ISSN 0378-4347

VOL. 307 NO. 2 MAY 11, 1984

(Biomedical Applications, Vol. 32, No. 2)

THIS ISSUE COMPLETES VOL. 307

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

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0378-4347/84/$03.00

Printed in The Netherlands

VOL. 307, NO. 2 JOURNAL OF CHROMATOGRAPHY MAY 11, 1984

(Biomedical Applications, Vol. 32, No. 2)

(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, Deep-Sea Research/Part B: Oceanographic Literature Review, Index Medicus, Mass Spectrometry Bulletin, Pharmaceutical Abstracts, Referativnyi Zhurnal, and Science Citation Index)

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Contents (continued)

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(Due to limitations of space, only the plenary and keynote lectures from the first conference and the topics of the second conference are listed below.)

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Journal of Chromatography, 307 (1984) 241—249
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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QUANTIFICATION OF A NUMBER OF BIOGENIC AMINES AND THEIR METABOLITES IN BRAIN HOMOGENATES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(First received June 13th, 1983; revised manuscript received December 6th, 1983)

SUMMARY

A sensitive method is described for the assay of various biogenic amines and their metabolites from brain tissue homogenates. Based on gas chromatography—mass spectrometry and selected ion monitoring, the method allows reliable low-level quantification of the compounds of interest. By careful study of reaction conditions both in the preparation of derivatives for gas chromatography and in the synthesis of deuterated analogues for internal standard use, relatively simple procedures could be applied with good analytical efficiency. Correlation of the results with those obtained by a high-performance liquid chromatographic procedure was also investigated, and a neurologic application of the method is briefly discussed.

INTRODUCTION

Various neurologic and psychiatric diseases seem to be characterized by alterations in brain levels of adrenaline, noradrenaline, dopamine, serotonin, 3-methoxy-4-hydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxy-3-indoleacetic acid [1—3]. Reliable quantification of the extremely low levels of such compounds in brain tissue necessitates application of sophisticated techniques, such as gas chromatography—mass spectrometry (GC—MS) [4], or electrochemical detection of compounds separated by high-performance liquid chromatography (HPLC) [5].

Using deuterated analogues as the corresponding internal standards, selected ion monitoring (SIM) provides an ideal basis for specific quantitative analysis by GC—MS, especially in association with quadrupole instruments [6, 7].

0378-4347/84/$03.00

The polar character of the compounds studied here — catecholamines, serotonin, and the four metabolites — necessitates their derivatization prior to gas chromatography. Several methods are available for reactions involving phenolic and alcoholic hydroxyl groups and primary or secondary amino groups present in these molecules [4, 8—13]. In the present investigation, acylation with pentafluoropropionic anhydride and esterification with pentafluoropropanol were used for derivatization, optimum reaction conditions being found by systematic selection and modification of appropriate methods.

Deuterium labelling was also investigated in detail, and relatively mild reaction conditions are described for an efficient conversion of the compounds of interest to the corresponding deuterated analogues by electrophilic substitution of the aromatic ring hydrogens [14—16].

EXPERIMENTAL

Chemicals used in the present investigation were obtained from the following sources: 3,4-dihydroxyphenylacetic acid (DOPAC), (—)-adrenaline (+)-bitartrate, 3-methoxy-4-hydroxy-phenethanol (MOPET), (—)-noradrenaline bitartrate, 3-hydroxytyramine (dopamine) hydrochloride and 3,4-dihydroxybenzylamine (DHBA) from Sigma (St. Louis, MO, U.S.A.); homovanillic acid (HVA), serotonin monooxalate, potassium salt of 3-methoxy-4-sulfonyloxyphenylethyleneglycol (MHPG) and 5-hydroxy-3-indoleacetic acid (5-HIAA) from Fluka (Buchs, Switzerland); 2,2,3,3,3-pentafluoropropanol from Koch-Light Labs. (Colnbrook, U.K.); acetic-d_3- acid-*d* (99.5 atom %) and deuterium oxide (100 atom %) from Aldrich (Milwaukee, WI, U.S.A.); deuterium chloride (20% solution in ^2H_2O) and sulphuric acid-*d* (98% solution in ^2H_2O) from EGA Chemie (Steinheim, F.R.G.); pentafluoropropionic anhydride (PFPA) from Pierce (Rockford, IL, U.S.A.); boron trichloride methanol (BCl_3—methanol, 10% w/v) from Applied Science Labs. (State College, PA, U.S.A.); Amberlite-XAD type 2 resin, particle size 0.3—1.0 mm from Serva Feinbiochemica (Heidelberg, F.R.G.). All other chemicals and solvents used were obtained from E. Merck (Darmstadt, F.R.G.).

Homogenization of brain tissue

Samples (0.8—1.0 g) of brain tissue were suspended in distilled water (1:3) containing 0.1% of $Na_2S_2O_5$ and 0.01% of sodium EDTA, and 400—700 μl of this homogenate were used for the assay. The pH was adjusted to 1—2 with a solution of 0.1 *M* hydrochloric acid, and 25 ng or 50 ng of appropriate internal standards were added. The suspension was finally vortex-mixed and centrifuged at 15,000 *g* for 20 min.

Extraction of adrenaline, noradrenaline and dopamine

The supernatant from homogenization (as above), 50 mg of alumina (Al_2O_3), and 2.5 ml of 0.5 *M* Tris buffer (pH 8.6) were mixed in a test tube. After centrifugation, the supernatant was removed and the alumina was washed with 10 × 1 ml of distilled water. The catecholamines were extracted from the alumina with 2 × 0.4 ml of a solution of 0.4 *M* acetic acid in methanol (1:45). After solvent evaporation with a stream of nitrogen, the residue was treated as described under Derivatization (see below).

Extraction of serotonin, DOPAC, HVA, MHPG and 5-HIAA

Either 25 ng or 50 ng of the appropriate internal standards were added to the supernatant from homogenization and this solution was slowly applied to a column (0.8 × 2 cm) containing Amberlite XAD-2 resin. After washing with 15 ml of diluted hydrochloric acid, the column was eluted with 3 × 0.7 ml of methanol. The eluate was evaporated to dryness under a stream of nitrogen and the residue was treated as described under derivatization. The extraction system was found to be quantitative in experiments using authentic compounds.

Derivatization

Standard solutions of adrenaline, noradrenaline, dopamine and serotonin (10 μg in 0.1 ml of methanol) were evaporated to dryness in test tubes under a stream of nitrogen. Then 0.1 ml of BCl_3—methanol was added and the tubes were maintained at 45°C for 15 min. After solvent evaporation, 0.1 ml of PFPA was added, and the reaction was allowed to proceed at 45°C for 1.5 h. The reagent was removed with a stream of nitrogen and the residue dissolved in 50 μl of ethyl acetate. The same procedure was used for the appropriate extraction residues (see above) and deuterated compounds (see below), while the metabolites DOPAC, HVA, MHPG and 5-HIAA, the corresponding extraction residues and deuterated compounds were derivatized according to the procedure reported in ref. 13, but using double reagent volumes.

Preparation of deuterated compounds for use as internal standards in SIM

Dopamine. 10 mg were dissolved in 2 ml of a solution of 20% ^2HCl in ^2H_2O, and maintained at 130°C for 21 h in a stoppered test tube. The product was recrystallized from methanol—diethyl ether (1:1) and stored at −20°C under nitrogen [14, 15].

Serotonin. 10 mg were dissolved in 2 ml of a solution of 20% $^2H_2SO_4$ in ^2H_2O and purged with nitrogen for 2 h. Aliquots (1 ml) of the solution were maintained at 45°C for 50 h or 72 h in stoppered test tubes. After cooling to room temperature, 150 mg of ascorbic acid were added to the solutions, which were then adjusted to pH 10.0 by dropwise addition of 2 *M* NH_4OH. Extraction with 3 × 2 ml of ethyl acetate* yielded stock solutions which were stored in stoppered test tubes under nitrogen at −20°C.

HVA. 30 mg were dissolved in a solution prepared by mixing 3.8 ml of 20% ^2HCl in ^2H_2O, 0.5 ml of $C^2H_3COO^2H$ and 1 ml of ^2H_2O. The solution was refluxed at 190°C for 30 min, with a stream of nitrogen directed into the cooler. After cooling to room temperature, the solution was extracted with 3 × 5 ml of ethyl acetate. The solvent was evaporated with a stream of nitrogen and the product recrystallized from 2 ml of benzene—diethyl ether (1:1). The crystals obtained were stored under nitrogen at −20°C.

DOPAC. 15 mg were dissolved in 0.7 ml of a soluton of 20% ^2HCl in ^2H_2O and incubated at 60°C for 5 h in a stoppered test tube. The solution was extracted with 3 × 4 ml of ethyl acetate. Evaporation of the solvent under a stream of nitrogen yielded a crystalline residue which was stored under nitrogen at −20°C.

*All ethyl acetate extractions were performed with freshly distilled, nitrogen-purged solvent.

5-HIAA. 5 mg was dissolved in 2 ml of a 10% solution of $^2H_2SO_4$ in ^2H_2O. The solution was purged with nitrogen for 2 h and divided into three aliquots which were incubated in stoppered test tubes under nitrogen at 50°C for 21, 50 and 72 h, respectively. After cooling to room temperature, 50 mg of ascorbic acid were added to each of the solutions, which were then extracted with 3 × 5 ml of ethyl acetate. The solutions obtained were stored under nitrogen at −20°C.

MHPG. Attempts to prepare deuterated analogues of this compound were unsuccessful.

Derivatization of deuterated compounds

The deuterated amines and metabolites were derivatized for gas chromatography according to the procedures described under Derivatization except in the case of 5-HIAA. The deuterated analogue of this compound was acylated with PFPA at 45°C instead of 75°C.

Gas chromatography—mass spectrometry

A Hewlett-Packard 5990 A quadrupole GC—MS system (with HP 9825 B calculator and HP 9876 A printer) was used. The gas chromatograph was equipped with an SE-30 fused-silica capillary column (25 m × 0.32 mm I.D., Orion Analytica). Helium was used as carrier gas with a flow-rate of 3 ml/min. The GC injector and the GC/MS interface were maintained at 220°C and 210°C, respectively. The column oven was maintained at 70°C during injection and raised to 90°C immediately after elution of the solvent peak. After 1 min, the temperature was increased by 10°C/min to 170°C, and this temperature was maintained until the analysis was completed. The electron impact source of the mass spectrometer was operated at 70 eV, and the detector multiplier voltage was 2600 V.

TABLE I

RESULTS FROM THE PREPARATION OF DEUTERATED ANALOGUES (INTERNAL STANDARDS)

Compound	MW*	Incubation conditions		Relative (%) yields of products**						Ion (*m/e*)
		Temperature (°C)	Time (h)	d_0	d_1	d_2	d_3	d_4	d_5	
Adrenaline	753	45	4	59	100	59	16	3	—	590
Adrenaline		45	27	—	23	91	100	18	2	590
Noradrenaline	767	45	4	54	100	68	19	3	—	445
Noradrenaline		45	16	3	34	90	100	17	—	590
Noradrenaline		45	38 (10% ^2HCl)	41	100	60	27	3	—	445
Dopamine	591	130	21	—	—	7	100	—	—	428
Serotonin	614	45	50	76	100	49	14	1	—	451
DOPAC	592	60	5	1	27	100	15	2	—	415
5-HIAA	615	50	21	40	71	100	55	11	—	438
5-HIAA		40	50	30	88	100	42	9	1	438
HVA	460	190	0.5	—	—	4	100	—	—	460

*MW = molecular weight.

**The values are only approximate since isotope effect may influence fragmentation.

High-performance liquid chromatography

The procedures and solvent systems described in ref. 5 were directly applied to the homogenate extracts (see above). The HPLC equipment consisted of an Altex 110 A pump, an Altex Ultrasphere ODS (C_{18}) column (25 × 0.4 cm, particle size 5 μm), a BAS LC 4 B amperometric detector with glassy carbon electrode, and a Shimadzu C-R1A Chromatopac integrator. Detector voltages of +0.60 V and +0.85 V were used in the analysis of amines and metabolites, respectively. The internal standard for amines was DHBA (0.2 μM), while MOPET (1.0 μM) was used as internal standard in the quantification of metabolites. The sample loop volume was 20 μl.

RESULTS AND DISCUSSION

Extraction of the compounds studied yielded consistent results; however, in the case of serotonin the procedure was unsatisfactory.

Esterification of the carboxyl groups of a number of catecholamine metabolites using BCl_3—methanol as the reagent has been previously reported [16,

```
** CONDITIONS FOR RUN #   100  dated: 5/ 6/1983  Friday
********************************************************************************
CAPILLARY CONDITIONS:  Injection Temp.=  70 deg.  Wait= 0.0 min.
   Ramp rate =10.0     Inject Mode Time = 0.0 min  Flush Mode Time =0.0 min
 TEMP1  TIME1    RATE    TEMP2  TIME2  INJ.PORT  MAN.OVEN  SOLVENT  RUN TIME
  Deg.   min.  Deg/min.   Deg.   Min.    Deg.      Deg.      Min.     Min.
  150    0.0    10.0     170    20.0    220       300       2.0      20.0

MS PEAK DETECT THRESHOLD =     12.0 linear counts
FLOW RATE =                     2   ml/min
SAMPLES PER .1 AMU =            2   SCAN SPEED = 690 amu/sec
ELECTRON MULTIPLIER =        2400   volts
GC PEAK DETECT THRESHOLD =   1000   TRIGGERED ON TOTAL ABUNDANCE
REAL TIME STRIPPING OF VALLEYS FROM PEAKS

SAMPLE NAME  DOPAC, HVA, 5-HIAA, MHPG
--------------------------------------------------------------------------------

                SE-30 silica capillary 25 m delay 30 s splitless
--------------------------------------------------------------------------------
```

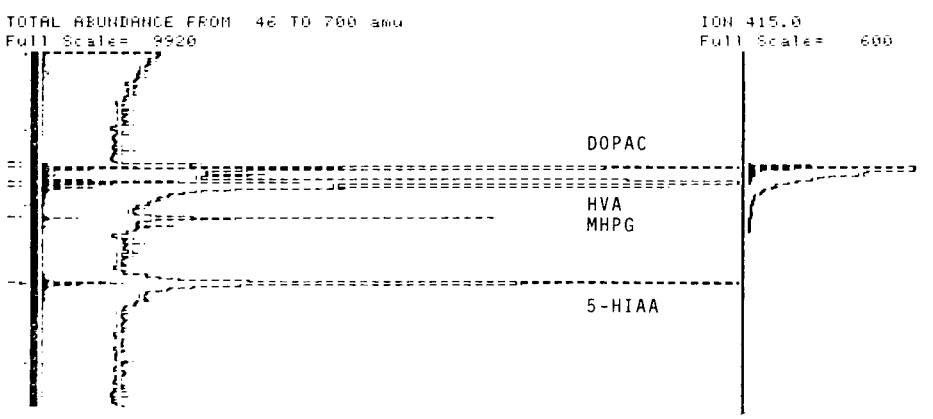


Fig. 1. Total ion current chromatogram showing the separation of derivatized DOPAC, HVA, 5-HIAA and MHPG.

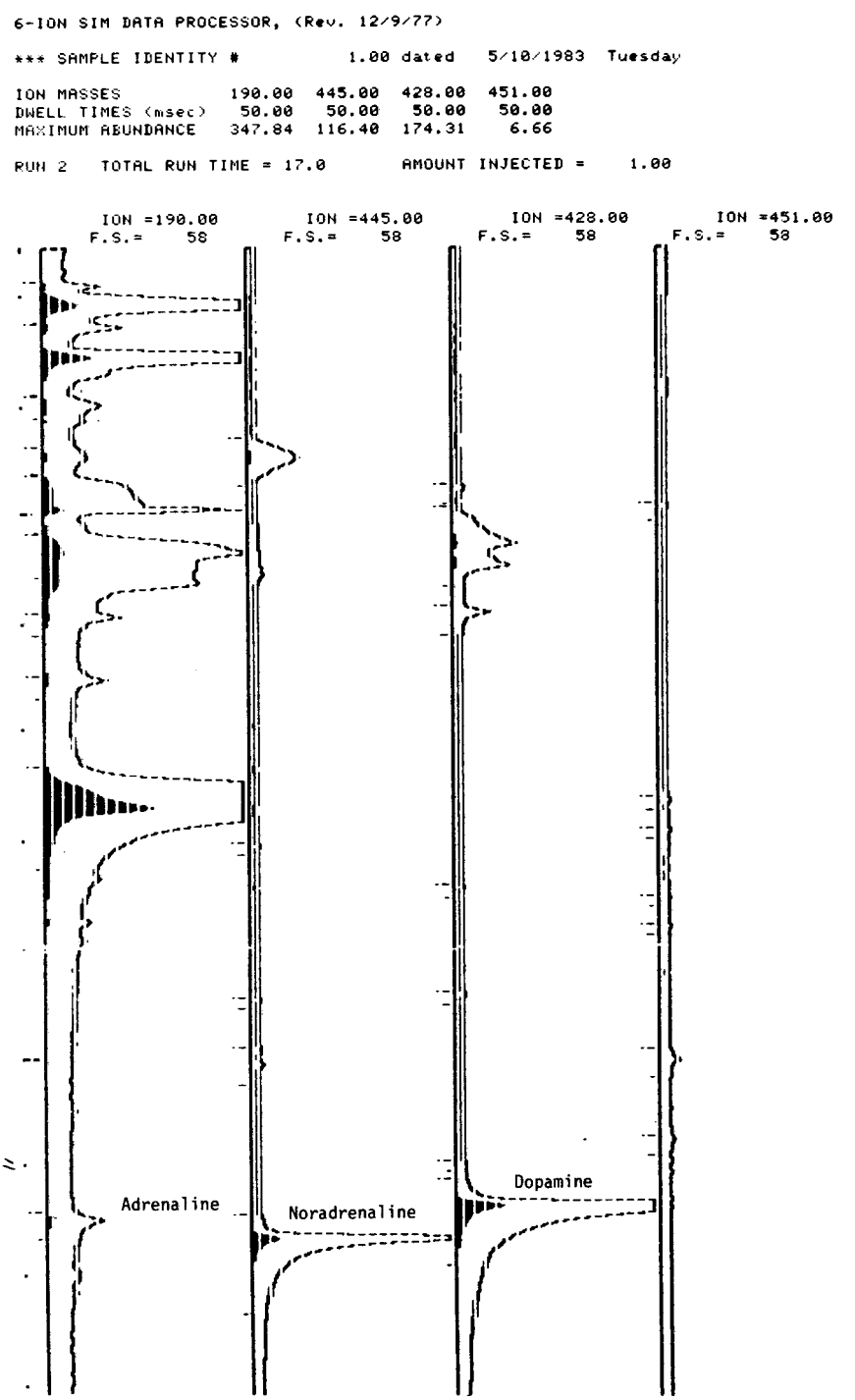


Fig. 2. SIM chromatogram traces for catecholamines from brain homogenate. Retention times (min): dopamine 11.3, adrenaline 11.4, noradrenaline 11.7 (uppermost trace is tuned for serotonin not present in the sample).

17]. In the present investigation, BCl_3—methanol was introduced as catalyst in the PFPA-acylation of amines. The reactions of all four amines studied proceeded smoothly and quantitatively at 45°C. A reaction temperature of 75°C, required for acceptable acylation rates in the absence of BCl_3, invariably led to partial decomposition of noradrenaline, as evidenced by MS.

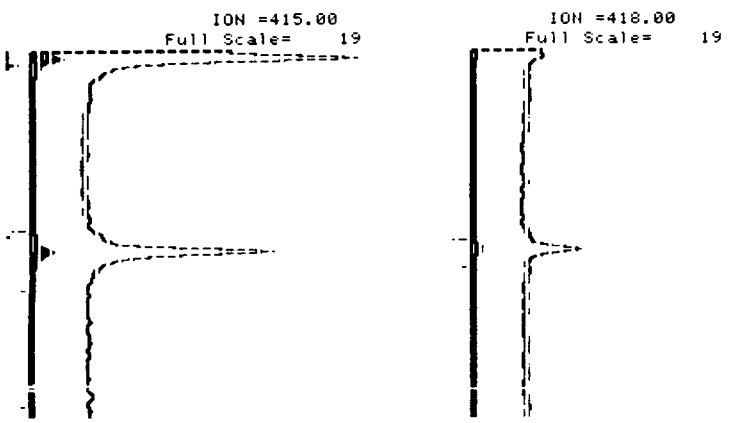
Mass spectrometric fragmentation of the derivatized compounds, as well as appropriate ions for SIM have been dealt with in refs. 13 and 16. In general, the compounds studied are prone to oxidative degradation during acid-catalysed synthesis of deuterated analogues. However, the simple procedures

```
6-ION SIM DATA PROCESSOR, (Rev. 12/9/77)

*** SAMPLE IDENTITY #          3.00 dated   5/23/1983  Monday

ION MASSES            415.00  418.00
DWELL TIMES (msec)     50.00   50.00
MAXIMUM ABUNDANCE      19.28    5.52

RUN 4   TOTAL RUN TIME =  7.9        AMOUNT INJECTED =     1.00
```




```
INTEGRATION SENSITIVITY =0.040  AREA THRESHOLD =      10  SMOOTHING FACTOR =0.80

1 ION 415.00  PEAK  RET. TIME     AREA  AREA/(SUM OF AREAS)  AREA/(LARGEST PEAK)
-------------------------------------------------------------------------------
              1     7.1          531.8        97.24936              100.00000
              2     7.8           15.0         2.75064                2.82844
          SUM OF AREAS =         546.8

2 ION 418.00  PEAK  RET. TIME     AREA  AREA/(SUM OF AREAS)  AREA/(LARGEST PEAK)
-------------------------------------------------------------------------------
              1     7.1          113.0       100.00000              100.00000
          SUM OF AREAS =         113.0

++++++++++++  DATA NORMALIZED ON AREA =          113.0

1 ION 415.00  PEAK  RET. TIME     AREA      % AREA
-----------------------------------------------------
              1     7.1          531.8      470.58577
              2     7.8           15.0       13.31023

2 ION 418.00  PEAK  RET. TIME     AREA      % AREA
-----------------------------------------------------
              1     7.1          113.0      100.03622
```

Fig. 3. SIM quantification of DOPAC from brain homogenate. Amount of internal standard 500 pg/μl (*m/e* 418), peak height ratio 4.71, calculated amount of DOPAC 969 pg/μl (*m/e* 415).

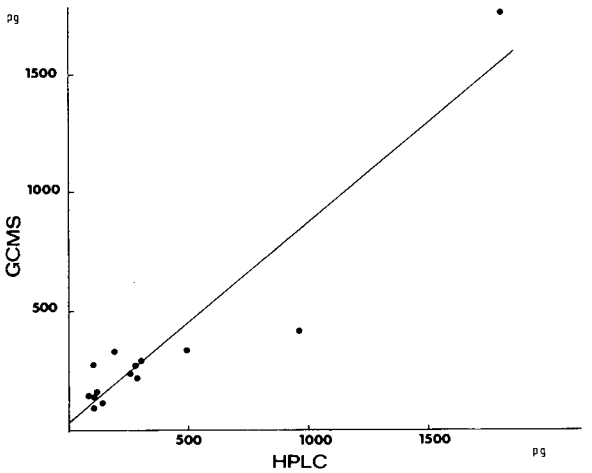


Fig. 4. Correlation of results obtained in the quantification of 5-HIAA by GC—MS and HPLC. Regression equation $Y = 0.83X + 27$, coefficient of correlation $r = 0.940$, $n = 14$.

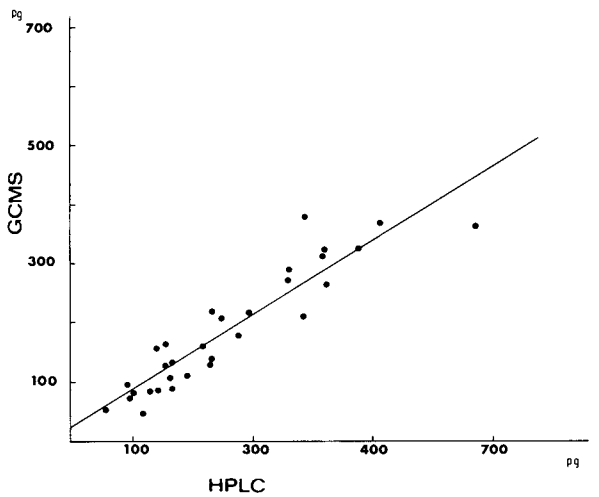


Fig. 5. Correlation of results obtained in the quantification of HVA by GC—MS and HPLC. Regression equation $Y = 0.62X + 25$, coefficient of correlation $r = 0.920$, $n = 31$.

used here are entirely satisfactory. It is noteworthy that deuterated serotonin and 5-HIAA could be obtained in good yields by using relatively high antioxidant concentrations.

Evaluation of the degree of incorporation of deuterium atoms in the compounds was undertaken by SIM, and the results are presented in Table I. In the case of adrenaline, the evaluation was hampered by a low relative intensity of the ion at *m/e* 590. The ion at *m/e* 445 would seem to provide a sufficiently reliable basis for the evaluation of deuterium incorporation in noradrenaline under the conditions used.

Fig. 1 shows a GC test run for the separation of DOPAC, HVA, MHPG and 5-HIAA, while selected-ion chromatogram traces for catecholamines in a brain sample are shown in Fig. 2. Typical calibration data for DOPAC and dopamine yielded correlation coefficients of $r = 0.997$ and $r = 0.984$, respectively.

TABLE II

NORADRENALINE AND DOPAMINE LEVELS MEASURED BY GC—MS IN VARIOUS BRAIN AREAS OF HISTOLOGICALLY VERIFIED PATIENTS WITH ALZHEIMER'S DISEASE AND DEMENTIA, AND OF THE CORRESPONDING CONTROL GROUPS

	Noradrenaline		Dopamine	
	ng/g ± S.D.	*n*	ng/g ± S.D.	*n*
Frontal cortex	12 ± 2	2*	171 ± 127	2
Temporal cortex	<20	4	187 ± 30	4
Hippocampal cortex	<20	2	155 ± 37	3
Thalamus	173 ± 96	6	184 ± 103	8
Nucleus caudatus	76 ± 86	6	1788 ± 188	8
Putamen	340 ± 336	4	713 ± 816	4
Pons	98 ± 45	2	114 ± 70	2

*Larger homogenate volume than usual.

Fig. 3 shows SIM responses for DOPAC and the corresponding internal standard. Correlation between the two analytical methods used, GC—MS and HPLC—electrochemical detection is evidenced by regression data in Figs. 4 and 5 for 5-HIAA and HVA, respectively.

The precision of the SIM method is relatively good; for example, for DOPAC, the mean amount per g of brain tissue is 399.8 ± 56.3 ng, $n = 5$, range = 326—457 ng, C.V. = 14%.

Table II summarizes the results from quantification of noradrenaline and dopamine from various brain areas of histologically verified patients with Alzheimer's disease, dementia, and of the corresponding controls. The high values of standard deviations are indicative of the very different amine levels associated with this type of material.

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Journal of Chromatography, 307 (1984) 251–260
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2046

QUANTITATIVE ANALYSIS OF PROSTAGLANDINS IN CELL CULTURE MEDIUM BY HIGH-RESOLUTION GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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(First received September 19th, 1983; revised manuscript received December 15th, 1983)

SUMMARY

Prostaglandins have been shown to be important modulators of haemostatis, immune responses, and growth of normal and neoplastic cells. In order to investigate the cell origin and metabolic profile of the endogenous prostaglandins in human tumours, a convenient extraction and gas chromatographic method for measuring the various classes of prostaglandins was developed. Infiltrating macrophages from human tumours were isolated using adherence to plastic. Macrophage-enriched and macrophage-depleted cell populations were then cultured in vitro and the media supernatant was studied for the presence of prostaglandins E_1, E_2, $F_{2\alpha}$, and 6-keto-$F_{1\alpha}$ (the spontaneous breakdown product of prostacyclin, PGI_2). Routinely, 1 ml of medium containing 10^6 cells was studied. The eicosanoids were extracted using commercially available octadecylsilyl silica reversed-phase columns prior to derivatization. Standards and samples were prepared as pentafluorobenzyl ester (methoxime) trimethylsilyl ether derivatives for analysis on an OV-101 (25 m × 0.2 mm) fused-silica capillary column. Recovery of standards ranged from 93% to 37%, with linear recovery in all instances (regression coefficients greater than 0.98). Detection limits were 20 pg for each of the prostaglandins. Analysis of cell subpopulations from six human tumours revealed that infiltrating macrophages produce various prostaglandin profiles and are largely responsible for the prostaglandin production in human cancer. The described analytical method is the first application of high-resolution gas chromatography with electron-capture detection to the quantitative profiling of prostaglandins from human cell culture.

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0378-4347/84/$03.00

INTRODUCTION

As the diversity of effects mediated by the various prostaglandins (PGs) becomes increasingly apparent, the need for a routine method capable of the simultaneous, quantitative determination of the profile of PGs is heightened. In biological systems, the different classes and types of PGs may cause different or opposing actions [1], thereby necessitating the simultaneous measurement of multiple PGs in the experimental setting. Additionally, the physiological concentration of PGs is in the nanomolar range, imposing strict demands on the sensitivity of the analytical method.

Detailed gas chromatographic profile analyses of PG standards have been done previously. Fitzpatrick [2] demonstrated the resolution of 24 different PG and thromboxane standards using glass capillary gas chromatography. This method was also used to study the metabolism of exogenously added PGH_2 by fibroblasts and lymphocytes in culture [3]. A similar method was used to analyze both cyclooxygenase and lipoxygenase products of arachidonic acid metabolism in platelets, endothelial cells, and mouse peritoneal macrophages [4]. Capillary column resolution of the various eicosanoids has been developed; however, quantitative measurements are not available. Arachidonic acid metabolism by mouse macrophages has been investigated using capillary chromatography and selected ion monitoring [5]; yet this methodology has also remained strictly qualitative in nature. Despite numerous developments in the analysis of PGs and other arachidonic acid metabolites by high-resolution gas chromatography, successful quantitative measurements from biological samples using this technology have yet to be performed.

We have developed a routine procedure for the quantitative analysis of a series of cyclooxygenase products using conventional fused-silica capillary gas chromatography with electron-capture detection. The method was employed for the detailed analysis of PG production in cell cultures derived from cells of cancer patients.

EXPERIMENTAL

Materials

Prostaglandin standards, obtained from Upjohn (Kalamazoo, MI, U.S.A.), were weighed, then dissolved in 70% ethanol; aliquots were stored in polypropylene tubes at −70°C. Organic solvents were of analytical grade, and purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Pentafluorobenzyl bromide and bis(trimethylsilyl)trifluoroacetamide were obtained from Pierce (Rockford, IL, U.S.A.). Methoxylamine · HCl in pyridine (4%, w/v) was purchased from Supelco (Bellefonte, PA, U.S.A.). Diisopropylethylamine was supplied by Aldrich (Milwaukee, WI, U.S.A.). Methyl formate was purchased from Eastman Kodak (Rochester, NY, U.S.A.). McCoy's 5A culture medium was supplied by Gibco (Santa Clara, CA, U.S.A.), and foetal calf serum was purchased from Flow Labs. (Inglewood, CA, U.S.A.). All in vitro culture was done using McCoy's 5A supplemented with 10% (v/v) foetal calf serum, which had been heat-inactivated at 56°C for 1 h. Tritiated prostaglandin E_2 {[5,6,8,11,12,14,15-^{3}H(N)]PGE_2, specific activity 160 Ci/m*M*} was obtained

from New England Nuclear (Boston, MA, U.S.A.). Bond Elut, octadecylsilyl silica columns, were purchased from Analytichem International (Harbor City, CA, U.S.A.).

Apparatus

Collected eluates from extractions were dried using a Speed-Vac Concentrator (Savant Instruments, Hicksville, NY, U.S.A.). Gas chromatography was performed by microlitre injection with a Hamilton microlitre syringe, Model 701N (Pierce) into a Hewlett-Packard Model 3700 gas chromatograph equipped with a purged splitless injector and ^{63}Ni-electron-capture detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). A fused-silica capillary column, 25 m × 0.2 mm, OV-101 WCOT, was obtained from Scientific Glass Engineering, Ringwood, Victoria, Australia). Instrument operating conditions were: injector temperature, 250°C; detector temperature, 300°C; column oven temperature programme: initial temperature, 50°C, maintained for 2 min after injection, followed by heating at 20°C/min to 200°C, immediately continuing at 10°C/min to 250°C, which was held isothermally for the remainder of the run. A post-run heating cycle of 300°C for 5 min was found to optimize the reproducibility of the chromatographic runs. Helium carrier gas was used at sufficient pressure to produce a linear column flow-rate of 28 cm/sec at 250°C. Make-up gas of argon—methane (90:10) was introduced at the detector base using a flow-rate of 20 ml/min. The detector signal was plotted on a strip chart recorder, as well as interfaced to a HP 3390A signal integrator (Hewlett-Packard).

Sample collection, preparation and storage

Human tumour ascites specimens were collected by paracentesis into preservative-free heparin (1000 units/ml). The cells were isolated by centrifugation at 900 *g* for 10 min, then washed once in culture medium. Cells were separated on the basis of adherence to plastic and phagocytosis of carbonyl iron particles. These methods generate subpopulations of cells depleted of macrophages (non-adherent and non-adherent—non-phagocytic) and a cell population enriched for macrophages (adherent). Cell-free supernatants from 24-h incubations of the collected cells were isolated by centrifugation, then stored at −70°C.

Sample clean-up

Prostaglandins were extracted from the culture medium by reversed-phase chromatography on octadecylsilyl silica [6]. A 1-ml volume of the supernatant was adjusted to pH 4.0 with 4% (v/v) formic acid. The sample was applied to a methanol-, and water-conditioned Bond Elut C_{18} column, which was slowly rinsed under vacuum sequentially with 10 ml each of distilled water, 15% ethanol, and hexane. The PGs were eluted into silanized conical tubes using two 0.5-ml flushes of methyl formate. The collected eluents were dried under vacuum by means of a Speed-Vac concentrator.

Recovery of prostaglandins from culture medium

To determine extraction recovery of the prostaglandins, [^{3}H]PGE_2 (25 pg) was added to 1 ml of culture medium, and processed using the extraction

protocol. Final recovery of the labelled PGE_2 was determined by scintillation counting, and compared to counts from the original aliquot. Additionally, authentic PG standards were used to verify the extraction recovery from culture medium. High-resolution gas chromatography of derivatized aliquots from standards and extracted standards was performed both to determine the purity of the extracted material and to measure the linearity of recovery.

Derivatization and sample analysis

For chromatographic stability and electron-capture response, the PGs were converted to their pentafluorobenzyl ester trimethylsilyl ether derivatives [7]. To secure stability of the keto group at the C-9 position of PGE_1, PGE_2, and 6-keto-$PGF_{1\alpha}$, a step producing the methoxime derivative was included prior to silylation [8]. Dried residues from extraction and standards were dissolved in 10 μl of pentafluorobenzyl bromide in acetonitrile (1:2, v/v) and 10 μl of diisopropylethylamine in acetonitrile (1:7, v/v), and the reaction vessel was securely capped. The solution was heated at 40° C for 5 min. The reactants were taken to dryness under nitrogen, and the esterification step was repeated again. To the dried residue were added 50 μl of methoxylamine · HCl in pyridine (4%, w/v), and the vial was heated at 60° C for 1 h, followed by evaporation under nitrogen. Sample purity was improved by partitioning the derivatized products in hexane (1 ml) and water (pH 3, 1 ml) twice. The hexane phases were combined and dried. Trimethylsilyl ether derivatives were made by reaction with 100 μl of bis(trimethylsilyl)trifluoroacetamide [9]. Routine chromatography was done after evaporating the silylating agent and dissolving the analyte in 100 μl of hexane to reduce the degree of solvent tailing. Verification of the final derivatized PGs was done by mass spectral analysis of standards.

Quantitation and peak identification

Precision of the assay was evaluated by repeated injections of standards. Linearity of extraction was measured by the analysis of diluted PG standards in culture medium, followed by the routine extraction and derivatization procedures. Peak identification was based on the retention times of sample chromatograms compared with standards which were run daily.

RESULTS

The mass spectrum for each prostaglandin standard was consistent with the fragmentation pattern of the anticipated derivative, as well as with reports in the literature [8].

A representative chromatogram from a 350-pg injection of each of the PG standards is displayed in Fig. 1. The major chromatographic peaks for PGE_1, PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ are clearly resolved from one another, and demonstrate good peak shape. The precision of the assay was determined by repeated 450-pg injections of the standards. The retention times and peak heights measured are presented in Table I. Retention times for the four prostaglandins tested are constant, with a coefficient of variation for each below 0.03%. The detector response to repeated injections of the same

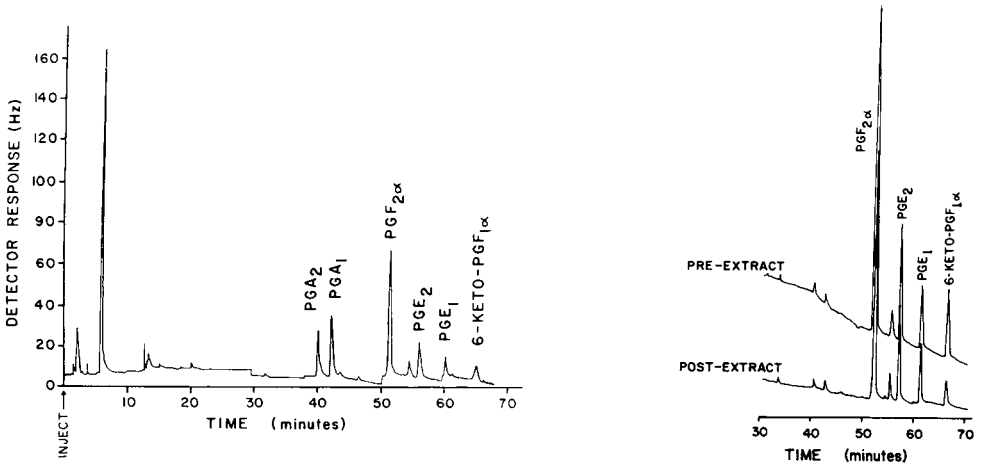

Fig. 1. Capillary chromatography of prostaglandins as pentafluorobenzyl ester (methoxime) trimethylsilyl ether derivatives. Splitless injection of 350 pg of each in hexane. Column: 25 m × 0.2 mm, OV-101, WCOT. ^{63}Ni-Electron-capture detector. Helium carrier gas at 28 cm/sec. Column temperature: 50°C × 2 min, 50°C/min to 200°C, 10°C/min to 250°C, 250°C isothermal for remainder of run.

Fig. 2. Extraction of prostaglandins from serum-supplemented culture medium. Equal aliquots of prostaglandin standards were analyzed before and after extraction using C_{18} reversed-phase. Chromatographic conditions as in Fig. 1. Approximately 450 pg.

TABLE I

PRECISION OF CAPILLARY CHROMATOGRAPHY AND ELECTRON-CAPTURE DETECTION OF PROSTAGLANDINS

Values shown are the means from four 450-pg injections. The coefficients of variation are given in parentheses.

	Retention time (min)	Peak height (mm)
PGE_1	61.50 ± 0.01 (0.008%)	32.5 ± 4.6 (14.2%)
PGE_2	57.31 ± 0.01 (0.017%)	25.3 ± 3.2 (12.6%)
$PGF_{2\alpha}$	52.39 ± 0.01 (0.019%)	72.0 ± 3.5 (4.9%)
PGI_2	66.51 ± 0.02 (0.027%)	69.3 ± 4.7 (6.8%)

standards was found to have the following coefficients of variation: PGE_1, 14.15%; PGE_2, 12.78%; $PGF_{2\alpha}$, 4.81%; and 6-keto-$PGF_{1\alpha}$, 6.84%.

Extraction recovery of the prostaglandins from the culture medium was tested with respect to isolation of the peaks of interest from interfering substances, as well as the linearity of extraction. Fig. 2 shows overlapping chromatograms, one from the stock mixture of the PG standards, the other from the same standards following extraction from culture medium. It is evident that the PGs are well isolated from any interfering peaks. Additionally, the PGs were recovered in a linear fashion from the culture medium which had been supplemented with decreasing concentrations of standards ranging from

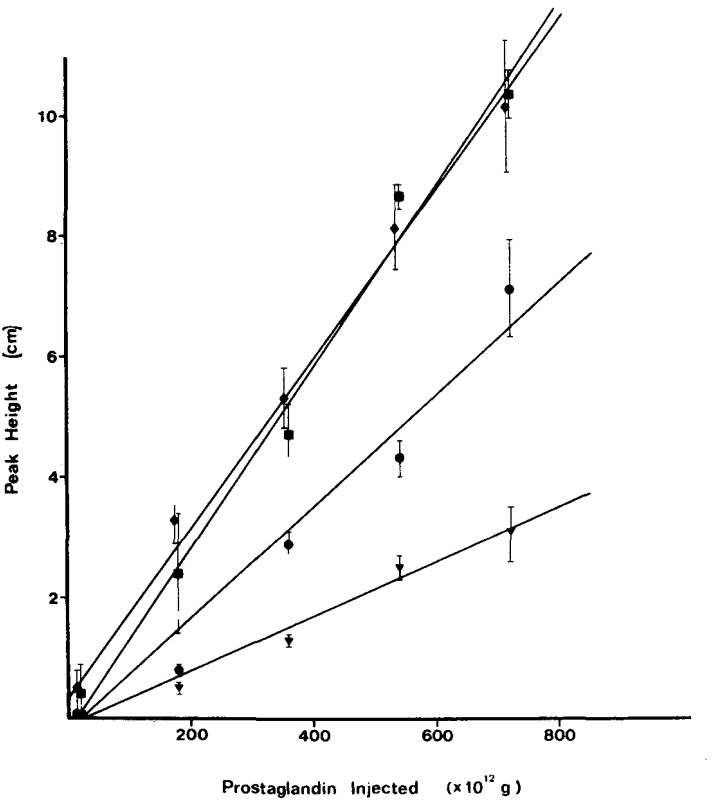

Fig. 3. Linearity of extraction and detector response of decreasing amounts of prostaglandins. Regression coefficients given in text. PGE_1 (♦); PGE_2 (●); $PGF_{2\alpha}$ (■); 6-keto-$PGF_{1\alpha}$ (▲).

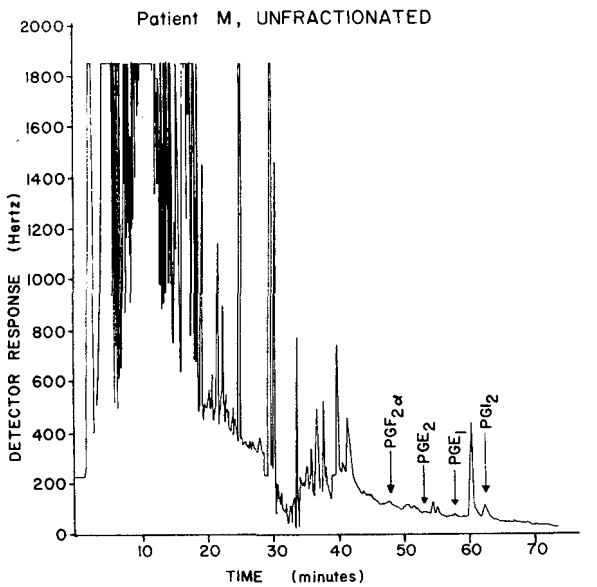

Fig. 4. Chromatographic analysis of prostaglandins in culture supernatant from 24-h incubation of dispersed cells from human ovarian cancer specimen.

TABLE II

PROSTAGLANDIN PRODUCTION BY CELL SUBPOPULATIONS FROM HUMAN TUMOUR SPECIMENS

Patient	Cell fraction*	Prostaglandin production (ng/ml)			
		PGE_1	PGE_2	$PGF_{2\alpha}$	PGI_2
M	UN	—	—	—	75.8
	NA	0.7	4.0	4.7	43.9
	AD	—	33.2	6.2	193.0
N	UN	—	9.5	—	3.0
	NA	—	4.4	—	8.5
	NA—NP	—	5.4	—	3.5
	AD	—	16.7	—	—
O	UN	—	13.7	—	3.6
	NA	1.1	10.5	—	—
	NA—NP	2.1	32.2	—	36.0
	AD	1.1	22.5	—	1.2
P	NA	14.1	210.0	75.8	1963.0
	AD	2.9	78.8	50.9	334.3
Q	UN	14.6	235.2	88.5	247.7
	NA	11.4	38.1	134.6	4.9
	NA—NP	—	51.2	—	—
	AD	60.4	14.0	—	34.4
R	UN	21.8	64.3	10.8	7.5
	NA	18.7	47.6	53.8	—
	NA—NP	—	51.8	45.5	—
	AD	14.5	240.6	48.2	—

*UN = unfractionated cells; NA = non-adherent cells; NA—NP = non-adherent—non-phagocytic cells; AD adherent cells.

40 to 1 ng/ml (Fig. 3). The regression coefficients for the extracted standards were: PGE_1, 0.997; PGE_2, 0.982; $PGF_{2\alpha}$, 0.995; and 6-keto-$PGF_{1\alpha}$, 0.993. Each of the regression lines has a negative y-intercept with the exception of PGE_1, which shows a positive intercept.

The analytical method was used to measure PGs in culture medium supernatants from cell populations isolated from human tumours (Table II). Chromatograms of the extracted supernatants from three subpopulations from one patient sample are shown in Figs. 4—6. The unfractionated cells (original, washed cells) produce low levels of PGs, with moderate levels of prostacyclin (PGI_2).

The non-adherent cells from the same patient produced marginally detectable amounts of the three PGs and also a low level of prostacyclin. Adherent cells from this specimen, however, showed significant PG synthesis compared to the other two cell subpopulations. This trend for predominant macrophage production of PGs is also evident in samples from patients N, O,

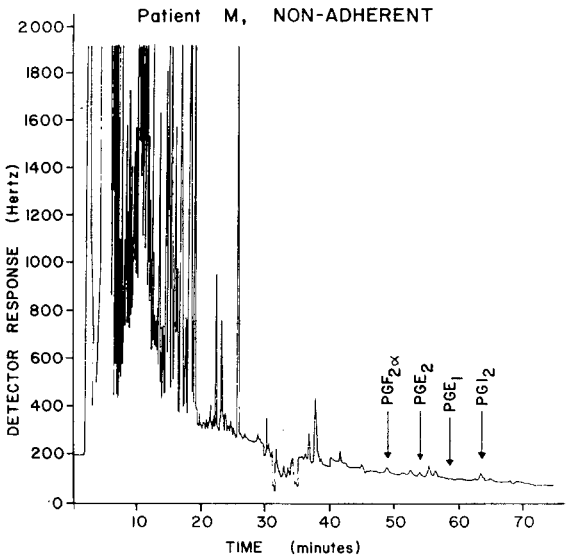

Fig. 5. Chromatogram from culture supernatant of non-adherent cells of human ovarian cancer specimen.

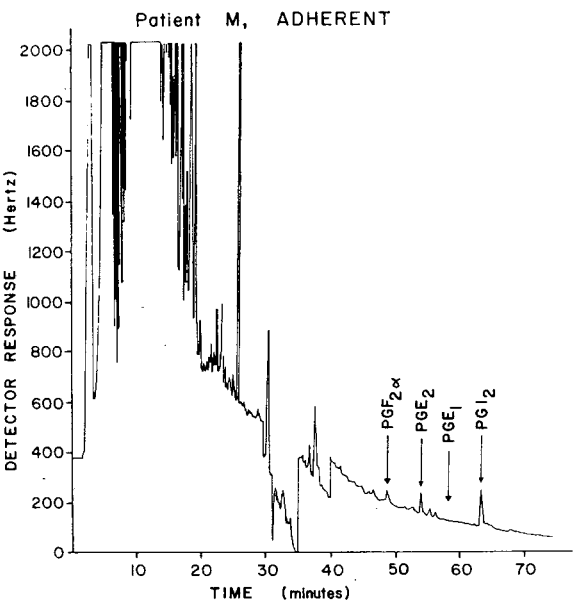

Fig. 6. Chromatogram from culture supernatant of adherent cells of human ovarian cancer specimen.

and R. In all patient samples tested, PGE_2 and PGI_2 were the dominant cyclooxygenase product measured, although for samples P, Q, and R, PGE_1 and $PGF_{2\alpha}$ were also found in moderately high concentrations. While the adherent cell population is the most homogeneous among the various samples with respect to cell composition, there is conspicuous lack of conformity between the PG profiles and levels of the PGs produced by the adherent cells from these

six patient specimens. Adherent cells from samples N, O, and R produced PGE_2 almost to the exclusion of the other compounds; the PG profiles of samples M, P, and Q show relative dominance by prostacyclin.

DISCUSSION

Prostaglandins in cell culture medium can be measured at the subnanogram level using reversed-phase extraction and electron-capture detection following derivatization to the pentafluorobenzyl ester (methoxime) trimethylsilyl ether derivatives. High-resolution capillary gas chromatography allows the simultaneous determination of prostaglandins E_1, E_2, $F_{2\alpha}$, and 6-keto-$F_{1\alpha}$ (the spontaneous breakdown product of prostacyclin, PGI_2). Furthermore, extraction using octadecylsilyl silica shows linear recovery characteristics sufficient for quantitative measurements of these important compounds. Although the extraction method does not isolate the PGs to the exclusion of other compounds, the precision of the chromatography is such that qualitative determination based on retention times is reasonable. The described method uses no instrument modifications, and is potentially amenable to automation.

The method has been successfully applied to the measurement of PGs in complex biological fluids. Cell culture supernatants from 24-h incubations of various cell subpopulations from human tumours were analyzed. These results indicate that there is considerable variation among the levels and profiles of PG production by human malignant tumours. The present study, however, includes insufficient numbers of patients to evaluate the possible clinical significance of prostaglandin synthesis by human tumours. Other investigators have reported that PG levels in human breast tumours may relate to the metastatic potential [10].

In the present investigation it was found that tumour macrophages display a wide range of cyclooxygenase activity which results in diverse levels and profiles of the PGs. It is known that macrophages possess a wide range of biological activities, one of which is prostaglandin synthesis [11]. Cyclooxygenase activity of macrophages has also been shown to be dependent on the level of macrophage activation [12] and the in vitro culture conditions [13]. The macrophages studied in this present report were isolated and cultured under identical experimental conditions; consequently, the divergent profiles and levels of PG production most likely reflect different functional states of these infiltrating host cells.

The convenient extraction and gas chromatographic analysis are presently used to investigate PG production by cell populations from a wide variety of human tumours including leukaemia, carcinomas, and sarcomas. The analytical procedure should prove useful in laboratories interested in routine studies of the profile of cyclooxygenase products. Attempts are under way to automate parts of the analysis.

ACKNOWLEDGEMENTS

This study was supported by Grants CA 17094, CA 21839, and CA 23074 from the National Institute of Health, Bethesda, MD, U.S.A.

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Journal of Chromatography, 307 (1984) 261—269
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in the Netherlands

CHROMBIO. 2035

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PHOSPHOLIPIDS USING DEUTERATED SOLVENTS FOR INFRARED DETECTION

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(First received September 19th, 1983; revised manuscript received December 8th, 1983)

SUMMARY

Infrared detection of chromatographic effluents offers the advantage of direct on-line quantitation of lipid fractions. However, infrared detection imposes limitations on the solvent systems that can be used for chromatography. Methanol and water, which are essential ingredients in the mobile phase for the successful chromatography of phospholipids, do not have spectral transmittance windows in the infrared region. Substituting deuterated methanol and deuterium oxide for methanol and water allowed infrared detection because they had lower infrared absorbance than their hydrogenated counterparts. We report a method that is suitable for the quantitative analysis of phosphatidylethanolamine, phosphatidylcholine and sphingomyelin in the tissue extracts. The lipid separation was accomplished on a micropraticulate silica gel column. Phosphatidylethanolamine and phosphatidylcholine were eluted isocratically with chloroform—acetonitrile—methanol—deuterium oxide (136:25:34:5.9) and detected at a wavelength of 5.75 μm. For the analysis of sphingomyelin, chloroform—acetonitrile—deuterated methanol—deuterium oxide (130:24:37.6:7.0) was used as the mobile phase, and the detection was at a wavelength of 6.15 μm.

INTRODUCTION

The traditional procedure for quantitative analysis of phospholipid composition in tissues involves extraction of total lipids, separation into lipid

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0378-4347/84/$03.00

classes by either thin-layer or column chromatographic methods, and quantitation by measuring the phosphorus content. The procedure is tedious and imprecise. Attempting to develop a simpler procedure, several investigators [1—8] have explored the possibility of using high-performance liquid chromatography (HPLC). HPLC provides fine separation of lipids. However, because of the problem of detection a satisfactory HPLC technique for direct quantitation of lipid classes is not yet available. The most popular method of detection is based on the change in ultraviolet (UV) absorbance. Being sensitive and nondestructive, UV absorbance detection is ideal for monitoring the separation of lipids by HPLC. But, it does not allow direct quantitation of fractions, because the 200-nm range of phospholipid absorbance reflects the number of double bonds rather than the number of molecules [1]. Usually the effluents from specific peaks are collected into the tubes and quantified by colorimetric methods. Detectors based on the principle of refractive index are relatively insensitive and incompatible with gradient elution techniques. Moving wire flame ionization detector allows direct quantitation of lipid fractions [7, 8]. However, it is also insensitive and the bulk of sample is destroyed by the flame. Since this type of detector lacks widespread popularity, they are no longer manufactured commercially.

Most lipids have specific structures that exhibit strong absorption bands in the infrared (IR) region of the spectrum. IR absorbance detection offers considerable potential for direct on-line quantitation of lipid fractions. It has been applied to the HPLC of neutral lipids [9, 10]. The major disadvantage is that it imposes limitations on the solvent systems that can be used in chromatography. Only a few solvents are transparent at the wavelengths where lipids absorb. Despite the limited choice of solvents, we have developed an HPLC technique using IR detection for the quantitative analysis of several major phospholipids in tissue extracts. The novelty of the technique is the use of deuterated methanol and deuterium oxide as substitutes for methanol and water in the mobile phase. The substitution clears the obstacle that methanol and water, essential ingredients for the chromatography of phospholipids, are not transparent in the spectral region of interest.

EXPERIMENTAL

Materials

Reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SPH) were derived from egg yolk. Phosphatidylserine (PS) was from bovine brain, whereas phosphatidylinositol (PI) was from soybean. Deuterium oxide (99.8 atom % ^{2}H) and deuterated methanol (99.5 atom % ^{2}H) were obtained also from Sigma. Chloroform, acetonitrile and methanol were of HPLC grade from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). [Dipalmitoyl-1-^{14}C]-phosphatidylethanolamine and [dipalmitoyl-1-^{14}C]phosphatidylcholine (100 mCi/m*M*) were purchased from New England Nuclear (Boston, MA, U.S.A.).

Tissue lipid extracts

Sprague–Dawley male rats weighing 150 g were used. They had access to the diet up to the time of sacrifice. Immediately after decapitation, heads and livers were placed in liquid nitrogen. Plasma was from a healthy human donor. A 1-g amount of rat tissue from cerebrum, right lobe of liver or 1 ml of human plasma was homogenized in 30 ml of chloroform–methanol (2:1). After filtration the lipid extract was separated into two phases according to the procedure of Folch et al. [11]. The upper phase was discarded and the lower phase was washed once with reconstituted pure solvent upper phase [11]. Lipid phosphorus in tissue extracts was measured by the Bartlett procedure [12].

Instruments

We used a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatographic system consisting of a Model 6000 solvent delivery system and a Model U6K injector. The column was a 30 cm × 3.9 mm I.D. prepacked μPorasil column (Waters Assoc.) which contained silica gel, particle size 10 μm. There was a guard column packed with Corasil (Waters Assoc.). The liquid chromatograph infrared detector was a DuPont product (Wilmington, DE, U.S.A.). The optical path length of the calcium fluoride cell was 1 mm and the internal cell volume was 463 μl (Analabs, Cat. No. 006-7026, North Haven, CT, U.S.A.). Chromatograms were recorded on a strip chart recorder. Peak areas were calculated by a Model 9874 digitizer interfaced with a Model 9830A calculator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Chromatographic conditions and IR detection

Phospholipid reference compounds and Folch [11] tissue lipid extracts were dried under nitrogen in a warm water bath. They were redissolved in a small amount of mobile phase, and an aliquot was injected onto the chromatograph. Sample sizes are indicated in figure legends. The elution was isocratic. For the analysis of PE and PC the solvent was chloroform–acetonitrile–methanol–deuterium oxide (136:25:34:5.9) and the detection was at a wavelength of 5.75 μm. For the analysis of sphingomyelin the solvent was chloroform–acetonitrile–deuterated methanol–deuterium oxide (130:24:37.6:7.0) and the detection wavelength was set at 6.15 μm. The mobile phase was delivered to the column at a flow-rate of 1 ml/min. The pressure was approximately 103 bars. The column temperature was that of the room temperature, 21°C. The IR detector was set at 2-mm slit width and 4 response meter. The range was either 0.1 A or 0.025 A as indicated in the figures.

RESULTS

IR detection

The separation of phospholipid classes, except for sphingomyelin, was monitored by IR detection at a wavelength of 5.75 μm, which corresponds to the absorbance of carbonyl group. The detection of sphingomyelin was based on the absorbance of amide group at 6.15 μm. Some of the best solvents for lipophilic samples, e.g., chloroform and acetonitrile, have spectral transmittance windows in these wavelength regions. Water and methanol are not IR

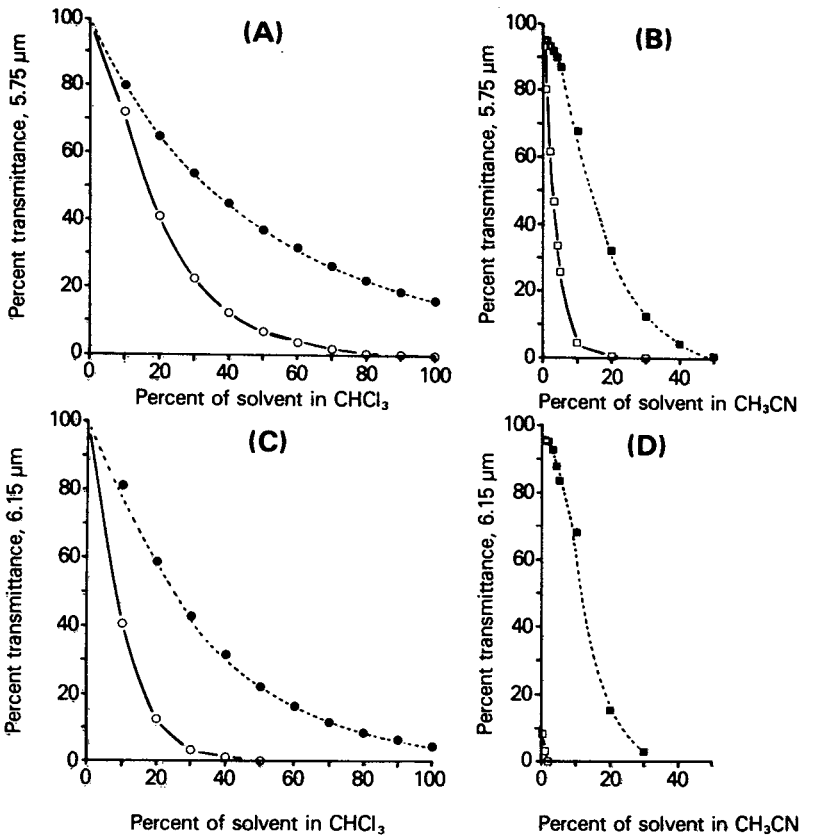

Fig. 1. (A) and (C) IR transmittance of deuterated methanol (●- - -●) and methanol (○—○) in chloroform; (B) and (D) IR transmittance of deuterium oxide (■- - -■) and water (□—□) in acetonitrile. In (A) and (B) the IR transmittance was measured at a wavelength of 5.75 μm, whereas in (C) and (D) the wavelength was set at 6.15 μm.

transparent, but they are essential ingredients in the mobile phase for the successful chromatography of phospholipids. Water absorbs the light at 5.75 μm and 6.15 μm so strongly that its presence even in a small percentage was incompatible with IR detection. In order to overcome this difficulty, deuterium oxide was used to replace water in the mobile phase. Deuterium oxide and deuterated methanol do not have spectral transmittance windows in the 5.75-μm and 6.15-μm regions. But, their IR absorbances were much less than those of their hydrogenated counterparts (Fig. 1). For IR detection at 5.75 μm the substitution of deuterated methanol for methanol was usually unnecessary, since the solvent (chloroform—acetonitrile—methanol—deuterium oxide, 136:25:34:5.9) allowed sufficient transmission of light for the detector to operate. The use of deuterated methanol could reduce the noise level and improve the detection sensitivity, but it is relatively expensive. For the detection of sphingomyelin at 6.15 μm deuterated methanol must be used to replace methanol in the mobile phase.

HPLC of major phospholipid classes

Because phospholipids vary greatly in polarity and charges, our attempt to develop a solvent system that could separate all the major classes isocratically in a single run was unsuccessful. Gradient elution method was not used, because it introduced a steep baseline which complicated the calculation of peak areas. We found a solvent system suitable for the analysis of PE and PC. Fig. 2 shows the chromatograms of reference compounds and various tissue extracts using this solvent system (chloroform—acetonitrile—methanol—deuterium oxide, 136:25:34:5.9). PE and PC were rapidly eluted and well separated from other lipids. Neutral lipids (NL) and diphosphatidylglycerol (DPG) were eluted with the solvent front. Sphingomyelin was not expected to be detectable at 5.75 μm, because the detection was based on the absorbance of carbonyl group. No other sharp peaks were detected on the chromatograms, even though PS, PI and LPC were present. Based on the phosphorus analysis of HPLC fractions, PS, PI, SPH and LPC were eluted during the following time intervals: PS from 12 to 17 min; PI from 15 to 20 min; SPH from 15 to 18 min; and LPC from 17 to 19 min. Evidently the mobile phase was not polar enough to resolve these lipids into sharp peaks. Poor detector sensitivity was another reason that phospholipids in small quantities might not be detectable. The appearance of a biphasic peak coincided with the completion of phospho-

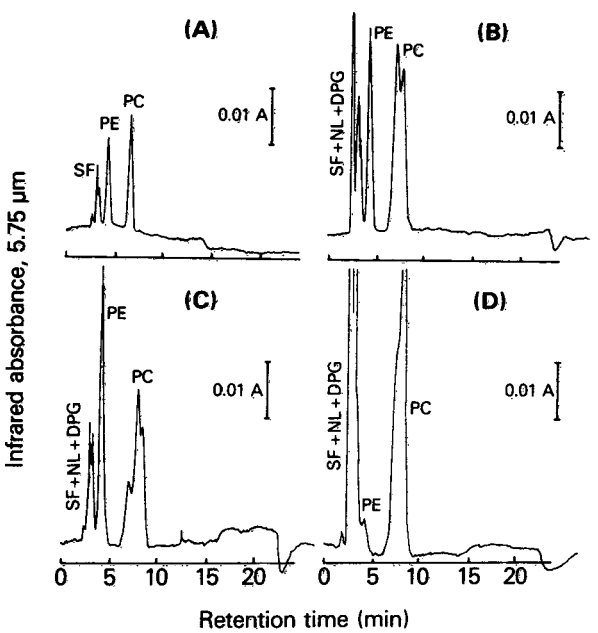

Fig. 2. HPLC analysis of reference compounds and tissue extracts. (A) Phospholipid standards; (B) rat liver; (C) rat brain; and (D) human plasma. The isocratic elution was with chloroform—acetonitrile—methanol—deuterium oxide (136:25:34:5.9), and the detection wavelength was set at 5.75 μm. The flow-rate was 1 ml/min. Other conditions were described under Experimental. The sample in chromatogram A contained 200 μg each of PE, PC, SPH, PS, PI and LPC. Aliquots of the total lipid extracts in B, C and D contained approximately 1.5 mg of total phospholipids. Peaks: SF = solvent front; NL = neutral lipids; DPG = diphosphatidylglycerol; PE = phosphatidylethanolamine; and PC = phosphatidylcholine.

lipid elution. The mechanism for this phenomena is unclear. For the analysis of sphingomyelin we employed chloroform—acetonitrile—deuterated methanol—deuterium oxide (130:24:37.6:7.0) as the mobile phase (Fig. 3). Sphingomyelin co-eluted with PS, but the presence of PS did not interfere with IR detection at 6.15 μm, because the absorbance was specifically due to sphingomyelin. It is noteworthy that in Fig. 3 the chromatograms show a partial separation of two fractions. The pattern was similar to the result obtained with silica gel thin-layer chromatography (TLC). It was previously reported that in the TLC of sphingomyelin the more rapidly moving component contained primarily C_{22} and C_{24} fatty acids, whereas the slower component contained mainly palmitic acid [13].

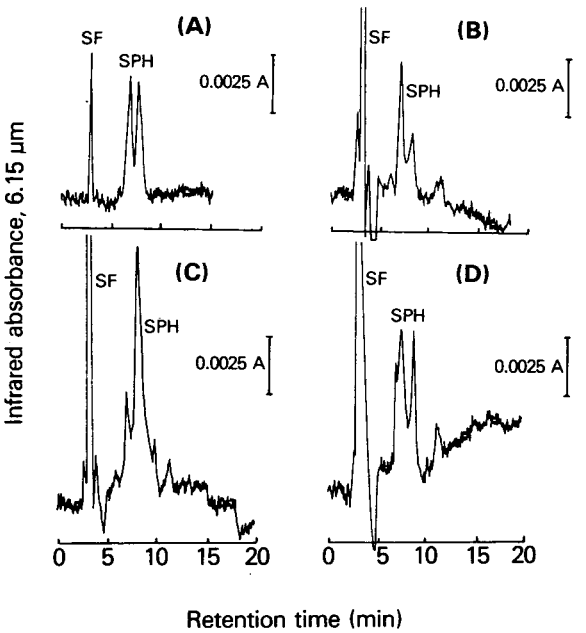

Fig. 3. HPLC analysis of reference compound and tissue extracts. (A) Egg yolk sphingomyelin, 200 μg; (B) rat liver; (C) rat brain; and (D) human plasma. Aliquots of total lipid extracts in B, C and D contained approximately 2 mg of total phospholipids. The isocratic elution was with chloroform—acetonitrile—deuterated methanol—deuterium oxide (130:24:37.6:7.0), and the detection wavelength was set at 6.15 μm. The flow-rate was 1.0 ml/min and the range of detector was set at 0.025 A. Peaks: SF = solvent front; SPH = sphingomyelin.

In the analysis of tissue lipid extracts, identification of peaks in Figs. 2 and 3 was accomplished by comparing retention times of lipid standards, by TLC analysis of individually collected fractions and by analyzing the IR spectra of the collected fractions.

The recoveries of phospholipids applied to the column were determined by the analysis of phosphorus content in the collected fractions. [^{14}C]PE and [^{14}C]PC were used both to evaluate the recoveries and to confirm the identity of peaks. The recoveries of DPG, PE, PC, PS, PI, SPH and LPC were respectively 94, 94, 93, 78, 84, 88 and 76% (average of at least three determinations for each phospholipid).

Standard curves

The standard curves relating peak area to concentration are shown in Fig. 4. The linearity of response between 30 and 250 n*M* phospholipid was evident. On a molar basis PE had a lower IR response than PC. This was related to the fact that the PE peak was rich in ethanolamine plasmalogen. Since the detection was based on the absorbance of carbonyl group, ethanolamine plasmalogen had a molar response factor of only one, whereas phosphatidylethanolamine had two. The PC peak also contained some choline plasmalogen but the proportion was very small.

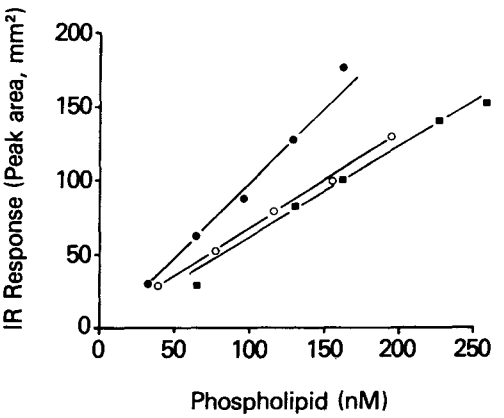

Fig. 4. Standard curves for phosphatidylethanolamine (○—○), phosphatidylcholine (●—●) and sphingomyelin (▪—▪). Known amounts of phospholipid standards were injected for HPLC analysis and the IR response in terms of peak area due to each phospholipid was measured.

Analysis of rat liver phospholipids

The method described here is suitable for the quantitative analysis of phospholipids in tissue extracts. An example is the analysis of PE, PC and SPH in rat liver (Table I). PE and PC determined by the IR peak area method agreed well with the results obtained by determining the amount of phosphorus in the eluted peaks. They were also in agreement with those results reported in the literature [14]. Within-run variability of the peak area method was small. Coefficients of variation (C.V.; S.D./mean) for PE, PC, and SPH were 0.5, 3.5 and 4.5% respectively. Day-to-day variations were 6.8% for PE, 6.1% for PC and 13.1% for SPH.

DISCUSSION

The major difficulty in using IR detection arises from restrictions on selecting solvents. In order to increase the sensitivity and minimize the noise level, the mobile phase should have a low absorbance at the wavelength employed. Phospholipid separation by a silica gel column requires the use of polar solvents. Unfortunately most polar solvents, such as methanol and water, do not have reasonable spectral transmittance windows in the IR region. In this present study we demonstrated that the problem of solvent absorbance could be greatly lessened by using deuterated compounds. The approach of

TABLE I

WITHIN-RUN AND DAY-TO-DAY PRECISION FOR THE DETERMINATION OF PER CENT COMPOSITION OF PHOSPHOLIPIDS IN RAT LIVER

For the analysis of PE and PC, aliquots of total lipid extracts from the rat liver, each containing 1 μM total phospholipids, were injected into the chromatograph as described in Fig. 2B. For the analysis of SPH, each aliquot of total lipid extracts contained 2 μM total phospholipids, and the chromatography was the same as that described in Fig. 3B. The values quoted, percentages of the total phospholipids, were determined by either the peak area method or measuring the phosphorus content in the collected peak effluents.

Phospholipids	Within-run (n = 3)				Day-to-day (n = 3)			
	Peak area method		Phosphorus assay		Peak area method		Phosphorus assay	
	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
PE	23.7	0.5	25.4	3.4	25.1	6.8	25.0	4.4
PC	53.7	3.5	50.4	2.7	50.6	6.1	50.1	2.0
SPH	3.3	4.5	—	—	3.6	13.1	—	—

using deuterated compounds expands the list of solvents that can be used for chromatography.

Another possible approach towards solving the problem of solvent restriction is to improve the design and the sensitivity of IR spectrophotometers. With conventional detectors it is preferable to use a mobile phase having over 30% transmittance at specified wavelength settings. Some specially designed detectors reportedly are so sensitive in detecting the solutes that solvents having only 5% transmittance can be used [15].

We have previously developed several HPLC methods for the analysis of phospholipids using either fluorescence or UV detection [3—6]. Each of these methods has its applications and limitations. Compared with UV detection, IR detection has the following advantages. First, since the detection is based on the absorbance due to either carbonyl or amide group, it imparts a degree of specificity to the detector response. Secondly, peak areas on the chromatogram reflect the amount of phospholipids eluted from the column. With reference to standard curves, direct quantitation of lipid classes can be rapidly accomplished. It should be emphasized that the PE standard employed should have the same proportion of ethanolamine plasmalogen as the samples tested. This is because in the chromatography ethanolamine plasmalogen co-elutes with phosphatidylethanolamine. Since the detection is based on the absorbance of carbonyl group, the response of the PE peak varies with the concentration of ethanolamine plasmalogen. If tissue extracts do not contain detectable amounts of lysoPE, this commercially available lipid can be added to the Folch [11] lipid extracts and used as an internal standard. This will improve the accuracy and the speed of analysis. Using the solvent mixture described in Fig. 2, the retention time of lysoPE is 13 min.

Disadvantages of IR detection include poor sensitivity and solvent restriction. The smallest amount of PE, PC or SPH we could measure was about 30 nM. The optimal sample size for the analysis was approximately 1 μM of

total phospholipids in the tissue extracts (31 μg of lipid phosphorus). This is about 100 times as large as the sample size for UV analysis. Sensitivity of analysis is a factor that minor phospholipids could not be detected. Solvent restriction hinders the development of a mobile phase that can satisfactorily separate PS, PI, SPH, and LPC from each other. Although PS co-elutes with sphingomyelin in chromatography, the method is still valid for the quantitation of sphingomyelin because of the specificity of detection at 6.15 μm.

In conclusion, this present study shows the potential that IR can be used for direct on-line quantitation of chromatographic effluents. The method described here is suitable for either the quantitation or the isolation of PE and PC from tissue extracts. The chromatography is rapid and free from other contaminants. However, the usefulness of this present method for the analysis of minor phospholipids appears limited. More work in improving the detection sensitivity and in the selection of columns and solvent systems is required in order to develop a satisfactory method for the analysis of minor phospholipids.

ACKNOWLEDGEMENT

This investigation was supported by the Veterans Administration.

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Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2041

SIMULTANEOUS DETERMINATION OF NOREPINEPHRINE, DOPAMINE, 5-HYDROXYTRYPTAMINE AND THEIR MAIN METABOLITES IN RAT BRAIN USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

ENZYMATIC HYDROLYSIS OF METABOLITES PRIOR TO CHROMATOGRAPHY

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(First received August 29th, 1983; revised manuscript received December 2nd, 1983)

SUMMARY

In order to measure turnover rates of the noradrenergic, dopaminergic, and serotonergic transmitter systems in rat brain, a method was developed by which norepinephrine, dopamine, and 5-hydroxytryptamine, and their main metabolites 3-methoxy-4-hydroxyphenylglycol, 3,4-dihydroxyphenylacetic acid, 3-methoxy-4-hydroxyphenylacetic acid, and 5-hydroxyindole-3-acetic acid, could be measured simultaneously. High-performance liquid chromatography in the reversed-phase mode, including ion pairing, separated the transmitters and their metabolites well. By means of enzymatic hydrolysis of the sample prior to chromatography, it was also possible to measure the conjugated forms of the metabolites. Since there was no prepurification step, the hydrolysed supernatants of tissue homogenates were injected directly into the chromatographic system; additional selectivity tests were necessary. Peak identification was confirmed by comparison of hydrodynamic voltagrams and capacity factors at different pH values of the mobile phase of the components in the sample and the standard solution. The method is demonstrated by analysing mediobasal hypothalamic tissues of probenecid-treated rats.

INTRODUCTION

The noradrenergic, dopaminergic and serotonergic transmitter systems play an important role in the control and regulation of many brain functions. In order to determine the activities of these transmitter systems, not only the tissue levels of the transmitters, but also the concentration of their main metab-

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olites should be measured. An estimation of the turnover in the transmitter systems can be obtained either from metabolite—transmitter relationships in steady-state conditions [1—3] or from the extent of metabolite accumulation after pharmacological blockage of their elimination from the brain by probenecid [4—6]. Before measurement, enzymatic hydrolysis of the sample is necessary, due to conjugation of the metabolites in the brain tissue [7—10].

The aim of this study was, therefore, to develop an analytical method in which norepinephrine (NE), dopamine (DA) and 5-hydroxytryptamine (5-HT) and the free and conjugated forms of their main metabolites, 3-methoxy-4-hydroxyphenylglycol (MOPEG) [7, 8], 3,4-dihydroxyphenylacetic acid (DOPAC), 3-methoxy-4-hydroxyphenylacetic acid (HVA) [11, 12], and 5-hydroxyindole-3-acetic acid (5-HIAA) [13] can be measured simultaneously. The use of high-performance liquid chromatography (HPLC) with electrochemical detection (ED) in this context is described in many papers (for review see refs. 14 and 15). Most of them include prepurification steps for the sample, which makes simultaneous measurement of the substances mentioned above impossible. The use of an internal standard is also necessary. To overcome these disadvantages we used the method of direct injection of the brain homogenate supernatant into the HPLC system [16—19]. In spite of the high resolution of HPLC, additional selectivity tests became necessary.

The development of the method and the test of selectivity are demonstrated by means of the analysis of mediobasal hypothalamic tissues of probenecid-treated rats.

EXPERIMENTAL

Reagents and drugs

Chemicals were obtained from the following sources: 3,4-dihydroxyphenylglycol (DHPG), norepinephrine · hydrochloride (NE), L-epinephrine (E), 3-methoxy-4-hydroxyphenylglycol (MOPEG), 3,4-dihydroxybenzylamine · hydrobromide (DHBA), DL-normetanephrine · hydrochloride (NMN), 3,4-dihydroxyphenethylamine · hydrochloride (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid · dicyclohexylammonium salt (5-HIAA), 5-hydroxytryptamine · creatine-sulphate complex (5-HT), 3-methoxy-4-hydroxyphenylacetic acid (HVA), probenecid, sulphatase (from *Helix pomatia*, type H-5), and dibutylamine from Sigma (St. Louis, MO, U.S.A.); sodium octyl sulphate from Fluka (Buchs, Switzerland); and 3-methoxy-4-hydroxyphenylglycol-4-sulphate potassium salt from Hoffmann-La Roche (Basel, Switzerland). Glass-distilled methanol (Merck, Darmstadt, F.R.G.) was used; the deionised water was separated from organic components by vacuum filtration through a Norganic-patrone (Millipore, Bedford, MA, U.S.A.).

