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CHROMBIO. 471

SMALL-VOLUME ELECTROCHEMICAL DETECTOR FOR MICROCOLUMN LIQUID CHROMATOGRAPHY

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(Received September 24th, 1979)

SUMMARY

A submicroliter electrochemical detector for liquid chromatography has been designed, using pressure-annealed pyrolytic graphite technology. The analytical performance of this detector was studied in connection with a reversed-phase packed microcapillary column at very low flow-rates. Although the miniaturized version of the electrochemical detector is less sensitive, a direct analysis of a number of urinary metabolites in 0.1—1.0 μl samples is feasible.

INTRODUCTION

Several recent communications have demonstrated the viability of capillary liquid chromatography (LC) [1—6]. Capillary LC is of great interest because of its potential for achieving a greater resolving power than conventional high-performance liquid chromatography (HPLC). Capillary LC and microbore HPLC [6—8] are also attractive in that consumption of the mobile phase is dramatically reduced; flow-rates employed in capillary LC are typically 0.5—5 μl/min. These extremely low flow-rates require that small-volume detectors be designed and this has been achieved for UV absorbance [7] and spectrofluorometric detection [5]. The chief limitation of capillary LC is that extremely small sample volumes must be used. Despite the great resolving power of the columns, this limitation requires that pre-concentration steps or very sensitive detectors be employed for analysis of trace components in complex mixtures. In this communication we report the use of electrochemical detectors with capillary LC.

Liquid chromatography with electrochemical detection has become a widely accepted technique. Detection of separated compounds with carbon electrodes has found a special utility in the analysis of body fluids and tissue samples for the determination of tyrosine and tryptophan metabolites [9]. The success of

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electrochemical detection arises from its sensitivity; changes in concentration of 10^{-8} *M* can readily be determined in flowing streams with carbon electrodes. Several different types of carbon materials have been evaluated for use as detectors for liquid chromatography [10—13]. One of these, pressure-annealed pyrolytic graphite (PAPG) [14], is an ideal material for designing a microelectrochemical cell since it is extremely flat and will not deform into the extremely small channel required for use with microcapillary columns.

We describe here performance of a low-volume electrochemical cell (approximately 0.1 μl) which responds in a linear fashion to changes in concentration at the flow-rates employed in capillary LC. Because of the reduced area of the electrode and the lower flow-rates employed, the detector is less sensitive than an electrochemical cell designed for use with conventional HPLC columns (approximately 2-μl volume). Nevertheless, the electrode is sufficiently sensitive to detect directly a number of compounds in human urinary samples without preconcentration. In addition, the response of the electrochemical detector is compared to the miniaturized fluorescence and absorbance detectors of comparable volume and is found to be far superior in this application.

EXPERIMENTAL

Electrochemical cells

The design of the conventional electrochemical detector is identical to that described previously [14]. In the low-volume cell, the dimensions were reduced to provide suitable operation with the extremely low flow-rates employed with microcapillary columns. The low-volume cell is illustrated in Fig. 1. The cell body is constructed from Lucite, and the two halves are held together by four bolts. The lower half contains a 0.16-cm deep groove, 0.95 cm wide, which extends the length of the block. A copper plate, which serves as a connection to the working electrode, is placed on the floor of the groove, and a 0.95 cm × 1.9 cm piece of pressure-annealed pyrolytic graphite (Union Carbide, Parma, Ohio, U.S.A.) is placed on top of the copper so that the basal plane will be exposed to the solution. The graphite extends approximately 0.5 mm above the top of the lower block. A polyethylene spacer is placed on top of the graphite. A hole cut in the spacer, which coincides with the inlet and outlet openings of the upper half, determines the length (0.30 cm) and width (0.10 cm) of the electrochemical cell. The spacer thickness is 50 μm, giving a cell volume of 0.15 μl. Polyethylene spacers do not compress as much as PTFE spacers, and thus, are preferable in this application. The inlet for the cell is fabricated by silver soldering three concentric stainless-steel tubes. The inner tube has an I.D. of 0.01 cm, and the outer tube is a 20-gauge needle (0.084 cm). The soldered tubes ensure that the inlet assembly is sufficiently rigid for manipulation. The inlet tube is press fit into the upper Lucite block and sanded so that the lower surface of the block is flat. The inlet is connected to the microcapillary column via Teflon capillary tubing. The outlet is a 0.064-cm hole bored in the Lucite block which leads to a compartment containing the saturated calomel reference and platinum wire auxiliary electrode. The entire cell is connected to a potentiostat which applies a constant potential and provides a voltage output proportional to the electrolysis current (Bioanalytical Systems, West Lafayette, Ind., U.S.A.).

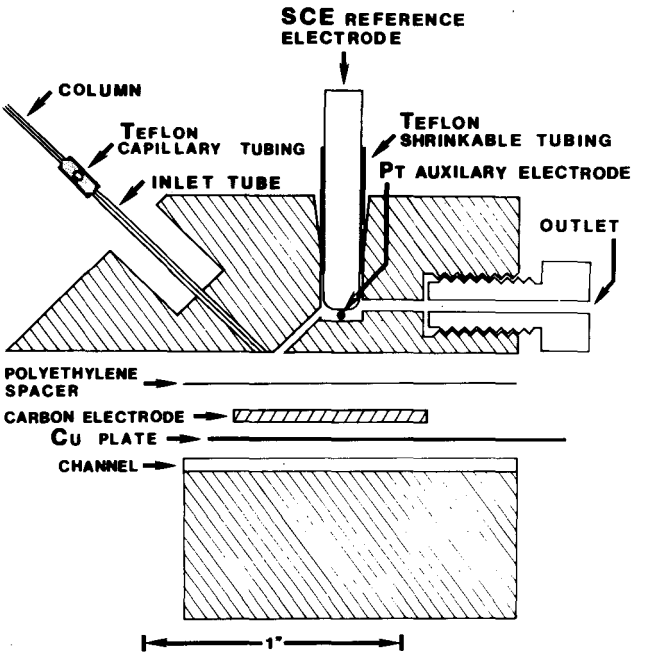

Fig. 1. Schematic diagram of the low-volume electrochemical cell.

The properties of pressure-annealed pyrolytic graphite have been previously discussed [14]. For sensitive and reproducible detection, the surface must be anodically oxidized before use. Complete electrolysis of the graphite is not possible with the low-volume cell since the auxiliary electrode does not oppose the working electrode (the miniature dimensions of the cell make this type of construction difficult). Therefore, the graphite is oxidized in a conventional flow electrochemical cell, and then incorporated in the low-volume cell.

Chromatographic system

Fabrication of microcapillary columns was identical to that described previously [4]. The packed columns contained 30-μm porous silica particles (LiChrosorb SI-100, Merck, Rahway, N.J., U.S.A.), and were drawn on a commercial glass drawing machine. The particles in the drawn column were reacted with octadecyltriethoxysilane (PCR Research Chemicals, Gainesville, Fla., U.S.A.). Reversed-phase columns based on packed alumina particles were also investigated; the resulting chromatograms exhibited skewed peaks for the oxidizable components in whole urine samples and, thus, this approach was not pursued.

The chromatographic system has also been previously described [5]. The pump was a Varian Model 8500 high-pressure syringe pump. In addition to the electrochemical detector, a UV monitor (Varian, Chicago, Ill., U.S.A.) operated at 254 nm and a spectrofluorometric detector (Schoeffel FS970 fluorometer, Westwood, N.J., U.S.A.) were employed. Both had an internal volume of approximately 0.1 μl. These detectors preceded the electrochemical detector in

dual detector applications. The "stop-flow" sampling method was used for sample injection [5]. Sample injection volumes varied from 0.1 to 1.0 μl.

Chemicals

Buffers for liquid chromatography were prepared from reagent grade chemicals dissolved in water that was distilled from basic permanganate and filtered through a 0.8-μm filter. Compounds for use as standards were from Sigma (St. Louis, Mo., U.S.A.); Regis (Morton Grove, Ill., U.S.A.); or Aldrich (Milwaukee, Wisc., U.S.A.). Urine samples from normal males were acidified with perchloric acid, centrifuged, and frozen until use. They were then directly applied to the column.

RESULTS AND DISCUSSION

Cell evaluation

Recently, Weber and Purdy [15] have developed an analytical expression for the current from an electrochemical cell of the type employed here as a liquid chromatographic detector. This expression relates the current to the cell dimensions, flow-rate, and concentration for an electrochemical cell that has the geometry of a rectangular channel and which operates under the conditions of laminar flow and at "infinite" potential. The carbon electrode forms the floor of the channel, and the height (b) and width (W) of the channel are determined by the polyethylene spacer. Opposing the electrode is either the auxiliary electrode or an inert material containing the inlet and outlet tubes. In the cells described here, the inlet and outlet tubes determine the length (L) of the channel. Since PAPG is an unusual electrode material, and since our cell geometry permits some electrolysis in the regions of non-laminar flow, we have evaluated the utility of this expression to describe the current from our conventional volume ($\approx$ 2 μl) detector.

To determine whether amperometric or coulometric conditions obtain, the following expression should be evaluated:

$$r = \frac{DWL}{Ub} \tag{1}$$

where D is the diffusion coefficient (cm^2/sec) and U is the volume flow-rate (cm^3/sec). For $r \ll 0.3337$, the expression derived by Weber and Purdy [15] reduces to an equation previously given by Posey and Meyer [16]:

$$I = 1.467\, nFC^{\circ} \left(\frac{DLW}{b}\right)^{2/3} U^{1/3} \tag{2}$$

where I is the Faradaic current (A), n is the number of Faradays per mole, F is the Faraday constant, C° is the concentration (moles/ml), and the cell dimensions are given in cm. For the large-volume cell, good agreement with this equation is obtained. A deaerated 10^{-6} M dopamine solution in pH 5.2 citrate—acetate buffer was pumped through various electrochemical cells at an applied potential of 0.8 V. Cells with electrode areas of 0.40 and 0.32 cm^2 were evaluated with spacers of 50 μm, 75 μm, 100 μm, 125 μm and 150 μm at several different

flow-rates from 0.04 to 3.8 ml/min. (The areas are geometric areas, the spacer thicknesses are from the manufacturers' specifications, and the flow-rates were determined separately for each experiment.) A plot of the resulting 56 current measurements in the form log I versus log $[UA^2/b^2]$ gives a slope of 0.3666, a correlation coefficient of 0.9971, and an intercept in good agreement with the known concentration and estimated diffusion coefficient.

The low-volume cell with an electrode area of 0.03 cm^2 and a 50-μm spacer has a value of r greater than 0.3337 at the flow-rates employed in capillary LC. These dimensions are in part determined by the mechanical limitations of construction of a small cell. Under these conditions Weber and Purdy [15] have shown that the current is given by

$$I = nFUC^{\circ}[1 - 0.3992 \exp\{2.505(0.3337 - r)\}] \quad (3)$$

Operation of the electrochemical detector where this equation applies is usually undesirable since the detector becomes more sensitive to changes in flow-rate and also because the sample is entirely decomposed precluding further analysis of the sample. For the low-volume cell used in this work, the response is given in Fig. 2. For this cell, the logarithmic plot gives a slope of 0.318 rather than

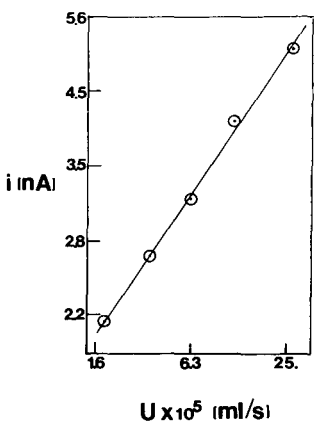

Fig. 2. Logarithmic plot of the amperometric response of the low-volume electrochemical cell as a function of flow-rate. Dopamine (10^{-6} M) in pH 5.2 citrate—acetate buffer was continuously flowed past the detector, and the increase in current over the residual current was determined.

the expected slope of 1 with a correlation coefficient of 0.9992. Although the current response of the low-volume cell is less than expected from eqn. 3, the reduced dependence of the current on flow-rate is advantageous. With this cell coulometric behavior is not observed until very slow flow-rates (70% electrolysis of 1 μl/min). Three different factors may be the cause of this discrepancy between theory and experiment. First, the distance required to obtain laminar flow occupies a significant portion of the length of the electrode with the low-volume cell. Second, the large diameter of the outlet tube increases the effective width of the channel over the last 20% of the cell length to an extremely large value. Third, the anodic oxidation of the carbon may not entirely acti-

vate the carbon surface; it has already been shown that an unoxidized basal plane of PAPG is a very inefficient electrode [14].

Since the conventional and low-volume cells are operating under entirely different flow-rates, direct comparison of the detector responses is difficult. Using typical values in eqn. 2 for the electrode area, spacer thickness, and flow-rate for each cell, the current from the low-volume cell should be 10% of the conventional cell for the same concentration. In fact, our detector is approximately three times less sensitive than theory predicts, so the output current from the small cell is $\approx$ 3% of the conventional cell when both are operating under usual conditions. Although the absolute current is less, the noise level of the current from the low-volume cell is also much reduced since it has a smaller surface area [10]. A comparison of signal-to-noise ratios with the two electrodes at $1 \cdot 10^{-4}$ ml/sec for the low-volume cell and $1 \cdot 10^{-2}$ ml/sec for the large cell indicates that the detection limit is reduced by approximately 10 for the low-volume cell.

Although construction of a low-volume cell with a platinum auxiliary electrode located opposite the working electrode is sufficiently difficult to preclude routine use, we have evaluated the flow-rate dependence of such a cell under conditions where $r \gg 0.337$. Under these conditions the current is flow-rate independent at very slow flow-rates for a reversible compound [15]:

$$I = nFUC(0.2671 + \frac{DLW}{Ub}) \quad (4)$$

and, indeed this is the case for the low-volume cell with 10^{-6} *M* dopamine in citrate—acetate buffer (pH 5.2) at flow-rates up to $3 \cdot 10^{-5}$ ml/sec.

Chromatographic detection

The low-volume electrochemical cell has been employed as a detector for microcapillary liquid chromatography in an investigation of the separation of acidic and neutral metabolites of tyrosine and tryptophan in human urine. During the course of this investigation, detectors were found to remain operable for about three weeks. Failure of the detector was evidenced by a high background current which results from leaking of the mobile phase under the polyethylene spacer.

Fig. 3 shows the separation of a 0.1 *M* acetic acid solution containing methoxyhydroxyphenyl glycol (MHPG), *p*-hydroxyphenylacetic acid (PHPAA), 5-hydroxyindoleacetic acid (5-HIAA), homovanillic acid (HVA) and vanillic acid (VA), each at a concentration of $1.6 \cdot 10^{-4}$ *M*. Four of these compounds can readily be identified in human urine; only MHPG is unresolved (Fig. 4). The urinary sample that was used to obtain the chromatogram in Fig. 4 was only acidified to prevent air oxidation of the phenolic compounds. A sample overload of 1 μl of acidified urine decreased somewhat the column efficiency as compared to standards where typically 0.1-μl samples were introduced (compare Figs. 3 and 4). The sample was not a 24-h collection, nor was it hydrolyzed, but it gives an outstanding example of the combined resolution and sensitivity that the microcapillary LC—electrochemical method provides.

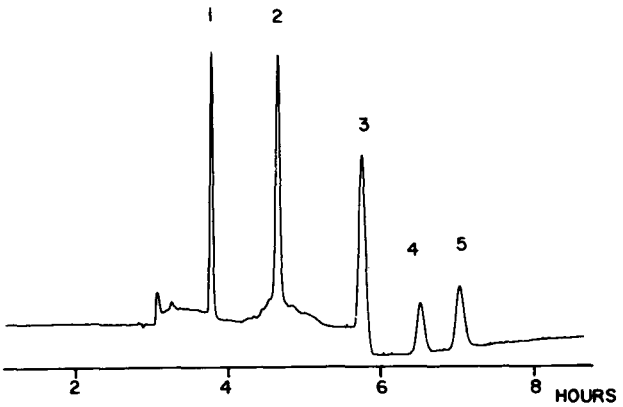

Fig. 3. Chromatogram of 0.3 μl of a solution containing $1.6 \cdot 10^{-4}$ *M* MHPG (1), PHPAA (2), 5-HIAA (3), HVA (4), and VA (5). Conditions: 60 m reversed-phase column, 0.2 *M* acetate buffer (pH 4.0), flow-rate of 1 μl/min, E_{app} = 1.0 V, 20 nA full scale.

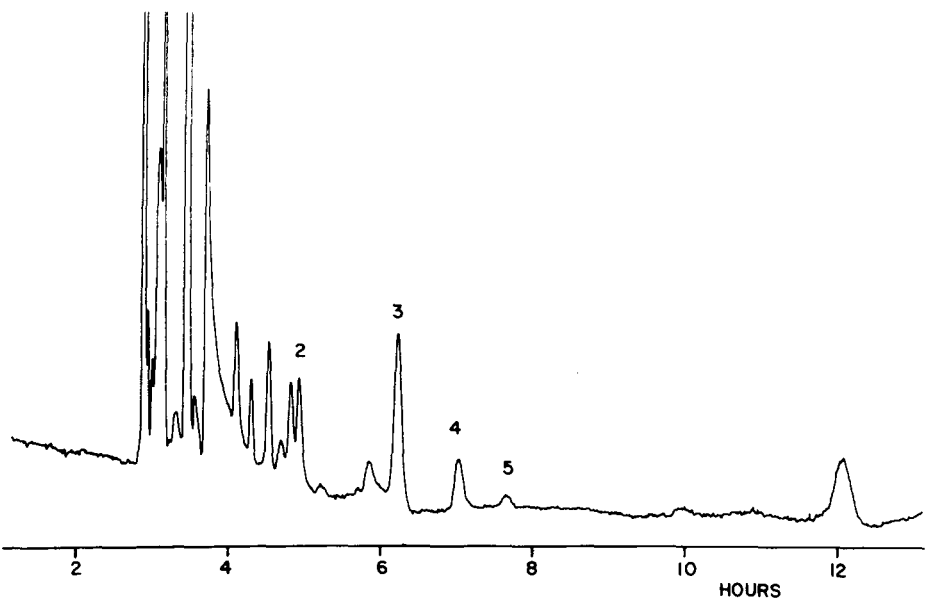

Fig. 4. Chromatogram of 1.0 μl of human urine, acidified to pH 2. Resolved components include PHPAA (2), 5-HIAA (3), HVA (4), and VA (5). Conditions as in Fig. 3 except 3 nA full scale.

Neither the fluorescence nor the UV detector were found sufficiently sensitive to provide any information on the eluting compounds in the urine samples. The electrochemical detector has been compared to the UV detector in the separation of 10^{-2} *M* standards; comparison of the chromatograms showed that band broadening is not introduced by interfacing the electrochemical detector to the column.

CONCLUSIONS

Electrochemical detection of compounds separated by microcapillary LC is a sensitive method for detecting oxidizable compounds. Because of the reduced dimensions of the cell and the lower flow-rates compared to conventional LC, the detector has a detection limit approximately 10 times higher than a large-volume cell. Nevertheless, the high column efficiency coupled with the sensitivity and selectivity of the detector provide a useful technique for the identification of compounds in a complex biological fluid. Sample pretreatment is not required, and, thus, the analysis scheme is much simpler than other methods. For the compounds discussed in this paper, the electrochemical method is far more sensitive than fluorescence or UV detection.

ACKNOWLEDGEMENTS

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CHROMBIO. 465

PROSTAGLANDINS E AND F, AND 19-HYDROXYLATED E AND F (SERIES I AND II) IN SEMEN OF FERTILE MEN

GAS AND LIQUID CHROMATOGRAPHIC SEPARATION WITH SELECTED ION DETECTION

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(First received July 13th, 1979; revised manuscript received October 12th, 1979)

SUMMARY

Low concentrations of prostaglandins (PG) could be related to male clinical infertility although relevant experimental data are scarce. The aim of this work is to establish reliable seminal PG levels in fertile men by rigorous sample control, to prevent degradation, and by rapid and simple extraction and assay procedures.

Single semen samples from healthy fertile men were immediately centrifuged (within 30 min of ejaculation) adding $PGF_{2\alpha}$ D_4 to the seminal plasma as internal standard. The samples were next ultrafiltered and the PGs in the ultrafiltrate were derivatized with a mixture of N,O-bis(trimethylsilyl)trifluoroacetamide and piperidine (1:1) at 60° for 30 min. Optimum gas—liquid chromatographic separation of all of the peaks of interest was achieved on 4 m × 1/4 in. I.D. Dexsil 300 packed columns at 280°. The detection and quantitation of all the peaks of interest depends on the selected ion monitoring of specific masses. The values obtained (in μg/ml, range in parentheses) were: PGEs, 63.5 ± 49.3 (9—164); PGFs, 2.6 ± 1.92 (0.95—6.63); 19-OH PGEs, 592.6 ± 312.5 (142.1—1047); and 19-OH PGFs, 12.66 ± 5.21 (4—19). Individual values for members of both series I and II are also presented.

The sample collection and extraction procedures were further checked by high-performance liquid chromatography on a μPorasil column, with individual isolation and collection of all of the PGs, including the 19-OH PGs not previously separated by liquid chromatography.

INTRODUCTION

The possibility of a relationship between low prostaglandin (PG) levels in human semen and clinical male infertility has been considered in the literature by different authors [1—7]. However, at the present time it is not known either how or which of the prostaglandins (PGs) may promote human fertility.

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Nevertheless, there are various interesting effects to consider, both in relation to the female as well as the male reproductive tracts [7]. Likewise, it has been suggested that PGs may affect spermatozoal metabolism through stimulation of cAMP [8], a possibility as yet unproven. Thus, any research effort in this direction could be potentially very rewarding, providing that the problem is well defined not only in relation to the infertility but also as to the nature and levels of the likely influencing factors, such as the PGs. It is now known that the recent re-examination of the type and extent of prostaglandins that occur in human semen has led to a significant reduction in the number of primary PGs identified in these samples from a high of thirteen, of which eight have been shown to be artifacts, to just five plus the recently identified 19-hydroxylated PGs (19-OH PGEs) and PGFs (19-OH PGFs) [7].

Briefly, at the present time, only nine types of endogenously synthesized prostaglandins can be considered as positively identified in semen, of which PGE_1 and PGE_2 together with their corresponding 19-hydroxylated analogs [9,10] are quantitatively the most significant.

On the other hand, it has also been indicated that even storage of samples at $-20°$ may not prevent the possible breakdown of PGEs and 19-OH PGEs into PGAs and PGBs [11]. However, this seems to be a variable and rather uncontrolled process which most likely could account for the remarkable discrepancies observed in the type as well as in the reported levels of seminal PGs [2,7,10,12], although there are reports in the literature which still attempt to establish the concentration of PGAs, PGBs, 19-OH PGAs and 19-OH PGBs in fresh human seminal plasma [13]. In contrast, there are very few descriptions of the endogenous levels of the 19-hydroxylated PGEs and PGFs in human semen [6].

For all of these reasons it would be interesting to establish on a firmer basis the number and concentrations of PGs in semen of fertile men. This has been attempted through strict control of the sampling and extraction procedures followed by a rapid simultaneous determination of all PGs and 19-OH PGs present in the extracts by a highly specific and accurate technique, combined gas chromatography—mass spectrometry (GC—MS) with multiple ion detection (MID) using a deuterated internal PGF standard for reliable quantitation. A parallel high-performance liquid chromatographic (HPLC) study of these major PGs in human semen demonstrated that no degradation took place under the experimental conditions described.

EXPERIMENTAL

Reagents, samples of seminal plasma and labelled reference compounds

Ethyl acetate, methanol, piperidine, chloroform, toluene, acetone, hexamethyldisilazane (HMDS), acetic acid and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) (chromatography grades) were used as described below.

The prostaglandin samples (A, B, E and F series I and II and F_2 D_4) were kindly supplied by Dr. J. Pike, Upjohn, Kalamazoo, Mich., U.S.A.

^{3}H-Labelled prostaglandins Fs, Es, As and Bs were purchased from the Radiochemical Centre, Amersham, Great Britain.

The samples of human seminal plasma were from the Instituto Urologico

Puigvert in Barcelona, except for the samples from fertile men, which were provided by volunteers.

Gas—liquid chromatography

The glass columns (4 m or 2 m × 2.5 mm I.D.) were washed with acetone, methanol and water and silanized for 24 h with a 5% solution of HMDS in toluene. After washing with methanol they were dried and immediately packed with 3% OV-17 or 3% Dexsil 300 on Gas-Chrom Q (100—120 mesh). The columns were usually conditioned for at least 48 h at 260—330° and were silanized by repeated injections of BSTFA before use. The separations were carried out on a Perkin-Elmer Model 900 gas chromatograph equipped with dual flame ionization detectors. Samples were injected with Hamilton syringes Models 701 (10 μl capacity) and 7101 (1 μl capacity).

High-performance liquid chromatography

The prostaglandins were separated on a 30 cm × 4 mm I.D. straight stainless-steel column packed with μPorasil. The packing of 10 μm silica particles was obtained from Waters Assoc., Milford, Mass., U.S.A. The liquid chromatograph was equipped with two solvent delivery pumps (Model 6000, Waters), a U6K injector and a solvent programming system (Model 660) also from Waters. The eluates were collected in an LKB 7000 fraction collector and analyzed either by liquid scintillation on an Intertechnique counter (Model SL 32) when they contained tritiated standards, or by gas—liquid chromatography as described above.

The liquid chromatographic separations of PGAs, PGBs, PGEs, PGFs, 19-OH PGE_2 and 19-OH PGF_2 were performed by a sequence of two programs. In the first non-linear program using gradient No. 4, which lasted 50 min, the initial solvent was 100% chloroform and the concentration of the second solvent (10% methanol, 2% acetic acid in chloroform) was increased to 50%. This first program was followed by a period of 10 min holding the final concentration of the first program. After the 10 min a second linear program (Waters gradient No. 6) was started until a final concentration of 100% of the second solvent was reached in 30 min. The flow-rate was maintained at 1 ml/min.

