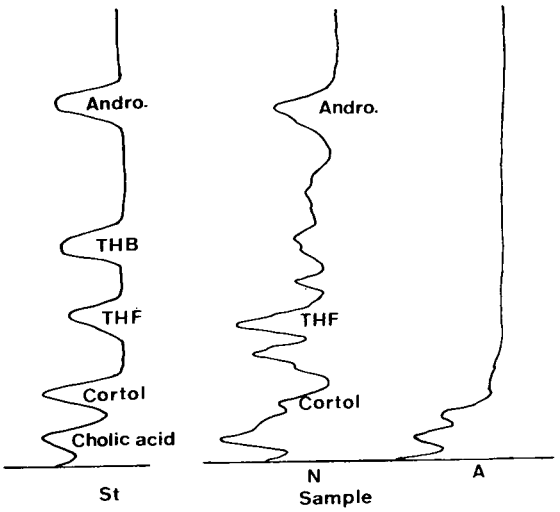


Fig. 3. Chromatograms of neutral and acidic fractions from a patient with a liver function disorder. N = neutral fraction; A = acidic fraction (may contain bile acids); St = standard.

REFERENCES

1 I. Makino, J. Sjövall, A. Norman and B. Strandvik, FEBS Lett., 15 (1971) 161.
2 I. Makino, K. Shinozuki and S. Nakagawa, Lipids, 8 (1973) 47.
3 B. Almé, A. Brennelgaard, J. Sjövall and P. Thomassen, J. Lipid Res., 18 (1977) 339.
4 H.L. Bradlow, B. Zumoff, C. Monder, H.J. Lee and L. Hellman, J. Clin. Endocrinol. Metab., 37 (1973) 811.
5 C. Monder, H.L. Bradlow and B. Zumoff, J. Clin. Endocrinol. Metab., 51 (1980) 312.
6 C.H.L. Shackleton, E. Roitman, C. Monder and H.L. Bradlow, Steroids, 36 (1980) 289.
7 Y. Yamaguchi, C. Hayashi and K. Miyai, J. Chromatogr., 182 (1980) 430.

Journal of Chromatography, 224 (1981) 116—121
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 846

Note

Analysis of trimethobenzamide in human saliva by gas chromatography—mass spectrometry

T.A. ROBERT*, A.N. HAGARDORN and E.A. DAIGNEAULT

Department of Pharmacology, East Tennessee State University, Johnson City, TN 37614 (U.S.A.)

and

R.D. BROWN

Department of Pharmacology, Louisiana State University Medical Center, Shreveport, LA 71130 (U.S.A.)

(First received November 12th, 1980; revised manuscript received January 26th, 1981)

In recent years there has been an increasing interest in the analysis of drugs in saliva. This interest has been inspired by the ease with which such non-invasively obtained samples can be collected and by the observation that many drugs are distributed in the saliva compartment in direct proportion to serum concentration [1—4]. For those drugs which do exhibit a proportional distribution between saliva and serum (and ultimately a relevant physiological compartment), analysis of their pharmacokinetic parameters allows a basis for comparing observed drug levels to therapeutic concentrations.

We report here a gas chromatography—mass spectrometry (GC—MS) analysis for an antiemetic drug, trimethobenzamide (TMB; N-[*p*-(2-dimethylamino-ethoxy)benzyl]-3,4,5-trimethoxybenzamide; 554-92-7; Tigan®, Beecham Laboratories, Bristol, TN, U.S.A.), in human mixed saliva specimens. In addition, we report upon the observed pharmacokinetics of this drug in the saliva of five healthy volunteers following an intramuscular dose of trimethobenzamide.

EXPERIMENTAL

Subjects

Five normal healthy adults volunteered to partake in the experiment, and informed consent was obtained from each. The subjects fasted from midnight the previous day until 2 h post medication, late the next morning. The experiment was initiated at time zero with an intramuscular injection of 200 mg TMB (Thera-Ject™ disposable syringes). Free flowing saliva specimens of approximately 2 ml were collected at time intervals of 0, 0.67, 1, 1.5, 2, 4, 6, and 8 h post drug injection. Saliva samples were stored at −60°C until analyzed. After the 2-h sampling period, all subjects were allowed to eat and drink.

Assay

Pure TMB·HCl for use as an analytical standard was a generous gift from Beecham Laboratories. Thioridazine·HCl (THZ), used as an internal standard, was obtained from Sandoz (Basle, Switzerland). Other reagents and spectroscopic grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.). Initially, GC—MS analytical conditions were established by using pure TMB and THZ dissolved in methanol. Analyses were performed using a Finnigan 4000 gas chromatograph—mass spectrometer with Incos data system. The chromatographic analysis was done on a 1.2 m × 2 mm I.D. glass column packed with 3% SE-30 on 100—120 mesh Supelcoport. The column oven was isothermal at 250°C and the injection port temperature was maintained at 260°C. Ultra-pure helium was used as the carrier gas at a flow-rate of 30 ml/min. The glass jet separator interface was held at 270°C. Electron impact (EI) mass spectra were recorded by multiscan monitoring of the GC effluent. The MS acquisition parameters were as follows: ion source temperature, 250°C; emission current, 0.25 mA; electron multiplier voltage, —1210 V; electron energy, 70 V. Multiscan parameters were set for masses 50—400 in a period of 1.95 sec with a hold time of 0.05 sec. The retention times of the test compounds were recorded and the base peak of each compound was chosen to determine the ion peak ratios used for TMB quantitation in saliva samples. The mass spectrometer was tuned using the calibration standard perfluorotributylamine (FC43, *m/e* peaks 69, 131, 219 and 264).

The following extraction procedure was employed for both unknown samples and saliva standards prepared with known amounts of TMB. To 1.0 ml saliva in a screw-capped PTFE-lined 15 × 120 mm glass tube were added 300 μl THZ internal standard (10 mg THZ base per l water). After the contents were mixed, 1 ml extraction buffer (1 *M* boric acid containing 1 *M* potassium chloride, titrated to pH 9.0 with 1 *M* sodium carbonate) was added and the tube contents were remixed. Diethyl ether (5 ml) was then added and the contents vigorously shaken for 2 min. The tubes were centrifuged at 1000 *g* for 5 min to separate phases. The organic phase was pipetted into a clean 50-ml glass centrifuge tube and set aside. The aqueous phase was re-extracted with a second 5 ml ether as before and the organic phase was added to that from the first extraction. The pooled organic phases were then evaporated to dryness under a stream of Zero Grade nitrogen (Air Products, Tamaqua, PA, U.S.A.) in a 60°C water bath. The dried residue was reconstituted with 25 μl methanol by vigorous vortexing for 30 sec. A 2-μl aliquot was then taken for injection into the gas chromatograph.

RESULTS

Standards

The EI mass spectrum of pure TMB is shown in Fig. 1. The base peak of *m/e* 58 was selected as the fragment for use in subsequent quantitative analyses. An example of a saliva standard reconstructed ion current (RIC) plot is shown in Fig. 2. THZ as internal standard exhibited a retention time of approximately 4.5 min and a base peak of *m/e* 98 [5], which was used in determining peak area ratios for TMB quantitation. The observed retention time for TMB was 6.2 min.

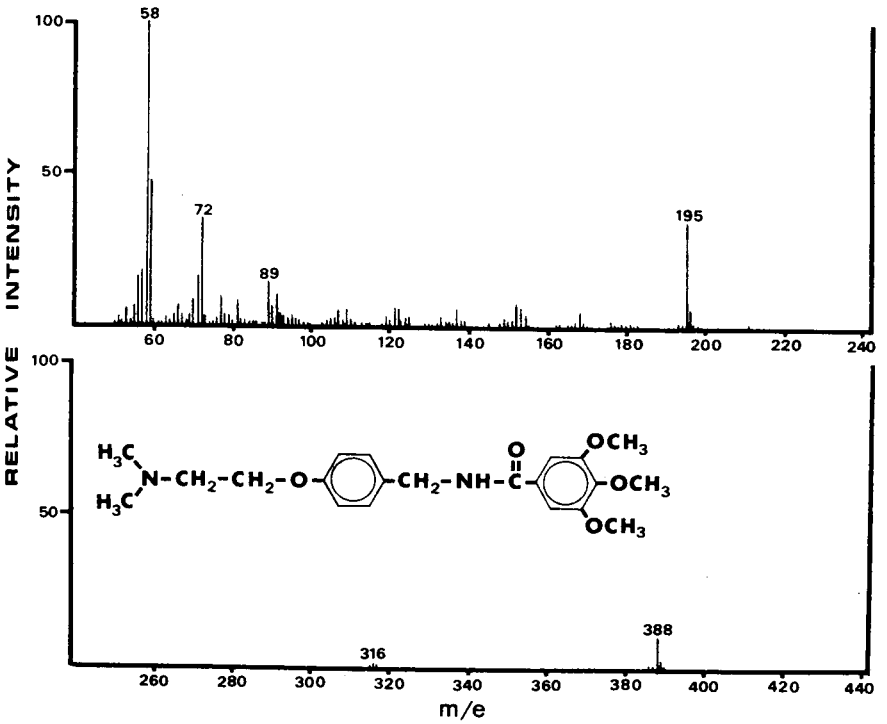


Fig. 1. EI mass spectrum of trimethobenzamide. The parent ion occurs at *m/e* 388, while the base peak at *m/e* 58 was used in quantitative analysis.

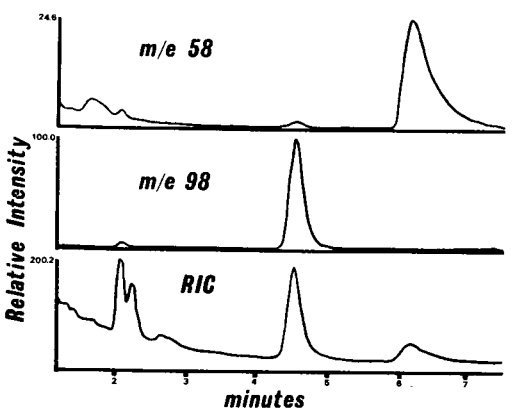


Fig. 2. Reconstructed ion current (RIC) plot and mass chromatograms of a 1 μg/ml TMB saliva standard. In the RIC tracing, the peak at 4.5 min retention time corresponds to the internal standard, THZ. The smaller peak with a retention time of 6.2 min is TMB. All peaks on the RIC plot represent all ions detected within the scanning range of 50—400 *m/e*. The two upper tracings show the *m/e* 58 (TMB) and *m/e* 98 (THZ) ion chromatograms, respectively, which were used in peak area ratio calculations.

A standard curve of base peak area ratios vs. known concentrations of TMB was prepared prior to analyses of the unknown specimens. Fig. 3 shows the curve used to quantitate TMB concentrations; the brackets indicate standard error of the mean from individual standards run each day the analysis was performed. Day-to-day precision of the 1 μg/ml saliva standard exhibited a coefficient of variation of 8% within observed base peak area ratios. Double ether extractions were used to increase the amount of TMB extracted from 40.5% with a single extraction to 60.6% of total TMB with two successive extractions. Ultimate sensitivity of the assay for TMB was not determined. However, for the lowest standard concentration (0.1 μg per ml TMB in saliva) the signal-to-noise ratio of the *m/e* 58 peak was typically greater than 63. The stability of TMB in solution and in normal saliva was checked and no deterioration of the drug was detected after one month at −60° C.

Pharmacokinetics

Saliva samples were analyzed and the observed concentrations were subjected to pharmacokinetic data analysis with the ESTRIP program of Brown and Manno [6]. Computerized fitting of the observed mean drug concentrations with sampling time interval produced the curve shown in Fig. 4. A two-compartment open model with linear absorption provides the polyexponential curve that best fits the data (r^2 = 0.956). Analysis of the function describing this curve provides the characteristic pharmacokinetic coefficients and ex-

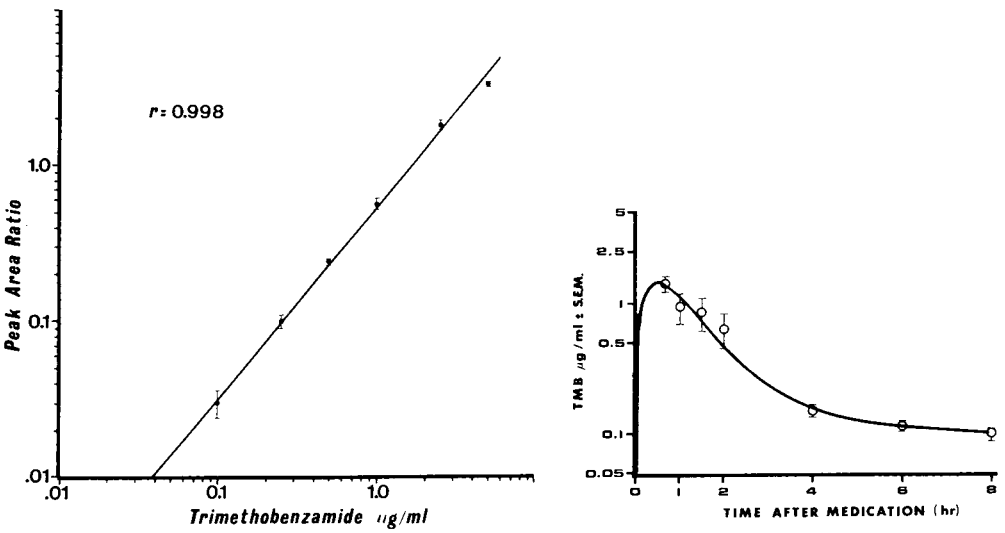


Fig. 3. TMB standard curve. The graph represents mean values ± S.E.M. of observed area ratios from known concentrations of TMB in saliva. The correlation coefficient (r = 0.998) indicates a high degree of linearity in the curve over the concentration range studied.

Fig. 4. Mean saliva TMB concentrations at each sampling interval. The open circles and associated brackets represent the mean ± S.E.M. saliva concentration of all subjects (n = 5). The curve through the concentration data points is the computer-generated polyexponential equation that best fits these mean concentration data.

ponents necessary for determining the drug distribution parameters. These calculated factors and the derived salivary half-lives of TMB are summarized in Table I. The elimination half-life for TMB from saliva appears to be 12.2 h.

TABLE I

MEAN SALIVARY PHARMACOKINETIC PARAMETERS OF TRIMETHOBENZAMIDE IN FIVE VOLUNTEERS AFTER A SINGLE 200-mg INTRAMUSCULAR INJECTION*

Subscript	Coeff. (A)	Exp. (B)	$t_{1/2}$**	Data points used***
1	0.157	0.057	12.2	2
2	3.941	1.231	0.56	4
3	−4.098	3.249	0.21	2
$r^2 = 0.956$ §				

*Based upon the following triexponential equation: $C_t = A_3 \exp(-B_3 t) + A_2 \exp(-B_2 t) + A_1 \exp(-B_1 t)$ ($B_1 = \beta$; $B_2 = \alpha$; $B_3 = k_{abs}$).

**Half-lives expressed in hours.

***Number of data pairs used in calculation of particular subscripted coefficient and exponent (counted backward beginning with last data).

§The r^2 value (squared correlation coefficient) provides an estimate of the fit between the calculated polyexponential curve and observed mean concentration—time values.

DISCUSSION

The current literature contains very few references [7—9] for the analysis of TMB. Indeed, there are no published accounts for the quantitative analysis of this drug in biological fluids. Since there are so little analytical data available regarding this clinically useful drug, our approach to its analysis by GC—MS, using the most fundamental methodology, was considered to be generally most useful. The use of THZ as an internal standard appears to be appropriate in this assay. While deuterated derivatives may possess some advantages as internal standards in GC—MS analyses, their lack of general availability can be a problem for other analysts. A phenothiazine, THZ, was selected for use since it is readily available and the likelihood of its concomitant use with TMB is small. Use of multiscan EI data acquisition in this assay is suitable because the therapeutic drug levels observed are sufficiently high to be easily detected by this type of GC—MS analysis and, because of the possibility of encountering this drug in cases of general drug screening (where EI is generally the instrumental mode employed), the spectrum and retention time reported may be of greatest interest. Derivatization of samples was unnecessary as the chromatographic analysis described was adequate for underivatized drugs, and drug screening techniques usually do not incorporate derivatizing steps. It must be pointed out, however, that our reported analytical technique is probably unsuitable for serum specimens since cholesterol and THZ have approximately equal retention times with our chromatographic conditions and therefore profound interference may be expected with quantitation based upon this internal standard. Some preliminary experiments in this laboratory indicate

that methoxypromazine may be a suitable internal standard for both serum and saliva samples. Using the assay conditions described here, we have found no interfering peaks due to the following clinically used antiemetics: prochlorperazine (Compazine®), thiethylperazine (Torecan®), trifluprom azine (Vesprin®), chlorpromazine (Thorazine®).

The pharmacokinetic analysis of mean saliva levels provides an estimate of the disposition of TMB in humans. Our reported analyses, in terms of both biological samples examined and kinetics, were far from exhaustive but were intended, rather, to provide some basic preliminary information regarding this drug. Extensive derivation of the more useful pharmacokinetic parameters awaits a more detailed and comprehensive examination of TMB disposition. There is a clear need for further study of TMB and we anticipate more fully characterizing the relationship between salivary and serum TMB kinetics at a later date.

ACKNOWLEDGEMENT

This work was supported in part by Beecham Laboratories, Bristol, TN, U.S.A.

REFERENCES

1 J.C. Mucklow, M.R. Bending, G.C. Kahn and C.T. Dolley, Clin. Pharmacol. Ther., 24 (1978) 563.
2 P.R.A. May, T. Van Patten, D.J. Jenden and A.K. Cho, Arch. Gen. Psychiat., 35 (1978) 1091.
3 G.J. DiGregorio, A.J. Pirano and E. Ruch, Clin. Pharmacol. Ther., 24 (1978) 720.
4 K.E.L. McColl, B. Whiting, M.R. Moore and A. Goldberg, Clin. Sci., 56 (1979) 283.
5 R. Ryhage and H. Brandenberger, Biomed. Mass Spectrom., 5 (1978) 615.
6 R.D. Brown and J.E. Manno, J. Pharm. Sci., 67 (1978) 1687.
7 K.W. Blessel, B.C. Budy and B.Z. Senkowski, in K. Florey (Editor), Analytical Profiles of Drug Substances, Vol. 2, Academic Press, New York, 1973, p. 551.
8 J.A. McIntyre, M. Black, M.E. McIntosh and J.K. Lynn, Clin. Chem., 24 (1978) 171.
9 H.G. Nowicki, J. Forensic Sci., 21 (1976) 154.