Apparatus

The HPLC system consisted of a 6000A solvent delivery pump, a U6K injection system and a 5-μm C_{18} column RCM 100 (I.D. 8 mm) protected by a Guard-Pak precolumn insert, all obtained from Waters Assoc. (Milford, MA, U.S.A.). The electrochemical detector E656/641 from Metrohm (Herisau, Switzerland) with a glassy carbon electrode was used.

The mobile phase entering the detector cell was held at a constant temperature of 20° C by means of a water bath. The detector potential was normally maintained at 0.80 V versus an Ag/AgCl reference electrode. In order to obtain hydrodynamic voltamograms, step-by-step reductions were made from 0.90 to 0.35 V.

Chromatography

The mobile phase was a mixture of 0.1 *M* sodium acetate, 0.1 *M* citric acid, 0.5 m*M* sodium octyl sulphate, 0.15 m*M* EDTA, 1 m*M* dibutylamine, and 10% methanol (v/v), pH 3.70. For some experiments the pH value of the mobile phase was varied between 2.63 and 6.74 by changing the ratio of sodium acetate to citric acid, keeping the ionic strength of the solution constant. The mobile phase was filtered through a 0.22-μm filter (GVWP 04700, Millipore) and degassed under vacuum by means of ultrasonic agitation. All separations were performed isocratically at a flow-rate of 1.0 ml/min at room temperature.

Standards and calculations

The stock solutions of the standards were prepared in 0.1 *M* sodium acetate, 0.1 *M* citric acid pH 3.68 at a concentration of 6 m*M* and stored at −80° C. They were diluted 1:40,000 with the mobile phase to give the working solution; 25 μl of this were injected into the HPLC system (3.75 pmol). In order to test the linearity of the detector signal, a series of seven standard solutions were diluted from the stock solution, containing 0.5—9.4 pmol per injected volume.

The tissue levels of the substances were calculated by comparing the heights of the peaks in the sample with the heights of the peaks in the standard solution of known content. They were related to the protein content of the samples, expressed as pmol per mg protein. For comparison with the literature values results were expressed as pmol per mg wet tissue. Statistical comparison of results of different animal groups was carried out using the Mann-Whitney *U*-test.

Sample preparation

Male Wistar rats (190—230 g, from Mus Rattus, Brunnthal, F.R.G.) were treated with probenecid (400 mg/kg, intraperitoneally) as described by Gibson and Wurtman [20]: probenecid was dissolved in a minimum volume of 1 *M* sodium hydroxide, and the pH value was adjusted to 7.4 by adding 4 *M* hydrochloric acid. The final concentration of 100 mg/ml of this suspension was obtained with saline (0.9%, w/v) and injected intraperitoneally. In some experiments untreated animals were also used for reference.

Sixty minutes after the injection the animals were decapitated, and the brains rapidly removed and frozen on dry ice. The basal hypothalami were punched out with a needle (I.D. 4 mm) placed behind the optic chiasma. The heights of the tissue cylinders were 2 mm. Each of the tissue pieces was then placed in 600 μl of 0.12 *M* sodium acetate, pH 5.0 obtained by adding citric acid, and homogenized with an ultrasonic cell disrupter (Model B15, Branson, Danberg, CT, U.S.A.). Aliquots were taken from the homogenates for the analysis of protein according to the method of Lowry et al. [21]. The homog-

enates were then centrifuged for 15 min at 4°C at 25,000 *g*; 400 µl of each supernatant were separated for further processing.

In recovery experiments these samples were spiked with either 225 pmol of MOPEG sulphate or with 18.7 pmol, 37.4 pmol or 74.9 pmol of free NE, MOPEG, DA, DOPAC, HVA, 5-HT and 5-HIAA.

In order to hydrolyse the conjugated metabolites 0.7 mg (19.7 units) of sulphatase (7 mg/ml in 0.12 *M* sodium acetate, pH 5.0) were added to each sample and all were incubated for 3 h at 37°C. The samples were subsequently cooled in an ice bath and adjusted to give them the same composition as the mobile phase by adding 40 µl of 1.3 *M* citric acid, 7.5 m*M* sodium octyl sulphate, 2.25 m*M* EDTA, 13.5 m*M* dibutylamine and 60 µl of methanol (final volume of the sample 600 µl). For purposes of comparison some samples were not hydrolysed; 0.12 *M* sodium acetate, pH 5.0, without sulphatase was added to them at the same volume (100 µl) as to the others and the incubation was omitted. The further processing is described above.

The samples were then centrifuged for 15 min at 4°C at 25,000 *g* to pellet precipitated protein; the supernatants were diluted 1:1.5 and filtered through a 0.22-µm filter (GVWP 01300, Millipore); 25 µl of filtrate were then injected into the HPLC system.

RESULTS AND DISCUSSION

Chromatographic conditions

The aim of this study — to detect NE, DA, 5-HT and their main metabolites simultaneously — makes considerable demands on the chromatographic system, since it entails separating compounds of different polarity, such as basic (NE, DA, 5-HT), neutral (MOPEG), and acidic substances (DOPAC, HVA, 5-HIAA). HPLC in the reversed-phase mode, including ion-pairing, offers the wides range of possibilities for method development. The composition and conditions of the mobile phase are the main determinants of solute separation [15]. By varying the pH, the concentration of the ion-pairing reagent and the content of the methanol, we found an optimal separation of NE, DA, 5-HT and many of their metabolites under conditions shown in Fig. 1a. At pH 3.70 the amino groups of the substances are fully protonated, and the dissociation of carboxyl groups is suppressed [22]. The uncharged acidic metabolites are retained long enough at the hydrocarbonaceous stationary phase. The charged functional groups of the amines interact with the ion-pairing reagent in the mobile phase and on the stationary phase prolonging the retention times of the amines [14]. With the methanol as an organic modifier the retention times of all substances were shortened to avoid a lengthy period of separation.

A flow-rate of 1.0 ml/min was found to give the best resolution.

After 80 injections the back pressure of the column had risen to 1.5 times the initial value. Exchanging the guard column diminished this increase. Up to about 400 injections the resolution of the column was sufficient. Then the retention times of the analytes had been shortened to such a degree that NE began to interfere with the front peak. Therefore the column cartridge had to be changed.

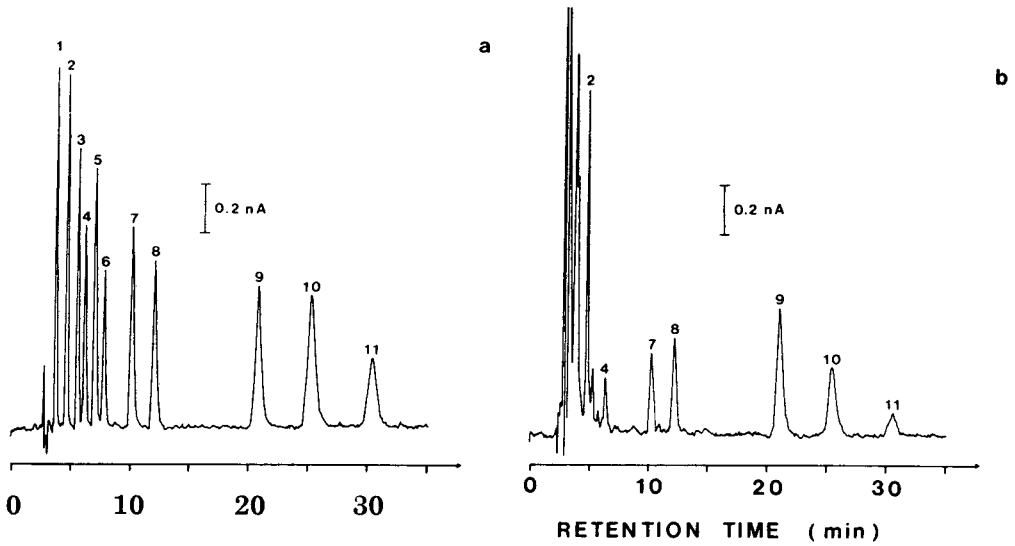

Fig. 1. (a) Chromatogram of a standard solution; 3.75 pmol of each standard dissolved in 25 μl of the mobile phase were injected. (b) Chromatogram of mediobasal hypothalamic tissue. The animals were treated with probenecid and the samples hydrolysed with sulphatase. Injection volume was 25 μl. Stationary phase, 5-μm C_{18} RCM 100; mobile phase, 0.1 *M* sodium acetate, 0.1 *M* citric acid, 0.5 m*M* sodium octyl sulphate, 0.15 m*M* EDTA, 1 m*M* dibutylamine, 10% methanol (v/v), pH 3.70; detector potential, 800 mV versus Ag/AgCl reference electrode. Peaks: 1 = DHPG, 2 = NE, 3 = A, 4 = MOPEG, 5 = DHBA, 6 = NMN, 7 = DA, 8 = DOPAC, 9 = 5-HIAA, 10 = 5-HI, 11 = HVA.

Linearity and sensitivity

The relation between the injected amount of standards and the detector response was linear for all analytes in the tested range (0.5—9.4 pmol). The sensitivity of the measurement varied mainly according to the condition of the detector. The noise of the detector cell was a limiting factor, which was primarily dependent on the condition of the reference electrode.

The detection limits, calculated by doubling the noise ratios, and expressed in terms of pmoles of the components injected were: 0.15 ± 0.02 (NE), 0.22 ± 0.02 (MOPEG), 0.28 ± 0.04 (DA), 0.33 ± 0.04 (DOPAC), 0.75 ± 0.10 (HVA), 0.43 ± 0.05 (5-HT), 0.38 ± 0.05 (5-HIAA) (n = 7).

Selectivity

Fig. 1b shows a chromatogram of mediobasal hypothalamic tissue of probenecid-treated rats. The samples had been hydrolysed with sulphatase. The long analysis time of 35 min was not reduced by increasing the methanol content of the mobile phase, since reduction of the retention times would lead to overlapping of the peaks in the first part of the chromatogram. The peaks were first identified by comparison of their retention times with those of the standards. Spiking the samples with standards provided additional reference points. Omitting prepurification steps in processing the samples renders this identification inadequate. Two additional tests were therefore carried out to ensure that correct peaks were identified and that there were no impurities coeluting with the compounds under investigation.

Variation of the potential of the detector cell (hydrodynamic voltamograms). By increasing the potential step by step, the catecholamines and indoleamines and their metabolites are oxidised at distinghishable potentials [23, 24]. Fig. 2 shows the comparison of the "relative current ratios" at different potentials between the provisionally identified components in the sample and the standard. The substances can be separated by their half-wave potentials ($E_{1/2}$) into three classes: dihydroxyphenols, indoles, vanillic compounds (in following the order of the increasing $E_{1/2}$). It is shown that the half-wave potentials of the provisionally identified components and their standards are almost identical.

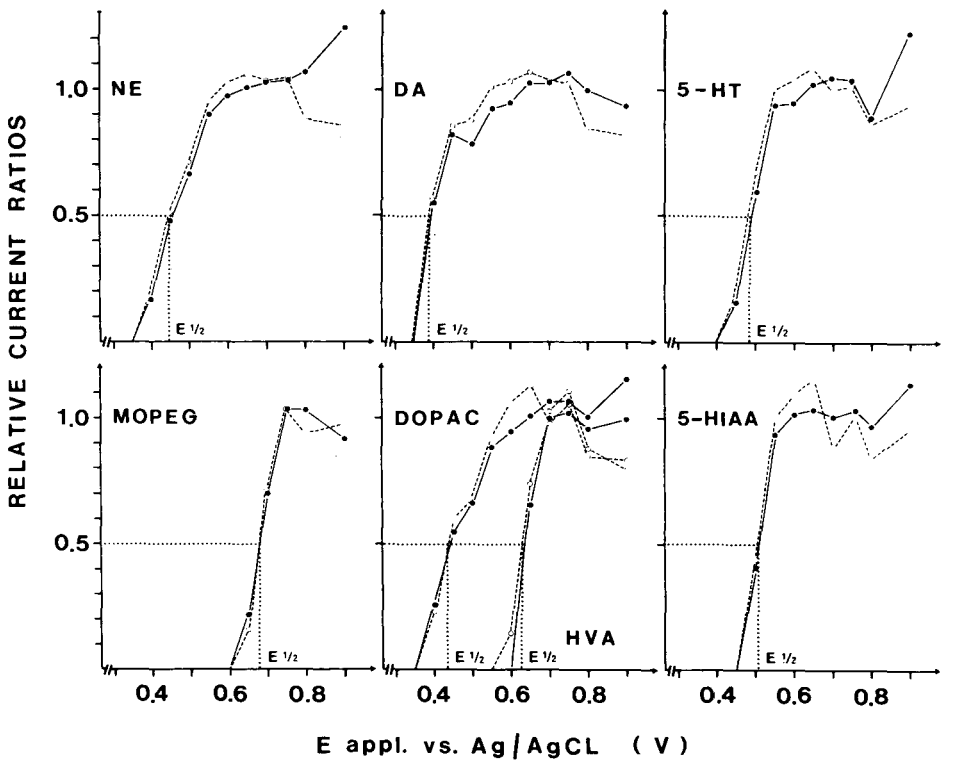

Fig. 2. Hydrodynamic voltammograms of the sample and the standard solution. The mediobasal hypothalami of probenecid-treated animals were pooled and hydrolysed with sulphatase; 25 µl of the processed samples were injected. The response (current) at several potentials was recorded, and the ratios of the current at any given potential to that of the average response at the plateau level were calculated and compared to the relative current ratios of the standards. Each point represents the mean of two determinations. (•——•), Sample; (○- - - -○), standards. The average deviation of the duplicates from their mean values was 2.0 ± 2.2%.

Variation of the pH value of the mobile phase. Depending on the pK_a values of the functional groups of the measured compounds, they are protonated to different degrees at certain pH values of the mobile phase. Fig. 3 shows the comparison of the capacity factors of the provisionally identified components of the sample with those of the standards dependent on the eluent pH. The

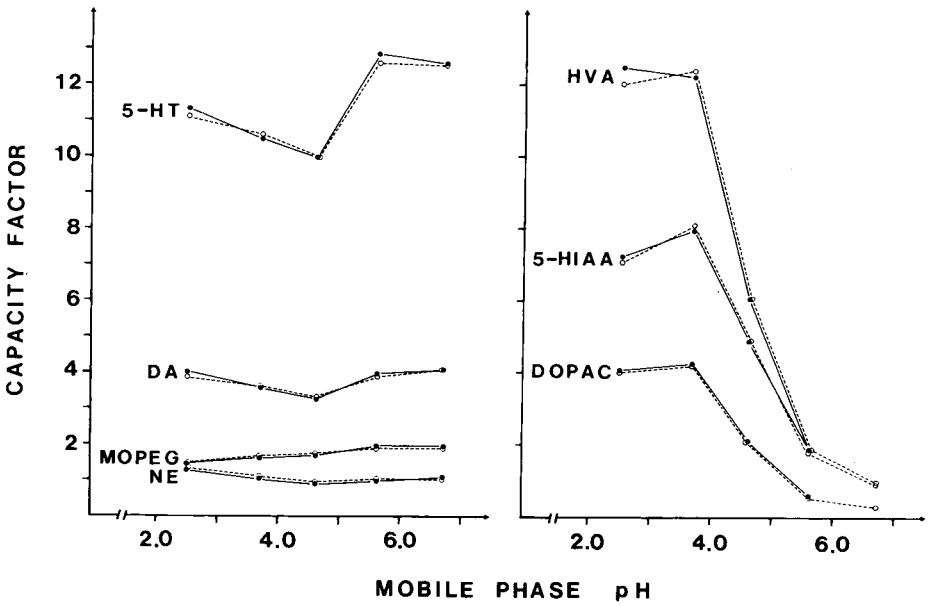

Fig. 3. Effect of mobile-phase pH on capacity factors of components in samples and standard solution. The mediobasal hypothalami of probenecid-treated rats were pooled and hydrolysed with sulphatase; 25 μl of the processed samples were injected. The pH of the mobile phase was varied by changing the ratio of sodium acetate—citric acid, maintaining a constant ionic strength. Each point represents the mean of two determinations. (•———•), Sample; (○- - - -○), standards. The average deviation of the duplicates from their mean values was 0.84 ± 0.63%.

capacity factors of the catecholamines and indoleamines do not change: their amine groups remain protonated over the pH range tested. The glycol MOPEG is not affected either. The carboxyl groups of the acidic metabolites dissociate at higher pH values and their capacity factors decrease. At pH values above 5.60 the capacity factors of the acidic metabolites in the sample could not be shown, since at these pH values their peaks interfered with the front peaks. Accurate identification became impossible. Comparison of the curves of the substances in the sample and of the curves of the standards showed almost no difference between the two.

In summary, the initial identification of the sample peaks was confirmed. Time-consuming prepurification of the sample to increase the selectivity of the method can therefore be omitted.

Precision and recoveries

The analysis of samples spiked with 18.7 pmol, 37.4 pmol or 74.9 pmol of free NE, MOPEG, DA, DOPAC, HVA, 5-HT and 5-HIAA gave recovery values independent from the added amount of analyte. Table I shows, therefore, the mean recoveries ± S.D. of all experiments. They ranged from 91% to 101%. This indicates that the substances are not affected by the sample processing, even during 3 h of incubation at 37°C. The good within-run precision of the estimations, given in the last column of Table I, together with the high recovery rates do not necessitate the use of an internal standard.

TABLE I

ESTIMATION OF THE PRECISION AND THE RECOVERIES OF SAMPLES SPIKED WITH KNOWN AMOUNTS OF STANDARDS

The mediobasal hypothalami of probenecid-treated animals were pooled and each of six samples was spiked with 18.7 pmol, 37.4 pmol or 74.9 pmol of standards. The samples were hydrolysed with sulphatase and 25 μl of each processed sample were injected.

	Endogenous amount (pmol per sample)	Mean recovery (%) ± S.D. (n = 18)	Precision, s_{rel} (%) (n = 24)
NE	167.4	99 ± 17	4.3
MOPEG	48.6	91 ± 9	5.3
DA	71.3	93 ± 7	3.3
DOPAC	85.3	100 ± 10	3.9
HVA	56.7	97 ± 10	5.4
5-HT	115.0	101 ± 10	3.1
5-HIAA	159.3	95 ± 16	3.4

Sample preparation

Enzymatic hydrolysis. The conjugated forms of the metabolites cannot be measured electrochemically. Hydrolysis is therefore necessary. The use of sulphatase preparation from *Helix pomatia* (type H-5, from Sigma) did not interfere with the chromatography of the sample. To test whether the hydrolysis of the conjugated compounds was complete, we spiked six samples

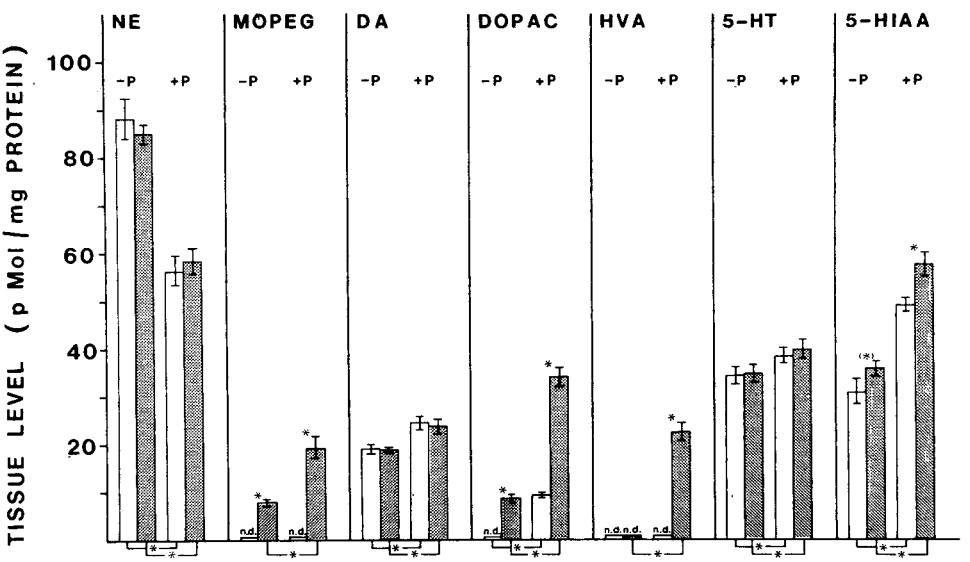

Fig. 4. Effect of probenecid treatment and enzymatic hydrolysis. The mediobasal hypothalami of probenecid-treated or untreated rats were pooled. The samples were then divided and independently processed by either incubating them with sulphatase or by omitting hydrolysis; 25 μl of each processed sample were injected. The heights of the columns represent the mean values ± S.D. of six determinations (n.d. = not detectable). —P = Untreated rats; +P = probenecid-treated rats; (□), unhydrolysed sample; (▨), hydrolysed sample. *: $p < 0.01$; (*): $p < 0.1$.

from probenecid-treated rats, as well as six samples from untreated animals, with quantities of MOPEG sulphate five to ten times higher than that found in mediobasal hypothalamic tissue. We estimated a recovery of 98 ± 5% and 101 ± 3%, respectively. We may assume, therefore, that under the conditions chosen all conjugated components will be hydrolysed completely. Fig. 4 compares data of samples with and without hydrolysis. It can be seen that the metabolites are partially or totally conjugated. Their levels were increased after hydrolysis. This is in agreement with the literature [7—10]. The catecholamines or indoleamines were not affected by this incubation procedure and their levels remained constant. After incubation the composition of the sample was adjusted to that of the mobile phase. This procedure resulted in very small front peaks in the chromatograms. The methanol content thus obtained was sufficient to precipitate protein, as also shown by others [17, 19].

Probenecid treatment of the animals. Fig. 4 demonstrates that probenecid causes an accumulation of all metabolites measured. It blocks the active transport system which eliminates them from the brain [5]. The dependence of the elimination on this transport system was demonstrated for MOPEG sulphate [4, 6], DOPAC sulphate [10], HVA and 5-HIAA [5, 6, 25]. The increase of unconjugated DOPAC after probenecid treatment shown here may not be due to inhibition of transport but is secondary to the end-product inhibition of the DOPAC-conjugating enzyme by the DOPAC conjugate [10]. The high dose of probenecid that we used (400 mg/kg) to guarantee complete blockage of the elimination system for MOPEG sulphate [4] may have been responsible for the significant changes of catecholamine and indoleamine levels after treatment. Other studies have shown that even at lower doses probenecid can change the metabolic rates of these transmitter systems [4, 26, 27]. An estimation of turnover rates by the probenecid method alone may therefore be problematic.

Accuracy

Evidence of the accuracy of this method has already been given by the performed selectivity tests and the high degree of recovery. Certainty in this connection could only be gained by a comparison of this method with a reference method, at present not available for this purpose. Therefore, a comparison of the values of the presented method with literature values of other studies has been made (Table II). The values obtained in this study are in general agreement with the others. None of the reported investigations determined all components simultaneously. Taylor et al. [28] used two different mobile phases to measure either the amines or their related metabolites.

Any comparison of metabolite determinations must take into consideration the fact that the results of the MOPEG, DOPAC and HVA determinations, presented in Table II in this investigation, include the free as well as the conjugated forms of the metabolites. The results of the HVA determination was additionally obtained from probenecid-treated animals. The results are therefore higher than the reported values. Only Kohno et al. [31] determined the free and the conjugated forms of MOPEG, and Smythe and co-workers [1, 2] included the sulphated form of metabolites in their determinations by using trifluoroacetic anhydride for derivatization of the samples. The apparent

TABLE II

RESULTS OF AMINE AND METABOLITE DETERMINATIONS BY THE PRESENT METHOD COMPARED TO LITERATURE VALUES FOR CERTAIN COMPOUNDS IN THE MALE RAT HYPOTHALAMUS

The values of the present method were obtained by six independent determinations of a pooled mediobasal hypothalamic homogenate. Mean values are given.

Investigation method	Concentration (pmol per mg wet tissue)						
	NE	MOPEG	DA	DOPAC	HVA	5-HT	5-HIAA
This investigation	9.71	0.87*	2.11	0.97*	2.53*,**	3.78	3.62
HPLC—ED [28]	14.91	0.43	2.65	0.59	0.14	4.68	1.10
HPLC—ED [29]	8.66	—	1.44	0.30	0.16	—	2.41
HPLC—ED [30]	8.93	—	2.72	—	—	2.91	—
Fluorometry [31]	8.25	1.12	—	—	—	—	—
Radioenzymatic assay							
[32]	9.54	—	3.06	—	—	—	—
[33]	—	—	—	—	—	2.53	—
Gas chromatography—mass spectrometry							
[2]	14.31	—	3.72	—	0.52	5.43	2.96
	13.40	—	2.66	—	0.47	7.56	2.48
	10.30	0.50	3.19	0.95	—	6.19	2.93
	10.10	—	2.35	—	0.64	4.21	2.17
	13.77	—	3.04	—	0.61	5.29	2.53
[1]	10.7	—	3.14	—	0.70	4.65	2.41
	—	—	4.09	—	0.84	6.2	3.5

*Determination of samples hydrolysed with sulphatase.
**Determinations of a pooled homogenate of probenecid-treated rats.

differences between the other values are probably a reflection of the differences in the methods as well as differences in the dissection technique of the hypothalamus. Furthermore, rats of different breed, age and sex were used.

CONCLUSION

This study offers a simple method for measuring NE, DA, and 5-HT and their main metabolites simultaneously, omitting time-consuming sample pre-purification. By means of enzymatic hydrolysis of the sample, which can be easily carried out prior to chromatography, it is also possible to measure conjugated metabolites. The additional tests of selectivity performed ensure adequate peak identification in the samples. This method is thus useful for the estimation of turnover rates in the selected transmitter systems.

ACKNOWLEDGEMENTS

The author thanks Dr. K.-M. Pirke for helpful discussions and for his critical revision of this manuscript, and Ms. Ursula Genning for her skilful assistance.

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Journal of Chromatography, 307 (1984) 283—294
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2049

DETERMINATION OF THIAMINE AND THIAMINE PHOSPHATES IN EXCITABLE TISSUES AS THIOCHROME DERIVATIVES BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON OCTADECYL SILICA

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(First received August 11th, 1983; revised manuscript received December 29th, 1983)

SUMMARY

The analysis of thiamine and thiamine phosphates by high-performance liquid chromatography owes its high sensitivity to the fluorescent derivatives or thiochromes obtained by chemical oxidation in alkaline medium. The possibility of performing precolumn oxidation with potassium ferricyanide instead of using the hazardous cyanogen bromide has been investigated. The derivatization step has been optimized with respect to the following parameters: concentration of alkali and oxidant, presence of methanol and stability of the thiochromes.

A gradient separation with 25 m*M* phosphate buffer (pH 8.4) and methanol as mobile phase components and an octadecyl silica column as stationary phase has been set up. The analytical run takes 14 min with the following elution order: thiochrome triphosphate, thiochrome pyrophosphate, thiochrome monophosphate and thiochrome. The minimum detectable amount is 0.05 pmol.

The method was found suitable for the determination of thiamine compounds in excitable tissues such as nerves and electric organs as well as in proteins extracted from membranes of these organs. It may be useful to study the role of thiamine in the electrical activity of these tissues at the molecular level.

0378-4347/84/$03.00

INTRODUCTION

Thiamine is present in animal tissues in different forms: thiamine (T), thiamine monophosphate (TMP), thiamine pyrophosphate or cocarboxylase (TPP) and thiamine triphosphate (TTP). Among these forms, TPP has a well known function as cofactor during enzymatic decarboxylation. However, considerable evidence has recently been accumulated in favour of a second function of thiamine compounds specific to the nervous system [1]. In addition to electrophysiological experiments more precise biochemical results suggest that the phosphorylated esters of thiamine could play a role in the electrical activity of excitable tissues, i.e. at the level of the voltage-dependent sodium-channel activity [2—4]. As this ion-gating mechanism has been described in terms of a phosphorylation—dephosphorylation cycle of a protein, TTP or TPP could be endogenous sources of phosphorus in this model [5—8]. To study this possibility we needed a rapid and sensitive technique to separate and quantify the thiamines at different stages of the sodium-channel preparation [9—11].

High-performance liquid chromatography (HPLC) seems to be the method of choice since its sensitivity reaches the subpicomole level using fluorimetric detection. Thiamines are converted into fluorescent derivatives or thiochromes by alkaline oxidation: Thc, ThcMP, ThcPP and ThcTP. In most of the recently published works [12—14], this derivatization is performed after the chromatographic separation, using $K_3Fe(CN)_6$ as oxidative reagent, which requires an additional experimental device: pump, mixing coil and incubator. In contrast, cyanogen bromide is used as oxidant in precolumn derivatization [15—17]. This chemical is very poisonous and its alkaline solutions are not stable. For these reasons, we have developed a simple method using $K_3Fe(CN)_6$ in precolumn derivatization. The separation is carried out with octadecyl silica as solid phase and mixtures of phosphate buffer and methanol as mobile phase. The technique is applied to the analysis of excitable tissue extracts.

MATERIAL AND METHODS

Instrumentation

The apparatus was an Altex (Berkeley, CA, U.S.A.) Model 334-50 programmable gradient liquid chromatograph consisting of two single-piston pumps (Model 110 A), a system controller (Model 421), a high-pressure solvent mixer and a sample injection valve (Model 210) with a 20-μl loop. Pump A was used to deliver the aqueous buffer and pump B the methanol.

The columns were also obtained from Altex (Ultrasphere-ODS, 5 μm, 150 or 250 × 4.6 mm). They were protected by precolumns (4.2 × 3.2 mm) dry-packed with Vydac-201RP (30—44 μm) from Macherey-Nagel (Düren, F.R.G.). At a flow-rate of 1 ml/min the resulting pressure varied from 7 to 17 MPa during the gradient elution.

Two detectors were connected in series. The Gilson Spectra/Glo filter fluorometer (Middelton, U.S.A.) was used with standard filters (excitation 390 nm, emission 475 nm) and cell (15 μl). The absorbance detector was an Altex Model 165 multichannel rapid scanning ultraviolet—visible apparatus.

Electrical signals were transfered to a double-pen recorder (10 mV) and/or a C-R1A Chromatopac Shimadzu integrator (Kyoto, Japan).

Reagents and solvents

T, TMP and TPP were obtained from Sigma (St. Louis, MO, U.S.A.) and used without further purification. TTP was prepared according to the method of Penttinen [18]. Aqueous solutions were prepared with water delivered by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.) and filtered on 0.45-μm ultrafilters. Organic solvents and other chemicals were of pro analysi grade (Merck, Darmstadt, F.R.G.).

Derivatization procedure

Standard mixtures of thiamines were dissolved in water. Aliquots were distributed in small tubes and lyophilized. The dried powder was stored at −20°C and the solution was reconstituted when necessary with 80 μl of water or water—methanol (1:1, v/v).

Optimal oxidation was obtained by adding 50 μl of fresh alkaline ferricyanide to 80 μl of methanolic standards, followed by a brief mixing (5—10 sec).

The alkaline ferricyanide solution was prepared daily as follows: 100 mg of $K_3Fe(CN)_6$ were dissolved in 10 ml of water; 50 μl of this solution were added to 2.5 ml of 15% NaOH. The resulting oxidant solution was found to be stable for one day if stored in the dark.

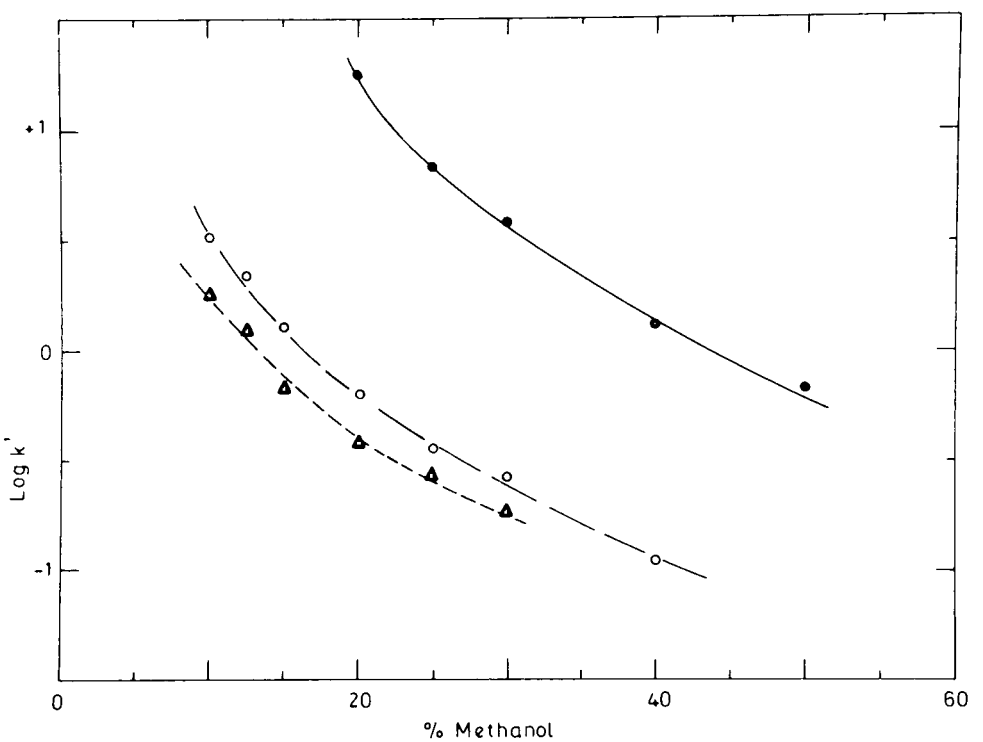

Fig. 1. Dependence of the logarithm of the capacity factor, k', on mobile-phase composition for thiochrome derivatives: (•), Thc; (○), ThcMP; (▲), ThcPP. Mobile phase: 25 mM potassium phosphate buffer (pH 8.4)—methanol. Stationary phase: Ultrasphere-ODS, 250 × 4.6 mm, 5 μm. Flow-rate: 1 ml/min.

Sample preparation

The procedure described by Ishii et al. [19] was followed except for the following points. Samples of 100 mg of tissue were homogenized in 3 vols. of 5% trichloroacetic acid at 4°C in a motor-driven glass homogenizer. After centrifugation (1 h, 5000 *g*, 4°C) the supernatant was extracted for 1 h with 3 vols. of water-saturated diethyl ether. Aliquots of the aqueous phase were oxidized as described above and injected 1 min after the addition of the $K_3Fe(CN)_6$—NaOH mixture.

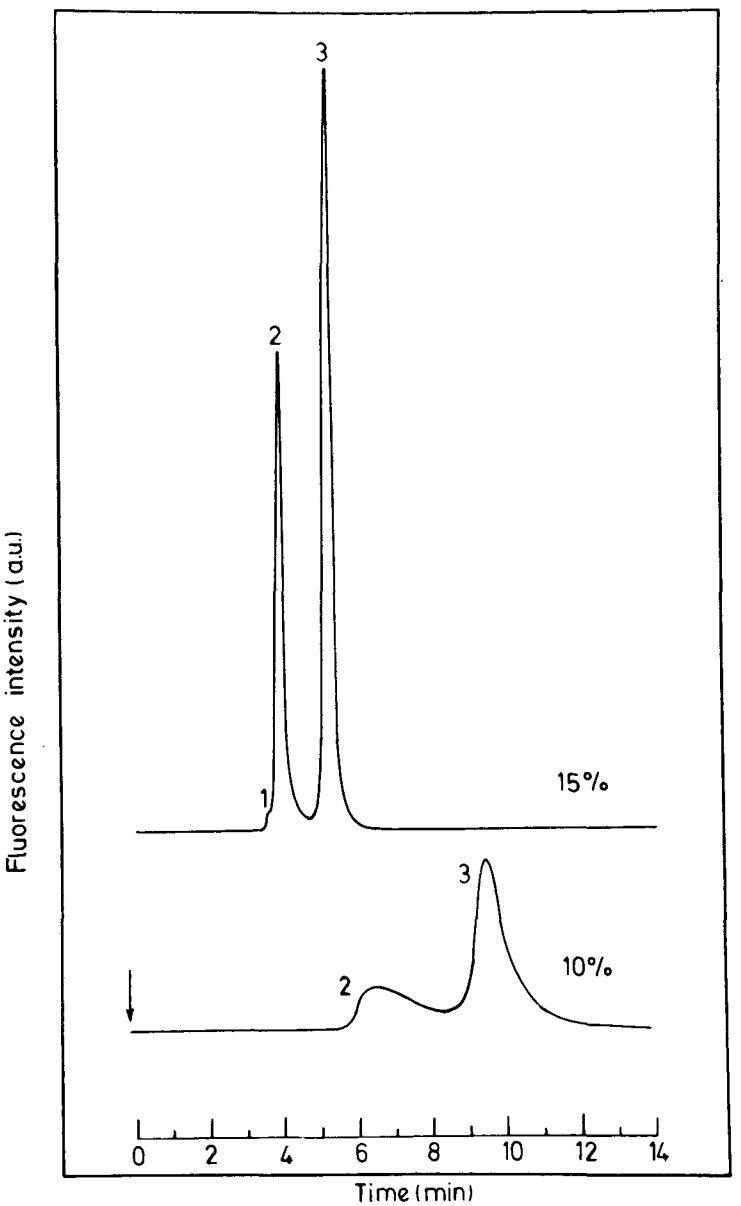

Fig. 2. Isocratic separation of ThcPP (peak 2) and ThcMP (peak 3) in 15% (upper part) followed by 10% methanol (lower part).

RESULTS AND DISCUSSION

Regulation of retention

Sanemori et al. [16] have shown that ThcTP, ThcPP and ThcMP can be separated isocratically in 20 min in the reversed-phase mode using 25 m*M* phosphate buffer (pH 8.4) and 2.5% N,N-dimethylformamide (DMF) as mobile phase. Thc could be subsequently eluted if the DMF content was increased to 50%. Our aim was to develop a method for the simultaneous determination of the four thiochromes by gradient elution using methanol as organic modifier instead of DMF.

The capacity factors were determined for the three major thiochromes at different methanol concentrations (Fig. 1). The higher retention of Thc compared to ThcMP and ThcPP clearly justifies the use of a gradient to shorten the analysis time. This experiment was carried out from a high methanol percentage (50%) to lower values. As the mobile-phase composition was changed from 15% to 10% methanol, a particular phenomenon was observed, both with phosphate (pH 8.4) or borate (pH 9.0) buffer (Fig. 2): considerably broader and deformed peaks were obtained without any modification of their areas. This effect could be suppressed either by repeated injections of the oxidized sample at short intervals (e.g. 1 min) or by a preinjection of the alkaline oxidant or of 15% NaOH alone.

Gradient set-up

The following gradient was programmed: after 1 min at 10%, the methanol concentration was raised to 100% in 3 min; 6 min after the injection the initial conditions were restored (Fig. 3B). Under these conditions a good resolution of the esters was obtained and thiamine eluted 1.5 min later. However, due to the very short re-equilibration time the methanol concentration is probably somewhat higher than 10% during the first step of the gradient. Obviously a first-gradient cycle without injection and a constant time interval between the injections (14 min) are required to obtain good reproducibility of the retention: $k' = 1.4$, 1.6, 2.0 and 3.8 for ThcTP, ThcPP, ThcMP and Thc, respectively. In the chromatograms presented in Fig. 3A, the small peak at 3.95 min (peak 1) was attributed to ThcTP as contaminant of the other products. This was confirmed later (Fig. 3B) when TTP was synthesized according to the procedure of Penttinen [18]. A frontal peak is observed by monitoring the absorbance at 254 nm (Fig. 3A) which is due to excess of the oxidant, $K_3Fe(CN)_6$. The minor peak between ThcMP (peak 3) and Thc (peak 4) is a fluorescent impurity present in the mobile phase since this peak also appears when the gradient is run without injection.

Optimization of the precolumn derivatization procedure

The oxidization conditions were found to be extremely variable in the literature [12—17, 20]: the final concentration of $K_3Fe(CN)_6$ varies from 1×10^{-4} to 30×10^{-4} *M*, that of NaOH from 0.04 to 1.9 *M*. We found it important to study by chromatographic means the influence of the following factors: reagent concentration, presence of methanol in the reaction medium and reaction time. The stability of the oxidized thiamines was also investigated. Peak areas were

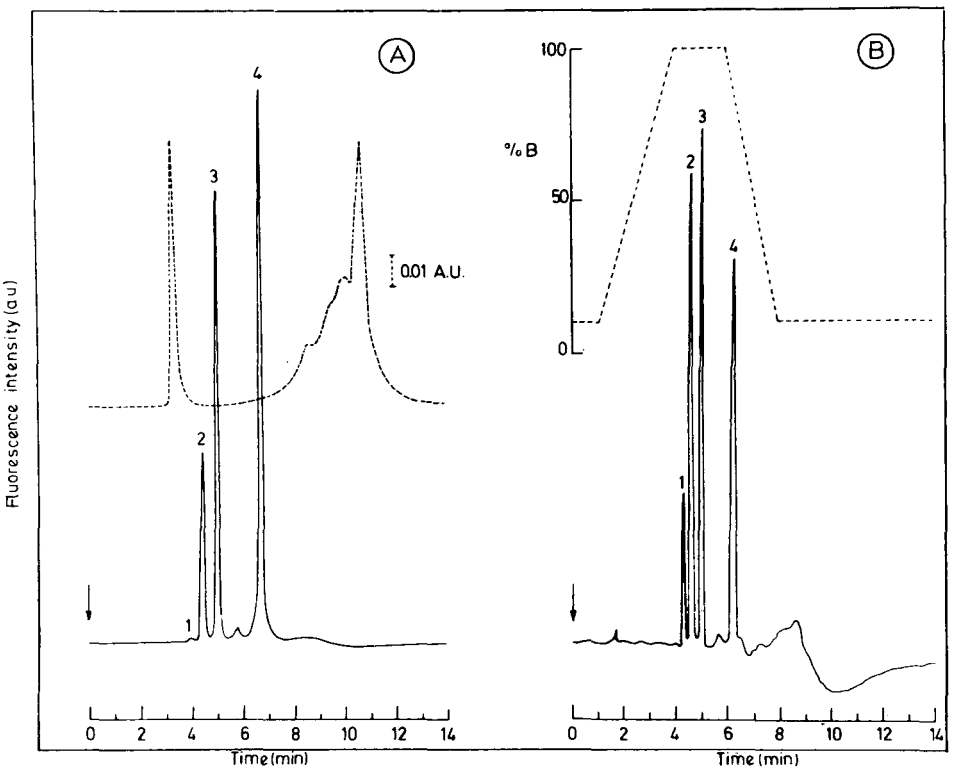

Fig. 3. Gradient separation of ThcTP (peak 1), ThcPP (peak 2), ThcMP (peak 3) and Thc (peak 4). Solid lines refer to the fluorescence signal and dotted lines to the UV trace at 254 nm (A) and to the profile of the programmed gradient (B).

measured from chromatograms corresponding approximately to 10 pmol of T and TMP and 6 pmol of TPP injected 1 min after the addition of the oxidizing mixture.

Fig. 4 illustrates the effect of the concentrations of the oxidizing reagents on the thiochrome production: $K_3Fe(CN)_6$ (Fig. 4A), NaOH (Fig. 4B) and combined chemicals (Fig. 4C). Constant values of peak areas are obtained at $K_3Fe(CN)_6$ concentrations higher than 0.3×10^{-4} *M* at a NaOH molarity of 1.44 (Fig. 4A). If the oxidant is maintained at a concentration of 1.54×10^{-4} *M* and NaOH varied, an important dependence upon NaOH concentration is observed (Fig. 4B): the peak areas increase to reach a maximum value above 1.5 *M*. This influence is also shown with more experimental data points in Fig. 4C where both factors are changed simultaneously but in a constant NaOH-to-$K_3Fe(CN)_6$ molar ratio of 10^4. Note that in the low concentration range the initial slope is decreasing from Thc to ThcPP. From these experiments optimal concentration values of 1.54×10^{-4} and 1.44 *M* were adopted for $K_3Fe(CN)_6$ and NaOH, respectively. These concentrations are indicated by arrows in the figures.

Methanol was mixed in increasing proportion with the mixture of thiamines before the oxidation (Fig. 5). Within a range of 10—50% methanol, retention times were perfectly stable but a linear increase in peak area was observed for each compound. This allows a 20% increase in sensitivity when the sample is in

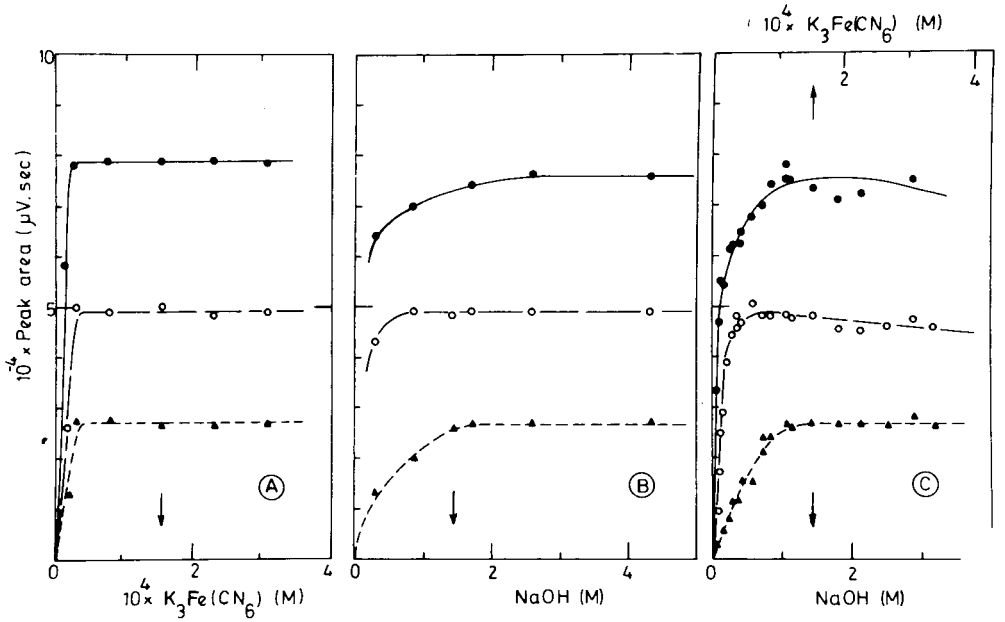

Fig. 4. Effect of $K_3Fe(CN)_6$ (A), NaOH (B) and combined reagents (C) on the oxidation reaction as measured by the peak areas of chromatograms obtained in gradient mode (see Fig. 2). The arrows indicate the optimal concentration adopted in the precolumn derivatization procedure: 1.54×10^{-4} *M* for $K_3Fe(CN)_6$ and 1.44 *M* for NaOH. (•), Thc; (○), ThcMP; (▲), ThcPP.

50% methanol leading to a final methanol concentration of 31% of the oxidized sample, as described in the derivatization procedure section. Higher methanol concentrations were not assayed in order to avoid a decrease of retention which can occur if the sample is dissolved in a medium of eluting strength higher than that of the mobile phase. The observed enhancement of 20% of the oxidation yield agrees with the data of Wostmann and Knight for thiamine [21]. In addition to the regulation of retention, methanol is thus advantageously used here to optimize the precolumn reaction.

In the above experiments, the mixture of thiamines was oxidized exactly 1 min before the injection. A reduction of the reaction time to 30 sec or an increase up to three days did not affect the chromatographic results. The derivatives were stable when stored in the dark at room temperature which allows the use of an autosampler for the analysis. Oxidized samples kept at −20°C in sealed vials were found to be unmodified three months after their preparation. This permits stable reference values for the analysis of biological samples.

Influence of sample composition and number on retention

Fig. 6 shows retention measurements for the experiments described in the previous section (Fig. 4). While changes in $K_3Fe(CN)_6$ concentration in the sample do not affect retention (Fig. 6A), the NaOH concentration has a significant influence on the retention of the phosphate derivatives (Fig. 6B and C). The magnitude of the effect seems to be related to the number of charged

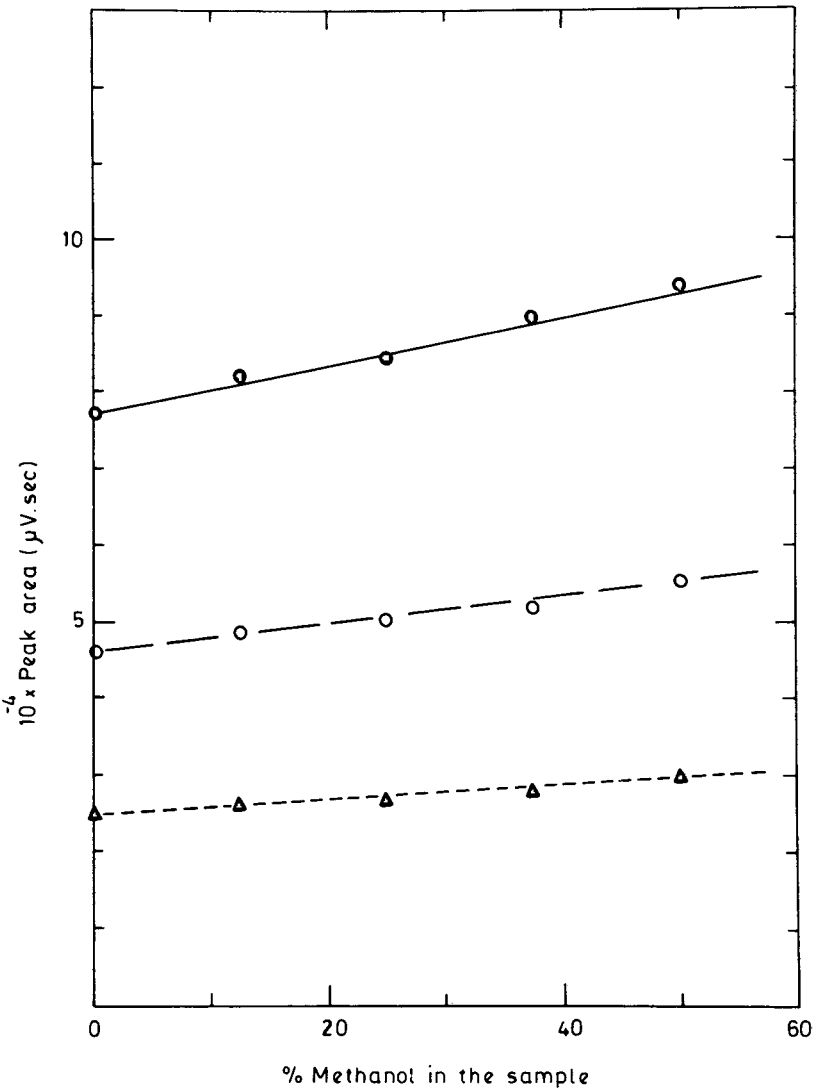

Fig. 5. Role of methanol on the oxidation reaction. ThcPP (▲), ThcMP (○) and Thc (●) were eluted at 4.56, 5.16 and 6.75 min, respectively, for each experimental condition.

phosphate groups. For NaOH concentrations equal or higher to the selected value (1.44 *M*) constant retention times are obtained together with a maximum selectivity.

The number of samples, injected at the same 14-min time interval and derivatized under optimal conditions, also affects the same chromatographic parameters, retention and selectivity, as shown in Fig. 7. The amplitude of the effect, however, is less pronounced than that described above for NaOH. In practice, two preliminary injections are required to obtain stable conditions. To shorten this stabilization time, three successives injections at 30-sec intervals could be made before starting quantitative determinations.

Such an influence of sample composition and number on chromatographic

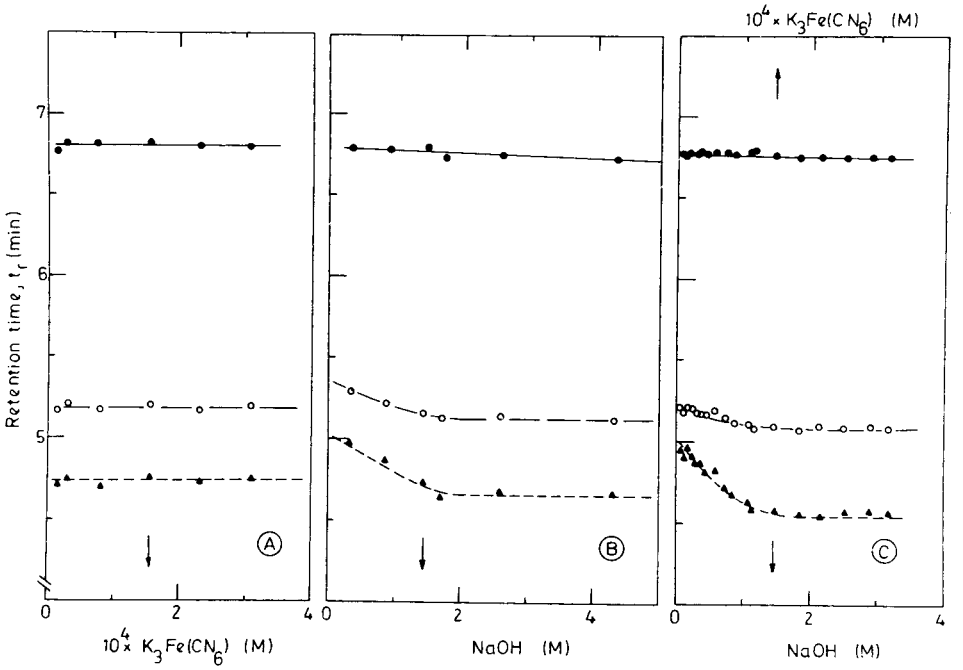

Fig. 6. Influence of the concentration of $K_3Fe(CN)_6$ (A), NaOH (B) and combined reagents (C) on the retention time of thiochrome derivatives (t_0 = 1.4 min). The experimental conditions are identical to those of Fig. 4. (•), Thc; (○), ThcMP; (▲), ThcPP.

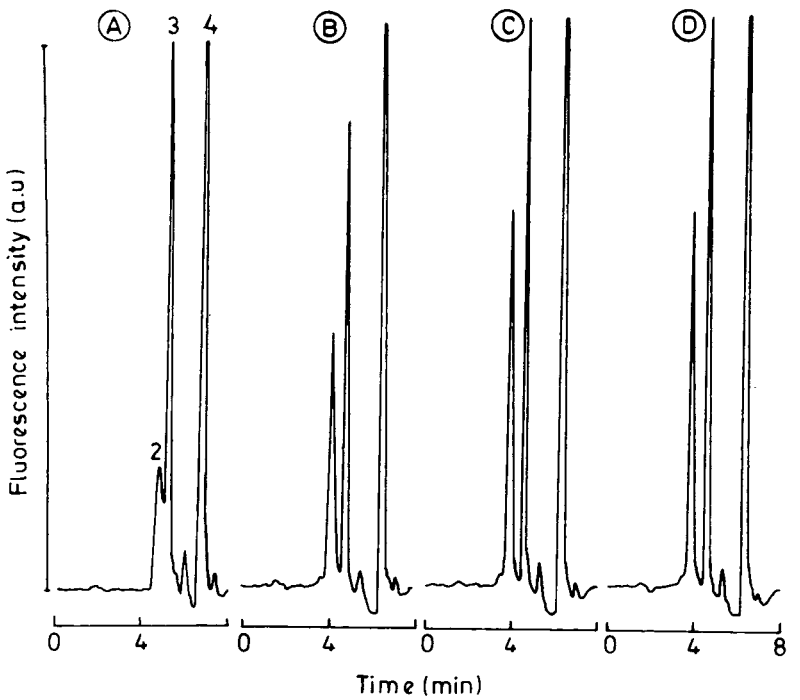

Fig. 7. Stabilization of the chromatographic system by repeated injection of an oxidized standard mixture of TPP (peak 2), TMP (peak 3) and T (peak 4).

behaviour should of course be avoided whenever possible in analytical techniques. However, highly reproducible results can be obtained if the time between successive injections is constant.

Column stability

The efficiency of a new column was maintained for one to two months in spite of intensive use (8 h per day) with an alkaline buffer (pH 8.4) as mobile-phase component and the injection of samples containing 1.44 *M* NaOH. The lifetime of the analytical column was not significantly increased by the use of precolumns. This drawback is not reported by the users of such an alkaline mobile phase for the analysis of thiamines [15, 16, 19].

Such a high pH was initially selected to obtain the best fluorescence intensity of the thiochromes [15]. Attempts were made to lower the pH of the buffer but resulted in a significant loss in sensitivity.

Quantitative analysis

A linear response was obtained over a large range, 0.1—20 pmol, with the same detection limit of 0.05 pmol reached by Sanemori et al. [16] who used cyanogen bromide as oxidant and 2.5% DMF as the organic mobile-phase component. It is worth noting that Thc gives a higher fluorescence signal compared to ThcMP and ThcPP. This results from an effect of methanol on the fluorescence quantum yield of the thiochrome during the gradient elution. Indeed, equimolar amounts of thiamine and its phosphate esters produce equal thiochrome fluorescence values [22] as we have also observed under isocratic elution conditions.

The concentration of thiamine and of its phosphorylated derivatives was measured in samples related to the study of the relationship between thiamine compounds and the sodium-channel activity. Additionally, rat tissues were assayed for comparison with the data in the literature [19, 23].

Fig. 8A shows a typical chromatogram obtained with a sample of sciatic nerve from *Rattus norvegicus*. The total thiamine content was equal to 3.4 pmol/mg wet weight, with the following distribution: 3% of TTP, 70% of TPP, 13% of TMP and 14% of T. Similar chromatograms were registered with membrane extracts prepared from electroplaxes of *Electrophorus electricus*. This tissue is widely used as starting material for the purification of the sodium-channel proteins [9, 24].

When the method was applied to the analysis of rat tissues other than the sciatic nerve, TTP, which only accounts for 1% of the thiamines, could not be quantified. In the chromatogram presented in Fig. 8B, the peak of TTP is masked by a non-thiochrome peak. The amount of TPP + TMP + T in these tissues was found to be 6.85, 14.0 and 8.1 pmol/mg wet weight in brain, heart and kidney, respectively. These results are in agreement with those of Ishii et al. [19] using HPLC and of Rindi and De Giuseppe [23] using a low-pressure technique. The first authors noted that approximately 20% of the samples did not show TTP peaks under the best chromatographic conditions. For this reason, they developed another HPLC separation for the analysis of thiamine phosphates without thiamine [16]. In our case, higher retention is required for the identification of TTP. Attempts to increase retention by reducing methanol

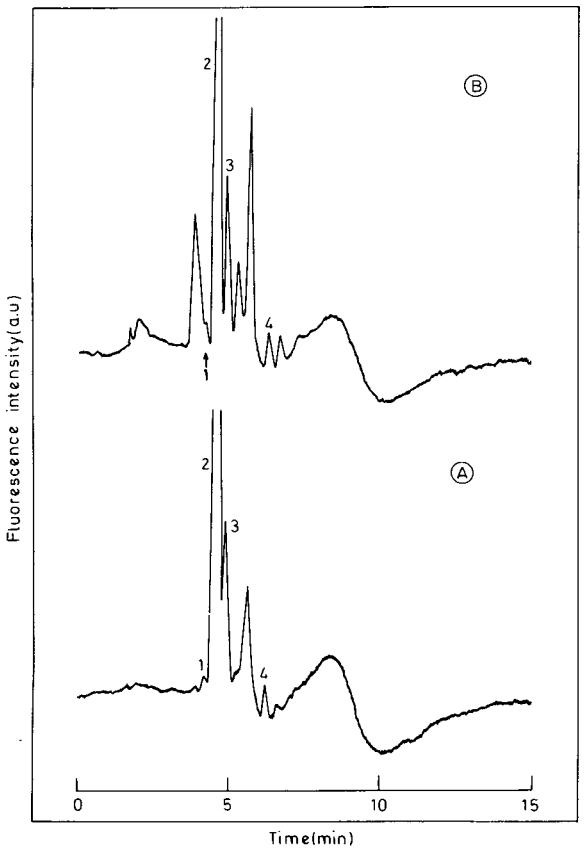

Fig. 8. Typical chromatograms of the analysis of rat sciatic nerve (A) and heart (B).

concentration gave rise to severe peak deformation (Fig. 2). Preliminary experiments have indicated that good peak symmetry can be obtained at low methanol concentration by using poly(styrene-divinylbenzene) instead of octadecyl silica. This should give more favourable conditions for the determination of TTP in such crude biological samples.

CONCLUSIONS

The results presented in this report clearly show that the oxidation of thiamine and thiamine phosphates by $K_3Fe(CN)_6$ can be used as a precolumn derivatization procedure instead of cyanogen bromide to reach the same order of sensitivity in chromatographic analysis. Moreover, the stability of $K_3Fe(CN)_6$ in NaOH allows the addition of the mixed reagents to the sample and this leads to a simpler and more accurate derivatization technique. The different parameters that affect the oxidation yield have been optimized. Among these factors, NaOH has been found to influence the chromatographic behaviour which requires carefully controlled analytical conditions: (1) a preliminary gradient cycle without injection; (2) three injections of oxidized standards at short intervals (30 sec); and (3) a constant time of 14 min between the sample injec-

tions. The role of NaOH seems to be related to the nature of the silica-based solid phase since it has not been observed in preliminary experiments on a poly-(styrene-divinylbenzene) column.

ACKNOWLEDGEMENTS

The excellent technical assistance of Mr. Louis Roland and Ch. Grandfils is gratefully acknowledged. This investigation received financial support from the Fonds National de la Recherche Scientifique (F.N.R.S.), Belgium.

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Journal of Chromatography, 307 (1984) 295—304
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2043

ISOTHERMAL GAS CHROMATOGRAPHIC ANALYSIS OF DIPHENHYDRAMINE AFTER DIRECT INJECTION ONTO A FUSED-SILICA CAPILLARY COLUMN

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(First received September 9th, 1983; revised manuscript received December 15th, 1983)

SUMMARY

The packed column injector of a gas chromatograph was modified to accommodate direct injection by syringe onto a wide-bore fused-silica capillary column. No changes were made to the nitrogen—phosphorus detector. The resultant configuration combines fast separations with precise quantitations. The analysis of diphenhydramine in serum is presented as an application. Chromatographic separation of diphenhydramine and orphenadrine (internal standard) from caffeine and other endogenous material takes 2 min. Serum diphenhydramine concentrations are presented for six volunteers following a 50-mg oral dose.

INTRODUCTION

Conventional capillary gas chromatographic analyses rely on split, splitless or on-column sample introduction. Each of these injection techniques usually involves cold trapping of the analytes onto the first few centimeters of the capillary column followed by temperature programming to effect their elution.

Grob and Grob, Jr. [1—3] have shown isothermal analyses to be reliable only under the influence of the solvent effect. To be successful, close attention must be paid to the rate of sample injection and the choice of solvent for a

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given stationary phase and oven temperature. The analytes must of course be soluble in the solvent selected.

Direct injection has been used with wide-bore borosilicate glass columns having inside diameters > 0.5 mm. Kits to convert chromatographs from packed column to wide-bore borosilicate column operation are commercially available from chromatography supply houses. Only two reports [4, 5] have described direct injection with fused-silica capillary columns.

Demedts et al. described the combined use of direct injection, a fused-silica capillary column and a nitrogen—phosphorus detector for the analysis of heroin in contraband [4] and for the analysis of codeine and ethylmorphine in biological materials [5]. In both reports, temperature programming was used.

In this report, direct injection onto a fused-silica capillary column is evaluated at isothermal conditions. It is shown to be a rapid means of sample introduction, resulting in fast separations without the need of the solvent effect.

EXPERIMENTAL

Equipment

A Perkin-Elmer Sigma 3 gas chromatograph equipped with nitrogen—phosphorus and flame ionization detectors, a Model R-100 chart recorder (Perkin-Elmer, Norwalk, CT, U.S.A.) were used. Quantitative results from peak areas were automatically obtained with a Model 3390A reporting integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.). A 007 bonded methyl silicone (0.5-μm film) wide-bore fused-silica capillary column, 15 m × 0.45 mm O.D., 0.32 mm I.D. (Quadrex, New Haven, CT, U.S.A.) was used in the analysis.

The injector of the gas chromatograph was modified as shown in Fig. 1. The packed-column injector rear fitting and glass insert were replaced by a 160-mm long borosilicate glass sleeve, 5.5 mm O.D., 0.65 mm I.D. The sleeve was chamfered at the inlet end to facilitate easy insertion of the 26-gauge (0.47-mm) needle of a 5-μl Hamilton 85 N syringe. Prior to mounting, the following deactivation procedure was used. The sleeve was soaked in 10% Decon 75 detergent (BDH Chemicals, Toronto, Canada) for 2 h, then rinsed with water, ethanol and hexane. It was silanized with 20% dimethyldichlorosilane (Pierce, Rockford, IL, U.S.A.) in toluene for 2 h. The sleeve was rinsed

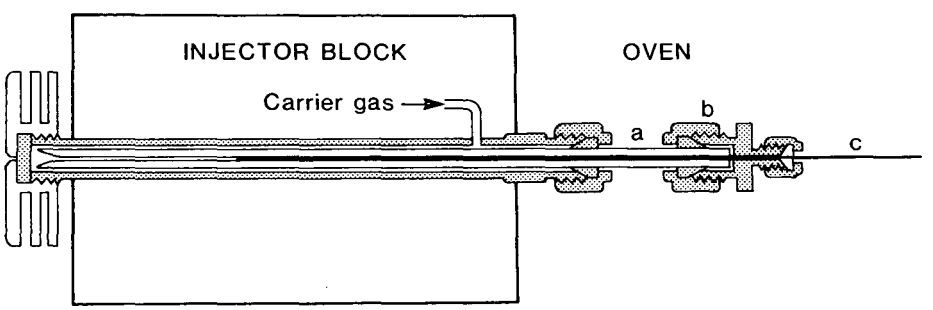

Fig. 1. Modified packed column injector to accommodate wide-bore fused-silica capillary column. (a) Borosilicate glass sleeve, 160 mm × 5.5 mm O.D., 0.65 mm I.D.; (b) 1/4—1/16 in. reducing union; (c) fused-silica capillary column 15 m × 0.45 mm O.D., 0.32 mm I.D.

again with ethanol and hexane, then air dried. A 6.35—1.59 mm (1/4—1/16 in.) stainless-steel Swagelok® zero dead volume reducing union facilitated connection of the fused-silica capillary column to the injector sleeve inside the oven. Vespel-graphite 6.35–1.59 mm (1/4—1/16 in.) reducing ferrules (Supeltex M2A®, Supelco, Bellefonte, PA, U.S.A.) were drilled out to 5.55 mm (7/32 in.). They were used to connect the injector sleeve to the reducing union and to the rear of the injector block.

The column inlet was positioned inside the injector sleeve 3—5 mm from the point of injection (needle tip). Thermogreen septa (Supelco) were used to avoid the addition of contaminant peaks to the chromatogram.

At the detector, the column was held in place by a 6.35—1.59 mm (1/4—1/16 in.) stainless-steel Swagelok reducer mounted onto the 1/4 in. packed-column fitting. The effluent column end was positioned flush with the detector jet tip, 1 mm from the rubidium bromide bead. Surface adsorption within the detector was thus avoided without the need of a make-up gas.

The gas chromatograph was operated isothermally at 180° C, the injector and detector temperatures set at 300° C. Helium carrier gas at 140 kPa (20 psi) provided a linear velocity of 60 cm/sec. Hydrogen and air flow-rates were 3 and 130 ml/min, respectively. The rubidium bromide bead potentiometer was set at 380; the attenuation was 64. Under these conditions, diphenhydramine and orphenadrine eluted at 1.30 and 1.69 min, respectively.

Equipment evaluation

Approximately 100 mg each of diphenhydramine · HCl and orphenadrine · HCl, internal standard (Sigma, St. Louis, MO, U.S.A.) were dissolved in 1 ml of water in a 20-ml extraction tube. The water was made basic with 100 µl of 1 mol/l sodium hydroxide and extracted with 5 ml of HPLC-grade hexane (Fisher Chemical, Winnipeg, Canada). The hexane layers were placed in clean tubes and dried under a stream of dry air. Stock solutions at 100 µg/ml in hexane were prepared from the free bases. Working dilutions of diphenhydramine were prepared at 80, 60, 40, 20, 15, 10, 5 and 1 µg/ml in hexane, each containing orphenadrine at 10 µg/ml. Quantities of 1 µl of these solutions were injected ten times in order to assess the precision, linearity and stability of the instrument.

Serum samples

Stock 100 µg/ml diphenhydramine and orphenadrine standards were prepared by dissolving 22.86 and 22.70 mg of the respective hydrochloride salts in 200 ml of 0.01 mol/l hydrochloric acid. Working orphenadrine internal standard solution, 1000 ng/ml, was a 100-fold aqueous dilution of 1.0 ml of the stock solution. A serum-based diphenhydramine solution (1000 ng/ml) was prepared by diluting 1.0 ml of aqueous stock standard to 100 ml with drug-free serum. Further dilutions were made with drug-free serum to give 50-ml standards of 800, 200, 100, 50 and 10 ng/ml. Aliquots stored at —20° C in 3-ml polypropylene vials were used for precision and recovery studies.

Extraction

New 20-ml Pyrex 16 mm × 150 mm PTFE-capped extraction tubes and 8-ml

Pyrex conical centrifuge tubes were washed, silanized and dried as described above for the custom-made injector sleeve. A 1-ml aliquot of serum was added to an extraction tube with 100 μl of orphenadrine internal standard (1000 ng/ml), 100 μl of 1 mol/l sodium hydroxide and 5 ml of hexane. The tube was gently mixed by inversion (60 rpm) for 15 min, then briefly centrifuged. The hexane layer was transferred to an 8-ml silanized conical centrifuge tube and reduced to about 10 μl under a stream of dry air. The tube was briefly vortexed, then 1 μl was injected onto the gas chromatograph.

Data analysis

Within-day and day-to-day diphenhydramine precision data were determined on 10 and 20 replicates, respectively. Data were obtained for the following serum concentrations: 800, 200, 100, 80 and 10 ng/ml. Recovery experiments at these five concentrations were carried out according to the above extraction protocol except that no internal standard was added to the serum. Rather, 80 ng of orphenadrine in hexane was added to 4.0 ml of the hexane extract just prior to its evaporation. Peak area ratios were compared to peak area ratios of the corresponding free base standards prepared in hexane. The same technique was used to calculate the recovery of orphenadrine at 100 ng/ml by using diphenhydramine as the reference material.

Six healthy volunteers participated in a diphenhydramine pharmacokinetic study. Each person ingested a 50-mg diphenhydramine · HCl capsule (Benadryl®, Warner Lambert/Parke Davis Pharmaceuticals, Ann Arbor, MI, U.S.A.) on an empty stomach. Blood samples were drawn before and at 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12 and 24 h following administration. The blood was centrifuged and the serum collected for analysis of diphenhydramine.

RESULTS AND DISCUSSION

A chromatogram following a 1-μl injection of hexane containing diphenhydramine and orphenadrine at 10 μg/ml is shown in Fig. 2. Symmetrical sharp peaks appear at 1.30 and 1.69 min, respectively, for these two drugs with little baseline disturbance from the solvent. For an OV-1 capillary column and similar chromatographic conditions, Grob and Grob, Jr. [2] have indicated hexane to be far too volatile to elicit a solvent effect. Here, the combined use of a narrow bore injector sleeve and a brisk carrier gas flow-rate (60 cm/sec) accommodate the relatively rapid transfer of analytes onto the fused-silica capillary column upon injection. Unfortunately the transfer rate is far from ideal and is most apparent in the early eluting peaks. Only 3500 and 4600 plates were calculated from the diphenhydramine (k = 6.6) and orphenadrine (k = 8.9) peaks, respectively. The optimal plate count is 38,000 for k = 10.3.

Carrier gas velocities below 40 cm/sec produced longer trailing solvent fronts and wider analyte peaks which sometimes split into doublets. Fortunately, typical van Deemter curves for helium are relatively flat [6] such that the column performance is not unduly compromised at a higher gas velocity.

During the investigative stages of this work, a 0.78-mm I.D. injector sleeve was tried and found to give acceptable chromatograms. However, the smaller

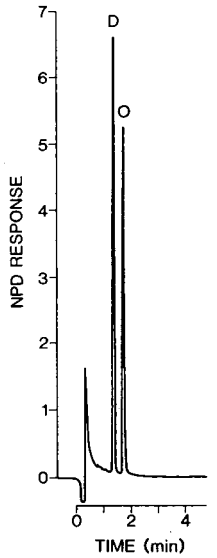

Fig. 2. Chromatogram of diphenhydramine (D) and orphenadrine (O), 10 ng each in hexane, 1 μl injection.

0.65-mm I.D. sleeve is preferable because it gave a two-fold increase in the peak height/peak area ratio. A yet narrower 0.50-mm I.D. sleeve resulted in only marginal further improvement. It appears that the choice of bore diameter is critical but not limiting for optimal performance of the injector.

The position of the capillary column inlet was varied along the length of the injector sleeve. Reproducibility was best with the column inlet positioned 3 to 5 mm from the point of sample deposition. The same inlet location was selected by Demedts et al. [4, 7].

The injector modification resulted in no memory effect. The rate of sample injection did not have to be rigidly controlled to avoid back flashing the sample from the injector sleeve. A convenient 1—2 sec delivery time was adopted for a 1-μl injection. A 10% carry-over of both drugs was inherent to the syringe needle, probably due to hexane distillation in the injector. The problem was eliminated by routinely baking the needle in the second injector for 10 sec after rinsing.

The nitrogen—phosphorus detector response was evaluated with the column effluent end positioned flush and withdrawn 10 and 20 mm from the jet tip. Each position was tested with and without nitrogen make-up gas. Make-up gas did not enhance the nitrogen—phosphorus detector response with the column end held flush with the jet tip. With the column end withdrawn inside the jet, a make-up gas was necessary to prevent peak broadening. Flush mounting is preferred because peak broadening is minimized without the need of a make-up gas, the nitrogen—phosphorus detector response remains optimized and the potential risk of drug adsorption within the detector is avoided.

Within-day retention time precisions (n = 80) were 0.7% for diphenhydramine (1.30 min) and 0.5% for orphenadrine (1.69 min). Over three months, however, the retention times of diphenhydramine and

orphenadrine gradually increased from 1.30 to 1.44 min and from 1.69 to 1.88 min, respectively. Approximately 1200 injections of serum extracts were made during this time. The injector sleeve was subsequently cleaned in detergent, resilanized and replaced. The retention times decreased to 1.33 min for diphenhydramine and to 1.73 min for orphenadrine, nearly identical to those originally observed. It would appear that the bonded methyl silicone stationary phase had not been modified by repetitive injections of hexane. The accumulation of septum particles and charred debris in the injector sleeve was responsible for the shift in retention times.

Repetitive injections of nanogram quantities of diphenhydramine standards in hexane resulted in desirable quantitative data (Table I). Precision ranged from 3.18% to 0.90% at 1 and 80 ng diphenhydramine injected, respectively. The instrument configuration gave a linear response and from the data in Table I, the correlation coefficient was 1.000; the equation of the straight line was $y = 0.099x + 0.053$, where y is the ratio of peak areas for diphenhydramine to orphenadrine and x is the mass of diphenhydramine injected. These results indicate the virtual absence of adsorption sites in the injector and column for these two drugs above 1 ng.

TABLE I

WITHIN-DAY INSTRUMENT PERFORMANCE

Diphenhydramine injected (ng) (n = 10)	Peak area diphenhydramine / Peak area orphenadrine	
	$\bar{X}$	C.V. (%)
1.0	0.119	3.18
5.0	0.533	2.15
10.0	1.041	1.48
15.0	1.558	1.37
20.0	2.039	0.59
40.0	4.002	1.08
60.0	5.984	0.97
80.0	7.910	0.90

Chromatograms obtained from serum extracts appear in Fig. 3. Baseline separations of caffeine, diphenhydramine and orphenadrine occurred at 1.05, 1.33 and 1.73 min, respectively. Extraneous serum peaks were small and did not interfere in the analysis. Caffeine peaks were disproportionately small because it is poorly extracted into hexane. Similar extracts were previously injected onto 1.83 m × 2 mm I.D. glass columns packed with either 3% OV-1 on 80--100 Chromosorb W HP or 3% SP2100 on 100—120 Supelcoport. Carrier gas was nitrogen at a flow-rate of 25 ml/min. With either packed column, caffeine separated poorly from diphenhydramine and tailed profusely. In the presence of > 1 μg/ml caffeine, quantitation of diphenhydramine below 50 ng/ml was unreliable. In addition, the calibration curve became somewhat alinear below 30 ng/ml, probably due to adsorption onto the column support.