Gas chromatography—mass spectrometry

The mass spectra and the multiple ion detection (MID) profiles were obtained with an Hitachi RMU-6H mass spectrometer coupled via a single-stage jet separator to a Perkin-Elmer gas chromatograph Model 3920. The MS operating conditions were: electron energy 70 eV, accelerating voltage 1800 V, emission current 80 μA, separator at 290° and ion source at 180°. The mass spectrometer was equipped with a four-channel monitoring system developed and built in our laboratory [14]. The GC parameters were adjusted so as to maximize the performance of the mass spectrometer. Thus, the retention times are not always directly comparable in absolute values to those measured on the gas chromatograph.

*Preparation of derivatives**

Amounts of 20—40 μg of synthetic PGEs, PGAs, PGFs, PGEs, 19-OH PGEs and 19-OH PGFs were simultaneously derivatized in a one-step reaction with 40 μl of BSTFA—piperidine (1:1) for 30 min at 60° [15] and directly injected into the gas chromatograph.

The dry residues obtained from the biological extracts were treated in the the same manner.

Extraction of prostaglandins from semen

The samples of human seminal plasma (1.5—2 ml) were deposited on the membrane of a 25 mm, stirred, 17 ml ultrafiltration cell (Millipore Cat. No. XX 4202510), equipped with a 25 mm diameter Pellicon PSAC membrane of approximately 39 cm^2. The nominal molecular weight limit of this molecular filter is 10^3. A filtration pressure of 8.5 kg/cm^2 was applied, to yield a very clear aqueous ultrafiltrate. The residual pellet left on the membrane was washed with 2 ml of methanol. The washings were taken to dryness under a gentle stream of purified helium and the residue was redissolved in the aqueous ultrafiltrate, which was then adjusted to pH 3 with 1 *N* HCl and extracted three times with an equal volume of ethyl acetate. The three extracts were combined and evaporated to dryness under a stream of purified helium. A detailed account of this simplified extraction procedure has been made [16].

RESULTS AND DISCUSSION

In a previous report [16] it was demonstrated that the simplified extraction process developed in this laboratory is remarkably efficient, with recoveries (referred to ^{3}H-labelled $PGF_{1\alpha}$) of the order of 88% in the ultrafiltrate and 99% in the ethyl acetate extract. It was also shown that, in practice, and judging from the reproducibility of the GC and MID profiles obtained from different extracts of semen samples of fertile men, these extractions are also sufficiently reliable from a qualitative standpoint, as illustrated in Fig. 1. A detailed examination of these three illustrative profiles shows that while there are the expected quantitative variations in the abundance of various components of these chromatograms, qualitatively all the samples contain the same major and even minor components, as reflected for instance in the twenty peaks labelled in the GC traces of Fig. 1. What needs to be known with sufficient certainty is which of these peaks are due to endogenous prostaglandins and their corresponding concentration levels.

*All prostaglandin (PG) derivatives described in this work were the trimethylsilyl (TMS) esters (TMSO indicates the trimethylsiloxy group) of the following acids: PGE_1, 9 enol TMS-11,15-bis TMSO prost-13-enoic; PGE_2, 9 enol TMS-11,15-bis TMSO prosta-5,13-dienoic; $PGF_{1\alpha}$, 9,11,15-tris TMSO prost-13-enoic; $PGF_{2\alpha}$, 9,11,15-tris TMSO prosta-5,13-dienoic; PGD_2, 11 enol TMS-9,15-bis TMSO prosta-5,11,13-trienoic; 15 keto PGF_2, 15 keto-9,11-bis TMSO prosta-5,13-dienoic; PGB_1, 15 TMSO, 9-keto prosta-8(12),13-dienoic; PGB_2, 15 TMSO, 9-keto prosta-5,8(12),13-trienoic; 19-OH $PGF_{2\alpha}$, 9,11,15,19-tetrakis TMSO prosta-5,13-dienoic; PGA_1, 9-enol TMSO-11-piperidyl-15 TMSO prosta-10,13-dienoic; PGA_2, 9-enol TMSO-11-piperidyl-15 TMSO prosta-5,10,13-trienoic; 19-OH PGA_2, 9-enol TMSO-11-piperidyl-15,19 bis TMSO prosta-5,10,13-trienoic; 19-OH PGE_2, 9-enol TMSO-11,15,19-tris TMSO-prosta-5,13-dienoic.

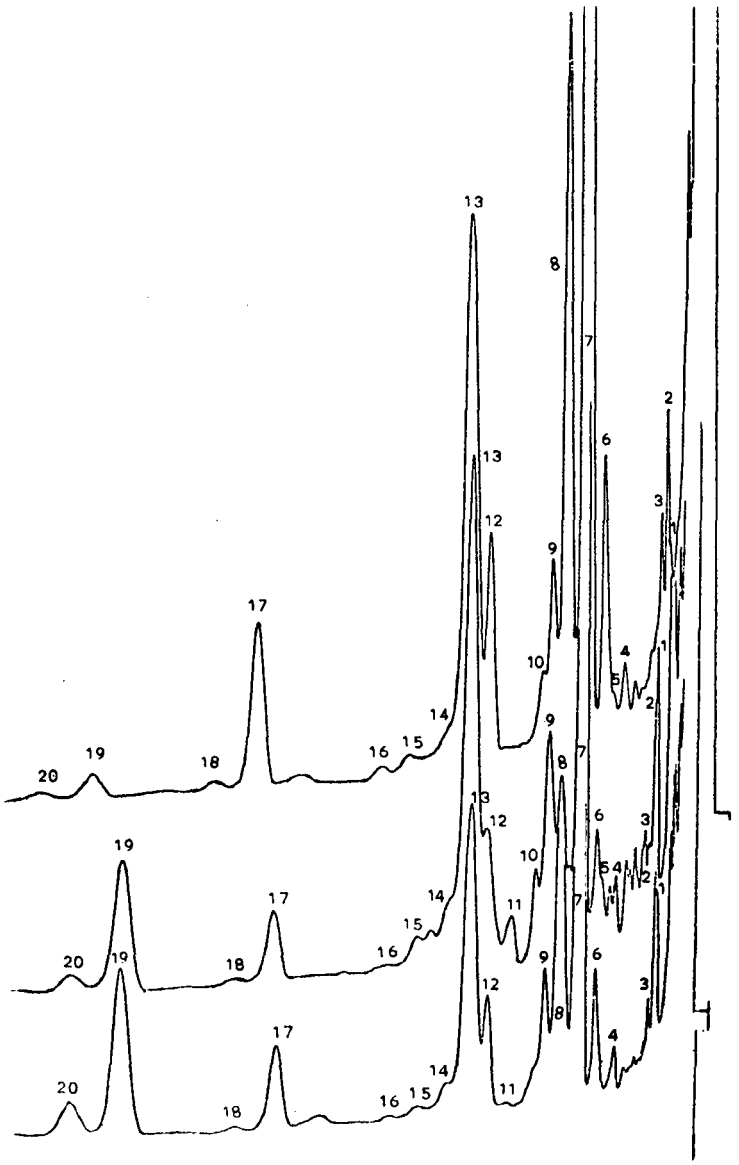

Fig. 1. GC profiles on Dexsil 300 at 280° obtained from the extracts of three different samples of seminal plasma.

Gas chromatographic separations

For GC purposes the dried extracts were derivatized in one step by direct silylation with BSTFA—piperidine (1:1). The structural identification of the persilylated derivatives obtained in this fashion has been described in detail [15]. It is interesting to note that like PGEs [17], the 19-OH PGEs also undergo an identical process of enolization with subsequent silylation of the ring keto function. The process is schematically illustrated in Fig. 2.

The GC retention indices of these silyl derivatives, calculated on two differ-

TABLE I

RETENTION INDICES OF SILYLATED PROSTAGLANDIN DERIVATIVES

The retention indices were calculated at 270° for the Dexsil 300 columns and at 260° for the OV-17 columns. Data given in parentheses correspond to theoretically calculated values. All PGs were derivatized by direct one-step silylation with a BSTFA—piperidine reagent mixture, obtaining the following derivatives (see footnote on p. 298). PGE_1, 9-enol PGE_1 $(TMS)_4$; PGE_2, 9-enol PGE_2 $(TMS)_4$; $PGF_{1\alpha}$, $PGF_{1\alpha}$ $(TMS)_4$; $PGF_{2\alpha}$, $PGF_{2\alpha}$ $(TMS)_4$; PGD_2, 11-enol PGD_2 $(TMS)_4$; 15-keto $PGF_{2\alpha}$, 15-keto $PGF_{2\alpha}$ $(TMS)_4$; PGB_1; PGB_1 $(TMS)_2$; PGB_2, PGB_2 $(TMS)_2$; 19-OH PGE_2, 19-OH PGE_2 $(TMS)_5$; PGA_1, 11-piperidyl PGA_1 $(TMS)_3$; PGA_2, 11-piperidyl PGA_2 $(TMS)_3$; 19-OH PGA_2, 11-piperidyl 19-OH PGA_2 $(TMS)_4$; 19-OH PGE_2, 9-enol 19-OH PGE_2 $(TMS)_5$.

	Series	PGEs	PGFs	PGDs	15-Keto PGFs	19-OH PGEs	PGBs	19-OH PGFs	PGAs	19-OH PGAs
Dexsil 300	I	2659	2730	—	—	(2845)	2880	(2929)	3026	(3241)
	II	2659	2693	2734	2827	2845	2872	2892	3038	3229
OV-17	I	2774	2807	—	—	(3002)	3006	(2982)	3185	(3371)
	II	2774	2780	2844	2908	3002	3006	2955	3185	3371

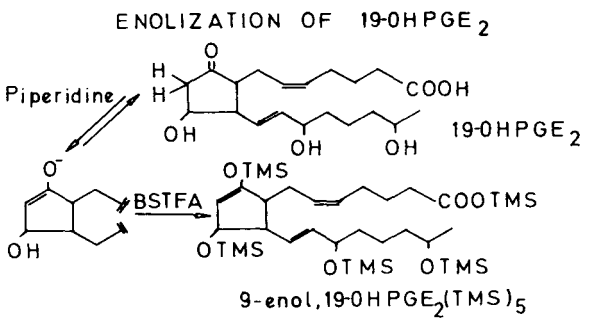

Fig. 2. Scheme of the enolization—silylation process for 19-OH PGE_2.

ent GC columns, are given in Table I. The conclusions that can be drawn from these data are that the Dexsil 300 GC column would elute all of these derivatives in less time than the OV-17 and, more importantly, with a better separation of PGEs and PGFs, which physiologically are the most significant prostaglandins. In other words, the separation of the two PGEs from $PGF_{2\alpha}$ is virtually impossible to achieve on these packed columns due to the small ΔI value of only six units (2780 vs. 2774), while the same ΔI is equivalent to 34 units on Dexsil (Table I). Likewise, the separation between both PGFs is much better on Dexsil (ΔI = 63 vs. 27). Furthermore, as Dexsil 300 is a high-temperature phase with a working range of 50—400°, it is specially suited for this application since the usual working temperature with persilylated PG derivatives is around 270°.

The practical benefits derived from the use of glass columns packed with Dexsil 300 is illustrated in Fig. 3. This profile shows the separation achieved at 270° on a 4 m × 1/4 in. I.D. column. In contrast to what happens on the OV-17 column, the peak of the $PGF_{2\alpha}$ $(TMS)_4$ derivative appears clearly resolved from the peak of the PGEs. Also the separation of both PGFs ($PGF_{1\alpha}$ and $PGF_{2\alpha}$) is better than on OV-17 [15].

Nevertheless, even with this improved GC system there is no possibility of resolving the tetrakis-TMS derivatives of PGE_1 and PGE_2, which would preclude in principle the simultaneous GC determination of their individual physiological levels in human semen samples. On the other hand, it is to be expected that the combined GC—MS technique in its selected ion detection mode of operation would allow, by proper selection of specific *m/e* ratios, the quantitation of these two prostaglandins as well as other PGs present in the samples analyzed.

Multiple ion detection

Table II gives the partial mass spectra of the TMS derivatives of the main PGs found in human semen. The table only includes the relative abundances of the molecular ions, as well as those of the structurally most significant fragment ions, whose origin has already been discussed in some detail in various publications [17—21]. In this respect, it is to be noted that the mass spectrum of the 19-OH PGE_2 $(TMS)_5$ derivative does not show, as predicted, fragment ions at M − 71 (loss of the C_{16}—C_{20} chain) of M − 161 (loss of the C_{16}—C_{20}

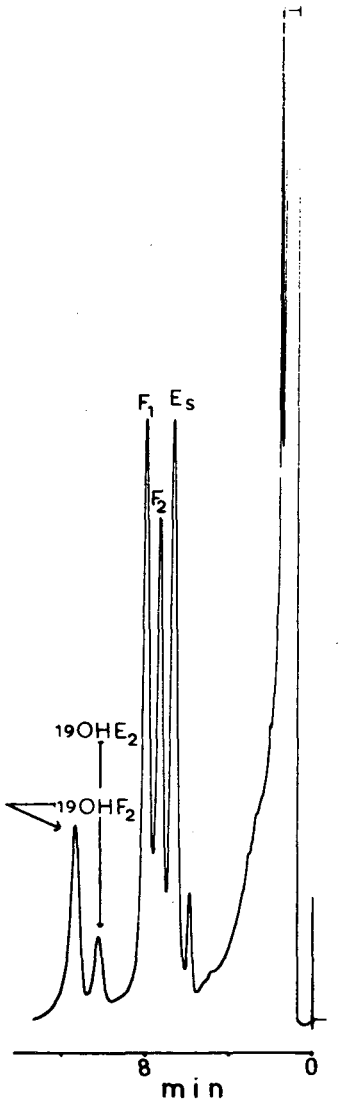

Fig. 3. GC separation of persilylated PG derivatives on a glass column packed with 3% Dexsil 300 at 280°. See Table I for retention indices.

chain plus the extra 19-HOSi$(CH_3)_3$ group) since, in this case, the terminal C_{16}—C_{20} chain is 89 mass units heavier due to the presence of the OTMS group on the C_{19} position. Also, the M — 131 ion is absent, as in the mass spectra of both PGE derivatives. The M — 249 ion, at *m/e* 479, would be the equivalent of the M — 161 fragment in the mass spectra of other PGs, considering that the loss of the C_{16}—C_{20} fragment entails here a loss of 249 a.m.u., corresponding to the C_{16}—C_{19} OTMS-C_{20} moiety. The mass spectra of the pentasilylated derivatives of 19-OH $PGF_{1\alpha}$ and 19-OH PGE_1 are not included as there were no reference standards available. In spite of this, a detailed comparative analysis of the MS patterns of the other related PGs indicates that the

TABLE II

PARTIAL MASS SPECTRA OF THE SILYLATED DERIVATIVES OF PGEs, PGFs, 19-OH PGEs AND 19-OH PGFs

See Table I for nomenclature of derivatives.

	PGF_2 and PGE_1			PGE_2		PGE_1		19-OH PGE_2		19-OH PGF_2	
	m/e	r.a.*	r.a.	*m/e*	r.a.	*m/e*	r.a.	*m/e*	r.a.	*m/e*	r.a.
M**	642	5	5	640	26	644	0.2	728	16	730	—
M − 15	727	11	31	625	36	629	6	713	21	715	—
M − 71	571	11	5	569	5	573	26	—	—	—	—
M − 90	552	32	100	550	86	554	39	638	100	640	64
M − 105	537	17	17	535	11	539	18	623	26	625	21
M − 131	511	10	—	—	—	513	18	—	—	—	—
M − 161	481	48	38	479	63	483	55	—	—	—	—
M − 180	462	33	59	460	100	464	21	548	73	550	55
M − 249	—	—	—	—	—	—	—	479	31	481	100

*r.a. = relative abundance.

**M = molecular ion; M − 15, loss of CH_3; M − 71, loss of C_{16}—C_{20}; M − 90, loss of $HOSi(CH_3)_3$; M − 105, loss of $HOSi(CH_3)_3$ and CH_3; M − 131, loss of $CH_2COOSi(CH_3)_3$; M − 161, loss of C_{16}—C_{20} and $HOSi(CH_3)_3$; M − 180, loss of two $HOSi(CH_3)_3$ groups (18—20); M − 249, in 19-OH PGs is equivalent to the M − 161 fragment of other PGs and corresponds to the loss of C_{16}—C_{19} OTMS-C_{20} fragment.

mass spectrum of the silylated derivative of 19-OH PGE_1 should contain a significant fragment at *m/e* 481, which would correspond to the mass of the M − 249 ion, the molecular weight in this case being 730 mass units. Accordingly, the mass spectrum of the 19-OH $PGF_{1\alpha}$ $(TMS)_5$ derivatives would show a major M − 249 ion at *m/e* 483 since the mass spectral patterns of its analog structure, the 19-OH $PGF_{2\alpha}$ $(TMS)_5$ derivative, is characterized by the appearance of a relatively abundant ion at *m/e* 481.

The data in Tables I and II provide the necessary information for selecting the most suitable, specific and characteristic ions to be monitored by the MID unit [14]. There is, however, a practical limitation to consider in the sense that this unit has only four channels available whereas the simultaneous determination of all PGEs and PGFs present in the samples would necessitate using a minimum of eight channels plus one for the monitoring of the deuterated internal standard. Nevertheless, this practical limitation could be obviated by a restrictive selection of as many common ions as possible for those derivatives that have been well separated by GC and of specific but different mass ions for those PGs not resolved by GC.

In this way, according to the scheme in Table III, the limitation imposed by the co-elution of PGE_1 and PGE_2, in terms of the simultaneous quantitation of these two prostaglandins, can be readily solved by monitoring the ions at *m/e* 481 for the quantitation of PGE_1 and at *m/e* 479 for the quantitation of PGE_2, as indicated in Table III. The same applies to the co-eluting 19-OH PGE derivatives, both monitored at the same masses but widely separated in retention times from the PGEs. The eight prostaglandins can be simultaneously detected and quantitated if the ions at *m/e* 483 and 485 are also monitored in the two other channels remaining. In the first case the silylated derivatives of $PGF_{1\alpha}$

TABLE III

IDENTIFICATION SCHEME OF SELECTED IONS VERSUS GC RETENTION TIMES

I values on Dexsil 300 at 270° (see Table I). In parentheses correction factor in percent due to the contribution of the isotopic ion cluster of the other co-eluting prostaglandin.

Recording channel	*m/e* selected						
I	485		F_2D_4				
II	483			$F_{1\alpha}$			19-OH $F_{1\alpha}$
III	481	E_1 (22.5)	$F_{2\alpha}$ (27)		19-OH E_1 (39.4)	19-OH $F_{2\alpha}$	
IV	479	E_2			19-OH E_2		
Retention index *I*		2659	2693	2730	2845	2892	
		PGEs	PGF_2	$PGF_{1\alpha}$	19-OH PGEs	19-OH PGF_2	19-OH $PGF_{1\alpha}$

and its 19-OH analog are detected, while in the second case the ion at *m/e* 485 is used for detecting the deuterated internal standard ($PGF_{2\alpha}$ D_4). $PGF_{2\alpha}$ and 19-OH $PGF_{2\alpha}$ are detected in the channel monitoring the ions of *m/e* 481.

The selected ion profiles obtained at 280° on the Dexsil 300 GC column with a derivatized mixture of standard prostaglandins of the E and F series plus the $PGF_{2\alpha}$ D_4 internal standard are illustrated in Fig. 4, showing that all of these PGs could be readily quantitated. However, taking into account the possible cross-channel contributions of the isotopic species of each of these selected ions, the only case that needs to be accounted for would be that due to the contribution of the isotopic cluster of the ion of *m/e* 479, selected for detection of PGE_2, to the profile of the ions at *m/e* 481, selected for detection of PGE_1, since both of these prostaglandins elute with the same retention time. This interchannel contribution, as well as that corresponding to the 19-OH counterparts, will be defined below.

The corresponding GC—flame ionization detection profile has been shown in Fig. 3. This sample also contained the TMS derivatives of the 19-OH PGE_2 and $PGF_{2\alpha}$, which appear well separated. This becomes a very important factor in the quantitative determination of these PGs in human semen.

The use of only one deuterated internal standard as reported by Perry and Desiderio [5] for the quantification of eight different PGs requires a preliminary study of the response curves corresponding to each one of the PGs relative to that of the internal standard. For this purpose, we carried out an appraisal of the response of the pentasilylated derivative of $PGF_{2\alpha}$ D_4 used as the internal standard, versus its corresponding protium form. Once the deuterated standard had been properly calibrated, we proceeded to check the relative responses of the PG derivatives relative to the internal standard by preparing three solutions of different concentrations for each PG and adding to them the same amount of deuterated internal reference. As an example, the graphical representation of the response ratio of $PGF_{2\alpha}/PGF_{2\alpha}$ D_4 vs. the corresponding concentration ratio gave a straight line of slope = 1.

Due to the unavailability of standard 19-OH $PGF_{1\alpha}$ and PGE_1, it was not possible to obtain the corresponding response curves relative to the deuterated

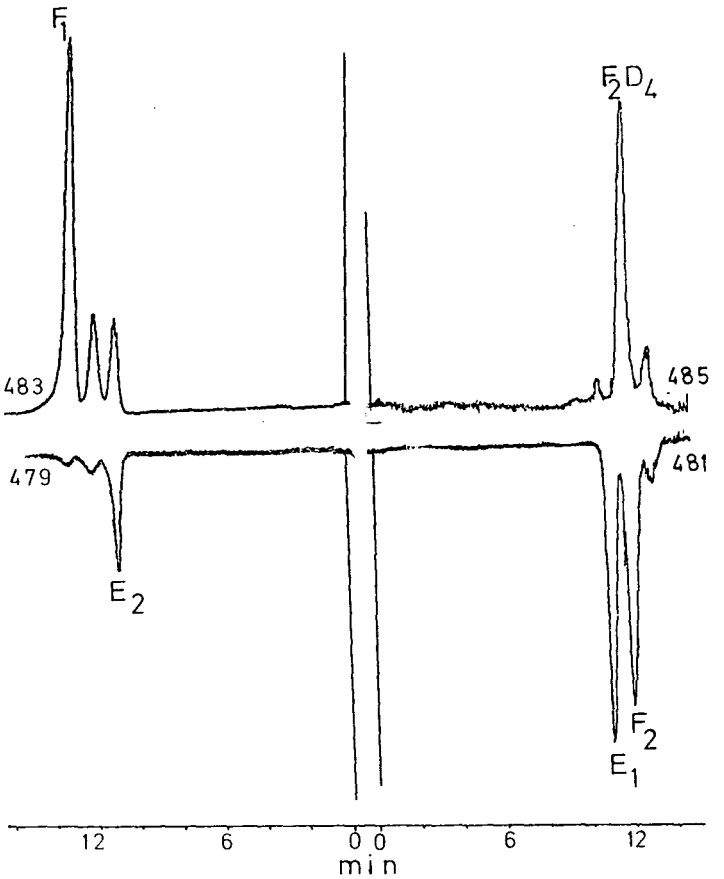

Fig. 4. Selected ion profiles of an authentic prostaglandin mixture derivatized according to the described procedure (see Tables I and II). As indicated in the text the two significant peaks eluting ahead of the PGF_1 derivative (*m/e* 483) are due to the response of the isotopic ion cluster from the *m/e* 481 ions of the PGE_1 and $PGF_{2\alpha}$ derivatives.

internal standard. However, it has been assumed that their chromatographic behaviour would be practically identical to that of their $PGF_{2\alpha}$ and PGE_2 analogs, in much the same manner as observed with the other series of PGs.

It has been already mentioned that the quantitative measurements taken on selected ion profiles, such as those shown in Fig. 4, may be influenced by the contributions of the isotopic clusters of neighbouring mass ions, themselves focused on other channels. For instance, as illustrated in Fig. 4, when the samples thus analyzed contain detectable amounts of the silylated derivative of PGE_1, monitored at *m/e* 481, there is also a response at the channel monitoring ions of *m/e* 483. In other words, the ions of *m/e* 483 are also natural components of the isotopic ion cluster of the fragment that appears at *m/e* 481 in the mass spectra of these PGE_1 derivatives. However, these responses will only have to be taken into account whenever the overlapping of GC retention times could cause a contribution of this nature to some of the ions monitored by the MID unit. Such would be the case, for instance, in relation to

PGE_2, whose response at *m/e* 479 would contribute to increase the actual height of the PGE_1 ion measured at *m/e* 481, to an extent that can be readily calculated as 22.5%. The same reasoning would apply to the TMS derivatives of the 19-OH PGEs where, as indicated in Table III, the contribution amounts to 39.4%. Also, since the deuterated internal standard contains a certain quantity of the corresponding protium form, its contribution to the response of the PGF_2 derivative would also have to be taken into account (see Table III).

HPLC separations

As the possibility of prostaglandin degradation is of great importance in this kind of study, a check was made to determine the level of degradation, if any, experimentally induced by the conditions under which we extract, derivatize and analyze these samples. For this purpose the ultrafiltrates were directly injected into a LC column and the various PGs eluted under the conditions described above. As indicated in Table IV, the combined non-linear/linear program herein described provides a good separation of all classes of authentic PGs, with the exception of PGAs and PGBs which elute together in cut No. 2. On the other hand, although the HPLC of free prostaglandins on silicic acid columns has been reported before [22,23], the elution parameters used in both cases (60 min linear gradient from chloroform to a chloroform—methanol—acetic acid mixture) are not adequate for the separation of the 19-OH PGEs and PGFs in a reasonable time. In contrast, and according to the cuts indicated in Table IV, the 19-OH PGE_2 can be readily isolated in cut No. 8 (52—57 ml) while the 19-OH PGF_2 can be recovered from cut No. 10 (81—87 ml).