Journal of Chromatography, 224 (1981) 122—127
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 842

Note

Rapid and sensitive gas chromatographic method for the determination of alfentanil and sufentanil in biological samples

R. WOESTENBORGHS*, L. MICHIELSEN and J. HEYKANTS

Department of Drug Metabolism and Pharmacokinetics, Janssen Pharmaceutica Research Laboratories, B-2340 Beerse (Belgium)

(Received November 21st, 1980)

Alfentanil (AF), or N-{1-[2-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)-ethyl]-4-(methoxymethyl)-4-piperidinyl}-N-phenylpropanamide, and sufentanil (SF), or N-{4-(methoxymethyl)-1-[2-(2-thienyl)ethyl]-4-piperidinyl}-N-phenyl-propanamide, are two novel narcotic analgesics with structures similar to that of fentanyl. Both compounds are currently under clinical investigation in anaesthesiology [1, 2].

The purpose of the present paper is to describe a rapid, sensitive and specific gas chromatographic (GC) procedure for the determination of AF and SF in plasma and other biological samples. The method will be used for pharmacokinetic studies in animals and humans.

EXPERIMENTAL

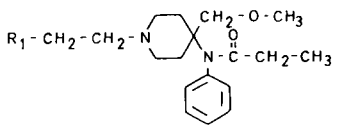
Standards and reagents

Alfentanil hydrochloride (R 39 209), sufentanil citrate (R 33 800) and the internal standard (R 38 527; IS) or N-{1-[3-(4-ethyl-4,5-dihydro-5-oxo-1H-tetrazol-1-yl)propyl]-4-(methoxymethyl)-4-piperidinyl}-N-phenylpropanamide hydrochloride hemihydrate were synthesized in our research laboratories and were of analytical grade. Chemical structures are shown in Fig. 1.

Spectrophotometric grade *n*-heptane and methanol were used; the isoamyl alcohol was of analytical grade. The Clin Elut™ tubes were analytically pure (CE 1003; Analytichem International, Lawndale, CA, U.S.A.).

Using the salt forms of AF, SF and IS, stock solutions were prepared in methanol corresponding to 1 mg/ml as the free base. Standard solutions were obtained by diluting the AF and SF stock solutions with methanol to a concentration range of 0.01—10 μg/ml. Internal standard solutions were prepared at a concentration of 1 μg/ml for IS, the internal standard for the determina-

0378-4348/81/0000—0000/$02.50



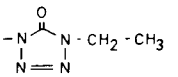
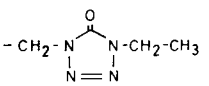
Compound	R_1
AF	
SF	
IS	

Fig. 1. Chemical structures of alfentanil (AF), sufentanil (SF) and the internal standard (IS).

tion of AF as well as for AF itself, which in turn was used as the internal standard for SF.

Apparatus

All the analyses were performed on a Varian Model 3700 gas chromatograph equipped with a thermionic specific detector, containing an electrically heated ceramic-alkali bead. The glass column (1 m × 3 mm I.D.) was packed with 3% OV-17 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The column, injector and detector temperatures were 290, 310 and 340°C, respectively. Nitrogen was used as a carrier gas at a flow-rate of 35 ml/min.

The detector was operated at a bias voltage of −4 V, the bead heating current was adjusted at 3 A and the hydrogen and air flow-rates were maintained at 4.5 and 175 ml/min, respectively, to ensure optimal detectability of the compounds. A Spectra-Physics Model 4000 data system was used for the integrations, the calculations and the plotting of the chromatograms.

Procedure

Extraction of plasma samples. To 1-ml aliquots of plasma, contained in 15-ml glass centrifuge tubes, were added 0.1 μg of the internal standard, 1 ml of 1 *M* sodium hydroxide and 4 ml of *n*-heptane—isoamyl alcohol (98.5: 1.5, v/v). The tubes were closed tightly with polyethylene stoppers, rotated for 10 min (25 rpm, Cenco rotary mixer) and centrifuged (5 min, 1000 *g*). The organic phase was carefully transferred to a second centrifuge tube by means of a disposable pasteur pipet and the plasma was extracted again with a 4-ml volume of the extraction solvent. The combined organic layers were back-extracted with 3 ml of 0.05 *M* sulphuric acid and removed after centrifugation. The acidic phase was made alkaline by the addition of 0.1 ml of concentrated ammonia and re-extracted twice with 2 ml of the heptane—

isoamyl alcohol mixture. The organic layers were combined into 5-ml glass test tubes and evaporated to dryness under nitrogen in a water bath of 60°C. The residues were reconstituted with 50 μl of methanol and 5 μl of the solutions were injected into the gas chromatograph.

Alfentanil standard curves were prepared by spiking blank human plasma with AF at concentrations ranging from 0.001 to 1 μg/ml, and with IS at a fixed concentration of 0.1 μg/ml. In the same way, sufentanil standard curves were prepared using 0.1 μg AF per ml as the internal standard. These samples were extracted and chromatographed as described above and the peak area ratios of AF and SF, relative to their corresponding internal standard, were plotted against the concentrations of AF and SF, respectively.

Preparation of tissue samples. Animal tissues were ground by means of a Waring commercial blender and homogenized (1:4, w/v) in distilled water using an Ultra-Turrax TP 18/10 homogenizer. To 2-ml volumes of these homogenates were added 0.1 μg of the internal standard (corresponding to 0.25 μg/g of tissue) and 2 ml of 1 *M* sodium hydroxide. The mixtures were extracted twice with 4 ml of the heptane–isoamyl alcohol mixture and re-extracted with 3 ml of 0.05 *M* sulphuric acid. The acidic phase was made basic with concentrated ammonia (pH 10) and added to the Clin Elut™ columns. Re-extraction into the heptane–isoamyl alcohol mixture was then achieved by adding two times 3 ml of extraction solvent to the extraction columns and allowing the organic phase to drip into 5-ml test tubes. The combined eluents were then evaporated to dryness, reconstituted with 50 μl of methanol and 5-μl aliquots were injected into the gas chromatograph.

Standard curves in tissues were prepared by spiking 2-ml aliquots of blank animal tissue homogenates with AF, SF and their corresponding internal standard as described for the plasma standard curves. The homogenates were then taken through the tissue extraction procedure described above to prepare the standard curves.

Urine samples. These were processed in the same way as the plasma samples.

RESULTS AND DISCUSSION

Typical chromatograms of a plasma extract from a rat treated with SF and of a heart tissue extract from a rat treated with AF are shown in Fig. 2. The retention times were 1.6, 2.7 and 3.6 min for SF, AF and IS, respectively.

Plasma extracts were pure and no interferences were noted. Extreme care must be taken not to withdraw any trace of the aqueous phase by aspirating the organic mixture in the last extraction step, since this results in interfering peaks and a severe loss of detector sensitivity.

In comparison to plasma, tissue extracts were more prone to the formation of emulsions, which obstructed the adequate removal of the organic phase. This problem was solved by using the Clin Elut™ disposable extraction columns, which resulted in clean extracts without noticeable loss of recovery.

The calibration curves for AF and SF, extracted from plasma and tissue were all linear in the range of the concentrations studied (Table I). The mini-

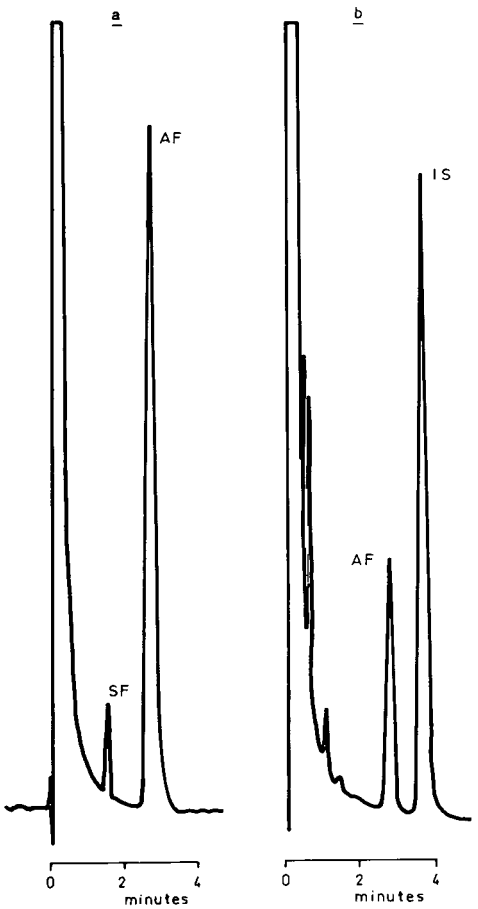


Fig. 2. Gas chromatograms of extracts from (a) rat plasma, 2 min after an intravenous dose of sufentanil (SF), spiked with AF as the internal standard, and (b) rat heart tissue, 8 min after an intravenous dose of alfentanil (AF), spiked with IS as the internal standard. GC conditions were as indicated in the text.

TABLE I

STANDARD CURVES FOR ALFENTANIL (AF) AND SUFENTANIL (SF) IN BIOLOGICAL SAMPLES

Compound	Sample	Internal standard (ng/sample)	Range (ng/sample)	Regression equation $y = ax + b$*		Correlation coefficient	
				a	b	r	n
AF	Plasma (1 ml)	100	1 —1000	1.046	—0.012	0.9998	11
	Tissue (1 g)	250	2.5— 500	1.071	—0.008	0.9998	9
SF	Plasma (1 ml)	100	1 — 100	0.628	+0.005	0.9994	5
	Tissue (1 g)	250	2.5— 250	0.650	—0.003	0.9996	8

*y = peak area ratios (AF/IS and SF/AF, respectively); x = concentration ratios (AF/IS and SF/AF, respectively).

mum detectable amount of AF and SF was 1 ng/ml of plasma and 2 ng/g of tissue. The reproducibility was checked by analyzing samples of different animal tissues (liver, kidney, pancreas, fat) spiked with several concentrations of alfentanil. The results are shown in Table II. The recovery over the

TABLE II

REPRODUCIBILITY DATA FOR THE DETERMINATION OF ALFENTANIL (AF) IN ANIMAL TISSUES

Added (ng/g)	Found, mean ± S.E.M.* (ng/g)	S.E.M. (%)
12.5	14.6 ± 1.4	9.6
25	25.5 ± 1.7	6.7
50	48.2 ± 1.4	2.9
125	118 ± 2.2	1.9
250	248 ± 10	4.0
500	503 ± 24	4.8

*S.E.M. = standard error of the mean (n = 4).

concentration range studied was 89± 4% (mean ± S.D., n = 6) and the precision was 3.0%.

The suitability of the method for AF was demonstrated by the analysis of plasma samples from a patient after an intravenous dose of 0.125 mg al-

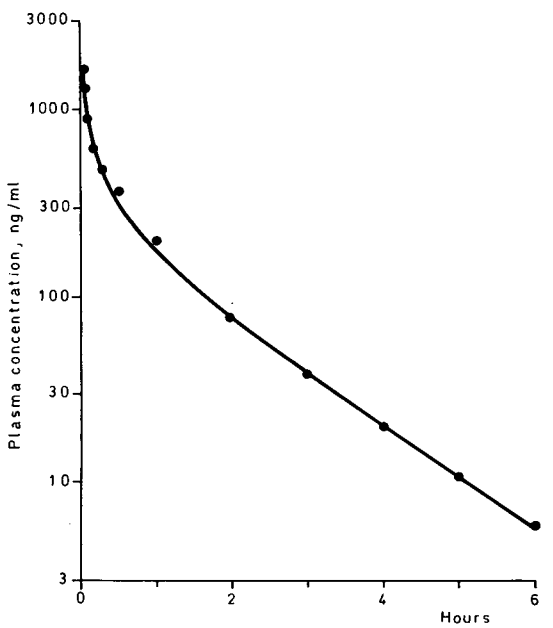


Fig. 3. Plasma levels of alfentanil (AF) in a patient after an intravenous dose of 0.125 mg/kg body weight.

fentanil per kg body weight. Plotting the AF plasma levels on a semilogarithmic scale (Fig. 3) permits the estimation of the biological half-life of the drug. It appears to be about 65 min.

REFERENCES

1 W.F.M. Van Bever, C.J.E. Niemegeers and P.A.J. Janssen, J. Med. Chem., 17 (1974) 1047.
2 J. De Castro, A. Van de Water, L. Wouters, R. Xhonneux, R. Reneman and B. Kay, Acta Anaesthesiol. Belg., 30 (1979) 5.

Journal of Chromatography, 224 (1981) 128—132
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 840

Note

Gas—liquid chromatographic procedure with alkali flame ionization detection for the determination of maprotiline in plasma

C. CHARETTE and I.J. McGILVERAY*

Drug Research Laboratories, Health Protection Branch, Health & Welfare Canada, Tunney's Pasture, Ottawa K1A 0L2 (Canada)

and

K.K. MIDHA

College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 (Canada)

(First received October 20th, 1980; revised manuscript received January 8th, 1981)

Maprotiline is a tetracyclic antidepressant drug with structural similarities to the tricyclic antidepressants. Steady-state plasma concentrations of 50—550 ng/ml were observed when 150 mg was given daily as reported by Riess et al. [1]. Several analytical procedures have been employed for the estimation of maprotiline in biological specimens such as the double radioisotope derivative technique by Riess [2], the gas—liquid chromatographic (GLC) procedures of Geiger et al. [3], who employed the electron-capture detector, and Gupta et al. [4] who used a nitrogen-specific detector, and recently the GLC—mass spectrometric (MS) assay of Alkalay et al. [5] who used selected ion monitoring in the chemical ionization mode with isotopically labelled internal standard.

A GLC procedure using an alkali flame ionization detector (AFID) was recently developed in our laboratories [6] for the determination of imipramine and desipramine. This method has been applied successfully to the determination of maprotiline from plasma and is described herein.

EXPERIMENTAL

Instrumentation

A gas chromatograph (Hewlett-Packard Model 5730A) equipped with an alkali flame ionization detector was used. The 1.83 m × 2 mm I.D. coiled-glass column was packed with 5% OV-17 on Gas-Chrom Q (100—120 mesh). The

0378-4347/81/0000—0000/$02.50

column was conditioned at 315°C for 48 h with a gentle flow of nitrogen and then the oven temperature was maintained at 255°C. Injection port and detector temperatures were 300°C while the detector bead current was adjusted for maximum sensitivity. Helium, was maintained at a flow-rate of 30 ml/min and the air and hydrogen flow-rates were adjusted for optimum sensitivity.

Extraction procedure

An analytical method used for tricyclic drugs [6] has been adapted for maprotiline. To a 0.5—2.0-ml plasma sample (control, spiked or from a dosed subject) were added 0.5 ml of an aqueous solution of the internal standard (desipramine) and 0.5 ml of a borate buffer solution (pH 9). The sample was extracted twice with 4-ml portions of cyclohexane. The combined organic layers were back extracted with a 2% methanolic solution of 1 *N* hydrochloric acid. After evaporation of the methanolic layer, the residue was redissolved in 50 μl amyl acetate and reacted for 2 min at 85°C with 10 μl acetic anhydride. The mono-N-acetyl derivatives of maprotiline and desipramine were analysed by GLC—AFID.

Standard solutions

Stock solutions of maprotiline and desipramine (100 μg base per ml) were prepared daily by dissolving their hydrochloride salts in distilled water. Appropriate dilutions in the concentration range required for the maprotiline calibration curve were prepared in control blank plasma using the stock solution. The internal standard working solution was prepared by diluting the stock solution of desipramine with distilled water to a concentration of 400 ng/ml.

Calibration curve

Peak height ratios were calculated by dividing the height of the peak due to mono-N-acetylmaprotiline by the height of the peak due to mono-N-acetyldesipramine, the internal standard. Calibration curves were assembled from results of spiked plasma samples by plotting the peak height ratios against the concentrations of the drug.

RESULTS AND DISCUSSION

When maprotiline and desipramine standards were derivatized with acetic anhydride and submitted to GLC—AFID analysis, sharp, symmetrical peaks were obtained, eluting at 11.5 and 9.8 min respectively. The authenticities of the derivatives thus formed were confirmed by GLC—MS to be the mono-N-acetyl derivatives of maprotiline and desipramine. Blank and spiked plasma samples were extracted, derivatized and submitted to GLC—AFID analysis. A typical chromatogram of blank plasma is shown in Fig. 1A and no peaks were seen beyond the 6-min retention time. The spiked plasma sample was analysed according to the procedure where the two acetyl derivatives eluted at the same retention times as the standards (Fig. 1B). When MS analysis was performed on these eluted peaks they were found to be identical to the authentic mono-N-acetyl derivatives, with no interferences from endogenous materials.

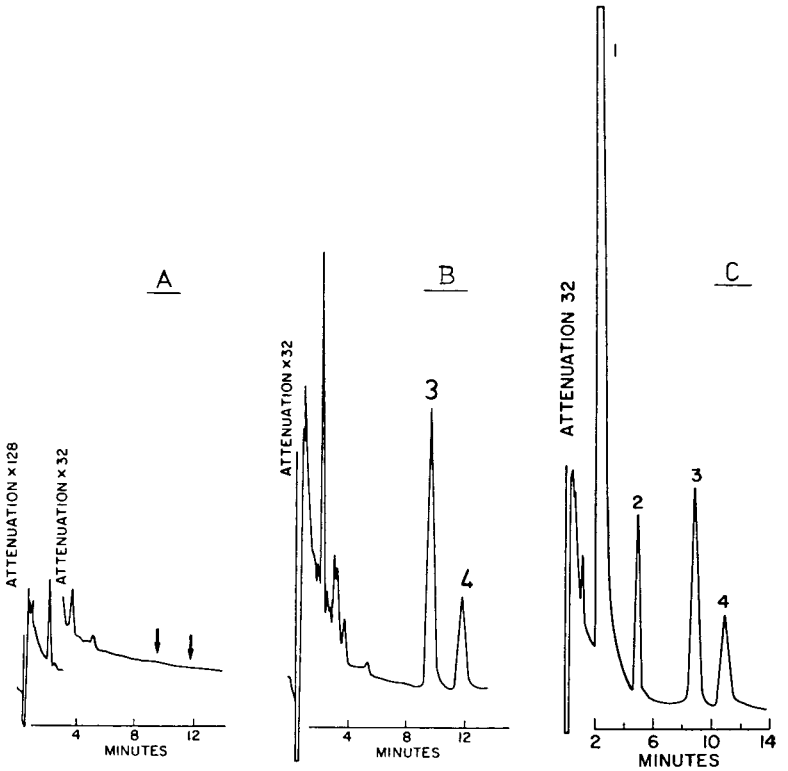


Fig. 1. Chromatograms of (A) blank plasma; (B) spiked plasma containing 200 ng of the internal standard desipramine, and 76.4 ng/ml of maprotiline (1 ml plasma) and (C) plasma from a patient overdosed on maprotiline (2 ml plasma were used and the concentration estimated was 50.3 ng/ml for maprotiline; 200 ng internal standard). Peaks: 1 = tris(2-butoxyethyl) phosphate (contaminant); 2 = unidentified; 3 = mono-N-acetyldesipramine; 4 = mono-N-acetylmaprotiline.

Typical calibration curves over the range 10—225 ng/ml gave a coefficient of determination, $r^2 = 0.997$ for the equation $Y = a + bX$ (where $a = 0.0193$, $b = 0.0041$ and $N = 32$). A calibration curve was run with every set of unknown samples.