Two brands of evacuated serum collection tubes were evaluated for their

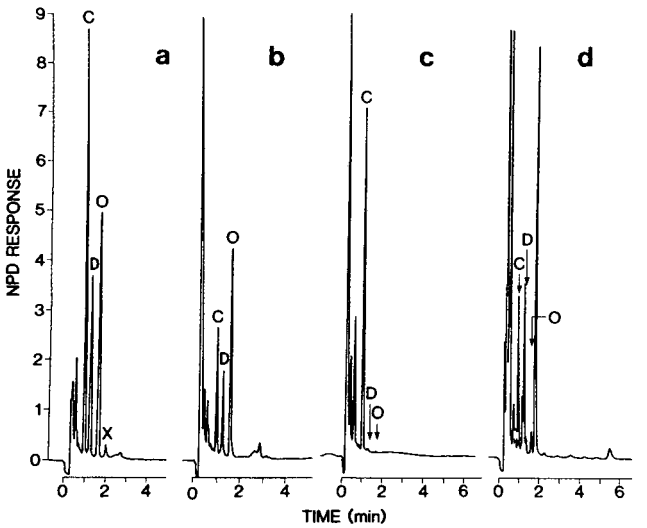

Fig. 3. Chromatograms from serum extracts. (a) Diphenhydramine 50 ng/ml standard; (b) volunteer 12 h post 50-mg oral dose of diphenhydramine · HCl, serum diphenhydramine = 27.4 ng/ml; (c) drug-free serum collected in a Vacutainer (plain) collection tube; and (d) drug-free serum collected in a Venoject (plain) collection tube. Peaks: C = caffeine, D = diphenhydramine, O = orphenadrine, X = unknown.

suitability in clinical studies. Drug-free blood was collected in 10-ml red-stoppered Vacutainer® (Becton-Dickinson, Mississauga, Ontario, Canada) and Venoject® (Kimble Terumo, Elkton, MO, U.S.A.) tubes. The serum was separated and extracted according to the above procedure except that an internal standard was not added. Resulting chromatograms appear in Fig. 3c and d. Numerous interfering peaks appeared in the serum extract from the Venoject tube but not the Vacutainer tube. The limit of detection for diphenhydramine collected in Vacutainer tubes was 0.9 ng/ml. Both collection tubes were further investigated for the presence of the plasticizing agent tris(2-butoxyethyl) phosphate (TBEP). Its presence in Vacutainer tubes has been shown to cause artifactually low serum concentration determinations for a variety of basic drugs, e.g. imipramine and alprenolol [8]. It is not known whether TBEP would affect diphenhydramine in the same way. TBEP if present extracts into hexane at any pH and elutes at 1.6 min at 220°C on this column. Both tubes were found to be TBEP-free.

Table II provides a summary of the precision and recovery data for serum-based standards at 10, 50, 100, 200 and 800 ng/ml diphenhydramine. The coefficients of variation (C.V.) are similar at corresponding serum concentrations for the within-day and day-to-day precision studies. However, from a comparison of corresponding diphenhydramine concentrations in Tables I and II, it is seen that the extraction step roughly doubles the imprecision compared to that observed with pure standards in hexane. To correlate the two tables, extraction of 1 ml of serum at 10 ng/ml results in an injection of about 1 ng of drug, etc. Recovery of diphenhydramine at all concentrations tested ranged from 98.0% to 106.7%. Recovery of orphenadrine at 100 ng/ml was 98.6%. For practical purposes, complete recovery for both drugs can be assumed.

TABLE II

ASSAY PERFORMANCE

Compound (ng/ml)	Precision						Recovery (n = 5)	
	Within-day (n = 10)			Day-to-day (n = 20)				
	$\bar{X}$ (ng/ml)	S.D. (ng/ml)	C.V. (%)	$\bar{X}$ (ng/ml)	S.D. (ng/ml)	C.V. (%)	$\bar{X}$ (%)	C.V. (%)
Diphenhydramine								
10	11.2	0.8	7.6	11.4	0.7	6.6	105.6	5.1
50	50.2	1.4	2.6	50.7	2.3	4.5	106.7	7.2
100	100.0	3.2	3.2	100.9	3.0	3.0	103.1	4.6
200	212.8	7.7	3.6	193.9	7.5	3.9	101.0	2.2
800	841.3	16.0	1.9	776.6	15.0	1.9	98.0	2.2
Orphenadrine								
100	—	—	—	—	—	—	98.6	3.4

Method linearity was evaluated from the within-day precision data presented in Table II by plotting observed diphenhydramine concentrations (y-axis) against the target values of the serum-based standards. Linear regression analysis gave a correlation coefficient of 1.000, a slope of 1.05 and a y-intercept of −1.36 ng/ml.

Single-step serum extractions with hexane provide gas chromatograms with clean backgrounds when a nitrogen–phosphorus detector is used. Hexane is a preferred solvent for drug analysis provided the recovery is sufficiently high. Unfortunately, hexane and biological fluids are notorious for forming emulsions which are not always broken under centrifugation. The addition of 2% isoamyl alcohol or any other low-molecular-weight alcohol to hexane prevents emulsion formation but also causes endogenous biological material to co-extract. Further time-consuming clean up steps such as back extraction into acid followed by re-extraction into an organic solvent from the alkalinized acid fraction are then required. It was discovered that when the silanized extraction tubes were simply rinsed with water after use rather than cleaned in detergent, emulsions rarely formed between hexane and serum. No diphenhydramine carry-over was detected with the following cleaning procedure: (a) rinse with 2 ml of methanol or ethanol; (b) rinse with four 10-ml portions of warm tap water; (c) rinse with two 10-ml portions of distilled water; (d) invert to drain dry.

Serum concentrations of diphenhydramine following a single 50-mg oral dose of the hydrochloride salt were measured in six healthy volunteers. The results are illustrated in Fig. 4. Peak concentrations ranging from 53 to 196 ng/ml occurred between 0.5 and 4 h. Similar serum concentration–time data have previously appeared [9, 10]. A thorough discussion of diphenhydramine pharmacokinetics is in press [11]. The major amine metabolite of diphenhydramine, 2-(benzhydroloxy)-N-methylethylamine, eluted between caffeine and diphenhydramine and hence would not potentially interfere with the

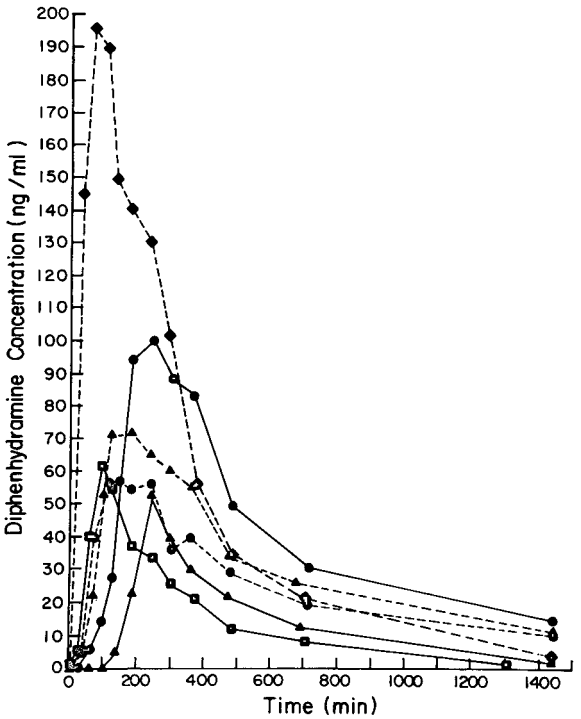

Fig. 4. Serum concentration—time curves for six volunteers following a single 50-mg oral dose of diphenhydramine · HCl. Each point is the average of duplicate determinations. Key: (●—●) subject 1; (● - - ●) subject 2; (▲—▲) subject 3; (▲ - - ▲) subject 4; (■—■) subject 5; (◆ - - ◆) subject 6.

quantitation of diphenhydramine. In fact, the metabolite appeared in only a few of the volunteer serum samples.

ACKNOWLEDGEMENTS

We thank Mr. D. Sansome, Perkin-Elmer Canada, Ltd. and Mr. I. Ward (Glass Shop), University of Manitoba, Canada for their technical assistance. We are grateful to Dr. Tsun Chang, Warner-Lambert Co., Ann Arbor, MI, U.S.A. for providing a pure sample of the monodesmethyl metabolite of diphenhydramine. This research was funded by grants from the Manitoba Health Research Council (MHRC-3215) and the Medical Research Council of Canada (MS-3250).

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Journal of Chromatography, 307 (1984) 305—321
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2052

SIMULTANEOUS QUANTITATION OF DISOPYRAMIDE AND ITS MONO-DEALKYLATED METABOLITE IN HUMAN PLASMA BY FUSED-SILICA CAPILLARY GAS CHROMATOGRAPHY USING NITROGEN—PHOSPHORUS SPECIFIC DETECTION

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(First received September 23rd, 1983; revised manuscript received December 30th, 1983)

SUMMARY

A nitrogen-specific detector gas—liquid chromatographic assay method is reported which provides improved selectivity and sensitivity for disopyramide and its mono-N-dealkylated metabolite using a crosslinked fused-silica capillary column. The quantitation of disopyramide and mono-N-dealkylated disopyramide was accomplished by injecting trifluoroacetic anhydride-treated samples containing derivatized internal standard *p*-chlorodisopyramide, into a gas chromatograph equipped with a nitrogen—phosphorus detector and an automatic liquid sampler. A 25 m × 0.31 mm crosslinked, 5% phenylmethyl silicone-coated fused-silica column was utilized and samples were injected using the splitless injection mode.

0378-4347/84/$03.00

Linearity was observed in the range 0.05—5.00 μg/ml for disopyramide and 0.02—3.00 μg/ml for the mono-N-dealkylated metabolite. The coefficient of variation was found to be within 10% for both compounds in the concentration range studied.

INTRODUCTION

Disopyramide, 4-(diisopropylamino)-2-(2-pyridyl)-2-phenylbutyramide, is an antiarrhythmic agent which shares a number of pharmacologic properties with quinidine and procainamide [1—9]. In patients with normal renal function, 50—60% of disopyramide is excreted unchanged in the urine with the remainder being excreted in urine and feces as the mono-N-dealkylated metabolite [4-(isopropylamino)-2-(2-pyridyl)-2-phenylbutyramide, MND] [10, 11]. Animal studies suggest that the antiarrhythmic potency of MND is about 80% that of disopyramide while the anticholinergic side effects are approximately 24-fold greater than that associated with the parent drug [12].

A number of analytical methods have been reported for the simultaneous measurement of disopyramide and MND in biological fluids including gas—liquid chromatography (GLC) [11, 13—23] and high-performance liquid chromatography (HPLC) [24—36] and a stable isotope dilution technique using gas chromatography—mass spectrometry (GC—MS) [37]. All GLC methods reported so far for disopyramide make use of conventional packed columns. The HPLC methods are convenient but sensitivity limitations necessitate injection of the entire extracted sample volume into the chromatograph.

The lack of adequately sensitive and selective assay methods has limited the disopyramide plasma protein binding determination to in-vitro binding studies [9, 38, 39]. In almost all cases the unbound percentage values are indirectly calculated from the individual binding curves rather than from measurements of binding to the proteins in plasma that was obtained from patients to whom drug had been administered [9, 38, 39].

The aim of the present report is to describe a sensitive and reproducible fused-silica capillary GCL—nitrogen—phosphorus detection (NPD) assay for the simultaneous quantitation of disopyramide and MND in human plasma in the presence of various antiarrhythmics and for use in binding studies.

Materials

Disopyramide, 4-(diisopropylamino)-2-(2-pyridyl)-2-phenylbutyramide (lot No. SC-7031), disopyramide phosphate (Norpace, lot No. SC-13957), mono-N-dealkyl disopyramide (lot No. ADAMEKCD3-185A) and *p*-chlorodisopyramide (lot No. SC-13957) were supplied by G.D. Searle of Canada (Oakville, Ontario, Canada). Trifluoroacetic anhydride (TFAA) was purchased from Pierce (Rockford, IL, U.S.A.). Toluene (distilled-in-glass) was purchased from Caledon Labs. (Georgetown, Ontario, Canada). Solutions of 1 *M* sodium hydroxide and 0.1 *M* hydrochloric acid were prepared from ACS reagent-grade chemicals (American Scientific and Chemical, Seattle, WA, U.S.A.). Deionised and distilled water was used in the preparation of stock solutions and throughout the analysis.

Instrumentation

A Model 5830A Hewlett-Packard (H-P) gas chromatograph equipped with a nitrogen—phosphorus selective detector and a Model 18835B capillary inlet system was used for all analyses. A Model 18850A H-P integrator system was used for peak area integration and quantitation. Capillary GLC electron impact mass spectrometry (EI-MS) and chemical ionisation mass spectrometry (CI-MS) were carried out utilizing GC—MS H-P Models 5970A and 5987A, respectively.

Chromatographic conditions

A 25 m × 0.31 mm I.D. crosslinked fused-silica capillary column (5% phenylmethyl silicone, ultra No. 2, film thickness 0.17 μm, siloxane-deactivated (Hewlett-Packard, Avondale, PA, U.S.A.) was used for all plasma analyses. The splitless injection mode employing a silanized fused-silica insert (78 mm × 2 mm I.D.) was used, with a 2-μl sample being injected. The operating conditions for routine analysis were: injection port temperature, 200°C; oven temperature 1, 160°C, programming rate, 5°C/min, oven temperature 2, 195°C; nitrogen—phosphorus detector temperature, 300°C; helium was used as a carrier at a flow-rate of 1 ml/min and make-up gas at a flow-rate of 30 ml/min; septum purge flow-rate was 3 ml/min; hydrogen—air flow-rate ratio was 3:50 (ml/min).

Stock solutions

Disopyramide phosphate (5 μg/ml, equivalent to base) was dissolved in distilled water; mono-N-dealkylated disopyramide (MND) (3 μg/ml) and the internal standard, *p*-chlorodisopyramide (PC-Dis) (5.5 μg/ml) were dissolved in 0.1 *M* hydrochloric acid. The solutions were stored at 4°C, following preparation, for up to two months.

Extraction and derivatization procedure

A 0.5-ml sample of blank human plasma was spiked with a volume (0.1, 0.2, 0.4, 0.6, 0.8 or 1.0 ml) of the prepared stock solutions of disopyramide (5 μg/ml) and MND (3 μg/ml). To this mixture, 0.5 ml of PC-Dis (5.5 μg/ml) and 0.5 ml of 1 *M* sodium hydroxide (pH 12) were added. The aqueous phase was adjusted to a total volume of 3.5 ml with distilled water. Toluene (6 ml) was added and the aqueous phase was extracted by shaking for 20 min on a rotary shaker (Labquake Tube Shaker, Model 415-110; Labindustries, Berkeley, CA, U.S.A.). After centrifugation for 10 min, 5 ml of the organic phase was removed and dried under a gentle stream of nitrogen in a 40°C water bath. The residue was reconstituted to a volume of 0.5 ml with toluene and 150 μl of trifluoroacetic anhydride (TFAA) were added. The sample was vortexed for 10 sec and incubated in an oven at 55°C for 45 min. The excess TFAA was removed by evaporating the sample under a gentle stream of nitrogen in a 40°C water bath. The residue was reconstituted with 100 μl of toluene and 2-μl aliquots were used for capillary GLC—NPD analysis. A similar extraction and derivatization procedure was carried out for diluted stock solutions of disopyramide (0.54 μg/ml), MND (0.23 μg/ml) and PC-Dis (0.55 μg/ml).

Quantitative analysis

A 2-μl aliquot of the toluene solution containing TFAA-treated disopyramide, MND and PC-Dis was injected into a gas chromatograph equipped with an automatic liquid sampler (H-P Model 7671A). Calibration curves for disopyramide and MND were constructed by plotting the area ratios against the known concentrations of disopyramide and MND. The calibration curves thus obtained were used for the estimation of the unknown concentration of disopyramide and MND in biological samples.

GLC—electron impact mass spectrometry

A computerized gas chromatograph—electron impact mass spectrometer H-P Model 5970A, equipped with a 12 m × 0.27 mm I.D. fused-silica column coated with methylsilicone fluid and a mass selective detector was used to study the fragmentation pattern of untreated and TFAA-treated samples of disopyramide, MND and PC-Dis. The following splitless, capillary GLC conditions were used: oven temperature 1, 225°C; time 1, 0.5 min; rate, 15°C/min; oven temperature 2, 250°C; time 2, 2.0 min; injection port temperature, 250°C; helium (carrier gas) flow-rate, 1.0 ml/min. For the mass spectrometer, the ionization beam energy was 70 eV, the electron multiplier voltage, 1400 V and the interface temperature was 280°C.

Chemical ionization mass spectrometry

An H-P Model 5987A gas chromatograph—mass spectrometer was used to determine the molecular ion of the parent, untreated compounds, as well as the TFAA-treated compounds. Samples were injected using the splitless mode and a linear scanning method was used. The instrument was equipped with a 12 m × 0.27 mm I.D. crosslinked methyl silicone fused-silica column. Methane was used as an ionizing gas and the following mass spectrometer conditions were used: injection port temperature, 250°C; interface oven temperature, 275°C; GC interface probe temperature, 275°C; ion source temperature, 100°C; oven temperature 1, 100°C; rate, 30°C/min; oven temperature 2, 260°C; multiplier voltage, 2007 V, emission current, 300 μA.

RESULTS

Representative GLC chromatograms from the extracts of blank plasma and spiked plasma are shown in Fig. 1. No extraneous (interfering) peaks from endogenous plasma constituents are apparent in the plasma extracts (Fig. 1A). Peaks with retention times (R_t) of 10.10 and 12.91 min are the dehydrated i.e. nitrile) forms of disopyramide and PC-Dis, respectively, and the peak at 10.59 min represents the TFAA derivative of the dehydrated form of MND. Baseline resolution is achieved between all peaks.

Fig. 2 represents the chromatogram of TFAA-treated mixture containing various antiarrhythmic drugs. All of the chromatographic peaks were resolved under the following splitless GLC conditions; oven temperature 1, 160°C; oven temperature programming rate, 5°C/min; oven temperature 2, 270°C; time 2, 25 min; injection temperature, 260°C; nitrogen—phosphorus detector temperature, 300°C; carrier gas (helium) flow-rate, 1.2 ml/min; make-up gas

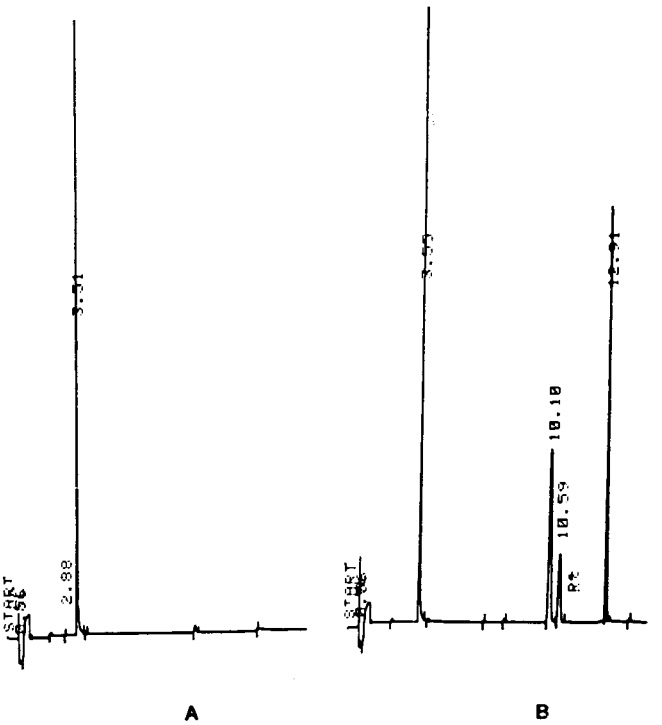


Fig. 1. Representative capillary gas—liquid chromatograms obtained from (A) blank and (B) spiked plasma extracts; the spiked sample before extraction contained disopyramide (R_t, 10.10 min), 1.0 ng/μl; MND (R_t, 10.59 min), 0.6 ng/μl and PC-Dis (R_t, 12.91 min), 2.7 ng/μl. Attenuation, 128; voltage 16 V; nitrogen—phosphorus detector collector was in use for ca. 200 h.

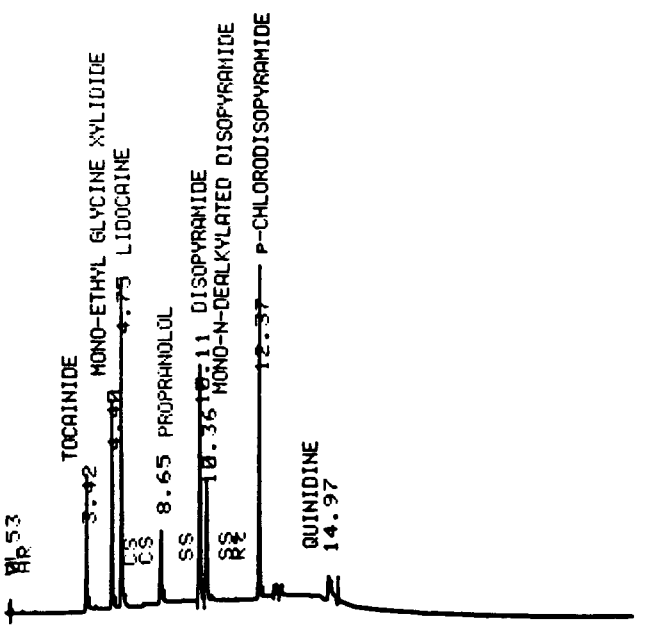


Fig. 2. Representative capillary GLC peaks of TFAA-treated antiarrhythmic agents.

(helium) flow-rate, 27 ml/min; hydrogen—air flow-rate ratio 3:50. Total analysis run time was 15 min.

Chromatographic responses for disopyramide and MND were linear in the range studied (0.05—5.00 μg/ml for disopyramide and 0.02—3.00 μg/ml for MND). The calibration curves were obtained by analyzing blank plasma samples

TABLE I

CALIBRATION CURVE OF DISOPYRAMIDE AND MND (HIGHER CONCENTRATION RANGE)

Statistics: linear regression lines for disopyramide; $y = (0.4689)x - 0.0463$; $r^2 = 0.999$; for MND; $y = (0.3061)x - 0.0157$; $r^2 = 0.996$.

Concentration of disopyramide (μg/ml)	Concentration of MND (μg/ml)	Area ratio disopyramide/PC-Dis range*	Area ratio MND/PC-Dis range*
0.5	0.31	0.206—0.219 (1.02)**	0.085—0.091 (4.98)**
1.0	0.61	0.408—0.421	0.175—0.182
1.99	1.23	0.879—0.891	0.357—0.368
2.99	1.84	1.235—1.398	0.485—0.549
3.98	2.45	1.748—1.880	0.668—0.751
4.98	3.07	2.12 —2.380 (2.53)**	0.902—0.983 (6.94)**

*Number of samples, $n = 2$—5 (one injection for each sample).
**$n = 5$, the numbers in the parentheses show the coefficient of variation (C.V., %).

TABLE II

CALIBRATION CURVE OF DISOPYRAMIDE AND MND (LOWER CONCENTRATION RANGE)

Statistics: linear regression lines for disopyramide; $y = (3.964)x + 0.001$; $r^2 = 0.980$; for MND; $y = (3.323)x - 0.025$; $r^2 = 0.986$.

Concentration of disopyramide (μg/ml)	Concentration of MND (μg/ml)	Area ratio* disopyramide/PC-Dis		Area ratio* MND/PC-Dis	
		Mean ± S.D.**	C.V.***	Mean ± S.D.	C.V.
0.054	0.023	0.229 ± 0.013	5.6	0.069 ± 0.007	10.2
0.109	0.046	0.454 ± 0.026	5.7	0.129 ± 0.010	8.1
0.217	0.092	0.877 ± 0.031	3.5	0.269 ± 0.026	9.5
0.326	0.139	1.160 ± 0.093	8.0	0.399 ± 0.033	8.2
0.435	0.185	1.811 ± 0.170	9.4	0.621 ± 0.054	8.7

*Number of samples, $n = 4$ (two injections for each sample).
**Mean ± one standard deviation.
***Coefficient of variation (%).

spiked with varying amounts of disopyramide and MND (Tables I and II). The best fit through the data points was obtained from linear regression analysis (Tables I and II). The coefficient of determination, r^2 was > 0.98 for all regression lines.

Reaction time

The optimum reaction time was evaluated by incubating samples containing equivalent amounts of disopyramide, MND, PC-Dis and TFAA for various times at 55°C. The peak areas of the dehydrated forms of disopyramide, PC-Dis and the TFAA derivative of the dehydrated form of MND were found to not change significantly over an incubation period of 2 h.

Excess TFAA was removed according to the method of Walle and Ehrsson [40], whereby the reaction mixture was evaporated on a water bath at 40°C under a gentle stream of nitrogen.

Extractability

Calibration curves for a serial dilution of the free base forms of disopyramide and MND in toluene were prepared. Following extraction of known quantities (Table III) of disopyramide and MND from human plasma, the recovery in the organic phase (toluene) was determined by using the free base calibration curves. The average percentages of disopyramide and MND extracted by toluene were 96% and 87%, respectively (Table III).

TABLE III

EXTRACTABILITY OF DISOPYRAMIDE AND MONO-N-DEALKYLATED METABOLITE

Disopyramide added (μg/ml)	Disopyramide measured (μg/ml) (mean ± S.D.)	Percent recovery* (mean ± S.D.)	MND added (μg/ml)	MND measured (μg/ml) (mean ± S.D.)	Percent recovery* (mean ± S.D.)
0.50	0.53 ± 0.01	106.1 ± 1.96	0.31	0.32 ± 0.01	104.2 ± 1.90
1.00	0.94 ± 0.02	94.08 ± 1.78	0.61	0.55 ± 0.01	90.3 ± 1.10
1.99	1.88 ± 0.02	94.55 ± 0.92	1.23	1.04 ± 0.02	84.4 ± 1.68
2.99	2.75 ± 0.23	92.08 ± 7.81	1.84	1.44 ± 0.11	78.2 ± 6.50
3.98	3.75 ± 0.19	94.25 ± 4.89	2.45	1.96 ± 0.11	79.9 ± 7.28
4.98	4.75 ± 0.23	95.34 ± 4.65	3.07	2.58 ± 0.17	83.5 ± 5.84

*Number of samples, $n = 3$.

Structural confirmation of compounds

Characterization of the structures of untreated and TFAA-treated compounds (disopyramide, MND and PC-Dis) was obtained using both EI and CI mass spectra. The total ion current mass chromatograms of disopyramide and PC-Dis under both ionization modes yielded single peaks, thus indicating the purity of these compounds. Fig. 3 shows the CI-MS results of TFAA-treated disopyramide and PC-Dis. In Fig. 4 can be seen the EI total ion current chromatogram of the underivatized MND. The MS data (both EI and CI) of untreated MND and its degradation products are shown in Fig. 5. Fig. 6 illustrates the MS data (EI and CI) of TFAA-treated MND (nitrile and amide forms).

DISCUSSION

The application of capillary column GLC in the analysis of biological

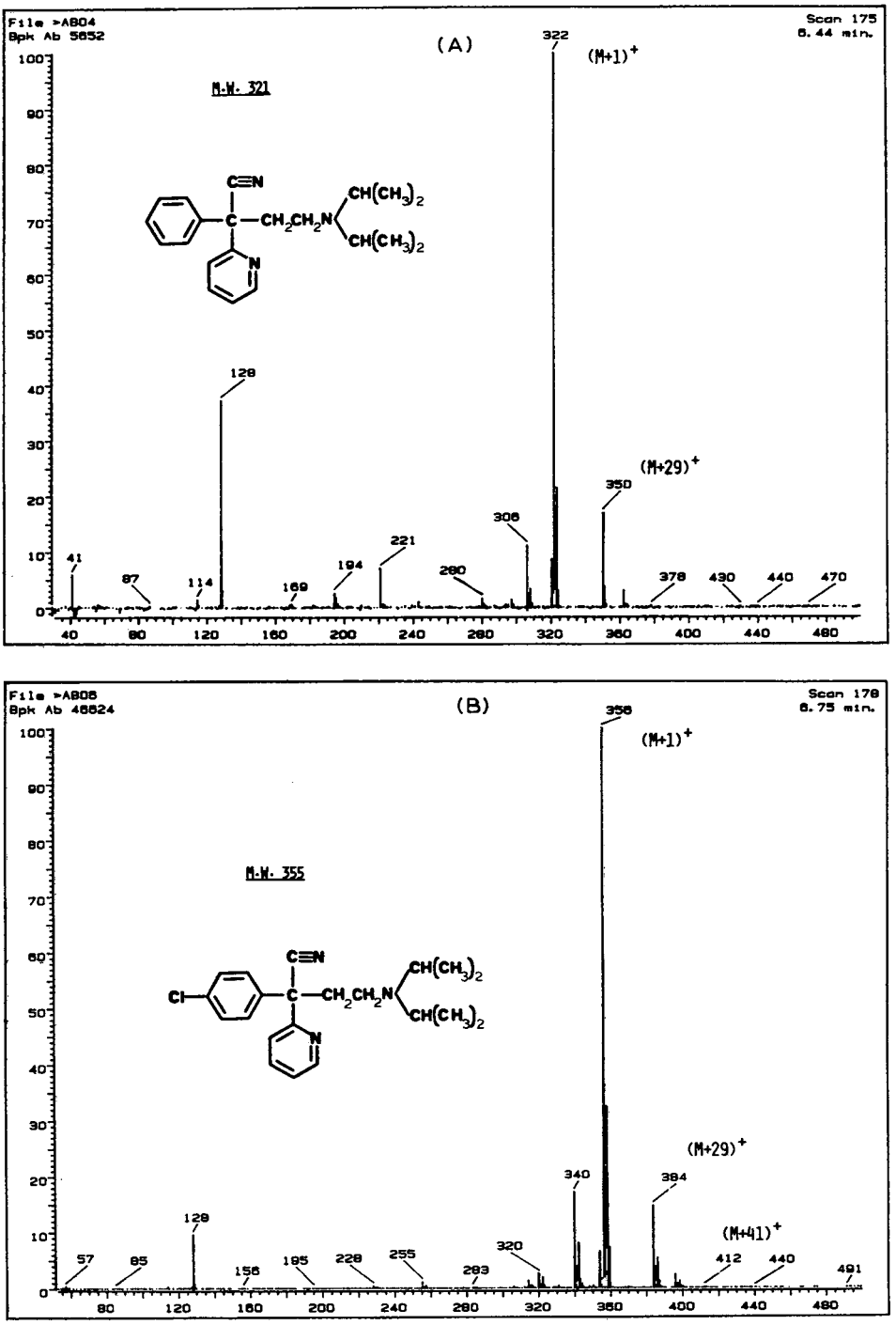


Fig. 3. Capillary GC mass spectral results. (A) CI (positive) mass spectrum of TFAA-treated disopyramide; (B) CI (positive) mass spectrum of TFAA-treated PC-Dis.

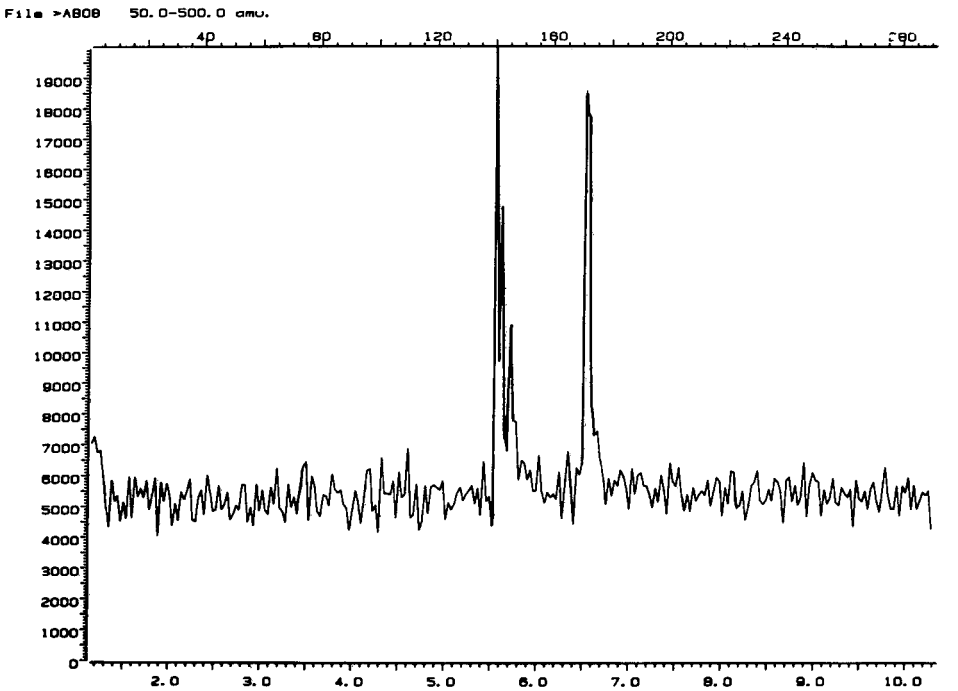
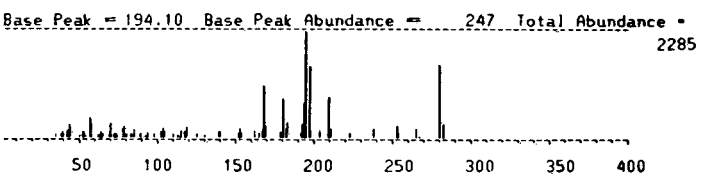
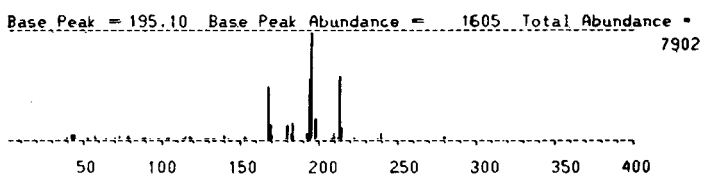


Fig. 4. Total ion current chromatogram of untreated MND.

(A)



(B)



PROMINENT FRAGMENT IONS :

MASS (m/e) :	195	212	194	167	196	182	213	239	43	44	280
ABUNDANCE(%) :	100	57	54	49	18	16	10	6	5	5	3

Fig. 5.

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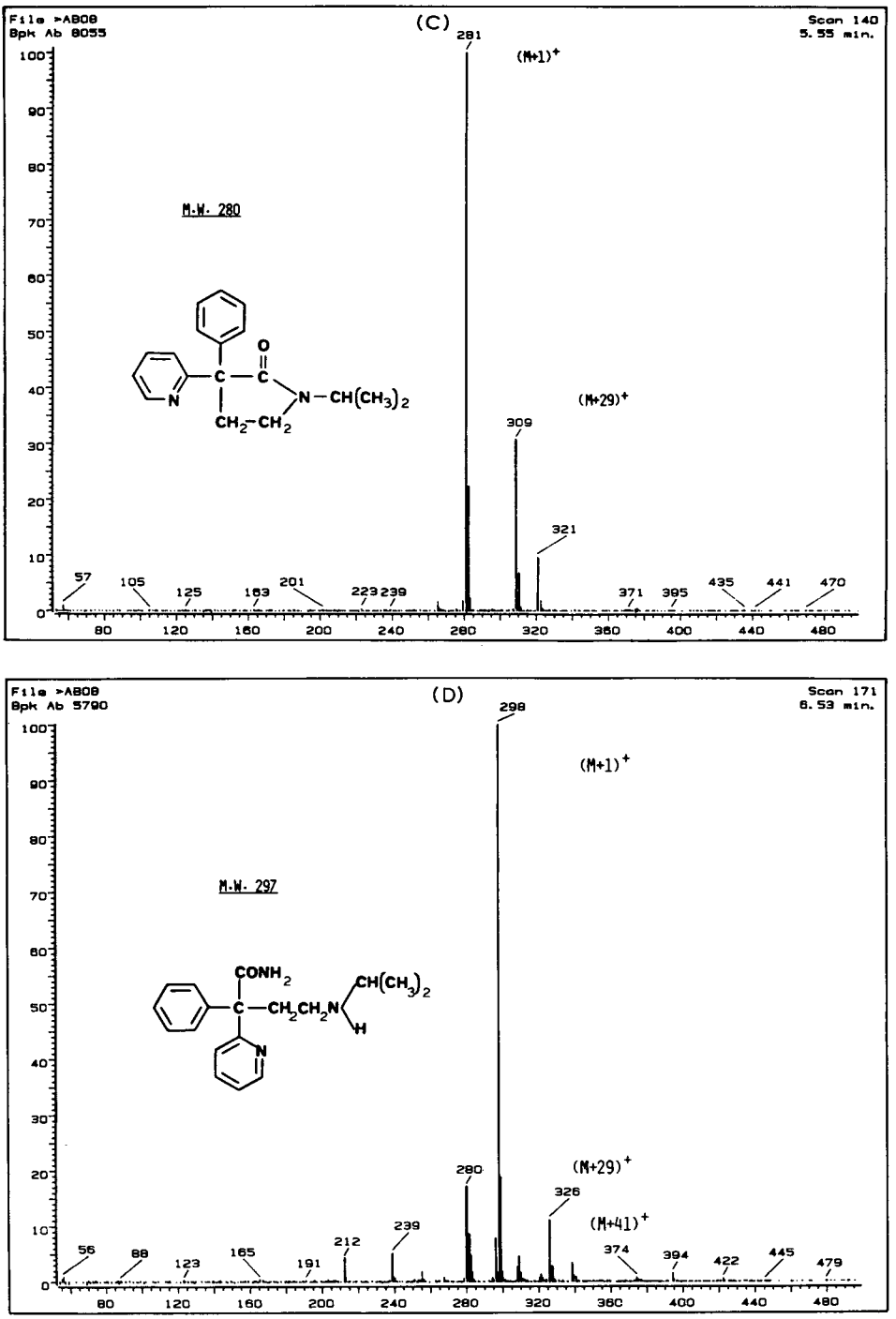
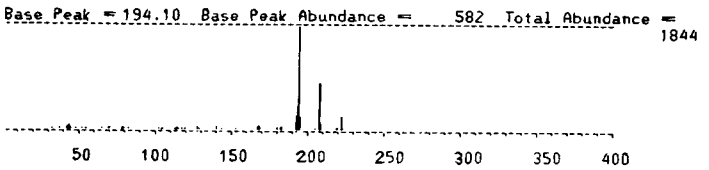


Fig. 5. Capillary GC mass spectral results. (A) EI mass spectrum and prominent fragment ions of degradation product of untreated MND; (B) EI mass spectrum and prominent fragment ions of intact MND; (C) CI mass spectrum of degradation product of untreated MND; (D) CI mass spectrum of intact MND.

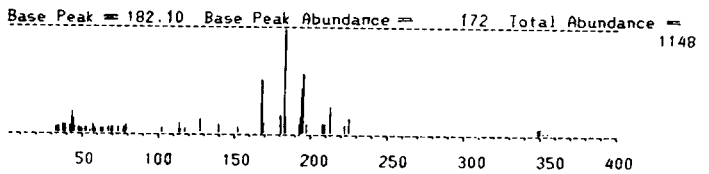
(A)



PROMINENT FRAGMENT IONS :

MASS (m/e) :	194	207	193	221	192	167	43	41	375
ABUNDANCE(%) :	100	45	21	13	13	5	5	4	1.2

(B)



PROMINENT FRAGMENT IONS :

MASS (m/e) :	182	195	167	194	212	43	225	193	44	69
ABUNDANCE(%) :	100	55	51	42	26	20	16	16	14	6

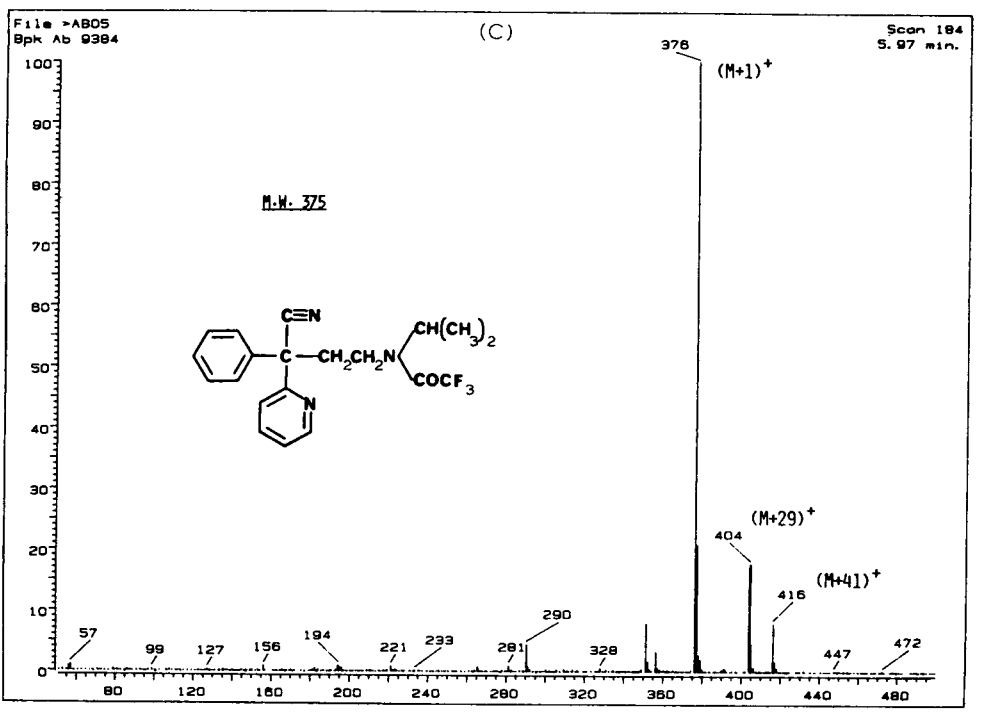


Fig. 6.

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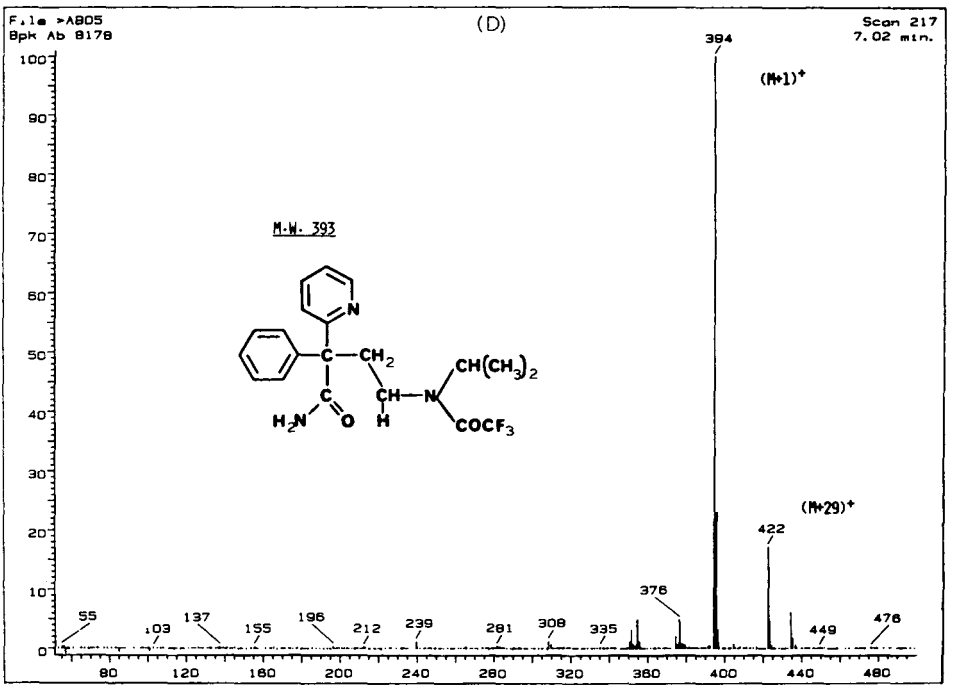

Fig. 6. Capillary GC—MS results. (A) EI mass spectrum and prominent fragment ions of TFAA-treated MND (derivatized nitrile form); (B) EI mass spectrum and prominent fragment ions of TFAA-derivatized MND (amide form); (C) CI mass spectrum of TFAA-treated MND (derivatized nitrile form); (D) CI mass spectrum of TFAA-derivatized MND (amide form).

samples has proliferated in recent literature. This technique has been applied in metabolic profiling of normal and diseased conditions [41—43], the measurement of drug concentration in human urine and plasma [43—45] and in pharmacokinetic drug studies in man [46]. With the advent of inert fused-silica capillary columns in 1979, more reliable and reproducible analysis of individualized drugs (acidic, basic, neutral, polar) became possible [47].

Before a pharmacokinetic study of disopyramide in coronary care patients could be undertaken, an analytical procedure with high selectivity and sensitivity was required to permit measurement of drug and metabolite in buffer (dialysate), plasma and serum.

A packed column GLC—flame ionization detection assay method developed for use in our laboratory using the technique of Hutsell and Stachelski [15] demonstrated potential for interference from other drugs and from endogenous substances in the plasma. Furthermore, the chromatographic peaks noted for disopyramide, MND and *p*-chlorodisopyramide exhibited excessive peak tailing and generally unsatisfactory symmetry using packed-column technology. Since the flame ionization detection measurement technique was generally insufficiently sensitive to permit measurement of free drug concentrations for disopyramide and MND during in vivo protein binding studies (0.10 μg/ml concentrations in 0.40 ml plasma volume), an attempt was made to develop a specific NPD assay method using splitless capillary GC.

A 25-m polysiloxane-deactivated, open-tubular fused-silica column with a crosslinked 5% phenylmethyl silicone phase was used for the development of a selective and sensitive assay for disopyramide and MND.

A complete baseline resolution between disopyramide and MND was obtained as illustrated in Fig. 1B and no interference from endogenous plasma components was observed (Fig. 1A) following a simple and single extraction step. The chromatographic peaks of disopyramide and MND under identical conditions were well separated (resolved) from several antiarrhythmic drugs, viz., tocainide, mono-ethyl glycine xylidide, lidocaine, propranolol and quinidine. The analysis time of the present assay is relatively short and replicate injections of plasma extracts every 15 min are possible without interference from plasma components. A series of samples containing 5 μg/ml of disopyramide, 3 μg/ml of MND and 4 μg/ml of PC-Dis were incubated at 55°C for periods ranging from 0—120 min to determine the optimum time required for reaction of the employed substances with TFAA. No apparent differences were observed in the areas for the three substances over the entire incubation time range; it would appear that the reaction was rapid. A 30-min reaction time was subsequently chosen to ensure complete dehydration of disopyramide, MND and PC-Dis as well as the derivatization reaction for MND. Excess TFAA was used (150 μl) to ensure completeness of the reactions.

Excess TFAA reagent was removed by evaporation rather than by the hydrolysis technique [40]. The hydrolysis of excess derivatizing reagent (heptafluorobutyric anhydride) and subsequent neutralization with an excess of ammonia has been reported to be a better technique [48]. However, the use of a small volume of toluene (100 μl) for reconstitution of dried sample extract did not permit an efficient separation of the organic layer from the aqueous phase during the hydrolysis procedure. Evaporation of the incubation mixture was carried out under a gentle steam of nitrogen, using a water bath at 40°C to prevent evaporation of the volatile TFAA derivatives. The TFAA-treated samples were found to be stable for at least ten days when stored at −4°C with repeat injections showing no significant decline in peak areas over the stored time period.

Toluene was found to be an efficient extraction solvent (Table III) with a recovery of 96.1% and 87.1% for disopyramide and MND respectively, over the entire concentration range of disopyramide (0.50—5 μg/ml) and MND (0.30 3 μg/ml). A single extraction step was sufficient for the quantitation of the compounds of interest without any interference from plasma constituents.

The present analytical technique has been found to show good linearity (Tables I and II) over the entire concentration range of disopyramide (0.05—5 μg/ml) and MND (0.02—3 μg/ml). These samples have been characterized by replicate calibration curves having a coefficient of determination (r^2) of at least 0.98. The reproducibility of the assay method is demonstrated in Tables I and II. The method is highly reliable and reproducible with a coefficient of variation within 10% for both compounds.

Identification of compounds and their derivatives by MS

Hutsell and Stachelski [15] in 1975 and subsequently other workers [21, 22] have reported that the mono-N-dealkylated metabolite of disopyramide

results in three unresolved chromatographic peaks when injected into a gas–liquid chromatograph equipped with a 3% OV-17 packed glass column. Unfortunately, no report has appeared in the literature regarding the identity of these peaks. A similar pattern of at least three peaks was observed when MND was injected into a gas chromatograph—mass spectrometer equipped with a 12-m fused-silica column coated with methyl silicone.

Previous studies have shown that acetylation of the secondary amino group of MND with acetic anhydride results in a single chromatographic peak [15, 21, 22]. However, recent findings in our laboratory have shown two limitations to this approach: (1) acetylation of the secondary amino group with acetic anhydride is not complete under usual conditions and (2) acetylated MND is associated with extensive peak tailing on columns conventionally used in disopyramide analysis, viz., 3% OV-17 and 5% phenylmethyl silicone.

Trifluoroacetic anhydride, a commonly used acylating agent, is more volatile than acetic anhydride. In addition to its acylating properties, TFAA has been reported to dehydrate aliphatic amide groups to form nitrile groups [49]. Gal et al. [23] in 1980 used TFAA as a dehydrating agent to accomplish such a conversion with disopyramide to improve its chromatographic properties. The EI-MS fragmentation of both disopyramide and PC-Dis in the present study followed a pattern similar to that reported by previous workers [23, 37]. The dehydrated derivatives (nitriles) obtained upon TFAA treatment of disopyramide and PC-Dis exhibited shorter retention times than the corresponding untreated amides. The prominent fragment ions for the nitrile derivative of disopyramide were at *m/e* 194, 221, 128, 114, 193, 43, 44, 72, 70, 195 (in order of decreasing intensity). The fragment ions at *m/e* 128,

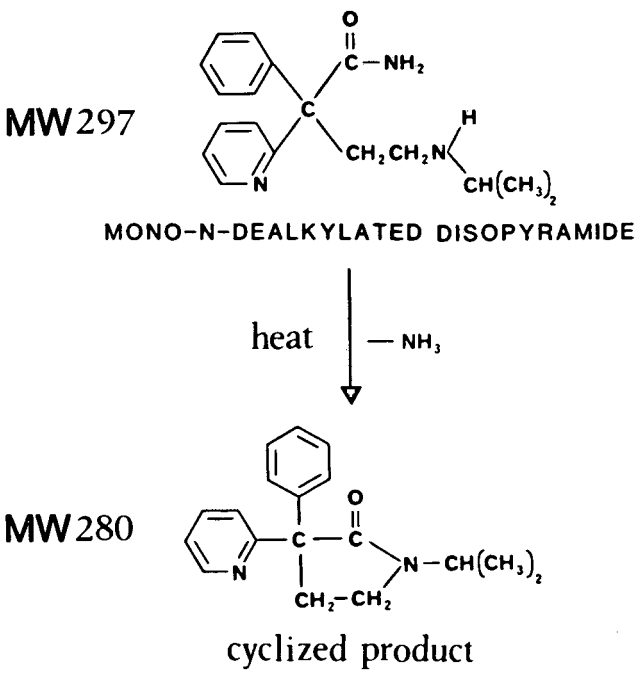

Fig. 7. Proposed degradation reaction of untreated MND at elevated temperature.

114, 72 and 42 were also present in the EI-MS profile of the nitrile derivative of PC-Dis. In addition, the latter contained ion pairs *m/e* 255:257, 228:230 and 229:231 in a 3:1 ratio, thus indirectly supporting the postulated structures reported for fragment ions of the nitrile derivative of disopyramide by Gal et al. [23].

Further evidence for dehydration of disopyramide and PC-Dis was obtained from the presence of pseudomolecular ions $(M+1)^+$ in the CI-MS mode (Fig. 3A and B). In addition to the molecular ions, characteristic ions of $(M+29)^+$ and $(M+41)^+$ were also found when methane was used as an ionizing gas.

Following the injection of untreated MND onto the GC—MS system, the total ion current chromatogram (Fig. 4) showed three unresolved MND degradation products from 5.5 to 5.8 min in addition to the peak for authentic MND at 6.5 min. It is proposed that untreated MND is unstable when exposed to high temperatures encountered in the injection port of the gas chromatograph. A comparison of the EI and CI spectra (Fig. 5A and C) of the three

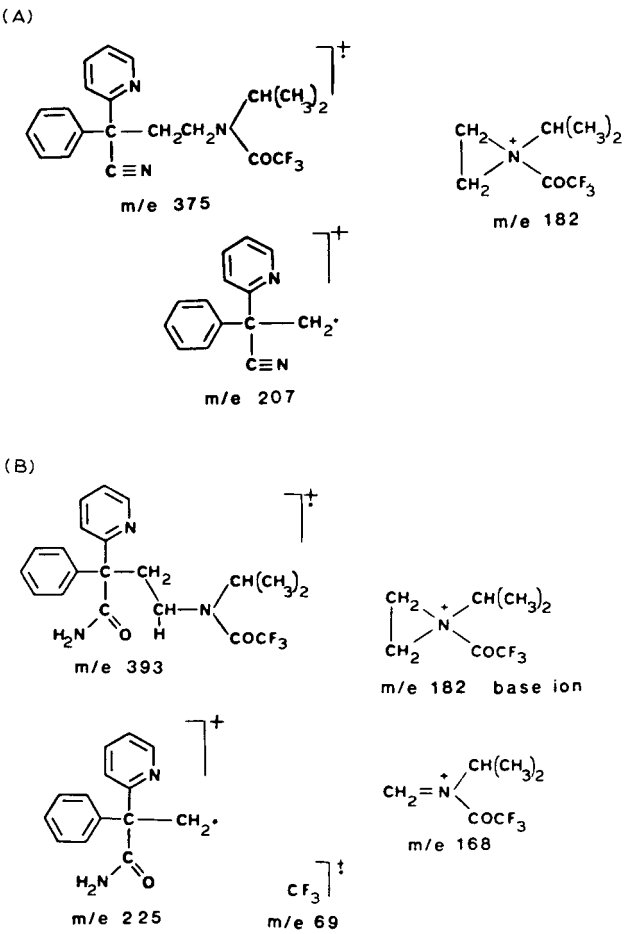

Fig. 8. Proposed prominent fragments based on the EI-MS of TFAA-treated MND. (A) dehydrated (nitrile) and trifluoroacetylated MND; (B) trifluoroacetylated MND (amide form).

peaks from 5.5 to 5.8 min, all show identical spectra. It appears that at least one of the degradation products may be a cyclized compound (Fig. 7) which could arise from MND through a loss of ammonia. This proposed structure is based largely on the presence of a strong molecular ion at *m/e* 280 in the EI spectrum and a corresponding pseudomolecular ion of *m/e* 281 in the CI spectrum. Confirmation of the cyclic structure requires further investigation. The fragment ions of MND in the EI mode (Fig. 5B) were similar to those reported by Haskins et al. [37]. The CI-MS profile (Fig. 5D) showing a pseudomolecular ion at *m/e* 298 confirms the identity of the MND chromatographic peak in Fig. 4.

Preliminary studies in our laboratory showed the presence of two peaks for TFAA (100 μl) treated MND. The EI and CI-MS data (Fig. 6A and C) indicated that the peak having the shortest retention time was the dehydrated form of the MND TFAA derivative whereas the later peak was that of the TFAA derivative of MND with the intact amide group. The EI- and CI-MS data of the amide are shown in Fig. 6B and D. Excess TFAA (150 μl), as used in the present assay, completely dehydrated the amide functional group of MND along with the complete acylation of the secondary amine group of MND. Structures for the characteristic fragment ions in the EI mass spectra of the two peaks are shown in Fig. 8A and B. Other ions present in the derivatized form of MND such as *m/e* 195, 194, 212 and 167 are also common to underivatized disopyramide [23, 37] or underivatized MND [37].

ACKNOWLEDGEMENTS

This work and Mr. Ram Kapil's Fellowship were supported by the British Columbia Heart Foundation. Dr. C.R. Kerr is a Scholar of the Medical Research Council of Canada.. Authentic drug, metabolite, and internal standard samples were graciously provided by G.D. Searle of Canada, Oakville, Ontario. The authors wish to thank Mr. R. Burton for his highly capable technical assistance with the mass spectrometer and Ms. Lisa Wong for her capable assistance in the preparation of this manuscript.

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Journal of Chromatography, 307 (1984) 323—333
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2038

CONTINUOUS EXTRACTION OF URINARY ANTHRACYCLINE ANTITUMOR ANTIBIOTICS WITH THE HORIZONTAL FLOW-THROUGH COIL PLANET CENTRIFUGE*

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(First received June 28th, 1983; revised manuscript received December 15th, 1983)

SUMMARY

Extraction of doxorubicin (adriamycin) and daunorubicin and their metabolites from human urine was attempted utilizing the horizontal flow-through coil planet centrifuge. Partition coefficients of the drugs for various combinations of non-aqueous phases and aqueous salt solutions were determined. Optimal coefficients for adriamycin and daunorubicin were achieved with *n*-butanol—0.3 *M* disodium hydrogen phosphate. Extraction efficiencies of the drugs from human urine comparable to those obtained by standard resin column techniques could be realized by employing the *n*-butanol—urine (containing 0.3 *M* disodium hydrogen phosphate) system in the coil planet centrifuge, at flow-rates of 500—600 ml/h, and at 650 rpm revolutional speed. Small quantities of drugs and metabolites could be continuously concentrated into small volumes of the *n*-butanol phase from large volumes of salted urine. The versatility of the technique was demonstrated by its application to extraction of aclacinomycin A, a novel anthracycline antitumor agent, and its metabolites from human urine.

*Presented, in part, at the 1981 Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, NJ, U.S.A.

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0378-4347/84/$03.00

INTRODUCTION

Basic and clinical pharmacological studies on the metabolism, pharmacokinetics, and mechanisms of action of anthracycline antitumor antibiotics require extraction, concentration, and purification of parent drugs and their metabolites from aqueous media [1—6]. The drugs and metabolites of interest are often present in low concentrations in biological fluids, such as urine. Several techniques for extracting and concentrating anthracyclines from urine have been described [3—7]. Liquid—liquid partition methods offer advantages in ready availability and low cost of reagents, and in usually yielding an organic phase containing the extracted materials. However, conventional liquid partition techniques, such as batch methods [8] or the Craig counter current distribution method, require fairly constant operator attention and large volumes of solvent, which necessitate significant additional processing and concentrating of the extract before the materials of interest are obtained in a form suitable for further analyses. A method which minimizes these disadvantages is desirable.

A new countercurrent chromatographic system, utilizing a compact horizontal planet centrifuge, has been introduced [9]. Flow-through design afforded continuous extraction, permitting processing of large volumes of the mobile phase, and organic extracts of small volume were obtained. The capability of the apparatus was demonstrated by the extraction of dinitrophenylated amino acids into ethyl acetate from an aqueous medium containing sodium dihydrogen phosphate [10]. The method allowed recovery of the amino acids, without contaminants, in a short period of time and at high efficiency.

We applied the horizontal flow-through coil planet centrifuge to countercurrent extractions of anthracyclines and their metabolites from human urine.

MATERIALS AND METHODS

Reagents

Doxorubicin (adriamycin, ADR) and daunorubicin (DNR) (Fig. 1) were obtained through the Division of Cancer Treatment, National Cancer Institute, and used without further purification. Anthracycline aglycones were prepared by acid hydrolysis of ADR and DNR [1,6]. Other reagents were analytical grade, and used as received. Urine samples were collected in dark bottles, with 10 ml toluene per 3 l urine as preservative, and stored at 4°C.

Partition coefficients

Partition coefficients, defined as the ratio of the concentration of drug in the non-aqueous phase to that in the aqueous phase, were determined for ADR and DNR in biphasic solvent systems. Chloroform—isopropanol (3:1) mixture, ethyl acetate, and *n*-butanol were individually tested against 1.0 *M* solutions of various salts. Equal volumes of organic and aqueous phases were placed in a test tube, 100 nmol of drug were added, and the tube was vigorously mixed for 30 sec. Quantities of drug in each phase were measured by fluorometry (SPF-125, American Instrument, Silver Spring, MD, U.S.A.; excitation, 470 nm; emission, 585 nm).

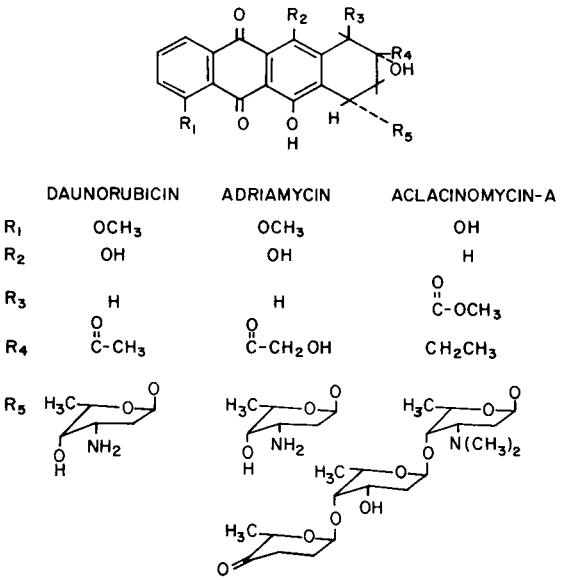

Fig. 1. Structures of adriamycin, daunorubicin, and aclacinomycin A.

Countercurrent chromatography

The design and principles of action of the horizontal flow-through coil planet centrifuge have been described in detail [9—12]. The chromatography column was prepared by winding PTFE tubing, 10 m × 2.6 mm I.D., around a holder 15 cm in diameter (column holder 1, Fig. 2); 18 helical turns were required. An appropriate counterweight was fixed to column holder II (Fig. 2). The capacity of the column was approximately 60 ml. The column was filled with *n*-butanol (stationary phase), and was subjected to planetary motion about two horizontal axes. The revolutional speed was 650 rpm. Disodium hydrogen phosphate (Na_2HPO_4) solution, 0.3 *M*, saturated with *n*-butanol, was pumped through the moving column to establish equilibrium conditions at 650 rpm. The stationary (butanol) phase volume was 32 ml. Prepared urine samples, 1—2 l in volume, were then pumped through the column at flow-rates of 500—700 ml/h, employing a multiple metering pump (Model 1508, Harvard Apparatus, Dover, MA, U.S.A.).

Urine was collected from patients or normal volunteers for 24 h during control periods, and patients for the first 24—48 h following anthracycline chemotherapy. Sufficient Na_2HPO_4 to yield a concentration of 0.3 *M* was added to several liters of urine. Salted urines were filtered (Whatman paper No. 802, Whatman, Clifton, NJ, U.S.A.) to remove precipitated material, and then saturated with *n*-butanol to prevent leaching of the columns's stationary phase. In some cases, filtration of the urine after butanol saturation was necessary. To test the efficiency of the countercurrent centrifuge extraction, known quantities of ADR and DNR were added to control urines. These spiked samples were processed in an identical fashion to post-therapy urines.

After each sample was chromatographed, the column was cleaned by elution with 100 ml of aqueous, *n*-butanol-saturated 0.3 *M* Na_2HPO_4. The *n*-butanol

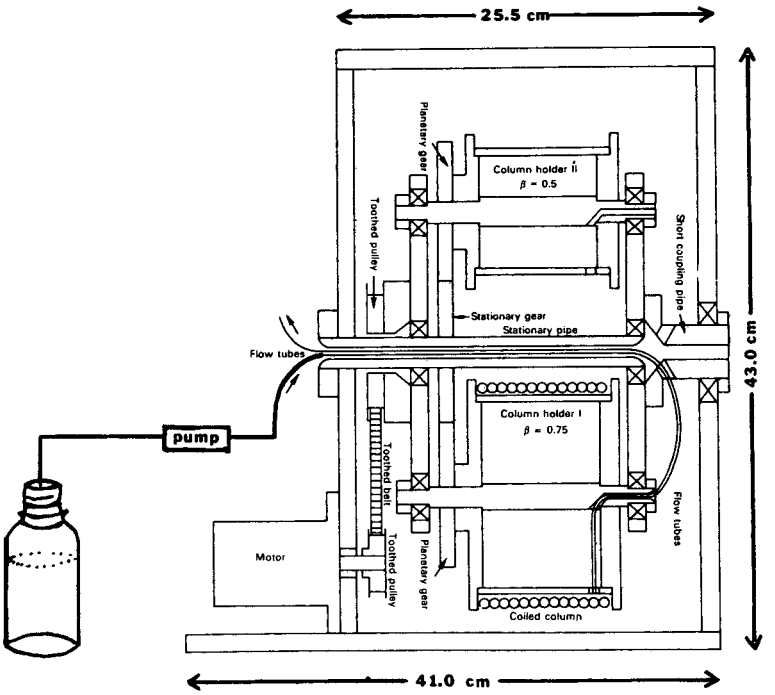

Fig. 2. Cross-section of the coil planet centrifuge. PTFE tubing is wound around column holder I (lower), and the counterweight is applied to column holder II (upper). A variable-speed pump delivers mobile phase from a large reservoir to the rotating coiled column while column effluent exits from the central stationary pipe as shown. The system eliminates the need for rotating seals. The dimensions of the apparatus, which is 45 cm deep, are indicated.

phase was drained from the column; several milliliters of *n*-butanol were passed through the column to recover any remaining sample. The *n*-butanol extracts were combined and evaporated to dryness by flash evaporation. The deep-red residue remaining after drying was redissolved in methanol.

Aliquots of pre-chromatography urine, column eluate (mobile phase), and the final extract from the column in methanol were analyzed for anthracycline content by fluorescence. A small volume of the aliquot was added to 2 ml of 1.8 *M* hydrochloric acid—ethanol (1:3), and concentration determined against known ADR standards in an Aminco-Bowman spectrophotofluorometer (American Instrument).

Solid—liquid chromatography

Urine samples identical to those subjected to countercurrent chromatography, but not prepared with Na_2HPO_4 or *n*-butanol, were studied by resin column chromatography. Glass columns, 250 × 11 mm, were packed with 150 mm of Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.). The chromatographic technique, a modification of the method of Fujimoto and co-workers [13,14], and preparation of the urine have been described [3,4]. Volumes (1 l) of patients' or spiked control urines containing ADR or DNR and their metabolites were chromatographed. Pre-chromatography urine,

column eluate, and final recovered samples were analyzed for anthracycline content as described above.

Thin-layer chromatography

Identification of parent anthracyclines and their metabolites in the sample fractions was accomplished with thin-layer chromatography (TLC). The methods have been previously detailed [3,4].

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) was also employed to identify parent drugs and metabolites in the column fractions. The method has been detailed [7]. The apparatus included a Waters instrument [Model 6000A solvent delivery system, Model 660 solvent programmer, Model 440 detector, and phenyl-μBondapak column (10-μm particle size), 300 × 3.9 mm, Waters Assoc., Milford, MA, U.S.A.] with fluorescence detection (Aminco Fluoromonitor, American Instrument. Gradient elution with tetrahydrofuran in 0.45 *M* ammonium formate buffer was undertaken at a flow-rate of 1.5 ml/min.

RESULTS

Various salts were studied for their effects on partitioning of adriamycin and daunorubicin between aqueous and organic phases (Tables I and II). Three organic solvents were selected for study based on extensive previous experiences with anthracycline extractions: *n*-butanol [1,2,5,6], chloroform—isopropanol (3:1) [7,11,15], and ethyl acetate [2,6]. In nearly all cases, partitioning of

TABLE I

PARTITION COEFFICIENTS FOR ADRIAMYCIN

Adriamycin (100 nmol) was added to each 4.0-ml phase system. The concentration of aqueous salt solution was 1.0 *M* in all cases. The systems were vigorously mixed and allowed to equilibrate. Concentration of drug in each phase was measured by fluorometry, and partition coefficients were calculated as the ratio of the concentration in the non-aqueous phase to that in the aqueous phase. Insoluble salt crystals were noted in mixing 1.0 *M* Na_2HPO_4 with all non-aqueous phases, and no coefficient could be determined for the chloroform—isopropanol—Na_2HPO_4 system.

Salt	*n*-Butanol—water (1:1)	Chloroform—isopropanol—water (3:1:2)	Ethyl acetate—water (1:1)
None	2.1	2.3	0.1
$(NH_4)_2SO_4$	2.2	0.4	0.1
NaH_2PO_4	4.3	0.3	0.4
NH_4Cl	7.6	1.1	0.1
KCl	12.3	2.4	0.8
LiCl	10.0	1.8	0.7
CH_3COONH_4	10.1	4.1	0.4
NaCl	11.7	1.8	0.9
Na_2SO_4	10.8	7.7	0.1
Na_2HPO_4	>40.0	–	>1.2

TABLE II

PARTITION COEFFICIENTS FOR DAUNORUBICIN

Experimental conditions were as described in Table I. Daunorubicin (100 nmol) was added to each system. Insoluble crystals of Na_2HPO_4 were encountered with all non-aqueous phases, and no coefficient could be determined for the chloroform—isopropanol—Na_2HPO system.

Salt	n-Butanol—water (1:1)	Chloroform—isopropanol—water (3:1:2)	Ethyl acetate—water (1:1)
None	2.7	3.2	0.1
$(NH_4)_2SO_4$	5.9	1.6	0.1
NaH_2PO_4	12.4	0.6	0.2
NH_4Cl	16.8	2.3	0.3
KCl	27.3	4.7	0.4
LiCl	20.9	4.4	0.3
CH_3COONH_4	21.6	12.1	1.3
NaCl	24.4	4.6	0.3
Na_2SO_4	36.3	12.5	0.6
Na_2HPO_4	>85.5	–	>6.9

anthracyclines into *n*-butanol was greater than into either of the other two systems. Partition coefficients were higher for DNR compared to ADR in nearly every biphasic system tested, because ADR is more polar than DNR (Fig. 1). The highest coefficients were obtained for the *n*-butanol—Na_2HPO_4 system at the 1.0 *M* Na_2HPO_4 concentration. However, precipitation of salt crystals was noted. Coefficients for a range of Na_2HPO_4 concentrations were measured

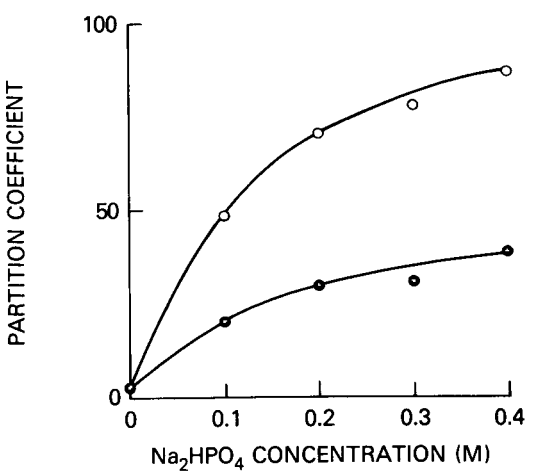

Fig. 3. Effect of Na_2HPO_4 on partition coefficients of adriamycin (•) and daunorubicin (○). Partition coefficients (ordinate) were measured in the *n*-butanol—aqueous Na_2HPO_4 system. Aqueous Na_2HPO_4 concentrations varied from 0—0.4 *M* (abscissa). A 100-nmol amount of drug was added to a test tube containing equal volumes of each phase, and the tube was vigorously mixed. The concentration of drug in each phase was measured by fluorometry, and the partition coefficients for adriamycin and daunorubicin were determined as ratios of concentrations in the non-aqueous and aqueous phases.

(Fig. 3), and a plateau was observed for concentrations greater than 0.4 *M*. No crystal formation occurred at Na_2HPO_4 concentrations less than 0.4 *M*. Further studies utilized the *n*-butanol—0.3 *M* Na_2HPO_4 system. At this Na_2HPO_4 concentration, partition coefficients for ADR and DNR were 31 and 78, respectively.

The capabilities of the technique were determined for extraction of daunorubicin and metabolites from a patient's urine (Fig. 4). A 24-h urine specimen (1.5 l) was collected from a patient who received DNR. Prior to countercurrent extraction, DNR and daunorubicinol (13-OH DNR) were the only identifiable drug species by reversed-phase HPLC; considerable interfering, non-anthracycline, polar materials were noted in the early column effluent (Fig. 4A). After preparation of the urine sample, as described in Materials and methods, the entire 1.5 l was chromatographed through the coil planet centrifuge. Only occasional operator attention was required to monitor pump and centrifuge conditions during the 3-h run. The retained *n*-butanol phase harvested from the column and two small *n*-butanol column washings were combined and evaporated to dryness. The residue was dissolved in methanol and subjected to

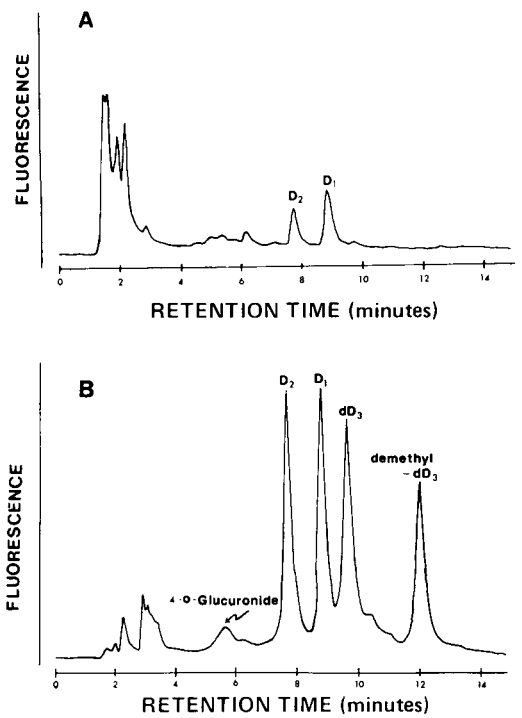

Fig. 4. Daunorubicin extraction from urine. (A) An aliquot of a 24 h urine sample from a patient treated with daunorubicin (DNR) was analyzed by reversed-phase HPLC. The early (2 min) column eluate contained considerable interfering materials. Only two anthracycline species, DNR (D_1) and 13-OH-DNR (D_2), were identifiable. (B) The same 24-h sample has been prepared and extracted by the coil planet centrifuge. The early interfering materials were largely excluded from the extract. Sharp, enriched peaks for D_1 and D_2 were evident, and three previously undetectable metabolites of DNR-1 conjugated species and two aglycones, dD_3 and demethyl-dD_3, were made apparent. HPLC conditions were given in Materials and methods; anthracycline detection was by fluorescence monitoring.

HPLC (Fig. 4B). In addition to DNR and 13-OH-DNR, which were greatly enriched in the extract, three metabolites not previously visible were demonstrable. The polar interfering materials had been largely eliminated from the extract. Similar enrichment could be shown for ADR and its metabolites (Fig. 5), although relatively greater amounts of interfering materials were recovered with the more polar ADR.

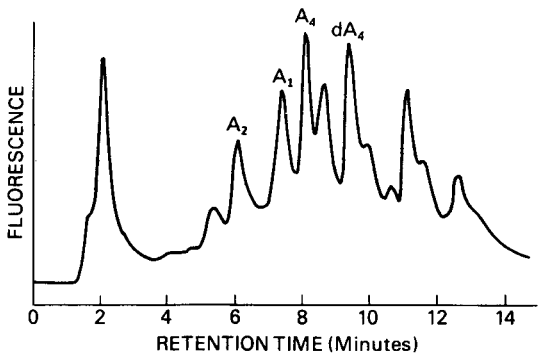

Fig. 5. Adriamycin extraction from urine. Urine from a patient receiving adriamycin was prepared, extracted, and chromatographed as in Fig. 4B. Adriamycin (A_1) and three metabolites (A_2, A_4 and dA_4) were enriched by coil planet centrifuge extraction. Increased amounts of polar interfering materials relative to the amounts of anthracycline were noted with adriamycin.

Aclacinomycin A (ACM), a novel anthracycline now undergoing clinical trials, [16,17], differs structurally from ADR and DNR both in the quinone system and the attached sugar moieties (Fig. 1). Urine was obtained from a patient who received ACM, and subjected to countercurrent extraction exactly as described for ADR and DNR. The *n*-butanol extract contained ACM and eight metabolites. The capacity factor (k') values calculated from reversed-phase HPLC values for these species are indicated in Table III [18].

Our standard technique for recovering anthracyclines from urine involves adsorption chromatography of pH-adjusted, filtered urine samples onto Amberlite XAD-2 resin [3,4]. We compared the XAD-2 method to the coil planet centrifuge for efficiency in extracting ADR and DNR from spiked control urines and authentic patients' specimens (Table IV). The XAD-2 technique yielded more efficient extraction of ADR than did the coil planet centrifuge, from spiked samples, but the methods were equivalent in their recoveries of the less polar DNR. The reverse was seen in the case of treated patients' urines, with about 50% improvement in recovery of ADR and metabolites using the coil planet centrifuge. This difference may have been due to altered partitioning of drug metabolites between the two phases, compared to parent drug. In particular, aglycone metabolites would be expected to distribute well into the *n*-butanol phase. Both TLC and reversed-phase HPLC confirmed that the coil planet centrifuge allowed recovery of relatively greater quantities of aglycones from urine than the XAD-2 column for ADR (60% aglycones with coil planet centrifuge versus 12% with XAD-2 column) and DNR (48% aglycones versus 6%).