The biological samples under study were subjected to the same LC separation system, and the identity of each of the compounds thus collected was confirmed by GLC analysis of the corresponding persilylated derivatives. For example, the gas chromatograms obtained from the cuts containing the 19-OH PGs from a sample of human semen are shown in Fig. 5. Quantitation was achieved by selected ion monitoring as described above. In this way it could be demonstrated that the cut that should contain the PGAs and

TABLE IV

HPLC SEPARATION OF FREE PROSTAGLANDINS

See text for experimental parameters.

Cut No.	Eluate fraction (ml)	PGs
1	5—9	—
2	10—16	As and Bs
3	19—22	—
4	23—28	Es
5	29—34	—
6	35—41	Fs
7	42—51	—
8	52—57	19-OH E_2
9	58—80	—
10	81—87	19-OH Fs

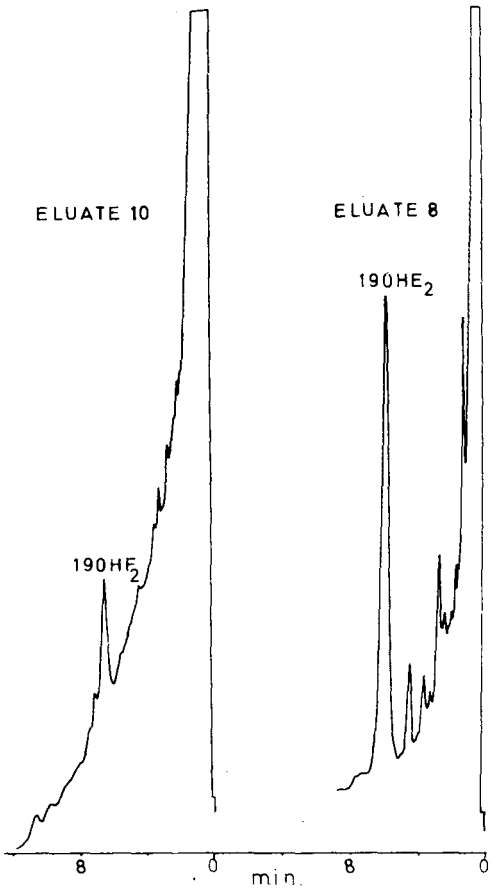

Fig. 5. Gas chromatograms of the persilylated HPLC eluate fractions (corresponding to the elution volume of 19-OH PGs) obtained from an extract of human semen. Same GLC conditions as in Fig. 3.

PGBs did not give any appreciable response with the ultrafiltered semen samples. The only cases where a relatively small GLC response was obtained for these two secondary PGs was in a few of the cuts containing the PGEs, thus demonstrating that they had been formed from PGEs upon derivatization and GLC analysis of these fractions. However, this can be prevented by careful handling of the collected eluates. A more thorough account of the HPLC of these PGs is given elsewhere [24].

Analysis of semen samples

Once the response parameters of these derivatives and their possible isotopic contributions to the MID traces had been established, the method was applied to a study of the physiological levels of the major prostaglandins in samples of human semen obtained from fertile donors. This study was undertaken under extremely controlled conditions, with regard to both sample collection and extraction procedures. The aim was to minimize any possibility of prostaglandin degradation leading to structural changes during the freezing, storage

with preservatives and defreezing procedures [7], and thus all samples were extracted without previously subjecting them to any freezing or storage. In fact, the time from ejaculation to the beginning of the extraction was never more than 30 min.

Single semen samples were obtained from seven volunteers, all of whom had been duly informed on the nature of the study and whose wives had recently given birth, except in one case whose wife was pregnant. All men were aged between 25 and 30 years. Six of them collected the sample by ejaculation into clean glass containers and one collected it by intercourse using a condom. Also, according to the responses given in a previously supplied questionnaire, this man was taking four aspirin tablets daily.

The extraction of the seven samples was carried out exactly under identical conditions and in the following manner: each sample of semen was centrifuged for 5 min at 17,500 *g*. The seminal plasma thus obtained was agitated and an aliquot of 1.5 ml was taken for ultrafiltration together with 50 μl of the solution containing the deuterated internal standard as described in the Experimental section. The final residue was dissolved in 40 μl of the BSTFA—piperidine mixture and allowed to react for 30 min at 60°. A total of five injections of each derivatized extract was made into the GC—MS combination, thus effectively obtaining five MID determinations for each sample. After subtracting the isotopic contributions to the response of the ions indicated in Table III, the mean of the five determinations was calculated.

Table V gives the prostaglandin concentrations found in the samples of semen from healthy fertile men. The mean concentrations and ranges shown are basically in good agreement with the data recently published by Templeton et al. [6], although these authors do not report the individual values of the two components (series I and II) of each family of prostaglandins. It must be noted, however, that the values corresponding to the sample obtained from the man taking daily doses of aspirin were in all cases remarkably low in relation to the other samples, an observation which can probably be explained considering the relationship between aspirin and the prostaglandins. In the last few years this relationship has been widely documented to the point where there seems to be a clear inhibitory effect of aspirin on the cyclooxigenase

TABLE V

PROSTAGLANDIN LEVELS (μg/ml) IN SEMEN OF HEALTHY FERTILE MEN

	$\bar{X}$ ± S.D. (n=7)	Range		$\bar{X}$ ± S.D. (n=7)	Range
PGE_2	33.26±28.68	5.02— 94.47	19-OH PGE_2	294.17±151.88	77.28— 498.60
PGE_1	29.63±20.55	3.99— 65.56	19-OH PGE_1	280.53±155.93	60.93— 515.01
PGEs	63.46±49.28	9.01—164.03	19-OH PGEs	574.70±302.71	138.21—1014.11
$PGF_{2\alpha}$	1.36± 0.83	0.75— 2.93	19-OH $PGF_{2\alpha}$	8.64± 3.87	2.5 — 12.46
$PGF_{1\alpha}$	1.22± 1.16	0.20— 3.7	19-OH $PGF_{1\alpha}$	5.48± 2.62	3.24— 9.97
PGFs	2.58± 1.92	0.95— 6.63	19-OH PGFs	14.12± 4.90	5.9 — 20.44

enzyme system catalyzing the formation of endoperoxides from arachidonic acid, these endoperoxides being the known precursors of all prostaglandins [25—27].

Under these strict conditions of sample collection and rapid analysis (samples extracted within 30 min of ejaculation), we did not detect any PGAs or PGBs, although in a recent publication from this laboratory [28] mass spectrometric proof was given on the identification in samples of human semen of PGA_1, PGA_2, 19-OH PGA_1 and 19-OH PGA_2. However, it was also acknowledged that no significant levels of PGAs had been detected in a few samples that had been frozen at —40° within 1 h of ejaculation [28]. In that report we did not take a stand on the "fact or artifact" issue regarding the presence of PGAs in semen [29,30], the aim at that time being just to demonstrate the advantages of the new piperidyl-TMS PGA derivatives for the detection of PGAs regardless of their origin. Nevertheless, our latest results obtained with recently ejaculated semen, as reported herein, would support the artifact theory [30]. Furthermore, in order to avoid even the minimum possible interconversion that may take place upon derivatization and/or on the GC injector or GC columns, some of the samples herein reported were previously qualitatively identified by HPLC and no PGAs or PGBs were found. The apparent contrast with a recent publication [13] reporting concentrations of PGAs, 19-OH PGAs and PGBs as well as PGEs and 19-OH PGEs in fresh semen, could be explained again by storage in cold ethanol at —20°, it having been reported that even at —20° there is the possibility of PGEs breaking down to PGAs [11].

Also, regarding the suggested relationship between PG levels in semen and male infertility, we tend to agree with Templeton et al. [6] as our results also show that the concentration ranges (Table V) are much too wide to allow for meaningful comparisons between fertile and infertile men. The values reported, for instance, for PGEs and 19-OH PGEs in men classified as azoospermic or oligospermic (see Table VI) would still fit very well within the normal range reported by Templeton et al. [6] and in this work.

Finally, it could be added that the data given in Table V can be considered very reliable because the process of extraction was carried out under exactly reproducible conditions in all seven cases, avoiding any freezing and/or storage of the samples and always within 30 min from ejaculation to sample extrac-

TABLE VI

PROSTAGLANDIN CONCENTRATIONS IN SEMEN FROM HEALTHY FERTILE MEN

Prostaglandin	Mean concentration (μg/ml) ± S.D. and range				
	Perry and Desiderio [5]			Templeton et al. [6]	This work
	Fertile	Azoospermic	Oligospermic	Fertile	Fertile
E_1—E_2	149± 91.7 (NR)*	113±114 (NR)	59.9±35.0 (NR)	73.2± 71.6 (2—272)	63.46± 49.28 (9—164)
19-OH E_1—19-OH E_2	526±367 (NR)	266±167 (NR)	163 ±63.6 (NR)	267 ±240 (53—1094)	574.7 ±302.7 (138—1014)

*NR = not reported.

tion. To our knowledge this is the first attempt at the direct extraction of semen immediately after its collection. Also to be noted is the possibility of determining simultaneously in a single extract all of the physiologically significant prostaglandins and 19-OH prostaglandins.

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Biomedical Applications

CHROMBIO. 500

HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF ULTRAVIOLET-ABSORBING CONSTITUENTS OF HUMAN URINE

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SUMMARY

A 100-μl urine sample was chromatographed on a column packed with a strongly basic macroreticular anion-exchange resin (Diaion CDR-10, 5—7 μm diameter with a nominal 35% cross linkage). The elution was performed with a linear acetate gradient from 0 to 6.0 *M* at an average flow-rate of 0.72 ml/min and at an average pressure of 104 kg/cm^2. The relative standard deviation of retention times and peak height was ± 4% or less. The properties of the macroreticular anion-exchange resin, the effect of the particle size, the pH of acetate buffers, and the effect of the flow-rate of the eluent on the separation were investigated. Thirty three components of urine were then resolved and named.

INTRODUCTION

Body fluids, such as urine and blood, contain hundreds of components, which have a positive correlation to pathological states [1, 2]. Scott and co-workers [3—8] developed an analytical system which separates the UV-absorbing components of human urine in less than 40 h. The microreticular

anion-exchange resin system, however, required a relatively long separation time [9—12], resulting in a low sample throughput. Thus several operational options were investigated in an effort to decrease the analysis time [9, 10, 13]. Therefore, we have studied a new anion-exchange chromatographic system using a macroreticular anion-exchange resin, which separates the UV-absorbing components of human urine in less than 120 min and with high resolution [14].

EXPERIMENTAL

Chemicals

Analytical grade ammonium acetate and acetic acid were purchased from Wako (Tokyo, Japan). The reference compounds creatine, creatinine, pyridoxine, uracil, histidine, theobromine, nicotinamide, hypoxanthine, adenosine, phenylalanine, xanthine, caffeine, tyrosine, tryptophan, theophylline, urocanic acid, uric acid, nicotinic acid, 4-aminohippuric acid, 4-hydrocy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenylacetic acid, *p*-aminobenzoic acid, hippuric acid, quinaldic acid, *p*-hydroxyphenylacetic acid, vanillic acid, kynurenic acid, *p*-hydroxyphenylpyruvic acid, benzoic acid, *p*-hydroxybenzoic acid, 3-hydroxyanthranilic acid, indoleacetic acid, and indoleacrylic acid, were also purchased from Wako.

Resin

The strongly basic macroreticular anion-exchange resins, Diaion CDR-10, with a particle size distribution of (A) 6 ± 1; (B) 11 ± 2 and (C) 25 ± 4 μm, were obtained from Mitsubishi (Tokyo, Japan).

Apparatus

A Hitachi Model 634 high-performance liquid chromatograph (Hitachi, Tokyo, Japan) was used for the urine analysis, and was coupled to a variable wavelength photometer and a 10-mV data recorder.

Column

A stainless-steel column (50 × 0.4 cm I.D.) was dynamically packed with Diaion CDR-10 at 150 kg/cm^2 using a high-pressure pump as described by Scott and Lee [15]. A slurry of the resin and water (50:50) was used for the rigid packing and then 6.0 *M* acetate buffer (pH 4.4) was run through the column for 1 h.

Sample preparation

Urine samples, usually 24-h collections, were refrigerated until complete, then frozen and stored at —20°. Before analysis, the sample was defrosted and passed through a 0.22-μm Millipore filter to remove particular matter.

High-performance anion-exchange chromatography

A 100-μl urine sample was introduced onto the column and eluted using a linear acetate gradient from 0 to 6.0 *M* at a flow-rate of 0.72 ml/min. The linear acetate gradient was formed by placing 30 ml of water in the first

chamber, and 30 ml of 6.0 *M* ammonium acetate buffer, pH 4.4, in the second chamber. During the first 25 min, the column temperature was increased from 22 to 60°, and then maintained at 60° until the end of the run. With the elevation of the temperature, the column inlet pressure dropped from 104 to 70 kg/cm^2, and with the increase of buffer concentration, the inlet pressure was raised from 70 to 135 kg/cm^2 and was maintained at 135 kg/cm^2 until the end of the run. After analysis, the column was washed with distilled water for 30 min at 150 kg/cm^2. The column was ready for the next sample when the pH of the eluate was tested to be neutral.

Assignment of the peaks

Peak assignments were carried out in three ways: firstly, by comparing the retention times of the peaks to those of known compounds; secondly, by injecting known compounds together with the samples; and thirdly, by measuring the UV spectrum with stopped-flow scanning spectrophotometry.

RESULTS AND DISCUSSION

Effects of particle size of macroreticular anion-exchange resins on the separation

A typical chromatogram of urine from a male subject is shown in Fig. 1. The column used was packed with Diaion CDR-10 having a particle size distribution of 5—7 μm (A). Other chromatograms of the same urine sample obtained when the columns were packed with resin of different particle size (B, 11 ± 2 and C, 25 ± 4 μm) were compared with Fig. 1. When these chromatograms were compared it was evident that the smaller the particle size of the resin the

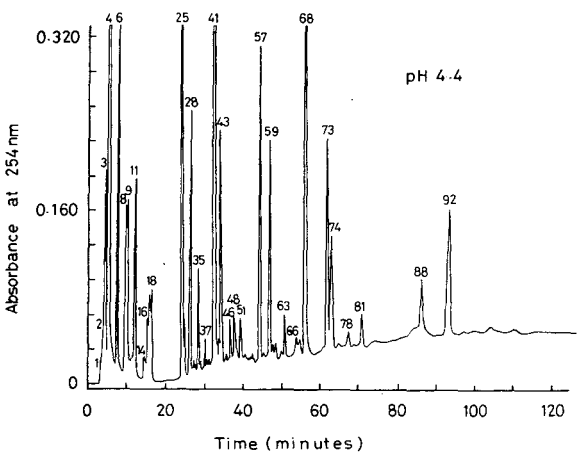

Fig. 1. A 120-min chromatogram of human urine from a normal male subject. Conditions: column, stainless steel (50 cm × 0.4 cm I.D.) packed with Diaion CDR-10; urine sample, 100 μl of a 24-h collection from a 60-kg man; temperature, increasing from 22 to 60° for the first 25 min, maintained at 60° until the end of the run; eluent, acetate buffer, pH 4.4, varying in concentration from 0 to 6.0 *M* by linear gradient, average flow-rate, 0.72 ml/min; average pressure, 104 kg/cm^2.

better the resolution. Thus, higher speed and resolution of the UV-absorbing components of human urine are dependent upon the use of resin particles of smaller size and narrower distribution.

The separation system used by Scott and Lee [9] was performed by using a longer column and high pressures; as the microreticular anion-exchange resin (12—15 μm diameter, Aminex A-27) gave severe flow-resistance and thus a limited flow-rate, even when high pressures were applied, this accordingly gave relatively longer run times. Then several operational devices, such as the use of coupled columns of anion-exchange resin followed by cation-exchange resin [9] or specific gradient programs [10], were attempted in an effort to increase the rate of sampling, but with very limited success.

Effects of the pH of acetate buffers on the separation

Chromatograms of the urine sample at different pH values of the buffer are shown in Fig. 2. Each separation was performed similar to that described above, using 6.0 *M* ammonium acetate buffer at pH 3.9, 4.4, and 4.9. At pH

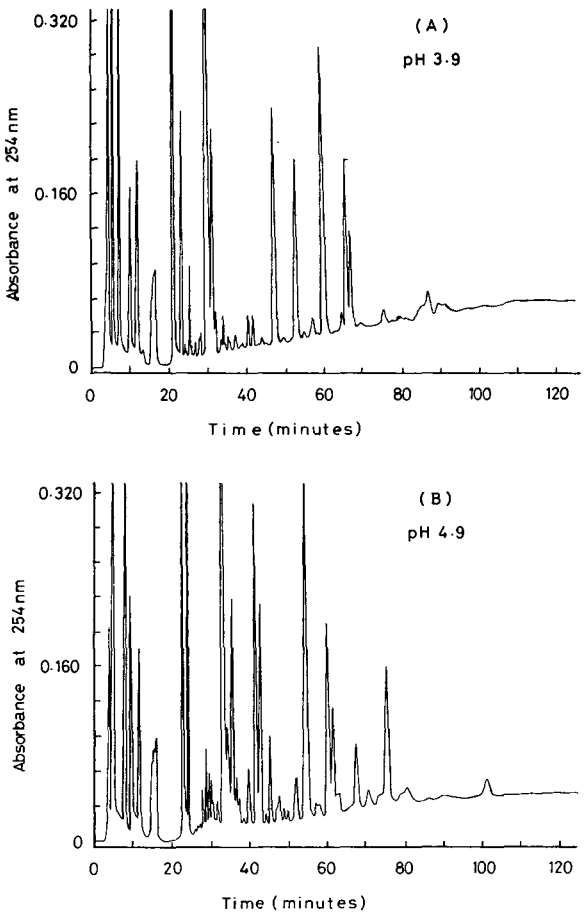

Fig. 2. Effect of the pH of the acetate buffer on the separation. Conditions are the same as in Fig. 1, except: (A) 6.0 *M* acetate buffer, pH 3.9; (B) 6.0 *M* acetate buffer, pH 4.9.

3.9 the chromatographic run required a longer elution time than at pH 4.4, and the resolution and elution after 70 min were very poor. On the other hand, the run time at pH 4.9 was shorter than at pH 4.4, but the resolution around 40 min elution was not satisfactory. Thus, the separation at pH 4.4 was used for the standard elution conditions, but the detection and estimation of a specific substance could be performed at pH 3.9 or pH 4.9.

Effects of flow-rate of eluent on the separation

Decreasing flow-rate of the eluent contributed to higher resolution, but resulted in a longer analysis time. In a run time of 120 min the number of detectable peaks was about 100, and in 200 min it was about 110, and when further increased to 12 h, 130 peaks. However, for the standard analytical system, we still adopted the 120-min system, since 100 peaks in 120 min is time-saving and more useful for practical purposes when compared with 110 peaks in 200 min or 130 peaks in 720 min.

Reproducibility of the separation of the standard separation system

To demonstrate the reproducibility of the standard separation system, quadruple chromatographic analyses were performed on the same urine sample. The elution times of eight of the major peaks were determined and were found to have a relative standard deviation of 0.81—1.08% (Table I). Thus, elution time is useful for the qualitative analysis of a substance. The peak heights of eight of the major peaks were determined and found to have a relative standard deviation of 1.04—3.55%. This peak height could be useful for quantitative analysis.

Assignment of the urine components in the chromatogram

The peaks that have been assigned so far are listed in Table II. Peak assignments were carried out by the use of reference compounds as described in Experimental. A chromatogram of uric acid and hippuric acid and their UV spectra are shown in Fig. 3. The compounds in Table II are listed in order of their elution from the separation system. These results indicate the good correlation between elution position and chemical structure. This correlation is

TABLE I

REPRODUCIBILITY OF THE SEPARATION OF HUMAN URINE

Means ± relative standard deviations of 4 determinations.

No.	Peak number in Fig. 1	Elution time (min)	Relative standard deviation (%)	Peak height (mm)	Relative standard deviation (%)
1	35	28.1	0.99	72.2	3.13
2	43	33.6	0.81	164.0	2.18
3	57	44.0	0.97	229.6	3.55
4	59	46.2	0.88	157.4	1.04
5	63	50.5	1.11	31.7	2.23
6	73	60.8	1.03	153.1	3.19
7	74	62.1	1.06	80.9	1.97
8	92	92.0	1.08	91.8	1.64

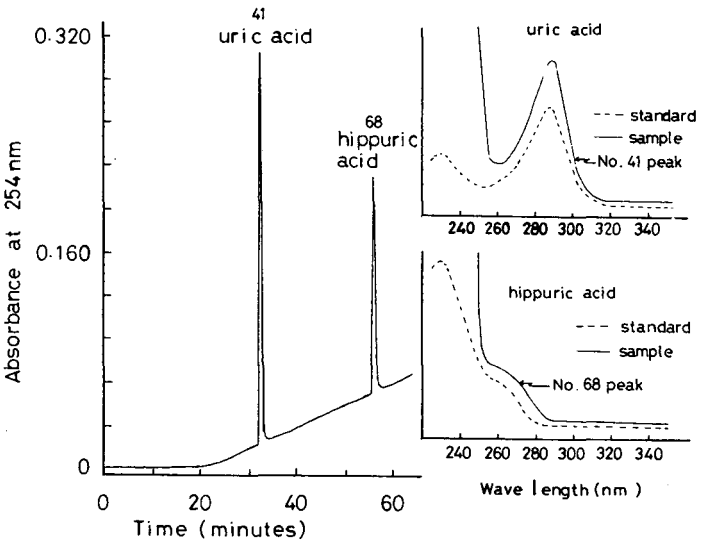

Fig. 3. Chromatogram of reference compounds and UV spectra of the peaks. Conditions are the same as in Fig. 1.

TABLE II

ASSIGNMENT OF THE MAIN PEAKS IN HUMAN URINE

Compound	Peak No.	Compound	Peak No.
Trigonelline	1*	Nicotinic acid	54
Creatine	3	4-Aminohippuric acid	55
Creatinine	3	2-Furoylglycine	57*
Pseudouridine	6*	5-Hydroxymethyl-2-furoic acid	59*
Pyridoxine	9	4-Hydroxy-3-methoxymandelic acid	65
Uracil	11	4-Hydroxy-3-methoxyphenylacetic acid	66
N'-Methyl-2-pyridone-5-carboxamide	17*	*p*-Aminobenzoic acid	67
Histidine	17	Hippuric acid	68
Theobromine	17	4-Hydroxy-3-methoxybenzoylglycine	70*
Nicotinamide	17	4-Hydroxybenzoylglycine	73*
7-Methylxanthine	17*	Quinaldic acid	73
Hypoxanthine	18	3-Hydroxybenzoylglycine	74*
Adenosine	19	*p*-Hydroxyphenylacetic acid	75
Phenylalanine	24	2,5-Furandicarboxylic acid	80*
Xanthine	25	Vanillic acid	76
3-Methylxanthine	26*	Kynurenic acid	85
Caffeine	29	*p*-Hydroxyphenylpyruvic acid	86
Tyrosine	29	Benzoic acid	88
Tryptophan	30	*p*-Hydroxybenzoic acid	88
Theophylline	30	3-Hydroxyanthranilic acid	89
1-Methylxanthine	32*	Indoleacetic acid	92
Urocanic acid	35	Indoleacrylic acid	101
Uric acid	41		
2-Amino-3-hydroxybenzoylglycine	43*		
Dimethyluric acid	48*		
5-Hydroxymethyl-2-furoylglycine	51*		

*Compounds assumed by comparison of the result established by Scott and Lee [9].

remarkably useful for the detection of the metabolites of the components of body fluids, such as purine, uronic acid, phenylalanine, tryptophan, benzoic acid, and their metabolites. The assignment of these normal urine components is important for application to the investigation or understanding of biological and pathological disorders.

Advantageous properties of the macroreticular anion-exchange resin

It is known that the macroreticular resins have a relatively higher cross-linkage, larger surface area, and larger porosity than the microreticular resins [16]. These macroreticular resins gave better adsorptivity, flow-resistance, mechanical stability, and chemical stability for ion-exchange chromatography.

With regard to the stability of Diaion CDR-10, it proved to be possible to analyze more than 200 urine samples over a 4-month period without any loss of resolution. Even when some decrease of resolution was noticed after applying a number of various types of samples, the column could be successfully recovered by washing with the dilute alkaline solution. Also, we have found that Diaion CDR-10 is mechanically stable when operated at high pressures and temperatures. We attribute this fact to a 35% cross-linkage which contributes to the mechanical stability of the resin and an easier chromatographic handling.

CONCLUSIONS

The purpose of this study was to use the macroreticular anion-exchange resin system for the separation of complex biochemical mixtures into their components and to examine the performance of the system [17]. The benefits of using macroreticular resin include a decrease in analysis time, an increase in resolution and a high degree of automation. Considerable work still remains to be done in the optimization of operating conditions, identification of the separated compounds, and also for application to clinical use. However, at this stage of development, the macroreticular anion-exchange resin system proved to be a more useful analytical tool than the previously developed systems of the microreticular or the pellicular anion-exchange resin columns, and is used already for routine analytical techniques in many medical research laboratories [18].

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CHROMBIO. 498

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PROTOPORPHYRIN AND ZINC PROTOPORPHYRIN IN BLOOD

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SUMMARY

Zinc protoporphyrin and protoporphyrin free acid in blood were determined by high-performance liquid chromatography on a C_{18} column. Results for 63 human blood samples obtained through a lead poisoning detection program compared favorably with the widely-used ethyl acetate—acetic acid extraction determination. Blood from 16 rats which had been maintained on water heavily spiked with chloroform or bromodichloromethane and blood from a lead-poisoned cow were examined by this procedure.

INTRODUCTION

It is difficult to determine any of the specific porphyrins in blood (Table I) because so many other absorbing and fluorescing species are present. Interlaboratory agreement for protoporphyrin (PP) in blood is lacking, probably because of the many spectroscopic interferences and the lack of a good primary standard [1]. To be accurate a method must be capable of efficiently separating the porphyrin of interest not only from the other porphyrins but also from these interferences.