The overall recoveries of maprotiline at 25, 75 and 150 ng/ml concentrations in plasma were 53.3, 51.4 and 50.7% with an overall relative standard deviation of 5.6%.

This method has been applied to plasma level determination for a patient suffering from a maprotiline overdose. The patient, a 67 year old male, was brought to the emergency unit in coma. During emergency treatment, including hemoperfusion, blood samples were taken and it was possible to monitor maprotiline plasma concentration for thirteen days following ingestion.

The authenticity of maprotiline was confirmed by GLC—MS. The highest maprotiline concentration attained was 449 ng/ml at the initiation of the hemoperfusion and this fell to ca. 300 ng/ml at the end of hemoperfusion; levels of ca. 28 ng/ml were found on days 12 and 13. The levels determined

were obtained from at least duplicate analysis of each sample (Table I).

A typical chromatogram of the patient's plasma sample is shown in Fig. 1C. Peak 1, eluting at 2.2 min, was due to tris(2-butoxyethyl) phosphate, a contaminant from the rubber stopper of Vacutainers. Peak 2, with a 5.0-min retention time, was not identified. Peaks 3 and 4 were due to mono-N-acetyldesipramine and mono-N-acetylmaprotiline, respectively. Other cases have also been monitored.

TABLE I

MAPROTILINE PLASMA LEVELS ESTIMATED

Time after suspected overdose		Concentration of maprotiline in plasma (ng/ml)
Hours	days	
40.0*	1.67	300.2
41.7	1.74	449.0
42.5	1.77	341.0
44.0	1.83	385.5
46.0	1.92	304.4
47.5	1.98	300.6
48.0**	2.00	
78.5	3.27	417.2
116.5	4.85	330.6
126.5	5.27	193.9
150.5	6.27	214.1
174.5	7.27	280.4
246.5	10.27	50.3
294.5	12.27	28.0
318.5	13.27	27.5

*Beginning of hemoperfusion.
**End of hemoperfusion.

The GLC—electron-capture detection procedure of Geiger et al. [3] is long (ca. 3 h) and tedious. Precautions have to be taken with lower plasma concentrations (less than 50 ng/ml) because of the electron-capture detection linearity response. The procedure of Gupta et al. [4], linear over the range 50—400 ng/ml, requires the use of silanized glassware, an initial washing step before extracting the drug, with additional drying being necessary to remove any pyridine traces. Use of the GLC—MS method of Alkalay et al. [5], although specific and sensitive (0.5 ng/ml), would be limited because the expensive sophisticated instrumentation is not readily available in all laboratories. For these reasons, an improved GLC procedure employing an AFID, which is simple, sensitive, specific and rapid, has been developed in our laboratories.

CONCLUSION

The described analytical procedure has been applied successfully to the determination of maprotiline from plasma with a detection limit of 2 ng/ml.

The method is applicable to therapeutic monitoring of the drug.

REFERENCES

1 W. Riess, L. Dubey, E.W. Fünfgeld, P. Imhof, H. Hürzeler, N. Matussek, T.G. Rajogopalan, F. Raschdorf and K. Schmid, J. Int. Med. Res., 3 (1975) 16.
2 W. Riess, Anal. Chim. Acta, 68 (1974) 363.
3 U.P. Geiger, T.R. Rajagopalan and W. Riess, J. Chromatogr., 114 (1975) 167.
4 R.N. Gupta, G. Molnar and M.L. Gupta, Clin. Chem., 23 (1977) 1849.
5 D. Alkalay, S. Carlsen, L. Khemani and M.F. Bartlett, Biomed. Mass Spectrom., 6 (1979) 435.
6 K.K. Midha, C. Charette, J.K. Cooper and I.J. McGilveray, J. Anal. Toxicol., 4 (1980) 237.

Journal of Chromatography, 224 (1981) 133—138
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 838

Note

Quantitative analysis of the cholinesterase inhibitor paraoxon in brain tissue using high-performance liquid chromatography

J.H. DE NEEF*, A.J. PORSIUS and H.H. VAN ROOY

Department of Pharmacy, University of Amsterdam, Plantage Muidergracht 24, Amsterdam (The Netherlands)

(First received October 14th, 1980; revised manuscript received January 13th, 1981)

In order to study pharmacokinetics and to obtain more information about the site of action of drugs, the availability of sensitive, precise and accurate analytical methods for the quantitative determination of drugs is essential. Various analytical methods have been developed for the analysis of small amounts of organophosphorus insecticides like parathion and its structurally related compound paraoxon (diethyl-4-nitrophenylphosphate, Fig. 1).

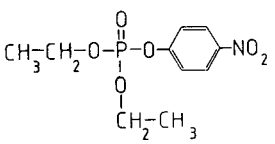


Fig. 1. Chemical structure of paraoxon.

Gas chromatography [1], using phosphorus-selective detection [2, 3], and high-performance liquid chromatography (HPLC) using UV detection [4], polarographic detection [5] or fluorimetric detection [6], have proved to be pre-eminently suitable for the analysis of residues in food.

Until now no HPLC method for the detection of the cholinesterase inhibitor paraoxon in animal tissues has been developed. In the present study a sensitive method for the determination of paraoxon in the presence of its hydrolysis product 4-nitrophenol in brain tissue of the cat using HPLC is described. In addition, concentrations of paraoxon in various brain tissues are presented after intravenous administration of various doses of paraoxon into the cat.

0378-4347/81/0000—0000/$02.50

EXPERIMENTAL PROCEDURES

Apparatus

The liquid chromatograph was custom-made and comprised a constant-flow pump (Varian 8500), a high-pressure injection valve (Valco) equipped with a 250-μl sampling loop, and a variable-wavelength UV detector (Pye Unicam LC3). The wavelength was set at 274 nm. All chromatograms were recorded on a linear potentiometric recorder (Goerz, Servogor). In all experiments stainless-steel 316 columns with dimensions 125 × 3 mm were used.

Materials

In all experiments double-distilled water was used. All solvents and chemicals were of analytical grade and used without further pretreatment. The column support was silica gel Si 60 (Merck, Darmstadt, G.F.R.) with a mean particle size of 5 μm. Paraoxon was obtained from Sigma, St. Louis, MO, U.S.A.

Chromatography

The HPLC columns were packed by a pressurized balanced slurry method. The slurry liquid consisted of a mixture of tetrabromoethane and chloroform of specific gravity of 2.09 for the bare silica (Si 60). After packing, the columns were washed with 200 ml of methanol and subsequently equilibrated with the eluent until constant retention of the solutes was obtained. Standard solutions of the drug and its metabolite (4-nitrophenol) were prepared from stock solutions of the compounds in methanol and were stored at 4°C. The mobile phase used for the analysis of paraoxon in extracts of brain tissue consisted of *n*-hexane containing 4% 2-propanol. The flow-rate was 60 ml/h.

Administration of drugs

Mongrel cats of either sex (weight 2—4 kg) were anaesthetized with α-glucochloralose (60 mg kg^{-1}). A femoral vein was cannulated for the intravenous administration of paraoxon. The trachea was cannulated for artificial respiration. After left-sided thoracotomy the aorta was ligated. Two or 10 min after dosing the animals were killed by occluding the aorta. The brain was removed and various brain regions were isolated on ice.

Sample preparation

The samples were homogenised with 0.1 *M* phosphate buffer (pH = 8.0) in appropriate glass tubes with Teflon pads at 0°C. For each 100 mg of tissue 1.0 ml buffer was added. After the addition of 0.2 ml of 2.5 *M* perchloric acid, the sample was diluted to 5.0 ml with 0.1 *M* perchloric acid. The mixture was homogenised and centrifuged at 2000 *g* for 15 min. Four millilitres of the clear supernatant were combined with another 4 ml of supernatant obtained after suspending and centrifuging the remaining pellet in 5.0 ml of 0.1 *M* perchloric acid. For the extraction of paraoxon the combined aqueous solutions (total volume 8.0 ml) were shaken vigorously with 2.0 ml of benzene in a glass stoppered centrifuge tube by means of a Vortex test-tube mixer. After centrifugation at 900 *g* for 5 min, the sample loop was filled with 250 μl of the organic layer.

RESULTS AND DISCUSSION

Chromatography

The retention behaviour of paraoxon and its metabolite 4-nitrophenol in liquid chromatography has been described for straight-phase [7] and reversed-phase systems [8, 9]. Of these two systems reversed-phase chromatography in combination with extraction and evaporation of the organic extraction fluid is not suitable for the analysis of low concentrations of these volatile solutes in aqueous solutions. Straight-phase liquid chromatography has also been described for a number of related organophosphorus insecticides [10, 11] in which hexane, containing 2-propanol or ethanol as the organic modifier, is used as the mobile phase. The main advantages of straight-phase chromatography are very stable separation systems and the compatibility of the system with organic extraction fluids. As can be seen from Fig. 2, a plot of $1/k_i'$ against percentage organic modifier yields a linear relationship which is commonly found in straight-phase systems. Excellent selectivity is obtained as is demonstrated by the test chromatogram of a solution containing paraoxon, 4-nitrophenol and benzene (Fig. 3).

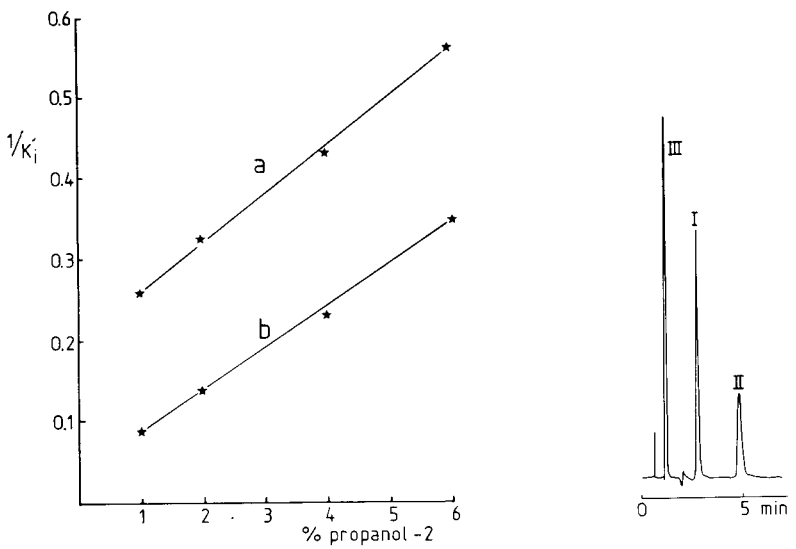


Fig. 2. Influence of the 2-propanol concentration in the mobile phase on the capacity ratio (k_i'). Stationary phase, Si 60. a = paraoxon; b = 4-nitrophenol.

Fig. 3. Separation of the solutes paraoxon (I), 4-nitrophenol (II) and benzene (III) in a test mixture.

Quantitative aspects

The precision and linearity of the method were determined by injecting 250 μl of solutions of paraoxon and 4-nitrophenol in different concentrations. The linear regression of peak height versus injected amount yields a correlation coefficient of 0.9993 for both solutes which indicates a high degree of linearity. The precision of the method was estimated by injecting solutions

of the solutes at high and at low concentration levels (n = 6). For both solutes the standard deviations for 250 ng and 25 ng were 1.45% and 1.82%, respectively. The peak-to-peak value of the baseline noise was determined as $5 \cdot 10^{-5}$ a.u. This leads to a calculated limit of detection for both solutes of 1.5 ng for a signal-to-noise ratio of 3. Consequently, for an injection volume of 250 µl the calculated limit of detection amounts to 12 ng of paraoxon per 100 mg of brain tissue.

Typical chromatograms were obtained from a blank brain tissue sample (Fig. 4a) and a sample obtained from a cat which received paraoxon (Fig. 4b). The possible degradation of paraoxon was studied by incubating the drug for 24 h in phosphate buffer (pH 7.4) at 37°C and in the presence of cat brain homogenates (at pH 7.4) at 37°C. The results are presented in Fig. 5. No chemical hydrolysis occurs, whereas a transient reduction of intact paraoxon can be observed under the influence of brain tissue. The degradation half-life in brain tissue homogenates was calculated to be 168 min.

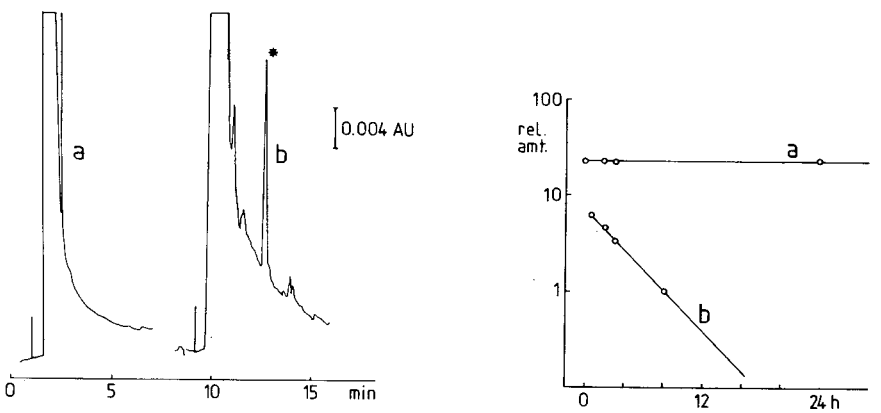


Fig. 4. Chromatograms of a blank brain tissue sample (a) and of a paraoxon(*)-containing tissue sample obtained after a pharmacological experiment (b). Mobile phase: 4% 2-propanol in n-hexane. Stationary phase: silica gel Si 60. Flow-rate: 60 ml/h.

Fig. 5. Semilogarithmic plot of the degradation of paraoxon in (a) phosphate buffer (pH 7.4, 37°C) and (b) in a brain tissue homogenate (pH 7.4, 37°C).

Brain concentrations in vivo

The developed method for the quantitative analysis of paraoxon was applied to brain tissue samples of cats which received paraoxon intravenously. Thus, the animals were given 550, 275 or 150 µg kg^{-1} via a femoral vein. Drug concentrations were measured in the medulla oblongata, pons and hypothalamus 2 and 10 min after dosing. The results are presented in Table I. With respect to the drug concentrations in the various brain parts, it should be emphasized that the relatively high standard errors of the mean are due to biological differences (blood pressure, blood flow) between the animals. Similar standard deviations were found after intravenous administration of radioactive compounds [12]. From Table I it is obvious that the amount of paraoxon decreases when lower doses are applied. Moreover, upon administration of 275 and 150 µg of paraoxon, equal concentrations are measured in

TABLE I

PARAOXON (Px) CONCENTRATIONS AND ACETYLCHOLINESTERASE (AChE) INHIBITION IN VARIOUS BRAIN REGIONS, 2 AND 10 min AFTER THE INTRAVENOUS ADMINISTRATION OF 550 μg, 275 μg and 150 μg OF PARAOXON PER kg
Values are presented as mean ± S.E.M. *n* represents the number of experiments.

Dose (μg/kg)	Brain part*	*t* = 2 min				*t* = 10 min			
		ng Px per brain part	ng Px per g tissue	AChE inhib. (%)	*n*	ng Px per brain part	ng Px per g tissue	AChE inhib. (%)	*n*
550	M	5076±386	3487±265	100±1	5	2054±356	1314±228	99±1	6
	P	2232±102	4501±206	100±1	5	964±174	1944±350	99±1	6
	H	1668±106	3691±235	99±1	5	415± 88	976±206	99±1	6
275	LM	937±164	1207±225	99±1	4	197± 24	270± 33	96±1	4
	RM	979±191	1332±260	98±1	4	203± 15	276± 20	96±2	4
	LP	463± 87	1907±358	98±1	4	105± 8	434± 32	95±1	4
	RP	446± 92	1763±364	97±1	4	107± 5	422± 19	94±1	4
	H	611±172	1352±380	98±1	4	127± 10	281± 22	95±1	4
150	LM	311± 97	427±133	86±4	4	n.d.**	—	—	
	RM	289± 74	292±100	85±4	4	n.d.	—	—	
	LP	95± 43	389±175	84±3	4	n.d.	—	—	
	RP	89± 33	352±132	82±4	4	n.d.	—	—	
	H	151± 43	335± 95	85±4	4	n.d.	—	—	

*M = medulla oblongata; P = pons; H = hypothalamus; LM = left side of medulla oblongata; LP = left side of pons; RM = right side of medulla oblongata; RP = right side of pons.
**n.d. = not detectable.

both sides of the pons and medulla. This is in agreement with previous observations that upon systemic application, drug distribution is uniform within the pontomedullary region [12]. Thus, this finding illustrates the validity of the method. The hydrolysis of paraoxon is demonstrated by the significantly smaller amounts measured 10 min after dosing. However, because of the detection limit, paraoxon could not be determined quantitatively 10 min after the infusion of 150 μg of paraoxon per kg. The presence of paraoxon was also established by the determination of acetylcholinesterase activity. The decrease of paraoxon concentration is accompanied by a reduction of the inhibitory effect on acetylcholinesterase activity. Conclusively, the developed method for the quantitative analysis of low amounts of paraoxon is a sensitive and accurate one. Its application to pharmacological experiments in vivo has been proven to be highly satisfactory. In a separate study [13] paraoxon concentrations in cat brain were measured also after central administration of the drug, and drug concentrations, enzyme inhibition and cardiovascular effects were compared.

REFERENCES

1 L. Fishbein, Chromatography of Environmental Hazards. Vol. III, Elsevier, Amsterdam, 1975.
2 J. Bäumler and S. Rippstein, Arch. Toxikol., 25 (1969) 57.

3 C.A. Bache and D.J. Lisk, Anal. Chem., 37 (1965) 1477.
4 J. Kvalvag, E.E. Ott and F.A. Gunther, J. Ass. Offic. Anal. Chem., 60 (1977) 911.
5 J.G. Koen and J.F.K. Huber, Anal Chim. Acta, 51 (1970) 303.
6 J.F. Lawrence, J. Chromatogr. Sci., 14 (1976) 557.
7 J. Kvalvag, D.L. Elliot, Y. Iwata and F.A. Gunther, Bull. Environ. Contam. Toxicol., 17 (1977) 253.
8 J.W. Dolan and J.N. Seiber, Anal. Chem., 49 (1977) 326.
9 D.C. Paschal, A.M. Jacobsen, R.G. Zayskie and V.J. Feil, J. Agr. Food Chem., 21 (1973) 804.
10 G. Szalontai, J. Chromatogr., 124 (1976) 9.
11 H. Jork and B. Roth, J. Chromatogr., 144 (1977) 39.
12 A.J. Porsius and G.M. Fronik, Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol., 311 (1980) R49.
13 J.H. de Neef and A.J. Porsius, in preparation.

Journal of Chromatography, 224 (1981) 139—143
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 839

Note

High-performance liquid chromatographic method for the simultaneous determination of iothalamate and *o*-iodohippurate

S. BOSCHI* and B. MARCHESINI

Department of Clinical Pharmacology, University Hospital, Via Massarenti 9, 40138 Bologna (Italy)

(First received October 7th, 1980; revised manuscript received January 13th, 1981)

The accurate evaluation of renal blood flow (RBF) and glomerular filtration rate (GFR) is often necessary to help physicians, physiologists and pharmacologists interpret clinical and experimental data [1]. To determine these parameters in patients it is preferable to make use of non-invasive and safe methods to avoid unjustified risks and complex diagnostic procedures.