TABLE III

CAPACITY FACTOR (k') VALUES FOR ACLACINOMYCIN A AND METABOLITES

Extracts of urine from ACM-treated patients were prepared by countercurrent chromatography with the coil planet centrifuge. The extracts were analyzed by reversed-phase HPLC as described in Materials and methods and k' values were calculated from the observed retention times. Aklavinone is the aglycone of ACM, and C_1, E_1, F_1, M_1, N_1, S_1, and aklavin are metabolites [18].

Compound	k'
F_1	8.9
E_1	9.8
C_1	8.2
Aklavinone	7.1
ACM	6.7
M_1	5.5
N_1	5.3
S_1	4.4
Aklavin	4.9

TABLE IV

COMPARISON OF ANTHRACYCLINE RECOVERIES FROM URINE

A. Adriamycin (ADR) or daunorubicin (DNR) was added to control urines which were then prepared and chromatographed as described in Materials and methods. The percentages of the added drug fluorescence which were recovered from the coil planet centrifuge or Amberlite XAD-2 column are displayed.
B. Urines from patients treated with ADR or DNR were chromatographed as in A. Recoveries, expressed as percentages of the initial anthracycline content, are indicated.

		Percentage recovery	
		Coil planet centrifuge	XAD-2 column
A.	ADR	27	65
	DNR	70	72
B	ADR	63	44
	DNR	33	35

DISCUSSION

The horizontal flow-through coil planet centrifuge has been proven to be valuable for analytical chromatographic applications [9—11]. We have demonstrated in this paper that efficient recovery of anthracyclines from large volumes of urine by countercurrent extraction with the apparatus is feasible.

n-Butanol was chosen for the non-aqueous (stationary) phase on the basis of the favorable partition coefficients observed for ADR and DNR with nearly every aqueous salt phase. Technical considerations fortunately render *n*-butanol a desirable solvent because it contributes to low interfacial tension and lower density in biphasic systems [12]. These properties favor adequate mixing of

the phases and good retention in the column. Ethyl acetate is retained better in the rotating coil, but has higher interfacial tension and inferior partition coefficients compared to *n*-butanol.

Reversed-phase HPLC proved that meaningful purification of ADR or DNR from urine of treated patients can be achieved by the coil planet centrifuge. This has relevance both for clinical pharmacology and preparative applications. The identification and quantification of urinary anthracyclines is a necessary adjunct to pharmacokinetic studies on these drugs [2—6, 15]. Urine also remains the cheapest and most productive source of human anthracycline metabolites, which, when extracted and purified, are suitable as cochromatographic standards and as substrates for in vitro studies [1,3,4,7]. The versatility of the coil planet centrifuge is substantiated by its ready application to extraction of ACM and its several metabolites from human urine. Similar results, confirmed by HPLC and TLC, have been obtained with batch extractions of urine for ACM [19].

The coil planet centrifuge appears to extract polar materials less efficiently than Amberlite XAD-2, which is our current standard method (Table IV). Both HPLC and TLC demonstrate the presence of polar anthracyclines (e.g., ADR and the glucuronides of ADR and DNR) in XAD-2 or coil planet centrifuge extracts of urine. However, when the centrifuge eluate (mobile phase) is examined, relatively more polar species than aglycones of ADR or DNR are noted. We speculate that the stationary *n*-butanol phase in the coil planet centrifuge has a finite capacity to retain polar anthracyclines, which is less than its capacity for aglycones. Conversely, irreversible binding of aglycones to the Amberlite XAD-2 resin probably occurs, contributing to the reduced recovery of ADR or DNR aglycones observed in extracts from that technique. Both methods suffer from the need for filtration of urine prior to its application to the XAD-2 column or rotating centrifuge, resulting in increased processing time and cost. Anthracyclines bind avidly to the filter paper, resulting in an obligate lowering of recovery. This disadvantage can be partially avoided by employing glass fiber filters, but at additional expense.

The compactness, facility of operation, and observed extraction efficiencies of the coil planet centrifuge recommend that studies of its ability to recover other anthracyclines and drugs of other classes from urine be undertaken. Suitable alterations of the components of the biphasic system and inclusion of newer technologies, such as ion-pair solvent extractions [20], may permit better extractions of polar compounds. Efforts to increase the capacity of the stationary phase, such as manipulations of column bore or length, or speed of column revolution, are worthy of consideration.

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Journal of Chromatography, 307 (1984) 335—342
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2051

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS OF 1-PHENYL-2-AMINOPROPANES (AMPHETAMINES) WITH FOUR CHIRAL REAGENTS

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(First received July 25th, 1983; revised manuscript received December 30th, 1983)

SUMMARY

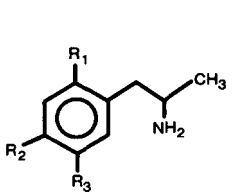
High-performance liquid chromatography (HPLC) was employed for resolution of enantiomers of chiral ring-substituted 1-phenyl-2-aminopropanes (amphetamines) and 1-phenylethylamine following derivatization with four chiral reagents: (*R*)-(+)-1-phenylethyl isocyanate (PEIC), (—)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA • Cl), 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), and 2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). Reactions were accomplished under mild conditions (25—70°C) and were complete for all substrates within 60 min. Derivatization with the sugar isothiocyanates (GITC and AITC) and the acyl chloride (MTPA • Cl) was carried out in methylene chloride or acetonitrile in the presence of a base catalyst while derivatization with the isocyanate (PEIC) was performed in methylene chloride. The diastereomeric derivatives were separated by reversed-phase HPLC (C_{18}) with a methanol—water mobile phase. In general, HPLC resolution of the diastereomeric reaction products of GITC or AITC, and MTPA • Cl with the amine substrates was more complete (usually greater than 98% baseline separation) than HPLC resolution of the diastereomeric reaction products of PEIC.

0378-4347/84/$03.00

INTRODUCTION

Enantiomers of many drugs possessing chiral centers have different pharmacological activities [1]. Included among these are a variety of amphetamines [2]. For example, (*S*)-(+)-1-phenyl-2-aminopropane (amphetamine) has greater activity than the (*R*)-(—)-enantiomer as a locomotor stimulant [3], hyperthermic agent [4], and ability to elicit stereotypic behavior [3]. The (*R*)-(—)-isomer of the psychotomimetic agent 1-(2,5-dimethoxy-4-methyl)phenyl-2-aminopropane (DOM) has approximately twice the hallucinogenic activity in humans when compared to the racemic mixture, and the (*S*)-(+)-enantiomer is devoid of activity [5]. Long-term neurotoxicity of the serotonergic neurotoxin 1-(4-chloro)-phenyl-2-aminopropane (*p*-chloroamphetamine) is greater for the (*S*)-(+)-enantiomer than for the (*R*)-(—)-enantiomer [6]. Enantiomers of drugs may also differ in their metabolism [7], and this may play a role in their differing pharmacological activities. For example, stereoselective in vitro metabolism of amphetamine [8], DOM [9], and *p*-chloroamphetamine [10] has been described in the literature.

Traditional methods for quantification of enantiomers, e.g. chemical resolution or rotation of polarized light, are not adequate for the determination of trace amounts of enantiomers in biological fluids. Increased interest in stereochemical aspects of pharmacological activity and drug disposition has led to the development of new sensitive and specific methods for the detection of enantiomers in biological fluids, including detection and quantification of one enantiomer in the presence of the other. Most common has been the use of chiral reagents which provide diastereomeric derivatives which can be resolved by chromatographic procedures. Several chiral reagents have been employed for gas chromatographic (GC) resolution of enantiomeric amines including N-pentafluorobenzoyl-S-prolyl-1-imidazolide [11], (—)-α-methyl-α-methoxy-pentafluorophenylacetic acid [12], N-trifluoroacetyl-(D)-prolylchloride [13], and (—)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA · Cl) [14]. Chiral reagents have also been employed for high-performance liquid chromatographic (HPLC) resolution of amine enantiomers. Chiral reagents used in these procedures have included 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC), 2,3,4,-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC) [15, 16], succinimidyl-*l*-α-methoxy-α-methyl-naphthaleneacetate [17], and O-methylmandelyl chloride [18]. Our interest in stereochemical aspects of



R_1	R_2	R_3
-H	-H	-H
-H	-Cl	-H
$-OCH_3$	$-CH_3$	$-OCH_3$
$-OCH_3$	$-OCH_3$	$-CH_3$
$-OCH_3$	$-SCH_3$	$-OCH_3$

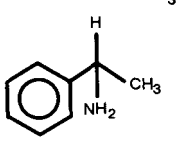


Fig. 1. Structures of the racemic amine substrates.

metabolism and disposition of amphetamines led us to investigate the use of four chiral reagents, (*R*)-(+)-1-phenylethyl isocyanate (PEIC), MTPA · Cl, GITC and AITC for the reversed-phase HPLC resolution of enantiomers of 1-phenylethylamine and the amphetamines shown in Fig. 1.

MATERIALS AND METHODS

Reagents

(*RS*)-(+)- and (*S*)-(+)-amphetamine sulfate were obtained from Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.). (*RS*)-(±)-*p*-chloroamphetamine hydrochloride was obtained from Regis Chemical (Morton Grove, IL, U.S.A.). (*RS*)-(±)- and (*S*)-(−)-1-phenylethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.). (*RS*)-(±)- and (*R*)-(−)-1-(2,5-dimethoxy-4-methyl)-phenyl-2-aminopropane hydrochloride, (*RS*)-(±)-1-(2,5-dimethoxy-4-thiomethyl)phenyl-2-aminopropane hydrochloride and (*RS*)-(±)-1-(2,4-dimethoxy-5-methyl)phenyl-2-aminopropane hydrochloride were generous gifts of Dr. Neal Castagnoli, University of California (San Francisco, CA, U.S.A.). (*R*)-(+)-1-Phenylethyl isocyanate was purchased from Fluka Chemical (Hauppauge, NY, U.S.A.). (−)-α-Methoxy-α-(trifluoromethyl)phenylacetic acid was purchased from Aldrich. 2,3,4,6-Tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate and 2,3,4,-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC) were obtained from Polysciences (Warington, PA, U.S.A.). N-acetyl-L-leucine was purchased from ICN Pharmaceuticals (Plainview, NY, U.S.A.) and N-acetyl-D-leucine was obtained from Sigma (St. Louis, MO, U.S.A.). All solvents were chromatographic or reagent grade.

(*R*)-(−)-*p*-Chloroamphetamine and (*S*)-(+)-*p*-chloroamphetamine were resolved as diastereomeric salts of N-acetyl-D-leucine and N-acetyl-L-leucine as previously described [10]. The acid chloride of (−)-α-methoxy-α-(trifluoromethyl)phenyl acetic acid (MTPA · Cl) was prepared by reaction with freshly distilled thionyl chloride as previously described [14]. The resulting oil was stored in the cold in methylene chloride (0.5 mmol/ml).

Derivatization procedures

PEIC. To 12-ml conical centrifuge tubes was added 0.1 mg of the amphetamine or 1-phenylethylamine dissolved in 100 μl methylene chloride. To each tube was added approximately 10% molar excess PEIC (100 μl, 6.8 μmol/ml solution in methylene chloride), and the reaction allowed to proceed at room temperature for 60 min. Solvents were then removed under a gentle stream of nitrogen. To each tube was added 1 ml of 0.1 *M* sodium hydroxide followed by vigorous shaking or vortexing for 15 min. Following addition of 1 ml of 20% sodium hydroxide and 3.0 ml methylene chloride, tubes were shaken on a mechanical shaker (15 min), and the aqueous layer separated by centrifugation (1000 *g*) for 15 min. The organic layer was removed and washed with 2 ml of 0.1 *M* hydrochloric acid. Aliquots (100 μl) of the methylene chloride extracts were diluted to 1 ml with methanol, and 20-μl aliquots of the diluted solution analyzed by HPLC. Diluted aliquots of the reaction mixtures were also directly injected for HPLC analysis.

MTPA · Cl. To 12-ml conical tubes was added 0.1 mg of the amphetamine or

1-phenylethylamine dissolved in methylene chloride (200 μl). To each tube was added 100 μl MTPA · Cl (100 μl, 0.5 mmol/ml solution in methylene chloride) and pyridine (50 μl). The reaction was allowed to proceed at 70° C for 30 min. Tubes were cooled in an ice water bath, 1 *M* hydrochloric acid added (1 ml), and tubes were shaken on a mechanical shaker for 5 min. Following centrifugation (1000 *g*, 15 min) the aqueous phase was discarded and 15% sodium carbonate (0.5 ml) added with additional methylene chloride (200 μl). The tubes were again shaken for 5 min and the organic layer removed following centrifugation (1000 *g*, 15 min). Aliquots (40 μl) of the methylene chloride extracts were diluted to 1 ml with methanol, and aliquots (20 μl) of the diluted solution analyzed by HPLC. The presence of pyridine precluded direct HPLC analysis of the MTPA · Cl reaction mixtures.

GITC and AITC. To 12-ml screw-capped centrifuge tubes was added 0.05 mg amphetamine or 1-phenylethylamine dissolved in 50 μl methylene chloride. To each tube was added approximately 10% molar excess AITC or GITC (50 μl, 7.6 μmol/ml solution in acetonitrile or methylene chloride containing 0.2% triethylamine). The reaction was allowed to proceed at room temperature for 60 min. To each tube was added 1 ml of 1 *M* hydrochloric acid, and tubes were shaken on a mechanical shaker for 5 min. Following centrifugation (1000 *g*, 15 min) the aqueous phase was discarded and 1 *M* sodium hydroxide (1 ml) added. The tubes were again shaken for 5 min and the organic layer removed following centrifugation (1000 *g*, 15 min). Aliquots (10 μl) of the extracts were diluted to 1 ml with methanol and 20—50-μl aliquots analyzed by HPLC. Diluted aliquots of the reaction mixtures were also directly injected for HPLC analysis.

To determine the amount of the underivatized amphetamines or 1-phenylethylamine remaining at the end of reaction with PEIC, MTPA · Cl, GITC, and AITC, aliquots of the reaction mixtures (1 μl) were analyzed directly by a nitrogen—phosphorus GC method [14] employing a 10% Carbowax—2% KOH column.

Derivatization time course studies

To samples of (*RS*)-(±)-amphetamine (0.5 mg, 0.5 ml methylene chloride) was added 0.5 ml of PEIC, GITC or AITC reagent solutions (see above). The reactions were carried out at room temperature. At the appropriate times, 40-μl aliquots were withdrawn and diluted with 960 μl of methanol. Aliquots (20 μl) of this diluted solution were then analyzed by HPLC.

For time course of derivatization studies with MTPA · Cl, individual samples of (*RS*)-(±)-amphetamine (100 μl of 1 mg/ml methylene chloride) and pyridine (40 μl) were prepared. MTPA · Cl reagent solution (100 μl, see above) was added to each sample and the reaction allowed to proceed at 70° C. After desired reaction times, samples were placed in an ice bath, 1 *M* hydrochloric acid (1 ml) and methylene chloride (200 μl) were added, the samples shaken for 15 sec and the mixtures frozen with a dry ice—acetone bath. After all reactions were completed, the samples were thawed and shaken for 5 min prior to centrifugation (10 min, 1000 *g*). The aqueous layer was discarded, 0.5 *M* sodium hydroxide (1 ml) added, and the mixture shaken for 5 min. Following centrifugation (10 min, 1000 *g*), 20-μl aliquots of the organic layer were analyzed by HPLC.

HPLC analysis

HPLC analysis of the diastereomeric derivatives was accomplished with a reversed-phase octadecyl column (250 × 4.5 mm, 5-μm particle size, IBM Instruments, Danbury, CT, U.S.A.) and methanol—water mobile phase with a flow-rate of 1—2 ml/min. An IBM CL/9533 ternary gradient liquid chromatograph, LC/934 or LC/9541 Data System, printer plotter, and Millipore Waters Assoc. sample processor (WISP) 710B (Milford, MA, U.S.A.) were used for HPLC analyses. An IBM LC/9523 variable UV detector was employed for UV absorbance signal detection. Mobile solvent conditions for analysis of the derivatives were as follows: PEIC, methanol—water (60:40) isocratic mobile phase; MTPA · Cl, methanol—water (60:40) isocratic mobile phase for 20 min followed by a linear gradient to 100% methanol at 40 min; GITC and AITC, methanol—water (55:45) isocratic mobile phase. Eluting materials were detected by UV absorbance at 220 nm or 254 nm.

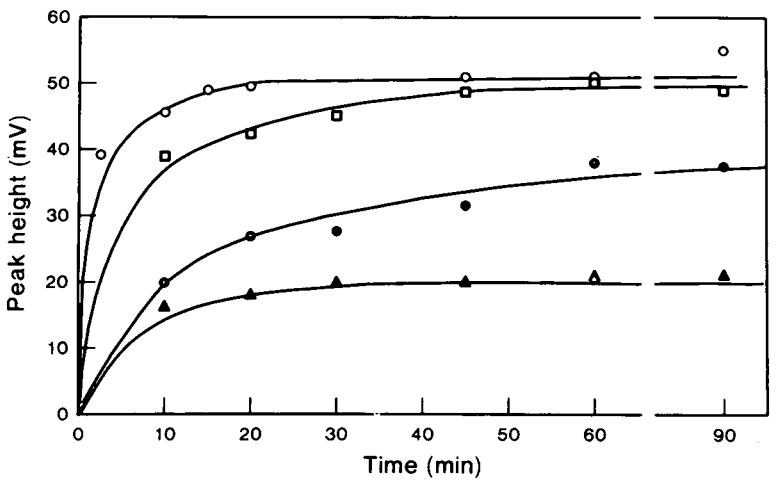
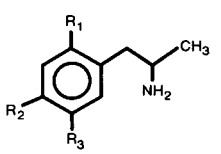


Fig. 2. Time course of derivatization of (*RS*)-(±)-1-phenyl-2-aminopropane (amphetamine) with MTPA · Cl (○); GITC (□); AITC (●); and PEIC (▲).



			R			
R_1	R_2	R_3	PEIC	MTPA·Cl	GITC	AITC
-H	-H	-H	0.36	1.20	1.17	1.14
-H	-Cl	-H	0.39	1.38	1.61	1.55
1-phenylethylamine			0.00	0.84	1.31	1.17
$-OCH_3$	$-CH_3$	$-OCH_3$	0.96	0.89	1.54	1.73
$-OCH_3$	$-OCH_3$	$-CH_3$	0.63	0.98	1.73	1.55
$-OCH_3$	$-SCH_3$	$-OCH_3$	--	1.08	1.19	1.07

Fig. 3. Tabular presentation of the optimum resolution achieved for all substrates with the four chiral reagents by reversed-phase (methanol—water) HPLC analysis as described in Materials and methods. Resolution factor (R) is defined as $2d/(w_1 + w_2)$ where d is the separation between maxima, and w_1 and w_2 are the widths of the peaks at baseline.

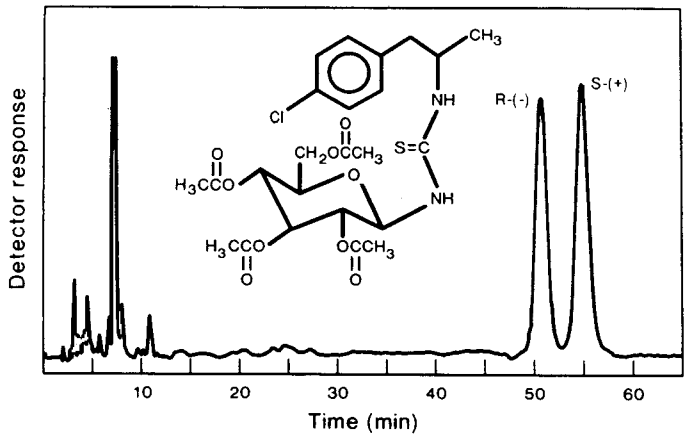


Fig. 4. Reversed-phase HPLC chromatogram of separation of the diastereomeric thiourea derivatives formed from (*RS*)-(±)-*p*-chloroamphetamine with GITC. Mobile phase is methanol—water (55:45) at 1 ml/min.

RESULTS

All amphetamines and 1-phenylethylamine (or the hydrochloride or sulfate salts) formed a pair of diastereomeric products with each of the four chiral reagents under mild reaction conditions. HPLC analysis did not reveal the presence of additional UV-absorbing reaction products following derivatization, and reaction products were stable under reaction and workup conditions. Reaction mixtures could be analyzed directly by HPLC without sample cleanup. Alternatively, hydrolysis and extraction procedures could be employed to remove excess reagent and to isolate underivatizated amines (if any) prior to HPLC analysis. Derivative formation was complete after 60 min, and no amine substrates were detectable at that time by HPLC or GC analysis (data not shown). The time course of derivatization of amphetamine with the four reagents is shown in Fig. 2.

Resolution of diastereomeric reaction products by reversed-phase HPLC varied widely according to substrate and reagent. In general, better resolution was achieved following derivatization with AITC, GITC and MTPA · Cl than with PEIC (data summarized in Fig. 3). For example, optimum resolution values (R) for the separation of the diastereomeric PEIC derivatives were less than 1 while values greater than 1 were obtained for the separation of the AITC, GITC and MTPA · Cl diastereomeric reaction products. The R values of 1.0 and 1.5 indicate 98% and 99.7% baseline resolution of diastereomeric pairs, respectively. The HPLC chromatogram of the diastereomeric products from one derivatization reaction (*p*-chloroamphetamine and GITC) is shown in Fig. 4. Actual retention times for the diastereomeric reaction products under optimum HPLC conditions (described in Materials and methods) are reported in Table I. In general, retention times may be reduced by one-half (by varying mobile phase conditions) with 3—20% loss in resolution with MTPA · Cl, GITC and AITC. Enantiomers were available for some substrates allowing determination of the order of antipode elution. When determined, the (*R*)-(—)-antipode was

TABLE I

RETENTION TIMES OF DIASTEREOMERIC REACTION PRODUCTS AT OPTIMUM RESOLUTION

Reagent	Substrate*			Retention time** (min)	
	R_1	R_2	R_3	*R*—	*S*—
PEIC	—H	—H	—H	13.5	14.0
	—H	—Cl	—H	23.6	24.2
	1-phenylethylamine			13.0	13.0
	$-OCH_3$	$-CH_3$	$-OCH_3$	26.3	28.0
	$-OCH_3$	$-OCH_3$	$-CH_3$	26.3***	27.2***
	$-OCH_3$	$-SCH_3$	$-OCH_3$	—	—
MTPA · Cl	—H	—H	—H	9.7	10.4
	—H	—Cl	—H	13.9	14.9
	1-phenylethylamine			22.1	22.8
	$-OCH_3$	$-CH_3$	$-OCH_3$	33.0	33.5
	$-OCH_3$	$-OCH_3$	$-CH_3$	32.5***	38.9***
	$-CH_3$	$-SCH_3$	$-OCH_3$	30.8***	31.4***
GITC	—H	—H	—H	26.4	28.5
	—H	—Cl	—H	49.2	53.1
	1-phenylethylamine			15.3	17.2
	$-OCH_3$	$-CH_3$	$-OCH_3$	46.4	51.0
	$-OCH_3$	$-OCH_3$	$-CH_3$	43.2***	47.0***
	$-OCH_3$	$-SCH_3$	$-OCH_3$	35.6***	38.0***
AITC	—H	—H	—H	18.2	16.8
	—H	—Cl	—H	35.4	32.3
	1-phenylethylamine			11.4	10.4
	$-OCH_3$	$-CH_3$	$-OCH_3$	33.1	30.2
	$-OCH_3$	$-OCH_3$	$-CH_3$	31.2***	28.7***
	$-OCH_3$	$-SCH_3$	$-OCH_3$	24.8***	23.5***

*See Fig. 3.

**Enantiomeric assignment based on retention times of pure enantiomers except where noted.

***Order of elution based on results with substrates for which enantiomers were available.

eluted prior to the (*S*)-(+)-antipode for PEIC, GITC, and MTPA · Cl derivatives. The opposite order of elution was observed for AITC derivatives. The results with AITC and GITC are consistent with those of Nimura et al. [16] for HPLC elution of AITC and GITC catecholamine derivatives.

DISCUSSION

HPLC resolution of enantiomers of amphetamines and related compounds has not been widely reported in the literature. Examples include resolution of DOM following derivatization with succinimidyl-*l*-α-methoxy-α-methyl-1-naphthaleneacetate [17], resolution of amphetamine amides with a chiral column [19], and resolution of catecholamines and amino acids with GITC and AITC [15, 16]. Our interest in stereochemical aspects of metabolism

prompted an investigation of methods which could be used for the resolution of amphetamines and which would be appropriate for biological studies. Based on the results of our studies, resolution of other amphetamines and related amines should be possible with one or more of the four chiral reagents studied. HPLC procedures may be preferred to GC procedures when derivatives are not adequately volatile or stable for GC analysis. The described HPLC methods are adequate for biological analysis as well. We can readily quantitate less than 100 ng derivative on column with detection at 254 nm, and sensitivity may be increased by detection at 220 nm (data not shown). An added advantage of HPLC resolution procedures such as these is the ability to recover analyzed materials. We are employing HPLC separation of amine enantiomers following derivatization with AITC or GITC as a preparative method for isolation of radiolabeled amine enantiomers [20].

ACKNOWLEDGEMENTS

This manuscript was supported in part by Grant DA02964, DHHS and a Research Career Development Award CA00755 awarded by the National Cancer Institute, DHHS to Matthew M. Ames, Ph.D.

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Journal of Chromatography, 307 (1984) 343—350
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2042

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF AMILORIDE IN PLASMA AND URINE

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(First received August 30th, 1983; revised manuscript received December 14th, 1983)

SUMMARY

A high-performance liquid chromatographic method has been developed for amiloride in rabbit plasma and urine which uses a reversed-phase C_{18} column, a mobile phase (flow-rate 2 ml/min) consisting of 32% acetonitrile in 0.15 *M* perchloric acid, pH 2.2, and spectrofluorometric detection via excitation at 286 nm. A simple extraction step with ethyl acetate eliminates interfering peaks. Short retention times of about 2.3 and 3.8 min are observed for amiloride and the internal standard, triamterene, respectively. The method can measure 4 ng/ml amiloride in plasma. This assay has been used to explore the pharmacokinetics of amiloride in rabbits. The plasma disposition profile is biexponential after a 50-mg intravenous bolus dose and there is no evidence for saturable elimination at zero-order infusion rates of 1.8, 3.6 and 7.2 mg/h.

INTRODUCTION

Amiloride, a pyrazinecarbonylguanidine, is a potassium-sparing diuretic which exerts its effect by blocking distal tubular exchange of sodium, potassium and hydrogen, in the presence or absence of aldosterone [1, 2].

Amiloride is usually administered orally and from 15 to 26% of the dose is reportedly absorbed [3]. However, in two studies using ^{14}C-labeled drug, urinary and fecal collections for 72 h accounted for 90% of the administered compound [4, 5]. Reports on the distribution of amiloride as well as its elimination profile are few in number and also filled with controversy. Better studies are needed to clarify the disposition of this drug, particularly in patients with impaired renal function, who will undoubtedly exhibit a decreased renal

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0378-4347/84/$03.00

clearance of the diuretic and thus may be disposed to adverse reactions unless properly dosed.

The primary reason for the lack of satisfactory pharmacokinetic data on amiloride is the absence of a suitable method for measuring amiloride in biological fluids. To this point the assays have included a fluorescence method [6] and measurement of radioactivity [5].

This report describes the development of a high-performance liquid chromatographic (HPLC) assay which has permitted us to examine the pharmacokinetics of amiloride in rabbits. The method has the required sensitivity and specificity to be used in human studies.

EXPERIMENTAL

Materials

Amiloride · HCl was supplied by Merck Sharp and Dohme (Rahway, NJ, U.S.A.). Triamterene, internal standard, was obtained from Smith, Kline & French (Montreal, Quebec, Canada). All chromatographic solvents were HPLC grade, and all other chemicals were analytical reagent grade.

Apparatus

An Altex Model 100A liquid chromatographic pump (Aviation Electric, Montreal, Canada) and a Rheodyne valve-loop injector fitted with a 175-μl loop were employed. A Spherisorb ODS 250 × 4.1 mm I.D. column (Altech, 10-μm particle size) was connected to a Schoeffel FS-970 fluorescence detector (Westwood, NJ, U.S.A.) whose excitation wavelength was set at 286 nm and which contained a KV370 emission cut-off filter. This filter transmits more than 99% and less than 1% of the incident light for wavelengths over 395 nm and under 345 nm, respectively. The mobile phase consisted of 32% acetonitrile in 0.15 *M* perchloric acid, pH 2.2 and was pumped at a constant flow-rate of 2.0 ml/min. The mobile phase was degassed by ultrasonication prior to use. The chromatograms were recorded on a potentiometric recorder (Westronics - MT 21, Fort Worth, TX, U.S.A.). All chromatography was carried out at ambient temperature.

Preparation of standard curve

Stock solutions of amiloride and the internal standard, triamterene, were prepared by dissolving the compounds in 1% (v/v) lactic acid. The plasma amiloride standard curve was prepared by diluting the above standard (300 μg/ml) with drug-free rabbit plasma so that 100 μl of plasma would correspond to a concentration range of 0.5—20 μg/ml. The urine amiloride standard curve was prepared similarly, yielding a concentration range of 10—50 μg/ml.

Sample preparation

After delivering 100 μl of a plasma sample to a clean, dry test tube, 1 ml ethyl acetate and 70 ng triamterene (10 μl) were added. While vortexing, 50 μl sodium hydroxide (5.0 *M*) were added. After vortexing a further 30 sec, the mixture was centrifuged at 3000 *g* for 2 min. Thereafter 0.8 ml of the upper organic layer was transferred to a second tube containing 0.5 ml hydro-

chloric acid (0.1 *M*). After vortexing again for 30 sec, the solution was subjected to a gentle nitrogen flow at 60° C in order to evaporate residual ethyl acetate and excess hydrochloric acid. An aliquot of the remaining solution (10—50 μl) was injected into the HPLC instrument.

Before being prepared for chromatography, all urine samples were first diluted ten-fold. Thereafter they were treated in an identical fashion to the plasma samples except that only 50 μl of the diluted urine was processed in conjunction with 105 ng triamterene.

Recovery and assay precision

The absolute recoveries of amiloride and triamterene from rabbit plasma and water were determined by extracting the agents using the aforementioned procedure and comparing the chromatograms to those obtained from an unextracted aqueous solution.

To determine the inter-assay precision for the plasma amiloride, six replicates at levels of 1.10 and 17.56 μg/ml were analyzed on different days. The inter-assay precision for urinary amiloride was determined in a similar way at concentrations of 1.61 and 18.25 μg/ml. These tests of precision were based upon calibration curves prepared on each day of the test.

The intra-assay precision was determined for the plasma and urine procedure by using six replicates of each of the above solutions. This test was based upon a single calibration curve constructed on the day of the test.

Quantitation

Standard curves for amiloride were constructed using peak height ratios obtained for the drug to the internal standard, versus the concentration of the drug. The concentration of amiloride in biological samples was determined by interpolation following linear regression of the standard curve.

Rabbit experiments

Intravenous amiloride was prepared by dissolving the drug in minimum 1% lactic acid with heating at 70° C and sonication. The solution was then adjusted to pH 3.5 with 5 *M* sodium hydroxide. Just prior to administration the solution was filtered (Swinex-25) and a portion retained to establish the exact dose administered.

New Zealand White male rabbits were weighed and placed in a restraining cage. Catheters (Angiocath, Deseret, Sandy, UT, U.S.A.) were placed in the marginal veins of each ear. The dose was administered in one ear while blood samples were obtained from the other. Urine samples were collected through a Foley catheter (No. 8 Fr., Acmi, Norcross, GA, U.S.A.) inserted into the bladder via the urethra.

Data analysis

The compartmental characteristics of the amiloride disposition in rabbits were established using standard pharmacokinetic procedures [7]. All computer fitting of data was performed on a Dec-10 Model 1090 computer using a non-linear computer fitting program originally described by D'Argenio and Schumitzky [8].

RESULTS AND DISCUSSION

Reversed-phase HPLC has become a popular tool for measuring drugs in biological fluids. Since amiloride is a weak base (p*K*a = 8.7) [9] it appeared logical that ion-pairing would be needed to achieve satisfactory chromatography using C_{18} columns. It was found that perchloric acid retarded the elution of amiloride such that potential interfering components in the solvent front would be avoided. By experimentation it was found that the mobile phase described previously yielded sharp, well resolved peaks.

Among eleven compounds tested as potential internal standards, only 4-bromoantipyrine and triamterene appeared to be suitable. Triamterene was chosen as the internal standard because its properties were quite similar to amiloride and therefore small fluctuations in extraction technique or chromatography would not lead to noticeable changes in peak height ratios.

In the preparation of plasma samples, acetonitrile or acetone were employed initially to precipitate proteins. However, the resulting supernatant gave rise to numerous undesirable peaks which in part interfered with amiloride quantitation. Therefore an extraction method was developed. Of the ten organic solvents investigated, ethyl acetate provided superior recovery of amiloride while excluding interfering components. The volume and concentration of alkalinizing agent was also selected to optimize amiloride recovery. Finally, it was found that in the absence of hydrochloric acid in the evaporation step, amiloride concentrations less than 1 μg/ml yielded a large variation in recoveries. The evaporation step was needed to remove a volatile component which otherwise interfered with amiloride fluorescence detection.

Figs. 1 and 2 exhibit chromatograms which reflect the analysis of amiloride in rabbit plasma and urine, respectively. The retention times of amiloride and triamterene were about 2.3 and 3.8 min, respectively. No interfering peaks

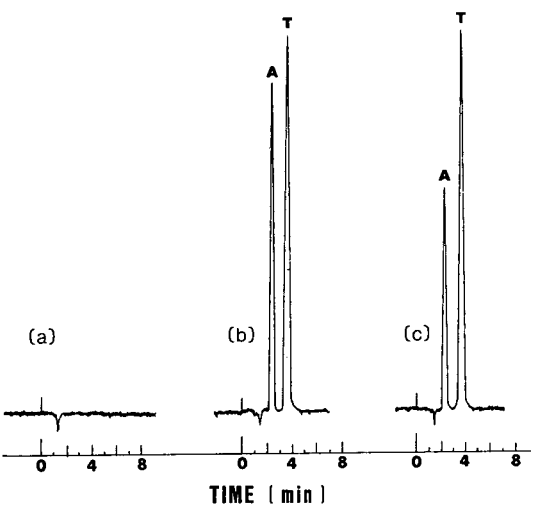

Fig. 1. Chromatograms obtained in the assay of plasma amiloride (A) using triamterene (T) as the internal standard. (a) Blank rabbit plasma, 0.05 ml injected; (b) blank rabbit plasma containing amiloride (2 mg/l) and triamterene (0.35 mg/l), 0.05 ml injected; and (c) plasma collected from a rabbit at 280 min during an intravenous infusion of 7.2 mg/h.

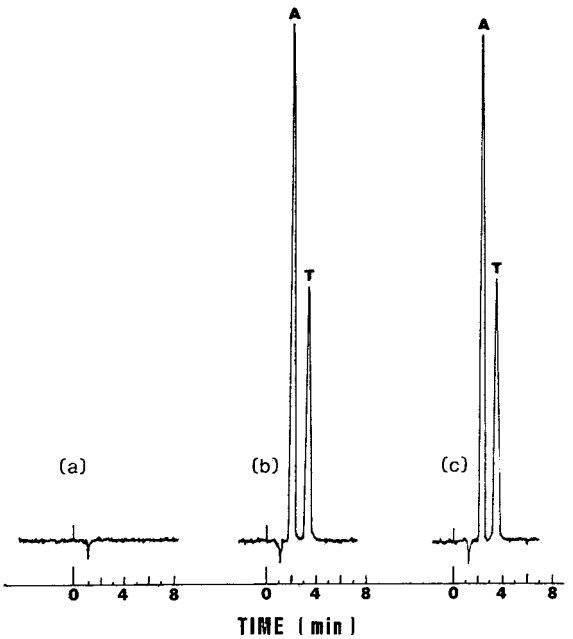

Fig. 2. Chromatograms obtained in the assay of urinary amiloride (A) using triamterene (T) as the internal standard. (a) Blank rabbit urine, 0.03 ml injected; (b) blank rabbit urine containing amiloride (40 mg/l) and triamterene (2.8 mg/l), 0.03 ml injected; and (c) urine collected from a rabbit during an intravenous infusion of 7.2 mg/h.

were observed using the aforementioned procedure for preparing biological samples. Calibration curves were linear over the concentration range used.

For the determination of amiloride in plasma (0.5—20 μg/ml) the average absolute recovery was found to be 65.9% with a 7.6% coefficient of variation. The corresponding results for the absolute recovery of amiloride from water were 71.5% and 7.0%, respectively. The absolute recoveries of triamterene at the standard concentration for plasma and water were 89.0% and 87.3%, respectively. Tables I and II present information on the inter- and intra-assay variability for the procedure. The small coefficients of variation confirm the reproducibility of the assay method.

The amiloride assay has been used to explore the pharmacokinetics of this

TABLE I

AMILORIDE INTER-ASSAY PRECISION (n = 6)

Sample	Theoretical concentration (mg/l)	Mean measured concentration (mg/l)	C.V. (%)
Plasma	1.10	1.19	5.43
	17.56	17.72	3.93
Urine	1.61	1.84	4.31
	18.25	18.39	1.79

TABLE II

AMILORIDE INTRA-ASSAY PRECISION (n = 6)

Sample	Theoretical concentration (mg/l)	Mean measured concentration (mg/l)	C.V. (%)
Plasma	1.10	1.18	2.46
	17.56	17.02	2.09
Urine	1.61	1.80	3.16
	18.25	17.88	2.64

agent in rabbits. The central purpose of these studies has been to examine drug interactions involving the renal disposition of selected compounds. The following preliminary data substantiate the applicability of the analytical method.

Rabbits receiving a 50-mg intravenous bolus of amiloride exhibited plasma disposition profiles as illustrated in Fig. 3. The biexponential disappearance curves yielded pharmacokinetic constants indicative of a two-compartment model as summarized in Table III. The distribution spaces observed were greater than the total blood volume (58—70 ml/kg [10]) in the rabbit, thereby indicating that the greater part of amiloride was located in extravascular sites. This finding is consistent with that noted in humans [4] and the dog [6]. From the urinary excretion of amiloride it was found that about 60% of the drug is removed renally by the rabbit. In order to test whether the two-compartment model adequately described the amiloride pharmacokinetics in rabbits, and to establish whether dose-dependent factors might become important, the agent was given as an infusion. On different days, three infusions (1.8, 3.6, and 7.2 mg/h) were preceded by initial loading doses of 7.5, 15, and 30 mg, respectively. The outcome of such experiments, as illustrated in Fig. 4, established the appropriateness of the two-compartment model, and the absence of saturable eliminating routes at the doses employed [7].

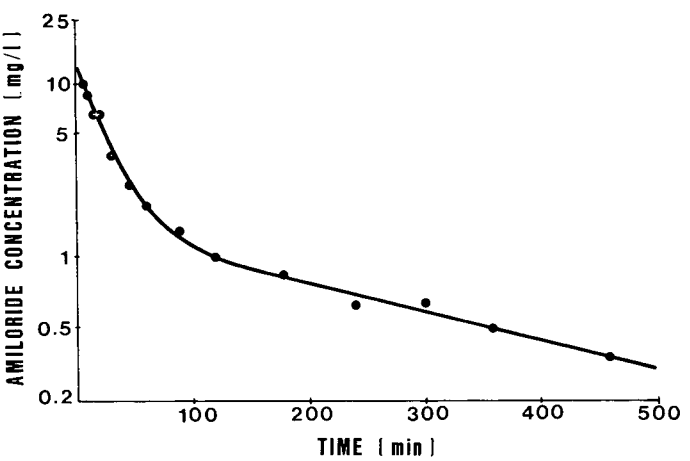

Fig. 3. Plasma amiloride concentrations observed in a rabbit (3.93 kg) following an intravenous bolus dose of 50 mg. The solid line represents a computer fit resulting in an equation, $C = 12.12e^{-0.0447t} + 1.83e^{-0.0036t}$

TABLE III

FITTED AMILORIDE PHARMACOKINETIC PARAMETERS OBTAINED FROM FOUR RABBITS RECEIVING A 50-mg BOLUS DOSE

Parameter	Mean ± S.D.		Range	
Vc (ml/kg)	815.4	± 157.9	610.1	—954.4
α (min^{-1})	0.046	± 0.009	0.038	—0.058
β (min^{-1})	0.0040	± 0.0007	0.0036	—0.0050
k_{10} (min^{-1})	0.0184	± 0.0015	0.0165	—0.0200
k_{12} (min^{-1})	0.0218	± 0.0067	0.0166	—0.0313
k_{21} (min^{-1})	0.0099	± 0.0016	0.0082	—0.0116
$t½\ \alpha$ (min)	15.41	± 2.67	11.91	—18.38
$t½\ \beta$ (min)	176.5	± 25.7	138.6	—192.5
TBC (ml/min/kg)	15.01	± 3.43	11.65	—19.09

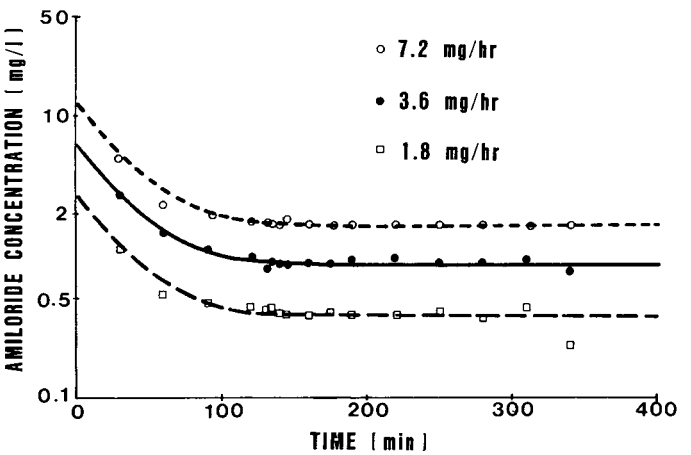

Fig. 4. Plasma amiloride concentrations observed in a rabbit on three different occasions following infusion of 1.8 (□ — — □), 3.6 (•—•), and 7.2 (○ — — ○) mg/h, preceded by loading dose of 7.5, 15 and 30 mg, respectively. The solid line is a computer fit while the other lines represent simulation based upon the fitted parameters of a two-compartment model (V_1 = 724.6 ml/kg, α = 0.0345 min^{-1}, β = 0.00149 min^{-1}, k_{21} = 0.00185 min^{-1}, weight = 3.31 kg).

The sensitivity of the HPLC assay was examined to determine its utility for human studies. In contrast to the aforementioned sample preparation method, 1 ml plasma, 6 ng triamterene, 5 ml ethyl acetate, and 0.5 ml sodium hydroxide were used. The final aqueous hydrochloric acid solution was reduced to about 100 μl. By injecting 50 μl, a measurable peak three times the baseline noise was observed for a plasma amiloride concentration of 4 ng/ml. Thus the assay could follow the pharmacokinetics of amiloride in man even after a 10-mg dose [11].

Clinically, amiloride has been used with other diuretics and beta-blockers. Some of these drugs have been tested with this assay to determine if they would interfere with the quantitation. Those tested were chlorothiazide, hydrochlorothiazide, furosemide, spironolactone, propranolol, and metoprolol. In each case, peaks for these drugs, if observed at all, did not interfere with

those of amiloride or triamterene. Further tests with metabolites of these compounds are yet to be done.

In conclusion, a new simple HPLC method has been developed for amiloride which is rapid, specific, and of requisite sensitivity to be used even in human studies.

ACKNOWLEDGEMENTS

This research was supported in part by the J.P. Bickell Foundation. We wish to acknowledge the excellent technical assistance of George Kieltyka.

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Journal of Chromatography, 307 (1984) 351—359
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2032

LIQUID CHROMATOGRAPHIC ASSAY OF HEPTAMINOL IN SERUM AND ITS ORAL PHARMACOKINETICS IN THE DOG

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(First received July 20th, 1983; revised manuscript received December 15th, 1983)

SUMMARY

In order to investigate the pharmacokinetics of heptaminol in dogs, a high-performance liquid chromatographic assay of the drug was devised and it was evaluated in a general purpose validation design through analysis of variance. Heptaminol and its internal standard *n*-propylamine were salted out from plasma together with acetonitrile, the previously proposed "solvent demixing" extraction procedure. Both amines were derivatised in acetonitrile with the *o*-phthaldialdehyde, 2-mercaptoethanol procedure of Roth. The adducts were quantitated by reversed-phase high-performance liquid chromatography on Radial-Pak® cartridges with ultraviolet detection. Peak height ratios were linearly related to concentrations up to 250 μmol l^{-1} with a 2% coefficient of variation. Sensitivity was 3.5 μmol l^{-1} (signal-to-noise ratio of 5).

Means of the usual pharmacokinetic parameters in four dogs were: elimination half-life 3.75 h, apparent distribution volume 2.18 l kg^{-1} and total clearance 0.402 l kg^{-1} h^{-1}, similar to the results obtained in humans by other authors using radiolabelled heptaminol.

INTRODUCTION

Heptaminol (6-amino-2-methyl-2-heptanol) is a cardiotonic drug used in Europe since 1953. Current drug regulations now require pharmacokinetic investigations which were not mandatory at that time and which were never done since then. In charge of animal studies, we had to devise a liquid chromatographic assay suitable for plasma levels lower than 10 mg l^{-1} (55 μmol l^{-1}).

We applied to heptaminol the solvent demixing extraction procedure [1] which we had found convenient and reproducible for valproic acid [2] and major anticonvulsants [3]. Low ultraviolet (UV) absorbance necessitated derivatisation. We adopted derivatisation with *o*-phthaldialdehyde (OPT) and

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mecaptoethanol according to the procedure of Roth [4] and already applied to pharmaceutical forms of the drug with UV detection by Nicolas et al. [5]. The whole assay procedure was evaluated through analysis of variance (ANOVAR) in an experimental design fitted to this general purpose, variations of which we have already used profitably in previous instances [2, 3].

EXPERIMENTAL

Materials

Reagents and solvents were of analytical grade: potassium chloride and disodium hydrogen orthophosphate from Rhône-Poulenc (France); *n*-propylamine from Merck (F.R.G.); *o*-phthaldialdehyde and 2-mercaptoethanol from Fluka; acetonitrile for far UV from Fisons (Loughborough, U.K.) through Touzart et Matignon (France). Heptaminol hydrochloride was from Finorga and tablets were from Richard (Sauzet, France).

The chromatographic apparatus was a Spectra-Physics SP 8000, equipped with a Valco loop injector (injected volume 10 μl). The column was a Radial-Pak cartridge from Waters, filled with C_{18} bonded reversed-phase, particle size 10 μm. The detector was a Model 770 variable-wavelength spectrophotometric detector from Schoeffel (F.R.G.).

Extraction procedure

Heptaminol was extracted from plasma or water into acetonitrile by a solvent demixing [1] procedure. To a 1.0-ml plasma sample were added 0.1 ml of 1 *M* sodium hydroxide and 1.0 ml of acetonitrile containing the internal standard at fixed concentration (*n*-propylamine, 3.6 mg l^{-1}), then the mixture was briefly mixed. An excess of solid potassium chloride was then poured in, followed by vigorous vortex-mixing and centrifugation (20° C, 1500 *g*, 15 min). A 500-μl aliquot of the acetonitrile supernatant was transferred into a second tube for pre-column derivatisation.

For assay validation, a blank plasma and water were used and the added acetonitrile contained both the internal standard at the fixed concentration and heptaminol hydrochloride at one of the following concentrations: 2.5, 5 or 10 mg l^{-1} (13.75, 27.5 or 55.0 μmol l^{-1}). Calibrations were made with the blank plasma and the highest heptaminol concentration.

Derivatisation and chromatography

The derivatisation solution was 0.075 mol l^{-1} *o*-phthaldialdehyde and 0.14 mol l^{-1} 2-mercaptoethanol in acetonitrile. To the 500-μl aliquot of acetonitrile extract, 200 μl of the derivatisation solution and 500 μl of a 1 *M* sodium hydroxide solution were added, and the two unmixed phases were briefly shaken together on a vortex mixer. The acetonitrile supernatant of the derivatisation mixture was injected through a 10-μl sample loop. The isocratic mobile phase was acetonitrile—disodium hydrogen orthophosphate buffer, pH 7, 12.5×10^{-3} *M* (50:50, v/v), flow-rate 1.5 ml min^{-1}, detection at 330 nm [5].

Method validation

The experiment was designed for an analysis of variance both factorial and

nested. The two fixed factors studied were heptaminol concentration (three levels) and sample composition (two levels: water and plasma).

For the nested analysis of extraction and chromatographic measurements, each water or plasma extract was duplicated and each derivatised duplicate was chromatographed twice. The acetonitrile solutions were also derivatised in duplicate in the same way as the extracts for evaluation of the extraction yield. Table I shows the structure of the validation design.

TABLE I

STRUCTURE OF THE FACTORIAL/NESTED VALIDATION DESIGN

	Factor: drug concentration			Total R_i
	$X'_1 = -1$	$X'_2 = 0$	$X'_3 = 1$	
Factor: sample composition				
Water		$\left.\begin{matrix} Y_{ij11} \\ Y_{ij12} \end{matrix}\right\} E_{ij1}$		
		$\left.\begin{matrix} Y_{ij21} \\ Y_{ij22} \end{matrix}\right\} E_{ij2}$ $\Big\} K_{ij}$		R_1
Plasma				$R_{r=2}$
Total C_j	C_1	C_2	$C_{c=3}$	G

Analysis of variance

Table II shows the corresponding variance analysis. Data y (peak ratios) were input as their decimal logarithms, Y, in order to warrant homoscedasticity under the hypothesis of a constant coeffient of variation.

Regression analysis

The hypothesis to be tested (assay linearity) that Y is proportional to the drug concentration x, i.e. $y = ax$, results in a linear relationship of unit slope $Y = X + \log a$, where $Y = \log y$, $X = \log x$ and the expected regression coefficient $b = 1$.

Calculations were simplified by using a coded abscissa

$$X' = 1 + \frac{\log x - \log \mathrm{Hi}}{\log 2}$$

where Hi = 10 mg l^{-1} is the highest of the three concentrations in ratio 1:2, and which is 1 when $\log x = \log \mathrm{Hi}$, 0 when $\log x = \log (\mathrm{Hi}/2)$, and -1 when $\log x = \log (\mathrm{Hi}/4)$.

Using the coded abscissa, regression calculations came down to: expected regression coefficient $b' = \log 2 = 0.30103$; sum of squares $SX' = \Sigma X'^2 = 8$ for each individual regression, 16 for common regression; sum of products $SYX' = \Sigma YX' = K_{3j} - K_{1j}$ for each regression, $C_3 - C_1$ for common regression.

TABLE II

ANALYSIS OF VARIANCE OF THE VALIDATION DESIGN (TOTALS E, K, R, C AND G AS SHOWN IN TABLE I)

Variance component	Sum of squares	Degrees of freedom
Correction factor	$C = G^2/4rc$	
Total	$ST = \sum^{4rc} Y^2 - C$	$NT = 4rc - 1 = 23$
Between all extracts	$SE = \frac{1}{2}\sum^{2rc} E^2 - C$	$NE = 2rc - 1 = 11$
Factorial		
Between cells	$SK = \frac{1}{4}\sum^{rc} K^2 - C$	$NK = rc - 1 = 5$
Between compositions	$SR = \frac{1}{4c}\sum^{r} R^2 - C$	$NR = r - 1 = 1$
Between concentrations	$SC = \frac{1}{4r}\sum^{c} C^2 - C$	$NC = c - 1 = 2$
Regression (common)	$SL = \frac{(C_3 - C_1)^2}{16}$	1
Interaction	$SRC = SK - SR - SC$	$NRC = NK - NR - NC = 2$
Nested		
Intra-cell	$SI = ST - SK$	$NI = NT - NK = 3rc = 18$
Betweeen duplications of measurements	$SM = ST - SE$	$NM = NT - NE = 2rc = 12$
Between duplications of extracts	$SX = SI - SM$	$NX = NI - NM = rc = 6$

Overall calculation procedure (all on logarithmic values)

(1) Homoscedasticity of log values was tested through Bartlett's test [6]. If not significant at the probability level $P = 0.1$, then:

(2) Variability of extraction was tested through a one-sided F test of SE vs. SM. If not significant at $P = 0.1$, then the intra-cell mean square was taken as the error variance s^2.

(3) Analysis of variance was performed on the whole data and separately on data from water and from plasma extractions.

(4) Separate regressions were calculated. Departure from linearity was tested versus the common error variance s^2 through a one-sided F test of (in each row) $SC - SL$ with 1 and 18 degrees of freedom (DF). If not significant at $P = 0.1$, departure from parallelism was tested through a two-sided t test as

$$t = \frac{|b_1' - b_2'|}{\sqrt{s^2_{\Delta b}}},\ 18\ \text{DF},\quad \text{where } s^2_{\Delta b} = s^2\frac{2}{SX'}.$$ If not significant at $P = 0.1$, then:

(5) Common regression was calculated and departure of the common slope b' from expected theoretical value 0.30103 was tested through a two-sided t test as

$$t = \frac{|b' - 0.30103|}{\sqrt{s^2_b}},\quad 18\ \text{DF},\quad \text{where } s^2 = s^2_b\,\frac{1}{SX'} = s^2/16$$

(6) The intra-assay coefficient of variation (C.V.$_i$) was calculated through the already proposed approximations [2]

$$\text{C.V.}_i \simeq \frac{dm}{m} = d \ln m = 2.306 \, d \log m \simeq 2.3026 \, s$$

which assimilate ($\simeq$) standard deviations with differentials and arithmetic with geometric means m, and which work as long as C.V. is not too large, say lower than 10%.

(7) The inter-assay C.V.$_e$ was determined separately. Two plasma samples (T2 and T8) were taken from the same dog 2 and 8 h, respectively, after heptaminol ingestion and were assayed in five separate assay sessions.

Pharmacokinetics in the dog

Four female beagle dogs (13, 16, 13 and 12.6 kg body weight) ingested 300 mg (two tablets) of heptaminol as 376.5 mg of heptaminol hydrochloride. This represented five times the usual unitary dosage for men on a mg/kg basis. Blood samples were taken 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 24 h later and plasmas were assayed for heptaminol. Pharmacokinetic parameters were calculated through fitting to an open two-comparment model.

RESULTS

Chromatography

Fig. 1 shows the chromatogram obtained from a blank plasma to which heptaminol and internal standard were added as described in Experimental at concentrations of 2.5 mg l^{-1} (17.25 μmol l^{-1}) and 3.6 mg l^{-1} (60 μmol l^{-1}), respectively.

Extraction yield

The volumetric yield of acetonitrile demixing (demixed/added volume) was estimated as 0.7. Comparison of peak heights on chromatograms obtained from calibration solutions and from plasma or water extracts, when combined with the volumetric yield, resulted in the following values of extraction yields: from water, drug = 0.634, internal standard = 0.662; from plasma, drug = 0.653, internal standard = 0.534.

Method validation

Homoscedasticity of log values was not ruled out by Bartlett's test. Variation from extraction was found not significant: the comparison SE vs. SM resulted in $F(6/12) = 1.52$, $P_{1\alpha} = 0.25$.

Linearity of the regression line was not denied: for water extracts $F(1/9) = 3.052$, $0.10 < P_{1\alpha} < 0.25$; for plasma extracts $F(1/9) = 0.136 < 1$, $P_{1\alpha} \gg 0.50$.

Parallelism was not denied: $t_{18} = 1.5725$, $0.10 < P_{2\alpha} < 0.20$.

The slope of the common logarithmic regression line did not differ significantly from expected value 0.30103: $t_{18} = 0.8109$, $0.40 < P_{2\alpha} < 0.50$.

Interaction between concentrations and sample composition was not significant: $F(2/18) = 2.546$, $0.10 < P_{1\alpha} < 0.20$.

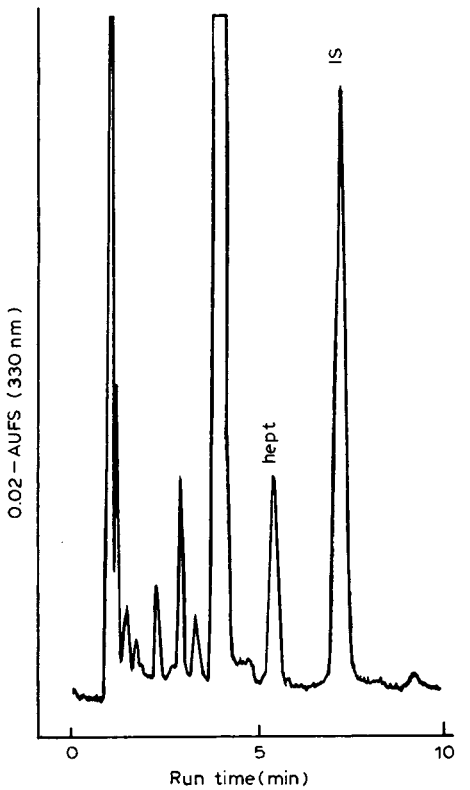

Fig. 1. Chromatogram of *o*-phthaldialdehyde adducts of heptaminol (hept) and propylamine (IS) in plasma.

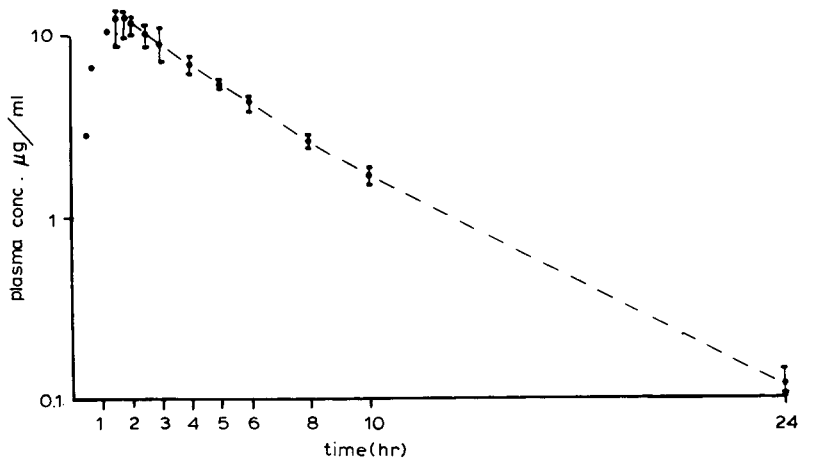

Fig. 2. Plasma concentrations of heptaminol versus time (mean ± S.D. from four dogs).

The intra-assay coefficient of variation was $C.V._i = 2.125\%$.

The five-fold separate determination of two plasma samples from the same dog resulted in: for T2, mean = 9.580 mg l^{-1}, $C.V._e = 3.3448\%$; for T8, mean = 1.857 mg l^{-1}, $C.V._e = 3.4603\%$.

TABLE III

PHARMACOKINETIC PARAMETERS OF HEPTAMINOL IN FOUR FEMALE BEAGLE DOGS (A—D)

	A	B	C	D	Mean
Body weight (kg)	13	16	13	12.6	13.65
Half-time (h)					
First (resorption)	0.319	0.042	0.050	0.009	0.105
Second (distribution)	0.724	1.112	1.338	1.423	1.149
Final (elimination)	3.417	3.828	3.455	4.316	3.754
T_{max} (h)					
Observed	1.5	1.5	1.75	0.75	1.375
Calculated	1.5	1.0	1.15	0.75	
C_{max} (mg l^{-1})					
Observed	16.04	14.29	18.04	19.71	17.02
Calculated	14.80	17.02	19.85	17.09	
Total clearance (l kg h^{-1})	0.374	0.421	0.429	0.385	0.402
Distribution volume (l kg^{-1})	1.844	2.330	2.142	2.401	2.179
K_a (h^{-1})	2.17	13.346	13.676	70.796	25.747
K_{el} (h^{-1})	0.49	0.37	0.327	0.259	0.3615

Dog pharmacokinetics

Fig. 2 is a graph of the mean serum concentration ± S.E.M. through time. Table III lists the corresponding calculated pharmacokinetic constants.

DISCUSSION

Extraction

No extraction method has been published for heptaminol to our knowledge. We extended successfully to this drug the solvent demixing technique which we are currently using for the quantitation of anticonvulsants.

The extraction yield is somewhat low, but highly reproducible as judged from the non-significant between-extract component of the error variance (see Method validation). This was to be expected, since solutes are salted out together with the organic phase and they partition between plasma water and acetonitrile at the molecular level instead of equilibrating through an interface. Good reproducibility has been observed also for valproic acid [2] and major anticonvulsants [3].

As mentioned elsewhere [1], the solvent demixing procedure is well adapted to routine use, since it allows calibrations to be performed by means of acetonitrile solutions of drug and internal standard instead of using spiked plasmas. This is true only under the assumption that plasma proteins, when denaturated by acetonitrile, reach the same adsorption equilibrium when the solutes are initially in the aqueous phase as when they are initially in the acetonitrile phase. Long experience with anionic drugs has shown us that this assumption

holds, and systematic studies now in progress show the same for cationic or highly lipophilic drugs.

Comparison of peak ratios shows that values obtained with plasma extracts are about 20% higher than with solutions or aqueous extracts. Comparison of peak heights shows that this discrepancy is due to a lower extraction yield of the presently used internal standard from plasma than from water, whereas heptaminol is equally extracted from both. Ideally, a better internal standard would be worth searching for, all the more as demixing technique makes such an optimisation easy to perform. However, in the present application of the method, this difference was considered sufficiently small that no significant systematic difference could reasonably be expected between different plasmas.

Derivatisation and chromatography

Derivatisation with *o*-phthaldialdehyde and mercaptoethanol according to the procedure of Roth [4] appears well suited to the assay of primary amines involving acetonitrile demixing. The reaction takes no measurable time to go to completion at room temperature in this solvent.

The UV absorbance spectrum of *o*-phthaldialdehyde adducts allows spectrophotometric detection at a wavelength where very few if any plasma components are detectable. The high absorbance affords a sensitivity evaluated at 3.5 μmol l^{-1}, corresponding to a signal-to-noise ratio of 5, which proved sufficient for the present study. Presumably, fluorescence measurements would afford higher sensitivity, but linearity of fluorescence emission would have to be checked.

Method validation

Lack of significance of Bartlett's test allowed for analysis of variance and unweighted regression to be made on logarithmic values. It may also be inferred from this test that measurements have a constant C.V. in the range of concentrations studied.

Lack of significance of comparison of S*E* vs. S*M* shows that extraction adds no measurable contribution to the instrumental error of chromatographic measurements. In other words, it has a better reproducibility than the measurements. Common regression analysis was valid since both lines could be considered straight and parallel. The slope of the common regression line of log values vs. log concentrations did not differ from unit value, which means that measured values are linearly related to concentrations in the range 0—10 mg l^{-1} (0—55 μmol l^{-1}). Results without internal standard not reported here show on visual inspection that linearity remains good up to 40 mg l^{-1}. Parallelism of the lines and absence of significant statistical interaction between concentrations and sample composition confirm that sample composition does not modify the assay relationship other than through logarithmic translation (i.e. arithmetic proportionality).

As can be seen, the validation design proposed here affords a wealth of precise information concerning analytical performance in return for the comparatively modest 24 individual determinations. We use it now whenever working up an analytical procedure [2, 3].

The hierarchical "intra-cell" organization of duplications allows evaluation of successive steps involved in the method (here extraction and chromato-

* J. Chromatogr. 309 (2) 1984 : 460 / A.U.

graphy of extracts). This nested pattern could be extended if desired to more than two levels (e.g. extraction, back-extraction and measurements) at the expense of doubling the number of measurements for every additional level.

Logarithmic transformation of values allows homogeneity of precision in the range of concentrations under study to be checked; otherwise, displaying a C.V. value has little meaning.

The factorial part of the design allows estimation of the contribution of selected factors to variability in addition to checking linearity. It was restricted here to the effect of sample composition, essentially plasma proteins which are known to affect extraction of basic drugs and which turned out indeed to affect differently the extraction yield of internal standard and heptaminol. A useful statistical factor is the repetition of the whole design such as described here in order to estimate a between-assay component of variance which is ascribable to variations in daily calibrations and which combines with the intra-assay component into the total inter-assay variability. Four-fold repetition of sessions was performed in the evaluation of an HPLC assay of anticonvulsants for clinical purposes [3] at the expense of four times more measurements. We restricted the present less-demanding work to the usual determination of the same two dog plasma samples in five assay sessions. The values so obtained of inter-assay C.V. are consistent with fairly equal intra-assay and between-assay variances.

Pharmacokinetics

The pharmacokinetics in the dog were compatible with an open two-compartment model, the half-lives being 1.14 h for the distribution phase and 3.7 h for the elimination phase. The average apparent distribution volume was as large as 2.18 l kg^{-1}, which agrees with the large tissue distribution found in rats [7]. Finally, the high 0.4 l kg^{-1} h^{-1} clearance approximates to renal plasma flow in mammals and is consistent with excretion through tubular secretion, as already described in man [8].

The whole administered dose of heptaminol was found in urine within 24 h, and 82—87% within 10 h. Presumably this corresponded to the parent drug. The rat has been shown to hydroxylate heptaminol on methyl side-chains at a rate of 4—7% [7]. Whether man metabolizes it is unknown. Indeed, our results do not support that metabolism even reaches such a low rate in the dog. A hydroxylated metabolite could be derivatised in the same way as heptaminol, but would presumably have a much shorter retention time in reversed-phase chromatography and could not be mistaken as heptaminol.

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Journal of Chromatography, 307 (1984) 361—369
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2034

QUANTIFICATION OF TIAZOFURIN IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received August 11th, 1983; revised manuscript received December 8th, 1983)

SUMMARY

A simple and sensitive method was developed for the separation and quantification of tiazofurin (TCAR) in plasma using 2-β-D-ribofuranosylthiazole-5-carboxamide (iso-TCAR) or [5-^{3}H]TCAR as an internal standard. The procedure uses C_{18} extraction columns to clean-up the plasma samples for measurement by high-performance liquid chromatography. A sensitivity of 0.33 μ*M* (0.08 μg/ml) was easily achieved for 0.5-ml plasma samples. When human whole blood was incubated in vitro with TCAR for 2 h, the plasma concentrations were decreased by 10% (4°C) and 25% (23°C). TCAR could still be measured 22 h after injection of 220 mg/kg into mice. For a two-compartment pharmacokinetic model, the half-lives of TCAR were 18.8 and 412 min.

INTRODUCTION

Tiazofurin (TCAR, 2-β-D-ribofuranosylthiazole-4-carboxamide, NSC 286193, Fig. 1), a thiazole-C-nucleoside, has shown promise as a new anticancer drug. It has demonstrated antitumor activity against several murine leukemias, as well as Lewis lung carcinoma [1,2]. TCAR has recently started Phase I trials in humans at several institutions. The pharmacokinetic studies associated with these Phase I trials require a simple and sensitive method for analysis of TCAR in plasma. Previously published methods for TCAR analysis are either inadequately sensitive for complete pharmacokinetic studies [3,4] or require multiple and/or lengthy procedures [4,5].

This paper describes a simple procedure for the analysis of TCAR in human plasma. The procedure utilizes C_{18} extraction columns for plasma clean-up and measurement by high-performance liquid chromatography (HPLC). Surface-

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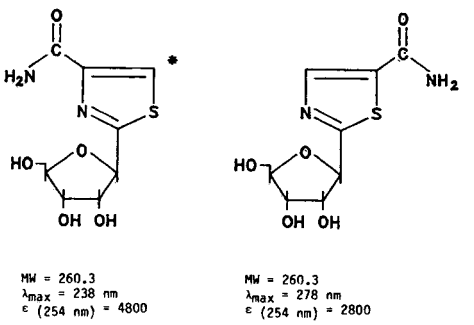

Fig. 1. Structures of TCAR (left) and iso-TCAR (right). The asterisk denotes the position of the tritium label on TCAR, when present.

modified materials (such as the C_{18} bonded phase) are commonly used for biological sample clean-up [6,7], and in some cases are more efficient than classical liquid—liquid extractions [8]. Iso-TCAR (NSC 363223, Fig. 1) is one of several structurally similar C-nucleosides which were synthesized concurrently with TCAR [9,10]. Either tritiated TCAR or iso-TCAR can be used as an internal standard for the procedure.

EXPERIMENTAL

Materials

TCAR, iso-TCAR and [5-^{3}H]TCAR were obtained from the Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD, U.S.A.). The organic solvents, methanol and acetonitrile, were HPLC grade (Burdick & Jackson Labs., Muskegon, MI, U.S A.). The glacial acetic acid was ACS grade (Fisher, Fair Lawn, NJ, U.S.A.). Double-distilled water was used for all solution preparations. The C_{18} extraction columns used were Sep-Pak (Waters Assoc. Milford, MA, U.S.A.) and Baker-10 SPE (J.T. Baker, Philipsburg, NJ, U S.A.).

HPLC conditions

The HPLC mobile phase contained 40 m*M* acetic acid with 1.5% (v/v) acetonitrile. The mobile phase was passed through a 0.45-μm filter to remove any particulate material and to degas the solvent. The solvent was pumped via a Waters Model 6000A pump, isocratically at 2 ml/min, through a Waters Radial-Pak C_{18} column, 5-μm particle size. The column was washed with approximately 450 μl acetonitrile after each injection of extracted plasma by increasing the acetonitrile concentration to 45% for 30 sec via a second pump. There was a 10-min delay after the acetonitrile wash before the next sample injection. A waters 440 fixed-wavelength detector was used to monitor the absorbance at 254 nm.

Sample preparation

On each day of use, a stock TCAR solution was diluted in water to the required concentrations. The standard curves in plasma were prepared by adding aliquots of the diluted stock solutions to pooled plasma obtained from normal

volunteers. The plasma standard solutions ranged from 0.1 μM to 1000 μM TCAR. The volume of plasma standards used for the extraction was 200 μl on the Baker columns and 500 μl on the Waters columns. Each point of the standard curve was processed in triplicate.

All plasma samples and standards received 50 μl of 10^{-4} M iso-TCAR. Alternatively, plasma standard curves were prepared with 50 μl [5-^{3}H]TCAR (100,000 dpm per 50 μl) as internal standard.

Samples of the mouse study with expected plasma TCAR concentrations of 100 μM or greater were diluted 10- or 100-fold in water to avoid overloading of the extraction or analytical columns.

Extraction procedure

A Baker-10 extraction system attached to an unregulated vacuum supply (house vacuum) was used for the extraction. All the washes and sample additions were eluted through the columns by vacuum.

Waters extraction columns

The columns (330 mg C_{18} resin) were first conditioned by passing 4 ml of methanol through the columns, followed by 4 ml of water. The samples were added to the columns and the columns were washed with 4 ml of water. TCAR and iso-TCAR were eluted from the C_{18} columns into collection tubes with 2 ml of methanol. The methanol collections were brought to dryness under a steady stream of nitrogen and redissolved in 150 μl of the HPLC mobile phase.

Baker extraction columns

The columns (107 mg C_{18} resin) were conditioned with 1 ml of methanol, followed by 1 ml water. The samples were added to the columns and 0.5 ml of water was used to wash the columns. Then 0.5 ml of a methanol—water (50:50) solution was eluted through the C_{18} extraction columns into the collection tubes. The methanol collection was dried under a stream of nitrogen and redissolved as before.

Recovery of TCAR and iso-TCAR

The extraction recovery of TCAR and iso-TCAR was determined by extracting a mixture of 100 μM TCAR and 100 μM iso-TCAR in water and normal plasma. For the aqueous samples TCAR and iso-TCAR were measured in each of the extraction column washes. The extraction efficiencies of TCAR and iso-TCAR in the methanol wash were measured for both the aqueous and the normal plasma samples.

To determine whether the extraction efficiency was concentration-dependent, normal plasma was spiked with TCAR and [5-^{3}H]TCAR to give final concentrations of 0.1 μM and 100 μM TCAR. The redissolved extraction residue was mixed with 15 ml liquid scintillation cocktail and counted on a Searle Analytic Mark III (Chicago, IL, U.S.A.) liquid scintillation counter.

TCAR stability in plasma and whole blood

TCAR was added to fresh human plasma to give plasma concentrations of

500 μM and 5 μM. The plasma was gently mixed and 0.5-ml aliquots were transferred into 4-ml soda-lime glass vials. Three of the vials at each TCAR concentration were frozen at −15°C and served as the controls at zero-time. Three samples, from each plasma concentration of TCAR, were gently shaken at 4°C and room temperature (23°C) for 24 h.

Fresh human blood was collected in heparinized tubes and used to prepare 500 μM and 5 μM TCAR solutions. The blood solutions were gently mixed and 1-ml aliquots from each were added to heparinized collection tubes. The tubes were gently shaken at either 4°C or room temperature. Three sample tubes at each TCAR concentration and each temperature were centrifuged at zero-time, 2 h and 24 h. The plasma was separated by centrifugation, transferred into glass vials, and stored frozen at −15°C until analyzed.

Mouse pharmacokinetic study

Plasma levels of TCAR were measured after a single bolus injection into the tail vein of CDF_1 mice, 24—27 g. TCAR powder was dissolved in 5% dextrose in water (Abbott Labs., North Chicago, IL, U.S.A.) and passed through a 0.45-μm filter. The dose of TCAR was 220 mg/kg, which is 1/13 of the single dose LD10 for CDF_1 mice [11]. Each mouse was sacrificed by decapitation and the blood was allowed to flow into a heparinized tube through a polypropylene funnel. Each tube was centrifuged immediately after blood acquisition. The plasma was separated and transferred to a 4-ml soda-lime glass vial. The samples were stored frozen at −15°C until they were analyzed. The volume of standards and samples used for the analysis was 150 μl.

Calculations

Both the TCAR standard curves and the plasma disappearance data from the mice were analyzed on MLAB, a non-linear-fitting program [12].

The standard curve data were fit to the straight-line equation of $y = mx + b$, where m is the slope of the line and b is the y-intercept. The y-value is the peak height ratio (PHR) of TCAR/iso-TCAR and the x-value is the concentration of TCAR. All the points of the standard curve were weighted using $1/(\text{PHR})^2$ as the weighting factor.

The plasma concentration—time data were fitted to biexponential and triexponentional equations:

$$C(t) = A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t}$$

$$C(t) = A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t} + A_3 e^{-\lambda_3 t}$$

where $C(t)$ is the plasma TCAR concentration at time t. The half-lives of each of the phases can be calculated by dividing the natural logarithm of 2 by each of the apparent first-order elimination rate constants (λ_1, λ_2, λ_3). The weighting factor used to fit the exponential equations was $1/(\text{concentration})^2$. The area under the concentration—time curve(AUC) and total body clearance (Cl_{TB}) were calculated by:

$$\text{AUC} = \Sigma\ (A_i/\lambda_i) \text{ and}$$

$$Cl_{TB} = \text{dose/AUC}.$$

RESULTS

Plasma extracts

Fig. 2 shows the chromatograms of normal human plasma extracts. In the

blank plasma (Fig. 2a), the peaks were unidentified plasma components. They did not elute, however, in the same region as TCAR or iso-TCAR (Fig. 2b). The retention times of iso-TCAR and TCAR were 7.8 and 8.6 min, respectively and had a peak-to-peak separation of 45 sec. The peaks of TCAR and iso-TCAR overlapped near their base but were separated enough such that the peak height measurement of either peak was not hindered by the presence of the other (Fig. 2b).

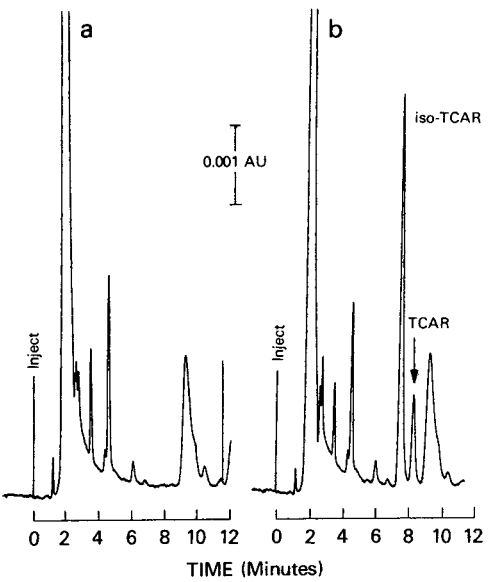

Fig. 2. Chromatograms for 0.5-ml samples of (a) normal human plasma and (b) normal human plasma with TCAR (1 μM) and iso-TCAR (10 μM). The absorbance was measured at 254 nm at an attenuation of 0.01 absorbance units (AU) full-scale. The retention times of TCAR and iso-TCAR were 7.8 min and 8.6 min, respectively. The extracted residue was redissolved in 150 μl HPLC mobile phase, and 50 μl were injected.

Linearity and sensitivity of extraction

The standard curve on the Waters columns with iso-TCAR as internal standard was linear between 0.1 μM and 1000 μM TCAR. The limit of detection was 0.33 μM (0.08 μg/ml). The standard curve obtained from the same standard solutions extracted on the Bakers columns was also linear over the same range. However, the limit of detection was 0.67 μM (0.17 μg/ml). The limit of detection for the two brands of extraction columns was proportional to the initial sample volume extracted on each brand. The standard curves that used [5-^{3}H]TCAR as an internal standard were also linear over the same range.