Most laboratories which assay blood samples to detect lead poisoning measure porphyrins routinely. However, they must perform double-solvent extractions in order to separate protoporphyrin from blood and hemin [2]. Many laboratories use a hematofluorometer, which measures porphyrin fluorescence directly from whole blood and therefore relies solely on optical filters for porphyrin specificity [3].

High-performance liquid chromatography (HPLC) has been successfully applied to the analysis of porphyrins [4—6]. Esterified 2-8-carboxylic acid-substituted prophyrins can be easily separated by adsorption on a 20-cm, 5-μm Partisil

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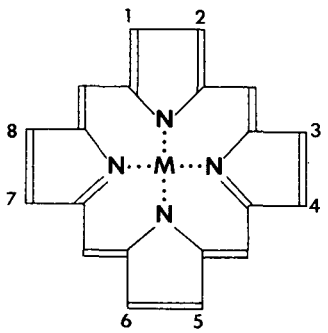
TABLE I

STRUCTURES OF SEVERAL PORPHYRINS IN HUMAN BLOOD



Porphyrin	M^{2+}	Substituents on positions		
		1,3,5,8	2,4	6,7
Uroporphyrin III	2H	$-CH_2COOH$	$-CH_2CH_2COOH$	$-CH_2CH_2COOH$
Coproporphyrin III	2H	$-CH_3$	$-CH_2CH_2COOH$	$-CH_2CH_2COOH$
Protoporphyrin IX	2H	$-CH_3$	$-CHCH_2$	$-CH_2CH_2COOH$
Zn protoporphyrin IX	Zn	$-CH_3$	$-CHCH_2$	$-CH_2CH_2COOH$
Fe protoporphyrin IX (heme)	Fe	$-CH_3$	$-CHCH_2$	$-CH_2CH_2COOH$

column with a solvent such as ethyl acetate–cyclohexane (60:40) [7]. However, the added esterification step is time-consuming, especially when large numbers of samples must be analyzed. Ion-pair separation of free acids has been reported [8]. This approach was rejected for the present work because of the added complexity which its use would introduce with regard to formation of the zinc complex.

We have developed a rapid HPLC method for accurate determination of porphyrin free acids in blood using a C_{18}-bonded column packing. Using a primary standard which has been assayed by this method, we have determined PP and zinc protoporphyrin (ZnP) in blood and compared the results with those obtained by conventional methods.

EXPERIMENTAL

Apparatus

A modified Waters Assoc. (Milford, Mass., U.S.A.) high-performance liquid chromatograph was used in the isocratic mode. The individual components included a Waters 6000A solvent delivery system, a Rheodyne 7105 continuous-flow septumless injector (Perkin Elmer, Norwalk, Conn., U.S.A.), a Waters 30 cm × 4 mm I.D. reversed-phase μBondapak C_{18} column (10-μm particle size), and a Dupont (Wilmington, Del., U.S.A.) Model 836 fluorescence detector (medium-pressure Hg source, R777 PMT) equipped with a λ_{ex} = 365-nm filter (Corning CS-7-60, Corning, N.Y., U.S.A.) and a λ_{em} = 595-nm filter (source unknown).

A Hewlett-Packard 3385A data system was used for all measurements. Integration was improved by shunting a 1-mF capacitor to the data system input.

A Dupont Sorvall Superspeed SS3 centrifuge equipped with an SS-34 rotor was used to separate the cellular debris.

Reagents

Methanol and ethyl acetate were purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.), acetic acid and zinc acetate from J.T. Baker (Phillipsburg, N.J., U.S.A.).

Uroporphyrin, coproporphyrin, protoporphyrin, and zinc protoporphyrin are separated (Fig. 1) using the mobile phase methanol—acetic acid—deionized water (83:2:15) (pH 3.9) which has been degassed by vacuum filtration through a 0.22-μm Teflon filter (Millipore, Bedford, Mass., U.S.A.). Blood samples, however, contain large amounts of hemin, which obscures the earlier peaks; therefore a more nonpolar 39:4:7 (pH 3.4) solvent mixture was chosen to speed up the analysis time for ZnP and PP.

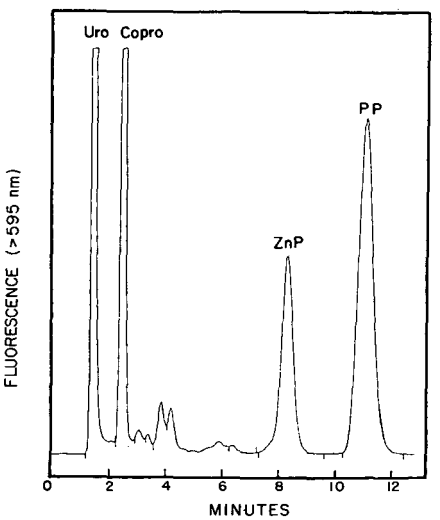


Fig. 1. HPLC separation of porphyrin free acids: uroporphyrin, coproporphyrin, ZnP, and PP. Sample, 100 μl of porphyrin standards (0.1—0.5 μg/ml) in the mobile phase; instrument, modified Waters; column, μBondapak C_{18} 10 μm; mobile phase, methanol—acetic acid—water (83:2:15); flow-rate, 2 ml/min; temperature, ambient; detector, Dupont 836 fluorescence, λ_{ex} = 365 nm, λ_{em} >595 nm.

A stock disodium protoporphyrin (Na_2PP) standard was prepared by dissolving 13.7 mg of the anhydrous salt (Sigma, St. Louis, Mo., U.S.A.) in 1 l of acetone—water (7:3) solvent. This standard was corrected for purity (see Discussion).

Dilute working standards were prepared daily as follows: for PP, 0.5 ml of the Na_2PP stock solution was diluted to 100 ml with chromatographic solvent. For ZnP, 3 ml of the Na_2PP stock solution, 3 ml of 0.3% zinc acetate in 1% acetic acid, and 50 ml of methanol were reacted for 1 h in a 100-ml volumetric

flask, then diluted to obtain a solvent composition of approximately 89:4:7.

The solvent used to extract porphyrins from blood was ethyl acetate—acetic acid (4:1).

Samples

Sixty-three human blood samples were chosen at random from among those submitted to this Divison's lead poisoning detection program.

Rat blood was obtained from Nya:NYLAR rats by drawing from the tail. The rats had been maintained for over 2 years on a water supply containing chloroform or bromodichloromethane (2 ml/l).

Cow blood was obtained from a cow which is regularly fed lead acetate for the production of quality-control samples for the New York State lead poisoning detection program.

All blood samples were collected into glass microcapillaries or Vacutainers (Becton Dickenson, Rutherford, N.J., U.S.A.), containing disodium ethylene dinitrilotetraacetic acid (EDTA) and stored away from light at 4°.

The freeze-dried pooled human blood was purchased from A.R. Smith Labs., Los Angeles, Calif., U.S.A.

Procedure

With an Eppendorf pipette 200 μl of blood are added to 100 μl of water in a glass centrifuge tube. The porphyrins are extracted by addition of 1 ml of ethyl acetate—acetic acid (80:20), followed immediately by vigorous mixing. The sample is then centrifuged for 1 min at 17,369 *g*, and 25 μl of the porphyrin-containing supernatant are injected onto the column.

The standards are injected separately with no extraction. Total analysis time for ZnP and PP is about 15 min/sample.

Chromatographic conditions

To minimize adsorption of hemin and porphyrins onto any polar silicic acid sites, the C_{18} column must first be washed with 50 ml of 0.01 *N* HCl. ZnP, PP, and hemin in blood extracts are then well separated as free acids in 5 min at ambient temperature, using the methanol—acetic acid—water solvent at a flow-rate of 2 ml/min. Between sample injections a 5-min washout time of sample residues is required, otherwise significant porphyrin adsorption will occur.

RESULTS AND DISCUSSION

Because it minimizes spectroscopic interferences, this HPLC method may be applied to many matrices containing porphyrin. However, blood samples were of particular interest because of their routine use for clinical assays.

Human, bovine, and rat blood samples were analyzed (Fig. 2) and found to contain varying amounts of ZnP and PP. Although about 90% of the protoporphyrin in whole human blood is found present as the Zn^{2+} complex, about 10% is not complexed. The 4-8-COOH-porphyrins may not be seen in the chromatograms because of hemin absorbance from 1.5 to 2.5 min, but an extraction solvent which eliminated hemin (possibly ethanol [9]) would permit analysis of these porphyrins.

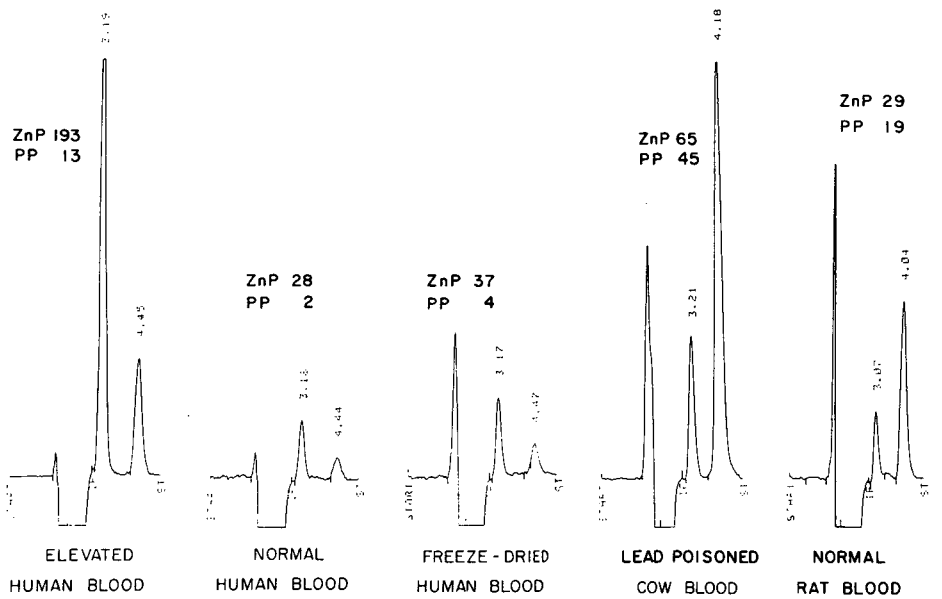


Fig. 2. Chromatograms of ZnP (3.1 min) and PP (4.4 min) for several different kinds of blood. Amounts are in μg/dl.

Sixty human blood samples were analyzed by HPLC for total protoporphyrin (ZnP + PP), commonly called erythrocyte protoporphyrin (EP). These samples were also analyzed by three established methods: for lead by a microsampling-cup atomic absorption method [10], for ZnP by a commercial filter hematofluorometer with fluorescent-dye calibration slides, and for total protoporphyrin (PP + ZnP) by a double-extraction fluorescence method [2]. These three established methods are routine in our laboratory, and our performance is checked routinely by participation in several interlaboratory surveys. A summary of these results is given in Table II.

The lead content of samples ranged from 9 to 63 μg/dl, total porphyrin content ranged from 18 to 204 μg/dl.

TABLE II

CORRELATION OF RESULTS FOR 60 BLOOD SAMPLES

R^2 = Correlation coefficient squared, indicates the proportion of the population represented by the regression [11]; b_1 = slope of line and b_0 = intercept on Y-axis.

Independent variable (X)	Dependent variable (y)	R^2	b_1	b_0
Double-extraction ZnP + PP	HPLC ZnP + PP	0.96	1.1	1.7
Double-extraction ZnP + PP	HPLC ZnP	0.96	0.97	1.8
Double-extraction ZnP + PP	Hemat. ZnP	0.88	0.85	6.5
Hemat. ZnP	HPLC ZnP	0.87	1.0	4.1
Hemat. ZnP	HPLC ZnP + PP	0.86	1.1	4.9
Double-extraction ZnP + PP	HPLC PP	0.43	0.11	−0.36
HPLC ZnP	HPLC PP	0.38	0.11	0.38
Hemat. ZnP	HPLC PP	0.37	0.11	0.42

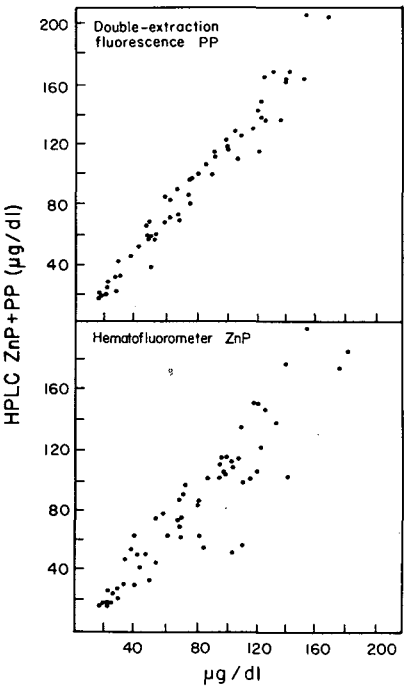


Fig. 3. Comparison of results of actual samples analyzed by HPLC and either the double-extraction fluorescence method or a hematofluorometer.

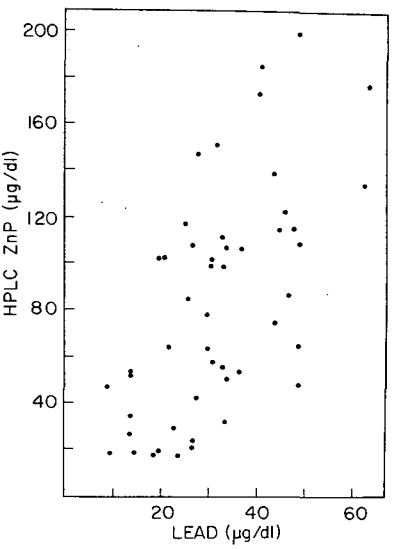


Fig. 4. Comparison of results of actual samples analyzed for ZnP by HPLC and for lead by microsampling-cup atomic absorption.

Consistently good agreement was found between HPLC and double-extraction fluorescence results for total porphyrins (Fig. 3). Hematofluorometric results correlated less well, but free uncomplexed protoporphyrin did not really correlate with the other measurements at all. Clearly the proportion of zinc-complexed to uncomplexed protoporphyrin is variable and may reflect the trace zinc content of the subject.

Because of our interest in detection of subclinical lead poisoning, we investigated the correlation between blood lead and HPLC ZnP results. In view of the good agreement between the HPLC ZnP + PP and double-extraction fluorescence ZnP + PP, it seems likely that the very poor correlation between blood lead and HPLC ZnP (Fig. 4) is physiological and not a product of methodology.

To obtain good quantitative results with the C_{18} columns currently available, the pH of the column, mobile phase, and sample must be controlled. The retention time of hemin was very sensitive to column acidity, requiring deactivation of the column by washing with strong acid. With predominantly hydrophobic packing, the best separation for uroporphyrin, coproporphyrin, ZnP, and PP was found (Fig. 1) as expected, when the mobile phase was maintained slightly below the isoelectric point of the porphyrins (pH 4—5). At significantly higher and lower pH values, porphyrin ionization caused a drastic loss of resolution. Injection of standards or samples containing less than 2% acetic acid is not re-

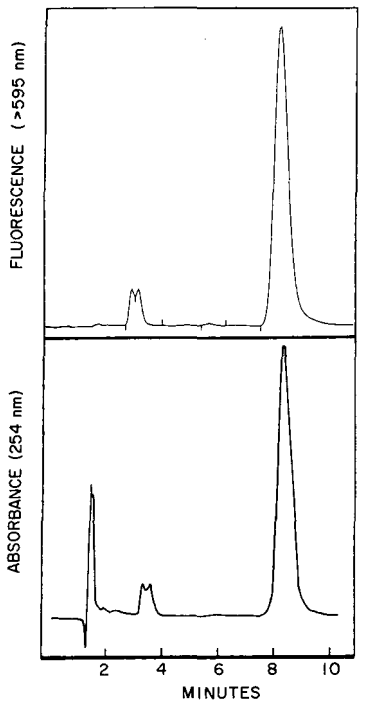


Fig. 5. Chromatogram of impure disodium protoporphyrin IX primary standard.

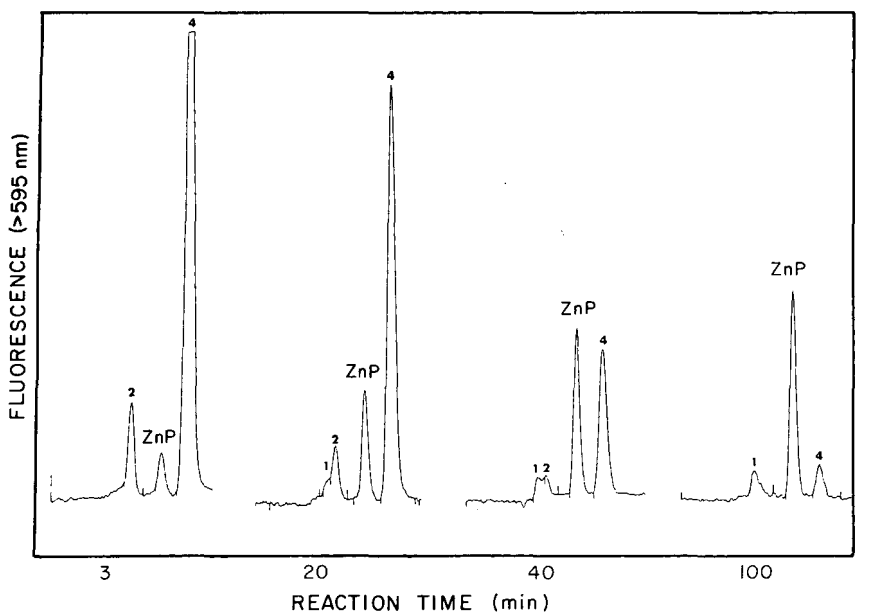


Fig. 6. Reaction of Zn^{2+} with protoporphyrin IX standard in methanol. Peaks: 1 = Zn complex of an unknown porphyrin impurity; 2 = unknown porphyrin impurity; 3 = ZnP; 4 = disodium protoporphyrin IX starting material.

TABLE III

PORPHYRIN (μg/dl) IN THE BLOOD OF RATS FED EITHER CHLOROFORM OR BROMODICHLOROMETHANE DAILY FOR 2 YEARS

$CHCl_3$		$CHBrCl_2$		Controls	
ZnP	PP	ZnP	PP	ZnP	PP
23	12	24	18	25	12
21	15	36	15	25	10
21	12	32	16	28	13
38	23	21	9	28	16
29	19	30	13	32	13
27	16	22	14	30	12
35	16	29	11	24	11
–	–	29	9	29	10
–	–	21	12	26	16
–	–	–	–	28	9
Mean					
27.7	16.1	27.1	13.0	27.5	12.2

commended, as up to 30% of the porphyrin may be lost by column adsorption. The Na_2PP IX standard was found to contain two small impurities (9% by area) by absorbance at 254 nm and by red fluorescence detection (Fig. 5). Efforts were made to prepare ZnP from Na_2PP and to remove the impurity by fractional crystallization, but Zn complexes of the impurities always appeared in the product (Fig. 6). Hence it was assumed that the impurities were porphyrin in nature, and the concentration of Na_2PP stock solution was corrected for 91% purity. Preparation of a working ZnP standard by reaction of the Na_2PP stock solution with Zn^{2+} in methanol (Fig. 6) yielded a ZnP standard which was stable for 1 day.

A number of instances have been reported in the literature where exposure to chlorinated organic compounds has produced hepatic porphyria. Administration of hexachlorobenzene [12], polychlorinated biphenyls [13], or 2,3,7,8-tetrachlorodibenzo-1,4-dioxin [14] produced hepatic porphyria in some mammals, explained by a decrease in the activity of decarboxylation enzymes. Currently we are conducting a chronic toxicology study with chloroform and bromodichloromethane in rats. Thus far an effect has not been reported in erythrocytes, and so the analysis was applied to blood samples from rats exposed for two years to these compounds at approximately 20 mg per kg body weight per day. Samples from rats exposed for two years to either chloroform or bromodichloromethane were analyzed for ZnP or PP by HPLC. Table III shows clearly that no effect on levels of ZnP or PP has been produced. More likely porphyrias, such as those found by San Martin de Viale et al. [15] in the liver or kidney, have not yet been studied.

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Biomedical Applications

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PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PLASMA CHOLINE*

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SUMMARY

Choline was isolated from deproteinized plasma by cation-exchange chromatography. Isolated choline was directly converted to the 3,5-dinitrobenzoate derivative and was analyzed by paired-ion high-performance liquid chromatography with UV detection at 254 nm. An internal standard, 3-hydroxy-N,N,N-trimethylpropanaminium iodide was used for quantitation of plasma choline.

Linearity was achieved from 1—500 nmole/ml with a reproducibility of ± 6%. Plasma choline concentrations below 1 nmole/ml could not be accurately measured while plasma choline concentrations in the μmole/ml range deviated from linearity.

INTRODUCTION

The quantitative analysis of choline in biological samples has been effected using a yeast culture [1], radioenzyme assays [2—5], a spectrophotometric assay [6], a polarographic assay [7], gas chromatographic assays [8, 9] and gas chromatographic—mass spectrometry assays [10—12].

Most of the procedures are highly esoteric and involve complicated and/or expensive instrumentation that are not normally found in clinical research laboratories. Gas chromatographs are readily accessible but the assays for choline involve either the pyrolysis of the quaternary salt [13] or the chemical demethylation of the quaternary salt with sodium benzenethiolate [8—11].

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Pyrolysis and chemical demethylation of esterified choline, i.e., acetylcholine or butanoylcholine [8, 9] have been reported [14—16] to cause the cleavage of the ester moiety from choline thus potentially leading to erroneous results. In addition, the chemical demethylation of choline with sodium benzenethiolate is a long, involved, process that requires highly trained personnel. The current surge of interest in the use of high-performance liquid chromatography (HPLC) in clinical research laboratories prompted the development of a simple, paired-ion HPLC assay for plasma choline.

Choline was isolated from deproteinized plasma by cation-exchange chromatography [9]. The isolated choline was directly converted to the 3,5-dinitrobenzoyl ester by treatment with 3,5-dinitrobenzoyl chloride in pyridine. The 3,5-dinitrobenzoate was analyzed by paired-ion HPLC. An internal standard, 3-hydroxy-N,N,N-trimethylpropanaminium iodide, was used for the quantitation of choline.

EXPERIMENTAL

Chemicals

Choline chloride, 98%, purchased from Eastman (Rochester, N.Y., U.S.A.) was recrystallized twice from absolute ethanol—diethyl ether [17]. 3-Hydroxy-N,N-dimethylaminopropane, iodomethane, sodium dodecyl sulfate and 3,5-dinitrobenzoyl chloride were purchased from Aldrich (Milwaukee, Wisc., U.S.A.) and were used without further purification.

Solvents

All solvents were distilled in glass grade purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Deionized water was distilled from alkaline permanganate and was filtered through a 0.22-μm Millipore filter before use.

Equipment

IR spectra were recorded on a Beckman IR 4240 spectrometer. UV spectra were recorded on a Beckman DB spectrometer. A Bioanalytical Systems LC-50 liquid chromatograph employing two μBondapak C_{18} (particle size 10 μm) analytical columns (30 cm × 4 mm I.D.) Waters Assoc., (Milford, Mass., U.S.A.) in tandem and an Altex 110 fixed wavelength (254 nm) ultraviolet detector were used for quantitative analysis. A chloride specific ion electrode (Cole-Parmer) was used for the determination of choline in the standard choline chloride solution.

Preparation of 3-hydroxy-N,N,N-trimethylpropanaminium iodide

To 10.0 g (97.1 mmole) of 3-hydroxy-N,N-dimethylaminopropane in 24 ml of absolute ethanol were added 18.5 g (130.1 mmole) of iodomethane. Upon stirring at ambient temperature, a white precipitate formed. The mixture was filtered. The supernatant was cooled and cold absolute diethyl ether was added producing a white precipitate. The mixture was filtered. The combined precipitate was recrystallized from absolute ethanol—diethyl ether affording 23.7 g (100%) of a white crystalline solid, m.p. 203—204° (literature [18] m.p. 201—202° from absolute ethanol).

The 3,5-dinitrobenzoate was prepared according to the procedure of McElvain [19] affording a golden solid, m.p. 172—174° (dec.) from absolute ethanol—diethyl ether: IR (KBr) 3125, 1740, 1550, 1354, 1286, 827, 739 and 725 cm^{-1}. UV $\lambda_{max}^{H_2O}$ = 230 nm ($\epsilon = 1.72 \cdot 10^4$).

A single peak was observed in the HPLC chromatogram at 15 min using 5 m*M* sodium dodecyl sulfate in 50% acetonitrile through the two μBondapak C_{18} analytical columns in tandem at a flow-rate of 2 ml/min.

Preparation of 2-(3,5-dinitrobenzoyl)-N,N,N-trimethylethanaminium chloride

The 3,5-dinitrobenzoate of choline chloride was prepared according to the procedure of McElvain [19] affording a pale yellow solid in 85% yield; m.p. 200—201° (dec.): IR (KBr) 3092, 2990, 1725, 1548, 1355, 1292, 950 and 730 cm^{-1}. UV $\lambda_{max}^{H_2O}$ = 230 nm ($\epsilon = 9.48 \cdot 10^3$).

A single peak was observed in the HPLC chromatogram at 13 min using 5 m*M* dodecyl sulfate in 50% acetonitrile through the two μBondapak C_{18} analytical columns in series at a flow-rate of 2 ml/min.

Analytical procedures

Standard curve preparation. To four sets of duplicate 0.25-ml aliquots of Red Cross human plasma were added 25 μl of 0.41 m*M* 3-hydroxy-N,N,N-trimethylpropanaminium iodide (internal standard) and:

A, 0 μl; B, 10 μl; C, 25 μl and D, 50 μl of 0.23 m*M* choline chloride.

Sample preparation. To duplicate 0.25-ml aliquots of plasma were added 25 μl of internal standard (0.41 m*M*). To each sample was added 0.75 ml of ice cold 1 *M* formic acid—acetone to precipitate the protein. The samples were mixed and were centrifuged for 15 min at high speed in a benchtop centrifuge. The supernatant was decanted and was used for the analysis.