The most widely used methods are those based on the "clearance" of some substance that is completely filtered or excreted by the kidneys [2]. Unfortunately, there are no endogenous substances suitable for accurate evaluation, and it is therefore necessary to administer a suitable compound to the patient before determining GFR and RBF [3].

Inulin and *p*-aminohippuric acid were used for many years before radioactive substances became available. Now, with radioisotopes, the procedure has become simpler and faster [3], but their diagnostic use has increased the risks for patients [4], and requires expensive equipment, more complex facilities and more highly specialized staff. Furthermore, for the simultaneous determination of GFR and RBF, two different isotopes are needed and often it is not easy to distinguish between them [5].

Some methods are described in the literature [6, 7] which outline the procedure for determining sodium iothalamate concentration. However, the sensitivity of these methods is insufficient for an accurate evaluation of GFR unless large amounts of iothalamate are given.

A high-performance liquid chromatographic method for *o*-iodohippurate (OIH) determination has been reported [8], but only for pharmaceutical formulations and not for diagnostic RBF evaluation. Thus we decided to evaluate the feasibility of a new simultaneous method for the determination of GFR and RBF using non-radioactive iothalamate and *o*-iodohippurate.

EXPERIMENTAL

Chemicals and reagents

Sodium iothalamate (3-acetamido-2,4,6-triiodo-5-methylcarbamoylbenzoic acid, sodium salt) and iodamide (3-acetamido-5-acetamido-methyl-2,4,6-triiodobenzoic acid) were kindly supplied by Bracco (Milan, Italy). *o*-Iodohippurate (OIH) and hippuric acid were obtained, respectively, from Sorin (Saluggia, Italy) and Carlo Erba (Milan, Italy). All other solvents were reagent grade except acetonitrile (LichroSolv) from Merck (Darmstadt, G.F.R.).

Instrumentation

A Perkin-Elmer Series 3B liquid chromatograph with Rheodyne valve was used equipped with a Perkin-Elmer ODS-HC Sil-X-1 reversed-phase column (25 × 0.26 cm, particle size 10 μm). The flow-rate was 1.0 ml/min; the detector was a Perkin-Elmer LC-75 variable-wavelength detector set at 235 nm.

Mobile phase preparation

The mobile phase was prepared by mixing water, acetonitrile and 85% phosphoric acid (960:40:0.3). The solution was filtered through a 0.22 μm Millipore filter (Millipore Corp., Bedford, MA, U.S.A.).

Extraction procedure

Plasma. Plasma samples (0.5—1 ml) were pipetted into glass stoppered tubes and internal standards (18 μg of iodamide and 2 μg of hippuric acid) were added. The samples were acidified with 1 ml of 1 *N* HCl and extracted twice by shaking for 10 min with 5 ml of ethyl acetate each time. After centrifugation, the organic layers were concentrated in a water bath at 60°C under a stream of nitrogen, and then 3 ml of 0.1 *N* NaOH were added. The samples were shaken for 10 min and centrifuged; the organic layer was then discarded. The aqueous layer was acidified with 0.5 ml of 1 *N* HCl and extracted twice with 5 ml of ethyl acetate each time repeating the previous step, and the organic layer was dried in a water bath at 60°C under a stream of nitrogen. The residue was reconstituted with 100 μl of mobile phase and an aliquot of 10—20 μl was injected.

Urine. To 1 ml of urine, 1 mg of iodamide (10 μl of a 10% aqueous solution) was added. Then 100 μl were transferred to a glass stoppered tube containing 1 ml of 1 *N* HCl and extracted twice with 5 ml of ethyl acetate each time. The organic layers were dried, the residue was reconstituted with 400 μl of mobile phase and 10—20 μl were injected.

RESULTS AND DISCUSSION

The retention times of iothalamate, iodamide, hippuric acid and *o*-iodohippurate are 2.0, 2.5, 5.6, 11.9 min, respectively, and a typical chromatogram of plasma spiked with known amounts of drugs is shown in Fig. 1. No interferences were observed in the plasma blank.

The quantitative determination of urinary *o*-iodohippurate and iothalamate was performed by adding known amounts of the drugs, in the range 0—2.0

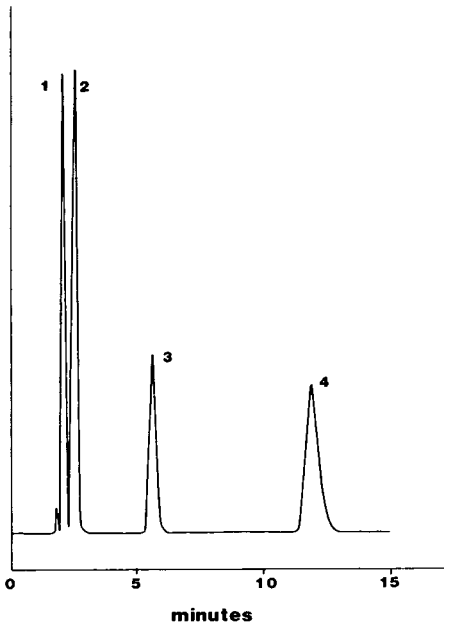


Fig. 1. Chromatogram of an extract of a 1-ml plasma sample spiked with 1 = iothalamate 16 μg; 2 = iodamide 18 μg; 3 = hippuric acid 2 μg; 4 = *o*-iodohippurate 10 μg. The chromatogram was recorded at 0.64 a.u.f.s. for peaks 1 and 2 and at 0.16 a.u.f.s. for peaks 3 and 4.

mg/ml, to urine using iodamide as internal standard for both substances. For plasma determination two internal standards (iodamide and hippuric acid) were used because greater accuracy was obtained using hippuric acid as internal standard for *o*-iodohippurate evaluation. It was not possible to use hippuric acid in urine determinations because it is an endogenous component of urine.

To analyze each 20-point calibration curve, regression analysis was first performed and the correlation coefficient r was obtained (0.999 for both curves). To test for linearity an analysis of variance was performed to isolate the following sources of variation: between concentration (linearity plus deviation from linearity), and within concentration (residual). For iothalamate the F value for deviation from linearity was 2.30 and for OIH it was 1.53. Both values were not significant as the tabled value for $F_{6,12}$ is 3.00. The average coefficient of variation from five standard curves was 6.0% for iothalamate and 6.9% for OIH. The sensitivity of the method was 0.5 μg/ml for iothalamate and 1.0 μg/ml for OIH. The reproducibility of the procedure was evaluated by analyzing replicate plasma samples to which known amounts of drugs had been added. The results are shown in Tables I and II. Good reproducibility was found and the average coefficient of variation was 0.6% for iothalamate and 1.3% for OIH.

The reliability of the method was tested in 15 normal subjects by plasma determination of the two compounds following intravenous administration. An example of the kinetics of the two substances with related parameters is shown in Figs. 2 and 3.

TABLE I

REPRODUCIBILITY AT A GIVEN PLASMA CONCENTRATION FOR IOTHALAMATE

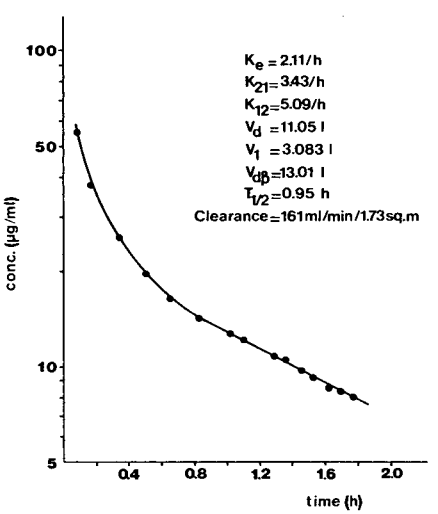
n = 5 in all cases.

Iothalamate (μg/ml)	C.V. (%)
5	0.6
9	0.4
15	0.3
19.5	1.1
Mean	0.6

TABLE II

REPRODUCIBILITY AT A GIVEN PLASMA CONCENTRATION FOR *o*-IODOHIPPURATE
n = 5 in all cases.

Concentration (μg/ml)	C.V. (%)
1.5	1.2
5	0.8
10	1.0
15	2.1
Mean	1.3



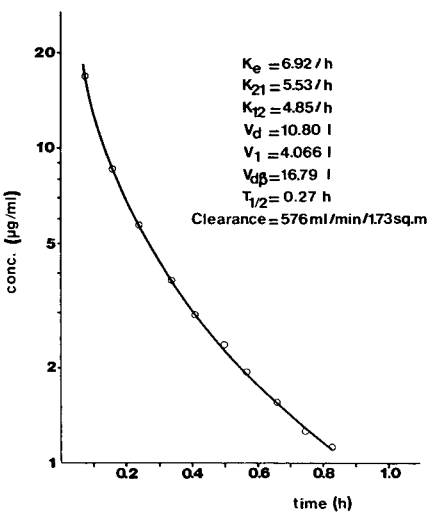


Fig. 2. Serum concentration curve following intravenous administration of iothalamate (364 mg) to a subject with normal renal function.

Fig. 3. Serum concentration curve following intravenous administration of *o*-iodohippurate (149 mg) to a subject with normal renal function.

The method described here has sufficient sensitivity and reproducibility to be used in determining simultaneously plasma concentrations of iothalamate and *o*-iodohippurate following single intravenous injections. Knowing the plasma concentrations of both substances and using the same pharmacokinetic model proposed for radioactive compounds, it is possible to calculate glomerular filtration rate and renal blood flow.

REFERENCES

1 D.C. Breater, Drugs, 19 (1980) 31—48.
2 E.E. Selkurt, Handbook of Physiology, Circulation n. 2, Am. Physiol. Soc., Washington, DC, 1963, pp. 1457—1516.
3 N.G. Levinsky and M. Levy, Handbook of Physiology, Renal Physiology, Am. Physiol. Soc., Washington, DC, 1973, pp. 103—117.
4 WHO Report No. 563, (1975) 14—15.
5 C. Martinenghi, L. Mombelli and G. Roncari, Radiol. Med., 54 (1968) 250.
6 T.B. Hunter, J.R. Thornbury, T.M. Silver and F. Medzihradsky, Invest. Urol., 14 (1977) 378.
7 F. Medzihradsky, P.J. Dahlstrom and T.M. Hunter, Invest. Radiol., 10 (1975) 532.
8 A.J. Falk, J. Pharm. Sci., 63 (1974) 274.

Journal of Chromatography, 224 (1981) 144—148
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 841

Note

Determination of guaiphenesin and its metabolite, β-(2-methoxyphenoxy)-lactic acid, in plasma by high-performance liquid chromatography

H.C.J. KETELAARS* and J.G.P. PETERS*

Department of Pharmacology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)

(Received December 5th, 1980)

Guaiphenesin [3-(2-methoxyphenoxy)propane-1,2-diol; I (Fig. 1)] is a widely used expectorant known to be a centrally acting muscle relaxant in high doses. Especially in equine anaesthesiology, guaiphenesin is accepted as a casting agent because of its negligible effect on respiratory function, cardiac action and haematological parameters [1]. In order to study the fate of guaiphenesin in the horse we needed a method for the quantitative determination of this drug in plasma. Because it is known from studies in man [2] that β-(2-methoxyphenoxy)lactic acid (II) is an important metabolite of guaiphenesin, the method should be capable of differentiating between these two compounds. Several methods have been described in the literature

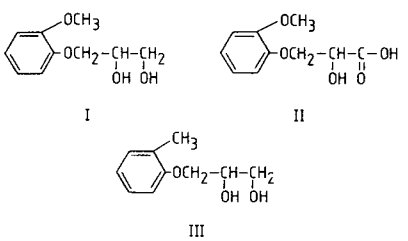


Fig. 1. Structural formulae of guaiphenesin (I), its metabolite β-(2-methoxyphenoxy)lactic acid (II), and the internal standard mephenesin (III).

*After preparation of the first draft of the present paper H.C.J. Ketelaars suddenly passed away. The paper was organized into its present form by J.G.P. Peters and C.A.M. van Ginneken.

0378-4347/81/0000—0000/$02.50

for the determination of guaiphenesin in biological fluids. Most of them [3, 4] are based on periodate oxidation of the number one atom of the propyl chain to formaldehyde and colorimetric determination with chromotropic acid. However, these methods are not specific. Gas—liquid chromatography has been used but derivatization is required [5].

This paper describes a high-performance liquid chromatographic (HPLC) method with a reversed-phase column (RP-8) for the separation and quantification of both guaiphenesin and β-(2-methoxyphenoxy)lactic acid using mephenesin (III) as internal standard.

MATERIALS AND METHODS

Materials

Guaiphenesin and mephenesin were commercially obtained from OPG (Utrecht, The Netherlands). β-(2-Methoxyphenoxy)lactic acid was isolated by ether extraction from acidified horse urine (pH 2—3) after intravenous injection of 100 mg/kg guaiphenesin. Its identity was verified by mass spectrometry, nuclear magnetic resonance and gas chromatography—mass spectrometry after derivatization with bis-(trimethylsilyl)-trifluoroacetamide (Pierce, Rockford, IL, U.S.A.).

LiChrosorb RP-8 (5 μm) was obtained from E. Merck (Darmstadt, G.F.R.). All other chemicals were of analytical reagent grade.

High-performance liquid chromatography

A Hewlett-Packard 1084B high-pressure liquid chromatograph equipped with a microprocessor and an automatic sampling system was used. The variable-wavelength UV detector was used and operated at 275 nm. The column was a 15 cm × 4.6 mm I.D. stainless-steel tube packed by the slurry technique with LiChrosorb RP-8 (5 μm) and operated at 30°C. The elution solvent consisted of a mixture (40:60) of methanol and citrate buffer (pH 6.5) (ultimate concentration 0.01 *M*). Elution was performed at a flow-rate of 1.0 ml/min. Under these conditions the retention times of I, II and III are about 2.8, 4.7 and 8.8 min, respectively.

EXPERIMENTAL

Sample preparation

Into a centrifuge tube with Teflon-lined screw cap are pipetted 0.5 ml of the plasma sample, 50 μl of the internal standard solution (1 mg/ml in water) and 60 μl of 1 *N* HCl. After homogenization the mixture is extracted once with 5 ml of freshly distilled diethyl ether by shaking for 30 min in an automatic shaker, and centrifuged for 15 min at 1000 *g*. The diethyl ether layer is transferred to another tube and evaporated to dryness under a gentle stream of dry air at 30°C. The residue is taken up in 0.5 ml of the elution solvent and transferred to a sample vial of the automatic sampling system of the HPLC system.

Calibration and recovery

Known quantities of I and II were added to blank plasma. The samples were then treated according to the sample preparation procedure described above. UV detection was carried out at a wavelength of 275 nm. Linear calibration curves could be constructed by plotting the absorbance of I or II in the samples as related to the absorbance of the internal standard (III) against the amount of I or II added to the plasma. In practice, the variable amounts of I and/or II added were calculated by the microprocessor according to Lambert—Beer's law. By plotting these calculated amounts against the added amounts linearity was confirmed unambiguously.

Recoveries of I and II were determined by adding known amounts to blank plasma. After extraction mephenesin was added and the relative peak area ratio was calculated. This value was compared with the ratio obtained by direct analysis of the same amounts of I, II and III in standard solutions.

RESULTS AND DISCUSSION

Typical chromatograms of a reference sample, a blank plasma sample and a spiked plasma sample are shown in Fig. 2. Guaiphenesin is well separated from its metabolite. The behaviour of guaiphenesin in our chromatographic system can be fully characterized by the following parameters: number of theoretical plates, 25,000—30,000/m; the capacity factor, 2.77; and the factor of symmetry (the width of the right half divided by that of the left half of the peak, as measured at 10% of peak height) is 1.5—1.8.

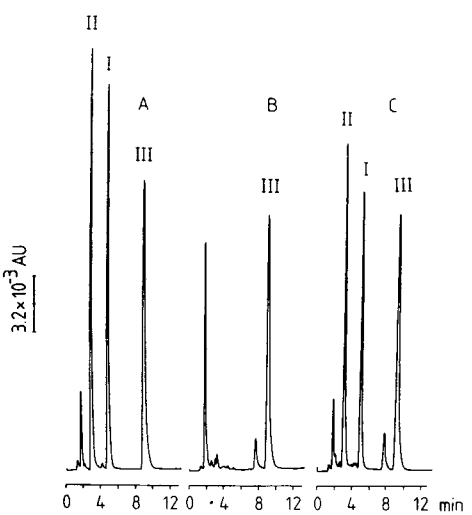


Fig. 2. High-performance liquid chromatograms obtained by the procedure described in the text for (A) a reference sample of standard solutions, (B) blank plasma spiked with internal standard (III) only, and (C) blank plasma spiked with guaiphenesin (I), its metabolite (II) and the internal standard (III). The plasma samples show an extra peak (retention time 7.4 min), the identity of which is unknown.

The plasma extract is clean except for a peak with a retention time of about 7.4 min, the identity of which is as yet unknown, but which is present in all blank samples. In the concentration range studied (5—200 μg/ml) there was no serious interference by endogenous compounds and peak areas were linear with concentration (correlation coefficient 0.997). The recovery was 91 ± 5% (S.D.) for I and 74 ± 2% (S.D.) for II.

The method described was used to study the pharmacokinetics of guaiphenesin in the horse. A typical plasma profile of a horse that received 100 mg/kg guaiphenesin intravenously is shown in Fig. 3. It appears that the decay of guaiphenesin can be adequately described by a two-compartment system [6]. In Fig. 3 also the plasma concentration of the acid metabolite II is plotted as a function of time after administration. As might be expected this concentration rises from zero to a maximum relatively shortly after administration.

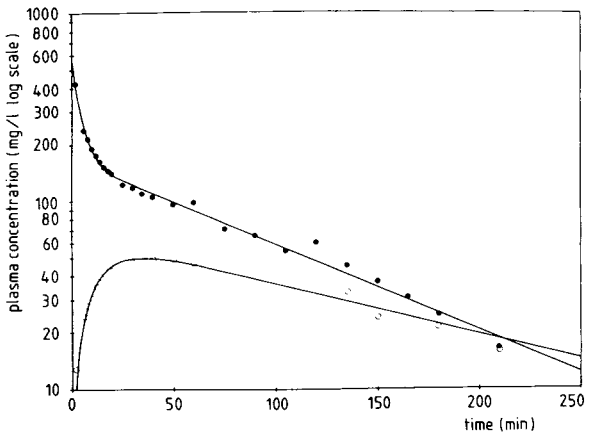


Fig. 3. Typical profile of a plasma curve of guaiphenesin and its lactic acid metabolite after intravenous administration of guaiphenesin to a horse. The closed circles (•) represent guaiphenesin, the open circles (○) the metabolite. See text for further explanation.