The size of the TCAR peak at the limit of detection for each of the procedures was 3.3—4% of the full-scale absorbance of 0.01 AU and was approximately ten times greater than the baseline noise.

The coefficient of variation for the standard curves was usually less than 5%. The maximum variation was 11% and occurred at concentrations less than 1 μM.

The y-intercept of the fitted line for each standard curve was less than 1/6

of the mean peak height ratio of the lowest TCAR standards. The deviation from the fitted line for 65 of 69 standard solutions was less than 6%. The maximum deviation from the fitted line was 15% and also occurred at concentrations less than 1 μM.

Extraction recovery

The aqueous 100 μM TCAR—iso-TCAR mixture was used for a total accounting of TCAR and iso-TCAR throughout the extraction procedure (Table I). The cumulative recovery of TCAR and iso-TCAR was close to 100% for both extraction procedures.

The mean recovery of TCAR and iso-TCAR in the methanol washes of the extracted normal plasma using both procedures was within 5% of the value for the methanol washes of the aqueous sample extraction. Also, the recovery of TCAR for each extraction procedure was not concentration-dependent. There was less than 5% difference in the recovery of TCAR from the 100 μM and 0.1 μM plasma solutions.

TABLE I

PERCENTAGE RECOVERY OF TCAR AND ISO-TCAR FROM EXTRACTION COLUMNS

	Waters column				Bakers column			
	Sample addition 0.5 ml	Water wash 4 ml	100% methanol wash 2 ml	Cumulative percent recovery	Sample addition 0.2 ml	Water wash 0.5 ml	50% methanol wash 0.5 ml	Cumulative percent recovery
TCAR	0	28	76	104	2	12	79	93
iso-TCAR	0	33	72	105	3	16	79	98

Dilution of samples

Six of the samples used in the in vitro blood study were measured, diluted 100-fold, extracted and remeasured. The undiluted samples were 500—700 μM. After remeasuring, and correcting for the dilution, there was a deviation of 6 ± 3% between the diluted and non-diluted measurements.

TCAR stability in normal human plasma

In normal plasma TCAR was stable for at least 24 h, regardless of the incubation temperature or the TCAR concentration. TCAR concentrations measured at the end of 24-h incubations were 100 ± 5% of the zero-time samples. In previous studies, TCAR was found to be stable for at least one week at room temperature in solution with mannitol and sodium chloride or 5% dextrose in water [13].

Apparent red blood cell uptake

Table II shows the concentrations of TCAR measured in the plasma of the blood samples. Even at zero-time, TCAR had entered red blood cells. If the red blood cells totally excluded TCAR, the expected plasma concentrations at

TABLE II

PLASMA TCAR CONCENTRATION (μM) OVER TIME FOR GENTLY MIXED HEPARINIZED HUMAN WHOLE BLOOD

Temperature (°C)	Incubation time		
	0	2 h	24 h
4	617 ± 8	556 ± 7	408 ± 4
23		463 ± 0	281 ± 2
4	4.82 ± 0.07	4.39 ± 0.01	3.14 ± 0.04
23		4.33 ± 0.04	2.56 ± 0.06

zero-time would have been 880 μM and 8.8 μM (for the measured hematocrit of 43%). At the end of the 24-h incubation, the mean concentration of TCAR in the separated plasma of the 4°C samples was 66% of the zero-time samples in the 500 μM case and 65% in the 5 μM case. At room temperature the 24-h 500 μM sample was 46% of the initial zero-time sample while the 24-h 5 μM sample was 53%.

Mouse pharmacokinetic study

Plasma samples from the mouse study had measurable TCAR concentrations for the entire 22-h sampling period (Fig. 3). A three-compartment fit to the data resulted in half-lives of 11.5, 38.4 and 497 min. At this dose, a two-compartment model also fit the data. The initial and terminal half-lives for the two-compartment model were 18.8 and 412 min. Both fits gave identical AUC and Cl_{TB} values (Table III) and similar values for the initial and terminal half-lives.

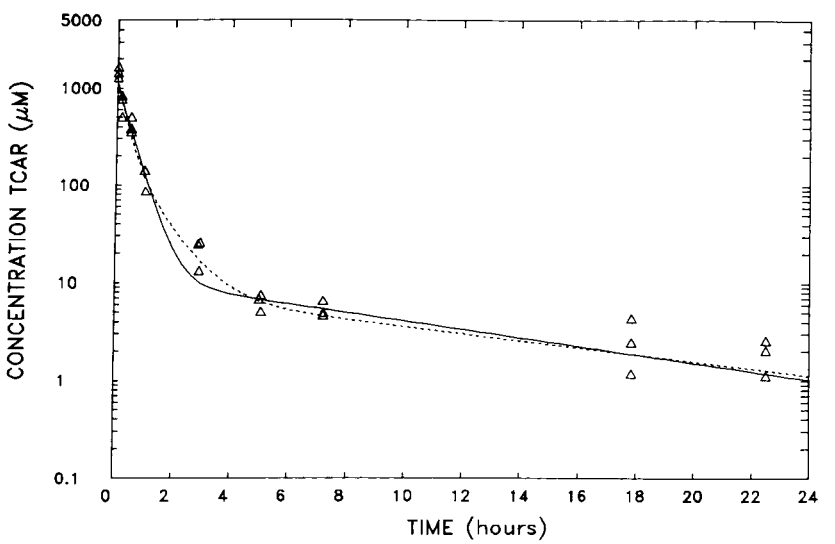

Fig. 3. The disappearance of TCAR in CDF_1 mouse after intravenous bolus injection (220 mg/kg). The triangles represent the measurement of plasma TCAR in a single mouse. The curves are the non-linear fits of the data for two-compartment (solid) and three-compartment (dashed) models.

TABLE III

SUMMARY OF THE PHARMACOKINETIC PARAMETERS OF TCAR IN CDF_1 MICE FOR BOTH TWO-COMPARTMENT AND THREE-COMPARTMENT MODELS

Model	AUC (m*M*—min)	Cl_{TB} (ml/min/m^2)	λ_1 (min^{-1})	λ_2 (min^{-1})	λ_3 (min^{-1})
Two-compartment	35.9	70.6	0.037	0.0017	—
Three-compartment	36.0	70.4	0.060	0.018	0.0014

DISCUSSION

The new analytical procedure for the measurement of TCAR in plasma is simple, sensitive, and reproducible. A large number of samples can be extracted and prepared for injection in a single day. An automatic injector allows the samples to be run on the high-performance liquid chromatograph overnight and the results can be calculated the following day. Either iso-TCAR or [5-^{3}H]-TCAR can serve as an internal standard, but iso-TCAR is more practical, especially for automated analyses.

The limit of detection for the extraction of 0.5 ml plasma is 0.33 μM (0.08 μg/ml). This is obtained by redissolving the extraction residue in 150 μl of HPLC mobile phase, injecting 50 μl into the HPLC system and measuring the absorbance at 254 nm. The limit of detection could be further lowered by injecting a larger fraction of the extract and/or by measuring the absorbance at the TCAR λ_{max} value of 238 nm.

In many clinical settings, blood samples are drawn and stored for several hours before the plasma is separated by centrifugation. If this occurs with blood samples of TCAR the results obtained from the analysis may be misleading. The fate of plasma TCAR in whole blood was shown in the in vitro blood study. After 2 h of incubation at 4°C the concentration of TCAR in the separated plasma was 10% less than the measured TCAR concentration in the zero-time sample, at the higher TCAR concentration. The room temperature sample showed a 25% decrease in plasma TCAR concentration.

The plasma disappearance of TCAR in animals has usually been described by a three-compartment model [14—16]. In the present study, there was little difference between the fits of the two-compartment and the three-compartment models. The major difference occurs at the 3-h time point, where the three-compartment model fit the data more accurately. However, the AUC and Cl_{TB} values were the same for either of the models.

Other HPLC procedures for the measurement of TCAR have been reported. One method proposes multiple liquid—liquid extractions for clean-up of plasma and uses and automated HPLC column-switching technique [5]. Although the column-switching technique can be a powerful tool, it is not readily available to the average researcher. The other methods are apparently less sensitive than the present method. Mouse plasma TCAR was detectable for less than 3 h [3] and 6 h [4] following doses of 200 mg/kg and 250 mg/kg, respectively. The present method measured TCAR in mouse plasma up to 22 h after injection of 220 mg/kg. The plasma TCAR was 1—10 μM in the samples between 3 and

22 h. The importance of this increased sensitivity is underscored by cell culture studies which have shown that TCAR concentrations as low as 5 μM produce 80% inhibition of its target enzyme, inosine monophosphate dehydrogenase [10].

ACKNOWLEDGEMENT

The authors thank Mildred Shoemaker for her technical assistance while performing the mouse study.

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Journal of Chromatography, 307 (1984) 371—379
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2053

SIMULTANEOUS DETERMINATION OF ZOMEPIRAC AND ITS MAJOR METABOLITE ZOMEPIRAC GLUCURONIDE IN HUMAN PLASMA AND URINE

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(First received September 30th, 1983; revised manuscript received December 22nd, 1983)

SUMMARY

A method is described for the simultaneous determination of zomepirac and its primary metabolite, zomepirac glucuronide, in plasma and urine. Reversed-phase liquid chromatography is used with an ion-pairing mobile phase of methanol—tetrabutylammonium hydrogen sulfate. Detection is by UV at 313 nm. Biological samples are cooled immediately, then adjusted to pH 3 to avoid zomepirac glucuronide degradation. Samples (0.5 ml) are then deproteinated with acetonitrile or acetone, the supernatant concentrated and dissolved in acetonitrile—acetate buffer, with up to one half of the sample injected onto the LC system. Recovery is greater than 70% and reproducible. The measurable concentration range is linear from 0.05 to 200 μg/ml. Total elution time of the assay is less than 10 min. Selectivity of zomepirac and zomepirac glucuronide is optimized. Sample preparation prior to analysis so as to prevent zomepirac glucuronide degradation is emphasized.

INTRODUCTION

Zomepirac (Z), 5-(4-chlorobenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid (Fig. 1) is a nonsteroidal anti-inflammatory drug (NSAID) with analgesic effects [1]. Its major metabolite in man is the glucuronic acid conjugate, zomepirac glucuronide (ZG) [2, 3]. A recent report from our laboratory has shown that ZG is unstable at physiological pH in vitro [4]. Preliminary results suggest that ZG is present in plasma at concentrations near that of the parent

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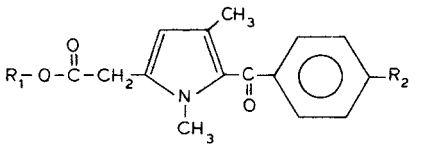

Compound	R_1	R_2
ZG	Glucuronic acid	CL
Z	H	CL
IS	H	OCH_3

Fig. 1. Structures of zomepirac, Z; zomepirac glucuronide, ZG; and the internal standard, IS.

Z. Interest in studying the disposition of ZG led to our development of an analytical method for direct measurement of intact ZG and Z in plasma and urine.

Several methods have been published for determination of Z in plasma and urine. Normal-phase [5], reversed-phase [6—8] and thin-layer chromatography (TLC) followed by gas chromatography—mass spectrometry (GC—MS) [9] have been used. Only one method included a direct analysis of ZG [8]. Using ^{14}C-labeled Z, ZG has been measured following TLC separation [2]. None of the above methods consider the possibility of ZG degradation which can be significant at physiological pH [4]. Not only do all but one of the previous methods not measure ZG, but because of ZG instability, these methods may be overestimating free Z concentrations.

In addition to simple hydrolytic cleavage of ZG to Z, our laboratory has documented the problem of intramolecular acyl migration of ZG [4]. This phenomenon is increasingly becoming recognized as a problem for acyl glucuronides with reports of acyl migration occurring with bilirubin [10], clofibric acid [11], probenecid [12], isoxepac [13], an investigational NSAID from Wyeth [14] and diflunisal [15]. The isomeric products formed from acyl migration of ZG have different liquid chromatographic (LC) retention times. No specific attempt was made to resolve the isomeric conjugates during development of the present LC method, although partial resolution occurred. As described below, the isomers are simply combined as total conjugates in our analysis.

EXPERIMENTAL

Developmental work

Methanol, acetonitrile and tetrahydrofuran were considered the organic modifiers of choice in this investigation. After preliminary tests only methanol proved to have a suitable selectivity between Z, ZG and the internal standard as was determined by measuring selectivity, $\alpha = K'_Z/K'_{ZG}$, where K' is the capacity factor. Further tests were done with methanol in order to obtain the most optimal mobile phase. Data are illustrated in Fig. 2. As is known from previous investigations in our laboratory [4], ZG is most stable

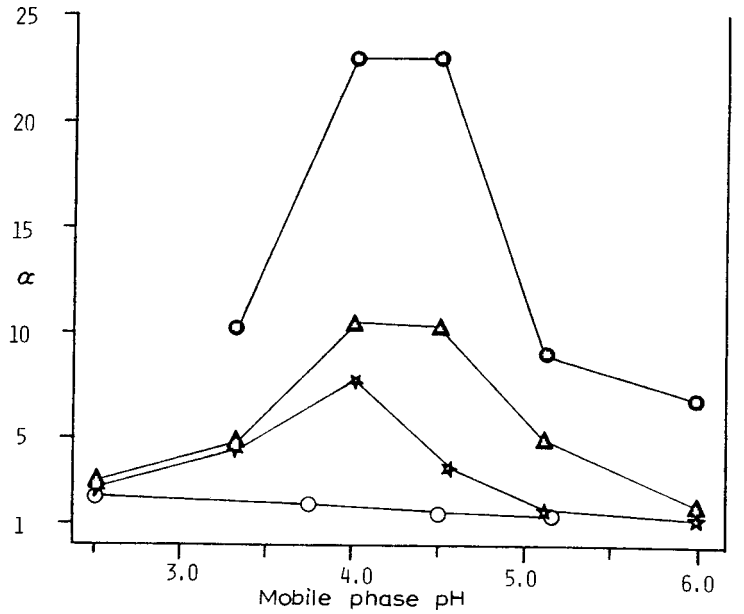

Fig. 2. Selectivity (Z—ZG) as a function of pH and mobile phase organic solvent. •, 40% acetonitrile; ⋆, 50% methanol; ▲, 30% tetrahydrofuran; ○, 58% methanol—ion pair. Column conditions and the ion-pairing buffer are as described in the text. All other buffers were 0.01 *M* acetate.

between a pH of 2 and 5. Because of this and because of a more favorable selectivity with the internal standard, the mobile phase was chosen to be 58% methanol—0.01 *M* tetrabutylammonium hydrogen sulfate and 0.05 *M* sodium acetate at pH 4.5. The facts that acetonitrile—water enhanced the stability of ZG and methanol—water caused loss of ZG to yield the methyl ester [4], accounted for the former being the solvent in which samples were reconstituted prior to injection. The short retention on the column (< 10 min) caused no significant loss of ZG due to the formation of the methyl ester in the methanol—water mobile phase.

Reagents and materials

Zomepirac sodium · $2H_2O$ and 5-(4-methoxybenzoyl)-1,4-dimethyl-1H-pyrrole-2-acetic acid, the methoxy analogue of zomepirac used as the internal standard (IS) (Fig. 1), were kindly supplied by McNeil Pharmaceutical (Springhouse, PA, U.S.A.). ZG was obtained by extraction and purification from urine [4]. Methanol, acetone and acetonitrile, all HPLC grade, were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). All other chemical reagents were of analytical grade. Quantities less than 100 μl were pieptted with glass syringes from Hamilton (Reno, NV, U.S.A.).

Sample preparation

A stock solution of zomepirac sodium · $2H_2O$ was prepared by dissolving 100 mg into 100 ml methanol. By appropriate dilution with methanol, standard solutions of 100 μg/ml and 10 μg/ml were also obtained. A stock solution of ZG was prepared by dissolving 25 mg of this compound into 25 ml

50% acetonitrile—0.01 *M* phosphate, pH 2. By appropriate dilution with 50% acetonitrile—0.01 *M* phosphate, pH 2, standard solutions of 100 μg/ml and 10 μg/ml were also obtained. A stock solution of IS was prepared by dissolving 50 mg of this compound into 100 ml acetonitrile. All stock and standard solutions were stored at −20° C. Standard curves at concentrations of 0.1, 0.3, 0.6, 1.0, 5.0 and 10.0 μg/ml for Z and ZG with a concentration of 3.0 μg/ml for the internal standard, IS, were prepared in plasma. Standard curves with concentrations of 0.1, 0.3, 0.6, 1.0, 5.0 and 10.0 μg/ml for Z, 1.0, 5.0, 10,0, 50.0, 100.0 and 200.0 μg/ml for ZG and a concentration of 10.0 μg/ml for IS were prepared in urine. The standard curves were prepared with blank plasma or urine, buffered to pH 2—4 with phosphoric acid and spiked with a small amount (2.5—100 μl) of the appropriate dilution of the standard solutions.

Each aliquot of 0.5 ml plasma or urine was spiked with the internal standard (plasma: 3 μg/ml IS; urine: 10 μg/ml). Then 1.0 ml acetone or acetonitrile was added and protein precipitation was done by vortexing on a whirlmixer for 30 sec. The precipitate was separated by centrifugation at 2250 *g* (3000 rpm) for 10 min. The supernatant was removed into a clean tube and evaporated under a gentle stream of nitrogen at 30° C. In the case of plasma the pellet was not discarded, but once more treated with 1.0 ml acetonitrile or acetone, vortexed and centrifuged. The supernatant was combined with that of the first sample treatment. The residue was reconstituted into 0.25—1.0 ml 25% acetonitrile—0.5 *M* acetate, pH 4.5, then 50—100 μl were injected onto the high-performance liquid chromatography (HPLC) column. Sample preparation and assay were done on the same day.

Special precautions in sample handling were taken because of the fast degradation of ZG at higher temperatures and pH [4]. During clinical trials blood samples were immediately cooled in ice after collection, red blood cells were separated from plasma in a refrigerated centrifuge and the plasma was transferred into a vial, prebuffered with 10 μl concentrated phosphoric acid (15 *M*) per ml plasma and immediately frozen at −20° C. Urine was immediately buffered with 5 μl concentrated phosphoric acid per ml urine and frozen at −20° C. In addition, volunteers were kept on cranberry juice throughout the study in order to maintain the pH of their urine below 6.

Chromatography

The HPLC system used consisted of an Altex Model 110A pump, a Waters Model 710A Wisp automatic injector, a Waters Model 440A fixed-wavelength UV detector set at 313 nm and an Altex Ultrasphere ODS reversed-phase column (15 cm × 4.0 mm I.D. with 5-μm particles). Chromatograms were recorded on a Spectra-Physics Model SP4100 computing integrator. Area ratios relative to the internal standard, IS, were obtained. The ion-pairing mobile phase (58% methanol—0.01 *M* tetrabutylammonium hydrogen sulfate and 0.05 *M* sodium acetate, pH 4.5) was prepared fresh daily and was filtered through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The column was conditioned with approximately 50 ml mobile phase prior to sample injection. The flow-rate was 1.3 ml/min. The retention times of IS, ZG and Z were approximately 4.5, 6.5 and 8.5 min, respectively (Figs. 3 and 4).

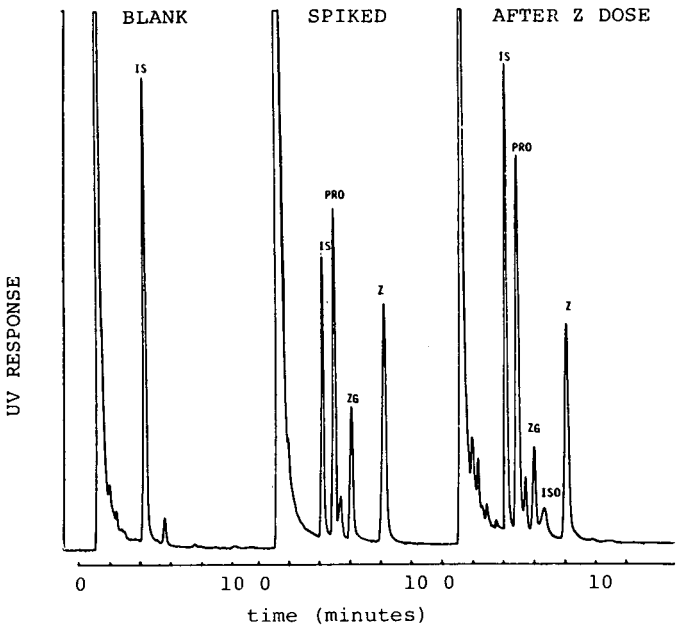

Fig. 3. Chromatograms of blank plasma spiked with 3 μg/ml IS; plasma spiked with IS and 1 μg/ml of Z and ZG with probenecid (PRO); and a plasma sample from a subject, pretreated with probenecid, 1 h after an oral dose of 100 mg of Z as its sodium salt. a.u.f.s. = 0.016.

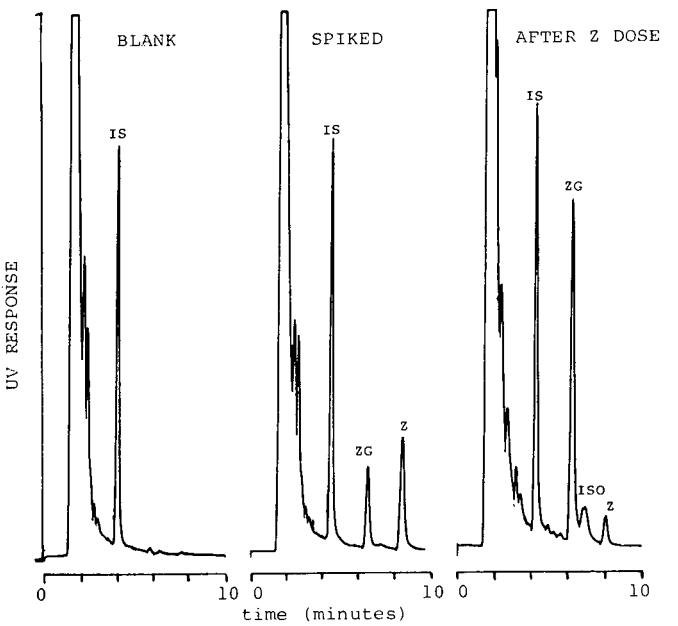

Fig. 4. Chromatograms of blank urine spiked with 10 μg/ml IS; urine spiked with IS and 5 μg/ml Z and ZG; and a urine sample from a subject collected between 8 and 12 h after an oral dose of 100 mg Z as its sodium salt. a.u.f.s. = 0.016.

Quantitation

Standard curves for Z and ZG in plasma and urine have been prepared according to the procedure described above. Area ratios of Z and ZG to the internal standard were plotted against concentrations of Z and ZG. A weighted (1/concentration) least-squares regression analysis was performed on the data. Linearity was tested and confirmed in the concentration range of at least 0.05—30 μg/ml for Z and ZG in plasma and 0.1—200.0 μg/ml for Z and ZG in urine.

RESULTS AND DISCUSSION

Recovery and variability

Recoveries were estimated by calculating the ratio of the slopes of standard curves obtained by protein precipitation, to untreated aqueous standard curves for three independently prepared sets of standards. An external standard was used to determine recoveries. Recoveries (± S.D.) were 74 ± 5% and 82 ± 7% for ZG and Z, respectively, in plasma over the concentration range of 0.1—10.0 μg/ml. In urine recoveries were 90 ± 3% and 95 ± 2% for ZG and Z, respectively, over the concentration range of the urine standard curves.

Intraday variability was calculated from the area ratios at three different concentrations: 0.1, 1.0 and 10.0 μg/ml in plasma and 0.5, 5.0 and 50.0 μg/ml in urine for both Z and ZG. Data are shown in Table I.

Interday variability is given in Table II, where the mean slopes of six,

TABLE I

INTRADAY VARIABILITY IN PLASMA AND URINE AT LOW, MEDIUM AND HIGH CONCENTRATIONS

In all cases $n = 6$.

	Concentration (μg/ml)	C.V. (%)	
		ZG	Z
Plasma	0.10	10.2	14.8
	1.0	12.4	11.8
	10.0	3.72	2.53
Urine	0.50	2.92	1.50
	5.0	0.45	0.40
	50.0	2.01	1.61

TABLE II

INTERDAY VARIABILITY OF THE STANDARD CURVES FOR PLASMA

In all cases $n = 6$.

	Mean slope	S.D.	C.V. (%)
ZG	5.38	0.359	6.67
Z	3.82	0.228	5.98

independently prepared, standard curves for Z and ZG in plasma are given together with the standard deviation and variability. These standard curves were prepared over a two-month time period.

Degradation and stability

Precautions undertaken to maintain an acidic urine pH and the immediate further acidification of plasma and urine samples together with the work-up procedure minimized the degradation of ZG in spiked samples due to sample handling, storage and work-up to less than 4%. However, analysis of clinical samples yielded greater fractions of the ZG degradation products. This could only result from degradation of ZG to the acyl migration products under the apparent in vivo physiological conditions. This degradation was accounted for by measuring the isomers peak (ISO, Figs. 3 and 4) and ZG as total conjugates. Degradation of glucuronides is not an unusual phenomenon among NSAIDs [13—16] nor other drugs that are extensively conjugated to acyl glucuronides [10—12]. However, degradation of NSAID glucuronides in vivo has not been extensively documented.

With Z no noticeable degradation occurred during sample handling, storage and sample preparation. In addition, the stock solutions of Z did not show significant changes in content over a period of five months. A small loss of ZG did occur in the stock solutions (3.2%) over a three-month period. A correction for this loss was made in each batch of samples that was analyzed. The stock solution of IS, the internal standard, also showed a slight degradation of approximately 2% per month. This, however, did not influence the assay since a fresh internal standard solution was prepared for each set of samples that was analyzed.

Application

This assay has been used successfully in a clinical study that investigated the influence of a steady-state drug level of probenecid on Z pharmacokinetics. Probenecid did not interfere with the plasma assay and could be measured together with Z and ZG. Plasma levels of probenecid were in the range of 20—100 μg/ml, much higher than Z or ZG. Because of a much lower molar extinction coefficient for probenecid at 313 nm compared to Z and ZG, the probenecid peak heights were of the same order of magnitude as for Z and ZG. No interference peaks due to endogenous compounds were observed with the clinical samples. In Fig. 5 typical plasma concentration—time profiles of Z and ZG with concomitant probenecid dosing are illustrated for one subject. Plasma concentration—time profiles of Z and ZG after ingestion of 100 mg Z with and without probenecid could be followed up to 25 h after Z administration. In all cases this part of the curve accounted for more than 95% of the total area under the curve.

Probenecid did cause problems with the analysis of urine. Putative metabolites of probenecid in urine eluted before probenecid, interfering with the IS measurement. When this occurred quantitation was done by normalizing peak areas with constant injection volumes. When probenecid metabolites were not interfering the use of constant injection volumes produced reproducible IS areas sufficient for quantitation. The simplicity of the sample preparation for

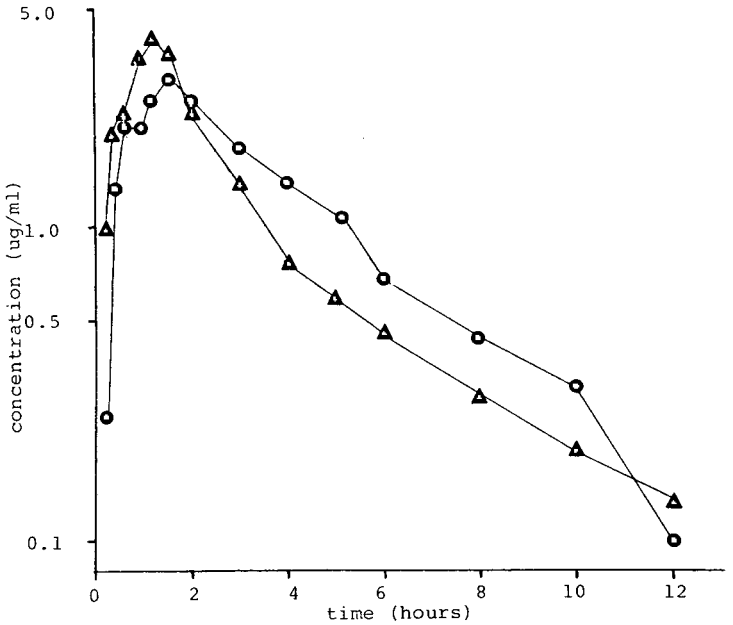

Fig. 5. Concentration vs. time of Z (▲) and ZG (●) in the plasma of a subject who had received 100 mg Z orally as its sodium salt after previous administration of 500 mg probenecid twice daily for four days. Ordinate is a log scale.

urine and the absence of protein in urine accounts for the reliability of this method of quantitation.

This assay was applied in a controlled clinical study. Volunteers were instructed not to take other drugs two weeks prior to and during the study. Other applications of this assay may involve patients who may also take products containing acetaminophen or salicylate. Urine samples collected from subjects after oral doses of acetaminophen and salicylate did not show any interference with this assay method.

Acyl migration

This direct injection assay method for Z and ZG has great advantages over previous reports. Although in a recent report ZG and Z are analyzed simultaneously in urine, no special precautions were taken prior to analysis to prevent loss of ZG [8]. These authors suggest that an anomalous peak in urine may be the putative hydroxylated metabolite of Z. We believe it likely that this peak results from an isomeric conjugate of ZG due to acyl migration. The fact that ZG is degrading, forming other isomers (not susceptible to β-glucuronidase) prior to liberation of the aglycone [4], means that estimation of the glucuronide simply by following formation of the free aglycone will result in error. To what extent degradation of glucuronides is affecting the results of clinical studies with drugs such as Z is not known, because usually no detailed information is given on sample handling or stability prior to analysis. The importance of this is emphasized in this assay and should be considered for further NSAID assays, since degradation of acyl glucuronides is probably a common occurrence among these drugs.

ACKNOWLEDGEMENTS

This research was supported in part by a grant from McNeil Pharmaceutical. During the course of this work, P.C. Smith was supported as an American Foundation for Pharmaceutical Education Merck Fellow.

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Journal of Chromatography, 307 (1984) 380—386
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2027

Note

High-performance liquid chromatographic determination of plasma lactate specific radioactivity

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(First received June 6th, 1983; revised manuscript received December 2nd, 1983)

The supply and utilisation of lactate in vivo can be studied by administration of radiolabelled lactate into the circulation and subsequent determination of the specific radioactivity of plasma L-(+)-lactate [1]. Several different approaches to this determination have been used, including ion-exchange chromatography [2, 3] and radioisotopic dilution analysis [4, 5]. However, methods involving the isolation of lactate from plasma by step gradient ion-exchange chromatography and subsequent enzymatic analysis of the isolated fraction for lactate and liquid scintillation counting of radioactivity content require at least 1.0 ml of plasma or blood and are of possibly variable specificity [2, 3, 6]. While radioisotopic dilution analysis with derivatisation of lactate is more specific, it requires a comparable volume of plasma, is more time-consuming and can involve the removal of a potentially labelled carbon atom, leading to errors in samples of unknown label distribution [5].

To study lactate metabolism in the sheep fetus [7], we attempted to develop a simple and rapid method for the determination of plasma lactate specific radioactivity, which would utilise smaller volumes of plasma than conventional methods, permitting repeated sampling of a limited blood volume. The application of high-performance liquid chromatography (HPLC) to the isolation of lactate seemed an obvious approach because separation of some organic acids by HPLC had already been achieved using octadecyl silica columns at low pH with acetonitrile—water gradients [8] or ion-exchange chromatography [9]. The first method was adopted because it had the adavantages of being inexpensive, simple and rapid. Detection of lactate is difficult, however, due to its lack of absorption in the ultraviolet—visible range. Although conversion of α-keto acids to their 2,4-dinitrophenylhydrazones, which absorb between 380 and 520 nm, has been used to overcome this problem, lactate cannot be

0378-4347/84/$03.00

derivatised in this fashion [10]. The feasibility of monitoring in the low ultraviolet region was therefore examined.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. L-(+)-lactate, Triton X-100, 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) and 2,5-diphenyloxazole (PPO) were obtained from Sigma (St. Louis, MO, U.S.A.). Trichloroacetic acid, orthophosphoric acid, diethyl ether and toluene were obtained from BDH (Port Fairy, Australia). Acetonitrile (HPLC grade) was obtained from Waters Assoc. (Sydney, Australia). Millipore Q reagent grade water was used throughout. L-[U-^{14}C]lactic acid, sodium salt (1.85—5.5 GBq/mmol) was obtained from Amersham Australia (Sydney, Australia).

Preparation of samples

Samples (5.0 ml) of heparinised blood were obtained from sheep fetuses with indwelling vascular catheters during late gestation [7]. Following centrifugation at 4°C and 2500 *g* for 15 min, plasma was removed and stored at —20°C until extraction.

Aliquots (0.1—0.5 ml) of plasma were deproteinised by the addition of 0.2 ml of 14% (w/v) trichloroacetic acid at 4°C. After vigorous mixing, samples were incubated at 4°C for 5 min, then centrifuged at 4°C and 2500 *g* for 20 min. The supernatants were poured into 1-ml disposable plastic syringes attached to pre-equilibrated C_{18} Sep-Pak cartridges (5 ml of acetonitrile followed by 15 ml of water) and washed through with water (see Fig. 1). Drops 11—55 were collected for further processing. The eluent was extracted three times with an equal volume of water-saturated diethyl ether in glass-stoppered tubes (10 ml) on a rotary mixer for 5 min. The water phase was then removed and freeze-dried. Lyophilised extracts were stored at —20°C until analysis.

Apparatus

A Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.) was used for solvent delivery. Injections were made using a Waters U6K universal injector. The eluent was monitored at 190, 220 and 245 nm using a Waters variable UV/Vis detector, Model 440, and the signal recorded on an Omniscribe recorder (Houston Intruments).

Chromatography

Plasma extracts (reconstituted in 50 or 100 μl of 0.01 *M* phosphate buffer of the same pH as the mobile phase) and lactate standards (in mobile phase) were chromatographed on a 4.6 × 250 mm μBondapak octadecyl silica (10 μm) column (Waters) with a mobile phase of 0.01 *M* phosphate buffer at varying pH. Aliquots of 1—10 μl were injected. The mobile phase of 0.01 *M* phosphate buffer was prepared by diluting 1.0 ml of orthophosphoric acid to approximately 50 ml, adjusting the pH appropriately with 2 *M* KOH, then diluting to 1000 ml with water, with a final adjustment made to give the required pH. A flow-rate of 2.0 ml/min was used with a back-pressure of 1950 p.s.i.

Protection of the column with a guard column (C_{18}/Corasil, Waters) was necessary to minimise the hydrolytic action of the low-pH mobile phase. Storage of the column in water only and use of the guard column resulted in little change of retention time with extensive use.

Peak areas were calculated by multiplying peak height by peak width at half height. All results are given as means with standard errors.

Measurement of radioactivity content in chromatography eluent

Chromatography eluent was collected in scintillation vials as 5-sec fractions for 4 min from the time of injection. Scintillation fluid (4 g PPO, 0.1 g POPOP, 330 ml Triton X-100 made up to 1 litre with toluene) (10 ml) was then added to each fraction. Samples were left to stand for 12 h in the dark before measurement of radioactivity in a Packard Tri-Carb liquid scintillation spectrometer. A quench curve was constructed using the external standards channels ratio method, and corrections for quenching were made.

RESULTS

Sample preparation

To prepare an extract suitable for HPLC, plasma was deproteinised, then hydrophobic components that would have accumulated on the analytical column were removed by C_{18} Sep-Pak chromatography (Fig. 1). The deproteinising agent, trichloroacetic acid, was itself removed by extraction into diethyl ether as it would have rendered lyophilisation and reconstitution in a small volume difficult. It also interfered with the chromatographic analysis and attacked the analytical column packing, reducing column efficiency with use. Deproteinisation with perchloric acid and neutralisation with potassium hydroxide were not feasible due to interference in detection of lactate. Recovery of lactate in the final reconstituted extract (as estimated by extracting ten plasma samples to which 67 pmol, 0.37 kBq L-[U-^{14}C]lactate had been added) was 94 ± 1.4% (S.E.M.).

Chromatographic analysis

To optimise the chromatographic separation of lactate from other compounds present in the plasma extracts, the pH of the mobile phase was varied from 2.3 to 3.5 and the eluent monitored at 190 nm. Baseline separation of a peak with the same capacity factor (k') as pure lactate was achieved with a mobile phase of pH 2.7 (Fig. 2). Accordingly, this optimum pH was adopted in subsequent studies.

Chromatography of pure amino acid standards — alanine, glutamine and glutamate — under these conditions indicated that they eluted just after the void volume at approximately 1.4 min (k' 0.001—0.065) as did the carbohydrates glucose and fructose (k' 0.064—0.069), and could be collected for further analysis if required.

Some trichloroacetic acid was still present in plasma extracts as indicated by a large peak eluting with the characteristic retention time of 4.5 min (k' 2.46). Elution of all compounds present was complete within 6 min of injection, allowing analysis of at least 60 samples plus standards per day.

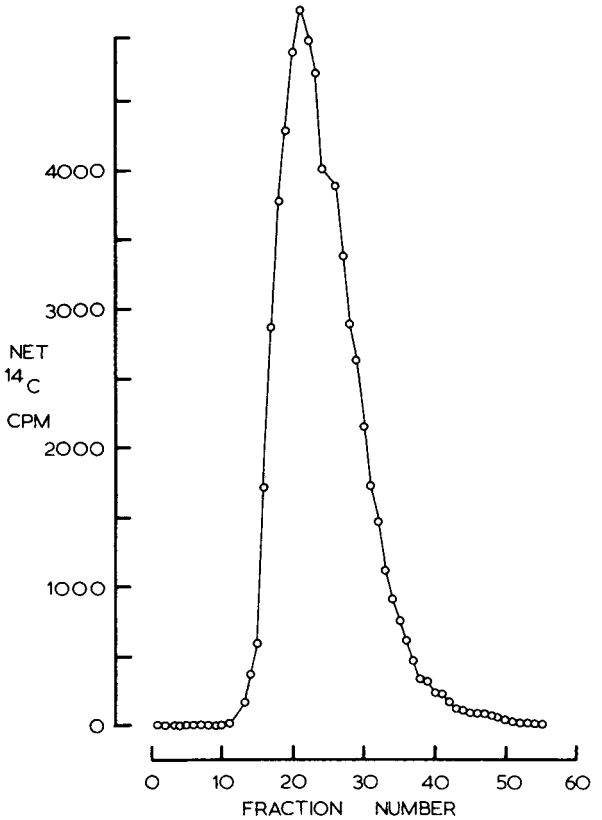

Fig. 1. Elution profile of L-[U-^{14}C]lactate in deproteinised plasma applied to a C_{18} Sep-Pak cartridge followed by washing with water. Fractions consisting of one drop were collected into scintillation vials and radioactivity content was measured as described for chromatography of eluent in text.

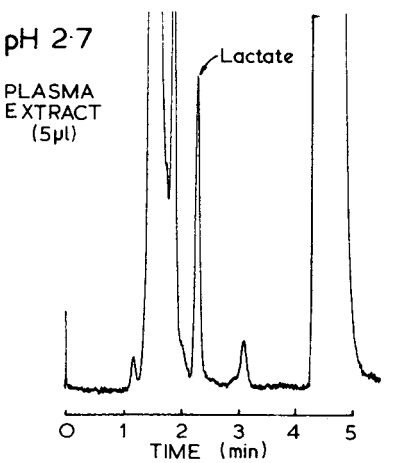

Fig. 2. HPLC of plasma extracts (reconstituted in 100 µl of appropriate mobile phase) at pH 2.7. Conditions: column, µBondapak C_{18} 4.6 × 250 mm; mobile phase, 0.01 *M* phosphate buffer of varying pH; flow-rate, 2.0 ml/min; detection wavelength, 190 nm; a.u.f.s., 0.04; temperature, 22°C.

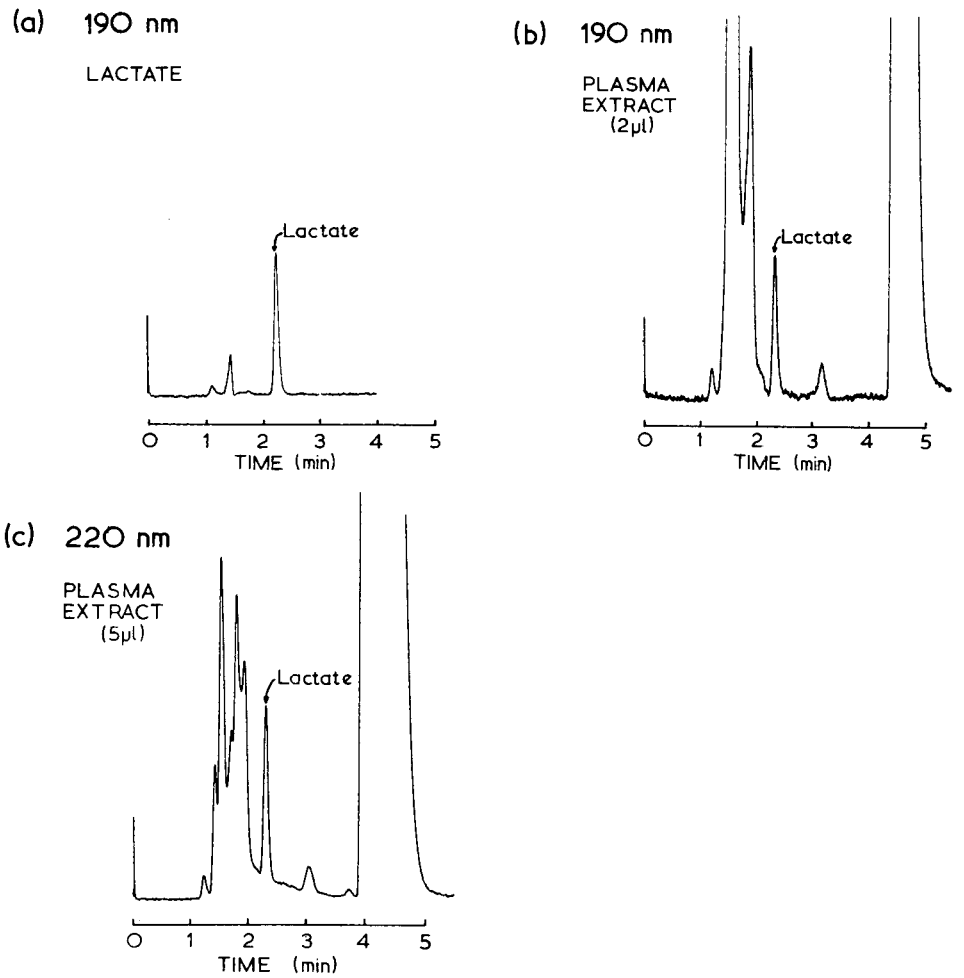

Fig. 3. HPLC of (a) lactate with detection at 190 nm, and of a plasma extract with detection at 190 nm (b) and 220 nm (c). Conditions: column, µBondapak C_{18}, 4.6 × 250 mm; mobile phase, 0.01 *M* phosphate buffer, pH 2.7; flow-rate, 2.0 ml/min; a.u.f.s., 0.04; temperature, 22°C.

The elution profiles of plasma extracts at different wavelengths were then examined (Fig. 3). As expected, significant absorbance by lactate in plasma extracts was seen only with the low-ultraviolet wavelengths, 190 and 220 nm (Fig. 3) and not with detection at 254 nm. The former was selected for use as it gave greater sensitivity and a more stable baseline (Fig. 3).

The specificity of the separation was examined by comparing retention times and peak area ratios (190 nm/220 nm) of lactate standards and the putative lactate peak in chromatographed plasma extracts (Table I). No significant differences in capacity factors or peak ratios were seen, indicating that lactate was in fact completely separated from other components in the extracts. Co-chromatography of pure lactate with plasma extract resulted in no change in retention time and capacity factor.

A linear response of peak area to lactate concentration was observed and the

TABLE I

IDENTIFICATION OF LACTATE IN PLASMA EXTRACTS FOLLOWING HPLC

Conditions were as described in Fig. 3. Results are expressed as mean ± S.D. (n = 5).

Sample	Capacity factor (k')	Peak area ratio (190 nm/220 nm)
Lactate	0.60 ± 0.02	2.35 ± 0.06
Plasma extract	0.59 ± 0.01	2.37 ± 0.10
Plasma extract plus lactate	0.59 ± 0.02	

limits of sensitivity under these conditions was 0.5 μg. The coefficients of variation of three determinations of the following lactate standards, 1, 4 and 10 μg, were 1.7, 0.9 and 0.8%, respectively.

Measurements of radioactivity content

The ^{14}C radioactivity content of lactate in chromatographed plasma extracts was estimated by summing all activity above background that eluted with the same retention time as the isolated lactate peak. For routine analysis, the peak was collected in bulk as one fraction. A quench curve was prepared using the external standards channels ratio method to correct for quenching. However, the efficiency of counting of eluent fractions only varied between 79.0 and 79.5%. The specific radioactivity of lactate in plasma to which 67 pmol, 0.37 kBq L-[U-^{14}C]lactate were added per 0.5 ml aliquot prior to extraction was 0.348 ± 0.028 kBq/μmol (n = 10), giving a coefficient of variation of 3.2% for the determination of plasma lactate specific radioactivity.

DISCUSSION

The determination of plasma lactate specific radioactivity by high-performance liquid chromatography on octadecyl silica is both rapid and precise. The technique requires only small volumes of plasma compared to conventional methods because of its high sensitivity in quantitation. Thus the minimum plasma volume extracted is usually limited only by the radioactivity content of lactate in the plasma sample. The addition of a suitable internal standard prior to extraction would also permit simultaneous determination of plasma lactate concentrations if required.

ACKNOWLEDGEMENTS

We thank Margaret Bell for typing the manuscript. This work was supported by the National Health and Medical Research Council of Australia.

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Journal of Chromatography, 307 (1984) 387—392
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2033

Note

Separation and determination of diacylglycerols as their naphthylurethanes by high-performance liquid chromatography

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(First received August 5th, 1983; revised manuscript received December 13th, 1983)

Natural glycerolipids are made up of complex mixtures of molecular species, which are believed to influence the biological properties of the cell membranes containing them [1, 2]. The immediate precursors in the biosynthesis of glycerolipids like phosphatidylcholine, phosphatidylethanolamine and triacylglycerol are the intramicrosomal diacylglycerols [3—5]. The species pattern of the phospholipids changes with physiological activity [6] and may be subject to metabolic regulation [7]. Much effort has therefore been expended in elaborating appropriate methods for the analysis of the species pattern and for detecting changes in it.

The present communication describes a quantitative resolution of the *sn*-1,2-diacylglycerol moieties by high-performance liquid chromatography (HPLC) in the picomole range after derivatization with α-naphthylisocyanate. Besides the separation of a mixture of commercial diacylglycerol species the separation of diacylglycerols obtained from phosphatidylcholine of rat liver microsomes by phospholipase C treatment is reported.

MATERIALS AND METHODS

Chemicals

Dimethylformamide (DMF) was purified by distillation with octadecylisocyanate. α-Naphthylisocyanate was purified by distillation in vacuo. The standard compounds 1,2-dimyristoyl-, 1,3-dimyristoyl-, 1,2-dipalmitoyl-, 1,3-dipalmitoyl-, 1,2-distearoyl-, 1,3-distearoyl and 1,2-dioleoylglycerol were purchased from Applied Science Labs. (State College, PA, U.S.A.).

0378-4347/84/$03.00

Separation of diacylglycerols from phosphatidylcholine

Rat liver microsomes were extracted according to the procedure of Bligh and Dyer [8] and the lipid extract was separated by two-dimensional thin-layer chromatography with chloroform–methanol—conc. ammonia (130:50:10) in the first dimension and chloroform—acetone—methanol—acetic acid—water (60:80:20:20:10) in the second dimension. The phosphatidylcholine-containing spot was extracted with chloroform–methanol (1:4). The solvent was evaporated in a stream of nitrogen at 40°C and the phosphatidylcholine was treated with phospholipase C (*Clostridium welchii*) from Sigma (St. Louis, MO, U.S.A.) as described in ref. 9. The diacylglycerols were purified by thin-layer chromatography with hexane—diethyl ether—acetic acid (70:30:4) as solvent.

Derivatization procedure

Ten to fifty nmol of the standard mixtures of diacylglycerols or diacylglycerols from the biological source were solubilized in chloroform—methanol (2:1) and transferred into the reaction vials. After evaporation to dryness in a stream of nitrogen the diacylglycerols were dissolved in 100 μl of DMF. A 200-fold molar excess of α-naphthylisocyanate and a four-fold molar excess of 1,4-diazabicyclo(2,2,2)octane were added to this solution. The stoppered vial was heated at 85°C for 2 h. After cooling to room temperature, excess reagent was destroyed by addition of 10 μl of methanol. After 10 min the reaction mixture was centrifuged and aliquots of 10—40 μl from the clear supernatant were applied to the column.

HPLC separation

The HPLC separations were carried out on a Hewlett-Packard Model 1084 B high-performance liquid chromatograph equipped with a variable-wavelength detector (190—600 nm). For fluorescence detection a Fluorichrom (Varian, Los Altos, CA, U.S.A.) was used (excitation 280 nm, emission 360 nm).

A Hewlett-Packard chromatographic column (200 × 4.6 mm) was used which was packed either with LiChrosorb RP-8 (10 μm) or with RP-18 (5 μm) supplied by Merck (Darmstadt, F.R.G.). The solvent system for the separation on the RP-8 column was acetonitrile—water (83:17). After 5 min the composition of the solvent was changed to 87% acetonitrile and 13% water. For the separation of the complex mixture of diacylglycerols an RP-18 (5 μm) column was used. According to the preconditions the solvent system was in this case a mixture of acetonitrile (solvent A) and acetonitrile—water (80:20) (solvent B). A linear solvent gradient was run from 50% B to 20% B between 0 and 75 min which produced a linear gradient of water running from 10% to 4%. The flow-rate was in all experiments 1 ml/min and the column temperature was set at 60°C.

Gas—liquid chromatography

For gas chromatographic analysis of the separated peaks a Varian 2100 instrument with a flame-ionization detector and a data system CDS 101 was used. A coiled glass column (1.8 m × 2 mm I.D.) was packed with 10% EGSS-X on Gas Chrom Q 100—120 mesh. The separation was carried out at a temperature of 150°C for 9 min and then with a temperature programme up to 190°C (1°C/min). The carrier gas was nitrogen (flow-rate 20 ml/min).

RESULTS AND DISCUSSION

Various methods for the analysis of diacylglycerol species are described in the literature. The most common method for the species analysis based on argentation thin-layer chromatography [5, 7, 10, 11] allows the separation of the molecular species according to the degree of unsaturation, but such techniques are time-consuming and require much larger amounts of sample than are conveniently available from routine preparations of cell compartments or bioptic materials. Another method is the analysis of diacylglycerol species as their *tert.*-butyldimethylsilyl ethers by a combination of capillary gas chromatography and mass spectrometry [12]; this, however, is very expensive and time-consuming. Also a method for the analysis of these species using capillary gas chromatography alone has been published [13]. The analysis of low concentrations of diacylglycerols by HPLC using sensitive ultraviolet or fluorescence detection is only possible after derivatization. The separation of *p*-nitrobenzoyl derivatives of diacylglycerols has already been described [14]. The detection limit of this method was in the nanomole range for each species and only commercial standard compounds were investigated. The reaction of alcoholic hydroxyl groups with α-naphthylisocyanate forming urethanes was used for the analysis of alcohols and drugs containing hydroxyl groups [15, 16].

Our aim was the application of this derivatization procedure to 1,2-diacylglycerols. In order to establish the optimum conditions for derivatization, the influence of excess reagent, reaction time, temperature and the usefulness of 1,4-diazabicyclo(2,2,2)octane as catalyst were determined. For the formation of the naphthylurethanes of alcoholic compounds other than diacylglycerols a 1.5- or 30-fold molar excess of the reagent was found to be optimal [15, 16]. For the less reactive diacylglycerols we found that a 200-fold excess of the reagent was the optimal condition.

Using these conditions the diacylglycerol urethanes have sufficient stability after centrifugation and no acyl migration was observed. The reaction with *sn*-1,3-diacylglycerols shows no differences compared with the reaction of *sn*-1,2-diacylglycerols.

Determination of the detection limit of diacylglycerols as well as linearity and reproducibility of the derivatization were carried out with *sn*-1,2-dipalmitoylglycerol separated on an RP-8 (10 μm) column. The detection limit with ultraviolet detection at 290 nm was 100 pmol of diacylglycerol. With the fluorescence detector the detection limit was at least ten times lower. The linearity of the derivatization ranged from 0.2 to 50 nmol, which is in the range of biochemical interest. A comparison of the different standard compounds, dioleoylglycerol and dipalmitoylglycerol, showed that there was no preference for either one of the species in the reaction with α-naphthylisocyanate. Investigation of five identical samples of 1,2-dipalmitoylglycerol demonstrated the good reproducibility of our derivatization procedure (coefficient of variation 3.1%). To exclude differences in the yield of the reaction, the quantitation of biological mixtures was based on 1,2-distearoylglycerol as internal standard. This diacylglycerol can be used because it was not present in our biological samples.

As shown in Fig. 1 the mixture of standard compounds was completely separated with good peak shape under our conditions. As expected from the

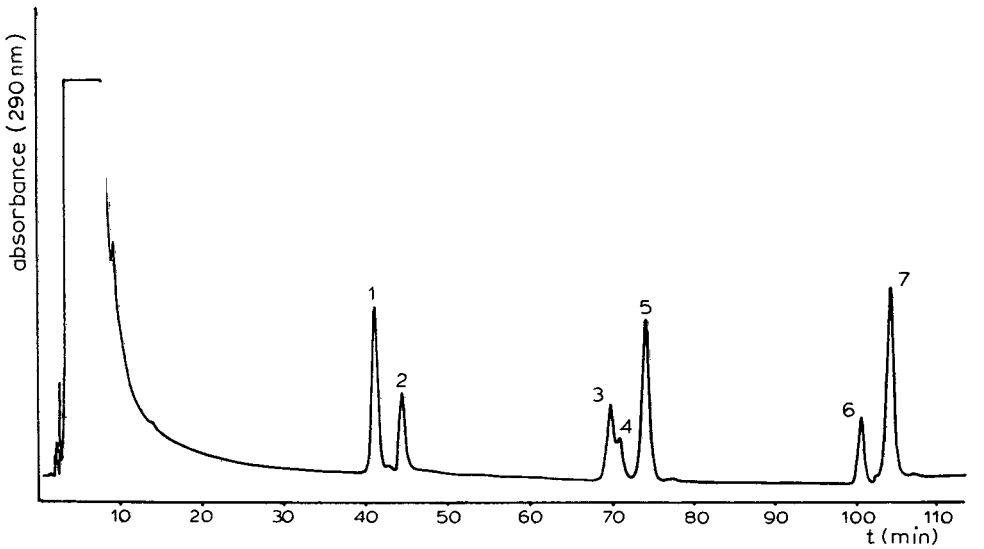

Fig. 1. HPLC separation of diacylglycerol naphthylurethane standards. 1 = 1,3-dimyristoyl-, 2 = 1,2-dimyristoyl-, 3 = 1,3-dipalmitoyl-, 4 = 1,2-dioleoyl-, 5 = 1,2-dipalmitoyl-, 6 = 1,3-distearoyl-, 7 = 1,2-distearoylglycerol napthylurethane.

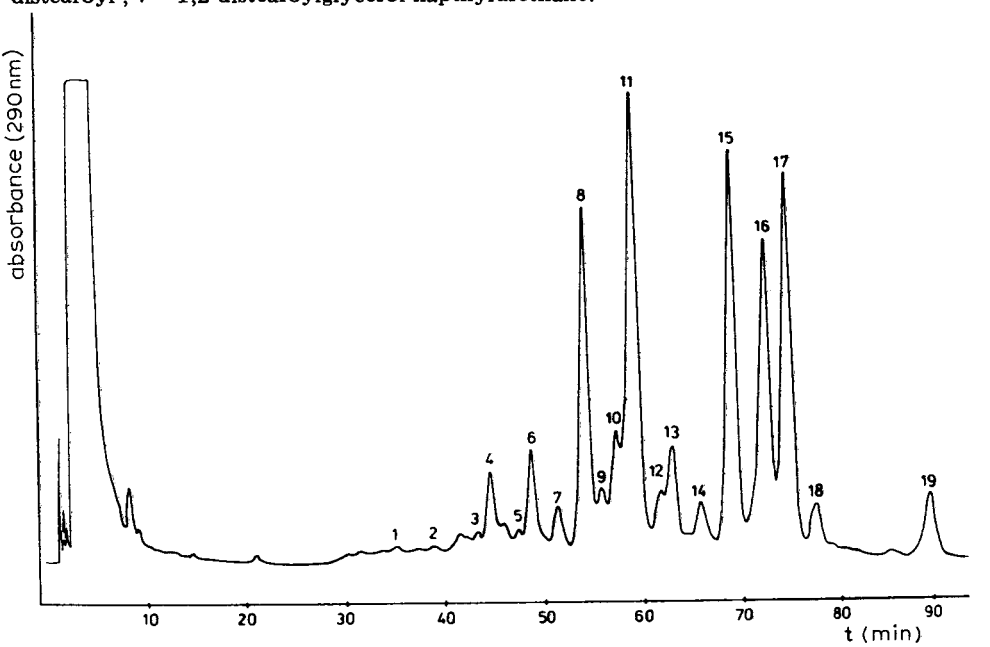

Fig. 2. HPLC separation of diacylglycerol naphthylurethanes from phosphatidylcholine of rat liver microsomes. Peaks are numered in sequence of elution time and are listed in Table I.

literature, the unsaturated diacylglycerols were eluted earlier than the saturated compounds with the same carbon number. In each case the 1,3-diacylglycerols were eluted before the 1,2-isomers. We applied our method to the analysis of a biological pattern of diacylglycerols using phosphatidylcholine from rat liver microsomes hydrolysed by phospholipase C. The species pattern of phospha-

tidylcholine from this source is well known by species separation with other analytical techniques. Identification of the single peaks of the separation pattern of biological diacylglycerols requires a second analytical method because only a few standard compounds were available. For a correct identification of the HPLC-separated species we collected each peak from at least five runs and determined the fatty acid composition of the single peaks by gas chromatographic analysis. As shown in Fig. 2, the diacylglycerol moieties of phosphatidylcholine from rat liver microsomes were fractionated into nineteen peaks related to 24 molecular species. This number was much higher than that obtained by conventional techniques such as argentation thin-layer chromatography. The distribution of the molecular species of phosphatidylcholine in rat liver microsomes is listed in Table I. As shown by gas chromatography not all peaks represent single diacylglycerol species. The major components were 16:0—20:4, 16:0—18:2, 16:0—18:1, 18:0—20:4 and 18:0—18:2. They represent nearly 70% of the detected peaks. As shown by comparison with standard compounds and by gas chromatographic fatty acid analysis, peaks 7, 9 and 14 represent 1,3-diacylglycerol species which were formed during the isolation of diacylglycerols. Comparison of our results with previously reported values [17, 18] shows a good agreement in the distribution of the major components, whereas Patton et al. [19] described a surprisingly low amount of the 16:0—18:1 species with 1.4% in phosphatidylcholine from rat liver.

TABLE I

DISTRIBUTION OF MOLECULAR SPECIES IN PHOSPHATIDYLCHOLINE FROM RAT LIVER MICROSOMES

Peak number*	Molecular species	Percentage composition
1	14:0—22:6	0.16
2	14:0—20:4	0.21
3	16:1—18:2	0.55
4	16:0—20:5, 18:2—18:2	2.5
5	15:0—18:2	0.55
6	16:0—22:6, 16:1—18:1	3.3
7**	16:0—20:4	1.5
8	16:0—20:4	11.3
9**	16:0—18:2	1.8
10	16:0—16:1	3.7
11	16:0—18:2, 18:0—20:5, 16:0—22:5, 18:1—18:2	19.1
12	16:0—20:3	1.8
13	18:0—22:6	3.8
14**	18:0—20:4	2.0
15	18:0—20:4	14.4
16***	16:0—18:1	13.9
17	18:0—18:2	14.3
18	18:0—20:3	2.0
19	18:0—18:1	3.0

*See Fig. 1.
**These peaks represent 1,3-diacylglycerol species.
***1,2-Dipalmitoylglycerol overlaps with this peak.

In agreement with the results of other groups [17, 18] we found in the fatty acid pattern of phosphatidylcholine from rat liver microsomes 8.5% of the oleic acid. We found also this percentage of 8.5% of the 18:1 in the fatty acid pattern of the diacylglycerols produced by phospholipase C treatment of the phosphatidylcholine from rat liver microsomes. The methods described in the literature to determine the species composition of phospholipids by HPLC were based on the quantitation of the peaks by analysis of the phosphorus content of each peak [18, 19]. The sensitivity of this method is limited by the sensitivity of the phosphate determination. This indirect way of quantitation was necessary because the absorption at 206 nm varied with the degree of unsaturation in the single species and their peak areas as a basis of quantitation were not comparable. We believe that because of the derivatization the importance of our method is the direct measurement of the species by ultraviolet absorption or fluorescence. This is combined with an increase in sensitivity to the picomole range.

Moreover, using radioactively labelled precursors, our method allows the measurement of the biosynthesis of diacylglycerols or of phospholipids which can be converted to the diacylglycerols by collection of the single peaks and determination of their radioactivity.

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Journal of Chromatography, 307 (1984) 393—398
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2055

Note

Automated anion-exchange chromatographic method for the quantitation of lactulose in physiological fluids and processed milks

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(First received October 3rd, 1983; revised manuscript received December 14th, 1983)

Lactulose (4-O-β-D-galactopyranosyl-D-fructofuranose) occurs in lactose containing solutions and is present in the body fluids of human subjects ingesting processed milks and milk products [1, 2]. It is formed by the non-enzymic conversion of the glucose half of the lactose molecule (4-O-β-D-galactopyranosyl-D-glucopyranose) to fructose, in alkaline solution especially if heated. It is not actively transported or hydrolysed by the human intestine and produces osmotic and fermentative diarrhoea when given in a concentrated syrup as a laxative [3]. Pre-packed liquid milks used for infant feeding are sterilised by heating, which promotes lactulose production and its content in these products may reach concentrations which cause or encourage diarrhoea in sensitive patients [4]. Accurate measurement of the small amount of lactulose which diffuses across the gut wall, and is excreted unchanged in the urine after an oral load, has been used to assess mucosal permeability in gastro-intestinal diseases [5].

Lactulose is difficult to separate from other commonly occurring sugars by thin-layer chromatography, but can be resolved by overnight paper chromatography and can be identified by its reaction with keto-specific reagents such as naphtho-resorcinol [1]. However, quantitation requires spectrophotometric scanning of the reacted spots under strict conditions [6], or elution, derivative formation and gas—liquid chromatography [7]. A rapid, automatic, quantitative method for the estimation of lactulose, even in the presence of massive amounts of lactose, has been developed using anion-exchange liquid chromatography of the borate complexes of sugars and a modified copper-bicinchoninate detection reagent [8]. The technique can be performed on an

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amino acid analyser with the appropriate resin and reagents, and minor modification to the reaction manifold of the instrument [9].

EXPERIMENTAL

Reagents

Lactulose and other reference sugars were obtained from Sigma London (Poole, U.K.), disodium bicinchoninate from Pierce and Warriner (Chester, U.K.) and the anion resin Aminex A-29 (8% divinylbenzene, 7 ± 1 μm diameter beads) from Bio-Rad (Watford, U.K.). The mixed resin used for sample preparation (Duolite MB 5113) and AnalaR grade chemicals for buffers and reagents were obtained from BDH (Poole, U.K.).

Sample preparation

The mixed ion-exchange resins were converted to the hydrogen and acetate forms and dried as previously described [1, 6]. Calibration and internal standard solutions of sugars were prepared in 0.25 m*M* thiomersal and stored at 4°C. This preservative was removed by the sample preparation resin and so desalted solutions were stored frozen. They were stable for at least three months if stored at −16°C. Dried milks (1 g) were homogenised with thiomersal solution (10 ml). These solutions and liquid milks (1 ml) were added to de-ionised water (7 ml) in a test tube. Internal standard (1 ml of 10 m*M* xylose) and saturated aqueous picric acid (1 ml) were added and the tube thoroughly mixed. After centrifuging (1500 *g* for 5 min), the extract (2 ml) was transferred to another tube and dry, mixed ion-exchange resin was added to approximately 60% of the final volume to remove excess picric acid and other ions including sugar phosphates, amines and uronic acids [1, 6]. After shaking (5 min) and centrifuging, the clear, colourless solution (0.4 ml) was mixed with the strong borate buffer (1 *M*) used for column elution (0.1 ml, see below) and loaded into the sampler cups of the apparatus. Frozen faeces (2 g) were homogenised with acetone (2 ml) and 5 m*M* palatinose internal standard (2 ml). After centrifuging, the extract (3 ml) was de-ionised with resin. A portion of the supernatant (1 ml) was shaken with an equal volume of chloroform to remove the final traces of lipid. Following centrifugation, part of the upper phase (0.4 ml) was transferred to a small flask, evaporated to dryness (50°C, rotary evaporator) and the residue dissolved in strong borate buffer (1.0 ml) which was loaded into the sampler cup.

Urine (2 ml) was mixed with 2 m*M* palatinose (1 ml) and this solution was de-ionised and borate added as described for milk. Calibration standards were treated as liquid samples and diluted, de-ionised and buffered as described above.

Chromatography

The analysis was performed on a Chromaspek J 180 automatic ion-exchange chromatograph with a high-pressure sample loop loader (Hilger Analytical, Margate, U.K.). The complete cycle time, including regeneration, was 1 h using a heated (69°C) 350 × 3 mm column of anion-exchange resin (A-29) eluted at 0.25 ml/min. The sample (100 μl) was loaded 18.5 min after the start of the

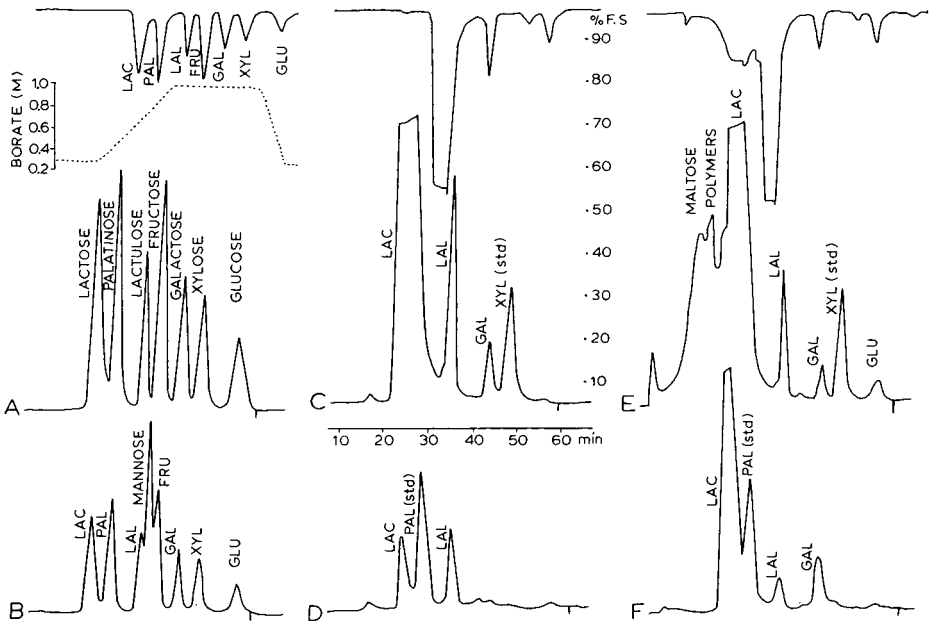

Fig. 1. Chromatograms of lactulose-containing mixtures. Upright traces 570 nm, inverted traces 440 nm. A and B, standard mixtures; C, pre-packed milk; D, faecal sample; E, pre-packed milk with added malto-dextrose; F, urine sample.

regeneration period of the cycle. Sugars were eluted using a gradient of increasing borate molarity and, to a less extent, pH, by mixing a weak (5.6 g borax, 4.4 g boric acid per litre, pH 8.5) and a strong (56 g borax, 19 g boric acid per litre, pH 8.7) buffer as determined by the profile of the rotating drum of the apparatus (Fig. 1).

Detection

The colour reagent of Sinner and Puls [8] was modified to maintain sensitivity in the presence of a higher concentration of borate ions. Potassium carbonate (200 g) was dissolved in approximately 900 ml of de-ionised water. This solution was heated to 70—80°C and 1.2 g of sodium bicinchoninate added. When this had completely dissolved, the solution was cooled and made up to 1 litre. Copper reagent (43 ml, 6.8 g copper sulphate + 26.4 g citric acid per litre) were added, the solution mixed and allowed to stand at least 30 min before use. If stored refrigerated this solution was stable for fourteen days. At room temperature the absorbance of this solution slowly increased after two days.

The column effluent (0.25 ml/min) was introduced into a stream of 2 *M* potassium carbonate (0.15 ml/min) which had been segmented with nitrogen (0.25 ml/min). This stream passed through a mixing coil, colour reagent (0.4 ml/min) was introduced and after a further preliminary mixing coil, it was heated at 90°C for 9 min whilst passing through a coil in an oil bath (Fig. 2). The absorbance of this partially cooled solution was measured at 570 and 440 nm in the flow colorimeter. The heights of the chart recorder peaks relative to

TABLE I

THE LACTULOSE CONTENT OF VARIOUS NATURAL SAMPLES

Source	Lactulose (m*M*)											
	n	Mean	Range	Reference values [2]	Recovery (%)				Between-batch imprecision			
					Conc. range	*n*	Mean	Range	*n* − 1	Mean	± S.D.	C.V. (%)
SMA — Gold Cap (Wyeth)	7*	11.82	10.13—13.54	10.0—11.40	2.0—20	12	99	94—103	10	12.62	0.24	1.90
SMA — Low birthweight	2*	—	4.72— 5.23	5.2— 5.3								
Premium (Cow & Gate)	7*	14.78	13.83—15.87	12.2—13.70								
Powdered Milks (concentration in 10% solution of dried product)	7**	1.17	0.51— 1.82	0.50								
Fresh cow's milk	3*		<0.10	—	2.0—20	5	98	95—102				
Infants (2 weeks to 6 months) receiving pre-packs												
Urine	30	0.41	0.15— 1.60	—	0.2— 5	10	101	95—106	8	0.86	0.02	2.60
Faeces	20	2.15	0.50— 8.02	—	0.2—10	5	102	92—105	5	2.71	0.15	5.60

*Each with a different batch number.

**One sample each of goat's milk, Ostermilk, Localsol, Complan, SMA, Premium and Marvel.

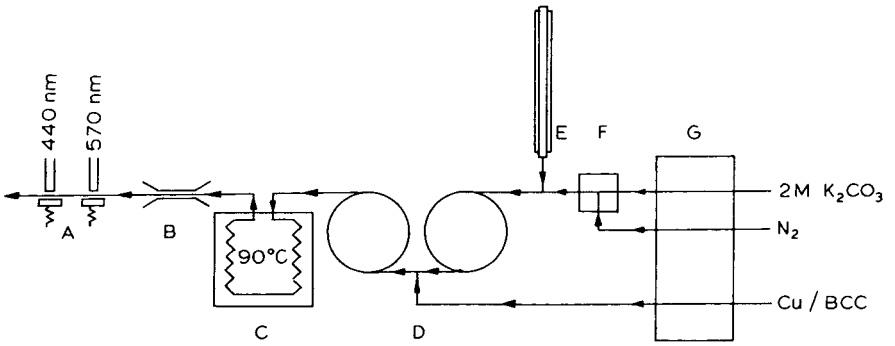

Fig. 2. Flow diagram for post-column detection of sugars using alkaline copper bicinchoninate reagent. A, Photometric detectors; B, vane cooler; C, oil bath; D, mixing coils (2 × 5 turns); E, column; F, gas injection block; G. peristaltic pump.

those obtained from calibration solutions and adjusted for internal standard results were used for quantitation.

RESULTS AND DISCUSSION

Typical chromatograms are illustrated in Fig. 1. The relationship between peak height and amount of lactulose loaded onto the column was linear between 0 and 150 n*M* at 570 nm ($r \geqslant 0.997$) and between 0 and 250 n*M* at 440 nm ($r \geqslant 0.998$). Typical lactulose concentrations in various specimens, its recovery when added to selected specimens, and the extent of between-batch imprecision are listed in Table I. The lactulose content of milk products was similar to that reported by Beach and Menzies [2] (Table I) who used a spectrophotometric scanning technique on paper chromatograms. A similar procedure [1], but using visual comparison with standards (C.V. ± 10%) specimens listed in Table 1. Lactulose concentrations as high as 30 m*M* were found in excessively sterilised or incorrectly stored pre-packed milks and similarly high concentrations were found in ileostomy fluids or faeces from patients with obvious intestinal hurry.

The position of lactulose in the chromatogram varied with the concentration of borate ions in the elution buffers. When the borate ion concentration of the buffer was less than 0.6 *M*, lactulose migrated after mannose but co-migrated with fructose. With buffers of higher molarity, it preceded mannose which partially overlapped both lactulose and fructose (Fig. 1B). The latter was considered preferable as mannose was not present in milks and seldom occurred free in body fluids [10]. A buffer of lower borate molarity was required for complete elution of large amounts of lactose before the emergence of lactulose. In this case, palatinose would appear on the tail of the lactose peak and xylose was a preferable internal standard.

Samples are made alkaline with borate buffer at the final stage of preparation and the possibility that lactose could be transformed to lactulose whilst the samples awaited analysis was investigated. Lactulose was not detected in a standard solution of lactose (10 m*M*) stored in borate for 48 h and the lactulose content of two extracts of prepacked milk did not increase

during the same time period after borate was added. This suggests that the borate complex of lactose is less reactive than the simple sugar.

The highest concentration of borate ions in the column elution buffer (pH 8.8) used by Sinner and Puls [8] was 0.5 *M*. As higher molarities were required to resolve lactulose, extra carbonate had to be added to the reaction mixture to maintain the optimum pH of 10.5. Sodium carbonate was replaced by the more soluble potassium salt but the 2,2'-bicinchoninate precipitated if the final carbonate concentration exceeded 1.5 *M*. Satisfactory conditions were best achieved by the two-stage addition illustrated in Fig. 2. This ensured smooth chart recorder traces (Fig. 1) although solutions of high ionic strength were mixed without the benefit of detergents which caused precipitation in the reaction manifold.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the generous loan of equipment by Hilger Analytical.

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Journal of Chromatography, 307 (1984) 399—403
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2030

Note

Determination of creatinine in human, dog and rat urine by high-performance liquid chromatography on a column of hydroxymethylated porous polystyrene

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(First received June 8th, 1983; revised manuscript received December 8th, 1983)

Creatinine in urine is commonly determined by assay methods based on the Jaffé reaction [1] in which creatinine reacts with alkaline picrate to form an amber-yellow colour. This reaction, however, is non-specific and many compounds are known to react with picrate [2]. Preliminary purification by ion-exchange chromatography has been attempted to overcome these difficulties [3—5]. More specific enzymatic methods have also been used [6].

Recently, high-performance liquid chromatography (HPLC) using a reversed-phase column was introduced for the determination of creatinine in human urine and plasma [7—13]. These HPLC separations are fast, reliable and require minimal work-up. However, a method for the determination of creatinine in urine of experimental animals such as dog and rat has not been reported.

In this paper we describe a method for measuring creatinine in human, dog and rat urine by HPLC on a column of hydroxymethylated porous polystyrene which is chemically more stable than the regular reversed-phase column.

EXPERIMENTAL

Materials

The column packing material was Hitachi Gel No. 3013-O (Hitachi, Tokyo,

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Japan) made of spherical porous particles of a hydroxymethylated styrene—divinylbenzene copolymer with an average particle diameter of 3 μm.

Creatinine was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Creatinine test kit based on the Jaffé reaction was from Wako Pure Chemical Industries (Osaka, Japan).

Apparatus

A high-performance liquid chromatograph Jasco Trirotar—II (Japan Spectroscopic Co., Tokyo, Japan) equipped with a variable-wavelength ultraviolet detector Uvidec-100-II (Japan Spectroscopic Co.) was used. Absorbance was continuously recorded at 236 nm.

The packing material, dispersed in distilled water, was packed into stainless-steel tubing at a pressure of about 150 kg/cm^2 by the slurry technique.

Sample urine

Human (male, age 29—38 years, weight 58—86 kg) urine was collected in a polystyrene bottle during 24 h. Rat Wistar ST (male, age 9 weeks, weight 210—262 g) urine was collected in a metabolic cage over a 24-h period. Beagle dog (male, age 8 weeks, weight 10—12 kg) urine was collected in a glass tube using a cannula. These urine specimens were stored at −80°C until analysis.

Procedure

The urine was diluted 25-fold with distilled water. Aliquots (5 μl) of the resulting solution were injected into the liquid chromatograph and analysed under the conditions described in the legend to Fig. 1. The urinary level of creatinine was quantitated from its peak height on the chromatogram.

The colorimetric determination of urinary creatinine based on the Jaffé reaction was performed using the creatinine test kit.