Ion-exchange chromatography [9]. Cation-exchange resin (AG 50W-X12), 300 mg per sample, was allowed to sit overnight in 1 ml of 0.1 *M* ammonium acetate pH 4.0. Pasteur pipets, 5 in., were fitted with glass wool plugs and the presoaked ion-exchange resin was added to each column. The solvent was allowed to pass through the resin. Each column was cleaned by the passage of 1 ml of 2 *M* NaCl in 50% methanol through the resin and was activated by the passage of 1 ml of 0.1 *M* ammonium acetate, pH 4.0 through the resin. The plasma supernatant was placed on the column. Each column was washed with 1 ml of 0.1 *M* ammonium acetate, pH 4.0. Choline and the internal standard were eluted from the resin by the passage of 1 ml of 2 *M* NaCl in 50% methanol, all liquid was forced out of the column by a rubber squeeze bulb.

Each sample was taken to dryness under a stream of nitrogen and kept under a vacuum for an hour. Choline and the internal standard were extracted from the salt residue by the addition of 1 ml of dry acetonitrile. The sides of each sample tube were thoroughly scraped with the tip of a Pasteur pipet.

Each sample was mixed and centrifuged. The supernatant was removed and saved. This process was repeated with an additional 1-ml aliquot of dry acetonitrile. The combined supernatant from each sample was taken to dryness under a stream of nitrogen.

Sample derivatization. To the residue from the evaporation of acetonitrile was added 0.30 ml of a 21.7 m*M* solution of 3,5-dinitrobenzoyl chloride in dry

(KOH) pyridine (prepared immediately prior to use). The samples were heated for 1 h at 105° in a hot sand bath. The pyridine was removed under a stream of nitrogen. The residue was first extracted with 0.30 ml of glass distilled water and then with 0.20 ml of glass distilled water. The combined aqueous extract was filtered through a 0.3-μm Millipore filter.

HPLC conditions. The filtered aqueous extract was placed onto two μBondapak C_{18} analytical columns in tandem via a 100-μl loop injector. The mobile phase, 5 m*M* sodium dodecyl sulfate and 0.1% acetic acid in 50% acetonitrile was pumped at a flow-rate of 2—2.3 ml/min. Detection was facilitated by a fixed wavelength (254 nm) UV detector. The retention time for dinitrobenzoyl (DNB)-choline was 13 min while the retention time for the DNB derivative of the internal standard was 15 min (Fig. 1).

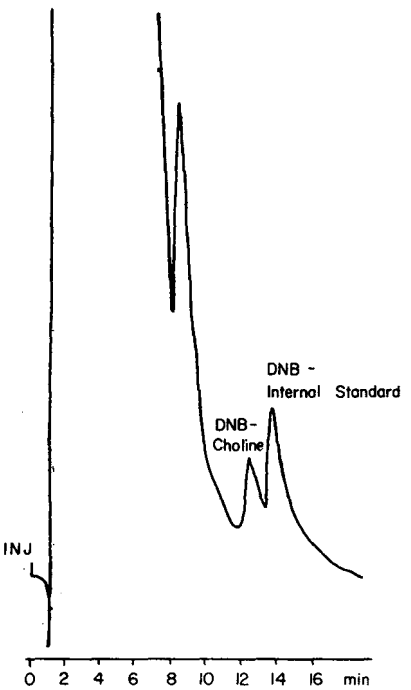

Fig. 1. HPLC chromatogram of human plasma choline (22 nmole/ml). Conditions: two μBondapak C_{18} (10 μm) analytical columns in tandem; mobile phase, 5 m*M* sodium dodecyl sulfate—0.1% acetic acid—50% acetonitrile; flow-rate, 2.0—2.3 ml/min; detection wavelength 254 nm. A level of 22 nmole/ml was assayed. DNB-choline and DNB-IS (DNB-3-hydroxy-N,N,N-trimethylpropaminum iodide) (40 nmole/ml) as the internal standard are shown.

Calculations

The method of standard addition was used to obtain the standard curve. The slope of the standard curve was calculated by linear regression analysis of choline concentration vs. peak height choline/peak height internal standard ratio. The slope of the standard curve was used as the working curve to calculate the choline concentration in the samples. The average value of two duplicate analyses was used to determine the choline concentration in each sample. Reproducibility was ± 6% of the mean. Linearity was achieved from 1—500

nmole/ml. Below 1 nmole/ml, the choline concentration could not be accurately determined. Above 500 nmole/ml, the choline concentration deviated negatively from linearity.

RESULTS

Choline was isolated from plasma by cation-exchange chromatography following the procedure of Zahniser et al. [9]. It was found that 300 mg of AG 50W-X12 resin per column quantitatively retained choline and the internal standard. Complete elution of these compounds from the column was effected by the passage of 1.0 ml of 2 *M* NaCl in 50% methanol. Precipitation of plasma protein with 3 volumes of 15% 1 *M* formic acid—acetone prior to ion-exchange chromatography hastened the passage of the sample through the ion-exchange column and did not result in the loss of choline or internal standard.

Extraction of choline and internal standard from the dried ion-exchange eluate was quantitative if the salt residue was completely dry. Extraction was accomplished by washing the salt residue twice with 1 ml of dry acetonitrile. The salt residue must be thoroughly scraped from the sides of the vessel and thoroughly mixed with the acetonitrile to ensure the quantitative removal of choline and internal standard.

The quantitative conversion of choline and internal standard to their 3,5-dinitrobenzoyl esters required an excess of 3,5-dinitrobenzoyl chloride in dry pyridine but a nitrogen atmosphere was not required. Heating the samples with 3,5-dinitrobenzoyl chloride—pyridine at 105° for an hour was found to afford the best results.

Quantitative extraction of derivatized choline (DNB-choline) and internal standard (DNB-IS) from the residue that resulted after the removal of pyridine was effected with water. In order to ensure good peak heights in the HPLC chromatogram and to allow Millipore filtration of the sample, the total volume of water used in the extraction was critical. We extracted the residue first with 0.3 ml water and then with 0.2 ml water.

Separation of DNB-choline from DNB-IS could not be effected on a μBondapak analytical reversed-phase column without using a paired-ion. Use of 5 m*M* sodium dodecyl sulfate in 50% acetonitrile afforded separation of the compounds (Fig. 1). The retention times for pure DNB-choline and pure DNB-IS were the same as the retention times observed when plasma samples containing the internal standard were processed and chromatographed.

The excess 3,5-dinitrobenzoyl chloride required for the quantitative derivatization of choline and the internal standard was hydrolyzed to 3,5-dinitrobenzoic acid during the water extraction procedure. This acid eluted from the column as a massive peak at the void volume of the system. When a single reversed-phase column was employed, the DNB-choline and DNB-IS eluted on the tail of the 3,5-dinitrobenzoic acid peak. This situation was rectified through the use of two reversed-phase columns in series. The use of a flow-rate of 2.0—2.3 ml/min kept the peaks sharp and maintained separation of DNB-choline and DNB-IS.

Using the method of standard addition to a Red Cross human plasma pool, linearity was achieved from 1 to 500 nmole/ml. Choline concentrations above

500 nmole/ml afforded negative deviations from linearity. When ten identical plasma samples were analyzed by this procedure, the standard deviation was ± 6% of the mean.

DISCUSSION

In order to test the effectiveness of our paired-ion HPLC choline assay, we used various plasma samples that we had stored in our laboratory freezer. These plasma samples were simultaneously analyzed by our HPLC procedure and by the gas chromatographic chemical demethylation procedure of Zahniser et al. [9]. The results are shown in Table I. The gas chromatographic procedure of

TABLE I

ANALYSIS OF HUMAN PLASMA FROM LITHIUM-TREATED PATIENTS VIA HPLC PROCEDURES AND GAS CHROMATOGRAPHIC PROCEDURES [3]

Patient No.	HPLC choline (nmole/ml)	Gas chromatography choline (nmole/ml)
1	35.65	23.40
2	21.82	5.54
3	33.82	18.90
4	16.00	15.60
5	36.36	10.40
6 (12/27/78)	16.07	22.00
6 (1/10/79)	17.85	7.95
7	21.38	15.10
8	8.00	12.10
9	7.56	17.50
10 (1/4/79)	25.78	21.00
10 (1/11/79)	23.11	12.20
11	19.56	20.80
12	10.22	13.00
13	13.33	15.10
14	20.89	11.30
15	12.89	9.78
Mean	20.02	14.80

Zahniser et al. [9] does not utilize a true internal standard (the internal standard is added after precipitation of plasma protein), while our HPLC procedure does. This does not entirely explain the low values that were obtained. The use of sodium benzenethiolate for the demethylation of esterified choline via an SN_2 mechanism, has been reported by Sheehan and Davis [16] to effect the base-catalyzed hydrolysis of the ester bond. This would result in low values of choline. Jenden and Hanin [8] reported that no cleavage of the ester bond took place in the chemical demethylation reaction, but they could not detect the presence of free choline by gas chromatography. Free choline is not volatile. Jenden and Hanin [8] instead looked for the presence of acetoxyethylphenyl sulfide which they did not find. Acetoxyphenylethyl sulfide would be formed if the benzenethiolate ion displaced the trimethylamino moiety from choline via an SN_2 mechanism but would not be formed if a base-catalyzed hydrolysis of the ester bond took place. In addition, any moisture present in

the system would convert sodium benzenethiolate to benzenethiol which would not react with esterified choline but which would react with any oxygen that was present to form diphenyl disulfide. In essence, the amount of sodium benzenethiolate used in the gas chromatographic assay is critical and absolutely anhydrous conditions must be employed. The use of anhydrous conditions would not hinder the hydrolysis of esterified choline to free choline.

The plasma samples that were used to compare the HPLC assay with the gas chromatographic assay had been obtained from patients on long-term lithium therapy. The mean value that resulted from the gas chromatographic procedure for the fifteen samples that were analyzed, agreed very well with the mean plasma value, 14.9 μmole/l, reported by Jope et al. [20], who did not report the procedure for plasma choline analysis. The mean value for these samples when analyzed by the HPLC procedure was 36% higher.

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CHROMBIO. 474

RAPID AUTOMATED ION-EXCHANGE ANALYSIS OF PLASMA TYROSINE AND PHENYLALANINE WITH DATA PRINT-OUT

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(First received September 10th, 1979; revised manuscript received October 22nd, 1979)

SUMMARY

A rapid automated method with data print-out is described for quantitation of plasma phenylalanine and tyrosine from 0.100 ml of sample. The system uses the Rank-Hilger Chromaspek amino acid analyser linked to a Digico M16E computer.

Amino acid concentrations up to 3000 μM can be quantitated without repeat dilutions and assessment of precision at the 500 μM level, produced coefficients of variation of 2.2% for tyrosine and 2.5% for phenylalanine. Recovery determinations from a plasma pool gave a mean recovery of 99.4% for tyrosine and 99.7% for phenylalanine.

Correlation with established fluorimetric techniques was excellent (r = 0.986 for tyrosine, r = 0.976 for phenylalanine). By using the same resin column for both the rapid separation of tyrosine and phenylalanine, and the standard physiological fluid separation, full analysis capability is retained with easy interchange between the two systems.

INTRODUCTION

Accurate quantitation of plasma phenylalanine and tyrosine concentrations are essential for diagnosis of phenylketonuria (PKU) and subsequent monitoring of dietary therapy.

Ion-exchange chromatographic techniques for simultaneous assay of both amino acids [1—4] allow accurate sequential estimations from one sample aliquot and offer certain advantages in specificity [3] over the commonly used fluorimetric techniques [5, 6], particularly in determining phenylalanine tyrosine molar ratios, which have been recommended for PKU heterozygote detection [7, 8].

This paper describes a rapid automated analysis for phenylalanine and tyrosine, using the Rank-Hilger Chromaspek amino acid analyser, linked to a Digico M16E computer.

INSTRUMENTATION

The Rank-Hilger Chromaspek amino acid analyser has been described more fully elsewhere [9, 10]. The main elements of the system are outlined below.

Auto sampler

The sampling module consists of a 60-place refrigerated turntable with programmed sampling time adjustable between 5 and 60 sec.

Programmer unit

The programmer unit uses two control devices. A steel drum rotating at constant speed carries a profile map in black tape, which defines the gradient elution system. This is scanned by an electronic reading head which controls mixing of acidic and basic buffers.

There is also a programmer plate which is scanned by a series of photoelectric sensors; this controls column temperature, ninhydrin/wash status, sampling time and computer print out.

Ion-exchange column

7% cross-linked cation-exchange resin of 8 μm nominal bead diameter is contained within a steel column (500 mm × 2.6 mm I.D.) surrounded by an aluminium heating block. By utilising the 500-mm column for the short analysis programme, the full system capability for analysis for complex physiological fluids is retained.

Pumping system

The Chromaspek uses two pump types: a high-pressure Milton-Roy pump delivering buffer to the resin column at a constant flow-rate of 0.150 ml/min (back pressure 3.5 MPa) and a peristaltic pump delivering buffer and aspirated sample to the high-pressure pump and nitrogen and colour reagents to the column outlet.

Colorimetry

The column eluate is mixed with a ninhydrin/cyanide reagent segmented with nitrogen and heated to 95°. The colour development is measured without debubbling by two photometers at 440 nm and 570 nm using fibre optic light guides from a grating monochromator.

Quantitation

Absorbance outputs from the two photometers are displayed on a dual pen potentiometric recorder (Vitatron Series 2001) and input to the interface of Digico Micro 16E computer (8K store of 16 bit words). Each measuring channel is supplied with three amplifier gain settings covering the ranges 0—0.05, 0—0.10, 0—0.2 absorption units. The Chromaspek interface uses an analogue to digital converter, stated to give full scale, at 10% overscale of the instrument range of 0—0.2 absorption. The interface converts analogue information to digital form and enters the digital data to the computer, which then, applies base line correction, determines peak area, relates to internal and

external standard peak areas and at the end of each run, prints out tabulated results in defined concentration units on the associated teletype.

REAGENTS

Acid buffer (pH 2.20)

Citric acid (AR)	10.5 g
1 *M* lithium chloride	150.0 ml
Thiodiglycol (25% v/v in water)	2.5 ml
Brij 35 (10% w/v in 5% methanol)	3.5 ml

Make up to 1000 ml with distilled water.

Basic buffer (pH 11.50)

Citric acid (AR)	10.5 g
Lithium hydroxide monohydrate (AR)	12.6 g
Boric acid (AR)	8.8 g
Ethylenediaminetetraacetic acid (disodium salt)	0.5 g
Brij 35 (10% w/v in 5% methanol)	3.5 ml

Make up to 1000 ml with distilled water.

Ninhydrin (pH 5.50)

Ninhydrin (AR)	10.0 g
Sodium acetate trihydrate (AR)	216.0 g
Glacial acetic acid (AR)	100.0 ml
2-Methoxyethanol	400.0 ml
Brij 35 (10% w/v in 5% methanol)	10.0 ml

Make up to 1000 ml with distilled water.

Wash solution

2-Methoxyethanol	400.0 ml
Brij 35 (10% w/v in 5% methanol)	10.0 ml

Make up to 1000 ml with distilled water.

Sodium cyanide

1% stock solution:

Sodium cyanide (AR)	1.0 g
Sodium carbonate	4.0 g

Make up to 100 ml with distilled water.

A 0.0005% working solution was then made up:

1% stock solution	0.50 ml
4 *M* sodium hydroxide	2.00 ml
Brij 35 (10% w/v in 5% methanol)	10.00 ml

Make up to 1000 ml with distilled water.

Protein precipitant (3% sulphosalicylic acid containing 120 μM norleucine)

Sulphosalicylic acid (AR)	30.0 g
Norleucine (10 m*M* stock solution)	12.0 ml

Make up to 1000 ml with distilled water.

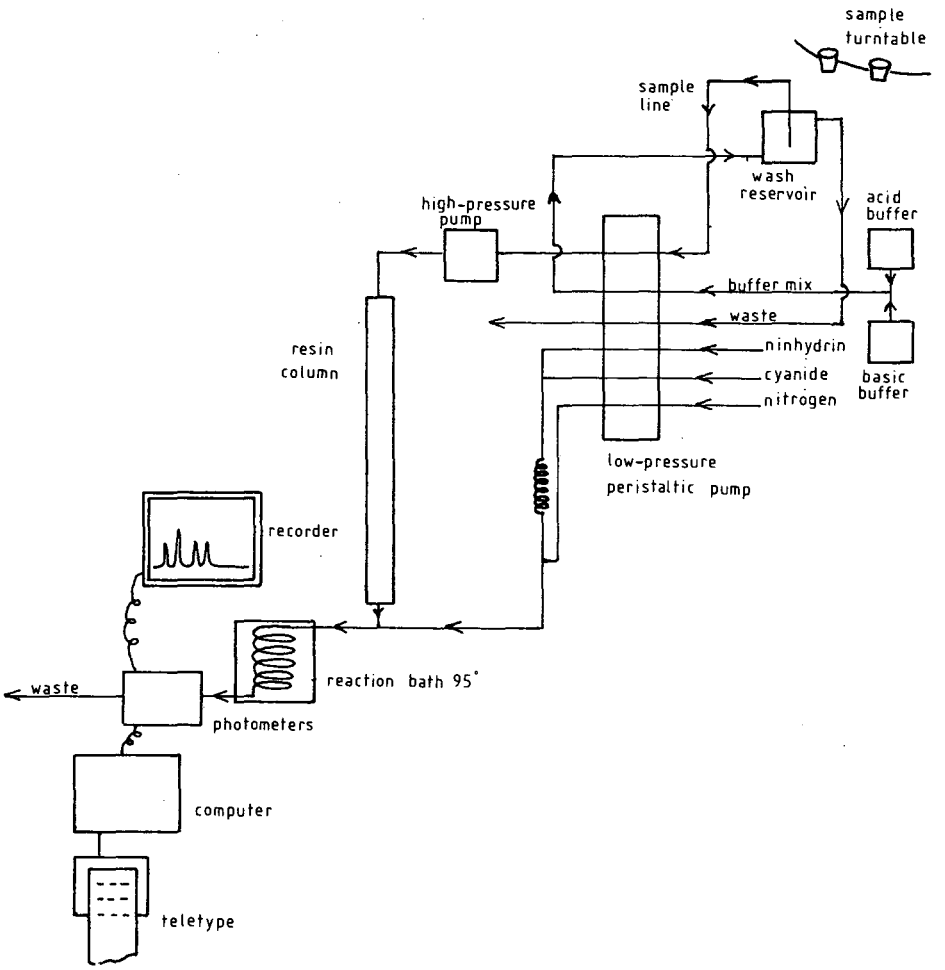

Fig. 1. System format of Rank-Hilger Chromaspek amino acid analyser.

Standard solution

The stock standard was made up of 10 m*M* each of tyrosine, phenylalanine, leucine and isoleucine in 0.025 *M* hydrochloric acid. The working standard comprised of 6.0 ml of the stock standard made up to 100.0 ml with pH 2.20 acid buffer.

SAMPLE PREPARATION

Plasma or working standard (100 μl) was mixed with protein precipitant (500 μl). After 10 min, this was centrifuged at 1500 *g* for 5 min. The supernatant (200 μl) was loaded into a sample cup and placed in position on the sample turntable.

Sample list and internal and external standard concentrations are input to the computer through the teletype. From this point until result print, analysis was totally automatic.

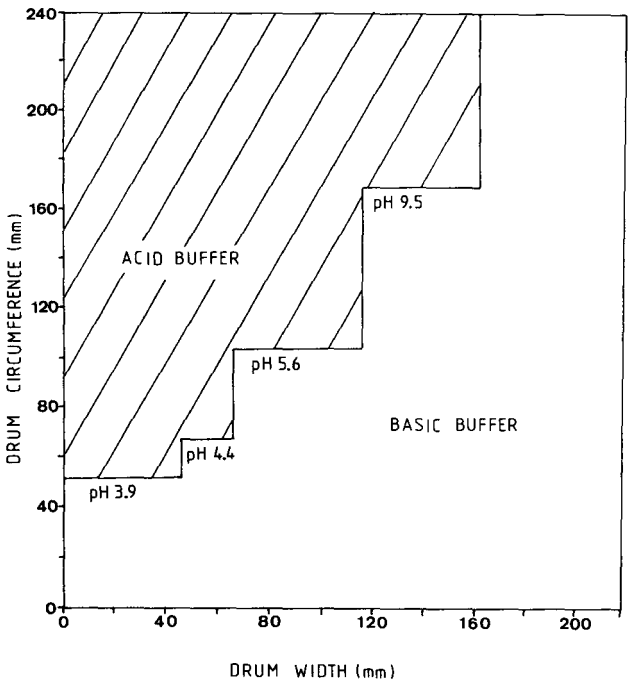

Fig. 2. Programme drum profile producing the pH gradient used in the rapid phenylalanine and tyrosine method.

ANALYSIS SYSTEM

The instrument format is shown in Fig. 1. Analysis conditions were as follows:

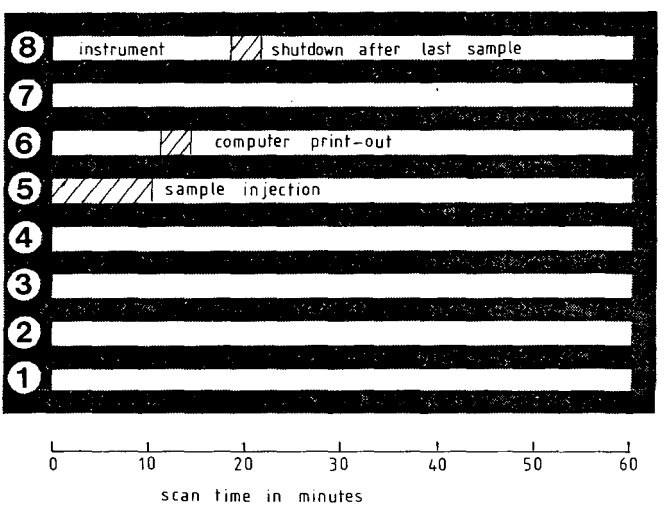

Fig. 3. Programme plate conditions used in the rapid phenylalanine and tyrosine method. Change from masked to clear area initiates function.

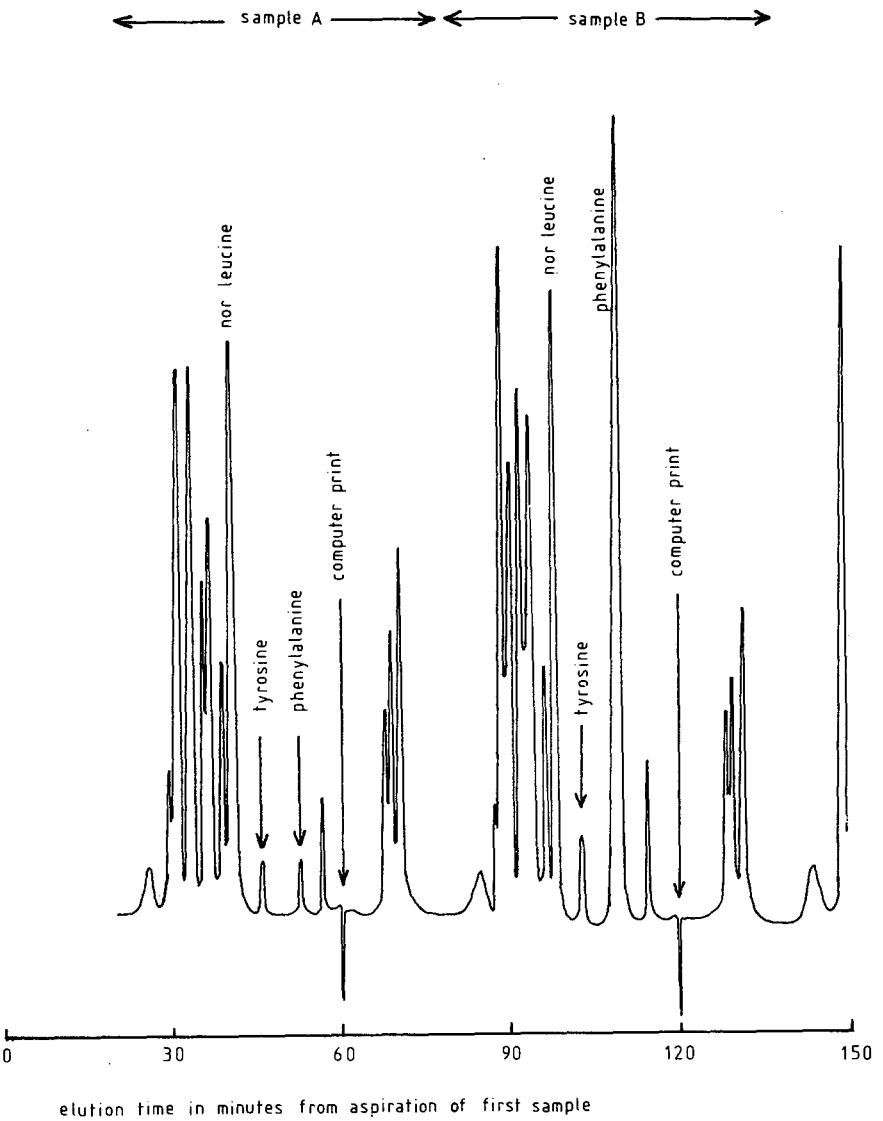

Fig. 4. Typical recorded amino acid profiles for (A) normal plasma sample and (B) PKU plasma sample.

column temperature, 60°; buffer elution rate, 0.150 ml/min; ninhydrin flow-rate, 0.200 ml/min; cyanide flow-rate, 0.050 ml/min; sampling time from analysis commencement, 10 min; total analysis time, 60 min and sample pick up volume (40 sec aspiration) 0.100 ml.

In routine use, one working standard precedes the first sample and every fifth cup contains a similar standard. The buffer profile and programme plate conditions are illustrated in Figs. 2 and 3 and examples of the recorded amino acid profile and computer print-out are shown in Figs. 4 and 5, respectively.