Thereafter its disappearance proceeds more slowly than that of the parent compound I. The total elimination of guaiphenesin and its metabolites from the body of the horse occurs largely via renal pathways. Substance I, however, hardly appears in urine as the free form, but almost wholly in the conjugated form, mainly as glucuronides. The analysis of urine samples therefore offers special problems, which will be described and discussed in a forthcoming paper.

ACKNOWLEDGEMENT

The authors wish to thank Prof. Dr. A. Lagerweij and Dr. J.S.M.M. van Dieten (Department of Veterinary Anaesthesiology of the State University of Utrecht, The Netherlands) for their kind co-operation and for performing the animal experiments.

REFERENCES

1 M. Westhues and R. Fritsch, Die Narkose der Tiere, Band II, Verlag Paul Parey, Berlin, Hamburg, 1961, p. 171.
2 W.J.A. VandenHeuvel, J.L. Smith and R.H. Silber, J. Pharm. Sci., 61 (1972) 1997.
3 A.M. Morgan, E.B. Truitt and J.M. Little, J. Amer. Pharm. Ass. Sci. Ed., 46 (1957) 374.
4 L.E. Davis and W.A. Wolff, Amer. J. Vet. Res., 31 (1970) 469.
5 W.R. Maynard and R.B. Bruce, J. Pharm. Sci., 59 (1970) 1346.
6 H.C.J. Ketelaars, J.S.M.M. van Dieten and E. Lagerweij, Berlin Muenchen. Tieraertzl. Wochenschr., 92 (1979) 211.

Journal of Chromatography, 224 (1981) 149—155
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 848

Note

Quantitative high-performance liquid chromatographic determination of antispasmodic trimebutine in human plasma

Pharmacokinetic studies after intravenous administration in humans

A. ASTIER* and A.M. DEUTSCH

Laboratoire de Toxicologie et Dosage des Médicaments, Département de Pharmacologie Clinique, Hôpital Henri Mondor, 51, avenue du Maréchal de Lattre de Tassigny, 94010 Créteil (France)

(First received November 3rd, 1980; revised manuscript received January 27th, 1981)

Trimebutine maleate, 2-dimethylamino-2-phenylbutyl-3,4,5-trimethoxybenzoate maleate (Debridat®) (Fig. 1), is an antispasmodic compound used in France in various gastrointestinal diseases and in radiological examinations [1].

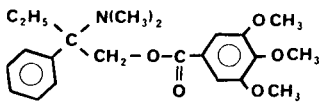


Fig. 1. Structural formula of trimebutine maleate.

Side-effects have been described [2, 3] after high doses. The pharmacokinetic behaviour of this drug in patients has been investigated using radiolabelled drug [4], but little is known about the characteristics of its absorption and elimination. The main reason for this paucity of information appears to be due to the lack of analytical methods applicable to human studies.

A simple, rapid, routine and non-radioactive assay of the drug in plasma was required for further investigations of its pharmacokinetics. This type of assay using reversed-phase high-performance liquid chromatography with C_{18} bonded-phase, is the subject of this report.

EXPERIMENTAL

Reagents

Methanol was obtained from Carlo Erba (Milan, Italy) and filtered (0.22 μm

0378-4347/81/0000—0000/$02.50

Fluoropore; Millipore, Molsheim, France) before use. Hexane and 2-pentanol (analytical grade) were purchased from Merck (Darmstadt, G.F.R.). The Pic-A reagent was obtained from Waters Assoc. (Milford, MA, U.S.A.). Trimebutine was generously supplied by Jouveinal Laboratories (Fresnes, France), procaine hydrochloride (internal standard) was obtained from the Pharmacie Centrale des Hôpitaux (Paris, France). All other solvents and reagents were of analytical quality.

Chromatography

A liquid chromatograph (Altex 380-Chromatem, Touzart et Matignon, Paris, France) equipped with a variable-wavelength detector (Pye Unicam, Cambridge, Great Britain) was used in a reversed-phase system with Partisil ODS-2 as the stationary phase (250 × 4.6 mm I.D.; particle size 10 μm) (Touzart et Matignon) and methanol—aqueous Pic-A (85:15, v/v; Pic-A = 0.005 *M*) as the mobile phase. The effluent stream was monitored at 265 nm and the volume of sample injected was 20 μl (Rheodyne, Berkeley, CA, U.S.A.).

The mobile phase was degassed by a helium stream during the determination and pumped at a constant flow-rate of 2.00 ± 0.02 ml/min. A chart speed of 5 cm/min was employed.

Standard curve and sample preparation

A 1-ml sample of patient plasma and 0.1 ml of a procaine hydrochloride solution (20 μg/ml; freshly prepared daily from a stock solution of 1 mg/ml in water) were added to a 20-ml culture tube with PTFE-lined caps (Prolabo, Paris, France).

The plasma was made alkaline (pH adjusted to 11) by adding 2 ml of 2.9% ammonia solution and was extracted with 3 ml of hexane containing 2-pentanol (0.1%) in a rotary shaker (Cenco, Breda, The Netherlands) for 15 min. The organic (upper) phase was separated by centrifugation at 600 *g* for 5 min and transferred to a clean evaporating tube.

The hexane was evaporated at 30°C under vacuum (Rotavapor, Buchi, Switzerland). The residue was taken up with 50 μl of methanol. Aliquots of 20 μl were injected into the chromatograph.

The concentration of trimebutine in the plasma was determined from a calibration curve of peak height ratio (drug/internal standard) versus drug concentration in spiked plasma (0.05—1 μg/ml) carried through the procedure.

In vivo study

Three in-patients with normal renal and hepatic functions participated voluntarily in the study. Trimebutine maleate (150 mg) was administered intravenously (right hand vein) in a rapid injection (2 min). Venous blood samples were collected via a Teflon catheter inserted in an antecubital vein of the contralateral arm in heparinized vials at various times and were centrifuged within 10 min at 1200 *g* (+ 4°C) to obtain plasma fractions (stored at −30°C until analysis).

RESULTS AND DISCUSSION

Method

A chromatogram obtained from a patient plasma sample containing trimebutine and procaine, extracted and assayed as described, is shown in Fig. 2. The retention times for procaine and trimebutine were 3.8 and 8 min, respectively.

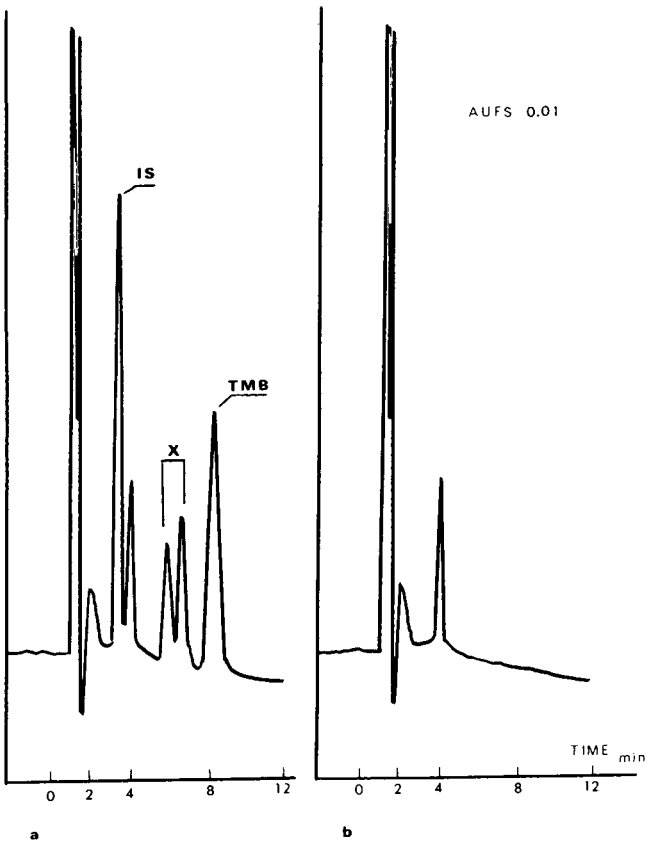


Fig. 2. Chromatograms of (a) a patient plasma sample (IS = procaine, 2 μg/ml; TMB = trimebutine, 250 ng/ml; X = unidentified peaks), 90 min after a single intravenous dose of 150 mg of trimebutine maleate, (b) a patient plasma before drug injection.

The trimebutine peak was well separated from all extraneous plasma peaks. The peaks marked "X" were unidentified compounds, probably biotransformation products of trimebutine. The calibration curves were linear over the concentration range studied. The least-squares regression line, which represents the mean of ten determinations for each point, has a slope of 1.21 and a y-intercept (y = peak height ratio of drug to internal standard) of 0.005 (R = 0.9979).

The precision of the method was determined by repeated analyses of spiked plasma samples containing low (0.5 μg/ml) and high (1 μg/ml) concentrations of trimebutine. The mean standard deviation and coefficient of variation for intra- and inter-day analyses are shown in Table I and were satisfactory. The

TABLE I

INTRA- AND INTER-DAY REPRODUCIBILITY OF TRIMEBUTINE ASSAYS IN PLASMA

		Amount added to plasma (μg/ml)	
		0.5	1.0
Intra-day (n = 10)	R^* (mean ± S.D.)	0.35 ± 0.015	0.80 ± 0.02
	Coefficient of variation (%)	4	2.5
Inter-day (n = 10)	R^* (mean ± S.D.)	0.38 ± 0.02	0.79 ± 0.03
	Coefficient of variation (%)	5	3.8

*R = (peak height of trimebutine)/(peak height of procaine).

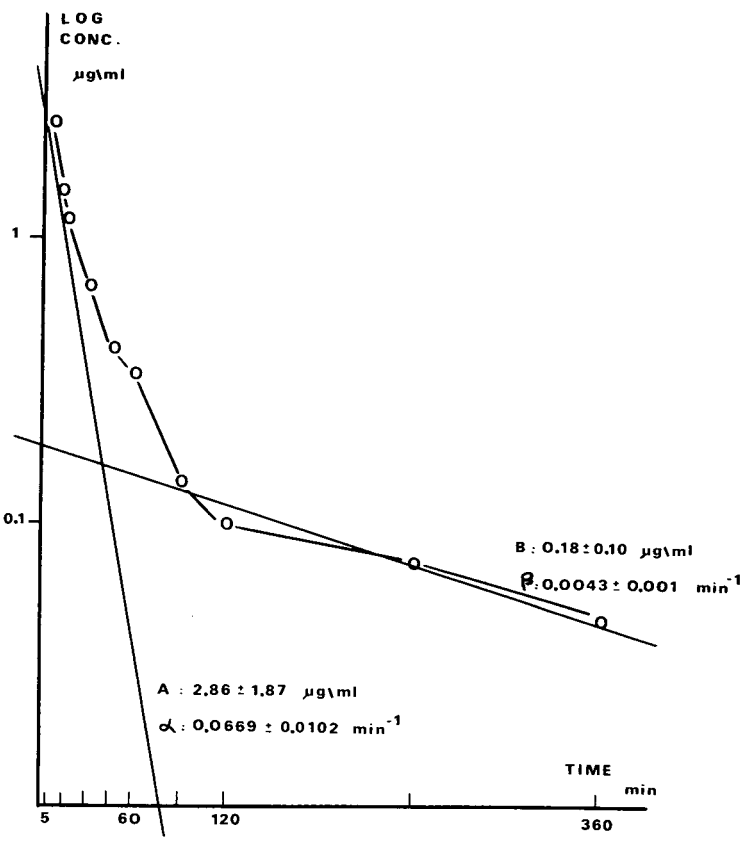


Fig. 3. Mean plasma concentrations of trimebutine after a single intravenous dose of 150 mg of trimebutine maleate.

TABLE II

PHARMACOKINETIC PARAMETERS* IN THREE VOLUNTEERS AFTER A SINGLE 150-mg INTRAVENOUS DOSE OF TRIMEBUTINE

Initials	A (μg/ml)	α(min^{-1})	$t_{1/2}\,\alpha$(min)	AUC_0^∞ (α) (μg per ml per min)	B (μg/ml)	β (min^{-1})	$t_{1/2}\,\beta$ (min)	AUC (β) (μg per ml per min)	AUC ($\alpha + \beta$) (μg per ml per min)
A.	1.43	0.0670	10.3	21.2	0.119	0.0048	144.3	24.8	46.0
L.G.	2.17	0.0565	12.3	38.5	0.308	0.0047	151.6	67.4	105.9
Y.	4.97	0.0769	9.0	64.7	0.133	0.0035	197.4	37.9	102.6
Mean	2.86	0.0669	10.5	41.5	0.187	0.0043	164.4	43.3	84.8
± S.D.	± 1.87	± 0.0102	± 1.6	± 21.9	± 0.105	± 0.0007	± 28.8	± 21.8	± 33.6

*Clearance (1800 ml/min) = $D \times \frac{\alpha\beta}{A + B}$, where α = constant of distribution phase, β = constant of elimination phase, D = dose administered, AUC = area under the plasma level curve, $t_{1/2}\,\alpha$ = distribution half-life, $t_{1/2}\,\beta$ = elimination half-life.

analytical recovery of trimebutine from plasma was determined by comparing the ratio of the peak heights of trimebutine to internal standard in spiked plasma specimens (trimebutine 0.5 μg/ml, internal standard 2 μg/ml), to the ratio in spiked plasma with only 2 μg/ml of internal standard (trimebutine added just before injection to chromatograph). Recovery (mean ± S.D.) was 91 ± 4% (n = 5).

The sensitivity of this method was estimated to be 0.04 μg/ml. Greater sensitivity may be possible by increasing the plasma volume or increasing the injection volume (50 μl).

In vivo study

No adverse effects were observed in any of the volunteers after the administration of trimebutine.

The plasma concentration kinetics of trimebutine (mean of three volunteers) are shown in Fig. 3. The various pharmacokinetic parameters were calculated with a non-linear least-squares regression program using a "feathering method" [5] written for a microcalculator (Hewlett-Packard HP 97). A summary of the pharmacokinetic parameters is shown in Table II. The AUC_0^∞ values were measured using the trapezoidal rule.

The data show that trimebutine kinetics follow a biexponential pattern, with an initial rapid phase (distribution) lasting for up to 10.5 ± 1.6 min after an intravenous infusion. This is followed by a slower (elimination) phase with a half-life of 164.4 ± 28.8 min. Maximum concentration (mean 2.6 ± 1.8 μg/ml) was obtained in the first sample taken 5 min after the end of the 2-min intravenous infusion.

The typical compartmental scheme corresponding to this model might be written as shown in Fig. 4, where k_{12} and k_{21} are first-order microconstants for transfer and k_e is the rate constant of elimination [5—7].

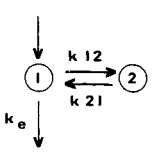


Fig. 4. Compartmental scheme (trimebutine, intravenous).

CONCLUSION

This assay provides an efficient and accurate method for the analysis of trimebutine in human plasma. We are currently using the method for pharmacokinetic studies on trimebutine following intravenous administration in humans. The method has been applied successfully in a study of the pharmacokinetics of trimebutine in liver dysfunction and these results will be presented elsewhere.

REFERENCES

1 M.F. Moshal, J. Int. Med. Res., 7 (1979) 232—235.
2 A. Bitoun, Acta Endoscop., 4 (1979) 309.

3 J.P. Galmiche, J. Vatier and M. Dubrasquet, in J.P. Giroud, G. Mathé and G. Meyniel (Editors), Pharmacologie Clinique, Expansion Scientifique, Paris, 1979, p. 644.
4 Jouveinal Laboratories, personal communication.
5 R.E. Notari, Biopharmaceutics and Pharmacokinetics, Marcel Dekker, New York, 1971.
6 J.G. Wagner, Fundamentals of Clinical Pharmacokinetics, Drug Intelligence Publications, Hamilton, IL, 1975.
7 M. Gibaldi, Biopharmaceutics and Clinical Pharmacokinetics, 2nd ed., Lea and Febiger, Philadelphia, PA, 1977.

Journal of Chromatography, 224 (1981) 156—161
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 853

Note

Convenient and sensitive high-performance liquid chromatography assay for cimetidine in plasma or urine

MICHAEL G. KUNITANI, DEBORAH A. JOHNSON, ROBERT A. UPTON* and SIDNEY RIEGELMAN

Department of Pharmacy, University of California, San Francisco, CA 94143 (U.S.A.)

(Received January 13th, 1981)

Cimetidine (Fig. 1) is a competitive histamine H_2-receptor antagonist which inhibits gastric acid secretion and is used for the treatment of peptic ulcer. For a review of the chemistry and pharmacology of cimetidine see Brogden et al. [1]. Previously reported high-performance liquid chromatographic (HPLC) methods of measuring cimetidine and its major metabolite cimetidine sulfoxide in blood and plasma require a triple extraction sample preparation utilizing either normal-phase or reversed-phase chromatography [2, 3]. This paper reports an HPLC cimetidine assay for plasma and urine that benefits from a simplified sample work-up and improved reversed-phase chromatography resulting in high accuracy, reproducibility and sensitivity.

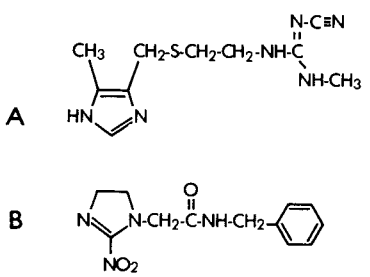


Fig. 1. Structures of (A) cimetidine and (B) internal standard R07-1051.

0378-4347/81/0000—0000/$02.50

MATERIALS AND METHODS

Reagents

Acetonitrile, methanol and ethyl acetate were HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Water was deionized and glass-distilled and reagents were of analytical grade. Cimetidine and cimetidine sulfoxide were obtained from Smith, Kline and French (Philadelphia, PA, U.S.A.) and the internal standard, R07-1051 (Fig. 1), from Hoffmann-La Roche (Nutley, NJ, U.S.A.). Stock solutions of cimetidine (2.5, 25, 250 μg/ml) used to construct standard curves were made with 1 m*M* hydrochloric acid and the internal standard stock solution (25 μg/ml) was made with water; both were stored at 4°C. The HPLC eluent was filtered through a 0.45-μm membrane filter (Type HA, Millipore, Bedford, MA, U.S.A.) immediately prior to use.

Chromatography

A dry-packed 150 mm × 4.6 mm pre-pre-column (Perisorb RP-2, 30—40 μm, Merck, Darmstadt, G.F.R.) was placed between the pump and injector. A slurry-packed 40 mm × 2.1 mm pre-column (LiChrosorb RP-2, 10 μm, Merck) was used to protect the analytical column (Ultrasphere ODS, 150 mm × 4.6 mm, 5 μm, Altex, Berkeley, CA, U.S.A.) from irreversible sample absorption. The HPLC eluent was composed of 10 m*M* phosphate buffer, pH 8.0 with 20% methanol. An Altex 100A pump was used to deliver eluent at 1.7 ml/min at a pressure of about 3500 p.s.i. An Altex-Hitachi 155-30 Vis—UV detector monitored the column eluent at 220 nm. A WISP 710A auto sampler (Waters Assoc.) initiated a Spectra Physics 4100 integrator which was used for data reduction. The integrator was operated in the peak height mode and was supplemented with customized multilevel calibration programs.