RESULTS AND DISCUSSION

Separation

Figs. 1, 2 and 3 show the typical results obtained for the analysis of human, dog and rat urine, respectively. Uric acid, trigonelline and pseudouridine were eluted as shown in Fig. 1. Hippuric acid largely excreted in urine was eluted at 26.4 min. Furthermore, creatine phosphate and also creatine were separated from creatinine. Thus, these constituents did not interfere with the determination of creatinine in this present HPLC method.

The separation could be carried out with better selectivity and resolution by using hydroxymethylated porous polystyrene of 3- or 5-μm particle diameter such as No. 3013-O than by using particles of 10—12 μm diameter such as No. 3011-O. No. 3013-O (3-μm particle diameter), which showed a slightly higher resolution than that of the 5-μm diameter particle, was adopted in our studies. The symmetry of the creatinine peak on this column was better than that obtained by employing the conventional C_{18} reversed-phase column. Retention time did not change over a period of ten months.

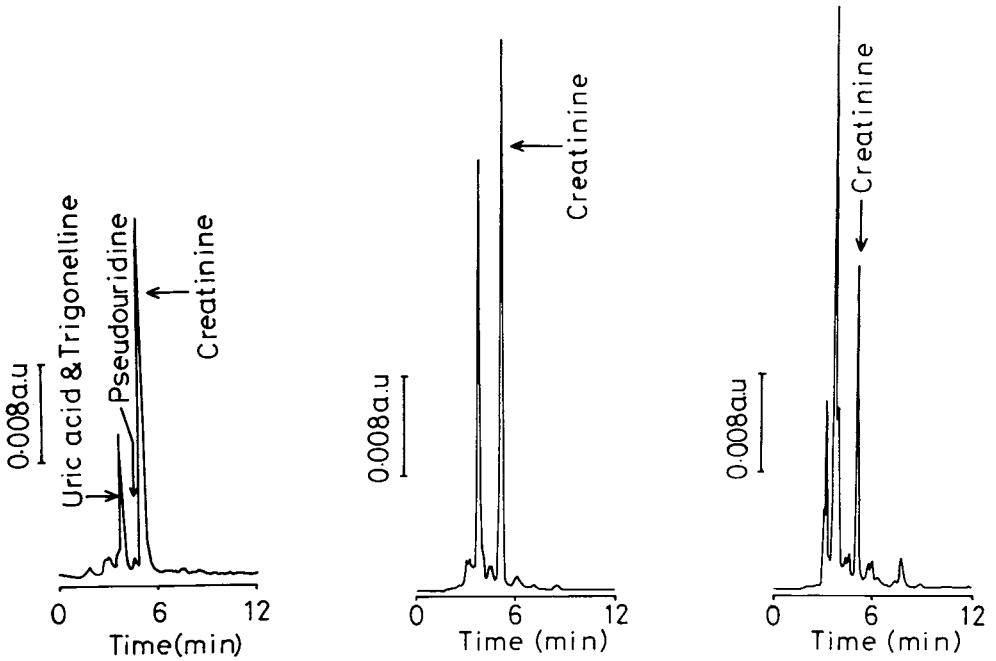

Fig. 1. Chromatogram of urine from a normal person. Urine was diluted 25-fold with distilled water. A 5-μl volume of this solution was applied under the following conditions. Column: stainless-steel tubing (250 × 4.6 mm I.D.) packed with Hitachi Gel No. 3013-O (particle diameter about 3 μm) at 30°C. Eluent: 0.02 *M* ammonium carbonate aqueous solution. Flow-rate: 0.8 ml/min. Detector: 236 nm, 0.08 a.u.f.s.

Fig. 2. Chromatogram of urine from a beagle dog. For conditions see legend to Fig. 1.

Fig. 3. Chromatogram of urine from a Wistar ST rat. For conditions see legend to Fig. 1.

Working curve, precision, recovery and accuracy

The relationship between peak height and amount injected into the chromatograph was linear over the range of 25—500 ng. The sensitivity of the assay was 38 mg/l with a signal-to-noise of 10.

The reproducibility of the present method summarized in Table I was fairly good compared with that of HPLC using the reversed-phase column [8, 13].

Portions of a urine containing 0.63 g/l creatinine were augmented to achieve final concentrations of 1.13 and 1.63 g/l. Analytical recovery (mean ± 1 S.D.) was 99.7 ± 0.5% (n = 5) and 100.3 ± 0.4% (n = 5), respectively.

Ultraviolet spectra of fractions corresponding to the chromatographic peaks of creatinine from column were identified for both urinary and reference creatinine as shown in Fig. 4. The correlation between the present method and the colorimetric method based on the Jaffé reaction was high (Fig. 5). For the urinary excretion of creatinine in dog and rat, however, the colorimetry gave results that were somewhat high in comparison with those obtained by HPLC. These results strongly support the present method as reliable for practical purposes and especially useful for the determination of creatinine in urine of various experimental animals.

TABLE I

PRECISION OF CREATININE ASSAY

	Conc. (g/l)	*n*	C.V. (%)
Within-day	0.63	6	0.7
	1.90	6	0.4
Between-day	0.63	10	2.7
	1.90	10	1.6

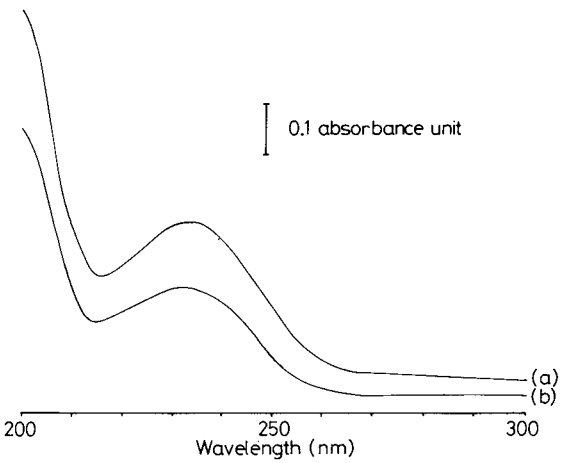

Fig. 4. Ultraviolet spectral comparison of urinary (a) and reference (b) creatinine. The same urine as in Fig. 1 was used. Fractions corresponding to chromatographic peaks of creatinine from the column were combined and freeze-dried to remove the ammonium carbonate. The samples were then dissolved in 4 ml of distilled water and their ultraviolet spectra measured. The spectrum was scanned from 300 to 200 nm at 120 nm/min scan speed and 2.0 nm slit width with a Hitachi 557 spectrophotometer (Hitachi, Japan).

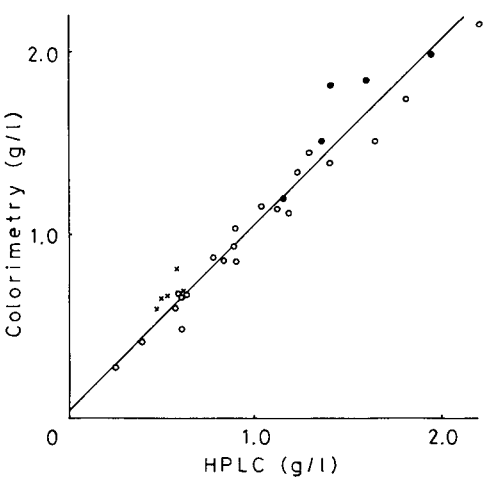

Fig. 5. Comparison of the present procedure with the method based on the Jaffé reaction for urinary creatinine. n = 31, r = 0.975, slope = 1.013, intercept = 0.032. (○), Human; (×), Wistar ST rat; (●), beagle dog.

Human urine

The urinary excretion (mean ± 1 S.D.) of creatinine in six normal humans was 1580 ± 150 mg/day (1410—1830 mg/day). The excretion (mean ± 1 S.D.) per g body weight was 23.3 ± 2.3 μg/day, which was consistent with values reported in the literature [14].

Dog urine

The concentration (mean ± 1 S.D.) of creatinine in urine of five beagles was 1490 ± 290 mg/l (1160—1950 mg/l).

Rat urine

The urinary excretion (mean ± 1 S.D.) of creatinine in five Wistar ST rats was 6.69 ± 1.60 mg/day (5.38—9.09 mg/day). The excretion (mean ± 1 S.D.) per g body weight was 28.0 ± 4.5 μg/day, which was larger than that excreted by humans.

CONCLUSION

The present method is very useful for the determination of urinary levels of creatinine not only in humans but also in dog and rat. The column used has a long life and is therefore economical for routine use.

ACKNOWLEDGEMENTS

We are deeply grateful to Dr. S. Mizogami and Mr. T. Higuchi of Mitsubishi Yuka Pharmaceutical Co. Ltd. for supplying dog and rat urine samples and to Dr. M. Uchiyama and Mr. T. Sakai of Mitsubishi Petrochemical Co. Ltd. for giving their support to this work.

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Journal of Chromatography, 307 (1984) 404—409
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2047

Note

Measurement of adenosine, inosine, and hypoxanthine in human plasma

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(First received September 22nd, 1983; revised manuscript received December 21st, 1983)

Adenosine has attracted much attention in recent years because of its possible role in vasodilation, neurotransmission, immune response and cyclic AMP formation [1, 2]. In addition, inosine and hypoxanthine were identified as endogenous ligands for benzodiazepine receptors [3] and were also shown to serve as a sensitive marker for tissue ischaemia [4, 5]. Several methods have been reported for the quantitation of adenosine [6—13], inosine [6—8, 13], and hypoxanthine [6, 8, 14, 15], using spectrophotometric [6, 7, 13, 15, 16], fluorometric [9, 10, 14], isotope dilution [9], and radioimmunological [11, 12] techniques. The reported plasma levels of the various purines, however, are highly variable. Part of this variability may be due to the fact that the metabolism of adenosine in blood is very rapid [13, 17]. Adenosine is avidly taken up by erythrocytes and is also degraded by plasma adenosine deaminase [18]. Thus, besides adequate methods for identification of each purine, reproducible measurements of plasma adenosine, inosine, and hypoxanthine require a reliable technique for blood collection.

This communication describes the quantitation of adenosine, inosine, and hypoxanthine in human plasma by high-performance liquid chromatography (HPLC) using the enzymatic peak shift method for verification. Furthermore, an effective technique for blood sampling is reported which almost completely prevents the further metabolism of adenosine and its degradative products during and after the sampling process.

EXPERIMENTAL

Chemicals and materials

Nucleosides, bases, adenosine deaminase (EC 3.5.4.4), and nucleoside phosphorylase (EC 2.4.2.1.) were obtained from Boehringer (Mannheim,

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F.R.G.). Dipyridamole (10 mg per 2 ml water) was purchased from Thomae (Biberach a.d. Riss, F.R.G.). Methanol and all other chemicals (analytical grade) were from Merck (Darmstadt, F.R.G.). Water was generated by a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.) Econo-Columns (100 mm × 3 mm I.D.) and anion-exchange resin AG 1-X2 (acetate) were from Bio-Rad (Richmond, CA, U.S.A.). Reversed-phase Nucleosil C_{18} (particle size 7.5 μm) was from Macherey-Nagel (Düren, F.R.G.). Plastic syringes (2 and 5 ml) were from Becton and Dickinson (Rutherford, NJ, U.S.A.).

Apparatus

Chromatographic analysis was carried out with a high-performance liquid chromatograph Model 5000 (Varian Assoc., Palo Alto, CA, U.S.A.) using home-made columns (300 mm × 4 mm I.D.) packed with reversed-phase Nucleosil C_{18}. Column temperature was maintained at 29°C using a thermostat (Haake Model FE). Absorbance changes were measured at 254 nm and recorded with a Model 56 two-pen recorder (Perkin Elmer, Norwalk, CT, U.S.A.).

Sample preparation

Blood was drawn using a specially developed syringe system (Fig. 1). A 5-ml "collecting" syringe was mechanically coupled with the 2-ml syringe containing 2 ml of an ice-cold "stopping" solution (mmol/l: dipyridamole, 0.2; NaCl, 154; KCl 5.6; $NaHCO_3$, 5.9; glucose, 5.6; $CaCl_2$, 2.15; Na_2HPO_4, 0.82; NaH_2PO_4, 0.17). Blood was drawn by pulling the plunger of the collecting syringe which caused immediate mixing of the aspirated blood with the stopping solution. Due to the dimensions of the syringes a constant mixing ratio of 1:1 was achieved. Following collection, blood was centrifuged at 8000 *g* for 2 min (Eppendorf table centrifuge). A 2-ml aliquot of the supernatant was removed, deproteinised with 100 μl of 60% perchloric acid and centrifuged (8000 *g*, 2 min) to remove the protein precipitate.

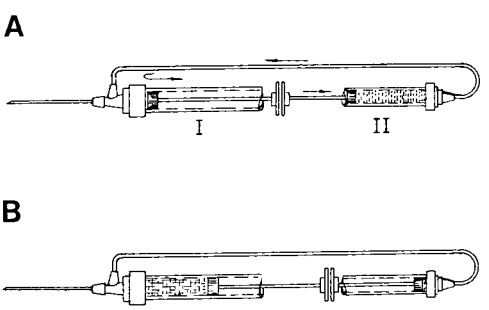

Fig. 1. Syringe system used for collecting blood. Syringe I (collecting syringe) was mechanically coupled to syringe II containing ice-cold 2 m*M* dipyridamole in Locke solution (stopping solution). Pulling the plunger of the collecting syringe causes the immediate mixing of blood with stopping solution at a constant ratio. (A) Before collection of blood; (B) after collection of blood.

Chromatography

Neutralisation of each sample was carried out by applying 1.5 ml of the acid extract on to columns (0.5 × 10 cm) filled with an AG 1-X2 anion-exchange

resin (acetate) equilibrated with water. Columns were washed with 9 ml of 5 m*M* acetic acid and the total column eluate was lyophilised. Residue was taken up in 250 μl of water of which 200 μl were injected onto the C_{18} column, equilibrated with water (flow-rate 1 ml/min). Following sample application a linear ascending gradient was started reaching 23% methanol after 25 min. Fractions containing adenosine, inosine, and hypoxanthine (fractions I, II, and III in Fig. 2A) were individually collected and evaporated to dryness. Compounds were identified by their retention time and coelution with standards. Enzymatic shifts were performed in each case according to the following principles:

$$\text{Adenosine} \xrightarrow{\text{adenosine deaminase}} \text{Inosine} + NH_3$$

$$\text{Inosine} \xrightarrow[(P_i)]{\text{nucleoside phosphorylase}} \text{Hypoxanthine} + \text{ribose-1-phosphate}$$

$$\text{Hypoxanthine} \xrightarrow[(\text{ribose-1-phosphate})]{\text{nucleoside phosphorylase}} \text{Inosine} + P_i$$

The evaporated column eluates were taken up either in 400 μl of water (adenosine samples), 400 μl of 2.5 m*M* phosphate buffer, pH 7.4 (inosine samples), or 400 μl of 10^{-3} *M* ribose-1-phosphate solution (hypoxanthine samples). Peak shift was initiated by the addition of 5 μl of adenosine deaminase (3.3×10^5 U/l) or 5 μl of nucleoside phosphorylase (2×10^4 U/l). All fractions were incubated for 30 min at 37°C, subsequently treated for 2 min at 100°C and then lyophilised. For rechromatography, each sample was taken up in 250 μl of water of which 200 μl were applied onto a C_{18} reversed-phase column as described above (Fig. 2B—D). In order to destroy completely enzymatic activity transferred to the C_{18} material, the columns were occasionally washed with 600 μl of 0.1 *M* hydrochloric acid.

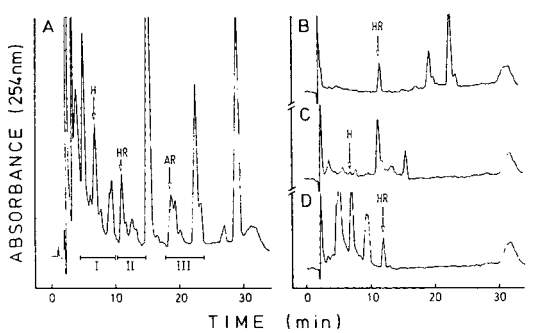

Fig. 2. Representative chromatograms of human plasma following sample preparation as described. (A) Original chromatogram showing retention of hypoxanthine (H), inosine (HR), and adenosine (AR). The different purine compounds were collected in fractions I, II, and III. In the case of adenosine, samples were treated with adenosine deaminase, in the case of inosine and hypoxanthine with nucleoside phosphorylase. (B) Rechromatography of adenosine (fraction III) yielding inosine (HR). (C) Rechromatography of inosine (fraction II) yielding hypoxanthine (H). ((D) Rechromatography of hypoxanthine (fraction I) yielding inosine (HR). For details see Experimental.

Calculation

Plasma levels of each purine were calculated according to the following formula: $c = \frac{m \times P_{sa} \times i}{P_{st} \times d}$

c = concentration of purine in plasma (μM)
m = amount of the injected standard (μmol)
P_{sa} = peak height of the sample (cm)
i = dilution factor (given the conditions described above: $i = 0.64$)
P_{st} = peak height of the standard (cm)
d = volume of plasma from which analysis was performed where

$d = \frac{1.43 \times (1 - h)}{(2 - h)}$ (h = haematocrit)

Values reported are not corrected for losses incurred during the different steps of the analysis.

RESULTS AND DISCUSSION

A new device was developed (Fig. 1) which permits the reliable sampling of blood for the measurement of adenosine and its degradative products inosine and hypoxanthine in plasma. It is based on the immediate mixing of blood with a solution containing dipyridamole (stopping solution) directly at the site of sample collection. Rapid inactivation of adenosine metabolism is absolutely necessary, since the half-life of adenosine in human blood is shorter than 10 sec and most of the nucleoside is removed within less than 30 sec (Fig. 3). Addition of dipyridamole almost completely prevented the disappearance of adenosine, and more than 90% of this nucleoside is still present in plasma after 5 min. Similar results were obtained with inosine; however, the loss of inosine from freshly drawn blood was much less dramatic.

The major route of adenosine removal in blood is by uptake into erythrocytes and incorporation into adenine nucleotides by the action of low K_m adenosine kinase [13, 17]. Furthermore, adenosine is removed by degradation to inosine and the enzyme responsible for this action, adenosine deaminase, is present in erythrocytes as well as in plasma [17]. In view of the rapid metabolism of adenosine, dipyridamole exhibits several features which make it particularly suitable for the sampling of human blood. It is well established that dipyridamole is a potent inhibitor of nucleoside transport in almost all tissues studied [17]. Dipyridamole was also shown to inhibit adenosine deaminase, however, only at higher concentrations [18]. Furthermore, this drug inhibits platelet aggregation [19] and thus the liberation of ADP during the release reaction. This latter action might be of particular importance, since degradation of ADP by cellular ectonucleotidases could give rise to the formation of adenosine during the sampling process. We have tested the possibility of adenosine being derived from extracellular adenine nucleotide breakdown by adding to the stopping solution 10^{-5} M α,β-methylene adenosine 5′-diphosphate (AOPCP), a potent inhibitor of ecto-5′-nucleotidase [20]. In two experiments we found no difference to the values given in Table I. Therefore, under our conditions, nucleotide breakdown in plasma during sampling does not contribute to the level of measured adenosine.

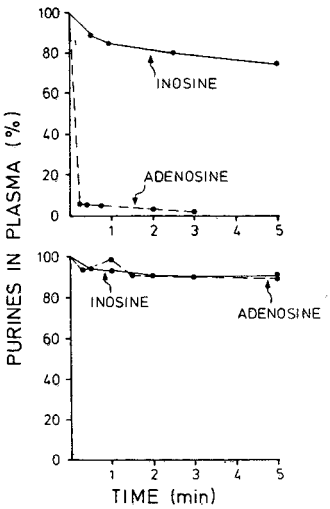

Fig. 3. Disappearance of adenosine and inosine in human plasma when blood was collected in the absence (upper panel) or presence (lower panel) of 0.2 m*M* dipyridamole. Blood was drawn from the cubital vein of a healthy individual using the syringe system shown in Fig. 1. The decrease in plasma purine levels was monitored by adding [8-^{14}C]adenosine (spec. act. 58 mCi/mmol, 10^5 cpm per tube), or [8-^{14}C]inosine (spec. act. 50 mCi/mmol, 2×10^5 cpm per tube) to syringe II (see Fig. 1). Following collection, blood was incubated for the time indicated. Sample preparation and separation by HPLC was the same as described under Experimental.

TABLE I

PLASMA LEVELS OF ADENOSINE, INOSINE, AND HYPOXANTHINE OF HEALTHY VOLUNTEERS

Blood was drawn from the cubital vein using the syringe system shown in Fig. 1. Analysis of purines was by HPLC (Fig. 2).

Subject	Adenosine (μM)	Inosine (μM)	Hypoxanthine (μM)
H.B.	0.404	0.099	0.502
J.O.	0.097	0.056	0.437
J.S.	0.340	0.113	0.315
B.B.	0.194	0.071	0.372
M.K.	0.388	0.099	0.388
$\bar{x}$ ± S.E.M.	0.285 ± 0.06	0.088 ± 0.01	0.403 ± 0.03

Values reported in the literature for adenosine, inosine, and hypoxanthine are highly variable. In the case of adenosine, they range between 0.07 and 1 μM [12, 21, 22], for inosine they are 0.7—0.9 μM [5, 23], while hypoxanthine is reported to be 0.4—1.3 μM [5, 15]. Reasons for this variability are not readily apparent in each case; however, a major source of error may be the conditions of blood sampling. Values for the various purines determined by us in plasma of healthy individuals by HPLC are shown in Table I. Recovery of adenosine and inosine during the different steps of analysis was 88% ± 2,67% ± 1, and 73% ± 1 ($\bar{x}$ ± S.E.M., n = 5), respectively.

The half-life of adenosine in whole dog blood was reported to be 3—6 min

[13]. These values, however, cannot be directly compared with our results, since micromolar amounts of adenosine were added to whole blood in these experiments. In our study, the disappearance of adenosine was investigated using only tracer amounts of labelled adenosine which mixed with adenosine endogenously present. Thus, the half-life reported here refers to the normal plasma level of adenosine.

We found it was necessary to identify adenosine, inosine, and hypoxanthine positively in every sample by enzymatic peak shift since the primary chromatogram (Fig. 2A) contained such a multitude of unidentified substances and the pattern of the chromatogram was not consistent between the individuals studied. Furthermore, in some but not all volunteers, ultraviolet-absorbing substances co-chromatographed with the purines of interest. It is conceivable that this interindividual variability may become of particular importance when plasma purines are measured under pathological conditions or at different nutritional states.

In summary, our method for collecting blood and compound identification permits the accurate measurement of adenosine, inosine, and hypoxanthine concentrations in plasma. Due to the syringe system used, which is mechanically coupled, this sampling method is also safe for use with humans.

ACKNOWLEDGEMENT

The authors wish to thank Dr. S. Nees for his helpful advice during the development of the chromatographic procedures.

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Journal of Chromatography, 307 (1984) 410—415
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2031

Note

High-performance liquid chromatographic analysis of hypoxanthine arabinoside in plasma

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(First received April 27th 1983; revised manuscript received December 8th, 1983)

Adenine arabinoside (ara-A) is an antiviral agent that has been used systemically in the treatment of herpes simplex and herpes zoster infections. In the body, ara-A is eliminated primarily by deamination to hypoxanthine arabinoside (ara-Hx). Following therapeutic doses, concentrations of ara-A in plasma are too low to accurately determine by conventional analysis, whereas concentrations of ara-Hx, being ten-fold higher, are often measured.

The determination of concentrations of ara-Hx in plasma has proven extremely difficult because the compound is structurally similar to many endogenous nucleosides that are found in relatively high concentrations in plasma. This structural similarity results in considerable difficulty in the chromatographic resolution of ara-Hx from potentially interfering endogenous compounds. Also, as with the endogenous nucleosides, ara-Hx is a polar molecule and cannot readily be extracted from plasma into organic solvents. As a result, it is necessary to inject plasma or deproteinized plasma directly on column, resulting in decreased column life expectancy as well as the appearance of many potentially interfering peaks from the endogenous nucleosides.

Few high-performance liquid chromatographic (HPLC) procedures for measuring concentrations of ara-Hx in plasma have been published, for the reasons just outlined [1—4]. All of the published procedures utilize ion-exchange chromatography which has important disadvantages compared to other HPLC separation techniques, including the need for column temperatures above ambient temperature to aid resolution, and the very short life expectancy of most ion-exchange columns.

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0378-4347/84/$03.00

This paper describes a sensitive and selective HPLC procedure for the analysis of ara-Hx in plasma using conventional reversed-phase chromatography. A pre-column sample preparation procedure is incorporated to rid the plasma of many interfering substances. Previous assays have not employed a method for sample clean-up, resulting in interferences during chromatography. The method has been used in our laboratories to measure ara-Hx concentrations in plasma after therapeutic doses of adenine arabinoside monophosphate (ara-AMP). Data are presented in one patient following therapeutic doses of ara-AMP.

EXPERIMENTAL

Materials

Ara-Hx was graciously supplied by Warner-Lambert/Parke-Davis (Ann Arbor, MI, U.S.A.). Standard solutions were prepared in water at concentrations of 10 and 100 μg/ml. Methanol (distilled-in-glass) was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). The water used throughout was glass-distilled. Sample preparation cartridges (C_{18} Sep-Pak®) were purchased from Waters Assoc. (Milford, MA, U.S.A.). All cartridges were pre-washed within 4 h of use with 10 ml of methanol followed by 10 ml of water using 10-ml glass syringes. The cartridges were then dried by flushing through four 10-ml syringe volumes of air.

Sample preparation

Plasma (2 ml) was deproteinized by the addition of 0.8 ml of 10% trichloroacetic acid. The mixture was vortexed for 10 sec and centrifuged for 10 min at approximately 1000 *g*. Subsequently, 1.2 ml of deproteinized plasma was transferred to a 10-ml capacity culture tube and brought to pH 2 with 0.5 *M* sodium hydroxide. The pH was checked by pH paper. The resultant mixture was then loaded onto a pre-washed sample preparation cartridge with a tuberculin syringe. Water, pH 5.5 (6 ml) was then pressed through the cartridge with a 10-ml glass syringe, then 4 ml of water (pH 10 with 1 *M* sodium hydroxide) were passed through the cartridge and collected. The collected pH-10 water was then neutralized with hydrochloric acid to a pH of about 7 and 100 μl were injected onto the chromatograph.

Apparatus

The modular high-performance liquid chromatograph consisted of a constant-flow pump (Model U6K, Waters Assoc.), a variable-wavelength absorbance detector (Model 450, Waters Assoc.) set at 254 nm, and a strip-chart recorder (Model 9176, Varian Instruments, Palo Alto, CA, U.S.A.). The column was a μBondapak C_{18} column (250 mm × 3.9 mm I.D.; Waters Assoc.).

Chromatographic conditions

The mobile phase was 15% methanol in water. Prior to mixing, sodium borate was added to both the methanol and water to achieve a concentration of 0.02 *M*. In addition, the water containing the sodium borate was acidified to pH 6.5 with hydrochloric acid. The flow-rate of the mobile phase was 1.5 ml/min resulting in a column pressure of 17 MPa. Overnight the flow-rate was reduced to 0.1 ml/min.

Calibration

The assay was calibrated by analyzing 2.0-ml aliquots of blank plasma to which 0.1—10 μg/ml of ara-Hx had been added. These standards were extracted and chromatographed identically to samples containing unknown quantities of ara-Hx. For each sample, the peak height of ara-Hx was measured and plotted against concentration to determine the linearity of the extraction and detector response for ara-Hx. In addition, each peak height was divided by the concentration of ara-Hx in that sample to give a normalized peak height. These normalized peak heights were averaged and the mean value was used to determine the amounts present in samples containing unknown concentrations of ara-Hx. Precision of the assay was estimated by calculating the coefficient of variation (C.V.) for each set of normalized peak heights.

Reproducibility

Aliquots (2 ml) of normal human plasma were spiked with 0.5, 1.0, 5.0 and 10.0 μg/ml ara-Hx and assayed in quadruplicate using the method described. These samples were extracted and chromatographed as described above. Absolute peak heights were measured and the coefficients of variation were calculated for each concentration.

Recovery

Aliquots (2 ml) of plasma were spiked with known quantities of ara-Hx (0.5 and 5.0 μg/ml). After the samples were prepared and chromatographed as described, the peak heights for ara-Hx were compared with the peak heights obtained when a standard containing either 0.5 or 5.0 μg/ml ara-Hx was injected directly onto the column. The recoveries were normalized for volume changes that occurred during the sample preparation procedures.

RESULTS AND DISCUSSION

Chromatography

Chromatograms of a blank plasma sample and a plasma sample containing approximately 2.0 μg/ml ara-Hx that were prepared according to the sample preparation procedure are shown in Fig. 1, together with a blank plasma sample that was not prepared. Plasma was collected from a single subject before and after administration of ara-AMP. As shown, the preparation procedure removed most of the peaks which could potentially interfere with the determination of ara-Hx. The retention time for ara-Hx under these conditions was approximately 4 min, but varied with changes in the composition of the mobile phase. Changes in composition of the mobile phase were necessary in some patients, to resolve ara-Hx from interfering peaks (see Discussion).

Calibration

A typical calibration curve relating peak height to concentration was linear for ara-Hx over the range of concentrations studied. The coefficients of variation for the estimation of ara-Hx averaged 9.3%. A standard curve was assayed with each set of samples containing unknown quantities of ara-Hx. The limit of detection of a prepared standard of ara-Hx, defined as five times baseline noise,

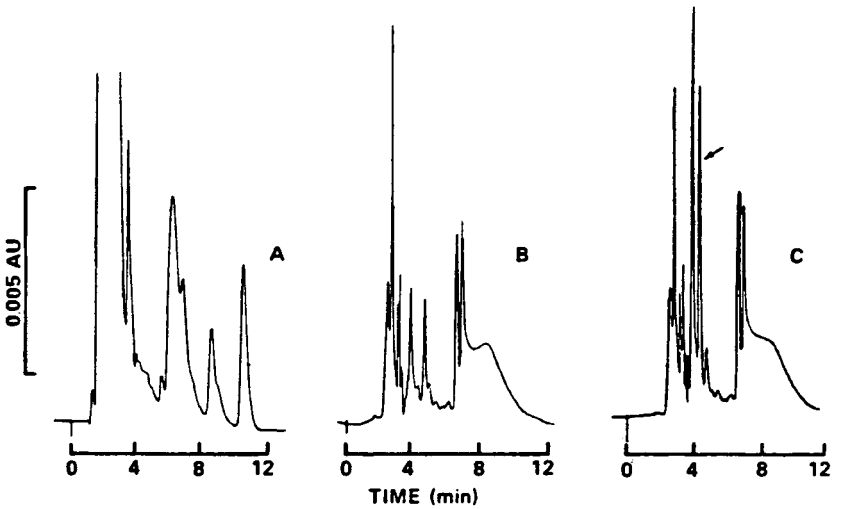

Fig. 1. Chromatograms of (A) blank plasma sample and two plasma samples (B and C) that were prepared according to the sample preparation procedure. Plasma samples were from a single subject who was treated with ara-AMP. (B) Represents plasma containing no ara-Hx (before drug administration), and (C) represents plasma from the same patient, containing approximately 2.0 μg/ml ara-Hx.

was 0.05 μg/ml. Again, this varied depending upon the presence of interfering peaks.

Reproducibility

Data showing the reproducibility of the assay are summarized in Table I. For any given concentration the determination of ara-Hx was highly reproducible. There was, however, a slight tendency of the average normalized peak height to decrease with increasing concentration.

Recovery

The total amount of ara-Hx recovered from the assay procedure was 34.5 ± 3.3% for 0.5 μg/ml, and 31.2 ± 3.1% for 5.0 μg/ml. These data represent the average of four determinations in each case.

TABLE I

REPRODUCIBILITY OF RECOVERY OF ARA-Hx FROM SPIKED PLASMA SAMPLES

Concentration (μg/ml)	Normalized peak height* (mm/μg)	Coefficient of variation* (%)
0.5	88.6	3.9
1.0	88.5	4.4
5.0	82.0	3.2
10.0	75.1	3.4

*Coefficient of variation of four determinations.

Patient data

Plasma concentrations of ara-Hx obtained in a single patient are presented in Fig. 2. The patient received 5 mg/kg ara-AMP intramuscularly every 12 h for three days followed by a single intravenous dose of 5 mg/kg ara-AMP. The plasma concentrations shown in Fig. 2 were obtained from plasma samples collected after the final intramuscular and the intravenous doses. As shown, the plasma concentration after both doses declined roughly in parallel with a terminal half-life of about 2 h.

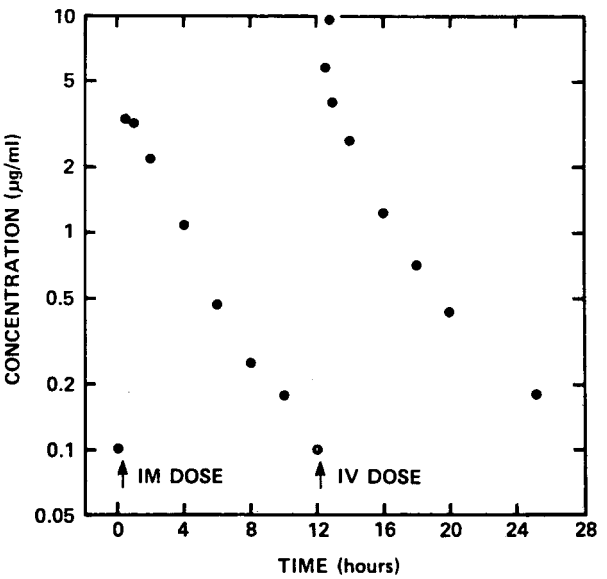

Fig. 2. Plasma concentrations of ara-Hx in a single patient after receiving ara-AMP intramuscularly (IM) and intravenously (IV) (5 mg/kg per 12 h). The points before the arrow indicate concentrations of ara-Hx just before drug administration.

DISCUSSION

Unlike other published procedures for ara-Hx that have employed ion-exchange chromatography [1—4], this assay uses conventional reversed-phase chromatography. Hence, column life is longer and the chromatography can be conducted at ambient temperature. In this study, a single column was used daily for six months before replacement was necessary.

Another important difference between this and the previously published HPLC procedures, in which deproteinized plasma is injected directly on column [1—4] is the incorporation of a sample preparation procedure. As shown in Fig. 1, the sample preparation procedure resulted in a substantial decrease in background noise, permitting the determination of ara-Hx at low concentrations. The volume and pH of the wash and elution steps were adjusted to achieve maximum signal-to-noise ratio. Maximum signal-to-noise ratio was achieved when recovery of ara-Hx was 32%, ie., at higher recoveries interfering substances ("noise") were present in even greater amounts in comparison with ara-Hx. The 32% recovery, although low, was reproducible and sufficiently sensitive to detect ara-Hx at concentrations as low as 0.1 μg/ml (Fig. 2).

The assay has been used to analyze plasma concentrations of ara-Hx after therapeutic doses of ara-A or ara-AMP and may be used with approximately equivalent sensitivity to assay ara-Hx concentrations from either 1 or 2 ml of plasma. Depicted in Fig. 2 are the plasma concentrations of ara-Hx obtained in a single subject using the method presented in this paper. There are many water-soluble endogenous compounds in plasma that absorb UV light at 254 nm and may potentially interfere with the determination of ara-Hx. However, resolution of ara-Hx from these peaks was obtained by varying the percent methanol in the mobile phase from about 12 to 18%, which changed the retention time of ara-Hx.

In summary, a sensitive, selective HPLC procedure for determining the concentration of ara-Hx in plasma is of considerable value in studies of the efficacy and toxicity of ara-A or ara-AMP. The procedure described here differs from previously published methods in that it incorporates a sample preparation step and uses conventional reversed-phase chromatography. The method has been used successfully to analyze concentrations of ara-Hx in plasma after therapeutic doses of ara-AMP.

ACKNOWLEDGEMENTS

This work was supported in part by Public Health Service Grant GM22209 from the National Institute of Health.

We would like to acknowledge Mr. Robert Pershe for his assistance in the analytical procedures, Ms. Kathleen Turner-Tamiyasu for helping collect blood samples, and Ms. Rebekah Levy for preparing the manuscript.

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Journal of Chromatography, 307 (1984) 416—419
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2029

Note

Electrophoresis of thiols in cellulose gels

III. Group analysis of urinary thiols

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(First received October 17th, 1983; revised manuscript received December 6th, 1983)

Urinary thiols and their disulphides comprise a rather long list of compounds that have been identified either as of natural origin including pathological excretion (e.g. cysteine, acetylcysteine, cysteinylglycine, mercaptoacetic acid, lipoic acid, cysteamine, methanthiol, mercaptolactic acid, homocysteine, glutathione), or as resulting from administered medicines (e.g. thiomalic acid, penicillamine, captopril [1, 2]. The analytical methods described in the literature are as a rule limited to the estimation of a single thiol, and no efforts have been encountered to demonstrate a general urinary thiol balance. An attempt to do so is given in this paper.

As previously suggested [3] the thiols may be separated into five groups on the basis of extraction of the free thiols and of their tributyltin (TBT) mercaptides. The thiols of groups 0 and 1 can be extracted with hexane, the thiols of group 2 with butanol; the free thiols of group 3 can not be extracted from aqueous solution. The TBT mercaptides of group 0 in hexane are not decomposed by alkalies whereas those of the group 1 are. The TBT mercaptides of group 2 are soluble in hexane and those of group 3 in butanol. The TBT mercaptides of group 4 are insoluble in organic solvents. Of course there is not a sharp distinction between the groups and the determined group composition depends on the procedure used.

In the present study the urinary thiols are separated into three groups by a procedure involving extraction of TBT mercaptides with butanol followed by stripping with alkalies. Group 4 thiols remain in aqueous solution, group 0 in butanol, and thus the sum of groups 1, 2 and 3 can be calculated by difference.

It has also been suggested to take account of the selective blocking of certain

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aminothiols by formaldehyde [4, 5]. Thiols such as cysteine, cysteamine, homocysteine, penicillamine and cysteine peptides with a free amino group of cysteine are referred to in this paper as active aminothiols. They can be determined as the difference between the total thiol content and the thiol content after adding formaldehyde using titration with *o*-hydroxymercuribenzoic acid (HMB) in the presence of dithiofluorescein as indicator [6].

To complete the thiol balance further separation is suggested on the basis of electrophoresis. In neutral media mercaptoacids can be separated from a mixture of neutral thiols and aminothiols and the mixture can be analysed using selective blocking with formaldehyde.

The thiols extracted as TBT mercaptides have to be recovered for electrophoresis as concentrated solutions, as far as possible free from electrolytes. In this study the thiols of groups 0, 1, 2, and 3 are extracted together as their TBT mercaptides with butanol and the extract is diluted with hexane. Stripping with acetic acid solution provides the samples for electrophoresis in acid solution, and stripping with a solution of barium hydroxide in glycerol followed by neutralization with sulphuric acid provides samples in neutral medium. To prevent oxidation a higher thiol (e.g. hexadecanethiol) has been added.

The thiols can be titrated with HMB at pH 8—11 using dithiofluorescein as indicator or in 0.05—0.1 *M* potassium (or sodium) hydroxide using dithizone as indicator. The titration can be carried out in a test tube even with a $2.5 \cdot 10^{-5}$ *M* solution of HMB. To avoid errors when dealing with urinary thiols the following points should be observed: (1) As the thiols of group 4 react slowly with the dithiofluorescein—HMB complex, the result of direct titration is too low. The HMB must be added in excess. This is not necessary when thiols of group 4 are absent. (2) In the presence of TBT only dithizone can be used as indicator. (3) In sodium hydroxide solution the urinary thiols are rapidly oxidized with air. The titration should be repeated and completed in as short a time as possible. The pure thiols are not so sensitive to oxidation.

EXPERIMENTAL

Reduction

The apparatus for reduction, described in another paper [7], is composed of a cooling water jacket, a lead cathode with each side of 125 cm^2 surface area, a ceramic tube and a lead anode in 2 *M* sulphuric acid. The urine was acidified with 1 ml of 98% sulphuric acid per 100 ml. To prevent foaming a drop of a silicone suppressor was added. The reduction of 100 ml of urine was carried out at room temperature for 2 h with a current of 0.5 A.

Analysis of the reduced urine

Procedure for total thiols. To 2 ml of the sample add 1 ml of 1 *M* triethanolamine and 1 ml of a solution of dithiofluorescein (it should consume about 1 ml of HMB solution per ml), and titrate with 10^{-4} *M* HMB until the blue colour disappears. Repeat the titration adding first the volume of HMB found previously with 20% excess, then add dithiofluorescein until the sample becomes blue and titrate until it becomes colourless again. The HMB should be

added in excess followed by an excess of dithiofluorescein. The result is the difference between the total volume of HMB and the consumption of HMB by the added dithiofluorescein.

Procedure for active aminothiols. Take 4 ml of the sample and titrate as above after adding 0.2 ml of 1 *M* formaldehyde. The concentration of active aminothiols is the difference between the amount of total thiols and the thiols found in this titration.

Extraction

Place 100 ml of reduced urine in a 250-ml separating funnel and dissolve in it 5 g of triethanolamine and 5 g of anhydrous sodium sulphite; the resulting pH should be between 7.5 and 8. Add 25 ml of a 0.06 *M* solution of TBT hydroxide in butanol and mix vigorously for 10 min by a stream of nitrogen. Separate the extraction layer and centrifuge to remove droplets of water and sediments. Add 1 g of sucrose to the extraction layer and store at 4°C with protection from light. Measure the volumes of the extraction and raffinated layers.

Determination of thiols of group 4

Extract 5 ml of the raffinated layer with 2.5 ml of 0.06 *M* TBT hydroxide in butanol. Add to 2 ml of the new raffinated layer 1 ml of 2 *M* potassium hydroxide and titrate with 10^{-4} *M* HMB using dithizone as indicator. Repeat the titration adding first the volume of HMB as found above, then add potassium hydroxide and indicator and finish the titration. The thiols found are assumed to belong to group 4.

Determination of thiols of group 0

Add to 2 ml of the extraction layer 2 ml of hexane and strip two times with 4 ml of 0.5 *M* potassium hydroxide. Take 2 ml of organic layer, add 3 ml of propanol and 0.2 ml of 2 *M* potassium hydroxide and titrate with 10^{-4} *M* HMB using dithizone as indicator. The result calculated for the total volume of the extraction layer corresponds to the group 0 thiols in 100 ml of urine.

Preparation of samples for electrophoresis

For acid medium. Add to 5 ml of extraction layer 4 ml of 0.1 *M* hexadecanethiol in hexane and 0.4 ml of solution prepared by mixing 1 ml of acetic acid with 10 g of glycerol. Shake vigorously for 5 min, remove the upper layer and wash the glycerol layer with 2 ml of hexane.

For neutral medium. Place in a polyethylene test tube 5 ml of the extraction layer, 4 ml of 0.1 *M* hexadecanethiol in hexane and 0.4 ml of a solution prepared by dissolving while heating 2 g of barium hydroxide octahydrate in 10 g of glycerol. Shake vigorously for 5 min, remove the upper layer and wash the glycerol layer with 2 ml of hexane. Neutralize the sample with 2.5 *M* sulphuric acid in 50% (w/w) glycerol in the presence of phenol red.

Determination of mercaptoacids, aminothiols and neutral thiols in neutralized glycerol layer

Carry out electrophoresis in a cellulose gel rod in neutral solution as described previously [3] using a 0.4-ml sample. When the mercaptoacids have

penetrated the gel, wash out the remaining thiols (aminothiols and neutral thiols) with water, dilute to 10 ml, extract with 1 ml of hexane to remove hexadecanethiol, if any, and analyse for total thiols and aminothiols as described above. Continue the electrophoresis with the gel rod in inverted position and control the emerging mercaptoacids by titration with $2.5 \cdot 10^{-5}$ *M* HMB using dithizone as indicator. From the results obtained calculate the percentage composition of thiols.

RESULTS AND DISCUSSION

The distribution of thiols in three samples of urine are given in Table I. Electropherograms obtained show at pH 1.8 one peak of cysteine, and at pH 7.2 two peaks — one corresponding to mercaptoacetic acid and the other to acetylcysteine.

TABLE I

DISTRIBUTION OF THIOLS IN REDUCED URINE (COLUMNS 2—6) AND IN THE SAMPLE PREPARED FOR ELECTROPHORESIS IN NEUTRAL MEDIUM (COLUMNS 7—9)

Sample	Total thiols (μmol/l)	Active aminothiols (μmol/l)	Group composition (%)			Thiol distribution (%)		
			0	1—3	4	Mercapto-acids	Amino-thiols	Neutral thiols
1	475	366	3	65	32	14	79	7
2	185	130	9	81	10	6	62	32
3	245	174	8	77	15	7	71	22

The active aminothiols form about 75% of the total thiols. The cysteine content can be roughly calculated from the percentage composition of thiols. As calculated from previously given results [8], when 25 ml of 0.06 *M* TBT in butanol are used for extraction from 100 ml of neutral solution, only 84% of cysteine will be extracted. Taking into account that cysteine belongs to group 3, its content in sample 1 will be given as $0.79 \times 0.65 \times 475/0.84 = 290$ μmol/l, and the values for samples 2 and 3 amount to 111 and 160 μmol/l, respectively, i.e. 61%, 60% and 65% of total thiols, or 79%, 85% and 92% of active aminothiols.

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Journal of Chromatography, 307 (1984) 420—425
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2045

Note

Determination of germicidal phenols in blood by capillary column gas chromatography

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(First received September 19th, 1983; revised manuscript received December 15th, 1983)

The investigation of two hyperbilirubinemia epidemics that affected infants in hospital nurseries suggested a causal association with the excessive use of a disinfectant containing *p-tert.*-amylphenol (3%), *o*-phenylphenol (0.5%), and *o*-benzyl-*p*-chlorophenol (6.4%). In one investigation, serum phenols were determined by gas chromatography (GC) with flame ionization detection, which gave a detection limit of 1 ppm in serum [1]. In the other, serum phenols were determined by high-performance liquid chromatography with dual ultraviolet detectors and a fluorescent detector [2]. Although the latter was more sensitive, quantification was difficult because of interferences in some samples with peaks of *p-tert.*-amylphenol and *o*-benzyl-*p*-chlorophenol.

The present method of determining germicidal phenols in serum is somewhat similar to our published method for determining pentachlorophenol [3]. The germicidal phenols differ from pentachlorophenol in that they are much less sensitive to electron-capture detection and also less lipophilic. Consequently, the present procedure differs mainly in that phenols are derivatized with an electrophoric reagent, and a more polar extraction solvent is used.

To enhance the electron-capture response of various phenols, Lamparski and Nestrick [4] derivatized them with heptafluorobutyrylimidazole and analyzed extracts by GC on a packed column. McKague [5] derivatized phenols with heptafluorobutyric anhydride and used a capillary column.

Some previous bioanalytical examples of the use of electron-capturing reagents for derivatizing phenolic groups can be cited. Trifluoroacetic anhydride was used in methods for diethylstilbestrol [6] and dopamine [7] in urine, and morphine [8] in serum. Pentafluoropropionic anhydride was reacted with

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catecholamines [9] and pentafluorobenzoyl chloride with phenolic acids [10] in urine analyses.

EXPERIMENTAL

*Reagents**

Heptafluorobutyric anhydride was obtained from Pierce (Rockford, IL, U.S.A.), Dowicide 1 (*o*-phenylphenol) from Dow Chemical (Midland, MI, U.S.A.), Pentaphen No. 67 (*p-tert.*-amylphenol) from Pennsalt (Philadelphia, PA, U.S.A.), Santophen 1 (*o*-benzyl-*p*-chlorophenol) from Monsanto (St. Louis, MO, U.S.A.), and *p*-phenylphenol from Chem Service (West Chester, PA, U.S.A.). Isooctane and diethyl ether were of distilled-in-glass quality from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Pyridine was supplied by Mallinckrodt (St. Louis, MO, U.S.A.). A lecture bottle of trimethylamine was obtained from Matheson Gas Products (Morrow, GA, U.S.A.). Buffer of pH 9.2 was prepared by diluting 1.236 g of boric acid and 53.4 ml of 0.2 *M* sodium hydroxide with water to 200 ml. Buffer of pH 8 was prepared by diluting 1.236 g of boric acid and 7.9 ml of 0.2 *M* sodium hydroxide with water to 200 ml.

Synthesis of heptafluorobutyrates of phenols

To 5 ml of diethyl ether containing 1.23 g (7.2 mmol) of *p*-phenylphenol were added 2 ml of heptafluorobutyric anhydride (8 mmol). Trimethylamine was bubbled into the mixture for a few minutes, after which it was allowed to stand 1 h at room temperature in a tube with a polytrifluoroethylene (PTFE)-lined cap. More trimethylamine was added, and 15 min later the mixture was washed three times with 10 ml of a pH 6 buffer and once with a pH 8 buffer. The organic layer was evaporated to a low volume under a stream of nitrogen. A product weighing 1.13 g was removed by filtration and recrystallized several times from isooctane. The *p*-phenylphenol heptafluorobutyrate melted at 72–73° C.

Similar procedures were used to prepare heptafluorobutyrates of the other three phenols. These derivatives were purified by distillation at reduced pressure to give boiling points at pressures (mmHg) as follows: *o*-benzyl-*p*-chlorophenol derivative, 85° C (0.05 mmHg); *o*-phenylphenol derivative, 68° C (0.15 mmHg); and *p-tert.*-amylphenol derivative, 53° C (0.10 mmHg).

Gas chromatography—mass spectrometry of phenol heptafluorobutyrates

The instrument used was a Finnigan 4000 equipped with a quadrupole spectrometer and a 1.8 m × 2 mm I.D. GC column packed with 3% OV-1. All four phenol derivatives showed only one GC peak. Details of the mass spectra are shown in Table I.

Extraction and derivatization

Each 0.5-ml sample of blood serum was spiked with 4 μl of an acetone

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*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

TABLE I

MASS SPECTRA OF PHENOL HEPTAFLUOROBUTYRATES

It is noteworthy that the spectrum of the *o*-phenylphenol derivative has a large peak at *m/z* 197 (from loss of C_3F_7) which is not in the spectrum of the *p*-phenylphenol derivative.

Phenolic compound	*m/z* (percent relative abundance)
o-Benzyl-*p*-chlorophenol	414[M^+](21), 217(100), 201(17), 181(19), 165(57), 152(32)
o-Phenylphenol	366[M^+](50), 169(100), 197(83), 153(25), 141(56), 115(50)
p-Phenylphenol	366[M^+](63), 169(100), 153(11), 141(51), 115(36), 69(17)
p-tert.-Amylphenol	360[M^+](3), 331(100), 303(23), 275(6), 169(10), 115(13)

solution of *p*-phenylphenol (internal standard). Calibration samples were spiked with acetone containing three germicidal phenols and internal standard. Spiked samples were rotated slowly for 1 h in tubes fitted with PTFE-lined screw caps. To each tube was added 0.5 ml of 2 *M* sulfuric acid, followed by 3 ml of 20% diethyl ether in isooctane. In order to decrease emulsification, the tube was rotated slowly for 2 h. Following centrifugation, 2 ml of each top layer was transferred to another tube and derivatized by adding successively 50 µl of pyridine and 50 µl of heptafluorobutyric anhydride, shaking for 1 min, and allowing the mixture to stand for 20 min at room temperature. By-products were removed by washing the extracts (shaking 30 sec each time) with 4 ml of pH 9.2 borate buffer followed by 4 ml of pH 8 borate buffer. The washed extracts were dried with anhydrous sodium sulfate before they were analyzed by GC.

Gas chromatography

The Tracor 560 gas chromatograph (Tracor, Austin, TX, U.S.A.) was equipped with a ^{63}Ni electron-capture detector and linearizer (constant-current pulsed mode). It was fitted with an 11 m × 0.25 mm I.D. fused-silica capillary column with methyl silicone coating (WCOT) obtained from Hewlett-Packard (Avondale, PA, U.S.A.). With a Varian 8000 autosampler, 1.5 µl was injected in the splitless mode for 1 min at 58°C, after which the temperature was programmed up to 178°C at a rate of 3°C/min, the last peak of interest emerging at 126°C. The detector temperature was 350°C. The injection port temperature was normally kept at 225°C but increased to 265°C during the 1-min injection period. The carrier gas was hydrogen at a flow-rate of 2.4 ml/min (linear velocity of 69 cm/sec with methylene chloride). The make-up gas was argon—methane at a flow-rate of 50 ml/min. The purge flow-rate from the injection port was 50 ml/min. Peak areas were quantified by means of the Supergrator-3 (Columbia Scientific Industries, Austin, TX, U.S.A.) placed between the detector and the recorder.

Quantitative analysis

Standard curves for each germicidal phenol were obtained by the method of standard addition with *p*-phenylphenol used as internal standard. The ratios of peak areas (germicidal phenol/internal standard) were plotted as a function of increasing concentrations of germicidal phenol in serum. The normal standard curves were obtained from analyses of eight samples spiked with

p-tert.-amylphenol at levels of 0—554 ng/ml, with *o*-phenylphenol at 0—405 ng/ml and *o*-benzyl-*p*-chlorophenol at 0—642 ng/ml. All samples were spiked with internal standard at 394 ng/ml. The average of correlation coefficients for standard curves from three experiments was 0.9979 ± 0.08% (relative standard deviation).

RESULTS AND DISCUSSION

Heptafluorobutyric anhydride (HFBA), pentafluorobenzoyl chloride, pentafluorobenzyl bromide, and 1-fluoro-2,4-dinitrobenzene have been tested as derivatizing agents in order to increase the electron-capture response. These agents have been used by McCallum and Armstrong [11] to prepare thymol derivatives and determine their relative sensitivity to electron-capture detection. The pentafluorobenzoate was found to be the most sensitive, being

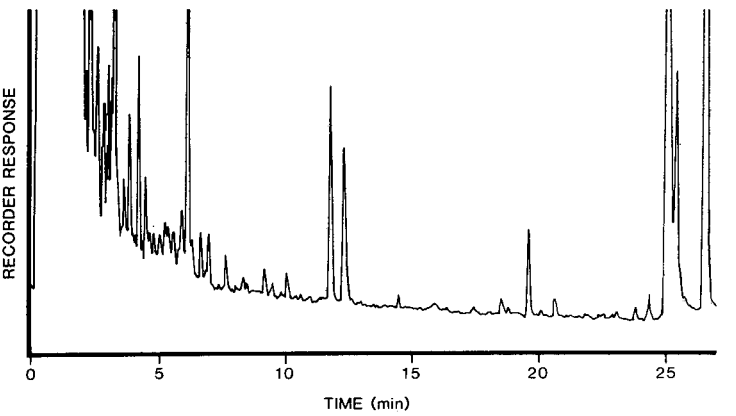

Fig. 1. Chromatogram of blank serum extract treated with HFBA. GC on capillary column as described in Experimental.

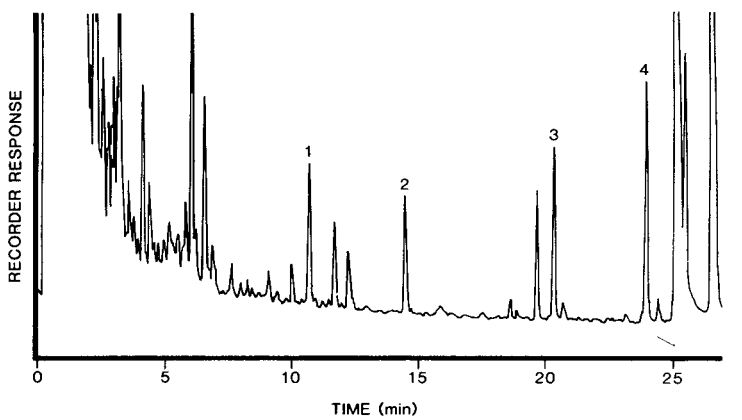

Fig. 2. Chromatogram of germicidal phenol-supplemented serum extract treated with HFBA. Heptafluorobutyrate peaks: 1 = *p-tert.*-amylphenol (101 ng/ml); 2 = *o*-phenylphenol (73 ng/ml); 3 = *p*-phenylphenol (141 ng/ml); 4 = *o*-benzyl-*p*-chlorophenol (102 ng/ml). GC conditions as described in Experimental.

seven times more potent than the heptafluorobutyrate, the second most sensitive. In our procedure, heptafluorobutyric anhydride appeared to be the most suitable reagent from the standpoint of percentage of recovery, nature of by-products, and volatility of derivatives.

The reaction of HFBA with an extract of serum unsupplemented with germicidal phenols produced a large number of electron-capturing compounds, as shown in Fig. 1. Most of these very early peaks were also found in 20% diethyl ether in isooctane reacted with HFBA and, therefore, result mainly from reagent impurities and/or reaction by-products. Fig. 2 shows a chromatogram of an extract of plasma spiked with germicidal phenols. A comparison of these two chromatograms shows that interferences with the germicidal phenols are minimal. However, a small amount of interference is present at the retention time of the HFBA derivative of *o*-benzyl-*p*-chlorophenol. Much higher interferences occurred in early experiments in which a column packed with 5% SP 2100 was used. The capillary column clearly offers advantages over packed columns in separating all the components of these complex mixtures.

Peak heights varied considerably for some of the unknown constituents of samples of the same serum pool, for example, the two peaks with retention times near 12 min in Figs. 1 and 2. The explanation may be that such constituents form unstable derivatives with HFBA. However, effects of reaction conditions have not been investigated.

Extraction solvents other than 20% diethyl ether were tried. Isooctane has a boiling point which makes it ideal for use in splitless injection on a capillary column, but it has less than the nearly 100% extraction efficiency of 20% diethyl ether in isooctane. Benzene or 50% diethyl ether in isooctane extracted much larger amounts of substances which interfered with peaks of interest.

Results of quantitative analyses in which we used the internal standard and calibration curves are shown in Table II. Each 0.5-ml sample of serum was spiked with 4 μl of acetone containing internal standard and 4 μl of acetone containing a mixture of three germicidal pehnols. Table II shows the amounts

TABLE II

RECOVERY OF GERMICIDAL PHENOLS FROM SERUM

Experiment	*p-tert.*-Amylphenol		*o*-Phenylphenol		*o*-Benzyl-*p*-chlorophenol	
	Added (ng/ml)	Recovery (%)	Added (ng/ml)	Recovery (%)	Added (ng/ml)	Recovery (%)
1	203	92	148	95	235	91
2	203	101	148	109	235	102
	406	100	299	101	470	105
3	51	94	37	135	59	103
	102	98	74	126	118	96
	203	86	148	106	235	88
	406	106	297	107	470	97
	610	82	446	98	706	71

of phenols added to the serum in ng/ml and the percentage of these phenols found by the analytical procedure (percent recovery). With a few exceptions, the recoveries ranged from 86 to 109%. The practical limit of detection for each phenol in serum appears to be near 20 ng/ml.

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Journal of Chromatography, 307 (1984) 426—431
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2026

Note

Quantification of antimalarial drugs

II. Simultaneous measurement of dapsone, monoacetyldapsone and pyrimethamine in human plasma

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(Received October 26th, 1983)

Maloprim® is widely used as a prophylactic agent against malaria, particularly in areas where chloroquine-resistant strains of *Plasmodium falciparum* are prevalent [1]. Each tablet of Maloprim contains 100 mg of dapsone (4,4'-diaminodiphenyl sulphone, DDS) and 12.5 mg of pyrimethamine [2,4-diamino-5-(4-chlorophenyl)-6-ethylpyrimidine, PYR]. DDS and PYR act at sequential steps in malaria parasite folate metabolism, resulting in a synergistic inhibition.

Analytical methods capable of simultaneously measuring the components of Maloprim and a major metabolite of DDS, monoacetyldapsone (4-acetamido-4'-aminodiphenyl sulphone, MADDS) are based on chromatographic methods. Jones and Ovenell [2] developed a high-performance liquid chromatographic (HPLC) method which was difficult to standardize because of the presence of volatile diisopropyl ether and ammonium hydroxide in the mobile phase. Ahmad and Rogers [3] reported a pharmacokinetic study using thin-layer chromatography with detection limits of 20 ng/ml for DDS and MADDS and 15 ng/ml for PYR, but the method required 3 h equilibration of glass tanks containing mobile phase before analysis was undertaken. A simple, reproducible and sensitive method for the estimation of Maloprim is required for measuring ng/ml concentrations of the drug combination found in plasma after a prophylactic dose (i.e. one tablet per week).

The purpose of the present work was to develop a rapid, selective and sensitive method for the simultaneous quantification of DDS, MADDS and PYR in human plasma using HPLC. The described method is similar to an HPLC procedure developed by the author [4] for the analysis of the antimalarial drug Fansidar® (sulphadoxine and PYR). Reversed-phase ion-pair

chromatography was used with pentane sulphonic acid as the counter-ion and with quinine as the internal standard.

MATERIALS AND METHODS

Chemicals and standards

HPLC grade acetonitrile, methanol and 1-pentane sulphonic acid (Waters Assoc., Australia) were used. Sodium hydroxide and ethylene dichloride were of analytical quality.

Reference compounds were donated as follows: DDS by Imperial Chemical Industries (Australia), MADDS by Parke Davis (Warner-Lambert, Ann Arbor, MI, U.S.A.), and PYR by Wellcome (Australia). A stock standard solution of DDS, MADDS and PYR was prepared containing 500 μg base of each compound per ml of methanol. Intermediate and working standard solutions were prepared by diluting the stock standard solution with water. A stock standard solution of quinine dihydrochloride was prepared containing 500 μg base per ml of water. All standard solutions were stored at 4°C in amber glass bottles.

Instrumentation and chromatographic conditions

The liquid chromatographic system comprised a Pye Unicam LC-XPD pump, a Model 710B sample programmer WISP (Waters Assoc.), a Pye Unicam DP88 integrator and a Waters M440 UV absorbance detector operated at 254 nm at a sensitivity of 0.005 a.u.f.s. The column was a 30 cm × 3.9 mm I.D., 10-μm particle size, μBondapak C_{18} column (Waters Assoc.).

The mobile phase consisted of methanol—acetonitrile—water (25:15:60, v/v) containing 0.005 *M* pentane sulphonic acid (pH 3.40). The flow-rate was 1.5 ml/min (approximately 155 bar) and the system was operated at ambient temperature. The mobile phase was filtered (FHUP04700 Millipore) prior to use and was purged with helium (50 ml/min) during analysis.

Extraction procedure

To a plasma sample (1 ml) in a 15-ml glass culture tube (Teflon-lined screw cap) were added 25 μl of quinine solution (125-ng base per 25 μl), 150 μl of 2 *M* sodium hydroxide and 6 ml of ethylene dichloride. The tube was agitated for 10 min on a Dymax shaker (100—120 strokes/min). After centrifugation at 1000 *g* for 5 min, the aqueous phase was removed and the organic phase was transferred to a clean glass tube. The organic phase was evaporated to dryness at 60°C using a gentle stream of air. The residue was dissolved in 100 μl of mobile phase and an aliquot (40 μl) injected. Analysis was done in duplicate.

To minimise adsorption of the compounds onto glass surfaces, glassware used in extraction was silanised using 0.2% Aquasil (Pierce, Rockford, IL, U.S.A.).

Calibration

Calibration curves were prepared by analysing 1.0-ml plasma samples spiked with known amounts of the compounds. The concentration range studied was

0.045—1.515 μg/ml for each compound. Peak area ratios of DDS, MADDS and PYR peaks to the internal standard peak were plotted against concentrations. Calibration standards were run on each day of analysis.

Recovery and reproducibility of the method

DDS, MADDS and PYR recovery was determined by comparing peak areas of each compound extracted from spiked plasma (range 0.023—0.909 μg/ml) with areas obtained by direct injection of pure compounds. Within-day reproducibility was determined for each compound over the range of 0.023—0.909 μg/ml of plasma, and day-to-day reproducibility was determined by assaying plasma standards over the range of 0.045—0.909 μg/ml for each compound on six occasions.

Stability of DDS, MADDS and PYR

The stability of each compound was determined by storing plasma standards and working standard solutions for six months at −15° C and 4° C, respectively. Concentrations were measured periodically using the described HPLC method.

RESULTS AND DISCUSSION

The described HPLC method uses the same instrumentation and chromatographic conditions as the simultaneous quantification of the components of Fansidar [4]. The major difference between the two methods is that Fansidar components are extracted under acidic conditions whereas Maloprim components are extracted under alkaline conditions. Pentane sulphonic acid was an essential component of the mobile phase for the separation and elution of quinine and PYR.

The separation of DDS, MADDS, quinine, and PYR extracted from plasma is shown in Fig. 1a. Retention times for DDS, MADDS, quinine and PYR were 3.4, 4.2, 7.1 and 9.3 min, respectively. Endogenous substances in plasma were found not to interfere with the assay (Fig. 1b). A typical chromatogram of the extract of a plasma sample obtained from a volunteer following one tablet of Maloprim is shown in Fig. 1c.

Calibration curves for the three compounds were linear, with correlation coefficients of 0.997 or better. Extraction recoveries were good with mean values of 96%, 94% and 93% for DDS, MADDS and PYR, respectively (Table I). Recovery of the weak base PYR was approximately 7.5% greater using alkaline extraction compared with the acidic conditions used in the analysis of Fansidar [4]. Within-day coefficients of variation averaged 7.8% for DDS, 6.2% for MADDS and 7.2% for PYR, and the day-to-day coefficient of variation averaged 8.5% for DDS, 6.5% for MADDS and 6.2% for PYR (Table II).

Jones and Ovenell [2], using HPLC, reported that metoprine [2, 4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine], a compound closely related to PYR, was a good internal standard. Metoprine and 4-nitro-4′-aminodiphenyl sulphone, an analogue of DDS, were investigated for suitability as internal standards under the described conditions. Metoprine and 4-nitro-4′-aminodiphenyl sulphone were found to coelute with PYR and an endogenous substance in plasma, respectively. As an alternative to using compounds of similar

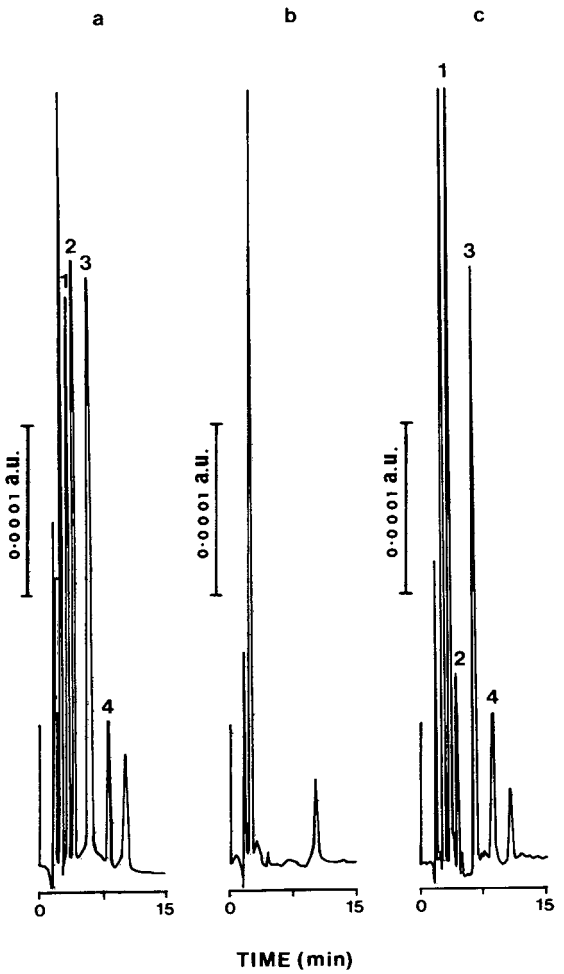

Fig. 1. Chromatograms of: (a) extracted spiked plasma sample containing 45 ng/ml each of DDS (1), MADDS (2) and PYR (4). Internal standard, quinine (3) = 125 ng base; (b) extracted drug-free plasma; and (c) extracted plasma sample obtained 72 h after administration of one tablet of Maloprim to a healthy volunteer (concentrations found in this sample were: DDS (1), 93 ng/ml; MADDS (2), 18 ng/ml; PYR (4), 40 ng/ml.

TABLE I

RECOVERY OF THE HPLC METHOD

Recovery (%) is expressed as mean % ± S.D. Number of observations = 5 per compound per concentration.

	Concentration (μg/ml)					
	0.023	0.045	0.182	0.413	0.909	Overall mean ± S.D.
DDS	94 ± 4.6	97 ± 5.0	95 ± 3.2	94 ± 2.3	98 ± 1.7	95.6 ± 1.8
MADDS	92 ± 3.2	98 ± 5.4	94 ± 0.9	92 ± 4.1	95 ± 1.1	94.2 ± 2.5
PYR	94 ± 4.7	97 ± 6.9	87 ± 3.1	92 ± 5.7	93 ± 6.1	92.6 ± 3.7

TABLE II

REPRODUCIBILITY OF THE HPLC METHOD: COEFFICIENTS OF VARIATION (%)

	Concentration (μg/ml)					
	0.023	0.182	0.909	1.515	Mean ± S.D.	
Within-day (n = 5)*						
DDS	13.6	7.1	5.1	5.2	7.8 ± 4.0	
MADDS	6.8	7.7	4.8	5.4	6.2 ± 1.3	
PYR	6.9	8.2	7.6	6.2	7.2 ± 0.9	
	Concentration (μg/ml)					
	0.045	0.091	0.182	0.413	0.909	Mean ± S.D.
Day-to-day (n = 6)*						
DDS	14.6	14.3	6.4	4.1	3.3	8.5 ± 5.5
MADDS	10.4	8.8	4.7	5.4	3.4	6.5 ± 2.9
PYR	12.6	6.7	3.5	4.4	3.8	6.2 ± 3.8

*n = number of observations per compound per concentration.

structure as internal standards, quinine was selected because it showed reproducible extraction, suitable retention and was well resolved from other peaks.

None of the following antimalarial drugs interfered with the determination of DDS, MADDS, quinine and PYR in plasma: chloroquine, mefloquine, primaquine and proguanil. Fansidar, because it contains PYR, does interfere with the analysis. No significant degradation of the three compounds was observed under the prescribed storage conditions.

The advantages of this new HPLC method over previously published methods [2, 3] are ease of sample preparation, a lower limit of detection and speed of analysis. The limit of detection for DDS and MADDS of 5 ng/ml is as sensitive as that reported by Jones and Ovenell [2], but the method is twice as sensitive for PYR determination with a detection limit of 5 ng/ml. The speed of the method was such that 50 samples could be analysed by one operator within 10 h.

Monitoring of antimalarial drug concentrations aids the assessment of possible drug resistance of the parasite. Lack of compliance with the recommended dosage regimen is a common cause of failure of malaria prophylaxis. The described HPLC method is used in our laboratory for routine monitoring of Maloprim levels and for pharmacokinetic studies.

CONCLUSION

In summary, a rapid, selective, and sensitive HPLC procedure for the simultaneous quantification of DDS, MADDS and PYR in plasma has been developed which is suitable for routine monitoring of Maloprim concentrations in man. The method is flexible in that by changing the extraction pH the principal components and the major acetylated metabolite of two widely used antimalarial drugs, Maloprim and Fansidar, can be quantified.

ACKNOWLEDGEMENT

I thank the Director General of Army Health Services, Australian Army, for approval to publish this paper.

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Journal of Chromatography, 307 (1984) 432—438
Biomedical Applications
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CHROMBIO. 2036

Note

Direct analysis of diflunisal ester and ether glucuronides by high-performance liquid chromatography

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(First received August 31st, 1983; revised manuscript received December 12th, 1983)

Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) is metabolised by conjugation with glucuronic acid to form both an ester and an ether glucuronide [1]. Previous methods for the quantitative analysis of diflunisal ester and ether glucuronides have employed β-glucuronidase or acid hydrolysis and have measured the glucuronide concentration as the difference in diflunisal concentration before and after hydrolysis [1, 2]. Difficulties in documenting the specificity of these hydrolytic methods and their lack of sensitivity in the presence of high concentrations of aglycone, such as those that frequently occur in plasma after aglycone administration, have previously been discussed by us [3].

In the case of diflunisal glucuronides, enzymatic and acid hydrolysis will yield diflunisal with both the ester and ether glucuronides and thus such analysis cannot be used to specifically quantitate each compound. Mild alkaline hydrolysis can be used to selectively hydrolyse ester glucuronides in the presence of the ether glucuronide [4] but such an approach would require three measurements of diflunisal: before and after both acid and alkaline hydrolysis in order to quantitate both glucuronides in the presence of diflunisal. Such an approach is time-consuming and frequently inaccurate.

It was our aim to develop a direct analysis for diflunisal ester and ether glucuronides that would be specific and capable of measuring low concentrations of one glucuronide in the presence of high concentrations of the other, or the aglycone.

0378-4347/84/$03.00

EXPERIMENTAL

Preparation of diflunisal glucuronides

Urine was collected from a volunteer prior to drug administration and over a 6-h period after ingestion of 125 mg diflunisal 6-hourly for one week.

A high-performance liquid chromatograph (Model 6000A) with a Model U6K injector and a variable-wavelength detector operated at 254 nm (Model 450) together with a 10-μm particle size, reversed-phase column (μBondapak C_{18}) all from Waters Assoc. (Milford, MA, U.S.A.) were used to examine the urine for diflunisal metabolites. The output from the detector was recorded using a dual-pen recorder with 10- and 50-mV voltage spans. The mobile phase of acetonitrile—0.01 *M* citrate buffer, pH 3 (250:750) had a flow-rate of 2.0 ml/min. Using the above conditions, the chromatographic profiles of pre-dose urine and diflunisal containing urine were compared prior to and after the following pre-treatments: (a) with 2000 Fishman units of β-glucuronidase (Type VII, Sigma, St. Louis, MO, U.S.A.) as previously described [3]; (b) as above but in the presence of 30 mg/ml of D-saccharic acid 1,4-lactone; (c) an equal volume of 0.2 *M* sodium hydroxide at room temperature for 30 min. Treatment a was designed to identify peaks due to glucuronides and the specificity of this approach was confirmed by treatment b. The ester, but not the ether glucuronide was hydrolysed by treatment c [4].

The urine (50 ml) was acidified to pH 2 with sulphuric acid and extracted with diethyl ether (100 ml). The diethyl ether was removed at room temperature and reduced pressure and the residue was taken up in 1 ml of 0.01 *M* citrate buffer, pH 3. This buffer was washed with hexane, which removed an interfering peak. The hexane was discarded and the buffer was filtered through a 0.45-μm filter. The filtered extract was then purified by preparative chromatography using the conditions previously described for the identification of the glucuronide peaks and the fractions containing the glucuronides were collected, based on retention time. An aliquot of each fraction was re-chromatographed prior to pooling the fractions, in order to confirm chromatographic purity. The pure fractions were pooled, lyophylised as previously described [5] and stored at −20°C prior to their use.

Samples of each lyophylised fraction were dissolved in water and their identity and purity were assessed using the following criteria: (a) the glucuronide fraction chromatographed as a single peak; (b) the glucuronide peaks were removed by β-glucuronidase hydrolysis, while additionally, the ester glucuronide peak but not the ether glucuronide peak was removed by mild alkaline hydrolysis; (c) the hydrolysis by glucuronidase was inhibited by D-saccharic acid 1,4-lactone; (d) hydrolysis of the pure glucuronide fractions yielded only diflunisal.

Chromatographic analysis of the fractions after the above pretreatments was carried out using a reversed-phase column as previously described but with a mobile phase consisting of methanol—0.01 *M* citrate buffer, pH 3 (40:60).

Assay procedure

To a 1.5-ml disposable centrifuge tube containing 0.15 ml of internal standard solution 0.05 ml of plasma or urine was added. The internal standard

solution consisted of desmethylnaproxen (50 mg/l) dissolved in acetonitrile—20%, w/v aqueous trichloroacetic acid (1:3). The samples were mixed on a vortex mixer and centrifuged at 9500 *g* for 2 min. An aliquot of supernatant (0.01 ml) was injected into the high-performance liquid chromatograph with a mobile phase of acetonitrile—0.01 *M* citrate buffer, pH 3 (300:700) flowing at 2 ml/min. The effluent was monitored at a wavelength of 254 nm. A Schoeffel (FS970 LC) fluorometer, at an excitation wavelength of 235 nm with a 370-nm emission cut-off filter was also used to monitor low concentrations of the glucuronides.

Calibration

Lyophilised fractions containing diflunisal ester and ether glucuronides were reconstituted in distilled water and the concentrations were established by hydrolysis of a sample of each of the fractions with β-glucuronidase. The amount of diflunisal liberated on hydrolysis was determined using a method previously described [6]. The ester and ether glucuronide containing solutions were mixed and serially diluted to give glucuronide concentrations in the range of approximately 3—75 mg/l for both compounds. Duplicates of these solutions were then analysed. The reproducibility of the method was assessed by determining the coefficient of variation (C.V.) of the normalised peak height ratio (peak height ratio divided by concentration).