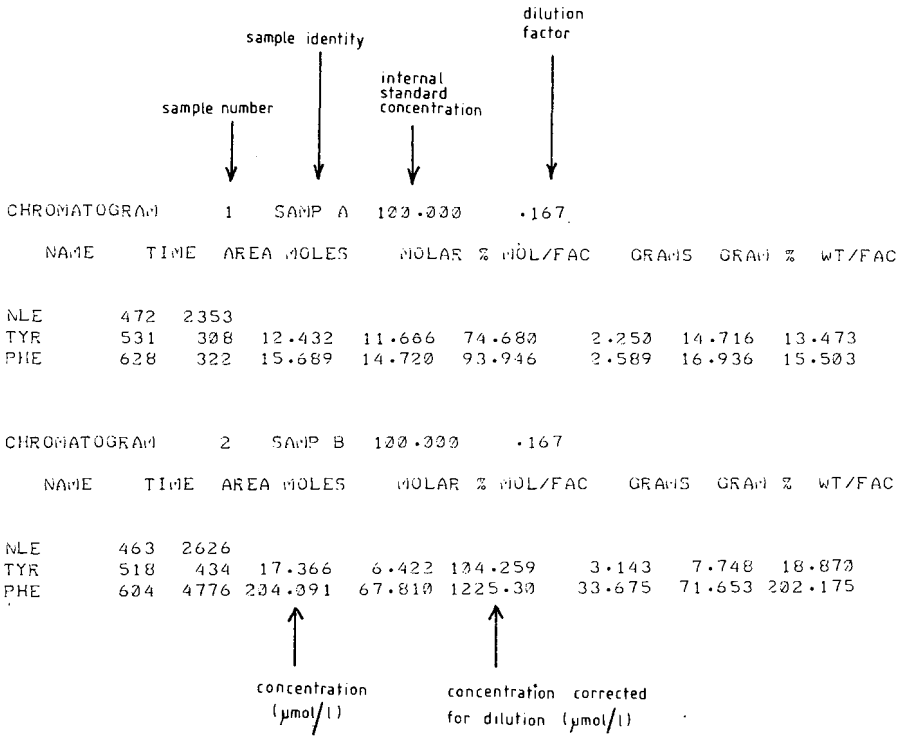

Fig. 5. Computer print-out data relating to profiles shown in Fig. 4. (A) Normal plasma sample and (B) PKU plasma sample.

METHOD ASSESSMENT

Linearity

A series of working standards covering the range 50—4000 μM were prepared and analysed by the method, including the sample preparation stage.

Precision

A plasma pool was divided into four samples. Phenylalanine and tyrosine were added to three of these to produce final concentrations covering the range 250—1000 μM. All four samples were analysed in replicate (20 assays).

Accuracy

A measure of the accuracy of the method was obtained from an assessment of the percentage recovery of the two amino acids, added to the pooled plasma used in the precision exercise.

In addition a commercially prepared control preparation containing known phenylalanine and tyrosine concentrations, was analysed in replicate and 50 plasma samples were analysed and compared with results obtained using the routinely used fluorimetric techniques [5, 6].

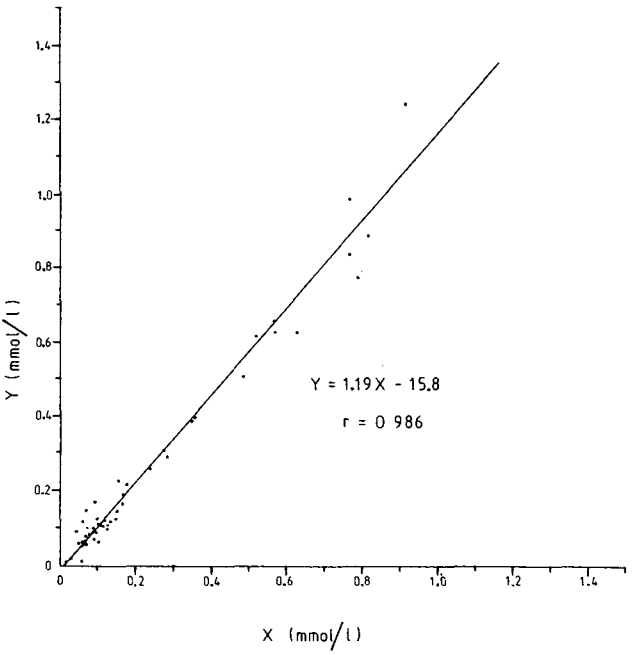

Fig. 6. Correlation between results obtained on 50 plasma sample analyses for tyrosine concentration, using the proposed method (*Y*-axis) and the fluorimetric method (*X*-axis).

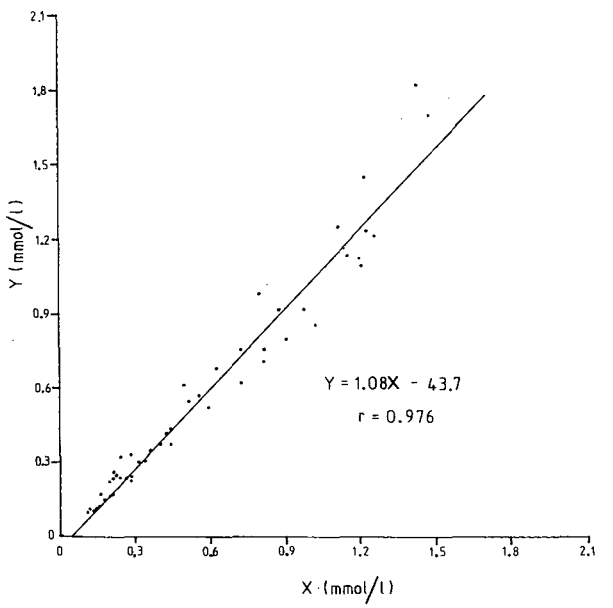

Fig. 7. Correlation between results obtained on 50 plasma sample analyses for phenylalanine concentration using the proposed method (*Y*-axis) and the fluorimetric method (*X*-axis).

RESULTS

Linearity

Using a recorder response range of 0—0.10 absorption units, i.e. mid gain

setting, it was found that although full-scale deflection on the recorder profile occurred at amino acid concentrations of about 1400 μM, computer response as printed sample concentrations was linear for both tyrosine and phenylalanine up to concentrations of 3000 μM.

Precision

Four pooled plasma samples containing varying concentrations of phenylalanine and tyrosine were analysed in replicate (20 assays). Mean phenylalanine concentrations were 79, 308, 532 and 984 μM, respectively, with corresponding coefficients of variation of 3.26%, 2.97%, 2.50% and 2.10%.

For the same samples, mean tyrosine concentrations were 81, 309, 536 and 987 μM, respectively, with corresponding coefficients of variation of 3.02%, 2.71%, 2.23% and 1.92%.

Accuracy

Recovery of phenylalanine and tyrosine added to a plasma pool containing 79 μM of phenylalanine and 81 μM of tyrosine, was determined at three concentration levels. For phenylalanine, percentage recovery (20 replicates) was 99.69 ± 2.94% at a concentration of 309 μM, 99.77 ± 2.51% at a concentration of 534 μM and 99.62 ± 2.10% at a concentration of 988 μM.

Corresponding figures for tyrosine were, 99.20 ± 2.71% at a concentration of 311 μM, 99.95 ± 2.23% at a concentration of 536 μM and 99.64 ± 1.92% at a concentration of 991 μM.

Analysis (20 replicates) of a Sigma Metabolite Control Type I (product No. S 3005), gave a mean phenylalanine concentration of 244.0 ± 7.9 μM and a mean tyrosine concentration of 265.4 ± 9.5 μM. The manufacturer's assigned values were 250 ± 20 μM for phenylalanine and 277 ± 22 μM for tyrosine.

A series of 50 samples from PKU patients at various levels of effective dietary control, were assayed by the proposed method and by the routinely used fluorimetric techniques (Sigma methods No. 60-F and No. 70-F). A number of these samples were supplemented with added tyrosine, to cover a wider range of concentrations. Correlation between the proposed method and the fluorimetric analyses was excellent over the range of concentrations studied (see Figs. 6 and 7); $r = 0.986$ for tyrosine, $r = 0.976$ for phenylalanine.

DISCUSSION

It was found that optimum separation of tyrosine from phenylalanine was achieved at pH 5.6 but that a stepwise elution system from an initial buffer pH of 3.9 through to a pH of 9.5 was necessary for adequate resolution of these two amino acids from preceding and subsequent plasma amino acid peaks (see Figs. 2 and 4). This ensured return to baseline conditions following elution of phenylalanine and prior to elution of basic amino acids and allowed sufficient resolution of norleucine internal standard from leucine and isoleucine peaks.

Using this system, elution and quantitation of norleucine, tyrosine and phenylalanine is completed within 60 min of sample aspiration. At this time, basic amino acids are still being eluted from the column by the pH 11.50 buffer

so that total removal of all amino acids contained in any sample is complete by 90 min from analysis commencement. In effect, a "slug" of regenerant buffer passes through the resin column between consecutive samples, so that at any given time, amino acids from two samples are being resolved on the same column. The system has been found to be reliable over batches of up to 40 samples with no deterioration in peak separation or resolution and no adverse effects on subsequent full physiological fluid analysis. The method achieves accurate, reproducible analysis of phenylalanine and tyrosine from 100 μl of plasma. The ability of the computer to determine peak area in relation to internal standard up to a sample concentration of 3000 μM, allows accurate determination over a wide range of concentrations, without recourse to further dilutions and repeat analyses.

Internal standard added to the sample at the deproteinisation stage compensates for concentration changes occurring in the protein-free supernatant inherent in this type of sample preparation and also compensates for any small variations in analysis conditions caused by fluctuations in pumping rates, sampling or colour development time. Of previously reported rapid chromatographic analyses of phenylalanine and tyrosine, only the recent system of Ersser [4] achieved sufficient resolution to employ an internal standard, but manual determination of peak height from a recorder trace rather than peak area is used for quantitation.

Although faster analysis systems have been published, such as those of Ersser [4] and Cooke and Raine [3], this system offers improved resolution and the ability to handle a wider range of sample concentrations. In addition, previous systems have been restricted to the analysis of phenylalanine and tyrosine, whereas in the system described here, the same resin column and instrumentation can be used for a full analysis of physiological fluid amino acids, interchange between analysis methods requiring a down time of less than 60 min.

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COMPARISON OF URINARY EXCRETION OF UV-ABSORBING CONSTITUENTS IN HEALTHY SUBJECTS AND PATIENTS WITH RHEUMATOID ARTHRITIS USING ANALYTICAL ISOTACHOPHORESIS

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SUMMARY

Analytical isotachophoresis has been applied to the separation of urinary constituents in healthy controls and patients with rheumatoid and osteoarthritis. Various methods of comparing isotachograms have been investigated. Significant differences have been demonstrated between the pattern of UV-absorbing components in patients with rheumatoid arthritis and healthy subjects.

INTRODUCTION

Isotachophoresis, where ions are separated on the basis of net mobility [1], has been widely used in the analysis of a variety of complex peptide and protein mixtures. Thus, analytical isotachophoresis has been employed for the quality control of peptide formulations [2, 3], in the analysis and isolation of peptides in the "middle molecule" fraction of uraemic body fluids [4, 5], and in the analysis of human serum, cerebrospinal fluid, urine and sweat proteins [6—10], enzymes [11, 12], tissue proteins [6] and soluble immune complexes [6, 13]. Conventional methods for analysing urine for UV-absorbing constituents, including proteins and peptides, are usually slow, insensitive and have a low resolving power. Isotachophoresis might be a better technique for studying these constituents. Proteinuria and peptiduria have been demonstrated in rheumatoid patients without renal involvement and are thought to mirror connective tissue involvement. Most studies have concentrated on particular proteins [14—20]. The pattern of excretion of urinary peptides and proteins in rheumatoid patients, and their diagnostic significance have not been investigated. The objective of this study was, therefore, to investigate the use of isotachophoresis in urine metabolic profiling and, in particular, to compare the pattern of excretion of constituents in the urine of patients with rheumatoid arthritis and in

healthy controls. A secondary objective was to investigate the use of various methods of data handling and pattern analysis for large numbers of samples.

EXPERIMENTAL

Clinical material

Urine samples (24-h collections using sodium azide as preservative) were obtained from ten healthy controls, ten patients suffering from rheumatoid arthritis, and six patients with osteoarthritis. Those suffering from rheumatoid arthritis were either housebound and contacted through their general practitioners, or had been admitted to hospital for assessment and treatment. Those with osteoarthritis were all hospital in-patients and acted as an "ill" control group. Informed consent, detailed medical and drug histories, and where possible results of any relevant investigations were obtained. Samples were stored frozen at −4° until analysed.

Controls

Creatinine levels of the 24-h urines were determined by the Jaffe reaction using a Technicon AutoAnalyzer II [21]. In order to ensure that complete 24-h collections had been obtained, no urine was used which had a creatinine level outside the mean ± 2 S.D. for the relevant subject group. Urines were tested for protein, haemoglobin, ketones, glucose and pH using Labstix (Ames Co.) in order to eliminate patients with renal involvement or other pathologies.

Analytical isotachophoresis

Urine samples to be analysed were first filtered through Millipore filters (0.45 μm, Millipore, London, Great Britain) to remove particulate matter. Then aliquots (10 μl) of an equivolume mixture of urine and aqueous Ampholine solution (2% v/v Ampholines, pH 3.5—10; LKB, Croydon, Great Britain) were analysed on the LKB Tachophor 2127. The leading electrolyte was hydrochloric acid (10 mmol/l) in methyl cellulose (2 g/l, high substitution; BDH, Poole, Great Britain) buffered to pH 8.5 with ammediol (2-amino-2-methyl-1,3-propanediol; Sigma, Poole, Great Britain). The terminating electrolyte was glycine (20 mmol/l; BDH) brought to pH 10.0 by the addition of barium hydroxide solid (BDH), and was filtered to remove particulate matter. The initial isotachophoresis run on the LKB Tachophor was at 150 μA until 10 kV was reached and then the current was reduced to 100 μA. A 40-cm capillary was used. Detection was by means of a UV detector (254 nm) connected to a chart recorder.

Data handling

The UV traces for each analytical run were converted to a numerical form (digitised) in the following manner. The UV trace was retraced using a chart recorder at constant speed with a variable resistor to alter the pen setting. The variable output from this retracing was fed to a data logger and readings were taken by the logger at intervals of the equivalent of one per second of the original run. From the data logger a paper tape of the digitised UV trace was then obtained.

The paper tape produced from the chart recording, i.e. the UV trace in numerical form, was then entered into a Data General Nova 2 computer and the UV isotachophoresis trace displayed on the storage oscilloscope. This was then checked against the original chart recorder output to ensure that all peaks had been entered and that none had been added. A second computer program designed to locate peaks in the digitised data, i.e. locate and measure the maxima in the peaks, was then run to produce (a) a "stick" diagram of peaks in the sequence in which they occurred in the run, and (b) a listing of the peak heights in the sequence in which they occurred in the run, with the peak height in absorbance units.

RESULTS

Typical isotachophoresis runs and "stick" diagrams from normal, rheumatoid arthritic, and osteoarthritic subjects are shown in Fig. 1. Up to fifty different UV-absorbing components were resolved in each of the urines. Whilst there was some variation in the isotachophoresis peaks in healthy controls, this was not great. No abnormal results were found on testing the urines with Labstix. The data obtained from each run were analysed statistically in three ways (Table I).

(1) In each analytical run, in all subject groups three distinct regions or groups of peaks could be recognised in the UV trace. The number of peaks in each region and the length of that region were measured and the area under all the peaks in each region was determined by cutting out and weighing. When comparing the results from the rheumatoid arthritic subjects with those from the normal controls, the most significant differences were found using the area/number of peaks in region II. The most significantly different parameter when comparing the results from the osteoarthritic subjects with the normals was the length/number of peaks in region III.

(2) When the sequences of UV peak heights in the isotachophoresis traces from patients with rheumatoid arthritis were combined with those from the normal subjects a master sequence was obtained. Then for each analytical run the sequence of peak heights was compared with the master sequence and the presence or absence of the peaks noted. Thus, for each disease or control group of subjects the frequency of the presence of each peak was found. Using the χ^2 test the significance of the differences in the presence or absence of each peak was analysed. The χ^2 values for approximately six peaks indicated that these peaks differed significantly in their presence in the two groups. However, because of the small number of runs, the total χ^2 value, i.e. the sum of all the χ^2 values, was not significantly different. Those peaks whose presence was shown to be significantly different in the two groups were then located in each analytical run.

(3) Using the listing of the peak heights in the sequence in which they occurred in the run, for each analytical run, each peak height was compared with the next. Thus, if the second of two adjacent peaks had a larger peak height than the first, this was regarded as an increase (+), and if the peak height of the second peak was smaller this was recorded as a decrease (−). This was attempted in order to define the overall shape of the UV isotachophoresis trace.

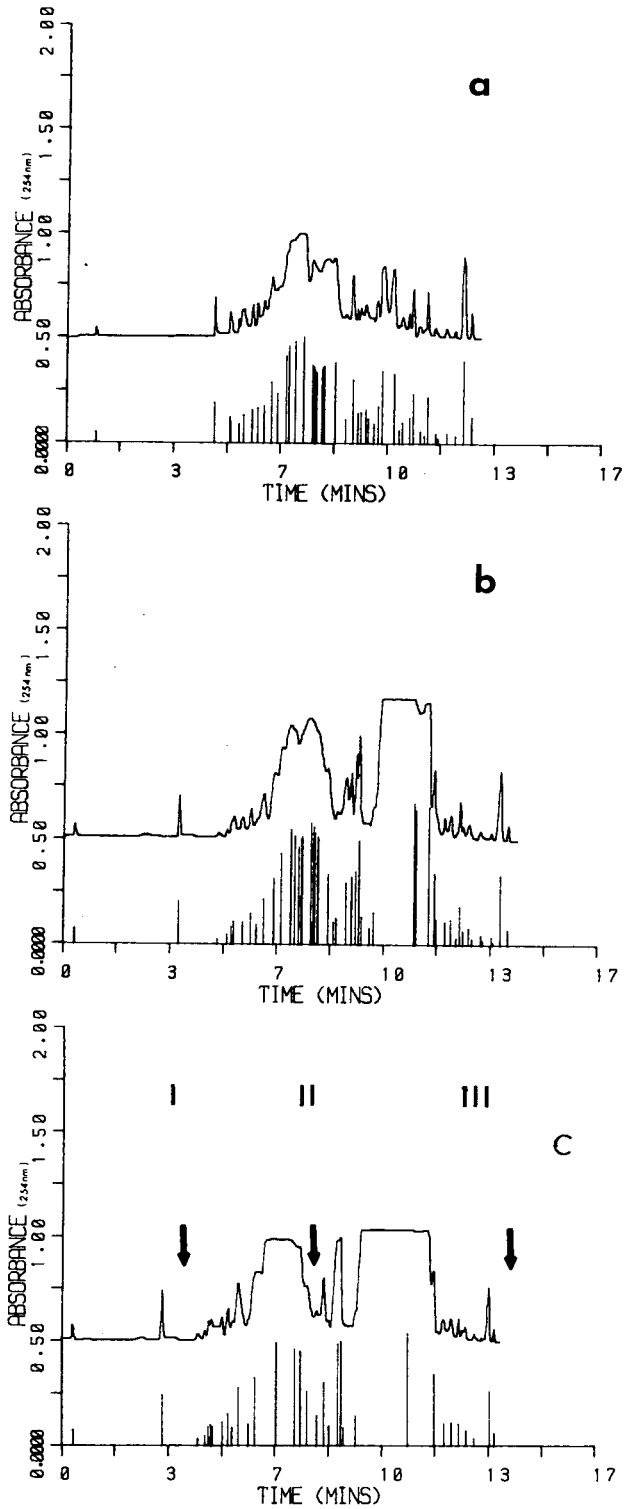
a
b
C
I
II
III
ABSORBANCE (234nm)
2.00
1.50
1.00
0.50
0.0000
0
3
7
10
13
17
TIME (MINS)

TABLE I

SIGNIFICANCE OF THE DIFFERENCES IN SELECTED VARIABLES IN ISOTACHOPHORETIC RUNS FROM RHEUMATOID ARTHRITIS AND OSTEOARTHRITIS URINES COMPARED TO NORMAL, HEALTHY CONTROLS (t-TEST)

Variable	Rheumatoid arthritis	Osteoarthritis
	P	P
Total length of run	0.1	0.1
Total number of peaks	0.1	0.02
Length of region		
I	0.05	0.1
II	0.1	0.1
III	0.1	0.1
Number of peaks in region		
I	0.1	0.1
II	0.1	0.05
III	0.1	0.1
Total length/total number of peaks	0.1	0.1
Length/number of peaks		
I	0.02	0.1
II	0.1	0.1
III	0.05	0.001
Area of region		
I	0.02	0.1
II	0.1	0.1
III	0.1	0.05
Area of region/number of peaks		
I	0.02	0.1
II	0.01	0.1
III	0.1	0.01

The total numbers of increases and decreases were noted for each analytical run for each subject group (Fig. 2). In some cases a series of increases (rises) or a series of decreases (falls) occurred together in a run; these were also noted (Fig. 3). These series were compared using the Kruskal Wallis Test [22] which showed no significant differences between the groups (Figs. 2 and 3).

Fig. 1. UV traces and "stick" diagrams from the isotachophoretic analysis of urines from (a) a healthy control, (b) a patient with rheumatoid arthritis, and (c) a patient with osteoarthritis. Arrows indicate the limits of regions I, II and III.

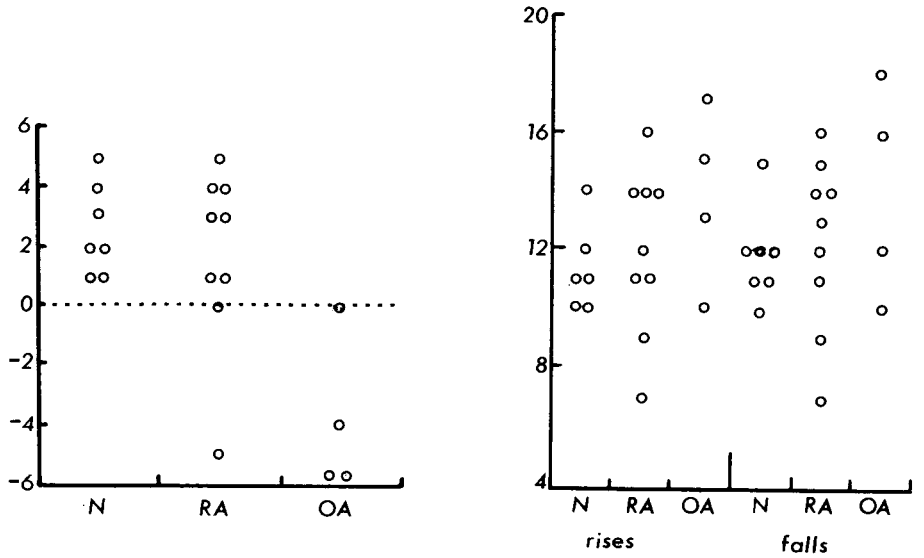

Fig. 2. Isotachophoresis: total number of increases (+) and decreases (−) in peak heights. N = Normal subjects; RA = rheumatoid arthritic patients; OA = osteoarthritic patients.

Fig. 3. Isotachophoresis: number of sequences of rises and falls in peak heights. N = Normal subjects; RA = rheumatoid arthritic patients; OA = osteoarthritic patients.

DISCUSSION

This study has shown that isotachophoresis is a high-resolution technique capable of separating urine into as many as fifty UV-absorbing components. Differences in the patterns of urine UV-absorbing constituents separated by isotachophoresis have been found between rheumatoid arthritic and osteoarthritic subjects as compared with normal, healthy controls. Six of the components showed significant differences between rheumatoid and normal subjects, but only one of these components was identified.

Comparison of UV traces from isotachophoretic runs is problematic since the location of a component on a trace is dependent upon the number and the concentration of other components present in the mixture being analysed. The presence of extra components will cause an expansion, and the absence of components a contraction of the trace.

If a large number of complex patterns from isotachophoretic runs are to be compared, which is necessary in order to overcome the problems of biological variation, some form of data handling is necessary. In this case, by digitising the data, locating the peaks in these data and measuring peak height, some data reduction was possible. Several statistical techniques were assessed in an attempt to define and compare the shapes of the UV traces. For small numbers of runs this is easily done by eye, but with a large number of runs with many peaks this becomes impossible. Whilst all three approaches tried in this study demonstrated differences between some of the UV-absorbing constituents of the subject groups, their main use was to indicate areas in the isotachophoretic run which differed between the groups. Further studies would be necessary to iso-

late and identify the constituents in these areas. More specific assays could then be developed to assess the value of measuring such constituents in the diagnosis and assessment of rheumatoid arthritis.

CONCLUSIONS

The use of isotachophoresis in the analysis of complex mixtures has, in the past, been hampered by problems inherent in the interpretation and comparison of results. The relatively simple data handling techniques described here have facilitated the comparison of UV traces from isotachophoretic runs and demonstrated significant differences in the pattern of urine UV-absorbing substances between patients with rheumatoid and osteoarthritis and healthy controls. It is envisaged that further refinement of the data handling techniques to include both UV and thermal traces will greatly enhance the usefulness of analytical isotachophoresis in metabolic profiling.