Plasma sample preparation

To a culture tube were added 250 μl of the plasma sample, 75 μl water, 75 μl of internal standard stock solution and 2.0 ml of acetonitrile. The mixture was vortexed for 30 sec and centrifuged for 5 min at 2000 *g* to precipitate protein. The liquid was decanted and evaporated to dryness in a vortex evaporator (Buchler, Fort Lee, NJ, U.S.A.) at 45°C with aspirator vacuum. Alternatively, a stream of nitrogen may be used for evaporation at 40°C. The dried samples were reconstituted in 275 μl of HPLC eluent, vortexed for 20 sec and transferred to polyethylene micro centrifuge tubes (250 μl, Brinkmann, Westbury, NY, U.S.A.). The samples were centrifuged in a Brinkmann centrifuge at 12,000 *g* for 2 min to pellet undissolved debris. The centrifuge tube caps were removed and the tubes used directly as disposable limited-volume sample holders in an automated WISP HPLC injector, facilitating injection of a large proportion of the sample. The injection volume is 150 μl. For calibration standards part of the 75 μl of water in samples was replaced by the appropriate volume of cimetidine stock solution and added to drug-free plasma.

Urine sample preparation

To 100 μl of urine sample in a screw-capped culture tube were added 250 μl

of internal standard stock solution, 300 μl water, 50 μl of freshly prepared 1 *M* sodium hydroxide solution, and 10.5 ml ethyl acetate. The extraction mixture was vortexed rapidly in a Buchler vortex evaporator for 5 min at room temperature and centrifuged at 2000 *g* for 3 min. The organic layer was transferred to a fresh culture tube, evaporated at 40°C with a stream of nitrogen and reconstituted with 275 μl of HPLC eluent. The injection volume was 50 μl. For calibration standards part of the 300 μl water in samples was replaced with the appropriate volume of cimetidine stock solution and added to drug-free urine.

RESULTS

Chromatography

An eluent pH of 8.0 yielded excellent peak shape of both cimetidine and internal standard (Fig. 2) by reducing peak tailing of these basic compounds.

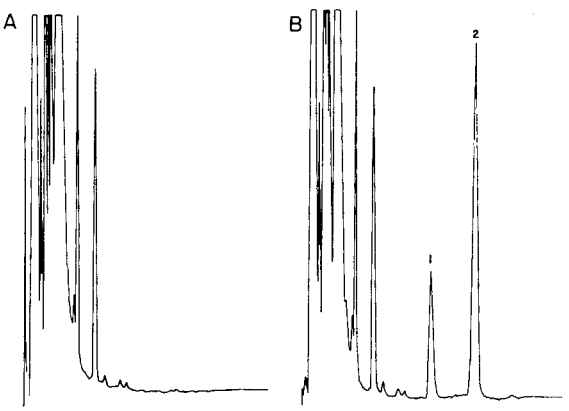


Fig. 2. Chromatograms of extracts of human plasma (A) before and (B) after spiking with 0.5 mg/ml cimetidine (1) and internal standard (2).

Column life was extended by the use of a pre-pre-column connected between the pump and injector. The purpose of such a column was to increase the concentration of dissolved silica in the eluent, thus reducing hydrolysis of silica on the analytical column [4]. Over 600 plasma and urine samples were analyzed on one Ultrasphere ODS column, indicating good stability under conditions of high pH and high pressure. Cimetidine and internal standard peaks fell in a clear area of the chromatogram (Fig. 2) with a few samples containing very small peaks occurring to either side of cimetidine. There was no interference from cimetidine sulfoxide, a primary metabolite [5] and several drugs tested: caffeine, theophylline, naproxen, ketoprofen and acebutolol.

Plasma

Extraction of cimetidine from plasma was found to suffer from poor reproducibility at low concentrations. In contrast, the procedure described (protein precipitation and direct injection of the reconstituted sample), gave

TABLE I

SPECIFICATIONS OF CIMETIDINE ASSAY IN PLASMA

	Concn. of cimetidine (μg/ml)				
	0.1	0.5	2.5	5.0	10
Precision					
C.V.%					
Intraday (6 samples)	3.7	1.2	1.7		2.2
Interday (16 days)		5.9		2.6	
Bias					
Deviation of mean from amount spiked (%)					
Intraday (6 samples)	−2.6	−1.8	−3.4		+3.4
Interday (16 days)		+3.2		−2.0	
Linearity		at 0.1—1.0		1.0—25 μg/ml	
Average C.V. of concentration-normalized peak height ratios (%)					
1 per day, 25 days; 5 points per range		4.3 ± 1.5		3.3 ± 1.7	

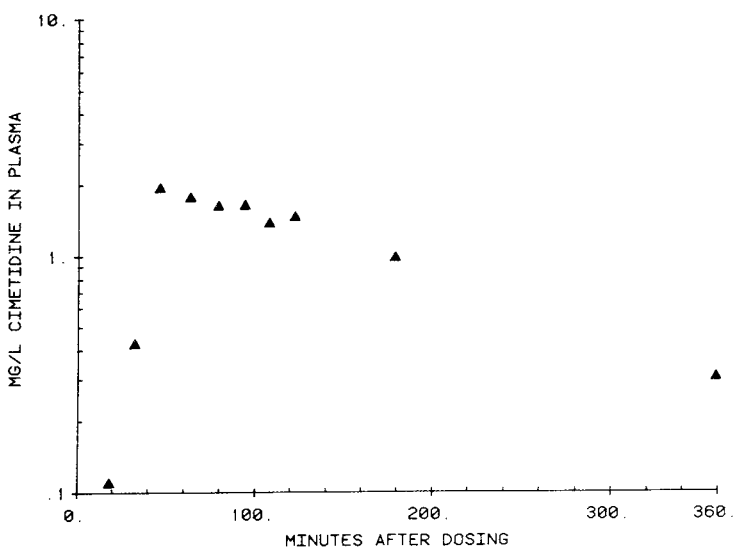


Fig. 3. Plasma concentration—time profile after a 500-mg dose of cimetidine.

excellent intra- and interday reproducibility, even at the limit of assay sensitivity (0.1 μg/ml, Table I). In order to improve reproducibility and accuracy the disposable pipet tips (Finntip, Finnpipette) were rinsed several times prior to use with the cimetidine stock solutions to be pipetted, as random absorption of cimetidine was found to be significant. Perhaps for the same reason a slightly convex standard curve of cimetidine peak height ratios was observed. The following calibrators were processed, covering a 250-fold range of concentrations: 0, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, 5.0, 7.5, 10, 25 μg/ml. To avoid excessive weight being given to the more concentrated calibrators, the standard curve was

divided into two ranges (0—1.0 μg/ml and 1.0—25 μg/ml). An unweighted least squares regression was independently fitted to each range with excellent linearity (Table I). A drug study in which eight individuals took 500 mg of cimetidine twice a day resulted in peak concentrations as high as 12.9 μg/ml falling to 0.25 μg/ml in 7 h. A typical plasma concentration—time profile obtained with this assay is shown in Fig. 3.

Urine

Because of interfering peaks in the chromatogram an extraction of cimetidine and internal standard was necessary for urine. A single basic extraction with ethyl acetate was effective in reducing interfering peaks to an insignificant level. Extraction efficiency was 65% for cimetidine and 97% for internal standard. Reproducibility above 100 μg/ml was excellent (Table II) but

TABLE II

SPECIFICATIONS OF CIMETIDINE ASSAY IN URINE

	Concn. of cimetidine (μg/ml)		
	150	300	500
Precision			
C.V.%			
Intraday (6 samples)	3.5	2.7	4.0
Interday (2 days)	8.4	2.3	4.9
Bias			
Deviation of mean from amount spiked (%)			
Intraday (6 samples)	+8.7	+1.9	—0.6
Interday (2 days)	+2.8	+3.5	—0.2
Linearity		at	100—750 μg/ml
Average C.V. of concentration-normalized peak height ratios (%)			
1 per day, 3 days; 11 points per range			7.9 ± 1.9

deteriorated below this concentration. In the drug study previously mentioned, no urine concentrations below this assay sensitivity were encountered. Samples having cimetidine concentrations greater than 750 μg/ml were diluted with drug-free urine prior to extraction. Standard curves calculated with an unweighted least squares regression yielded the linearity figures in Table II.

DISCUSSION

The cimetidine HPLC plasma assay described is a significant improvement over those previously reported [2, 3] in terms of range, sensitivity and ease of sample preparation. Larsen et al. [3] reported an assay range of 0.5—4.0 μg/ml for 750 μl plasma with a potential sensitivity limit of 0.1 μg/ml with 10% reproducibility. The range of the assay described in this paper is 0.1—25 μg/ml

for 250 μl plasma with a sensitivity of 0.1 μg/ml with 4% reproducibility. The requirement of only 250 μl of plasma may be clinically advantageous as little more than a pin prick may be used for cimetidine quantitation in the therapeutic range. Alternatively, the use of 1.0 ml of plasma will increase assay sensitivity to 25 ng/ml with no loss in reproducibility or accuracy. Previous cimetidine assays require triple extractions as the sample purification procedure prior to chromatography. In contrast the assay described herein involves a single protein precipitation step thus greatly reducing sample preparation time and manipulative errors. With the aid of the automated sample injector as many as 70 plasma samples a day could easily be processed and chromatographed by a single technician. The high degree of chromatographic resolution of cimetidine from endogenous peaks is responsible for the abbreviated sample work-up as prechromatographic extractions of plasma are not necessary. In addition, the excellent peak resolution aids in identification and quantification of low concentrations. The urine assay also benefited from the good chromatographic resolution but required a single alkaline extraction to remove acidic urine compounds. The excellent reproducibility of the urine extractions provided good assay accuracy despite a relatively low extraction efficiency of cimetidine.

ACKNOWLEDGEMENTS

The authors acknowledge Ms. Jean Buskin for her contribution of customized multilevel calibration programs for the SP 4100 integrator.

REFERENCES

1 R.N. Brogden, R.C. Heel, T.M. Speight and G.S. Avery, Drugs, 15 (1978) 93.
2 W.C. Randolph, V.L. Osborne, S.S. Walkenstein and A.P. Intoccia, J. Pharm. Sci., 66 (1977) 1148.
3 N.-E. Larsen, P. Hesselfeldt, S.J. Rune and E.F. Hvidberg, J. Chromatogr., 163 (1979) 57.
4 J.G. Atwood, G.J. Schmidt and W. Slavin, J. Chromatogr., 171 (1979) 109.
5 R.M. Lee and P.M. Osborne, J. Chromatogr., 146 (1978) 354.

Journal of Chromatography, 224 (1981) 162—167
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 854

Note

Trace determination of trimetazidine in plasma by high-performance liquid chromatography using fluorescence detection

S. COURTE and N. BROMET*

Département de Pharmacocinétique, Technologie Servier, 45 000 Orléans (France)

(First received August 18th, 1980; revised manuscript received February 2nd, 1981)

Derivatives of 5-dimethylamino-1-naphthalenesulphonyl chloride (dansyl chloride) have been widely used for thin-layer chromatographic determination of a large variety of amines [1—3]. The method is particularly useful for the analysis of trace components due to the high sensitivity of the products. The derivatives are usually separated by high-performance liquid chromatography (HPLC) using a fluorescence detector.

In the present paper, we describe preliminary results of the HPLC separation of dansylated trimetazidine and its fluorimetric determination in the nanogram range. Previous studies carried out by gas—liquid chromatography (GLC) with flame ionisation and nitrogen—phosphorus detectors enabled us to determine about 100—200 ng of trimetazidine per ml of plasma as the limit of detection. The level of determination was insufficiently sensitive to follow plasma pharmacokinetics in man.

Trimetazidine hydrochloride [1-(2,3,4-trimethoxybenzyl)-piperazine dihydrochloride] regulates ionic and extracellular exchanges, correcting the abnormal flow of ions across the cell membrane caused by ischemia, and preventing cellular oedema caused by anoxia.

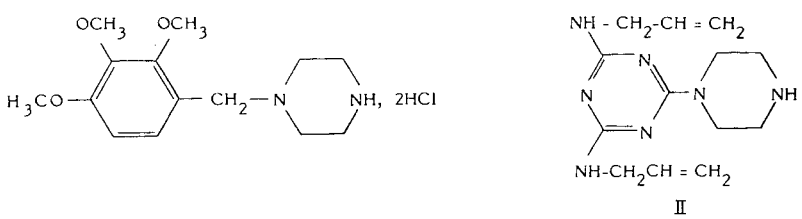


Fig. 1. Structures of trimetazidine hydrochloride (I) and internal standard (II).

0378-4347/81/0000—0000/$02.50

The structures of trimetazidine hydrochloride [I] and bis-2,4-(allyl-amino)-6-(1-piperazinyl)-triazine [II], used as the internal standard in the assay, are shown in Fig. 1.

MATERIALS AND METHODS

Reagents

Trimetazidine hydrochloride and its internal standard were supplied by Science Union & Compagnie, Suresnes, France. Acetone, cyclohexane, chloroform, isooctane, diisopropyl ether, methanol, 2 *N* sodium hydroxide and sodium hydrogen carbonate were supplied by E. Merck, Darmstadt, G.F.R. We used pro analysis quality. Dansyl chloride was supplied by Pierce, Rockford, IL, U.S.A.

High-performance liquid chromatography

The modular liquid chromatographic system used consisted of a Waters 6000A pump, a Rheodyne loop injector and a Schoeffel FS 970 LC fluorimeter (excitation wavelength, 252 nm; emission wavelength, >370 nm).

The chromatography column (15 cm × 2 mm I.D.) was slurry-packed with LiChrosorb Si-60 (5 μm). The mobile phase [4] was a mixture of solvents; namely, 950 ml of solvent A [isooctane—diisopropyl ether (50 : 50)], and 50 ml of solvent B [methanol—diisopropyl ether (50 : 50) + 2.6% of water]. The mobile phase was degassed with helium.

Fluorescence was measured using excitation at 252 nm and with an emission cut-off filter at 370 nm. The flow-rate (1 ml/min) was chosen to separate dansyl derivatives of reagents and endogenous plasma constituents from dansylated trimetazidine and dansylated internal standard.

Extraction procedure

Plasma samples (2 ml) were made alkaline with 300 μl of 2 *N* sodium hydroxide and extracted by stirring with three times 5 ml of cyclohexane—chloroform (3 : 1, v/v) for 15 min. The organic phases were separated and evaporated under a gentle stream of nitrogen. Before the last evaporation, 50 μl of internal standard solution (1 μg/ml in acetone) were added.

To dissolve the residue, 100 μl of aqueous sodium hydrogen carbonate solution (0.1 *M*) and 100 μl of dansyl chloride solution (0.4 mg/ml in acetone) were added. The tubes were capped and incubated in a water bath at 50°C for 30 min. After this time, the caps were removed and solutions were evaporated under a gentle stream of nitrogen. A 200-μl volume of mobile phase was then added and the mixture shaken for 2 min. A 20-μl aliquot of the organic phase was injected into the liquid chromatograph for the HPLC analysis.

The retention times for dansylated trimetazidine and dansylated internal standard were 10 min and 8 min, respectively (Fig. 2).

Calibration curve and reproducibility

A calibration curve was constructed by adding known amounts of trimetazidine hydrochloride and the internal standard to plasma, and these were then taken through the analytical procedure. The peak height ratio of dansylated tri-

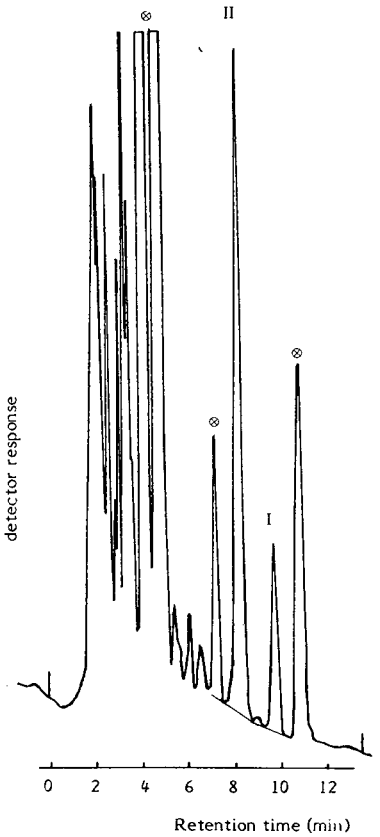


Fig. 2. Chromatogram obtained with plasma spiked with 25 ng/ml trimetazidine. I = Trimetazidine hydrochloride; II = internal standard. ⊗ = reagents and endogenous plasma constituents.

metazidine to the internal standard was plotted against the amount of trimetazidine added to construct the calibration curve. The linear regression equation $y = 0.0101x + 0.028$ describes the curve, where $r = 0.999$.

Replicates (six samples) of 20 ng of trimetazidine were carried through the procedure to determine the reproducibility of the method expressed by the standard deviation. Values of R [= (h trimetazidine)/(h internal standard)] were 0.284, 0.262, 0.271, 0.270, 0.295, 0.296, with $\bar{m} = 0.279 \pm 0.014$ equivalent to 20 ± 1 ng.

The reproducibility was further determined under the same conditions with plasma containing about 50 ng/ml of trimetazidine and with plasma containing about 2 ng/ml of trimetazidine. The results found were, respectively, 50 ± 2 ng/ml and 2 ± 0.5 ng/ml.

RESULTS

Limit of sensitivity

If the limit of sensitivity is defined as that signal which is three times higher

than the background signal, this method can be used to determine plasma containing about 1 ng of trimetazidine per ml.

Identification of dansylated trimetazidine

The structure of the compound that is analysed by HPLC has been confirmed as dansylated trimetazidine by GLC coupled with mass spectrometry. It shows a molecular peak at *m/z* 499 (Fig. 3). The molar mass of this derivative was also confirmed by chemical ionisation with the peak ($M^+ + 1$) = 500 *m/z*.

The structure of dansylated internal standard has been confirmed by GLC coupled with mass spectrometry. It shows a molecular peak at *m/z* 508. The molar mass of this derivative was also confirmed by chemical ionisation with the peak (M + 1) = 509 *m/z* (Fig. 4).

Conditions of derivatisation

The conditions for reaction of trimetazidine with dansyl chloride were optimised for pH, reagent concentration, temperature and time using published data for the reaction of the reagent with amines [2].

Pharmacokinetic profile in man

The method was applied to the determination of the pharmacokinetic profile of the drug during the clinical evaluation of trimetazidine in patients dosed orally. Fig. 5 shows results obtained for a patient who received a single 40-mg oral dose of trimetazidine hydrochloride.