RESULTS AND DISCUSSION

Characterisation of diflunisal glucuronides

Fig. 1 shows chromatograms of the reconstituted lyophilised ester glucuronide fraction without pretreatment (Fig. 1A), after hydrolysis with sodium hydroxide (Fig. 1B), after treatment with β-glucuronidase (Fig. 1C) and after treatment with β-glucuronidase in the presence of its inhibitor, D-saccharic acid 1,4-lactone (Fig. 1D). Under these conditions the retention times of diflunisal ether glucuronide, diflunisal ester glucuronide and diflunisal were 3.3, 5.9 and 10.1 min, respectively. When pure extracts of diflunisal ester and ether glucuronide were hydrolysed both by β-glucuronidase and acid there was a maximum of 2.8% difference between these two methods in the amount of diflunisal liberated suggesting complete hydrolysis by both methods. Similarly Fig. 2 shows chromatograms of the reconstituted ether glucuronide fraction without pretreatment (Fig. 2A), after treatment with sodium hydroxide (Fig. 2B), after treatment with β-glucuronidase (Fig. 2C) and after β-glucuronidase treatment in the presence of its inhibitor D-saccharic acid 1,4-lactone (Fig. 2D). The pattern of peaks resulting from the above pretreatments is consistent with the interpretation that the fraction shown in Fig. 1 contains pure diflunisal ester glucuronide and the fraction shown in Fig. 2 contains pure ether glucuronide, using the criteria previously described (see Experimental). The retention time of diflunisal liberated by these pretreatments of 10.1 min was the same as that of authentic diflunisal under these conditions.

Quantitative analysis

Fig. 3A shows a chromatogram of urine containing diflunisal ether glu-

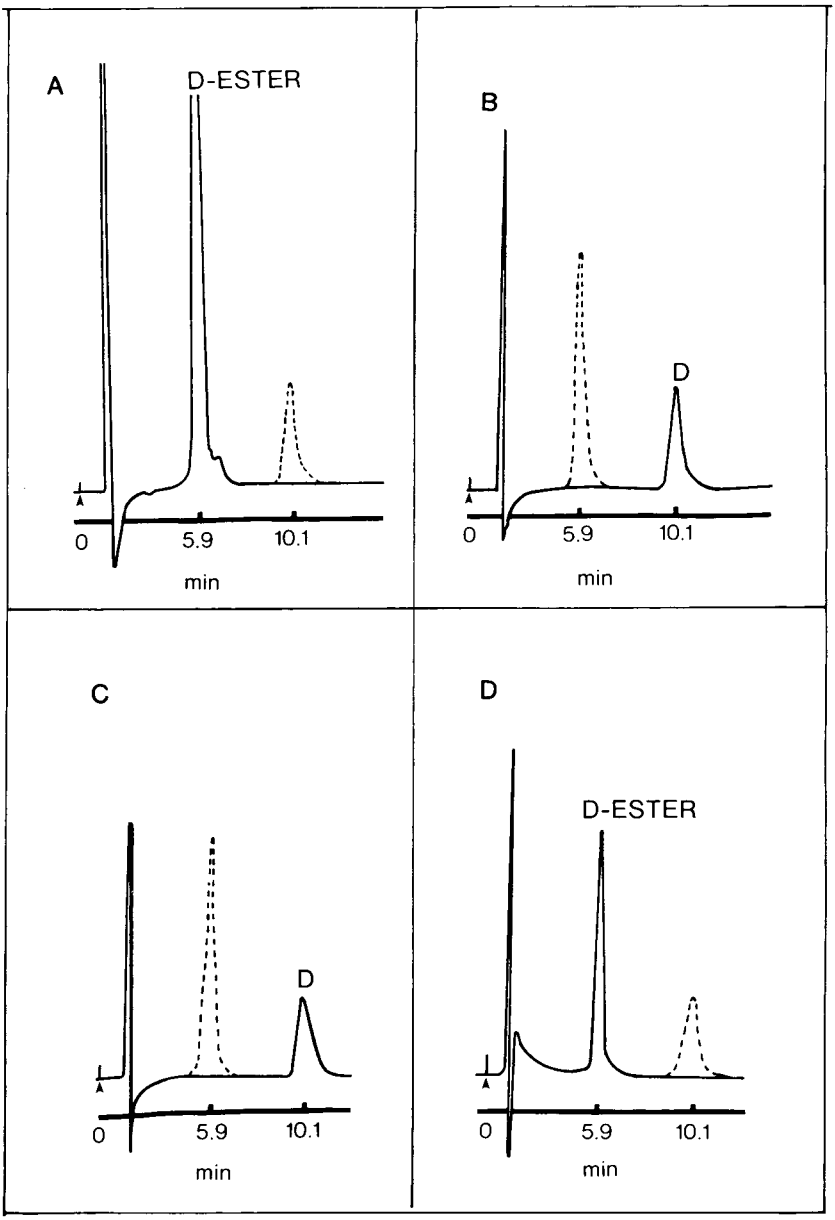

Fig. 1. Chromatograms of the reconstituted lyophilised ester glucuronide fraction. (A) No pretreatment; (B) treatment with 0.1 *M* sodium hydroxide; (C) treatment with β-glucuronidase; and (D) treatment with β-glucuronidase in the presence of D-saccharic acid 1,4-lactone. Peaks: D = diflunisal; D-ester = diflunisal ester glucuronide. In samples where no diflunisal or diflunisal ester glucuronide was detected, their retention times are indicated by a broken line.

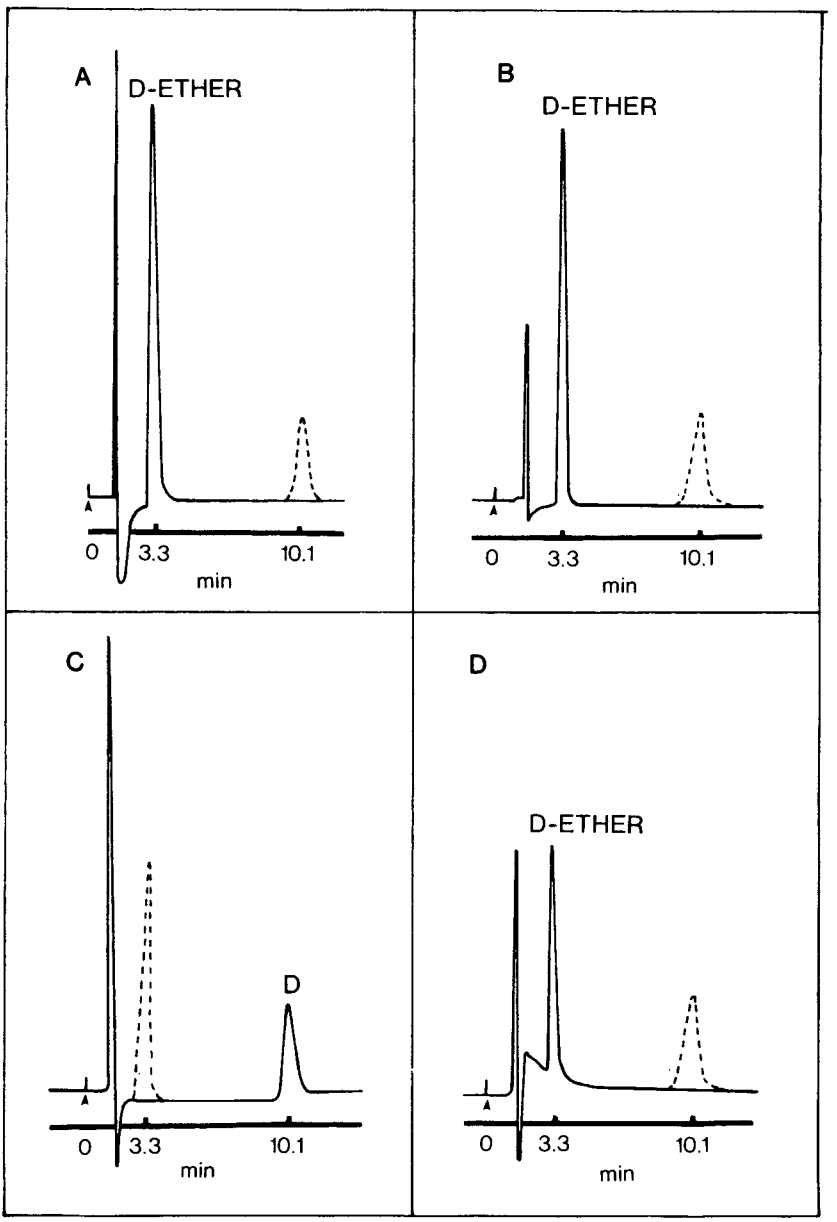

Fig. 2. Chromatograms of the reconstituted lyophylised ether glucuronide fraction. (A) No pretreatment; (B) treatment with 0.1 *M* sodium hydroxide; (C) treatment with β-glucuronidase; and (D) treatment with β-glucuronidase in the presence of D-saccharic acid 1,4-lactone. Peaks: D = diflunisal; D-ether = diflunisal ether glucuronide. In samples where no diflunisal or diflunisal ether glucuronide was detected, their retention times are indicated by a broken line.

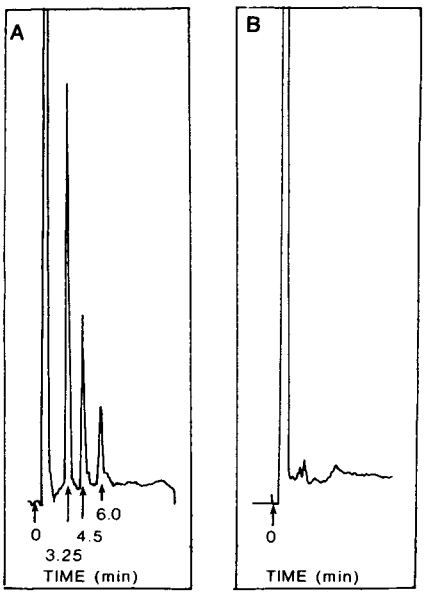

Fig. 3. Chromatograms of urine containing (A) ether glucuronide (3.25 min), internal standard (4.5 min) and ester glucuronide (6.0 min) and (B) control urine.

curonide (59 mg/l) with a retention time of 3.25 min, diflunisal ester glucuronide (20 mg/l) with a retention time of 6.0 min and the internal standard (50 mg/l) with a retention time of 4.5 min. A chromatogram of diflunisal-free urine is also shown (Fig. 3B). There were no peaks in control plasma or urine which coincided with those of the diflunisal glucuronides or the internal standard.

The reproducibility of the method over the calibrated range is shown in Table I. In the range 7.4—59.2 mg/l for the ether glucuronide and 2.8—22.0 mg/l for the ester glucuronide the coefficient of variation for the normalised peak height ratio is approximately 5%. In the range 4.7—75.2 mg/l for both ester and ether glucuronide in urine, the coefficient of variation is about 3%.

Fluorescence detection was used in a qualitative manner to examine the pre-

TABLE I

CALIBRATION DATA

Biological fluid	Concentration range (mg/l)	Mean C.V. of normalised peak height ratio (%)	
		Diflunisal ether glucuronide	Diflunisal ester glucuronide
Plasma (n = 20)	7.4—59.2	5.2	–
	2.8—22.00	–	5.1
Urine (n = 15)	4.7—75.2	2.9	3.2

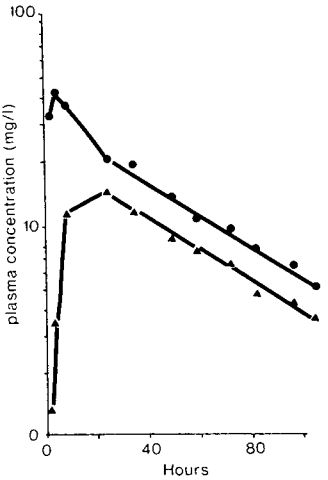

Fig. 4. The concentration of diflunisal (•) and diflunisal ether glucuronide (▲) in plasma of a patient who took a single 250-mg oral dose of diflunisal. No ester glucuronide was detected in plasma.

sence or absence of glucuronide in plasma but no calibration data are available for this mode of analysis. It is estimated that the minimum concentration detectable by fluorescence was in the order of 0.3 mg/l for the ester glucuronide and 0.1 mg/l for the ether glucuronide.

The method has been applied in the analysis of human samples (Fig. 4). Both diflunisal ether glucuronide and diflunisal, measured by a previously described method [6], are present in the plasma of a patient who took a single 250-mg dose of diflunisal, but no diflunisal ester glucuronide could be detected.

The data in Fig. 4 illustrate the difficulty of accurate glucuronide quantitation by indirect hydrolytic methods in the presence of high concentrations of aglycone. Hydrolysis of the glucuronides would result in an increase of approximately 5—10% in diflunisal concentration and if acid or β-glucuronidase and alkaline hydrolysis were carried out in order to estimate each glucuronide separately, this small increase would have to be partitioned between both compounds with a resulting accumulation of errors.

ACKNOWLEDGEMENT

This work was supported by the National Health and Medical Research Council of Australia.

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Journal of Chromatography, 307 (1984) 439—444
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2037

Note

High-performance liquid chromatographic method for the measurement of mexiletine and flecainide in blood plasma or serum

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(First received July 18th, 1983; revised manuscript received December 21st, 1983)

Mexiletine and flecainide are orally effective Class 1 antiarrhythmic agents. Plasma mexiletine measurement is an accepted aid in the administration of the drug, which has a narrow therapeutic range [1, 2]. Flecainide has been used as an investigational compound in the U.S.A. and U.K. [3] and a simple, selective assay is needed for use in the investigation of its clinical pharmacology.

Two high-performance liquid chromatographic (HPLC) methods for the measurement of mexiletine in biological fluids have been published. Both use a relatively large sample size (0.2—0.4 ml [4], 1 ml [5]), a lengthy extraction procedure, and UV absorption detection either following derivatisation with 2,4-dinitrofluorobenzene [4] or at 254 nm [5]. Published methods for the measurement of flecainide in biological fluids use HPLC with either fluorescence [6] or ultraviolet absorption detection at 308 nm [7]. Both use a relatively large sample size (1 ml) to achieve limits of accurate measurement of 50 μg/l and 22 μg/l, respectively.

The method described here for the measurement of mexiletine and flecainide is both sensitive and selective, and is based on the extraction of a small (50 μl) sample volume with an organic solvent at alkaline pH, followed by the direct analysis of the resulting extract. The chromatographic system used employs a microparticulate (5 μm) silica column together with a non-aqueous ionic eluent [8] and fluorescence detection.

EXPERIMENTAL

Mexiletine hydrochloride [1-methyl-2-(2,6-xylyloxy)ethylamine hydrochloride] and flecainide acetate [2,5-bis-(2,2,2-trifluoroethoxy)-N-(2-piperidylmethyl)benzamide acetate] were obtained from Boehringer Ingelheim U.K.

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(Bracknell, U.K.) and Riker (Loughborough, U.K.), respectively. The internal standard, benzimidazole, was obtained from BDH (Poole, U.K.) and was used as a 0.3 mg/l solution in glass-distilled water, this latter solution being prepared by dilution from a 1.0 g/l methanolic solution. Methanol, 2,2,4-trimethylpentane and methyl-*tert.*-butyl ether were all HPLC grade (Rathburn, Walkerburn, U.K.). *d*-10-Camphorsulphonic acid monohydrate was obtained from Aldrich (Gillingham, U.K.). Tris-(hydroxymethyl)-methylamine (Tris) was analytical reagent grade (BDH) and was used as a 2 *M* solution in glass-distilled water.

High-performance liquid chromatography

The solvent delivery system was a constant-flow reciprocating pump (Applied Chromatography Systems, Model 750/4) and sample injection was performed using a Rheodyne Model 7125 syringe-loading valve fitted with a

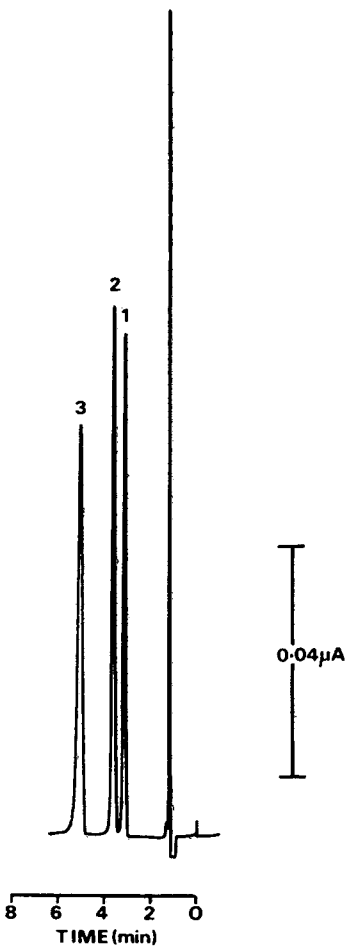

Fig. 1. Chromatogram obtained on analysis of an extract of a standard solution prepared in heparinised human plasma containing mexiletine (1) and flecainide (2) at concentrations of 1.5 and 0.5 mg/l, respectively; 100 μl injection. The initial benzimidazole (3) concentration was 0.3 mg/l.

100-μl sample loop. Stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet port of the valve to the analytical column, a stainless-steel tube 125 × 5 mm I.D. packed with Spherisorb S5W Silica (Hichrom, Woodley, U.K.) which was used at ambient temperature (normally 22°C). The mobile phase was methanol—2,2,4-trimethylpentane (80:20, v/v) containing 1 mmol/l (0.25 g/l) *d*-10-camphorsulphonic acid and was used at a flow-rate of 2.0 ml/min. The column effluent was monitored using a Schoeffel Model FS 970 fluorescence detector at an excitation wavelength of 200 nm, without emission filter, and at a time constant of 0.5 sec. Integration of peak areas was performed using a Hewlett-Packard 3390A recording integrator.

The chromatography on this system of an extract of a plasma standard containing both mexiletine and flecainide is illustrated in Fig. 1. The retention times, measured relative to the internal standard, of mexiletine, flecainide and some additional compounds of interest are given in Table I.

TABLE I

RETENTION TIMES OF MEXILETINE, FLECAINIDE AND SOME OTHER COMPOUNDS RELATIVE TO BENZIMIDAZOLE

Compound	Relative retention time	Compound	Relative retention time
Desalkylflurazepam	0.55	Dipyridamole	0.82
Nitrazepam*	0.61	Trimipramine	0.88
Mexiletine	0.61	Desipramine	0.90
4-Hydroxypropranolol	0.65	Norverapamil	0.90
Ajmaline	0.68	Prazosin	0.93
Nadolol	0.68	Terazosin	0.94
Pyrimethamine*	0.68	Protriptyline	0.97
Pindolol	0.69	Benzimidazole	1.00
Triamterene	0.69	Dextropropoxyphene*	1.10
Penbutolol	0.71	Trazodone	1.16
Propranolol	0.71	Orphenadrine	1.47
Nordextropropoxyphene*	0.71	Butriptyline	1.48
Metoprolol	0.71	Mianserin	1.65
Oxprenolol*	0.75	Verapamil	1.68
Flecainide	0.75	Imipramine	1.85
Prajmalium	0.79		

*Poor fluorescence under the conditions of the assay.

Sample preparation

Plasma or serum (50 μl) was pipetted into a small glass (Dreyer) test tube (Poulton, Selfe and Lee, Wickford, U.K.). Internal standard solution (20 μl), Tris solution (50 μl) and methyl-*tert.*-butyl ether (200 μl) were added using Hamilton gas-tight glass syringes fitted with Hamilton repeating mechanisms. The contents of the tube were vortex-mixed for 30 sec and centrifuged at 9950 *g* for 2 min in an Eppendorf 5412 centrifuge (Anderman, East Molesey, U.K.). Subsequently, a portion (approximately 110 μl) of the extract was taken and used to fill the sample loop of the injection valve. Duplicate sample analyses were performed and the mean result taken.

Instrument calibration

Standard solutions containing mexiletine at concentrations equivalent to 0.5, 1.0, 2.0 and 3.0 mg/l free-base were prepared in heparinised human plasma by serial dilution of an aqueous solution of mexiletine hydrochloride equivalent to 1.0 g/l free-base. Standard solutions containing flecainide at concentrations equivalent to 0.1, 0.25, 0.5, 0.75 and 1.0 mg/l free-base were prepared in heparinised human plasma by serial dilution of an aqueous solution

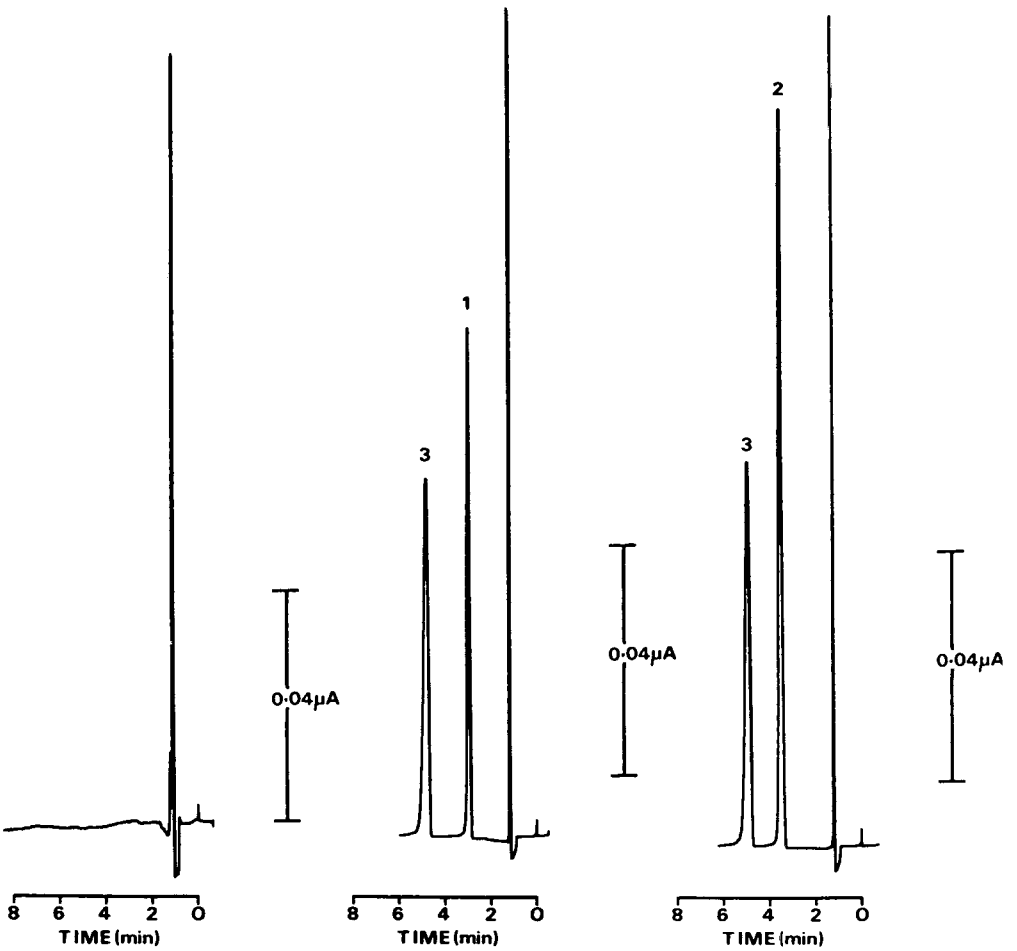

Fig. 2. Chromatogram obtained on analysis of an extract of mexiletine- and flecainide-free human plasma without addition of an internal standard; 100 μl injection.

Fig. 3. Chromatogram obtained on analysis of an extract of a plasma sample obtained from a patient receiving mexiletine (600 mg per day); 100 μl injection. The initial benzimidazole (3) concentration was 0.3 mg/l, and the plasma mexiletine (1) concentration was found to be 1.7 mg/l.

Fig. 4. Chromatogram obtained on analysis of an extract of a plasma sample obtained from a patient receiving flecainide (200 mg per day); 100 μl injection. The initial benzimidazole (3) concentration was 0.3 mg/l, and the plasma flecainide (2) concentration was found to be 0.75 mg/l.

of flecainide acetate equivalent to 1.0 g/l free-base. In addition, an internal quality assurance sample containing mexiletine and flecainide at concentrations of 1.5 and 0.5 mg/l, respectively, was prepared in heparinised human plasma by dilution from independent stock solutions of the drugs.

These standards were stable for at least three months if stored in 250-μl aliquots at −20°C in tightly closed containers in the absence of light. On analysis of these solutions the ratio of the peak area of the analyte to the peak area of the internal standard, when plotted against analyte concentration, was linear and passed through the origin of the graph in each case.

RESULTS AND DISCUSSION

No endogenous sources of interference have been observed. A chromatogram obtained on analysis of an extract of mexiletine- and flecainide-free human plasma is illustrated in Fig. 2, whilst those obtained on analysis of specimens from patients receiving either mexiletine or flecainide are shown in Figs. 3 and 4, respectively.

Compounds which were extracted under the conditions of the assay were studied further as potential sources of interference (Table I). Desalkylflurazepam, ajmaline, prajmalium, nadolol, pindolol, triamterene, penbutolol, propranolol and metoprolol elute close to either mexiletine or flecainide but were, at least, partially resolved. Although nitrazepam elutes with mexiletine and oxprenolol elutes with flecainide (Table I), no interference from these compounds was seen at the concentrations attained during normal therapy owing to their relatively poor fluorescence under the conditions of the assay. In addition, 4-hydroxypropranolol and nordextropropoxyphene were not detected owing to the instability of the former and the poor fluorescence of the latter. Finally, although protriptyline and dextropropoxyphene elute close to benzimidazole, both were partially resolved and dextropropoxyphene has a poor fluorescence relative to benzimidazole.

Some other cardioactive drugs and metabolites studied (amiodarone, desethylamiodarone, disopyramide, lignocaine, procainamide, tocainide, quinidine, sotalol, atenolol, labetalol, nifedipine, lorcainide and methyldopa) were not detected on this system.

TABLE II

THE INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION FOR MEXILETINE AND FLECAINIDE

	Mexiletine		Flecainide	
	Concentration (mg/l)	C.V. (%) (n = 10)	Concentration (mg/l)	C.V. (%) (n = 10)
Intra-assay	1.5	1.2	0.88	1.6
	0.5	2.8	0.40	2.3
	0.2	3.7	0.10	3.1
Inter-assay	2.0	2.9	0.50	2.9

The intra- and inter-assay coefficients of variation (C.V.) for replicate analyses of standard solutions of mexiletine or flecainide, prepared in heparinised human plasma, are shown in Table II. Using a sample size of 50 μl, the limits of accurate measurement for mexiletine and flecainide were 50 μg/l and 20 μg/l, respectively (intra-assay C.V. at these concentrations 8.4% and 8.9%, respectively; n = 10 in both cases).

CONCLUSIONS

The method described here has been used for the measurement of the plasma concentrations of both mexiletine and flecainide attained during therapy. Only 100 μl of specimen are required for a duplicate analysis, which can be completed together with the analysis of a quality control specimen, within 30 min and few potential sources of interference have been identified. An increase in sample size would facilitate the measurement of both compounds at the concentrations attained following single oral dosage.

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Journal of Chromatography, 307 (1984) 445—450
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2039

Note

High-performance liquid chromatographic determination of hydrochlorothiazide in plasma and urine

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Hydrochlorothiazide (HCTZ: 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide) is a potent diuretic widely used in the treatment of systemic hypertension.

Several methods to determine HCTZ in plasma and urine have been described, e.g. in patient compliance studies. These methods include thin-layer chromatography [1], colorimetry [2], gas—liquid chromatography [3,4] and high-performance liquid chromatography (HPLC) [5—10].

The method described by Tisdall et al. [8] is suitable only for qualitative determination of thiazide diuretics in urine. Other recent papers on HPLC procedures [9,10] point out limitations of previously available methods. Each of the more recent methods, however, has disadvantages as well: in the method described by Soldin et al. [9], substantial amounts of interfering substances appear in the chromatograms of both serum and urine; the method described by Barbhaiya et al. [10] requires different procedures for plasma and urine.

For these reasons and because we needed to determine plasma and urinary HCTZ levels in a clinical trial, we developed a new, simple, rapid and sensitive HPLC method to measure HCTZ in plasma and urine, using chlorothiazide (CTZ) as an internal standard.

MATERIALS AND METHODS

Reagents

Ethyl acetate, acetic acid, sodium bicarbonate, tris(hydroxymethyl)aminomethane and methanol were obtained from Merck (Darmstadt, F.R.G.). Anhydrous sodium acetate and tetrabutylammonium hydrogen sulphate were obtained from Baker Chemicals (Deventer, The Netherlands) and Janssen Chimica (Beerse, Belgium), respectively. All reagents were of analytical grade quality. HCTZ and CTZ were kindly supplied by Merck, Sharp and Dohme (Haarlem, The Netherlands).

Biological fluids

Human blood plasma stored at −20°C was obtained from the local blood bank. Human urine (pH adjusted to 5.0 ± 0.5) was collected from a male volunteer shortly before analysis.

Apparatus and chromatographic conditions

We used a Hewlett-Packard HP 1084B liquid chromatograph equipped with a variable-wavelength detector and autosampler. The detection wavelength was 272 nm. The stainless-steel column (15 cm × 4.6 mm I.D.) was packed with LiChrosorb RP-18, particle size 5 μm (Merck). The oven temperature was 38°C and the injection volume was 10 μl for urine and 30 μl for plasma.

The mobile phase was a mixture of methanol and twice-distilled water (20:80) containing 0.01024 *M* tetrabutylammonium hydrogen sulphate and 0.00976 *M* tris(hydroxymethyl)aminomethane (pH 5.5). This was delivered at a rate of 1.2 ml/min, producing a pressure of 178 bars.

The mobile phase was prepared as follows: 3.820 g of tetrabutylammonium hydrogen sulphate (mol. wt. = 339.54) were dissolved in 450 ml of twice-distilled water, and 1.363 g of tris(hydroxymethyl)aminomethane (mol. wt. = 121.14) were dissolved in 450 ml of twice-distilled water. The latter solution was added to the tetrabutylammonium hydrogen sulphate until pH 5.5 was attained (429 ml were required). Next, the mixture was filtered through a 0.45-μm Millipore filter and 200 ml of methanol were subsequently added to 800 ml of the filtered mixture.

Procedure

We pipetted 100 μl of a solution of CTZ in methanol (50 mg per 100 ml methanol for urine, and 1 mg per 100 ml methanol for plasma) into a screw-capped extraction tube. The methanol was evaporated with a gentle stream of dry-filtered air, whereupon 0.5 ml of plasma (or urine), 0.5 ml of 0.1 *M* acetate buffer (pH 3.8 for plasma and pH 5.0 for urine) and 5 ml of ethyl acetate were successively pipetted into the tube. The tube was then closed and shaken mechanically for 30 min; after centrifugation at 1300 *g* for 15 min the organic layer was transferred to a clean extraction tube containing 400 mg of sodium bicarbonate.

The tube was closed again and placed on a whirl mixer for 20 sec. After centrifugation at 1300 *g* for 10 min the organic layer was then pipetted into a clean tube and evaporated to dryness at 30°C with dry filtered air. Subsequently the residue was dissolved in 0.5 ml of the mobile phase (1 ml for urine sample), and 30 μl (for plasma) or 10 μl (for urine) were injected onto the column.

RESULTS

Figs. 1 and 2 show typical chromatograms for blank plasma and blank urine samples with or without sodium bicarbonate treatment, for plasma containing 0.106 μg/ml HCTZ and 2.024 μg/ml CTZ and for urine containing 2.66 μg/ml HCTZ and 1.012 μg/ml CTZ (both treated with sodium bicarbonate). The retention time of HCTZ is about 7.0 min, and that of CTZ about 9.9 min.

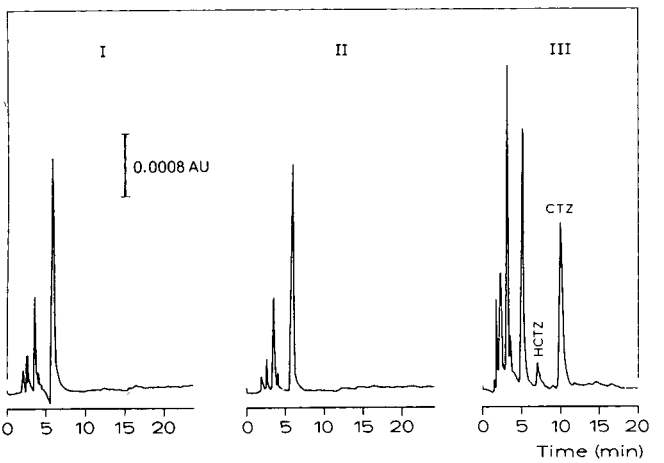

Fig. 1. Chromatograms obtained from a blank plasma sample not treated with sodium bicarbonate (I), a blank sample treated with 200 mg of sodium bicarbonate (II), and a sample containing 0.106 μg/ml HCTZ and 2.024 μg/ml CTZ, treated with sodium bicarbonate (III).

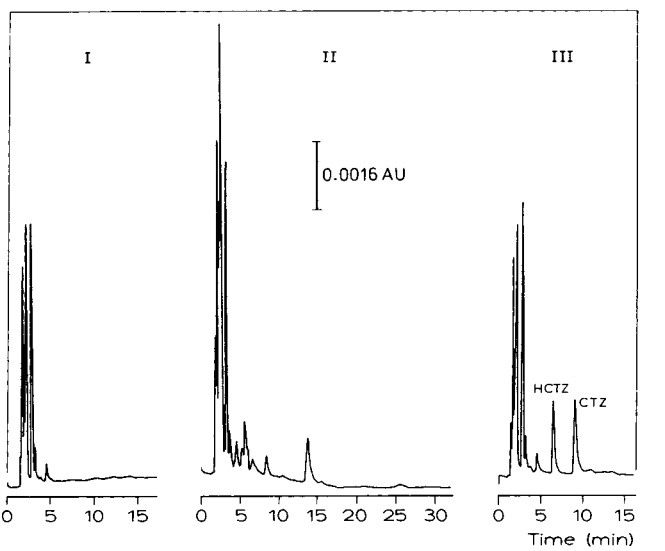

Fig. 2. Chromatograms obtained from a blank urine sample treated with 400 mg of sodium bicarbonate (I), a blank sample not treated with sodium bicarbonate (II), and a sample containing 2.66 μg/ml HCTZ and 10.12 μg/ml CTZ, treated with sodium bicarbonate (III).

Calculation

The HCTZ concentration in a sample was determined by comparing the peak height ratio (HCTZ/internal standard) with a standard curve of peak height ratio versus HCTZ concentration. Whenever a sample containing HCTZ was measured, a standard curve was generated by adding different amounts of HCTZ to blank plasma or blank urine and analyzing them by the method already described.

A linear relationship was found between the peak height ratio (PHR) of HCTZ to CTZ (Y) and the plasma HCTZ concentration (X), as given by the equation $Y = 1.4117\ X + 0.007$ ($r = 0.9997$, $n = 6$) for the plasma HCTZ concentration range 0.025 — 1.00 μg/ml. For urine the equation was $Y = 0.03930\ X + 0.00047$ ($r = 0.9998$, $n = 6$) for the range 2.66 — 53.20 μg/ml (internal standard concentration 101.2 μg/ml), and $Y = 0.3703\ X - 0.00183$ ($r = 0.9999$, $n = 5$) for the range 0.25 — 5.39 μg/ml (internal standard concentration 10.12 μg/ml).

Recovery

Overall recovery was determined by comparing the peak heights of HCTZ and CTZ obtained after injection of standard solutions with peak heights obtained after injection of extracted standard solutions (Table I).

CTZ recovery from plasma was 80 ± 4% at a concentration of 2.0 μg/ml and that from urine was 64 ± 2% at 50 μg/ml.

TABLE I

RECOVERY OF HCTZ IN PLASMA AND URINE

$n = 10$ for all concentrations.

Plasma			Urine		
HCTZ concentration (μg/ml)	Recovery (%)	C.V. (%)	HCTZ concentration (μg/ml)	Recovery (%)	C.V. (%)
0.1	92	2	0.5	95	3
0.3	95	4	5.0	92	3
1.0	96	4	15.0	88	2
			50.0	87	1

Sensitivity and precision

The detection limit with the analysis described was 0.025 μg/ml for plasma and 0.5 μg/ml for urine. Tables II and III show the within-day precision of HCTZ analysis in plasma and urine, respectively. The between-day precision for spiked HCTZ plasma samples (in the range 0.05 — 1.00 μg/ml) was 2.8% ($n = 9$), and that for spiked HCTZ urine samples (in the range 5 — 50 μg/ml) was 1.6% ($n = 6$).

DISCUSSION

As pointed out, none of the published methods to determine HCTZ met our requirements. Our method is simple and rapid, and does not require different procedures to measure HCTZ in plasma and in urine.

TABLE II

ANALYSIS OF SPIKED HCTZ PLASMA SAMPLES

Sample	Concentration (μg/ml)	n	C.V. (%)
1	0.021	10	8.3
2	0.107	10	2.4
3	0.320	10	3.7
4	1.080	10	2.7

TABLE III

ANALYSIS OF SPIKED HCTZ URINE SAMPLES

Sample	Concentration (μg/ml)	n	C.V. (%)
1	0.51	10	2.2
2	5.23	10	3.3
3	15.46	10	2.0
4	51.20	10	2.0

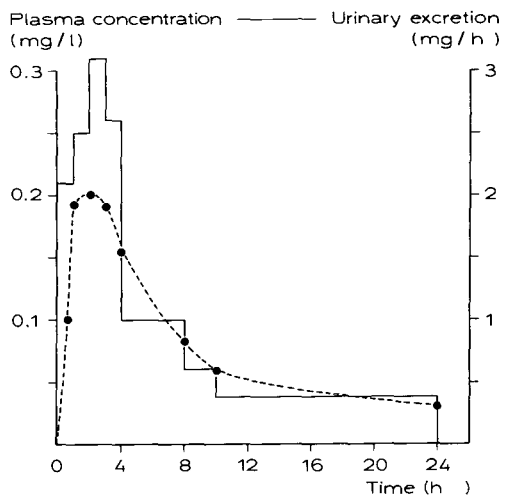

Fig. 3. Plasma concentration (- - -) and urinary excretion (——) of HCTZ in a healthy adult male volunteer given 50 mg of HCTZ orally.

Recovery of our internal standard, CTZ, is admittedly only about 70%; however, it is very constant. Like Cooper et al. [5], we used sodium bicarbonate. We found the HCTZ retention time in plasma to be constant after addition of sodium bicarbonate. For urine, we used sodium bicarbonate to eliminate interfering substances.

Our method was applied in pharmacokinetic studies. As a typical example Fig. 3 shows the plasma concentration and urinary excretion of HCTZ in a healthy adult male volunteer after ingestion of one 50-mg tablet at 08.00 a.m.

The fall in plasma concentration was biphasic, confirming results reported by others [11,12]. The elimination half-life is 4 h, and a peak plasma level is reached at 2 h. The other kinetic parameters were also within the reported range.

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Journal of Chromatography, 307 (1984) 451—456
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2044

Note

High-performance liquid chromatographic—electrochemical assay method for primaquine in plasma and urine

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(First received September 6th, 1983; revised manuscript received December 27th, 1983)

Primaquine, an 8-aminoquinoline, is currently the only available antimalarial drug which is a true tissue schizontocide. In 1950 it was first reported to be the drug of choice for the radical cure of vivax malaria [1] and a true causal prophylactic agent not only against vivax malaria but also the frequently lethal falciparum malaria. More than a quarter of a century later a less toxic replacement for primaquine has not been forthcoming nor have the mechanisms of its antimalarial and hemolytic effects been elucidated [2]. Although several methods have recently been reported using gas—liquid chromatography—mass spectrometry [3], gas—liquid chromatography—electron-capture detection [4] and high-performance liquid chromatography (HPLC)—UV [5—7] techniques, they require extraction, derivatization with partial recovery or lack sensitivity for monitoring therapeutic dosage regimens. Here we would like to report a simple, sensitive assay for primaquine, and its metabolites utilizing HPLC with electrochemical detection (ED).

MATERIALS AND METHODS

Chemicals

Primaquine (PQ) was obtained from Aldrich (Milwaukee, WI, U.S.A.) as the diphosphate salt. 6-Methoxy-8-aminoquinoline was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.). PQC, 8-(3-carboxy-1-methyl-propylamine)-6-methoxyquinoline (Fig. 1) was graciously provided by Dr. J.D. McChesney, University of Mississippi, U.S.A. The chemicals used in preparing the buffer are reagent-grade anhydrous citric acid (Sigma, St. Louis, MO, U.S.A.) and potassium hydroxide (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Acetonitrile and

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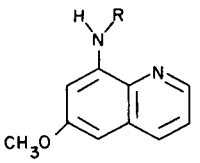

PRIMAQUINE R = $-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-CH_2-NH_2$

8-(4-amino-1-methylbutylamino)-6-methoxy quinoline

PQC R = $-\underset{\underset{CH_3}{|}}{CH}-CH_2-CH_2-COOH$

8-(3-carboxy-1-methylpropylamino)-6-methoxy quinoline

Fig. 1. Structures of primaquine (PQ) and the oxidative deamination product PQC.

methanol are HPLC grade obtained from Fisher Scientific. Amberlite XAD-2 is obtained from Bio Rad Labs. (Richmond, VA, U.S.A.). Deionized water is prepared with a Milli RO/Milli Q water purification system from Millipore (Bedford, MA, U.S.A.).

Instrumentation

The HPLC system (Fig. 2) consists of a Waters WISP (Waters Intelligent Sample Processor) (Waters Assoc., Milford, MA, U.S.A.), a Waters 6000A pump and a Waters μ Bondapak CN (250 mm × 3.9 mm I.D., particle size 10 μm) reversed-phase column. Mobile phase is acetonitrile—0.08 *M* citrate (77:23, pH 5.0). Flow-rate is 1 ml/min at ambient temperature.

Detection is by ESA Model 5100 dual-electrode electrochemical detector (ESA, Bedford, MA, U.S.A.) and with an ESA guard cell placed between the pump and injector. There are in line ESA 0.2-μm graphite filters before the guard and analytical cells (Fig. 2). The analytical cell contains two electrodes in series configuration, with the upstream electrode being designated D_1 and the downstream electrode being designated D_2. The potentials used are 0.56 V at the guard cell and 0.3 V and 0.50 V at D_1 and D_2. The gain at D_2 is 50 × 10 with a response time of 10 sec. The signal from D_2 is recorded on a Fisher Recordall Series 5000 dual-pen recorder.

Procedure

Preparation of plasma and urine samples are carried out according to a previously reported procedure [7] except that smaller amounts of standard were used in construction of the standard curve. PQ and PQC, 10 ng/ml to 1.0 μg/ml and 0.20 μg/ml to 2.0 μg/ml, respectively, were added to control plasma and urine samples.

Quantitation

The calibration curves are obtained each day by plotting detector response against the concentration for each of the standard solutions. Slope of the calibration curves and coefficients of determination are then calculated and utilized in quantitating PQ and PQC in unknown samples.

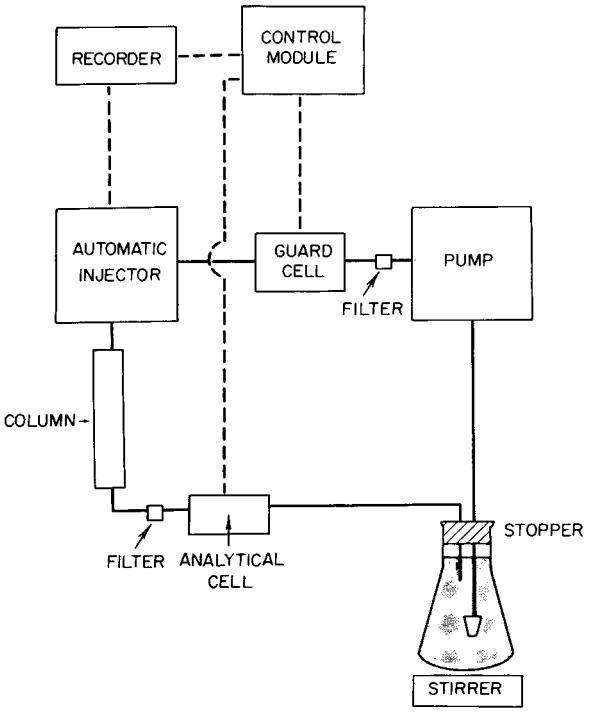

Fig. 2. Schematic presentation of the HPLC—ED system used. The fluid path is shown by the solid line and the electrical connections are shown by the hatched line.

Recovery

Recoveries of PQ and PQC at different concentrations are determined by comparison of spiked plasma R_1 to direct injection of PQ and PQC in mobile phase solvent R_2. Recovery (%) = $R_1/R_2 \times 100$.

Recovery of spiked urine primaquine samples are determined by passing known solutions of primaquine through the Amberlite XAD column and analyzing the methanol eluent for primaquine content. The detector responses are then compared using the above equation.

Dosing and specimen collection

After Institutional Review Board approval, single doses of primaquine diphosphate were administered with four ounces of water to normal healthy male volunteers. Doses given ranged from 30 to 180 mg calculated as the base. At 3 h after the dose the volunteers were allowed to eat and drink ad libitum.

Venous blood was collected at 0, 0.5, 0.75, 1, 2, 2.5, 3, 4, 6, 8, 10, 12, 15, 18 and 24 h. Urine is collected at time zero and at each void for up to 48 h.

RESULTS

Standard curves are linear over a concentration range of 10 to 1000 ng/ml and 0.2 to 2.0 μg/ml for PQ and PQC, respectively. The coefficients of determination (r^2) of the calibration graphs are 0.995 ± 0.004 (n=10). The lower

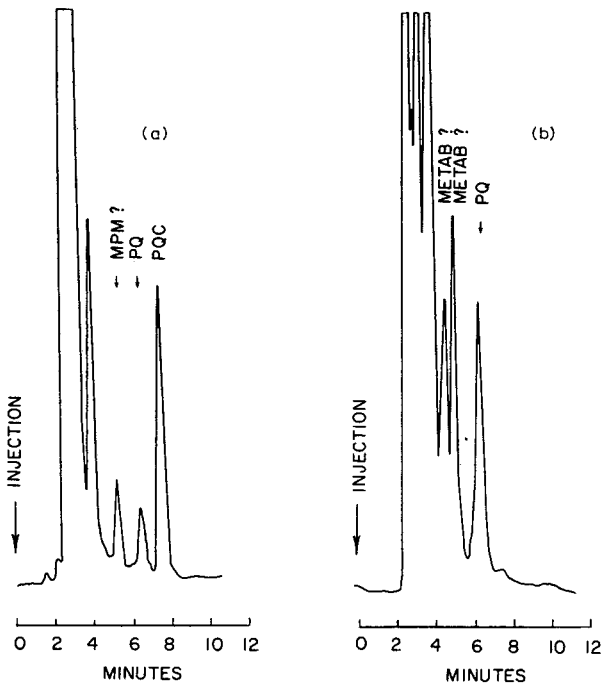

Fig. 3. Chromatograms of (a) plasma sample; and (b) urine extract done on a 0—24 h urine pool. Peaks: MPM = more polar metabolite.

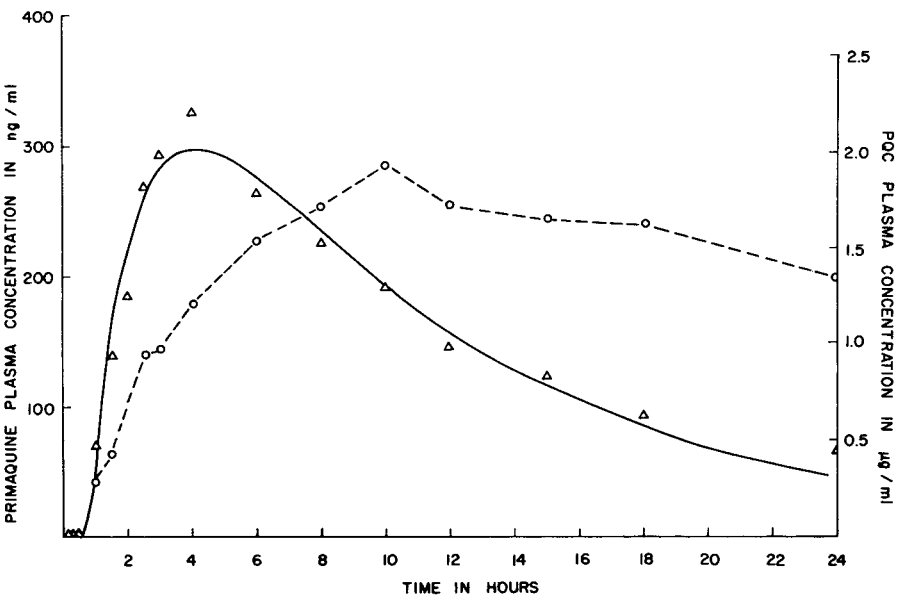

Fig. 4. Plasma concentrations of PQ and PQC for the first 24 h from a volunteer taking a single dose of 120 mg. PQ (△—△) measured concentrations with curve fitted using NONLIN; PQC (o- -o) measured concentrations with interpolated curve.

limit in routine primaquine quantitation is 0.2 ng on the column using a signal-to-noise ratio greater than 3.

Chromatograms resulting from the analysis of plasma and urine from a volunteer after ingestion of a single dose of primaquine may be seen in Fig. 3. The plasma chromatogram shows the PQ and PQC peaks as well as a more polar peak (MPM), which is ascribed to an as yet unknown metabolite. The urinary chromatogram shows the presence of PQ and two peaks which are thought to be metabolites. Using our HPLC—ED conditions PQ has a k' of 2.0 and PQC has a k' of 2.5. The known metabolite (PQC) and presumed metabolites are not present in control samples.

Fig. 4 shows typical plasma levels for PQ and PQC after a 120-mg oral primaquine dose. Data were fitted to a one-compartment open model using a non-linear least-squares regression computer program (NONLIN) [8], which estimated the following pharmacokinetic parameters: lag time, 0.78 h; absorption half-life, 1.08 h; elimination half-life, 6.72 h. These parameters were used to generate the fitted curve for PQ. Superimposed above the PQ concentration curve is the interpolated curve for the metabolite PQC.

DISCUSSION

The use of the citrate mobile phase buffer at pH 5 effects a good separation among residual plasma components, PQ, PQC and other presumed metabolites. However, it does adversely affect column life and will result in chromatograms with different retention times and even peak reversal with other manufacturers columns. Reduction of the incubation precipitation period to less than 30 min yields samples with more plasma contaminates which result in a larger signal-to-noise ratio. Effects of other substances may be minimized by setting D_1 on the electrochemical detector at 0.3 V. Thus more easily oxidizable components are oxidized before reaching the electrode at D_2.

Primaquine appears in plasma shortly after ingestion with duration of detection being dose-dependent. This period is usually less than 24 h. Visual inspection of the plasma chromatograms indicates the presence of two primaquine metabolites. One is an oxidative deamination product identified as 8-(3-carboxy-1-methylpropylamino)-6-methoxyquinoline (PQC) in rats [5] and now shown by us to be a human plasma metabolite. Subsequently this was confirmed by mass spectrometry [9]. The other plasma metabolite has not been identified.

Small amounts of primaquine are found in urine (2—5% of the dose) and urinary chromatograms have two peaks in addition to primaquine. These peaks appear after oral ingestion of primaquine and are presently thought to be metabolites. PQC does not appear to be present in urine samples and attempts to find conjugation products of PQC in urine samples have been unsuccessful. Other primaquine metabolites have been reported by Strother et al. [10]. As yet we do not know if any of these correspond to our unidentified metabolites.

In contrast to our HPLC—UV method [7] the HPLC—ED technique has seven-fold increased sensitivity sufficient for measuring primaquine after oral ingestion of therapeutic as well as experimental doses. It will permit the elucidation of primaquine pharmacokinetics and allow us to follow its metabolism more closely. It is now possible to determine if there are drug—drug inter-

actions between primaquine and other antimalarials and may lead to a better understanding of primaquine antimalarial and hemolytic effects.

ACKNOWLEDGEMENTS

This investigation received the financial support of the UNDP/World Bank/WHO Special Programme for Research Training in Tropical Disease, intramural grants and funds from the BRSG to the Rush Medical College and from the Otto S.A. Sprague fund.

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Journal of Chromatography, 307 (1984) 457—463
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

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Note

Assay of plasma thioridazine and metabolites by high-performance liquid chromatography with amperometric detection

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(First received September 27th, 1983; revised manuscript received December 14th, 1983)

The clinical utility of monitoring plasma levels of phenothiazines and their metabolites is not yet firmly established, although past work suggests that there is a relationship between plasma level and clinical effects [1]. Adequate assay methods are necessary to further elucidate this relationship and to investigate the pharmacokinetics of this important class of antipsychotic agents. Thioridazine (Mellaril) is a particularly interesting antipsychotic agent in this regard because it is commonly used clinically and its clinical effects may be highly dependent on the relative proportions of its individual metabolites in blood and tissue [2]. Since the introduction of thioridazine in the late 1950s, several analytical procedures have been described for its determination in human plasma [3—5]. Most frequently, a gas or liquid chromatographic method has been employed. Among the liquid chromatographic assays, both adsorption (normal-phase) [6] and reversed-phase [7] techniques have been used. Detection of the eluted compounds has typically been accomplished with ultraviolet (UV) absorbance methods [8], although electrochemical detection is becoming more popular for the phenothiazine class of drugs [9, 10]. This report describes a reversed-phase high-performance liquid chromatographic (HPLC) system coupled to an amperometric detector for the determination of thioridazine and three pharmacologically active metabolites in human plasma. The assay described provides several advantages over existing methods: (a) increased chromatographic resolution and efficiency, (b) increased detector selectivity and sensitivity, and (c) better sample recovery.

0378-4347/84/$03.00

EXPERIMENTAL

Apparatus

The HPLC system consisted of an Altex Model 110A pump, an Altex Model 210 injector equipped with a 500-μl sampling loop (all from Rainin Instruments, Woburn, MA U.S.A.), and a cyano-bonded reversed-phase column (30 cm × 3.9 mm I.D. Waters Assoc., Milford, MA, U.S.A.). An amperometric detector was employed (Model LC-4) using a glassy carbon working electrode maintained at 0.9 V relative to a silver/silver chloride reference electrode (all from Bioanalytical Systems, West Lafayette, IN, U.S.A.). The detector signals were plotted on a strip-chart recorder (10-mV full-scale pen deflection; Houston Instruments, Austin, TX, U.S.A.). Other columns used during methods development (μBondapak C_{18}, μBondapak Phenyl, and μPorasil; all with 10-μm particle size) were also purchased from Waters Assoc.

Materials

All solutions were made with ultrapure water, prepared by a reverse osmosis, deionization, and charcoal filtration system (Hydro Service, Weymouth, MA, U.S.A.) Tetrahydrofuran (THF), hexane, and diethyl ether were all HPLC grade (Fisher Scientific, Medford, MA, U.S.A.). All buffer salts and other reagents were analytical grade or better.

Authentic standard compounds (thioridazine·HCl, mesoridazine·besylate, sulforidazine, northioridazine, thioridazine-5-sulfoxide, and thioridazine-2,5-disulfoxide) were all generously provided by Sandoz Pharmaceuticals (Basel, Switzerland). Chlorpromazine·HCl was donated by Smith, Kline and French Labs. (Philadelphia, PA, U.S.A.).

No special surface treatment was found to be necessary for the Pyrex glassware. All tubes and caps used in the sample preparation and storage were polypropylene (Elkay Products, Shrewsbury, MA, U.S.A.).

Chromatography

The mobile phase consisted of 70% (v/v) 20 m*M* monosodium phosphate containing 0.1 m*M* disodium ethylenediaminetetraacetic acid (Na_2EDTA), prefiltered through a 0.45-μm pore cellulose microfilter (Millipore, Bedford, MA, U.S.A.), and 30% THF. This mixture was adjusted to pH 4.0 and degassed by ultrasonication for 10 min. The chromatographic flow-rate was maintained at 1.5 ml/min for all separations.

Sample preparation

Plasma samples were prepared from whole blood drawn into heparinized tubes (Venoject, Kimble-Terumo, Elkton, MD, U.S.A.). Following a low-speed (2000 *g*), refrigerated (4°C) centrifugation, the clear plasma was transferred to, and stored in capped polypropylene tubes at −20° C until assay.

For the phenothiazine extraction, 1.0 ml of plasma was pipetted into a 12 × 75 mm polypropylene tube containing 100 μl of 10 *M* sodium hydroxide and 100 μl (1.0 μg) of authentic chlorpromazine in water as the internal standard. Following brief vortex-mixing, 2.5 ml of freshly prepared diethyl ether—hexane (3:1, v/v) was added. This mixture was vortexed vigorously for

1 min and then centrifuged at 6000 *g* for 5 min. The sample tubes were then placed on crushed dry ice. When the lower, aqueous phase was frozen, the organic phase was decanted into a 12-ml capacity polypropylene tube containing 2.5 ml of 0.1 *M* citric acid. The tubes were capped, vortexed for 30 sec, and then centrifuged at 6000 *g* for 5 min. The organic phase, now devoid of phenothiazines, was aspirated to waste. To the aqueous phase, 250 μl of 10 *M* sodium hydroxide and 0.9 g of sodium chloride were added, followed by 1.0 ml of hexane. The samples were agitated on a rotary inversion mixer for 15 min, after which time they were centrifuged for 5 min at 6000 *g*. Samples were then placed on crushed dry ice, as before. The hexane phase, containing the phenothiazines, was decanted from the frozen aqueous layer into a 12 × 75 mm polypropylene tube containing 1.0 ml of 0.1 *M* citric acid. This new mixture was vortexed and centrifuged as before. Following aspiration of the hexane phase to waste, 500 μl of the citrate phase was injected into the HPLC system.

Standard solutions of authentic phenothiazines in plasma and water were always assayed in parallel with unknown samples. Concentrations of thioridazine, mesoridazine, sulforidazine, and northioridazine of 50, 100, 500, and 1000 ng/ml were found to be appropriate for comparison to clinical samples.

RESULTS AND DISCUSSION

Chromatography

Several chromatographic schemes were tried without success before discovery of the system discussed in Experimental. Our first attempt, using a modification of a previously reported normal-phase method [6], employed a mobile phase of isooctane—water-saturated methylene chloride—methanol (9:1:1, v/v/v) containing 200 μl/l diethylamine. The column used was a 30 cm × 3.9 mm I.D. stainless-steel μPorasil column. The column provided excellent resolution of the drug standards thioridazine, northioridazine, sulforidazine, mesoridazine, thioridazine-5-sulfoxide, and thioridazine-2,5-disulfoxide. Due to the UV-absorbing characteristics of our mobile phase, the UV detector was tuned to a wavelength of 285 nm, instead of 254 nm as employed by Kilts et al. [6]. Although the detector provided adequate sensitivity for clinical samples, a large interfering peak, present only in patient blood specimens and coeluting with mesoridazine, yielded erroneously high mesoridazine values. This interfering signal may represent an additional, ring-oxidized metabolite* of thioridazine amplified preferentially at the selected detector wavelength.

The UV detector was abandoned in favor of the more sensitive and selective amperometric detector. Since electrochemical detectors rely on electrically conductive mobile phases, a suitable reversed-phase system was sought. In early trials we used previously reported methods incorporating acidic buffers, polar organic solvents, and trace organic amine modifiers in the mobile phases. Octadecylsilica (ODS or C_{18}) and phenyl-bonded silica (μBondapak C_{18} and

*Of the many theoretically possible thioridazine metabolites, this is most likely a ring-sulfoxide or sulfone because it is not detected electrochemically; such detection requires a reduced ring-sulfur atom in the oxidation process at the carbon electrode.

μBondapak Phenyl, respectively) stainless-steel columns were employed with these mobile phases. The chromatographic results were disappointing with each mobile phase combination tried. Individually, each of the four compounds tested (mesoridazine, sulforidazine, thioridazine, and northioridazine) gave usable peaks, but, unfortunately, the pairs mesoridazine—sulforidazine and thioridazine—northioridazine coeluted exactly.

ODS columns have been found to exhibit excellent resolving power with moderately polar organic molecules, such as the monoamine neurotransmitters and their metabolites. The phenothiazines, as well as other relatively large drug molecules, are much less polar, however, so that the ODS column characteristics are less appropriate. Additionally, ODS colums usually require a small amount of organic amine modifier in the mobile phase to prevent peak tailing. These organic amines react with and passivate glassy carbon electrode surfaces, thereby causing large losses of sensitivity over time [11].

Recently, other bonded phases have become available. These columns exhibit diverse and unique chemical characteristics. Wallace et al. [12] reported an assay for the non-antipsychotic phenothiazine drug promethazine using a normal-phase column (MicroPak CN-10) with cyanopropyl as the phase bonded to silica and a mobile phase of acetonitrile—20 m*M* KH_2PO_4 (45 : 55). Thus a cyano-bonded column seemed a promising choice.

A Waters Assoc. reversed-phase cyano-bonded column was tried with the mobile phase used by Wallace et al. [12]. Although mesoridazine gave a sharp,

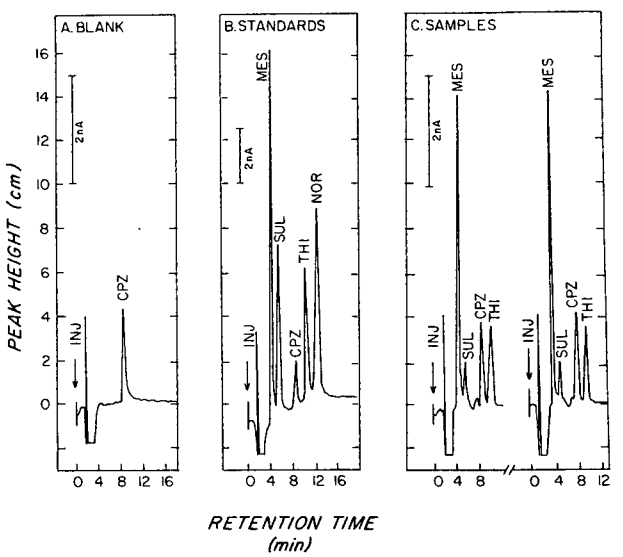

Fig. 1. Sample chromatograms. (A) Blank, extracted human plasma containing 1000 ng/ml chlorpromazine (CPZ) as the internal standard. The initial biphasic solvent front is due to the citric acid in the injection vehicle. (B) Normal human plasma extract, containing authentic standards (1000 ng/ml) of mesoridazine (MES), sulforidazine (SUL), chlorpromazine (CPZ), thioridazine (THI), and northioridazine (NOR). Note that sensitivity is 50% that in examples A and C. (C) Duplicate assays of plasma extracts from a patient receiving thioridazine (200 mg per day, orally). Blood was drawn at 12 h after the last daily dose following two weeks of this treatment. Note typical absence of northioridazine at this detector sensitivity.

early-eluting peak, thioridazine eluted much later, as a broad asymmetric hump. Methanol and butanol, at various pH values, were tried unsuccessfully as solvent modifiers. THF was then tried, with better results.

A final mobile phase composed of 20 m*M* monosodium phosphate containing 0.1 m*M* Na_2EDTA—THF, pH 4.0 (70:30, v/v) was chosen. This system resolved mesoridazine, sulforidazine, thioridazine, and northioridazine in less than 14 min at a flow-rate of 1.5 ml/min (see Fig. 1). Virtually symmetrical peaks were obtained without any organic amine modifier in the mobile phase. The elution order indicated mixed reversed- and normal-phase chromatographic behavior of the system. The sulfoxyl metabolites of thioridazine, mesoridazine, and sulforidazine eluted before the less polar parent compound, as is expected in a reversed-phase system. Thioridazine, however, eluted before its desmethylated and thus more polar metabolite northioridazine.

The ring-sulfoxyl metabolites of thioridazine are not detectable by amperometry, presumably because a reduced ring-sulfur atom is necessary for oxidation at the glassy carbon electrode. Since the ring-sulfoxyl metabolites of thioridazine are believed not to be active as antipsychotic agents [2], the added selectivity of the present assay, which excludes them, is welcome. Furthermore, amperometric detectors, which can reliably detect less than 1 ng of a phenothiazine injected on a column, give the present method other potential clinical applications since highly potent phenothiazines, such as fluphenazine (Prolixin), are typically present in blood at levels below 1 ng/ml [13].

Sample preparation

A back-extraction method was found to be necessary for the phenothiazines due to the poor recovery experienced when evaporation—concentration steps were used. Although some previous authors did not report this problem [14, 15], we found that this class of drugs adhered strongly to the sample tubes following solvent evaporation under a gentle stream of nitrogen gas. Recovery of the phenothiazines did not exceed 20% following reconstitution of the residue in various buffers or methanol—buffer mixtures. This effect persisted even when tubes composed of polypropylene, polyethylene, PTFE, stainless steel, Pyrex, or flint glass were used for the evaporation—reconstitution step. Coating the inside walls of sample tubes with an organosilane surface-treating agent (Prosil-28, PCR Research Chemicals, Gainesville, FL, U.S.A.) had little or no effect on the recovery of thioridazine.

The use of large injection volumes eliminated the need to concentrate samples. Little or no loss of resolution occurred, nor was any interference from the void volume observed, with injected samples volumes of up to 500 µl. Presumably, even larger volumes can be used, if necessary.

Recovery, precision and linearity

The recovery of the water-based standards averaged 76% for standards at 50, 100, 500, 1000, 5000, and 10,000 ng/ml (one sample at each concentration). The plasma-based standards, however, exhibited somewhat lower recoveries, averaging a high of 71% for thioridazine, but 62% for chlorpromazine, and only 39% for mesoridazine (one sample each at 10, 50, and 100 ng/ml), presumably due to lipophilic interactions between drugs and plasma constitu-

ents. Nevertheless, these values were reliable and the use of peak height ratios, relative to the internal standard, chlorpromazine, adequately corrected for the recovery losses in the plasma samples. This conclusion is supported by the linearity of the ratio of peak heights for thioridazine and its metabolites to chlorpromazine across a wide range of concentrations likely to be encountered in clinical specimens.

Both water- and plasma-based standards yielded highly linear relationships based on peak height or on the ratio of peaks for thioridazine or mesoridazine to that of the internal standard, chlorpromazine, in the range 10—1000 ng/ml ($r > 0.99$). This range can be extended downward to the limit of detection of thioridazine and other phenothiazines, about 0.1 ng per injection. Above 1000 ng, however, the detector signals began to level off, presumably due to the configuration of the working and reference electrode pair.

In a study of precision of the method, plasma samples from each of eight patients receiving thioridazine were assayed in duplicate. Mean coefficients of variation for thioridazine, sulforidazine, and mesoridazine were 7.97% 8.12%, and 7.89%, for mean concentrations of 250.3, 81.44 and 258.2 ng/ml, respectively.

CONCLUSIONS

Reversed-phase HPLC with electrochemical detection is a powerful and useful tool in the determination of plasma thioridazine, its metabolites, and other phenothiazines. The use of a cyano-bonded silica column with tetrahydrofuran as the organic modifier in the mobile-phase buffer allowed clear resolution of thioridazine and three of its pharmacologically active metabolites: mesoridazine, sulforidazine, and northioridazine. The use of large sample injection volumes eliminated the need for sample concentration. Thus, an extraction—back-extraction procedure was used to avoid recovery problems associated with solvent evaporation—reconstitution steps. Electrochemical detection provided excellent selectivity and sufficient sensitivity to detect less than 1 ng of phenothiazine injected onto a column. Interestingly, no northioridazine was observed in any patient sample, so that northioridazine may not be an important metabolite of thioridazine in human subjects. The present method has the capability to quantitate reliably even highly potent phenothiazines, which are typically present in blood at levels below 1 ng/ml. Compared to other HPLC methods for thioridazine and other phenothiazines, this method has several advantages in terms of improved recovery, resolution, and sensitivity.

ACKNOWLEDGEMENTS

Supported, in part, by NIMH Career Award MH-47370, and Program MH-31154, as well as an award from the Bruce J. Anderson Fund. Dr. Finklestein is the recipient of a Clinician-Scientist Award from the American Heart Association. Sandoz Pharmaceuticals kindly provided authentic drug standards. The manuscript was prepared by Mrs. Mila Cason.

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Journal of Chromatography, 307 (1984) 464—468
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2050

Note

Separation of 5-fluorouracil and uracil by ion-pair reversed-phase high-performance liquid chromatography on a column with porous polymeric packing

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(First received September 29th, 1983; revised manuscript received December 13th, 1983)

The pyrimidine antimetabolite 5-fluorouracil (5-FU) is widely used in clinical practice for the treatment of various forms of cancers. However, its applicability is limited by myeloid and gastrointestinal toxicity. Recently, delayed uridine administration was shown to rescue mice from high-dose 5-FU toxicity and to enhance the antitumour activity of 5-FU [1—3]. To evaluate this combination in a clinical phase I study, pharmacokinetics of 5-FU and uridine were determined [4].

Uridine and its catabolite uracil could be determined in plasma and urine by high-performance liquid chromatography (HPLC) using an Aminex A-29 column as described previously [5]. In addition, 5-FU was well resolved from uridine and uracil. However, low concentrations of 5-FU in the micromolar range could not be determined due to the presence of uric acid. HPLC separation of 5-FU and uracil is difficult with commonly used reversed-phase columns. Miller et al. [6] tested several of such columns for their ability to separate 5-FU and uracil and found that with every column tested these compounds were poorly resolved. Since 5-FU and uracil have relatively high pK_a values of 7.98 and 9.50, respectively [7], their separation could theoretically be achieved at high pH values. With most reversed-phase columns HPLC is restricted to the use of eluents at pH < 7. However, PRP-1 columns packed with poly(styrene-divinylbenzene) can be used over a wide pH range

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0378-4347/84/$03.00

from 1 to 13 [8]. With this column, different separations of nucleosides and bases are possible at various pH values. In the present study, we compared the separation of uridine, uracil and 5-FU with a LiChrosorb 10-RP-18 column and a PRP-1 column at high pH with the ion-pairing agent cetrimide.

MATERIALS AND METHODS

HPLC was carried out using instrumentation from Perkin-Elmer (Norwalk, CT, U.S.A.) consisting of a Series 2/2 solvent delivery system connected to two LC75 variable-wavelength detectors set at 254 and 280 nm. Kontron (Zürich, Switzerland) apparatus was also used, consisting of an LC410 pump connected to two Uvikon 740 LC fixed-wavelength detectors (254 and 280 nm). At 280 nm, the absorbance by interfering peaks was lower. The 280/254 ratio enabled identification of the compounds. A LiChrosorb 10-RP-18 column (150 × 4.6 mm) was obtained from Chrompack (Middelburg, The Netherlands). The PRP-1 material (particle size between 8 and 12 μm) was obtained from Hamilton (Reno, NV, U.S.A.) and was packed in a 150 × 4.1 mm column as described by Zygmunt et al. [9]. Uracil, uridine and 5-FU were purchased from Sigma (St. Louis, MO, U.S.A.), and cetyltrimethylammonium bromide (cetrimide) was from BDH (Poole, U.K.). Other chemicals were of the highest quality commercially available. Eluents were prepared with de-ionized water which was further purified with a Millipore Milli Q system, filtered through a 0.22-μm filter and sonicated. The PRP-1 column was routinely run at 0.5—1 ml/min (pressure about 100 bars). The column was run overnight at 0.1 ml/min and stored in methanol—water (1:1) between use.

Plasma obtained from heparinized blood was deproteinized for 20 min on ice using trichloroacetic acid (8% final concentration) or perchloric acid (0.4 *M* final concentration) The extracts were clarified by centrifugation and neutralized by thoroughly mixing 1 vol. of supernatant with 2 vols. of trioctylamine—Freon (1:4, v/v) as described previously [10, 11]. After centrifugation, the aqueous phase was pipetted off and stored at −20°C until analysis. No difference between the two extraction procedures was found.

The following equation was used to calculate the capacity factor (k'): $k' = (V_p - V_0)/V_0$, where V_0 is the void volume and V_p the volume required to elute the compound. The relative capacity factors for uracil and 5-FU were expressed as the selectivity factor r, defined as $k'_{5\text{-FU}}/k'_{\text{uracil}}$.

RESULTS AND DISCUSSION

5-FU concentrations in plasma could be determined with a LiChrosorb 10-RP-18 column (Fig. 1A). This system could also be employed to study the pharmacokinetics of uridine, since uridine and uracil could be separated. The compound which was coeluted with uracil did not interfere with the determination of uracil at concentrations of uracil higher than 10 μM. 5-FU and uracil were also resolved. However, 5-FU could not be detected when the uracil concentration (about 100 μM) exceeded that of 5-FU as in the treatment of patients in whom 5-FU administration is followed by uridine. Such conditions resulted in the detection of the 5-FU peak as a shoulder on the uracil peak

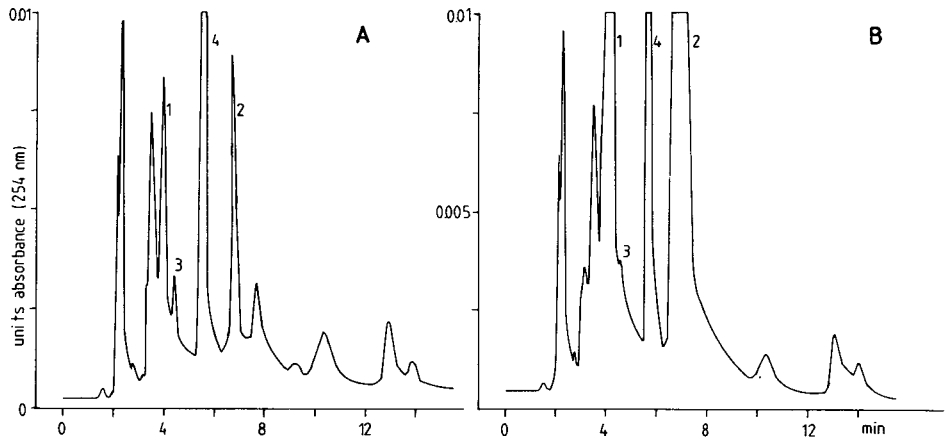

Fig. 1. Chromatograms of plasma of patients, using a LiChrosorb 10-RP-18 column with isocratic conditions. The elution solvent was 0.01 *M* $NH_4H_2PO_4$, pH 3.8, containing 2.5% methanol. The flow-rate was 1 ml/min. Retention times were: uracil (1) 4.0 min; 5-FU (3) 4.4 min; uric acid (4) 5.6 min; and uridine (2) 6.7 min. Uracil coeluted with an unknown plasma compound. The equivalent of 40 µl of undiluted deproteinized plasma (total injection volume 50 µl) was injected. (A) plasma of a 5-FU-treated patient (700 mg/m², bolus injection), 2 h after injection; (B) plasma of the same patient at the end of a 1-h uridine infusion (5 g/m², intravenous, 2 h after 5-FU).

(Fig. 1B). Phosphorolysis of uridine led to concentrations of uracil 100 times higher than those of 5-FU. With other reversed-phase HPLC columns improvement of separation was not expected [6]. Since the PRP-1 column could be used with buffers over a wide pH range, we attempted to improve the separation of 5-FU and uracil by varying the pH of the elution solvent. Ammonium phosphate buffers between pH 7 and 9 were tested. Although 5-FU is ionized to a greater extent than uracil at pH 8 [7], the resolution of 5-FU and uracil at this pH was unsatisfactory. Also, no separation was achieved at the other pH values. Subsequently, ion-pair HPLC was performed to increase the retention of ionized compounds. With cetrimide as the ion-pairing agent, 5-FU and uracil could be separated although coelution of uridine and uracil occurred (Fig. 2A).

In drug-free plasma, no interfering peaks were present at the retention time of 5-FU (Fig. 2B). Uric acid, the main interfering peak in plasma, was separated from 5-FU. An anion-exchange system using an Aminex A-29 column [5] at pH 9.1 (0.01 *M* disodium hydrogen phosphate, 0.005 *M* citric acid, 19% ethanol; at 0.3 ml/min and 70°C) was also capable of separating 5-FU from uracil; however, 5-FU was poorly resolved from uric acid (data not shown). The concentration of 5-FU determined in plasma of 5-FU-treated patients with the PRP-1 column was similar to the concentration determined with the LiChrosorb 10-RP-18 column. The presence of high concentrations of uridine and uracil in plasma of patients treated with 5-FU—uridine did not interfere with the determination of 5-FU (Fig. 2C). The detection limit of the method was 20 pmol (in 20 µl of plasma), and the peak area was linear with concentration to at least 500 pmol. Since the 5-FU dose may be higher in the 5-FU—uridine regimen than in the conventional 5-FU schemes, the period during which plasma drug concentrations can be measured may be extended.