ACKNOWLEDGEMENTS

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CHROMBIO. 467

DETERMINATION OF OXPENTIFYLLINE AND A METABOLITE, 1-(5'-HYDROXYHEXYL)-3,7-DIMETHYLXANTHINE, BY GAS—LIQUID CHROMATOGRAPHY USING A NITROGEN-SELECTIVE DETECTOR

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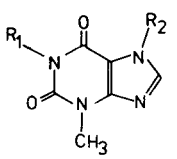
SUMMARY

A gas chromatographic method for the determination of oxpentifylline and a metabolite, 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine is described. Oxpentifylline, metabolite and internal standard are extracted from basified plasma into dichloromethane, then the metabolite and internal standard are converted to their O-trifluoroacetates. Analysis by gas—liquid chromatography using a nitrogen-selective detector allows quantification of oxpentifylline and 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine down to levels of 3 ng/ml and 3—10 ng/ml, respectively. The assay had been applied to plasma samples from volunteers after both intravenous and oral administration of oxpentifylline. The need to separate plasma from erythrocytes immediately after venipuncture sampling to prevent further metabolism of oxpentifylline is emphasized.

INTRODUCTION

Oxpentifylline (Trental®, pentoxifylline, I in Fig. 1) is widely used in the treatment of peripheral vascular disease. It increases blood flow by acting as a vasodilator and by reducing blood viscosity [1—5]. Metabolism of oxpentifyl-



	R_1	R_2
I	$CH_3COCH_2CH_2CH_2CH_2^-$	CH_3^-
II	$CH_3CH(OH)CH_2CH_2CH_2CH_2^-$	CH_3^-
III	$CH_2(OH)CH_2CH_2CH_2CH_2CH_2^-$	$CH_3CH_2CH_2^-$
IV	$CH_3CH_2CH_2^-$	$CH_3COCH_2CH_2CH_2CH_2^-$

Fig. 1. Structural formulae of oxpentifylline (I), metabolite I (II) and internal standards (III and IV).

line is extensive and a major metabolite found in blood is metabolite I, 1-(5'-hydroxyhexyl)-3,7-dimethylxanthine (II in Fig. 1) [6].

A sensitive assay was required to determine plasma levels of oxpentifylline in the presence of metabolites so that the bioavailability and pharmacokinetics in man could be studied. Previously reported assays have used both thin-layer chromatography [7, 8] and high-performance liquid chromatography [9]. This paper describes an assay using gas—liquid chromatography (GLC) which enables plasma levels to be determined from at least 3 μg/ml down to 3 ng/ml for oxpentifylline and 3—10 ng/ml for metabolite I. Whole blood freshly obtained by venipuncture (to which oxpentifylline had been added) metabolises oxpentifylline to metabolite I. No formation of this metabolite was observed in plasma. Thus erythrocytes should be separated from plasma immediately after sampling to prevent further formation of metabolite I.

MATERIALS AND METHODS

Reagents

All chemicals were of analytical grade and were used without further purification, unless otherwise stated. Dichloromethane (Fisons, Loughborough, Great Britain) was redistilled before use. A hexane solution of 5% (v/v) trifluoroacetic anhydride (TFAA; Aldrich, Gillingham, Great Britain) was freshly prepared for each batch of samples.

Standard solutions

A standard solution of oxpentifylline (100 μg/ml) in 0.01 *M* HCl was prepared by dissolving the solid material in 1 *M* HCl and making up to the required volume with distilled water. This solution was diluted with 0.01 *M* HCl to provide a calibration standard containing 10 μg/ml oxpentifylline. Standard solutions containing 10 μg/ml of metabolite I and the internal standard, 1-(6'-hydroxyhexyl)-3-methyl-7-propylxanthine (III in Fig. 1) were prepared in the same way. These solutions were stored at 0—5° and were stable for at least one month.

Extraction from plasma and derivatisation

Internal standard (1 μg in 100 μl of 0.01 *M* HCl) and plasma (2 ml) are pipetted into a 10-ml conical tube fitted with a screw cap having a PTFE-faced rubber liner (Sovirel, Paris, France), and thoroughly mixed. Dichloromethane (5 ml) and 1 *M* NaOH (0.5 ml) are added and the plasma extracted for 15 min using a mechanical rotary inversion mixer operating at a fixed speed of 20 rpm (Heto Rotamix, V.A. Howe, London, Great Britain). The phases are separated by centrifugation at 2000 *g* for 5 min then the upper plasma phase is aspirated and discarded. The remaining dichloromethane emulsion is broken by briefly shaking the tubes and centifuging again. After careful aspiration of the lipid plug, the dichloromethane is transferred to a 10-ml tapered test tube (Quickfit, Fisons, Great Britain) and evaporated under a gentle stream of nitrogen with the tubes in a water bath at 40°.

A freshly prepared solution of 5% (v/v) TFAA in hexane (1 ml) is added and the stoppers secured in place by means of spring clips (HWS, Labap, Hudders-

field, Great Britain). The residues are taken up in solution with the aid of a vortex mixer and esterification is completed by heating the tubes in a water bath at 60° for 10 min. Excess reagent is removed by evaporation under a stream of nitrogen with the tubes immersed in a water bath at 60°. The residues are taken up in toluene (50 μl) and aliquots (5 μl) of this solution are analysed by GLC.

Gas—liquid chromatography

Analyses were performed on a Perkin-Elmer F17 gas chromatograph equipped with a nitrogen—phosphorus detector. The inside of the glass column (2 m × 1.75 mm I.D.) was treated with a 5% (v/v) solution of dimethyldichlorosilane in hexane, then rinsed with methanol and acetone, and dried before packing with 3% OV-25 on Chromosorb W HP (100—120 mesh). The column inlet was repacked with fresh material after the analysis of each batch of samples and conditioned overnight before re-use. The polarising voltage on the nitrogen detector control box was set to position 3, and the bead current potentiometer set between 500 and 700 depending on the age of the bead. Helium was used as the carrier gas at a flow-rate of 25 ml/min and the air and hydrogen flow-rates to the detector were 80 and 3 ml/min, respectively. The oven temperature was 235°, and the injector/detector block was maintained at 250°.

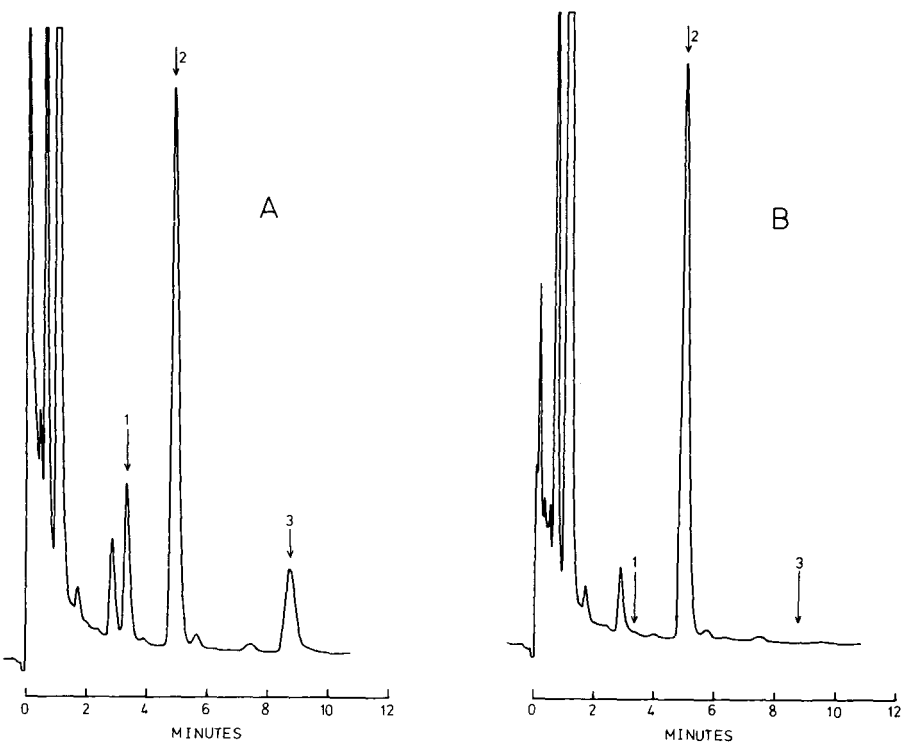


Fig. 2. Examples of chromatograms: A, extract of plasma (2 ml) to which had been added oxpentifylline (106 ng/ml), metabolite I (99.6 ng/ml) and internal standard (497 ng/ml); B, extract of blank plasma (2 ml) to which had been added internal standard (497 ng/ml) alone. The arrows 1, 2 and 3 indicate the retention times of metabolite I (O-trifluoroacetate), internal standard (O-trifluoroacetate) and oxpentifylline, respectively.

Under these conditions typical retention times of the O-trifluoroacetate of metabolite I, the O-trifluoroacetate of the internal standard, and oxpentifylline were 3.4, 5.0 and 8.8 min, respectively.

Chromatograms obtained from plasma extracts are shown in Fig. 2.

Quantification

Plasma levels of oxpentifylline and metabolite I were calculated from peak height measurements using response factors obtained by analysing, in parallel with the unknowns, blank plasma to which had been added oxpentifylline (1 μg in 100 μl of 0.01 *M* HCl) and metabolite I (1 μg in 100 μl of 0.01 *M* HCl).

Extraction efficiency

Oxpentifylline or metabolite I (1 μg) in 0.01 *M* HCl (100 μl) and human plasma (2 ml) were pipetted into screw-top test tubes and thoroughly mixed. 1 *M* NaOH (0.5 ml) and dichloromethane (5 ml) were added and the plasma was extracted for various times between 2 and 20 min on a rotary inversion mixer operating at 20 rpm. The phases were separated by centrifugation, the plasma aspirated, and a portion (4 ml) of the dichloromethane phase transferred to a tapered test tube containing a dichloromethane solution of internal standard (1 μg). The solvent was evaporated, the residue taken up in toluene (50 μl) and analysed by GLC. The recovery of oxpentifylline and metabolite I was estimated by comparison with non-extracted standards.

Metabolism of oxpentifylline by whole blood

Whole blood was obtained from a human volunteer by venipuncture and placed in a heparinised container to prevent coagulation. Plasma was obtained from a sample of the whole blood by centrifugation, and, within 30 min of sampling, portions (2 ml) of whole blood or plasma were added to a solution of oxpentifylline (0.8 μg) in 0.01 *M* HCl (100 μl), thoroughly mixed, and incubated for up to 2 h at 37°. Internal standard (1 μg) was added and the levels of oxpentifylline and metabolite I were then determined by the standard extraction and analysis procedure, except that the volume of 1 *M* NaOH added was increased to 1 ml to reduce the viscosity of the whole blood samples.

RESULTS AND DISCUSSION

Oxpentifylline and metabolite I have similar retention indices when chromatographed on OV-1, OV-17 and OV-25 stationary phases (table I). The use of more selective stationary phases other than the siloxanes was prevented because of the relatively high analysis temperature. Therefore it was necessary to derivatise one of the compounds to achieve separation by GLC. Metabolite I forms a trimethylsilyl ether and a trifluoroacetate, both of which are easily separated from oxpentifylline on siloxane stationary phases (Table I). The latter derivative was chosen as it was formed quantitatively and the excess reagent was easily removed.

The esterification of metabolite I and the internal standard was complete after 5 min, and no difference was observed between chromatograms of plasma extracts which had been reacted with 5% TFAA in hexane for 5 or 60 min.

TABLE I

RETENTION INDICES OF XANTHINE DERIVATIVES ON OV-1, OV-17 AND OV-25

Xanthine as shown in Fig. 1	Retention index on OV-1 at 240°	Retention index on OV-17 at 250°	Retention index on OV-25 at 250°
I	2420	3000	3230
II	2430	2995	3215
II O-trifluoroacetate	2300	2720	2875
II O-trimethylsilyl ether	2485	2915	—
III	2575	3125	3345
III O-trifluoracetate	2450	2850	3000
III O-trimethylsilyl ether	2635	3045	—
IV	2475	3000	3200

TABLE II

IN VITRO METABOLISM OF OXPENTIFYLLINE

Oxpentifylline (402 ng/ml) was added to each sample, and each result is the mean of two determinations.

Sample	Incubation time (min)	Oxpentifylline found (ng/ml)	Metabolite I found (ng/ml)
Plasma	120	421	Not detected
Whole blood	2	434	<1
Whole blood	120	337	75

Dilution of TFAA with hexane prevented the formation of side-reaction products.

Two compounds were available as possible internal standards; namely, 1-(6′-hydroxyhexyl)-3-methyl-7-propylxanthine (III in Fig. 1) and 1-propyl-3-methyl-7-(5′-oxohexyl)xanthine (IV in Fig. 1). The latter is the most suitable for the analysis of oxpentifylline, but it was not fully resolved from oxpentifylline on methylsiloxane or methylphenylsiloxane stationary phases. Thus, 1-(6′-hydroxyhexyl)-3-methyl-7-propylxanthine, analysed as its O-trifluoroacetate, was chosen as the internal standard, although it is more closely related to metabolite I than to oxpentifylline.

Human plasma containing either oxpentifylline or metabolite I (0.5 μg/ml) was basified and extracted with dichloromethane for various times. The results from this experiment, which are uncorrected for any volume change of the dichloromethane phase which may have occurred during extraction, show that an extraction time of only 2 min is required to extract oxpentifylline and metabolite I with efficiencies of 99% and 96%, respectively.

Incubation of oxpentifylline with either whole blood or plasma shows that the formation of metabolite I occurs only in the erythrocytes (Table II). It is therefore necessary to separate and remove the plasma from whole blood immediately after sampling to prevent further metabolism of oxpentifylline.

Accuracy and precision

The accuracy and precision of the method was determined by analysing blank human plasma to which had been added known amounts of oxpentifyl-

line and metabolite I. The results of six separate determinations at each plasma level are summarised in Tables III and IV. They show that plasma levels can be accurately determined from approximately 3 μg/ml down to 3 ng/ml for oxpentifylline, and from 3 μg/ml down to 3—10 ng/ml for metabolite I. The actual limit of the assay is determined by background interference which has been found to vary between batches of samples.

TABLE III

DETERMINATION OF OXPENTIFYLLINE ADDED TO BLANK PLASMA

Each result is the mean of six determinations.

Oxpentifylline added (ng/ml)	Oxpentifylline found (mean ± S.D., ng/ml)	Relative standard deviation	Mean recovery (%)
0	0.8 ± 0.6	0.8	
1.0	1.0* ± 0.4	0.4	100
3.1	3.0* ± 0.8	0.3	97
10.3	9.3* ± 0.4	0.4	90
30.6	27.6* ± 0.3	0.1	90
106	100 ± 3	0.03	94
309	304 ± 9	0.03	98
1050	1070 ± 40	0.04	102
3017	3063 ± 66	0.02	102

*These values have been corrected for a background level of 0.8 ng/ml.

TABLE IV

DETERMINATION OF METABOLITE I ADDED TO BLANK PLASMA

Each result is the mean of six determinations.

Metabolite I added (ng/ml)	Metabolite I found (mean ± S.D., ng/ml)	Relative standard deviation	Mean recovery (%)
0	Not detected		
2.9	3.4 ± 2.9	0.9	117
9.7	11.0 ± 1.1	0.1	113
28.9	30.5 ± 1.0	0.03	106
99.6	101 ± 1	0.01	101
291	300 ± 3	0.01	103
989	1022 ± 21	0.02	103
2843	2863 ± 85	0.03	101

Application of the method

The assay has been applied to the analysis of plasma from volunteers and patients after intravenous infusion of oxpentifylline and after single or multiple oral administration of various oxpentifylline preparations. In a typical experiment a volunteer was given oxpentifylline (200 mg) in a capsule and blood samples were collected over the following 24 h. Plasma was separated from the red blood cells by centrifugation immediately after withdrawal and stored deep frozen until analysed. A plasma profile obtained from one such experiment is shown in Fig. 3.

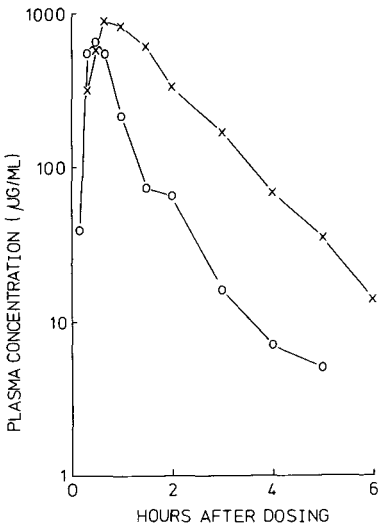


Fig. 3. Plasma levels of oxpentifylline (O) and metabolite I (×) in a volunteer after an oral dose of 200 mg of oxpentifylline.

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CHROMBIO. 473

DETERMINATION OF HEXAMETHYLMELAMINE AND METABOLITES IN PLASMA OR SERUM BY GAS—LIQUID CHROMATOGRAPHY WITH A NITROGEN-SENSITIVE DETECTOR

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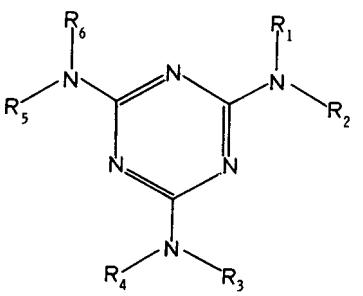
SUMMARY

A gas chromatographic method for the quantitative determination of hexamethylmelamine (HMM) and five of its metabolites in plasma (or serum) is described. After adjustment of the pH of the plasma sample to about 9.5, the compounds are extracted with chloroform containing 5% of isopropanol. Amyl alcohol is added to the extract, which is then evaporated until a small volume remains. An aliquot of this solution is injected into a gas chromatograph equipped with a nitrogen—phosphorus flame ionisation detector. Separation of the methylmelamines is achieved with a 10% Carbowax 20M—2% KOH column. By programming the oven temperature unnecessarily long retention times are avoided.

Using 1 ml of plasma, concentrations as low as 5 ng/ml of HMM and its metabolites can be quantitated. The method has been applied to the determination of HMM and metabolites in plasma of patients who received oral doses of HMM.

INTRODUCTION

Hexamethylmelamine (HMM) is an orally administered anti-tumor agent, which is active in different human malignancies and is used in the treatment of ovarian adenocarcinoma [1—4]. In vivo it is quickly converted into demethylated metabolites [5, 6], of which pentamethylmelamine (PMM) is also considered for therapeutic use [7]. With the exception of N^2,N^2,N^4,N^4-tetramethylmelamine ($TeMM_1$) all of the possible demethylated analogues of HMM have been recovered from urine samples of patients treated with HMM [6] (Fig. 1).



	Compound	R_1	R_2	R_3	R_4	R_5	R_6
1	Hexamethylmelamine (HMM)	CH_3	CH_3	CH_3	CH_3	CH_3	CH_3
2	Pentamethylmelamine (PMM)	CH_3	CH_3	CH_3	CH_3	CH_3	H
3	N^2, N^2, N^4, N^4-Tetramethylmelamine ($TeMM_1$)	CH_3	CH_3	CH_3	CH_3	H	H
4	N^2, N^2, N^4, N^6-Tetramethylmelamine ($TeMM_2$)	CH_3	CH_3	CH_3	H	CH_3	H
5	N^2, N^2, N^4-Trimethylmelamine ($TriMM_1$)	CH_3	CH_3	CH_3	H	H	H
6	N^2, N^4, N^6-Trimethylmelamine ($TriMM_2$)	CH_3	H	CH_3	H	CH_3	H
7	N^2, N^2-Dimethylmelamine (DMM_1)	CH_3	CH_3	H	H	H	H
8	N^2, N^4-Dimethylmelamine (DMM_2)	CH_3	H	CH_3	H	H	H
9	Monomethylmelamine (MMM)	CH_3	H	H	H	H	H
10	Melamine (MM)	H	H	H	H	H	H

Fig. 1. Structural formulas of the methylmelamines.

In a recent study the peak plasma concentrations of HMM obtained after oral administration of the drug were found to differ markedly between patients [8]. Varying (incomplete) absorption from the gut or differences in metabolic rates (first pass effect) could account for this. The metabolism of HMM might be influenced by other drugs, such as 5-fluorouracil, methotrexate and cyclophosphamide, when co-administered with HMM in combination chemotherapy. The aim of the present investigation was to develop a method for the quantitative determination of HMM and a number of its metabolites in plasma. The metabolic conversion of HMM can then be followed after administration of the drug in various dosage forms, and in different therapy schemes.

A number of chromatographic methods for the analysis of HMM and/or the other methylmelamines have been described [5, 6, 8—16]. In recent studies [8, 10], the determination of HMM and of PMM in plasma by gas chromatography with nitrogen—phosphorus detection was reported. In the present study we also used a gas chromatograph equipped with a nitrogen-sensitive detector. We were

able to quantitate HMM and five of its metabolites in concentrations as low as 5 ng/ml plasma. The method was applied to plasma samples from patients following oral administration of HMM.

MATERIALS AND METHODS

HMM was obtained from Ofichem (Gieten, The Netherlands; batch No. 790205). The metabolites of HMM used in this investigation were a gift of Dr. D.E.V. Wilman (Institute of Cancer Research, Royal Cancer Hospital, London, Great Britain). Chloroform and methanol (both nanograde quality) were from Mallinckrodt (St. Louis, Mo., U.S.A.). Isopropanol, zur Analyse (Merck, Darmstadt, G.F.R.) and amyl alcohol (Brocacef, Maarssen, The Netherlands) were distilled from glass prior to use. Sodium hydroxide (zur Analyse, Merck) and pethidine hydrochloride (Brocacef) were used without further purification. Centrifuge tubes of 7 ml capacity, with glass stoppers and the conical ends drawn to a fine point, were used.

Gas chromatography

A Hewlett-Packard Model 5710A gas chromatograph equipped with a Model 18789A dual nitrogen—phosporus flame ionisation detector was used. The glass column (120 cm × 1.9 mm I.D.) was silanized and packed with 10% Carbowax 20M—2% KOH on 80—100 mesh Chromosorb W AW (Chrompack, Middelburg, The Netherlands) and conditioned overnight at 230°.

The operating conditions were: injection port temperature, 285°; detector temperature, 360°; carrier gas (nitrogen) flow-rate, 30 ml/min; hydrogen flow-rate, 3.6 ml/min; air flow-rate, 55 ml/min. The detector voltage (d.c.) was set at about 14 V.

An oven temperature program was maintained following each injection: 2 min isothermal heating at 194°, then 4°/min from 194—230°, and 8 min isothermal heating at 230°. A 4-min period of cooling and stabilizing was maintained between injections.

Procedure

A 1.0-ml volume of plasma or serum (stored at −20° until analysis) was transferred to a centrifuge tube and mixed with 100 μl methanol and 10 μl 2.0 *N* sodium hydroxide (resulting pH 9.5—9.7). After the addition of 3.0 ml chloroform—isopropanol (95:5), containing 290 ng/ml pethidine hydrochloride as internal standard, the contents of the tube were shaken manually for 1 min. Following centrifugation (2500 *g*, 5 min) the upper aqueous layer was removed; the organic phase was transferred to a centrifuge tube and 50 μl amyl alcohol was added. After mixing, the solvent was removed under a stream of nitrogen until a volume of about 50 μl remained, of which 2 μl were injected into the gas chromatograph.

Calibration curve

Pooled serum samples (1 ml) were transferred to centrifuge tubes and spiked with 5—100 μl of a methanolic solution containing HMM (5.05 μg/ml), PMM (2.50 μg/ml), $TeMM_1$ (0.97 μg/ml), $TeMM_2$ (2.50 μg/ml), $TriMM_1$ (1.08 μg/ml)

and $TriMM_2$ (4.94 μg/ml). After the addition of methanol to a total amount of 100 μl, the samples were treated further as described under Procedure. Following chromatography the peak heights were measured and the peak-height ratios of HMM and its metabolites to the internal standard were plotted against the concentration in the samples.

Determination of the absolute recoveries of HMM and metabolites

To 1-ml pooled serum samples in centrifuge tubes, 100-μl portions of the methanolic solution described above were added. After addition of 10 μl 2 *N* sodium hydroxide and mixing, 3.0 ml chloroform—isopropanol (95:5) were added and the tubes were shaken manually for 1 min. Following centrifugation (2500 *g*, 5 min), the aqueous layer was removed and the organic phase transferred to a centrifuge tube. The solvent was blown off (nitrogen), taking care that the dried samples did not remain under the nitrogen stream.

The residues in the tubes were reconstituted in 50.0-μl methanol portions, containing 17.2 μg/ml pethidine hydrochloride as internal standard. Of this solution 2 μl were injected. From the resulting peak-height ratios (HMM and metabolites to the internal standard), and the peak-height ratios obtained after chromatography of a standard solution of HMM and its metabolites and pethidine hydrochloride, the absolute recoveries of each of the methylmelamines under investigation were calculated.

RESULTS AND DISCUSSION

The Carbowax—KOH stationary phase provides excellent chromatographic properties, not only to HMM and PMM as reported previously [10], but also to seven other methylmelamines and the internal standard, pethidine. The peaks of all compounds mentioned in Fig. 1 and of pethidine were completely separated under the prevailing conditions. The retention times are given in Table I.

TABLE I

RETENTION TIMES AND ABSOLUTE RECOVERIES FROM SERUM OF METHYLMELAMINES AND PETHIDINE

Compound*	Retention time (min)	Recovery (%)** ± S.D.
HMM	2.7	85.8 ± 4.3
PMM	4.8	82.9 ± 2.3
$TeMM_1$	6.6	85.3 ± 3.0
$TeMM_2$	7.6	84.6 ± 2.9
$TriMM_1$	9.6	83.8 ± 4.0
$TriMM_2$	10.8	74.4 ± 3.2
DMM_1	11.8	
MMM	13.1	
MM	20.9***	
Pethidine	3.8	

*The notation of the compounds is explained in Fig. 1; N^2,N^4-dimethylmelamine (DMM_2) was not available.

**The mean values and standard deviations (S.D.), obtained from 6 experiments, are given.

***The isothermal period at the end of the oven temperature program was extended to 12 min.

A chromatogram obtained after injection of the methanolic solution of HMM and five of its possible metabolites is shown in Fig. 2A.