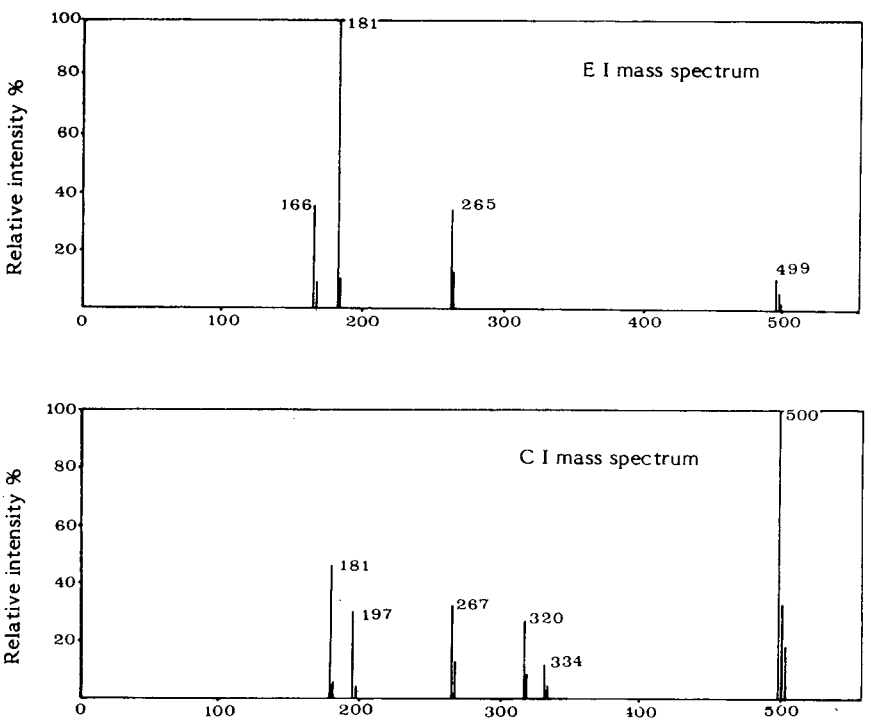


Fig. 3. Identification of dansylated trimetazidine by mass spectrometry.

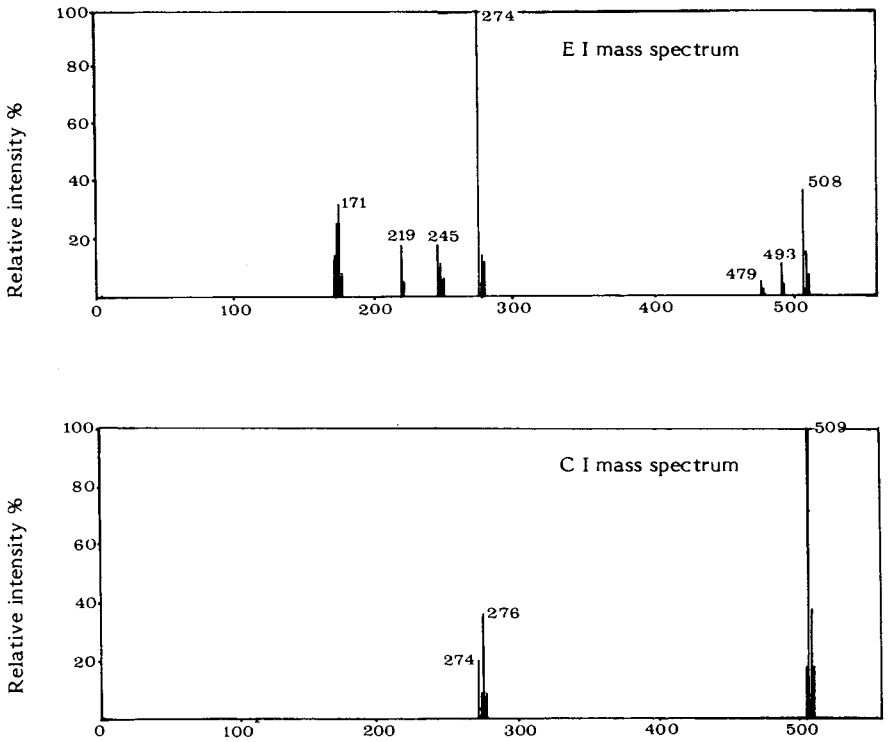


Fig. 4. Identification of dansylated internal standard by mass spectrometry.

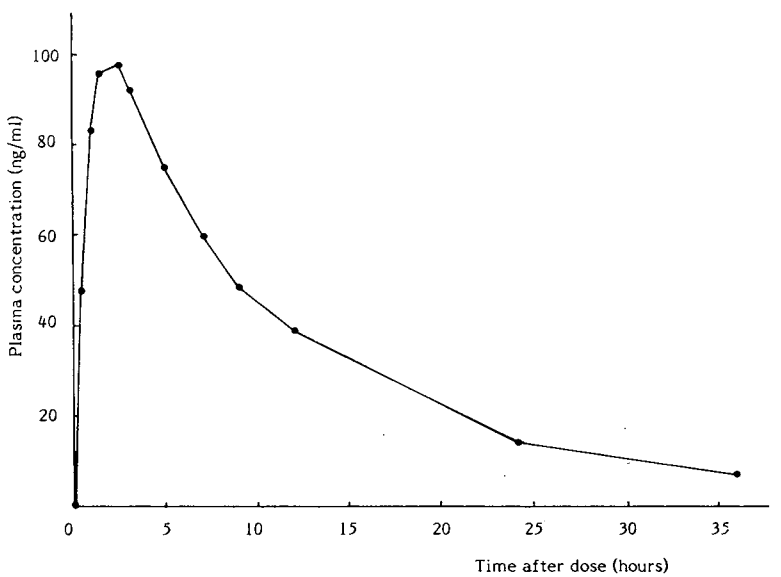


Fig. 5. Plasma trimetazidine profile after a single oral dose administered to a patient.

CONCLUSION

An HPLC procedure has been used successfully for the determination of trimetazidine in plasma at the ng/ml level. The sensitivity of the method described will allow plasma kinetics to be followed in hospitalized patients treated with trimetazidine, when plasma concentrations may range from 10 to 500 ng/ml.

REFERENCES

1 R.W. Frei, W. Santi and M. Thomas, J. Chromatogr., 116 (1976) 365—367.
2 J.F. Lawrence and R.W. Frei, Chemical Derivatization in Liquid Chromatography, Elsevier, Amsterdam, Oxford, New York, 1976, p. 153.
3 L. Di Cesare, Trends in Fluorescence, 2, Perkin-Elmer, Norwalk, CN, 1978.
4 J.P. Thomas, A. Brun and J.P. Bounine, Analusis, 7 (1979) 22.

Journal of Chromatography, 224 (1981) 168—174
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 855

Note

Analysis of the anticancer drugs etoposide (VP 16-213) and teniposide (VM 26) by high-performance liquid chromatography with fluorescence detection

R.J. STRIFE*

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, W. Lafayette, IN 47907 (U.S.A.)

I. JARDINE*

Department of Pharmacology, Mayo Foundation, Rochester, MN 55905 (U.S.A.)

and

M. COLVIN

Pharmacology Laboratory, The Johns Hopkins Oncology Center, 601 North Broadway, Baltimore, MD 21205 (U.S.A.)

(First received October 8th, 1980; revised manuscript received January 26th, 1981)

In a previous paper [1] we reported on an assay for the important anticancer drugs etoposide (VP 16-213) and teniposide (VM 26) (Fig. 1) by reversed-phase high-performance liquid chromatography (HPLC) using fixed-wavelength UV detection at 254 nm, and utilizing each drug as the internal standard for the other. The assay allowed the analysis of clinical drug plasma levels to 24 h, at which time drug levels are commonly about 500 ng/ml (850 pmol/ml) [2].

The potential of using fluorescence detection of these compounds for increased sensitivity was also demonstrated [1]. Detection of these relatively weakly fluorescing compounds was accomplished by placing a 30-μl flow-cell in the cuvette block of a typical spectrofluorometer, exciting the compounds at

*Present address: Department of Pharmacology, Mayo Foundation, Rochester, MN 55905, U.S.A.

0378-4347/81/0000—0000/$02.50

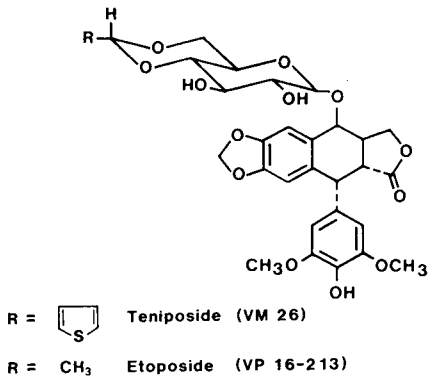


Fig. 1. Structures of etoposide (VP 16-213) and teniposide (VM 26).

288 nm and observing the emission at 328 nm. The transition from this experiment to that described here of using a commercial dedicated HPLC fluorescence detector was not straighforward, particularly regarding optimum conditions for sensitivity and selectivity. However, an improved assay for these drugs to 50 ng/ml (85 pmol/ml) of plasma was developed and is reported here. The advantages of this lower detection limit are demonstrated with a patient plasma concentration profile for etoposide over 48 h in which drug concentrations of considerably less than 500 ng/ml of plasma were encountered, primarily because a small dose of the drug was administered. The example also illustrates the advantage of the selectivity of fluorescence detection since the patient also received numerous other drugs during the 48-h time course and some of these interfered with the UV assay.

EXPERIMENTAL

Materials

Etoposide and teniposide were generously provided by Drs. H. Friedli and H. Stähelin (Sandoz, Basle, Switzerland), and by Dr. R.L. Buchanan (Bristol Labs., Syracuse, NY, U.S.A.). Chloroform and methanol (distilled in glass) were obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). All water used was distilled. Extraction of plasma was carried out in 16 × 125 mm culture tubes with PTFE-lined caps. All glassware used was routinely washed in chromic acid.

Apparatus

The HPLC system consisted of a Model M-6000A pump, a Model U6K injector, and a prepacked 30 cm × 3.9 mm I.D. μBondapak C_{18} (10 μm) column, all from Waters Assoc. (Milford, MA, U.S.A.). A short precolumn (7 cm × 2.1 mm I.D.) packed with Co:Pell ODS (30 μm) from Whatman (Clifton, NJ, U.S.A.) protected the analytical column. Detection was accomplished using the Model FS 970Q dedicated HPLC fluorescence detector with a quartz window photomultiplier tube, and the Model SF-770 dedicated

variable-wavelength UV detector, both from Kratos, Schoeffel Instrument Division (Westwood, NJ, U.S.A.). Cut-off filters at 300 and 320 nm, and a 7-54 broad band filter (ca. 200 nm wide centered at ca. 320 nm) were supplied by Schoeffel. A narrow band pass interference filter, 8.2 nm wide at half height and centered at 328 nm, was supplied by Spectrofilm (Winchester, MA, U.S.A.).

HPLC conditions

Analyses were performed with a flow-rate of 1.0 ml/min at an inlet pressure of about 48 bars. For samples from plasma extracts the flow-rate was increased after the compounds of interest were eluted, in order to elute retained background peaks as quickly as possible. The solvent used was methanol—water (60:40), and was filtered through a 0.5-μm MF millipore filter (Millipore, Bedford, MA, U.S.A.) and briefly degassed prior to use. The injection volume was 25 μl.

Calibration and analysis of etoposide from plasma

A stock solution of about 5 mg (accurately weighed) of etoposide per ml was made up in methanol. Ten-fold dilutions were made through 5 μg/ml (5 ng/μl). Teniposide was made up in a similar manner at 1 and 0.1 mg per ml of methanol. In spiking plasma, no more than 20 μl total methanol were added to 1 ml of plasma. One μg of teniposide was used as internal standard in spiked 1-ml plasma samples containing up to 5 μg of etoposide. For spiked samples containing more than this amount of etoposide per ml, 10 μg of teniposide were used as internal standard and this was taken into account in the calibration curve. These concentrated samples were also diluted ten-fold with methanol prior to injection in order to prevent overloading of the detector photomultiplier tube. All clinical samples analysed for the patient profile contained 1 μg of teniposide as internal standard. The samples were extracted and prepared for injection as described previously [1].

The excitation wavelength was set at 215 nm and the 10-nm band pass interference filter was mounted centrally in a piece of 2.5 × 2.5 cm cardboard, which was placed in the emission filter holder of the fluorescence detector. The sensitivity setting corresponded to about 1500 V applied to the photomultiplier tube.

Calibration points were determined in triplicate over the range 50 ng to 50 μg of etoposide per ml of plasma. No significant background was observed for the internal standard teniposide at 1.0 μg/ml of plasma. Therefore, the observed background at the retention time of etoposide, which was significant at the 50 ng/ml plasma level, was able to be internally standardized in blank samples as a peak height ratio with respect to the internal standard teniposide. This ratio was substracted from the observed peak height ratio of etoposide to teniposide in spiked samples. The zero time sample served as the blank in clinical samples.

RESULTS AND DISCUSSION

In determining the optimum instrumental configuration of the fluorescence

detector for this analysis, various excitation wavelength and emission filter combinations were employed to examine samples of pure etoposide and teniposide, as well as extracts of plasma samples spiked with these drugs. It was found that the previously used [1] excitation wavelength of 288 nm provided insufficient sensitivity for analysis. For example, with a 320-nm cut-off filter on the emission side, a 200-ng injection of pure etoposide only provided a signal-to-noise ratio of about 15:1. However, because the light source was now a deuterium lamp instead of the previously used xenon lamp, advantage could be taken of the stronger absorption of these drugs at 215 nm to gain sensitivity. A xenon lamp provides insufficient light intensity at this wavelength. Thus, with a 300-nm cut-off filter along with a 7-54 broad band filter on the emission side (to reduce stray white light reaching the PMT), 15 ng of etoposide provided a signal-to-noise ratio of about 40:1 using 215 nm excitation. Using these filters, sensitivity in the assay from plasma was ultimately limited by the background from the biological matrix. A 10-nm band pass emission filter provided only slightly better selectivity and sensitivity for plasma analysis.

The calibration curve from plasma was determined in triplicate at 51.85, 181.5, 285.2, 5185 and 51,850 ng of etoposide per ml of plasma by plotting the peak height ratio of etoposide/teniposide versus plasma concentration of

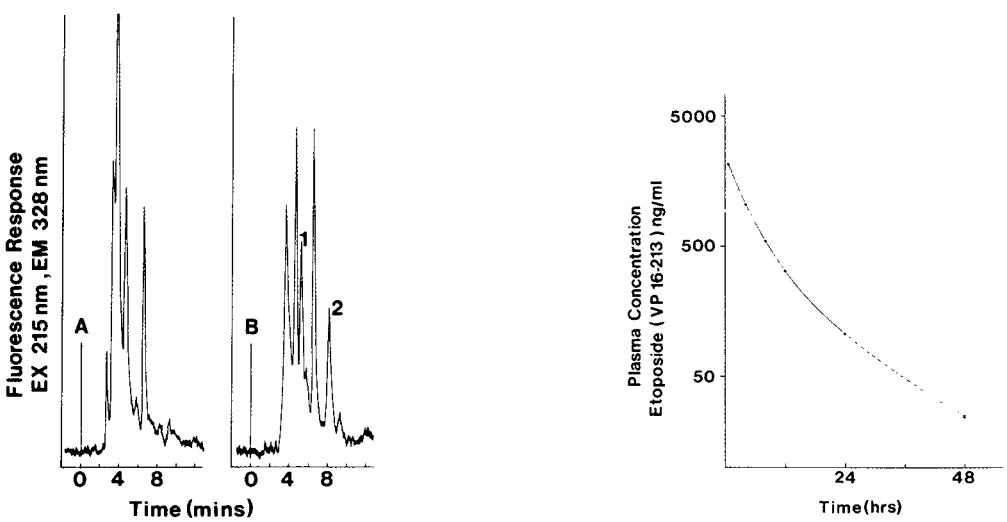


Fig. 2. HPLC fluorescence traces of plasma samples spiked with 50 ng/ml etoposide and teniposide, and treated by the chloroform extraction procedure. Chromatograms of (A) blank sample and (B) spiked sample. The endogenous substances eluting prior to 4 min varied from sample to sample but peaks after this time were reproducible. Chromatographic conditions: 30 cm × 3.9 mm I.D. μBondapak C_{18} (10 μm) column; solvent, methanol—water (60:40); flow-rate, 1.0 ml/min at an inlet pressure of ca. 48 bars. Peaks: 1 = etoposide (51.8 ng); 2 = teniposide (50 ng). EX = excitation wavelength, EM = emission wavelength.

Fig. 3. Post-infusion plasma decay curve of etoposide for a patient receiving the drug. All points represent the average of two determinations. The point labelled * was obtained from two separate determinations and is shown as an estimate since the actual calibration was performed down to 50 ng etoposide per ml plasma.

TABLE I

COMPARISON OF UV ABSORBANCE VS. FLUORESCENCE DETECTION OF ETOPOSIDE (VP 16-213) EXTRACTED FROM PLASMA WITH TENIPOSIDE (VM-26) AS INTERNAL STANDARD (I.S.)

Sample plasma concentration	Detector*	n	Average peak height ratio, $\bar{x}$ etoposide/ teniposide	S_x	Coefficient of variation $S_x/\bar{x}$ (%)	95% Confidence limit (ng)
500 ng/ml etoposide 10 μg/ml teniposide (I.S.)	UV (254 nm)	6	0.0747	0.0042	5.6	± 75
500 ng/ml etoposide 1 μg/ml teniposide (I.S.)	Fluorescence (215 nm EX, 328 nm EM)	6	0.797	0.0124	1.5	± 21
50 ng/ml etoposide 1 μg/ml teniposide (I.S.)	Fluorescence (215 nm EX, 328 nm EM)	7	0.0767	0.0063	8.2	± 10

*EX = excitation wavelength, EM = emission wavelength.

etoposide. The correlation coefficient was r^2 = 0.9999 with a y intercept of —0.006 and a lowest peak height ratio determined at 0.080.

From Table I it can be seen that the fluorescence assay at 500 ng of etoposide per ml of plasma provides a considerable improvement versus the UV assay [1]. The fluorescence assay can be used with confidence to 50 ng of etoposide or teniposide per ml of plasma (Fig. 2), and the presence of the drug can be confirmed at concentrations as low as 25 ng/ml plasma.

The analysis of etoposide in the plasma of a 62-year-old woman with oat cell carcinoma of the lung is shown in Fig. 3. The patient received a constant intravenous infusion of only 51 mg of etoposide over 0.5 h. The patient also received concurrently the other antineoplastic drugs doxorubicin (adriamycin), carmustine (BCNU), and procarbazine. Over the 48-h plasma collection period the patient also received doses of the diuretic furosemide, the antiemetic prochlorperazine (compazine), and the sedative—analgesic meperidine (demerol). This patient profile (Fig. 3) demonstrates the need to be able to detect plasma levels of less than 500 ng of etoposide per ml, which would not have been measurable by the UV method. The peak identity for etoposide at the first time point was confirmed by stopped flow UV scanning while at 4 h interferences occurred in the UV traces. Injection of appropriate extracts of the pharmaceutical preparations of the drugs coadministered with etoposide, showed that some produced peaks that could have interfered with the analysis of etoposide using UV detection. However, none of these drug extracts showed any peaks by fluorescence detection and this specificity permitted accurate analysis of etoposide even at long times in patient samples (Fig. 4). The excellent profile for etoposide actually obtained reassures that no interfering compounds were co-eluted with etoposide or teniposide and detected by fluorescence.