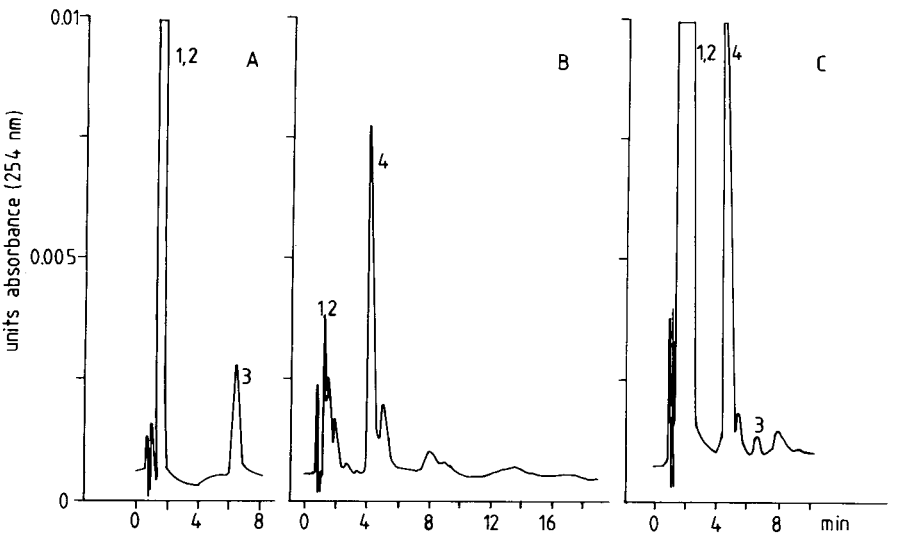

Fig. 2. HPLC separation of uracil (1), uridine (2), 5-FU (3) and uric acid (4) on a PRP-1 column under isocratic conditions. The elution solvent was 0.05 *M* Tris—HCl, 0.025 *M* cetrimide (pH 8.0), and the flow-rate was 1 ml/min. Retention times were: uracil and uridine, 1.8 min; uric acid, 4.5 min; and 5-FU, 6.8 min. (A) 20 μl of a mixture of standards (10 μM uridine, 10 μM uracil and 25 μM 5-FU); (B) 20 μl diluted deproteinized patient plasma (1 part plasma, 2 parts water) before treatment; (C) 20 μl diluted deproteinized plasma (1 part plasma, 1 part water) of the same patient described in Fig. 1B at the end of the 1-h uridine infusion.

TABLE I

CAPACITY AND SELECTIVITY FACTORS FOR 5-FU, URIDINE, URACIL AND URIC ACID

Capacity (k') and selectivity (r) factors were calculated for separations with the eluents described in Figs. 1 and 2.

	LiChrosorb 10-RP-18	PRP-1
k'		
5-FU	2.9	12.1
Uracil	2.5	3.1
Uridine	4.4	3.1
Uric acid	3.7	7.9
r		
5-FU—uracil	1.2	3.9
5-FU—uridine	1.5	3.9
5-FU—uric acid	1.3	1.5
Uridine—uracil	1.8	1
Uridine—uric acid	1.2	2.5
Uracil—uric acid	1.5	2.5

The column performance decreased during prolonged use, but could be regained by careful refilling the top of the column with the packing slurry. Table I shows a comparison of the separation characteristics of the LiChrosorb

10-RP-18 and the PRP-1 columns. It is apparent that the PRP-1 column gives a good separation of 5-FU and uracil.

This paper shows that the use of the PRP-1 column at high pH allows an enhanced separation of 5-FU and uracil which has not been possible with other reversed-phase columns. Furthermore, this separation which takes advantage of modest differences in ionization is only possible with ion-pairing. Resolution of 5-FU and uracil is applicable in clinical studies of the combination of 5-FU and uridine. Also, this HPLC method may serve as an alternative in the separation of 5-FU from other plasma components.

ACKNOWLEDGEMENT

This work was supported by Grants AUKC 80-3 and NKI 83-16 from the Netherlands Cancer Foundation — "Koningin Wilhelmina Fonds".

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Journal of Chromatography, 307 (1984) 469—474
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2054

Note

Simultaneous determination of allopurinol, oxipurinol, hypoxanthine and xanthine in biological fluids by high-performance liquid chromatography

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(First received October 3rd, 1983; revised manuscript received December 21st, 1983)

Allopurinol [4-hydroxypyrazolo(3,4-*d*)pyrimidine] is increasingly used for the treatment of hyperuricaemia. Allopurinol and its major metabolite, oxipurinol, are potent inhibitors of xanthine oxidase, the enzyme that converts hypoxanthine to xanthine, and xanthine to uric acid. As a result, allopurinol decreases uric acid production and increases hypoxanthine and xanthine formation in the blood and urine of patients treated with this drug. Several high-performance liquid chromatographic (HPLC) methods for the determination of allopurinol and oxipurinol have been published [1—5]. In this paper is presented an HPLC procedure for the simultaneous determination of allopurinol, oxipurinol, hypoxanthine and xanthine, and its application to the analysis of plasma and urine from hyperuricaemic patients treated with allopurinol.

EXPERIMENTAL

Reagents

Allopurinol, oxipurinol, hypoxanthine and xanthine were purchased from Sigma (St Louis, MO, U.S.A.). 9-Methylxanthine was obtained from Fluka

0378-4347/84/$03.00

(Buchs, Switzerland). Potassium dihydrogen phosphate, orthophosphoric acid, trichloroacetic acid and perchloric acid were obtained from Merck (Darmstadt, F.R.G.).

Apparatus and chromatographic conditions

Chromatographic analyses were done with an integrated unit, an SP 8000 high-performance liquid chromatograph (Spectra Physics, Orsay les Ullis, France). The column (15 cm × 4.6 mm I.D.) and the precolumn (5 cm × 4.6 mm I.D.), used as a guard column, were packed with Hypersil ODS, 3 μm (Shandon, Cheshire, U.K.) by the slurry packing technique as described by Coq et al. [6]. The mobile phase consisted of 0.02 *M* potassium dihydrogen phosphate, the pH of which was adjusted to 3.65 with orthophosphoric acid. The flow-rate was 1.5 ml/min. Detection was carried out at 254 nm.

Sample collection and treatment

One millilitre of blood was collected in a heparinized tube. In accordance with our previous findings, this tube was immediately centrifuged to prevent an increase of hypoxanthine concentration in plasma samples left in contact with the erythrocytes [7]. Plasma was decanted and stored at −20° C until analysis. A 500-μl aliquot of plasma was spiked with the internal standard (9-methylxanthine, 12.5 μmol/l). The samples were then deproteinised by 150 μl of trichloroacetic acid (12%) or 50 μl of perchloric acid (35%).

Twenty-four-hour urine samples were collected and stored at −20° C. Urine samples were analysed without further treatment.

RESULTS AND DISCUSSION

A chromatogram obtained from a standard solution containing hypoxanthine, xanthine, 9-methylxanthine, oxipurinol and allopurinol is shown in Fig. 1. Fig. 2a and c show typical chromatograms of blank plasma and blank urine, respectively.

Chromatograms of plasma and urine samples from a patient under allopurinol therapy, 300 mg once daily, are presented in Fig. 2b and d, respectively. As can be seen by comparison of chromatograms from blank samples (Fig. 2a and c) and from biological samples (Fig. 2b and d) there are no endogenous interfering peaks at retention times corresponding to those of allopurinol and oxipurinol. Furthermore, the chromatograms of biological samples show that uric acid is separated from compounds of interest in this study.

Calibration curves were linear from 0.15 to 20 mg/l for allopurinol and oxipurinol and from 0.50 to 50 μmol/l for hypoxanthine and xanthine with intercepts not significantly different from zero. Furthermore, slopes corresponding to aqueous solutions and plasma are identical; this confirms that there was no loss of any of the compounds under study during deproteinisation.

Under the conditions employed in this study, the minimum quantity detectable is 1.5 ng for allopurinol and oxipurinol, 2.5 pmol for hypoxanthine and 5.0 pmol for xanthine.

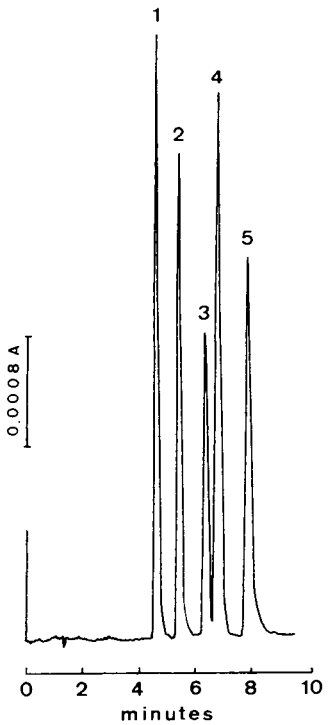

Fig. 1. Chromatogram of a standard solution containing (1) hypoxanthine (10.0 μmol/l), (2) xanthine (10.0 μmol/l), (3) 9-methylxanthine (12.5 μmol/l), (4) oxipurinol (4.0 mg/l) and (5) allopurinol (2.5 mg/l). Injection volume: 10 μl. Column: Hypersil ODS, 3 μm. Mobile phase: 0.02 *M* potassium dihydrogen phosphate, pH 3.65. Flow-rate: 1.5 ml/min. Detection: 254 nm.

TABLE I

REPRODUCIBILITY AND ACCURACY OF ALLOPURINOL AND OXIPURINOL DETERMINATION IN SPIKED INTRA-ASSAY PLASMA

	Amount added (mg/l)	Amount found (mg/l; mean ± S.D.; n = 5)	C.V. (%)
Allopurinol	0.5	0.48 ± 0.02	4.2
	2.0	2.06 ± 0.05	2.4
	5.0	5.09 ± 0.11	2.2
Oxipurinol	0.5	0.49 ± 0.02	4.1
	2.0	2.01 ± 0.08	3.9
	5.0	5.01 ± 0.15	2.9

Reproducibility and accuracy were determined on spiked plasma. The intra-assay results for allopurinol and oxipurinol are given in Table I. The inter-assay was performed by analysing spiked plasma samples on different days over one week (n = 5). The inter-assay coefficient of variation for the analysis of allopurinol and oxipurinol over the concentration range 0.5—5 mg/l was

found to be about 3% for both allopurinol and oxipurinol. The results obtained for hypoxanthine and xanthine have been listed in a paper published previously [8]; for these two compounds, the coefficient of variation was found to be about 1.5%.

Contrary to some methods published [4, 5] for the determination of allopurinol and oxipurinol, a rapid sample clean-up procedure (plasma deproteinisation) was used in order to obtain baseline separations, symmetric peak shape and constant retention times.

This paper describes a rapid and sensitive HPLC method for the monitoring of allopurinol and oxipurinol concentrations in plasma and urine to obtain a

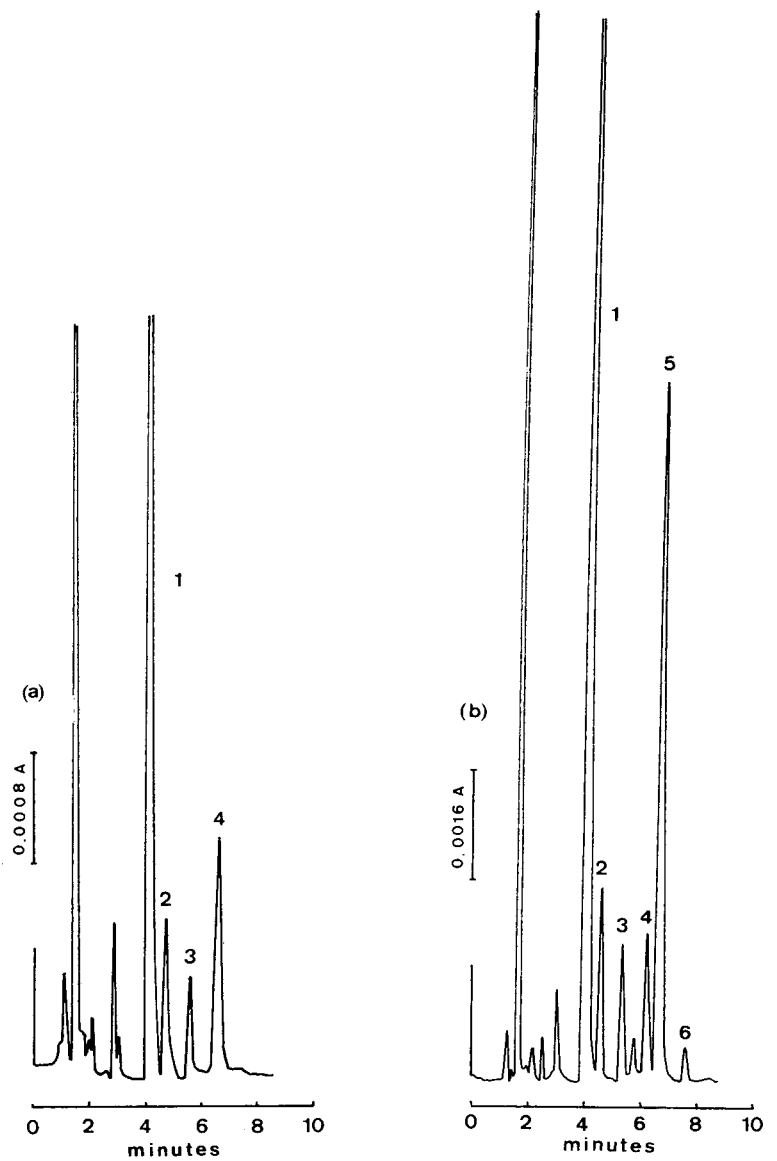

Fig. 2.

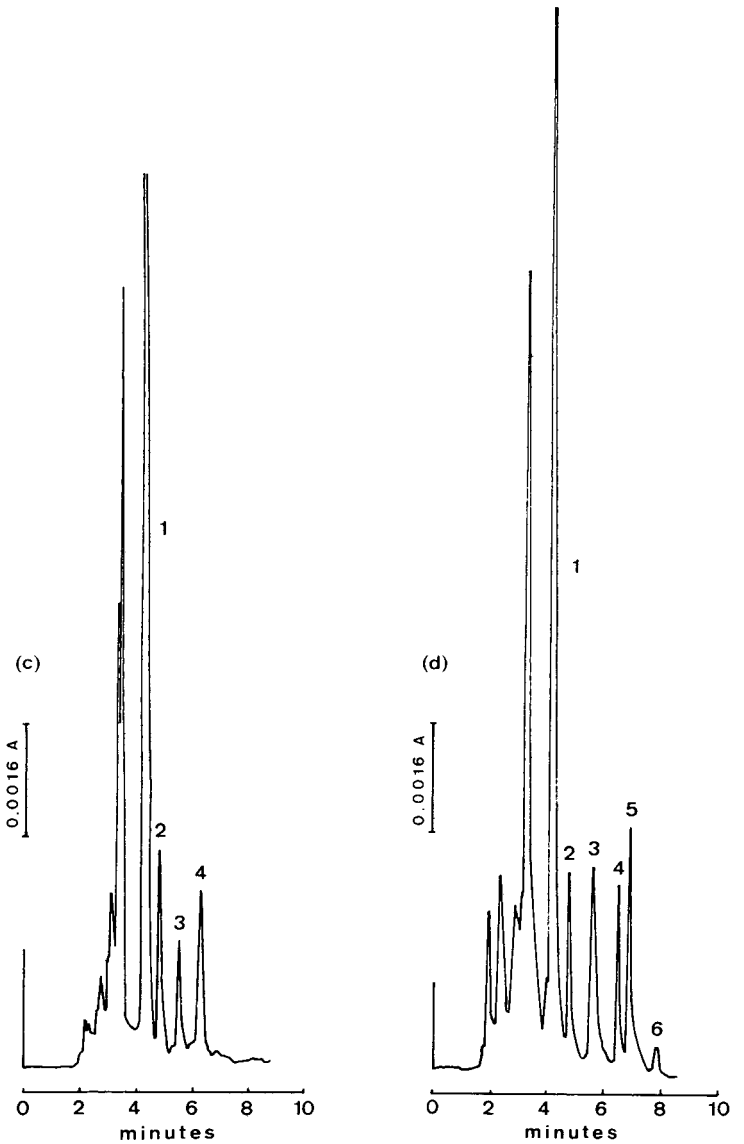

Fig. 2. Chromatograms of (a) blank plasma, (b) plasma sample from a patient under allopurinol therapy, 300 mg once daily, (c) blank urine diluted five times, and (d) urine sample, diluted ten times, from the same patient under allopurinol therapy. Peaks: 1 = uric acid; 2 = hypoxanthine [(a) 1.5 μmol/l, (b) 3.5 μmol/l, (c) 29.5 μmol/l (44 μmol per 24 h), (d) 52.0 μmol/l (99 μmol per 24 h)]; 3 = xanthine [(a) 2.0 μmol/l, (b) 5.5 μmol/l, (c) 25.0 μmol/l (37.5 μmol per 24 h), (d) 90.0 μmol/l (171 μmol per 24 h)]; 4 = 9-methyl xanthine (12.5 μmol/l); 5 = oxipurinol [(b) 12.0 mg/l, (d) 35.0 mg/l (66.5 mg per 24 h)]; 6 = allopurinol [(b) 0.5 mg/l, (d) 4.0 mg/l (7.6 mg per 24 h)]. The concentrations of the compounds of interest in urine samples are given taking dilution into account.

significant pharmacological effect and also to prevent toxic side-effects of the drug, in particular, the formation of oxipurinol stones in patients with renal failure.

Using this method, we observed that the hypoxanthine and xanthine levels in plasma and especially in urine from patients under allopurinol therapy are higher than those obtained in our study of healthy subjects [7]. With this HPLC method, the effect of allopurinol treatment on hypoxanthine and xanthine levels in biological fluids can easily be studied, while monitoring allopurinol levels in patients under allopurinol therapy.

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Journal of Chromatography, 307 (1984) 475—480
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2057

Note

Simple reversed-phase high-performance liquid chromatographic determination of antipyrine in rabbit plasma for pharmacokinetic studies

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In order to study the influence of dilution on the pharmacokinetic behaviour of antipyrine after oral administration, a rapid, sensitive, precise and accurate method of analysis was needed. The sensitivity required had to be at least 0.1 μg/ml antipyrine in rabbit plasma for sample volumes ranging from 0.25 to 0.5 ml.

Antipyrine, which is normally used as an indicator of hepatic drug metabolism [1—5], was selected for this experiment on the basis of its simple pharmacokinetic behaviour: first-order kinetics, no first-pass effect, one-compartment distribution [6] and practically no protein binding [7].

Eichelbaum and Spannbrucker [8] were the first to present a rapid and sensitive method of analysis for antipyrine by high-performance liquid chromatography (HPLC) on silica gel. The most prevalent disadvantage of this type of chromatography, however, especially in bioanalyses, is column inactivation. Usually the presence of water in the injection sample or in the mobile phase is responsible for this. As a result retention times of the compounds decrease and resolution is lost. According to our experience with the above method, a change of retention volume was often observed after the ammonia-containing mobile phase was renewed. More recently, Guinebault and Broquaire [9] described an interesting straight-phase HPLC method in which neither mobile phase nor injection sample contained any water and no variation of retention times of antipyrine and phenacetin was observed.

We also considered reversed-phase HPLC methods to resolve our problem. Campell et al. [10] reported a reversed-phase HPLC method. Remarkable in their method is the ten-fold dilution of the plasma samples, taking into account the injection of only 25 μl of filtrate. They thus have to select a maximum detector sensitivity to measure even high concentrations. As to the choice of

0378-4347/84/$03.00

internal standard, benzoic acid is used, but a structurally related compound would be more convenient. Shargel et al. [11] described a reversed-phase HPLC method for antipyrine in 0.1 ml of rat plasma samples with methanol—water as mobile phase. Too much peak tailing was observed, however, while the sensitivity was not higher than 1.0 μg/ml.

In this paper we have tried to overcome most of these inconveniences. Special attention was paid to a solvent combination to minimize peak tailing as much as possible.

MATERIALS AND METHODS

Chemicals

When available, all the chemicals and solvents were of analytical grade (Merck, Darmstadt, F.R.G.). Antipyrine and aminopyrine (aminophenazone), conforming to the European Pharmacopoeia, were used as standard and internal standard, respectively. The HPLC solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Apparatus

The samples were analysed on an HPLC system consisting of a Pye Unicam LC3-XP pump (Cambridge, U.K.), an Altex Model 210 injection valve, fitted with a 20-μl loop and a Pye Unicam PU 4020 ultraviolet detector operated at 254 nm with the sensitivity range at 0.08 a.u.f.s. A reversed-phase 10-μm μBondapak C_{18} column (300 × 3.9 mm I.D., stainless steel) (Waters Assoc., Milford, MA, U.S.A.) was used at ambient temperature. Isocratic elution was performed with a mixture of acetonitrile—water (32.5:67.5, v/v) containing 0.5% acetic acid at a flow-rate of 0.75 ml/min. The chromatograms were recorded at a chart-speed of 120 mm/h.

Procedure

In a glass tube 0.1—0.25 ml of plasma, 0.1 ml of sodium hydroxide (0.1 mol/l) and 0.1 ml of internal standard solution of aminopyrine in water (25 μg/ml) were mixed on a vortex mixer. Then 1 ml of dichloromethane was added, the mixture shaken on a IKA-Schüttler S50 apparatus for 5 min and the two liquid phases were separated by centrifugation (1500 *g*) for 5 min. By means of an eppendorf pipette, 500 μl of the dichloromethane phase were transferred into a test tube, evaporated under nitrogen and the residue was dissolved in 100 μl of water, 20 μl of which were injected into the HPLC system. At the same time 0.1 ml of sodium hydroxide (0.1 mol/l), 0.1 ml of internal standard solution and 0.1 ml of aqueous solutions of increasing amounts of antipyrine (0.223 μg, 0.446 μg, 0.892 μg, 1.784 μg, 2.230 μg, 4.460 μg and 7.805 μg) were mixed with 0.25 ml of water and analysed together with the plasma samples.

RESULTS AND DISCUSSION

Aminopyrine (internal standard) and antipyrine exhibited two symmetrical, well resolved peaks under the described chromatographic conditions (Fig. 1).

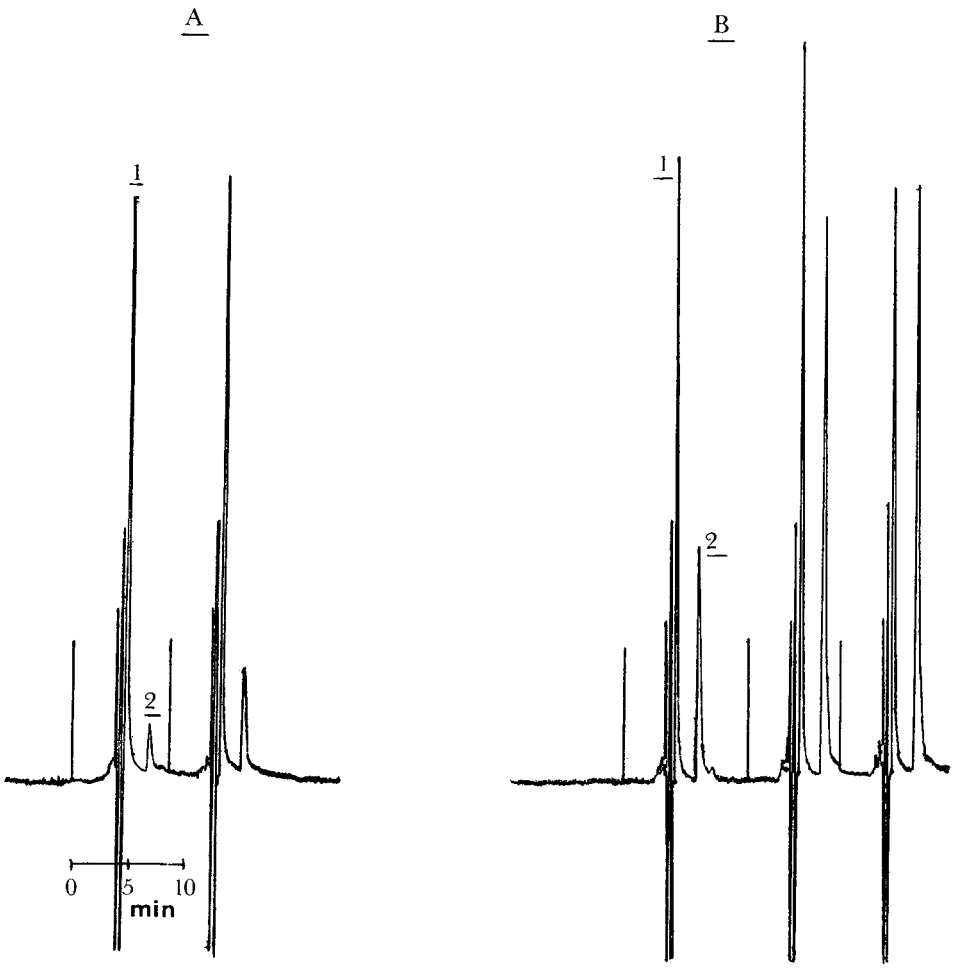

Fig. 1. (A) Chromatograms of rabbit plasma extracts spiked with 0.2 and 0.4 μg/ml antipyrine. Detector sensitivity is 0.08 a.u.f.s. Peaks: 1 = aminopyrine; 2 = antipyrine. (B) Chromatograms of rabbit plasma extracts after administration of antipyrine to the rabbit.

Peak height ratio was used to quantitate detector response. A linear relationship was obtained for the range tested (0.2—25 μg/ml).

For spiked plasma the coefficient of variation was ± 4.8% for 2.23 μg of antipyrine per ml of plasma and 5.1% for 0.223 μg of antipyrine per ml of plasma (n = 6). The recovery was 104% and 97%, respectively. The retention times were 4.75 and 6.25 min for aminopyrine and antipyrine, respectively. Day-to-day precision obtained by analysing pooled rabbit plasma, containing 2.23 μg/ml antipyrine, on different days was 4.2% (n = 10). In a sample volume of 0.5 ml plasma, antipyrine concentrations of 0.1 μg/ml could still be detected with a reasonable precision. No interfering peaks of plasma constituents appeared on the chromatograms of blank plasma samples. All the plasma extracts could be injected on the HPLC system successively without disturbing the baseline. Standard curves were drawn daily by linear regression. The correlation coefficients obtained were at least 0.99.

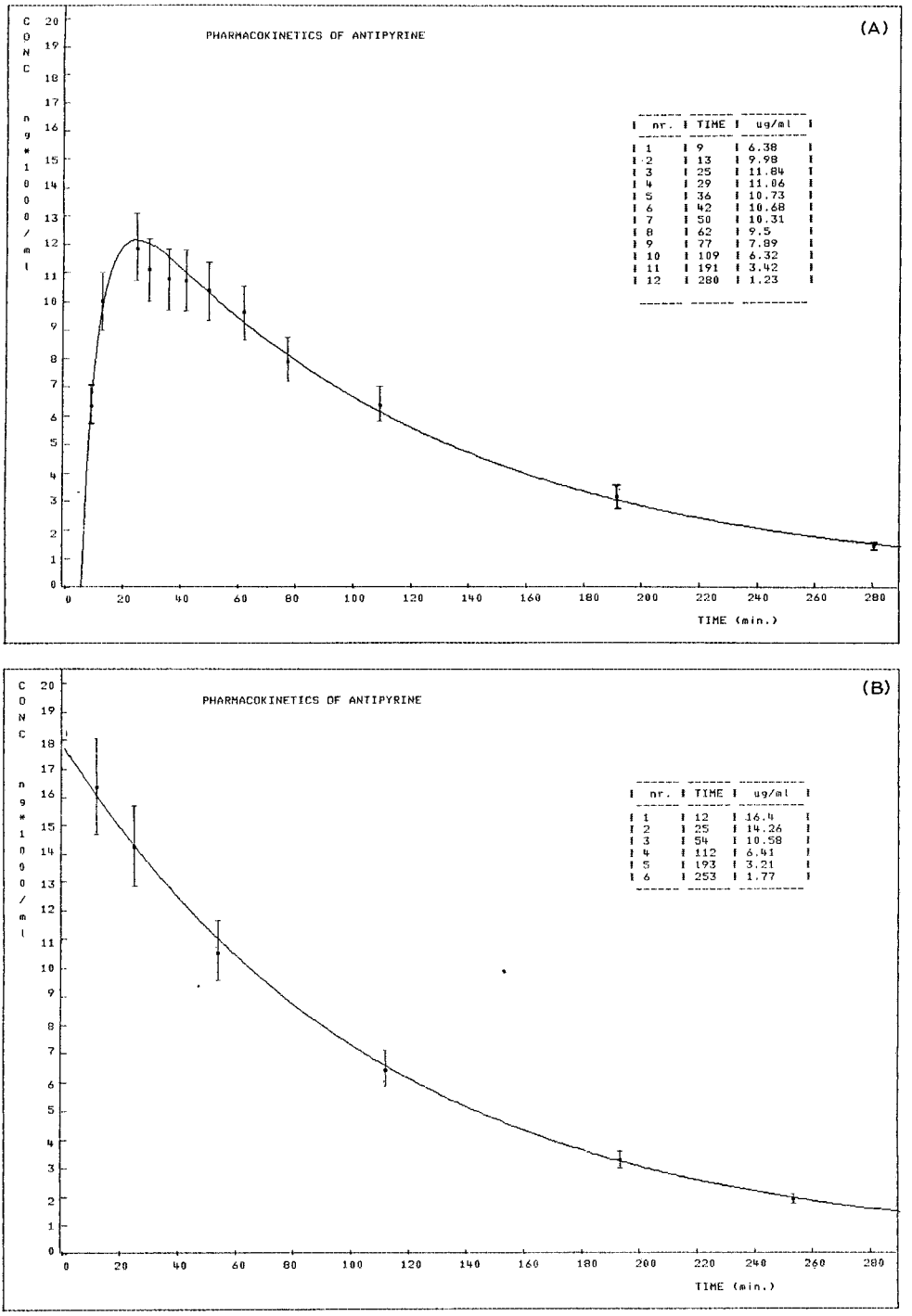

Fig. 2. (A) Rabbit plasma antipyrine concentrations following peroral administration of 12.5 mg/kg as a concentrated aqueous solution. (B) Rabbit plasma antipyrine concentrations following intravenous injection of 12.5 mg/kg as a concentrated aqueous solution.

The HPLC method developed was applied in a pharmacokinetic study of antipyrine, administered by oral intubation and intravenous injection to a rabbit in a dose of 12.5 mg/kg. Venous blood samples were drawn into heparinized tubes by venipuncture at various times for about 6 h after administration. Plasma concentrations of antipyrine in all samples were determined using the described method to demonstrate its performance. Absorption and/or excretion curves obtained are shown in Fig. 2A and B, together with the 95% confidence interval of the plasma concentrations measured.

TABLE I

KINETICS OF INTRAVENOUS AND ORAL ANTIPYRINE

Kinetic variables	Route of administration	
	Peroral	Intravenous
Absorption half-life (min)	4.4	—
Elimination half-life (min)	77.1	76.4
Absorption constant k_a (min^{-1})	0.157	—
Elimination constant k_e (min^{-1})	0.00899	0.009073
Maximum plasma concentration C_{pmax} (μg/ml)	12.55	17.87 ($C_{p,\,t=0}$)
t_{max} (min)	25	—
Lag time t_0 (min)	5.3	—
Distribution volume V_d (ml/kg)	836	700
Area under the curve (AUC) (μg/ml min)	1660	1969.1
Total clearance (ml/min/kg)	—	6.35

In Table I the absorption half-life, the elimination half-life, the absorption constant k_a, the elimination constant k_e, the maximum plasma concentration C_{pmax}, the time t_{max} corresponding to C_{pmax}, the lag time t_0, the distribution volume V_d and the area under the curve (AUC) are listed. All these kinetic variables for antipyrine were determined using standard pharmacokinetic methods [12, 13]. Values of elimination half-life were 77 and 76 min for the peroral and intravenous route of administration, respectively. These values agree with reported values [14]. After a lag time of 5.3 min, oral antipyrine was absorbed with an apparent half-life of 4.4 min. Based on comparison of areas under the curve, following oral and intravenous administration, the fraction of the dose absorbed was 84%. The plasma elimination of antipyrine was first order. Results on the influence of dilution on the pharmacokinetic behaviour after oral administration will be published elsewhere.

CONCLUSION

As illustrated, the described method achieves the level of sensitivity and accuracy required to obtain meaningful data about the single-dose pharmacokinetic behaviour of antipyrine in rabbit.

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Journal of Chromatography, 307 (1984) 481—487
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2058

Note

A high-performance liquid chromatographic method for methohexital and thiopental in plasma or whole blood

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(First received October 12th, 1983; revised manuscript received December 23rd, 1983)

Methohexital (methohexitone, Brietal®) is an ultra-short-acting barbiturate used for induction of anaesthesia or as an intravenous sedative [1]. Gas chromatographic methods for the determination of methohexital in biological samples have been reported, the most sensitive of which utilize nitrogen-selective detection [2, 3] or electron-capture detection of a pentafluorobenzyl derivative [4].

For the purposes of pharmacokinetic work, we have developed a rapid and sensitive liquid chromatographic analysis permitting the determination of methohexital in plasma or blood in the nanogram per millilitre range. The method is also applicable to thiopental, for which a number of high-performance liquid chromatographic (HPLC) methods have, however, already been reported [5—13]. During the preparation of this manuscript a brief outline of another HPLC method for methohexital appeared in the literature [14].

EXPERIMENTAL

Reagents and chemicals

Methohexital was supplied by Eli Lilly Sweden (Stockholm, Sweden). Hexobarbital was of European Pharmacopoeia quality. These compounds were each dissolved in 6.7 m*M* phosphate buffer (pH 7.4) and the appropriate stock solutions were then prepared by dilution with distilled water. Thiopental sodium (Pentothal®, Abbott, Campoverde, Italy), containing 86.1% free acid, was dissolved in water. The methohexital and hexobarbital solutions were kept at room temperature and the thiopental solutions in the refrigerator. Toluene

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0378-4347/84/$03.00

(8325; Merck, Darmstadt, F.R.G.), acetonitrile (30; Merck) and $NaH_2PO_4 \cdot H_2O$ (6346; Merck) were used without further purification. The water was freshly distilled and collected in a stainless-steel vessel.

Instrumentation

The liquid chromatography system consisted of an LDC/Milton Roy (Riviera Beach, FL, U.S.A.) Minimetric II pump, a Rheodyne 7125 loop injector and an LDC Spectro Monitor III variable-wavelength UV detector. A LiChroCart RP-18 7-μm column (250 × 4 mm) was used in conjunction with a Hibar precolumn (30 × 4 mm) filled with Perisorb RP-18 (all from Merck). The mobile phase was a 1:1 mixture of 0.050 *M* NaH_2PO_4 solution (pH 4.6) and acetonitrile. The flow-rate was 1.00 ml/min and the detection wavelength was 195 nm. Ultraviolet spectra were recorded on an LKB Beckman Model 25 scanning photometer (LKB, Stockholm, Sweden) with the barbiturates dissolved in aliquots of mobile phase.

Methods

To 1.00-ml (or smaller) samples of plasma or haemolysed (frozen and thawed) whole blood containing methohexital (0—16.0 μg/ml = 0—61.2 μM) or (and) thiopental (0—16.0 μg/ml = 0—66.3 μM) were added 0.500 ml of internal standard solution (hexobarbital, 2.00 μg/ml = 8.46 μM in water), 0.5 ml of 0.25 *M* hydrochloric acid and 0.04 g of sodium chloride. The samples were extracted with 3 ml of toluene on a Hook and Tucker rotamixer and the solvent layers were separated by centrifugation. The toluene layer was transferred to another tube and the solvent was evaporated on a sand bath (50 ± 5° C) under a stream of dry air. The residue was taken up in 0.20 ml of acetonitrile, and 0.20 ml of 0.050 *M* sodium dihydrogen phosphate solution was added; 20 μl of this solution were injected into the chromatograph.

Extraction recoveries were determined by adding methohexital and thiopental to 1.0 ml of plasma or whole blood (final concentration of methohexital 0.50 μg/ml and thiopental 1.0 μg/ml), freezing and thawing the blood and extracting by the standard procedure with 4.00 ml of toluene. Of the toluene phase, 2.00 ml were transferred to another tube, 0.500 ml of hexobarbital (1.00 μg/ml in methanol) was added and the solvents were evaporated. The residue was taken up in acetonitrile—0.050 *M* sodium dihydrogen phosphate solution and injected. A mixture of methohexital—thiopental—hexobarbital (1:2:2) prepared from the same stock solutions served as reference. Four samples each of plasma, blood and reference were analysed.

The quality of the stock solutions was checked by addition of internal standard and direct injection into the chromatograph. Freshly made solutions served as references for old ones.

The stability of methohexital and thiopental in blood samples was assessed by spiking 6 ml of freshly drawn, heparinized blood with 50 μl of methohexital or thiopental solution, 100 μg/ml (final concentration in the blood 0.7 μg/ml). To mimic careless sample handling, the tubes were then left on the bench for 24 h. Aliquots were withdrawn at 0, 1, 2, 4, 8, 12 and 24 h, frozen (at −20° C) and analysed on the following day.

For the pharmacokinetic verification of the method, blood samples were

drawn from consenting day patients undergoing minor (orthopaedic) surgery under nitrous oxide—halothane anaesthesia. The induction doses of intravenous barbiturate were titrated individually and ranged from 1 to 2 mg/kg for methohexital and from 3 to 5 mg/kg for thiopental. The blood samples were collected in heparinized tubes, kept in the refrigerator and centrifuged after a few hours. The plasma samples were then stored at −20°C until analysed.

RESULTS

General considerations

Typical chromatograms from methohexital and thiopental analysis are shown in Figs. 1 and 2. The retention times of six representative barbituric acids in this system are: barbital 2.9 min, phenobarbital 3.6 min, pentobarbital 4.5 min, hexobarbital 4.8 min, thiopental 6.8 min and methohexital 7.3 min. Of possible interfering compounds, salicylic acid and acetylsalicylic acid are not retained while caffeine and indomethacin appear at 2.5 and 8.6 min, respectively.

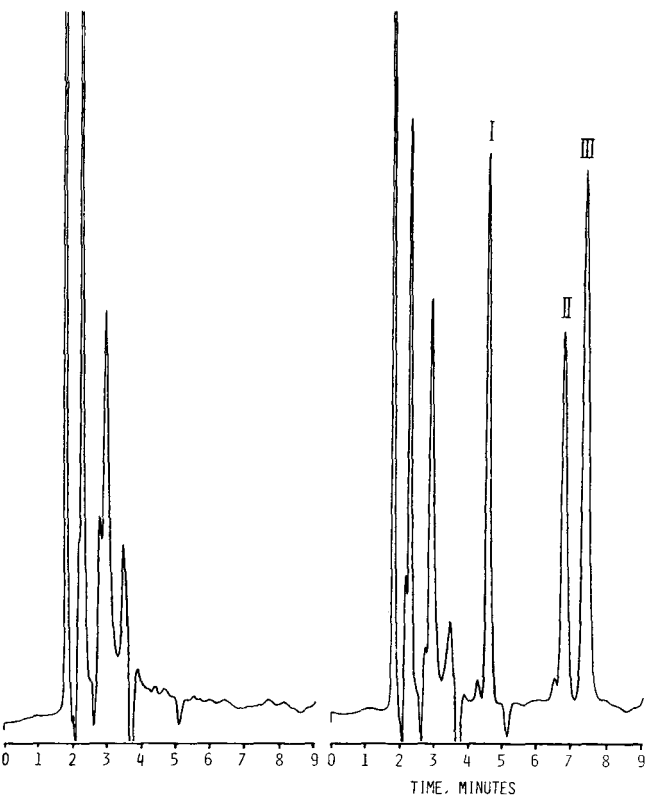


Fig. 1. Chromatograms of an extract of haemolysed blood and of a similar sample spiked with hexobarbital (I, 1.00 μg/ml), thiopental (II, 4.00 μg/ml) and methohexital (III, 2.00 μg/ml). The small peaks just in front of the hexobarbital and thiopental peaks are impurities in the thiopental (Pentothal) preparation. Extracts of plasma give similar chromatograms. Some samples also give large, unidentified peaks at 10 and 13 min, and in practice the interval between injections is around 14 min. Detector: 0.02 a.u.f.s. Recorder: 10 mV, 10 mm/min.

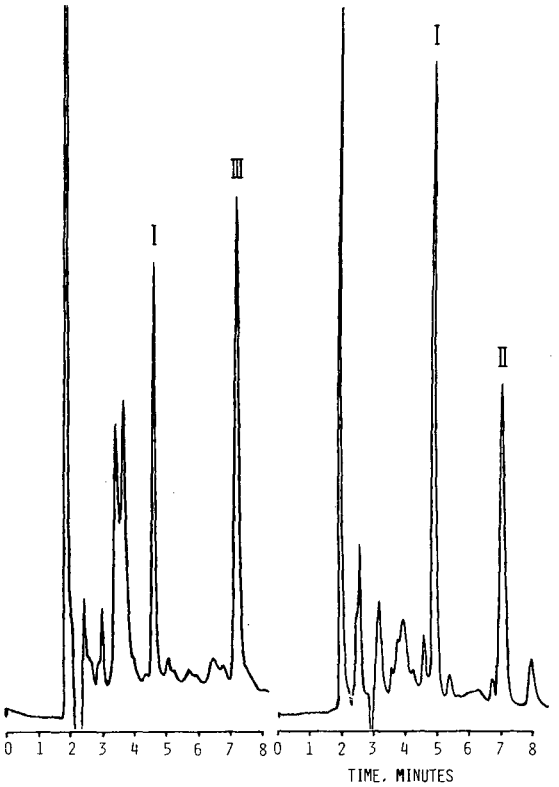


Fig. 2. Chromatograms of plasma extracts from patients. Left: a sample taken 5 min after induction of anaesthesia with methohexital sodium, 1.7 mg/kg. The methohexital concentration is 1.2 μg/ml. Right: a sample taken 30 min after induction of anaesthesia with thiopental sodium, 4.1 mg/kg. The thiopental concentration is 3.2 μg/ml. Peak identification numbers and detector and recorder settings are as in Fig. 1.

The ultraviolet spectra of hexobarbital and methohexital (as free acids) show absorption peaks at 195 nm, with shoulders at 220 nm. Also thiopental gives an absorption maximum at 195 nm, albeit a lower one than at 236 and 286 nm. The relative detector responses of the three compounds, measured as peak areas at 195 nm in a chromatogram of an equimolar mixture, are 1:0.70:0.32. The lower ultraviolet absorption of thiopental compared to methohexital is reflected in a lower sensitivity of the analysis.

The stock solutions of methohexital and hexobarbital were stable for at least six months at room temperature, and the thiopental stock solutions for at least one month at +8° C.

Methohexital

Standard curves drawn on the analysis of duplicate samples containing 0.125, 0.250, 0.500, 1.00, 2.00, 4.00, 8.00 and 16.0 μg methohexital per ml were linear (generally $r = 0.999$ on a six-point standard curve). Quantitation was by peak height. In addition, samples of very low concentration were analysed with 0.500 ml of 0.50 μg/ml hexobarbital as internal standard. Under these conditions, linear (typically $r = 0.998$) standard curves could be obtained

TABLE I

ANALYSIS OF SPIKED PLASMA AND BLOOD SAMPLES (n = 8)

Amount added (μg/ml)		Amount found (μg/ml)	Relative S.D. (%)
In plasma			
Methohexital	1.00	0.993 ± 0.026	2.6
Methohexital	0.250	0.250 ± 0.0083	3.3
Methohexital	0.0625	0.0625 ± 0.0028	4.4
Thiopental	2.00	2.04 ± 0.055	2.7
Thiopental	0.50	0.54 ± 0.032	6.1
In blood			
Methohexital	1.00	0.963 ± 0.032	3.3
Methohexital	0.250	0.246 ± 0.0049	2.0
Methohexital	0.0625	0.0641 ± 0.0018	2.8
Thiopental	2.00	1.99 ± 0.036	1.8
Thiopental	0.50	0.503 ± 0.015	3.0

on duplicate samples containing 31.3, 62.5, 125, 250, 500 and 1000 ng/ml. The precision of the analysis is given in Table I.

Shifting the detector wavelength to 220 nm lowered the sensitivity of the detection without improving the selectivity. Sample clean-up by back-extraction into 0.01 M sodium hydroxide [4] tended to decrease the peak-to-noise ratio of the chromatograms and did not improve the sensitivity of the analysis.

The recovery of methohexital was 78 ± 3% from plasma and 78 ± 3% from haemolysed whole blood, as measured at a sample concentration of 0.50 μg/ml. At 2.00 μg/ml the recoveries were 93 ± 1% and 88 ± 5% [4].

Thiopental

Standard curves drawn from the analysis of duplicate samples containing 0.250, 0.500, 1.00, 2.00, 4.00, 8.00 and 16.0 μg/ml were linear (generally r = 0.999 on a five-point standard curve). Quantitation was by peak height. The precision of the analysis is given in Table I. The recovery of thiopental (at 1.0 μg/ml sample concentration) was 91 ± 1% from plasma and 82 ± 2% from haemolysed blood.

Stability of blood samples

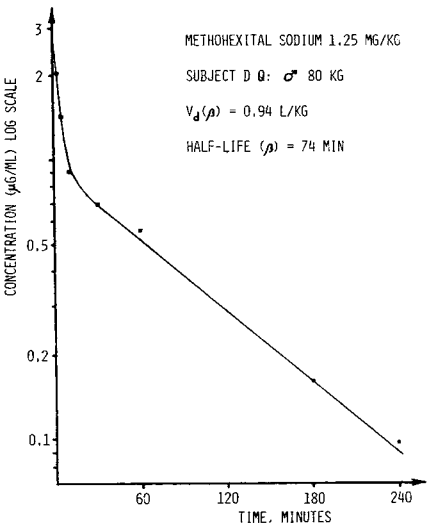
There was, within the limits of error of the method, no apparent degradation of methohexital or thiopental in blood samples left on the bench (at 21 ± 1°C) for 24 h.

Pharmacokinetic verification of the method

Representative plasma concentration curves from two patients given intravenous bolus doses of barbiturates are shown in Figs. 3 and 4.

DISCUSSION

Separation of barbiturates on chemically bonded octadecylsilyl phases is highly dependent on the pH of the eluent, and for good peak shapes and



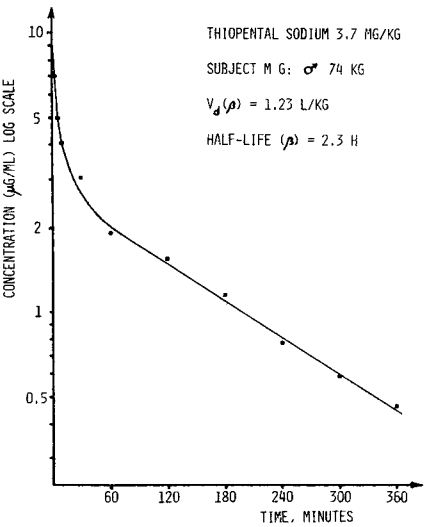


Fig. 3. Plasma concentration of methohexital in a patient under methohexital—halothane—nitrous oxide anaesthesia. The dose of methohexital sodium (Brietal) corresponds to 1.15 mg/kg free acid. The apparent volume of distribution (V_d) and the half-life (β) given in the figure were calculated from the six-point regression line.

Fig. 4. Plasma concentration of thiopental in a patient under thiopental—halothane—nitrous oxide anaesthesia. The dose of thiopental sodium (Pentothal) corresponds to 3.4 mg/kg free acid. The apparent volume of distribution (V_d) and the half-life (β) given in the figure were calculated from the six-point regression line.

efficient separations acidic systems are often preferable [15]. Determination of barbiturates as free acids requires short-wavelength ultraviolet detection, typically 195 nm, which precludes the use of alcohols in the mobile phase. Water—acetonitrile mixtures are consequently employed. In spite of the low wavelength the detection is quite selective and permits determination of the barbiturates in the ng/ml range [14, 16].

In conclusion, we have developed an HPLC method for the determination of methohexital or thiopental or, if need should arise, the simultaneous determination of both compounds. Its sensitivity, for methohexital, is at least as good as that of the gas—liquid chromatographic methods utilizing nitrogen-selective detection [2, 3]. It also compares favourably with our gas—liquid chromatographic method with electron-capture detection [4], with a roughly two-fold higher precision and sensitivity and no need for sample derivatization. The barbiturates proved sufficiently stable in blood to permit sample handling without special precautions. The feasibility of the method for pharmacokinetic work is demonstrated.

ACKNOWLEDGEMENT

We thank Miss Ulla Jönsson for skilful technical assistance.

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Journal of Chromatography, 307 (1984) 488—492
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2056

Note

Quantitative analysis of the metabolites of saikosaponin a using high-performance liquid chromatography

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(First received October 3rd, 1983; revised manuscript received December 13th, 1983)

Kampo-Hozai have been used clinically in China for 2000 years and their efficacy was clinically established. Among various Kampo-Hozai, Saiko-zai and Kuoketsu-zai have been used for chronic diseases, and, above all, their therapeutic effects on chronic hepatitis and chronic nephritis have well been recognized; their frequency of application in these chronic diseases is increasing rapidly in Japan and China.

The pharmacological effects of Saiko-zai may be due to that of the main crude drug, Bupleuri Radix, and several reports have been published concerning the chemical structure of the main constituent, sakosaponin [1—6]. Some pharmacological effects of saikosaponins concerning the anti-inflammatory action have also been reported [7, 8]. But there is no report about the actual active constituents — whether they are the glycosides themselves or aglycones. Thus, we have examined the absorption of the glycosides, saikosaponin a and saikosaponin b_1, and of their aglycone (non-sugar part), saikogenin A, in blood after oral administration, using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Standard and reagents

Saikosaponin a, saikosaponin b_1 and saikogenin A were extracted and identified with authentic samples in our laboratory. Oleana-11,13(18)-diene-3β-21α-28-triol (ODT), internal standard, was synthesized in our laboratory [9, 10].

0378-4347/84/$03.00

Animals

Male ddY strain mice of 25—35 g body weight were used. The animals were housed in an air-conditioned room for a week after purchasing.

Apparatus

The apparatus used in the present study was a Shimadzu Model 4A chromatograph with a Shimadzu Model SPD 2A ultraviolet detector. A stainless-steel column (25 cm × 4 mm I.D.), packed with reversed-phase Hypersil ODS (5 μm; Erma Optical Works, Tokyo, Japan) was used. The mobile phase was acetonitrile—water (46:54). The column temperature was 45°C, the flow-rate was 1.0 ml/min. Detection wavelength was 210 nm for the saponins and 254 nm for the aglycone. The sensitivity of the detector was set at 0.005 a.u.f.s. The peak area was measured using a Shimadzu C-R2A computing integrator.

Sample preparation

To 1.9 ml of artificial gastric juice (hydrochloric acid solution, pH 1.2) were added 50 μg of saikosaponin a dissolved in 100 μl of water. The mixture was incubated at 37°C, and a 50-μl aliquot of the incubation mixture was withdrawn after 5, 10, 20, 30, 60, 90, 120, 180, 240 and 300 min. After adding 200 ng of ODT, as the internal standard, in 50 μl of 1% ethanol solution, the aliquot was injected into the C_{18} cartridge column (Waters Assoc., Milford, MA, U.S.A.). After washing with 15% of methanol, elution was carried out with 3 ml of methanol. The eluate was concentrated to dryness under reduced pressure, and the residue was dissolved in 100 μl of methanol. After filtering the solution through a 0.45-μm membrane filter (Toyo Kagaku Sangyo, Osaka, Japan), 40 μl of filtrate were subjected to HPLC analysis.

At 15 and 30 min, and 1, 2, 3 and 5 h after oral administration of 40 mg/kg saikosaponin b_1, or 20 mg/kg saikogenin A, blood specimens (100 μl) were collected from the veniplex of the fundus oculi, and the collected blood was allowed to stand at room temperature for 30 min. Then it was centrifuged at 3000 rpm (6000 *g*) for 10 min and 30 μl of serum were obtained. To 30 μl of serum thus obtained, 25.0 ng of ODT were added as the internal standard, and 50 μl of methanol were added. The mixture was centrifuged at 11,000 rpm (22,000 *g*) to remove protein. The supernatant was injected into the C_{18} cartridge column and eluted with 3 ml of methanol. The eluate was evaporated to dryness, the residue was dissolved in 50 μl of methanol, and the suspension was filtered through a 0.45-μm membrane filter; 30 μl of the aliquot were subjected to HPLC analysis.

Calibration curve

A calibration graph was obtained with standard material ranging from 2 to 50 μg of saikosaponin a and saikosaponin b_1 using 200 ng of ODT as the internal standard according to the extraction procedure for the artificial gastric juice.

For the analysis of saikogenin A in blood, a calibration curve was prepared with the standard material, saikogenin A, ranging from 2 to 50 μg using 25.0 μg of ODT as the internal standard according to the extraction procedure for blood specimens.

RESULTS AND DISCUSSION

Saikosaponin a was incubated in artificial gastric juice, and an aliquot of the mixture was subjected to HPLC. The chromatogram obtained after 20 min of

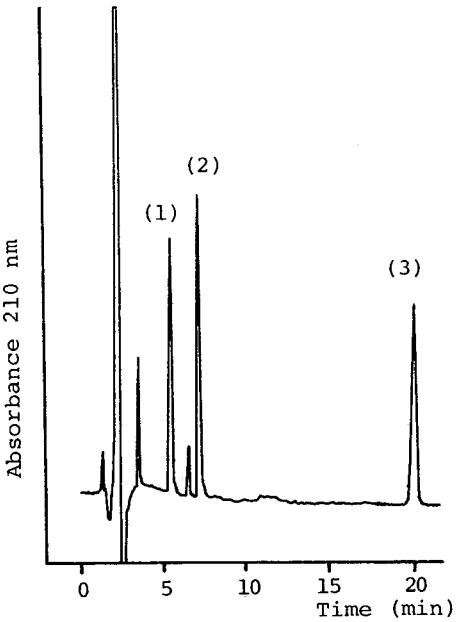

Fig. 1. Chromatogram of the artificial gastric juice 20 min after the addition of saikosaponin a. Peaks: 1 = saikosaponin a, 2 = saikosaponin b_1, 3 = ODT (internal standard).

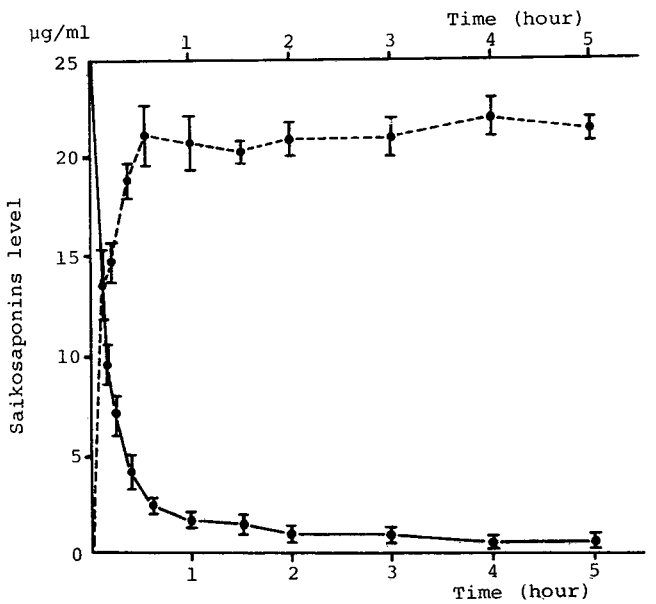

Fig. 2. Time course change of saikosaponin a to saikosaponin b_1 in the artificial gastric juice. The level of saikosaponin a is represented by a solid line and that of saikosaponin b_1 by a dotted line. Each point indicates the mean of four samples, vertical bars indicate S.E.

incubation is shown in Fig. 1. The peak of saikosaponin a corresponds to the peak appearing at a retention time of 5.8 min. A new peak, corresponding to saikosaponin b_1, appeared at 7.0 min with an unknown peak, 15—20% of the amount of saikosaponin b_1, at 6.3 min. The time course of the change of saikosaponin a to saikosaponin b_1 in the artificial gastric juice is shown in Fig. 2. The amount of saikosaponin a was rapidly decreased in acidic conditions (pH 1.2) to give saikosaponin b_1 which has a heteroannulardiene structure (see Fig. 5). The amount of saikosaponin b_1 was not decreased in acidic medium after 5 h, showing that no hydrolysis takes place at 37°C to yield sugar and saikogenin A (Fig. 5).

Then, uptake of saikosaponins into blood was examined. It may be well assumed that orally administered saikosaponin a would be changed in vivo to saikosaponin b_1 in the stomach by the action of acidic gastric juice, and the saikosaponin b_1 thus formed might be moved to the intestine. For such a reason, saikosaponin b_1 was administered to mice orally to examine its uptake into the blood stream from the intestine. A chromatogram of serum obtained 2 h after the oral administration of saikosaponin b_1 is shown in Fig. 3. Saikogenin A, the aglycone of saikosaponin b_1, was identified as the main peak at 2 h after administration. The time course of change of blood saikogenin A is shown in Fig. 4.

When saikogenin A was orally administered instead of saikosaponin b_1, the blood level of saikogenin A reached a maximum in 15 min, and then declined reaching half level at 40 min. The time lag may be interpreted as follows: saikogenin A, an aglycone of saikosaponin b_1, can be absorbed in the intestine and taken up rapidly into the blood stream. On the ohter hand, saikosaponin b_1, a glycoside, might be hydrolysed by the intestinal bacteria in the blind gut and in the large intestine to sugar and saikogenin A.

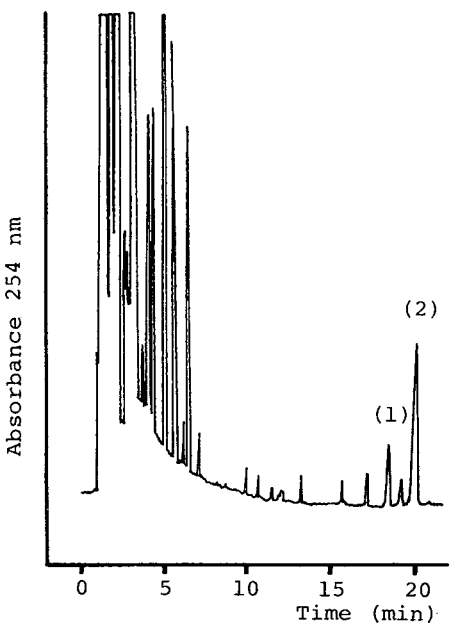

Fig. 3. Chromatogram of saikogenin A in blood 2 h after the oral administration of saikosaponin b_1. Peaks: 1 = saikogenin A, 2 = ODT (internal standard).

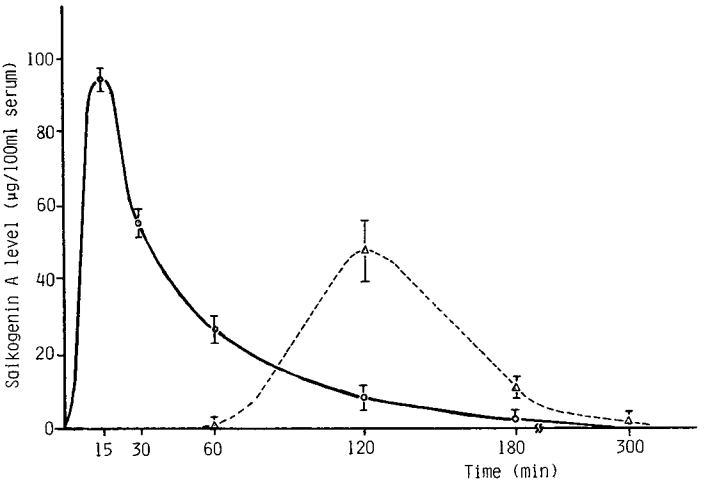

Fig. 4. Time course change of blood level of saikogenin A after oral administration of saikosaponin b_1 and saikogenin A. The blood level of saikogenin A after oral administration of saikogenin A is represented by a solid line and the blood level of saikogenin A after oral administration of saikosaponin b_1 by a dotted line. Each point indicates the mean of four samples, vertical bars indicate S.E.

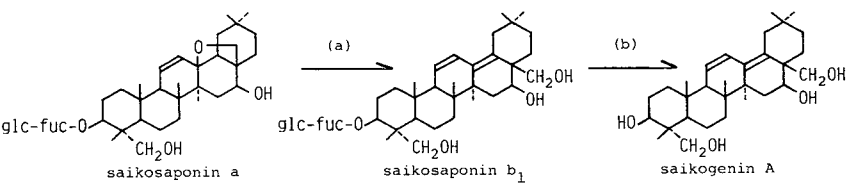

Fig. 5. Mechanism of saikosaponin b_1 and saikogenin A formation from saikosaponin a: (a) under acidic conditions in the artificial gastric juice; (b) hydrolysis of saponin by the intestinal bacteria.

The recovery of saikogenin A from serum ranged from 84.4% to 91.2% with an average of 88.0% for four determinations.

The change in chemical structure from saikosaponin a to saikogenin A, observed in the present study, is shown in Fig. 5.

It may be strongly suggested that the anti-inflammatory effect and other pharmacological effects observed by the oral administration of saikosaponin a would be the effect of its aglycone, saikogenin A.

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Journal of Chromatography, 307 (1984) 493—499
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2040

Note

Detection and quantitation of tetrahydrocannabinol in serum using thin-layer chromatography and fluorometry

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(First received September 29th, 1983; revised mansucript received December 19th, 1983)

Much work is being performed in the cannabis field due to the popularity of marijuana among the young and because marijuana is being used in medical research in the areas of cancer and glaucoma treatment. The concentration of Δ^9-tetrahydrocannabinol (THC), the active principle in marijuana, in blood is believed to correlate with some psychological and physiological responses [1, 2] and thus, an analysis of THC in blood or serum is important for pharmacological studies. Recent evidence [3] indicates that of approximately 1800 blood specimens from motorists stopped by the California Highway Patrol for erratic driving, analysis revealed that 14.8% were positive for THC. Analysis of THC would be useful for forensic purposes since its concentration in plasma is related to impaired motor function [4].

THC occurs in plasma in very low concentrations (ng/ml) with the peak level of about 50 ng/ml about 10 min after smoking [5]. The physiological effects persist for several hours after smoking when the plasma levels are only about 2 ng/ml. Thus, a method is needed which is capable of detecting and quantitating THC down to 1 ng/ml. There are several analytical methods available for analysis of THC in serum. These have been recently reviewed [6] and include gas chromatography, radioimmunoassay, mass spectroscopy, and high-performance liquid chromatography. Most recently, an immunoassay technique has come into widespread use for urine analysis but lacks the sensitivity and specificity to be used for blood or serum [7].

Thin-layer chromatography (TLC) has been shown by us to be a sensitive and selective qualitative method for detecting THC in serum [8]. We wish to report that a modification of this TLC procedure followed by solution fluorometry is suitable for quantitative analysis of THC in serum.

0378-4347/84/$03.00

EXPERIMENTAL

Reagents and chemicals

Tetrahydrocannabinol (THC) was obtained as a solution in ethanol from the National Institute of Drug Abuse and was 99+% pure as analyzed by gas chromatography. The derivatizing reagent, 2-*p*-chlorosulfophenyl-3-phenylindone (DIS·Cl) was available from Polysciences (Warrington, PA, U.S.A.). Acetone and benzene were distilled-in-glass solvents from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Dimethylformamide, methanol, hexane, isoamyl alcohol and acetonitrile were Fisher reagent grade (Pittsburgh, PA, U.S.A.). Potassium methoxide was also obtained from Fisher. Dibenzo-18-crown-6 was available from Aldrich (Milwaukee, WI, U.S.A.).

Supplies and instrumentation

Membrane filters were Alpha Metricell-450 (25 mm) from Gelman (Ann Arbor, MI, U.S.A.). Bakerflex IB2 silica gel sheets, 5 × 20 cm or 20 × 20 cm (J.T. Baker, Phillipsburg, NJ, U.S.A.) were used for the chromatography. All glassware was siliconized with Dri-Film SC-87 from Pierce (Rockford, IL, U.S.A.). Reacti-Vials were also obtained from Pierce. The fluorescence was measured with an uncorrected SPF-125 fluorometer (American Instruments, Silver Springs, MD, U.S.A.).

Extraction from serum

Methanol (4 ml) was added to 2.0 ml of sample serum and vortexed for 1 min in a screw-capped siliconized test tube. The tube was then centrifuged for 5 min with a tabletop centrifuge. The clear solution was decanted into another tube. The remaining precipitate was washed with 4 ml of methanol and the centrifugation and decantation repeated. The pooled extracts were evaporated to a volume of 3 ml in an 80°C water bath with a slow stream of air. The THC was then extracted with 8 ml of hexane—isoamyl alcohol (97:3) by vortexing followed by centrifugation. The hexane layer was transferred to another tube and evaporated to dryness. A 2-ml volume of a modified Claisen alkali [8] (37 g of potassium hydroxide in 20 ml of water followed by addition of 100 ml of methanol) was added to dissolve the dried residue. The solution was washed with 1 ml of hexane which was discarded. The Claisen alkali was evaporated to 0.5 ml and 2 ml of water were added. Then 8 ml of hexane were added and the mixture vortexed. The hexane layer was quantitatively removed and evaporated to dryness. The residue was transferred to a 0.3-ml Reacti-Vial with hexane which was then evaporated. The extraction was repeated on a pooled serum blank which was known to be free of THC.

Standard

The pure DIS·THC derivative was prepared as follows. A 14-μl volume of 1.0 *M* sodium hydroxide was added to 4 mg of THC. To this mixture was added 0.6 ml of an acetone solution of 4.9 mg of DIS·Cl. After 5 min, the product was precipitated with water, filtered and taken up in 1 ml of methanol. The solution was spotted as a band on a 20 × 20 cm silica gel IB2 plate which was then developed for 10 cm in methanol—water (95:5). The yellow band at

4.8 cm was removed and eluted with acetone. The gummy residue after evaporation was recrystallized twice from methanol, dissolved in benzene, and stored at −15°C until use.

Derivatization and chromatography

Of a solution of 1 mg/ml of DIS·Cl in acetonitrile 10 μl were added to the sample extract and blank extract. The vials were vortexed to insure proper mixing. After adding 5 μl of a 0.2 *M* aqueous sodium carbonate solution, the vials were sealed. The mixture was allowed to react for 30 min at 40°C. Inorganic solids were precipitated by the addition of 25 μl of acetone to the reaction mixture. After centrifugation, the salt-free solution was spotted with a microsyringe on a 5 × 20 cm or 20 × 20 TLC plate about 1 cm from the bottom of the plate. A 20-ng amount of pure DIS·THC (10 μl of a 2 μg/ml solution) was also spotted on the plate as a standard.

After drying of the spots, the TLC plate was developed for 3 cm in an unsaturated chamber with dimethylformamide—water (80:20). The plate was removed, dried with a hair dryer, and then redeveloped for 6 cm. The drying was repeated and the plate redeveloped for 9 cm. The plate was dried and then sprayed lightly with a fresh solution of 4 g of clean sodium dissolved in 100 ml of methanol. The plate was immediately observed under long-wave ultraviolet light. The blank contained two spots, a reagent spot near the solvent front and slightly below an unknown endogenous material. The DIS·THC had an R_F of 0.68 and fluoresced yellow—green. If the sample was positive, then the DIS·THC standard, and the DIS·THC spot were lightly circled with a lead pencil.

Elution and fluorometry

A 1-cm^2 section of the silica gel plate was carefully scraped into a siliconized centrifuge tube. Sections were taken for DIS·THC standard, samples positive for THC and blank serum. For the latter, the silica gel was scraped at the same R_F as the DIS·THC standard. Each silica gel section was vortexed for 1 min with 1 ml of acetone. After centrifugation, the supernatant was decanted onto a membrane filter and the vacuum applied. The filter was previously washed with 5—10 ml portions of acetone. The filtrate was collected directly in a fluorometry tube. The residual powder was washed twice with 1 ml of acetone. The combined filtrates were evaporated with a slow stream of air in an 80°C water bath.

The fluorometry reagent was prepared by dissolving 0.2 g of dibenzo-18-crown-6 in 100 ml of benzene. To a 10-ml portion of this solution was added 0.1 g of potassium methoxide. The solution was allowed to stand for 1 h, centrifuged and transferred to a screw-capped tube. This final solution was prepared daily.

To the fluorometry cells was added 1 ml of fluorometry reagent by means of a pipette. The fluorescence of samples and standard were read immediately at an excitation of 430 nm and an emission of 500 nm using a mercury lamp. The blank serum plate eluate was used to zero the fluorometer.

RESULTS AND DISCUSSION

The necessity of siliconizing glassware when analyzing for trace quantities of THC was shown by Garrett and Hunt [9] who found that 20—40% of THC in aqueous solution bound to glass. Plastics and rubber were found to be even more effective than glass. Thus, all glassware was siliconized in this work and contact with plastics was minimized.

The extraction procedure was a modification of that published by Rosenfeld et al. [10]. The addition of methanol to serum effectively extracts the THC while precipitating the proteins which bind the THC. The Claisen alkali [8] selectively extracts phenols such as THC while leaving the lipids in the hexane. The absolute extraction efficiency was found by spiking duplicate serums and comparing the fluorescence with THC standards which were also derivatized, chromatographed and eluted. The results are shown in Table I. The extraction efficiency was found to be 96.4 ± 6.5%.

The extent of THC derivatization with DIS·Cl was found to be extremely dependent on the solvent and the base used to ionize the THC. With sodium hydroxide, the derivative forms rapidly and degrades rapidly as evidenced by extra spots on the TLC plate. Sodium bicarbonate gave a slower reaction than sodium carbonate and thus the carbonate was chosen as the base. As a solvent, acetonitrile gave a reaction rate more than twice as fast as acetone which is the commonly used solvent in the analogous dansyl chloride derivatizations. In acetonitrile, using sodium carbonate as the base, the reaction is complete after 30 min at 40°C. Reaction times of up to 12 h have shown no degradation of the DIS·THC derivative. The extent of derivatization of amounts of THC varying from 4—50 ng is shown in Table II. The results were calculated by comparing the amount of fluorescence produced by THC which was derivatized with DIS·Cl, chromatographed and eluted to that of DIS·THC which was also chromatographed and eluted. The average derivatization was 87.3 ± 4.3%.

TABLE I

DETERMINATION OF THC EXTRACTION EFFICIENCY OF THC FROM SERUM

Spiked THC concentration (ng/ml)	Found THC concentration (ng/ml, ± S.D.)
2.0	1.9 ± 0.1
10.0	9.5 ± 0.6
50.0	49.0 ± 6.2

TABLE II

EXTENT OF DERIVATIZATION OF THC WITH DIS·Cl USING SODIUM CARBONATE IN ACETONITRILE FOR 30 min AT 40°C

Amount of THC (ng)	Percent derivatized (± S.D.)
4.0	87.0 ± 1.6
20.0	90.7 ± 1.9
50.0	84.3 ± 4.8

Recovery of DIS·THC from the plate was very critical in order to obtain the maximum sensitivity of the method. It was found that acetone, among the many solvents tried, provided the maximum recovery with a minimum of contamination from the silica gel. More polar solvents gave slightly greater recoveries but left behind a white residue on evaporation which was probably the silica gel binder. This residue caused a large amount of blank fluorescence. Distilled-in-glass acetone was used to minimize fluorescence due to solvent impurities. The average recovery of 10 ng of THC as DIS·THC which was spotted four times, chromatographed and eluted was 93.0 ± 3.9%.

The search for a suitable, strongly basic reagent to render the DIS·THC fluorescent in solution proved to be a difficult task. The reagent used to visualize the DIS·THC on the plate, sodium dissolved in methanol, was found to give too great a blank fluorescence. Potassium methoxide in methanol, a reagent used to render the DIS derivative of vitamin B_6 fluorescent in solution [11], also gave too high a blank fluorescence. Sodium methoxide in dimethyl sulfoxide, an extremely basic medium, produced reasonable fluorescence of DIS·THC but the solution was quite viscous and was difficult to pipet and a search for other solvents was begun. It is known that fluorescent intensity is favored by a solvent of low viscosity. Hydrocarbons are of extremely low viscosity but it is impossible to dissolve strong base in them. However, Pedersen [12] has found that potassium hydroxide could be solubilized in toluene using compounds known as crown ethers. These macrocyclic polyether compounds complex with the cation leaving a "naked" anion dissolved in the hydrocarbon. Dibenzo-18-crown-6 is a specific crown ether for potassium ion. This crown

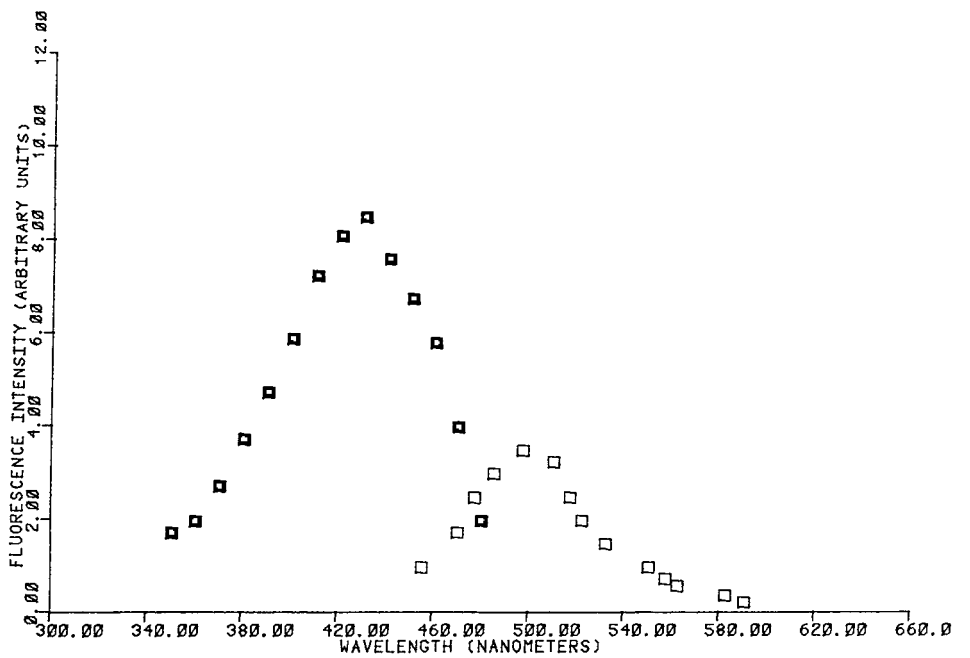

Fig. 1. Excitation (▪) and emission (▫) spectra of DIS·THC in a solution of sodium methoxide in benzene solubilized with dibenzo-18-crown-6.

ether is suitable for solubilizing potassium methoxide in benzene. With this system, the fluorescence of DIS·THC is about four times that in sodium methoxide in dimethyl sulfoxide. A 0.2% solution of the crown ether produces a maximal DIS·THC fluorescence with a minimum of blank fluorescence. The excitation and emission spectra of DIS·THC are shown in Fig. 1. A fluorescence calibration curve of 2—10 ng of THC as DIS·THC gives a straight line with a correlation coefficient of 0.9974 which passes through the origin.

Five "blind" serum samples from a human marijuana study were received from the National Institute of Drug Abuse. Each sample was analyzed by the reference gas chromatographic—mass spectrometric (GC—MS) method at the Battelle Institute and by the present THC procedure. The data are shown in Table III. A correlation plot gave a slope of 1.099 and a correlation coefficient of 0.981.

TABLE III

VALIDATION OF THE TLC METHOD FOR ANALYSIS OF THC IN SERUM AFTER SMOKING 10 mg OF THC

Time (h)	THC concentration GC—MS method (ng/ml)	THC concentration TLC method (ng/ml, ± S.D.)
Pre-smoking	0	0
0.25	22.0	25.5 ± 1.0
1	9.2	7.8 ± 0.2
2	2.1	5.5 ± 1.4
5.5	0	0

The TLC method has only minimal interferences. DIS·Cl has been shown to be specific for phenols [8]. It does not react under the experimental conditions to produce fluorescent derivatives with a variety of drugs which have amine functional groups such as amphetamines, tranquilizers, and psychoactives. Barbiturates and common analgesics such as salicylic acid, salicylamide and acetaminophen also do not react with DIS·Cl. Apomorphine, morphine, pentazocine, levorphenol and bufotenine, all of which have phenolic functional groups, give fluorescent derivatives with DIS·Cl but are either not extractable under the experimental conditions or give a different R_F value in the THC system and thus do not constitute an interference. No spots other than DIS·THC and the endogenous spot have been seen in serum samples analyzed to date. The metabolites of THC such as the hydroxy and carboxy compounds were not seen in any authentic sample positive for THC. They are probably too acidic to be extracted under the experimental conditions.

The detection limit for THC in serum by this method is approximately 1 ng/ml when 2 ml of serum are used. If no DIS·THC spot is seen after spraying, then the quantitation does not need to be performed — a considerable saving in time and labor. Other screening methods such as radioimmunoassay and GC require all samples to be put through the entire process in order to detect negatives.

In summary, the TLC procedure has been shown to be sensitive, selective and suitable for routine screening and quantitation.

ACKNOWLEDGEMENTS

The authors would like to thank R. Stillman for supplying authentic serum samples. This research was supported by a contract from the Insurance Institute for Highway Safety.

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Errata

J. Chromatogr., 278 (1983) 387—395

Page 389, text line 3 from bottom, "1 mg/l" should read "1 g/l".

Page 391, text line 2, "1 mg/l" should read "1 g/l".

Page 393, Fig. 4, the quantity of *p*-nitrobenzyloxyamine injected is 1000 times greater than indicated in the graph.

J. Chromatogr., 306 (1984) 394—397

Page 396, Fig. 1, the applied voltages are ten times smaller than indicated in the graph.

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ISBN 0-444-42015-0.
Price: US $ 89.25 (U.S.A. & Canada)
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