Because of the high sensitivity capable with fluorescence detection, extractions were also carried out on 100 μl of spiked plasma samples using 500 μl of chloroform. At 500 ng etoposide per ml of plasma, about 20 ng is actually loaded on column. Recoveries were still nearly quantitative but the 95%

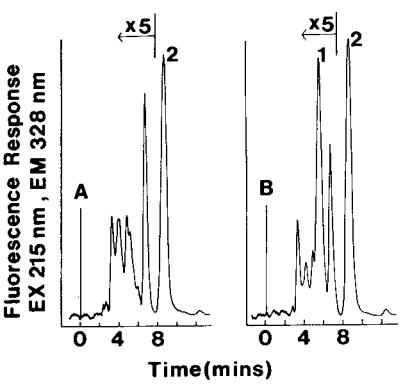


Fig. 4. HPLC fluorescence traces of patient plasma samples treated by the chloroform extraction procedure. (A) pre-infusion and (B) 24-h post-infusion. Peaks: 1 = etoposide (109 ng/ml); 2 = teniposide, internal standard (1.01 μg/ml). Chromatographic conditions and abbreviations as in Fig. 2.

confidence levels previously obtained using 1 ml of plasma (Table I) were slightly wider (518 ± 50 ng/ml, $n = 5$, $S_x/\overline{x} = 3.1\%$) because baseline noise was becoming significant. Thus it is feasible to perform the assay on patient plasma samples of only 100 μl to the 500 ng/ml level.

A problem with this fluorescence assay is that fluorescing substances are detected at long retention times. These peaks can interfere with subsequent injections. At concentrations of 500 ng of etoposide per ml plasma, samples can be safely overlapped to avoid these relatively small interferences so that three samples can be run in 90 min. However, it is suggested that for routine clinical use column switching technology [3] should be implemented to allow samples to be run every 10—15 min. In any case, since the internal standard is so similar to the drug being measured in its extraction, chromatographic and fluorescence behavior, it is expected that instrumental variations in analyses which take some time to complete, will be minimal. This is supported by consistent peak height ratios for pure samples injected and observed in day-to-day operations. For this reason, it is expected that frequent recalibration of the instrument will not be necessary.

The interferences at long retention times can also be minimized by operating at 235 nm for excitation, but some sensitivity is lost with a comparable detection limit for etoposide from plasma being about 150 ng/ml. Similar linearity over a comparable analysis range was obtained as with analysis at 215 nm.

CONCLUSION

An improved reversed-phase HPLC assay for the antineoplastic drugs etoposide (VP 16-213) and teniposide (VM 26) has been developed for clinical analysis of plasma levels of these drugs. Fluorescence detection of these compounds increases the sensitivity and selectivity of analysis compared to UV detection. The improved assay is useful for the analysis of these drugs to about 50 ng/ml (85 pmol/ml) of plasma and will allow analysis of plasma levels to about 48 h even with administration of low drug dosages.

ACKNOWLEDGEMENTS

The authors thank Drs. H. Friedli and H. Stähelin of Sandoz Basle, Switzerland and Dr. R.L. Buchanan of Bristol Laboratories, Syracuse, NY, U.S.A., for extremely generous gifts of etoposide and teniposide. We also thank the Mayo Foundation for research support and, in particular, for funds to purchase the HPLC equipment. Dr. Colvin is supported by NIH grant CA 00103.

REFERENCES

1 R.J. Strife, I. Jardine and M. Colvin, J. Chromatogr., 182 (1980) 211.
2 L.M. Allen and P.J. Creaven, Eur. J. Cancer, 11 (1975) 697.
3 G.C. Davis and P.T. Kissinger, Anal. Chem., 51 (1979) 1960.

Journal of Chromatography, 224 (1981) 175—176
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 834

Book Review

Drug level monitoring — Analytical techniques, metabolism, and pharmacokinetics, by W. Sadée and G.C.M. Beelen, Wiley-Interscience, New York, 1980, XIII + 495 pp., price £22.20, ISBN 0-471-0488-1.

Although several encyclopaedic works in drug analysis such as "GLC and HPLC determination of therapeutic agents", in three volumes, edited by Tsuji (1978, 1979), "Drug fate and metabolism — Methods and techniques", edited by Garrett and Hirtz (4 volumes), and "Analysis of drugs and metabolites by gas chromatography—mass spectrometry", edited by Gudzinowicz and Gudzinowicz (9 volumes) are available as authoritative reference works, the present work like its immediate predecessor entitled, "Drug assay: The strategy of drug monitoring", by Kalman and Clark (1979), represents a more manageable reference for the analytical chemist involved in routine drug analysis. The authors have drawn extensively from their teaching experience and have developed a textbook suited both to the graduate student majoring in clinical biopharmaceutics, pharmacokinetics, and to the bioanalytical chemist or toxicologist at large.

The book is divided into five chapters; the first four cover the principles of drug metabolism, pharmacokinetics, clinical pharmacokinetics and therapeutic drug level monitoring and analytical techniques. Chapter 4 covers sample preparation, clean-up, derivatization prior to analysis and the principles underlying the different analytical techniques available for the final quantitative determinate step, and therefore contains the bulk of the physico-chemical analytical information. The scope of these four chapters is quite general and is intended as an overview, with pertinent literature citations given for more in-depth study. They do contain useful tables of information which summarize data on the pharmacokinetic parameters and metabolic profiles of 102 selected drugs (Tables I and II) and Table III, which is a survey of different analytical techniques available for the selected drugs previously mentioned.

The bulk of the book, Chapter 5, comprises a series of "monographs" of 100 drug compounds that any clinical hospital laboratory involved in therapeutic drug monitoring and/or accidental overdosage analysis is likely to encounter. The monographs alphabetized by generic name, are compiled by a variety of authors (graduate students) in a set format and contain essential information on therapeutic drug concentration range, metabolism, analogous compounds and the scope of analytical methods used for their quantitation.

The most useful and/or generally applicable method is discussed in some detail with a literature citation, while other useful ancillary methods are critiqued and treated superficially. Each monograph for a particular drug is fairly complete in the extent of information contained, and is the best feature of the book. The monographs per se provide valuable information to the clinical chemist responsible for the Bioanalytical-Clinical Laboratory, hence serves as a useful reference. Indeed, the book should have been titled, "Bioanalytical Monographs of Drug Substances".

Of the multiplicity of methods presented for each compound and/or class of compounds, either high-performance liquid chromatography (HPLC) or gas—liquid chromatography (GLC) using a specific detector (flame ionization, electron-capture, nitrogen—phosphorus) or gas chromatography—mass spectrometry methods predominate, and justifiably so. The HPLC and GLC methods are described in greater detail and indicate to the reader their overall utility in this field; however, the selection of one technique over another as the one of choice is left to the reader's discretion. The authors appear to favor HPLC methods in each monograph, probably based on their own experience in the clinical pharmacokinetics laboratory, since they are described in greater frequency and detail, and in many cases, they are either the most appropriate or straightforward in use, e.g. for the anticonvulsants, antibiotics, and others such as quinidine, chloroquine, etc. The text is up to date as of November, 1979, by virtue of an appendix, is remarkably free of typographical errors in the text and in the chemical structures given (except on p. 172 — structure of chlordiazepoxide, the double bond should be in the 1—2 position rather than the 2—3 position of the benzodiazepine ring), denoting an excellent job of proof reading. The book would be a useful addition to the library of the practicing bioanalytical chemist or toxicologist involved in therapeutic drug monitoring.

Nutley, NJ (U.S.A.) J.A.F. de SILVA

Vol. 224, No. 1

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

NEWS SECTION

CALENDAR OF FORTHCOMING EVENTS

July 22–24, 1981 Manchester, Great Britain	**Biochemical Society Annual General Meeting and Joint Society/Nucleotide and Nucleic Acid Group Colloquium on Biochemical Interactions of Plasmids with their Hosts** Contact: The Biochemical Society, 7 Warwick Court, High Holborn, London WC1R 5DP, Great Britain.
July 26–31, 1981 Liverpool, Great Britain	**6th International Symposium on Carotenoids** Contact: Dr. G. Britton, Department of Biochemistry, University of Liverpool, POB 147, Liverpool L69 3BX, Great Britain.
Aug. 23–28, 1981 Espoo, Finland	**Euroanalysis IV – Triennial Conference of the Federation of European Chemical Societies** Contact: Professor L. Niinistoe, Department of Chemistry, Helsinki University of Technology, SF-02150 Espoo 15, Finland.
Aug. 25–28, 1981 Noordwijkerhout, The Netherlands	**2nd Noordwijkerhout IUPAC–IUPHAR Symposium "Strategy in Drug Research"** Contact: Secretariat 2nd Noordwijkerhout IUPAC/IUPHAR Symposium, c/o Merck Sharp & Dohme B.V., Professional and Government Liaison, Waarderweg 39, P.O. Box 581, 2003 PC Haarlem, The Netherlands. (Further details published in Vol. 223, No. 2)
Aug. 30–Sept. 5, 1981 Vienna, Austria	**XI International Congress of Clinical Chemistry – IV European Congress of Clinical Chemistry** Contact: Congress Secretariat: Interconvention, P.O. Box 35, A-1095 Vienna, Austria. Tel. (0222) 421352.
Sept. 1–3, 1981 Houston, TX, U.S.A.	**"EXPOCHEM '81"** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749–2623.

Sept. 1–4, 1981 Siofok, Hungary	**3rd Danube Symposium on Chromatography** Contact: Hungarian Chemical Society, H-1368 Budapest, P.O.B. 240, Hungary. Tel. Budapest 427–343. (Further details published in Vol. 189, No. 2).
Sept. 7–10, 1981 Guildford, Great Britain	**4th International Bioanalytical Forum** Contact: Dr. E. Reid, Wolfson Bioanalytical Unit, Robens Institute, University of Surrey, Guildford GU2 5XH, Great Britain. (Further details published in Vol. 223, No. 1)
Sept. 9–11, 1981 Chicago, IL, U.S.A.	**2nd International Symposium on Radiopharmacology and Exhibition** Contact: Dr. Lelio G. Colombetti, Pharmacology Department, Loyola University, Stritch School of Medicine, Maywood, IL 60153, U.S.A.
Sept. 20–25, 1981 Philadelphia, PA, U.S.A.	**8th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies (FACSS)** Contact: Richard J. Knauer, Publicity Chairman, ARMCO INC., P.O. Box 1697, Baltimore, MD 21203, U.S.A.
Sept. 28–Oct. 1, 1981 Barcelona, Spain	**16th International Symposium Advances in Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel. (713) 749-2623. (Further details published in Vol. 222, No. 2)
Sept. 29–Oct. 2, 1981 Basle, Switzerland	**ILMAC 81; 8th International Exhibition of Laboratory, Chemical Engineering, Measurement and Automation Techniques in Chemistry** Contact: D. Gammeter, Secretariat ILMAC '81, Postfach, CH-4021 Basle, Switzerland. Tel. 061 26 20 20.
Oct. 22–23, 1981 Montreux, Switzerland	**Workshop on Liquid Chromatography – Mass Spectroscopy** Contact: Prof. Dr. R.W. Frei, Free University, Department of Analytical Chemistry, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands (Further details published in Vol. 207, No. 3)
Nov. 9–10, 1981 Berlin, G.F.R.	**Symposium on Practical Aspects of HPLC** Contact: Dr. I. Molnár, Wissenschaftliche Geratebau Dr. H. Knauer GmbH, Hegauer Weg 38, D-1000 Berlin 37, G.F.R. (Further details published in Vol. 207, No. 2)
Nov. 16–17, 1981 Washington, DC, U.S.A.	**The International Symposium on HPLC of Proteins and Peptides** Contact: Shirley E. Schlessinger, Symposium Manager, International Symposium on HPLC of Proteins and Peptides, 400 East Randolph, Chicago, IL 60601, U.S.A. (Further details published in Vol. 208, No. 2)
Nov. 23–25, 1981 Barcelona, Spain	**2nd International Congress on Analytical Techniques in Environmental Chemistry** Contact: Dr. J. Albaigés, General Secretary, Plaza de Espana, Barcelona–4, Spain. Tel: 223–31 01.
Dec. 2–3, 1981 Paris, France	**Journées de Chromatographie en Phase Liquide** Contact: H. Colin, Laboratoire C.A.P., Ecole Polytechnique, Route de Saclay, 91128 Palaiseau Cedex, France.

March 28–April 2, 1982 Las Vegas, NV, U.S.A.	**National American Chemical Society Meeting** Contact: A.T. Winstead, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 14–16, 1982 Amsterdam, The Netherlands	**12th Annual Symposium on the Analytical Chemistry of Pollutants** Contact: Prof. Dr. Roland W. Frei, The Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 206, No. 1)
April 19–22, 1982 Barcelona, Spain	**International Congress on Automation in Clinical Laboratory** Contact: Dr. R. Galimany, Sección de Automatización, Laboratorio de Analisis Clinicos, C.S. "Principes de Espana", Hospitalet de Llobregat, Barcelona, Spain.
April 21–23, 1982 Neuherberg near Munich, G.F.R.	**Second International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft fuer Strahlen- und Umweltforschung, Institut fuer Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstaedter Landstrasse 1, D-8042 Neuherberg, G.F.R.
April 27–30, 1982 Munich, G.F.R.	**Biochemische Analytik Conference** Contact: Prof. I. Trautschold, Medizinische Hochschule Hannover, Karl-Wiechert-Allee 9, 3000 Hannover 61, G.F.R.
July 11–16, 1982 Washington, DC, U.S.A.	**6th International Conference on Computers in Chemical Research and Education (ICCCRE)** Contact: Dr. Stephen R. Heller, Chairman, 6th ICCCRE, EPA, MIDSD, PM–218, 401 M Street, S.W., Washington, DC 20460, U.S.A. Tel: (202) 755–4938, Telex: 89–27–58.
Aug. 15–21, 1982 Perth, Australia	**The 12th International Congress of Biochemistry** Contact: Brian Thorpe, Department of Biochemistry, Faculty of Science Australian National University, Canberra A.C.T. 2600, Australia
Aug. 31–Sept. 2, 1982 Vienna, Austria	**5th International IUPAC Symposium on Mycotoxins and Phycotoxins** Contact: Prof. P. Krogh, Department of Veterinary Microbiology, Purdue University, West Lafayette, IN 47907, U.S.A.
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.
Aug. 28–Sep. 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

NEW BOOKS

Clinical Biochemistry Review, Vol. 2, edited by D.M. Goldberg, Wiley, Chichester, New York 1981, ca. 416 pp., price ca. US$ 30.00, £ 12.60, ISBN 0-471-08297-X.

Protein–protein interactions, edited by C. Frieden and L.W. Nichol, Wiley, Chichester, New York, 1981, ca. 400 pp., price ca US$ 37.50, £ 16.00, ISBN 0-471-04979-4 (to be reviewed in *J.Chromatogr., Biomed. Appl.).*

How To Comply With The New Copyright Law

Participation in the Copyright Clearance Center (CCC) assures you of legal photocopying at the moment of need.

Libraries everywhere have found the easy way to fill photocopy requests legally and instantly, without the need to seek permissions, from more than 3000 key publications in business, science, humanities and social science. You can:

Fill requests for multiple copies, interlibrary loan (beyond the CONTU guidelines), and reserve desk without fear of copyright infringement.

Supply copies from CCC-registered publications simply and easily.

The Copyright Clearance Center is your one-stop place for on-the-spot clearance to photocopy for internal use.

Its flexible reporting system accepts photocopying reports and returns an itemized invoice. You send only one convenient payment. CCC distributes it to the many publishers whose works you need.

And, you need not keep any records, the CCC computer will do it for you. Register now with the CCC and you will never again have to decline a photocopy request or wonder about compliance with the law for any publication participating in the CCC.

To register or for more information, just contact:

Copyright Clearance Center
21 Congress Street
Salem, Massachusetts 01970
(617) 744-3350

a not-for-profit corporation

NAME _____ TITLE _____

ORGANIZATION _____

ADDRESS _____

CITY _____ STATE _____ ZIP _____

COUNTRY _____ TELEPHONE _____

PUBLICATION SCHEDULE FOR 1981

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1980	D 1980	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography			203 204 205/1 205/2	206/1 206/2 206/3	207/1 207/2 207/3	208/1 208/2 209/1	209/2 209/3 210/1	210/2 210/3 211/1	211/2 211/3 212/1 212/2	The publication schedule for further issues will be published later.				
Chromatographic Reviews							220/1							
Biomedical Applications	221/1	221/2	222/1	222/2	222/3	223/1	223/2	224/1	224/2					

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. A free reprint can be obtained by application to the publisher)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (e.g., Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.

JOURNAL OF ANALYTICAL AND APPLIED PYROLYSIS

Editors:
H. L. C. MEUZELAAR
Biomaterials
Profiling Center,
University of Utah,
391 South Chipeta
Way,
Research Park,
Salt Lake City,
UT 84108, U.S.A.

H.-R. SCHULTEN
Institut für Physikalische Chemie der
Universität Bonn,
5300 Bonn,
Wegelerstrasse 12,
G.F.R.

Associate Editor:
C. E. R. JONES,
36 Green Lane,
Redhill, Surrey RH1 2DF, U.K.

This new international journal brings together, in one source, qualitative and quantitative results relating to:

- Controlled thermal degradation and pyrolysis of technical and biological macromolecules;
- Environmental, geochemical, biological and medical applications of analytical pyrolysis;
- Basic studies in high temperature chemistry, reaction kinetics and pyrolysis mechanisms;
- Pyrolysis investigations of energy related problems, fingerprinting of fossil and synthetic fuels, coal extraction and liquefaction products.

The scope includes items such as the following:

1. Fundamental investigations of pyrolysis processes by chemical, physical and physicochemical methods.
2. Structural analysis and fingerprinting of synthetic and natural polymers or products of high molecular weight.
3. Technical developments and new instrumentation for pyrolysis techniques in combination with chromatographic or spectrometric methods, with special attention to automation, optimization and standardization.
4. Computer handling and processing of pyrolysis data.

Pyrolysis is applied in a wide range of disciplines. This journal is therefore of value to scientists in such diverse fields as polymer science, forensic science, soil science, geochemistry, environmental analysis, energy production, biochemistry, biology and medicine.

The journal publishes original papers, technical reviews, short communications, letters, book reviews and reports of meetings and committees. The language of the journal is English. Prospective authors should contact one of the editors.

Subscription Information:
1981: US $86.25/Dfl. 168.00, including postage.

Ask for a free sample copy.

P.O. Box 211,
1000 AE Amsterdam
The Netherlands

52 Vanderbilt Ave
New York, N.Y. 10017

The Dutch guilder price is definitive.
US $ prices are subject to exchange rate fluctuations.

ELSEVIER