Chromatograms of a control patient plasma sample and of one, taken from a patient 3 h after administration of an oral dose of 300 mg HMM, are shown in Fig. 2B and C, respectively. Peaks with retention times corresponding to HMM, PMM, $TeMM_2$, $TriMM_1$ and $TriMM_2$ were apparent in the chromatogram of the patient sample (Fig. 2C). Using a 2% OV-225—1% OV-17 column, the retention times of the relevant peaks in the chromatogram of the plasma sample were the same as those obtained after injection of a solution of HMM and its analogues onto the same column.

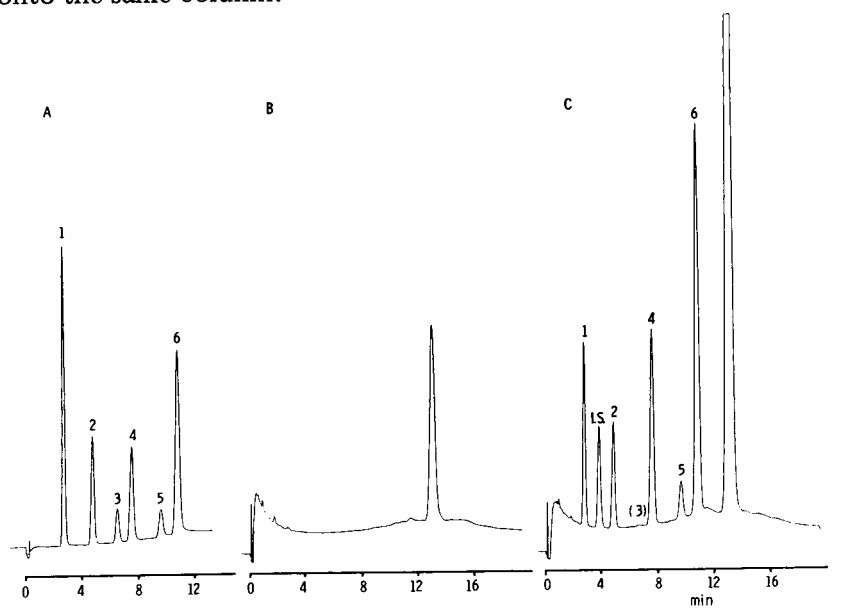


Fig. 2. Chromatograms obtained from (A) a methanolic solution of HMM and 5 demethylated analogues, (B) a control patient plasma sample, and (C) a patient plasma sample 3 h after the oral administration of 300 mg HMM. The peak numbers correspond to the compounds as denoted in Fig. 1 (I.S. = internal standard).

After extraction of aqueous solutions of DMM_1, MMM and MM, following the procedure described under Materials and methods, about 30% of DMM_1, 1% of MMM and no measurable amounts of MM were recovered. No significant peaks of MMM and MM could therefore be expected. The DMM_1 peak, if at all present, coincides with a large plasma peak.

In most chromatograms obtained from patient samples no $TeMM_1$ peak could be discerned. In some of the chromatograms a small peak appeared with the same retention time as the $TeMM_1$ peak. Assuming the corresponding compound to be $TeMM_1$, its concentration always remained under the 5 ng/ml plasma level.

Ames and Powis [10] stated that oven temperatures higher than 190° can not be used because of the limited stability of the stationary phase. The upper temperature limit of 230° in our investigation is, however, consistent with the

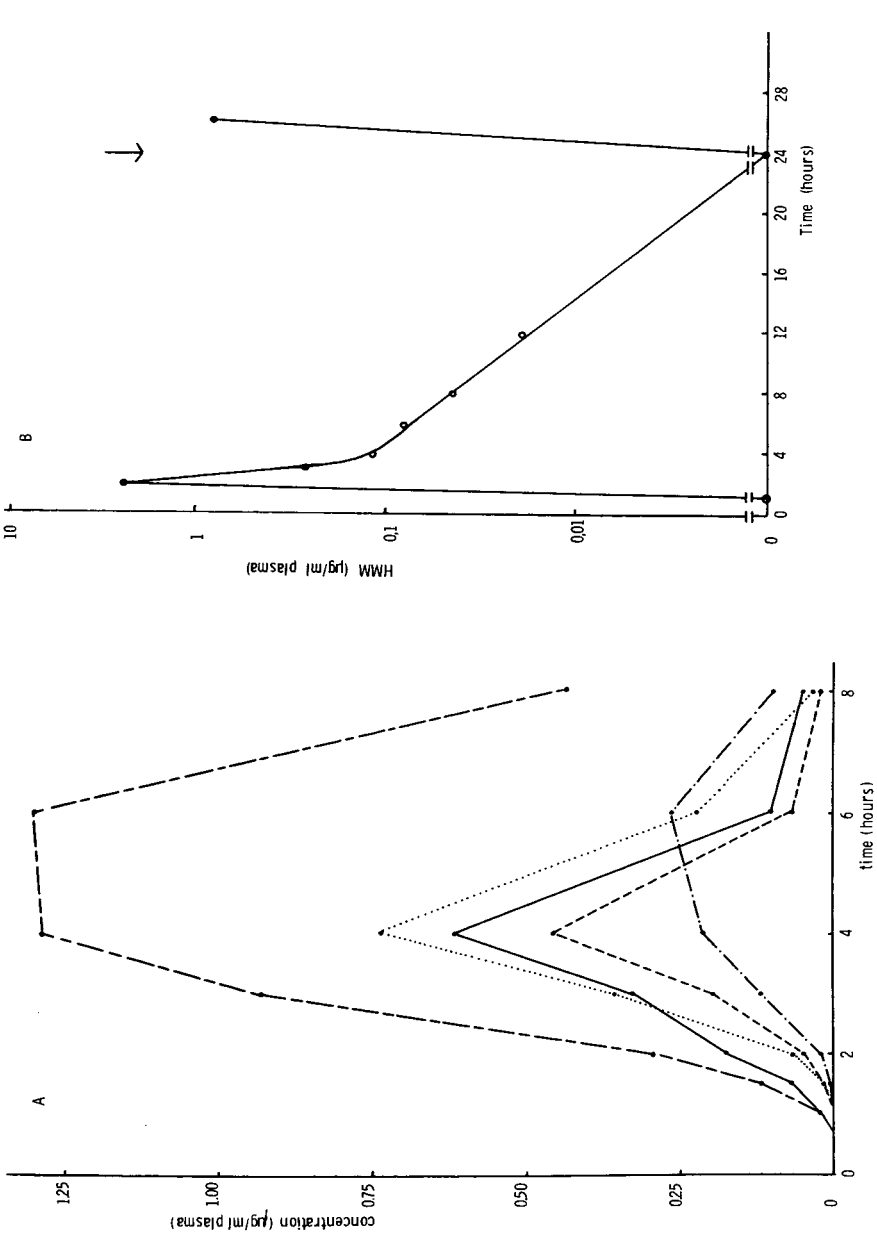


Fig. 3. (A) Plasma concentration—time curves for HMM (1), PMM (2), $TeMM_2$ (3), $TriMM_1$ (4), and $TriMM_2$ (5) from a patient after an oral dose of 300 mg of HMM. (B) Plasma concentration—time curve for HMM from the same patient after oral doses of 300 mg, given in a second medication sequence. The arrow denotes administration of second dose.

highest allowable temperature as mentioned by the manufacturer. We have used the same column daily for over three months without any perceptible loss of performance.

Except for the chromatographic column no glassware was silanized prior to use. Irregular recoveries of HMM, its metabolites and pethidine, due to adsorption onto the glass, were prevented by the addition of small volumes of an alcohol (methanol, isopropanol or amyl alcohol) during the various phases of the procedure.

Before extraction the pH of the plasma was adjusted to pH 9.5—9.7, which is more than four units above the reported pK_a value of HMM [17]. Mixing the serum samples with the extraction solvent by means of a vortex type mixer often resulted in the formation of emulsions, which could not be broken by centrifugation. No disturbing emulsification occurred if the tubes were shaken by hand. In view of the somewhat volatile nature of HMM, PMM, and particularly of the internal standard, pethidine, amyl alcohol was added to the organic extraction mixture before evaporation of the solvent; evaporation was stopped before the samples became entirely dry.

Calibration curves for HMM, PMM, $TeMM_1$, $TeMM_2$, $TriMM_1$ and $TriMM_2$ were constructed using the data obtained after the analysis of 10 pooled serum samples, spiked with HMM and its metabolites (5 different concentrations; 2 samples of each concentration). The calibration curves proved to be straight lines ($r^2 > 0.99$), passing through the origin. The curves could be extended to much higher concentrations of HMM and its metabolites than those obtained by adding to the samples 100 μl of the methanolic solution described under Materials and methods.

Five of the compounds which were investigated showed absolute recoveries of more than 80% (Table I). The absolute recovery of $TriMM_2$ was somewhat less, 74.4%, but still acceptable.

The reproducibility of the assay was examined by analyzing two series of six serum samples each, to which were added 10-μl and 100-μl portions of the methanolic solution (see Materials and methods) of HMM and metabolites, respectively. The results are shown in Table II.

TABLE II

PEAK-HEIGHT RATIOS (PHR) OF HMM AND METABOLITES TO PETHIDINE (INTERNAL STANDARD), AND COEFFICIENTS OF VARIATION (C.V.)

Results obtained with serum samples (1 ml) spiked with 10 μl or 100 μl of a methanolic solution of HMM and metabolites.

Compound	10 μl			100 μl		
	Concentration (ng/ml)	PHR*	C.V. (%)	Concentration (ng/ml)	PHR*	C.V. (%)
HMM	50.5	0.249	3.4	505	2.50	1.3
PMM	25.0	0.118	5.1	250	1.26	4.8
$TeMM_1$	9.7	0.040	5.0	97	0.409	3.2
$TeMM_2$	25.0	0.112	5.3	250	1.19	1.6
$TriMM_1$	10.8	0.038	7.9	108	0.327	4.3
$TriMM_2$	49.4	0.187	5.9	494	2.05	2.9

*Each value is the mean of 6 determinations.

The sensitivity of the method is such, that about 5 ng/ml plasma of each of the compounds could be quantitated with a coefficient of variation not exceeding 20% (results not shown). When analyzing plasma samples containing 5 ng/ml HMM, about 200 pg HMM is finally injected into the gas chromatograph. The detection limit for HMM under the prevailing chromatographic conditions was 30—40 pg HMM (signal-to-noise ratio, 5).

In order to examine the selectivity of the method, solutions of a number of compounds were injected and chromatographed following the same oven temperature program. These compounds were: metoclopramide, triethylperazine, prochlorperazine, 5-fluorouracil, glaphenine, triazolam, nitrazepam, flurazepam, doxorubicine, methotrexate, cyclophosphamide, cis-platinum, acetaminophen, acetophenetidin, caffeine, phenobarbital and bisacodyl. Of these compounds only acetophenetidin (retention time, 11.3 min) showed some interference with one of the methylmelamine peaks in the chromatogram.

The stability of the final amyl alcohol solutions obtained after extraction and evaporation was examined. Following chromatographic analysis some of the samples were stored for 24 h at room temperature, and then re-injected into the gas chromatograph. The peak-height ratios always remained constant within the experimental error, indicating that the amyl alcohol solutions are stable long enough to allow automation and the processing of large numbers of samples.

In Fig. 3A plasma concentration—time curves are shown for HMM, PMM, $TeMM_2$, $TriMM_1$ and $TriMM_2$, constructed from the analysis results of plasma samples of a 47 year old patient (55 kg) with normal renal function, who had received an oral dose of 300 mg HMM. After about 0.5 h, HMM and its metabolites appeared in the plasma samples. PMM, $TeMM_2$ and $TriMM_2$ levels were higher than the $TriMM_1$ and $TeMM_1$ levels, the latter compound being virtually absent. After having received no HMM for several weeks, a 14-day period started in which this patient were given daily doses of HMM. The plasma concentration of HMM, plotted on a logarithmic scale against time, after the first dose of 300 mg is shown in Fig. 3B. As reported before [8], the maximum plasma concentration was reached within a few hours, after which HMM disappeared from the plasma following a biphasic curve, indicating that drug absorption was fast as compared with drug distribution over the central and peripheral compartments. The halflife of the β-phase of elimination, determined graphically, was 2.9 h. HMM had practically disappeared from the plasma after 24 h, when the next dose was administered.

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SIMULTANEOUS LIQUID CHROMATOGRAPHIC ANALYSIS FOR CARBAMAZEPINE AND CARBAMAZEPINE 10,11-EPOXIDE IN PLASMA AND SALIVA BY USE OF DOUBLE INTERNAL STANDARDIZATION

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SUMMARY

High-performance liquid chromatography (HPLC) was used for simultaneous quantitation of carbamazepine (CBZ) and carbamazepine 10,11-epoxide (CBZ-EP) in plasma and saliva. Because concentrations of CBZ can greatly exceed those of CBZ-EP after single doses, two internal standards, lorazepam and N-desmethyldiazepam were added to all samples. Following extraction with chloroform, the components are separated on a μBondapak CN column with a mobile phase composed of 30% acetonitrile in water. Total chromatography time is 10 min. Concentrations of CBZ and CBZ-EP as low as 18 and 56 ng/ml, respectively, can be detected using 0.5 ml of plasma or saliva. The maximum within-day and day-to-day coefficients of variation for both compounds are 6.3 and 7.0%, respectively. Specificity of the method was supported by a significant correlation ($r = 0.99$) between assay results of the present method and those of a previously published HPLC assay. Application of the method to protein binding and salivary measurements in a single-dose CBZ disposition study is demonstrated.

INTRODUCTION

Carbamazepine (CBZ) is an anticonvulsant drug that has been shown to be as effective as phenytoin or phenobarbital in the treatment of grand mal and complex partial seizures [1]. The utility of plasma CBZ measurements for therapeutic monitoring of patients with convulsive disorders has been demonstrated [2]. Optimum seizure control usually occurs when plasma CBZ

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concentrations are maintained in the range of 4—12 μg/ml, while concentrations greater than 12 are associated with minor and major toxicities [3]. Adjustment of the dosage, based on the determination of plasma CBZ concentrations is therefore considered very important.

The relationship between CBZ plasma concentrations and therapeutic effect is complicated by the formation of a pharmacologically active metabolite, carbamazepine 10,11-epoxide (CBZ-EP). In rats, this metabolite has been found to display anticonvulsant properties comparable to those of the parent drug [3]. Although this activity has not been confirmed in man, it may be possible that measurement of both CBZ and CBZ-EP in plasma or saliva would be more useful in the clinical management of epileptic patients [4, 5].

Previously reported techniques for measuring CBZ and/or CBZ-EP in biological fluids include gas—liquid chromatography (GLC) [6, 7], enzyme-mediated immunoassay [8], and more recently high-performance liquid chromatography (HPLC) [9—11]. Because of its simplicity, specificity and ability to separate thermally labile compounds at room temperature, HPLC is often the assay method of choice in a clinical laboratory. This report describes a rapid and sensitive HPLC method for simultaneous analysis of CBZ and CBZ-EP in both plasma and saliva. Because concentrations of CBZ-EP can be much smaller than those of the parent drug following single doses of CBZ, two internal standards were employed to facilitate more reliable quantitation of both compounds simultaneously. Application of the method to a single-dose CBZ disposition study is demonstrated.

EXPERIMENTAL

Chemicals and reagents

CBZ and CBZ-EP were gifts from Geigy Pharmaceuticals (Ardsley, N.Y. U.S.A.). Lorazepam (LOR) was donated by Wyeth Laboratories (Philadelphia, Pa., U.S.A.) and N-desmethyldiazepam (ND) was obtained from Roche Laboratories (Nutley, N.J., U.S.A.). Acetonitrile and chloroform, ultraviolet grade, were purchased from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.) and reagent grade tribasic sodium phosphate was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.).

Drug standards

Stock solutions of CBZ and CBZ-EP were prepared in methanol at concentrations of 400 and 100 μg/ml, respectively. These solutions were further diluted with water to produce solutions of the desired concentration. Stock solutions of the internal standards, LOR and ND, were prepared in methanol at concentrations of 100 μg/ml and a working internal standard solution was prepared in water to contain 10 μg/ml of LOR and 30 μg/ml of ND. A chromatography drug reference mixture containing 25 μg/ml of CBZ and CBZ-EP, 5 μg/ml of LOR and 15 μg/ml of ND was prepared in methanol. All solutions were stored in amber-colored glass bottles at 4°.

Apparatus

A Model 841 high-pressure liquid chromatograph (DuPont Instruments,

Wilmington, Del., U.S.A.) equipped with a 254 nm ultraviolet detector and fitted with a Rheodyne Model 7120 sample injection valve (Perkin-Elmer, Norwalk, Conn., U.S.A.) was used. Analyses were performed on a μBondapak CN (particle size 10 μm) column from Waters Assoc. (Milford, Mass., U.S.A.). Detector output was recorded at 1 mV with an Omniscribe recorder (Houston Instruments, Austin, Texas, U.S.A.).

Other equipment included 13 × 150 mm PTFE-lined screw-capped culture tubes, 15-ml conical centrifuge tubes, a reciprocating shaker, bench-top vortex-type mixer, high-speed centrifuge, and an analytical evaporating bath with a nitrogen gas source.

Extraction procedure

Saliva, plasma or buffer solution (0.5 ml) was transferred to a glass culture tube containing 25—50 μl of the aqueous internal standard mixture and 0.5 ml of aqeuous saturated tribasic sodium phosphate solution. After gentle mixing, 7 ml of chloroform was added and the mixture shaken for 15 min. After centrifugation (1200 *g*, 10 min) the aqueous (top) layer was aspirated and discarded. The remaining chloroform layer was transferred to a conical glass tube and evaporated at 40° under a gentle stream of nitrogen. The sides of each tube were rinsed with an additional 1 ml of chloroform and evaporated again. The residue was dissolved in 100 μl of mobile phase, vortex-mixed for 30 sec, and 50 μl of the extract injected into the chromatograph.

Chromatography and quantitation

All chromatography was performed at room temperature. The mobile phase consisted of 30% acetonitrile in distilled deionized water and the flow-rate was 1.2 ml/min. Column effluent was monitored at 254 nm with detector sensitivity set at 0.04—0.16 a.u.f.s. Chart speed of the recorder was 20 cm/h.

Plasma standards were prepared by adding CBZ and CBZ-EP to drug-free pooled plasma to give final concentrations ranging from 0.5 to 30 μg/ml. The standards were processed according to the procedure, peak heights measured, and the peak height ratios of CBZ-EP:LOR and CBZ:ND were calculated. Standard curves relating peak height ratios to respective drug concentrations were used to calculate each drug concentration in the unknown samples.

Recovery

CBZ, CBZ-EP, LOR and ND were added to drug-free plasma, saliva and phosphate buffer and then analyzed according to the procedure but without any added internal standards. Carefully measured aliquots of the reconstituted extracts were injected and peak heights corresponding to each compound were measured. Absolute recovery was calculated by comparing these peak heights with peak heights obtained by direct injection of pure drug standards.

Precision

Precision of the method was evaluated by analysis of plasma standards containing both CBZ and CBZ-EP at concentrations of 0.5 and 30 μg/ml. Within-day precision was obtained by analyzing plasma standards 10 times in one day. The same plasma standards were stored in aliquots at —20° in glass

tubes and analyzed once daily for 11 days over 2 months to assess day-to-day precision.

Interferences

Seven anticonvulsant and sedative drugs, commonly used with CBZ, were tested for potential interferences with our procedure by comparing the retention times for methanolic standards of these drugs with those of CBZ, CBZ-EP, LOR and ND.

Comparative assays

Twenty saliva and plasma samples were randomly obtained from 15 epileptic patients and analyzed for CBZ and CBZ-EP by the HPLC method of Kabra and Marton [12] and by our method. According to the former method, components were extracted from acidified plasma and separated on a C_{18} μBondapak column (Waters Assoc.) with a mobile phase of 37% acetonitrile in water. Although this method was originally developed for quantitation of CBZ only, it was possible to quantitate the epoxide metabolite as well.

Clinical application

One volunteer took 400 mg of CBZ (Tegretol®, Geigy Pharmaceuticals) orally. Samples of plasma and saliva were obtained over a 72-h period and stored at −20° until analyzed. Protein binding of CBZ was determined by equilibrium dialysis of plasma against an equal volume of isotonic phosphate buffer, pH 7.4, in a water bath maintained at 37°. Analysis of buffer and plasma after 24 h allowed calculation of free CBZ concentrations.

RESULTS

Resolution and sensitivity of the chromatographic system were determined daily by injection of 25 μl of the standard drug reference mixture. A typical chromatogram of the mixture is shown in Fig. 1A. This same mixture was extracted from drug-free plasma according to the procedure and chromatographed; the resulting chromatogram is shown in Fig. 1C. Chromatographic peaks are sharp and symmetrical allowing use of peak heights rather than peak area to quantitate detector response. Extracts of drug-free plasma yielded no interference from endogenous plasma components with the exception of a small peak having a retention time slightly longer than that of CBZ-EP, as shown in Fig. 1B. Although complete separation of this component from CBZ-EP can be obtained by decreasing the flow-rate, it only represents 0.06 μg/ml of CBZ-EP, thus contributing minimal interference with plasma CBZ—EP concentrations during chronic dosing of CBZ. Retention times for CBZ-EP, CBZ, LOR and ND under our conditions are 4.0, 5.2, 6.1, and 9.5 min, respectively. With use of a mobile phase at pH 5, column-life is estimated to be at least 9 months with daily use.

Linearity of detector response was evaluated by injecting 50 μl of various methanolic standards containing amounts of CBZ and CBZ-EP ranging from 0.3 to 30 μg. Detector response (peak height) was linear over this range for both compounds, with both curves passing through the origin. Peak height ratios of

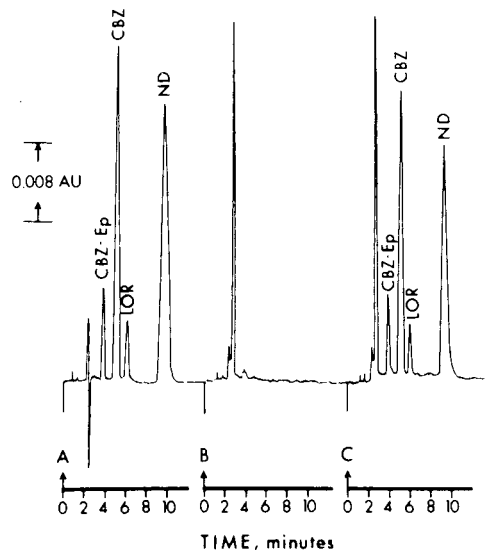

Fig. 1. Chromatograms of carbamazepine and its 10,11-epoxide: (A) standard mixture of compounds, CBZ and CBZ-EP, 0.625 μg; LOR, 0.125 μg; ND, 0.375 μg; (B) extract of drug-free plasma; (C) standard mixture extracted from plasma.

CBZ-EP:LOR and CBZ:ND from extracted samples were also linearly related to concentration over the range of 0.5—30 μg/ml.

The limits of detection, allowing a signal-to-noise ratio of 4, are 9 and 28 ng for CBZ and CBZ-EP, respectively. The sensitivity allows for quantitation of at least 0.02 μg/ml of CBZ using 0.5 ml of sample. Because of the plasma impurity that is partially eluted with CBZ-EP, the sensitivity of detection for this metabolite is 0.12 μg/ml using 0.5 ml of plasma and 0.06 μg/ml using buffer or saliva.

TABLE I

ANALYTICAL RECOVERY

Drug	Concn. (μg/ml)	*n*	Recovery (%)		
			Saliva	Buffer	Plasma
Carbamazepine 10,11-epoxide	1.0	5	103	99.8	109
	10	5	100	96.3	100
Carbamazepine	1.0	5	106	100	103
	10	5	100	97	99.6
Lorazepam	0.2	4	101	101	103
	2.0	4	97.6	109	97.6
N-desmethyldiazepam	0.6	4	104	100	104
	6.0	4	102	107	98.7

TABLE II

PRECISION OF ASSAY FOR CARBAMAZEPINE AND ITS 10,11-EPOXIDE

	Carbamazepine			10,11-Epoxide		
	Concn. (μg/ml)	*n*	C.V. (%)	Conc. (μg/ml)	*n*	C.V. (%)
Within-day	0.5	10	4.4	0.5	10	6.3
	30	10	2.4	30	10	5.0
Day-to-day	0.5	11	7.0	0.5	11	5.0
	30	11	3.1	30	11	4.6

TABLE III

RETENTION TIMES FOR SELECTED DRUGS

Drug	Retention time (min)
Primidone	3.2
Ethosuximide	3.4
Carbamazepine 10,11-epoxide	4.0
Phenobarbital	4.7
Carbamazepine	5.2
Oxazepam	5.5
Phenytoin	5.9
Lorazepam	6.2
Clorazepate	7.0
N-Desmethyldiazepam	9.5
Diazepam	10.6

Analytical recoveries at two concentrations of CBZ-EP, CBZ, LOR and ND are given in Table I. Recovery of all compounds ranged from 96 to 109% with no perceivable dependence on drug concentration or sample media. As a result, analytical standards of CBZ and CBZ-EP were prepared in plasma and used to quantitate unknown concentrations in plasma, buffer or saliva.

As shown in Table II, within-day precision of CBZ and CBZ-EP varied from 2.4 to 6.3% while day-to-day precision for these compounds in frozen plasma ranged from 3.1 to 7.0%. The stability of CBZ and CBZ-EP in plasma stored at 20° in glass tubes was thus determined to be at least 2 months.

Table III lists the retention times for methanolic standards of various anticonvulsant and sedative drugs detected by the chromatographic system. Of those drugs tested, oxazepam and phenytoin showed potential interference with analysis of CBZ and LOR, respectively. Although interference by phenytoin was eliminated by our basic extraction procedure, potential interference by oxazepam remains.

Fig. 2 shows the results of the two HPLC analyses for CBZ and CBZ-EP in 20 plasma and saliva samples from patients receiving CBZ maintenance therapy for convulsive disorders. Due to obvious interferences with quantitation of CBZ-EP using the method of Kabra and Marton [12] only 10 out of 20 patient samples were analyzed for this compound. No obvious interferences with either

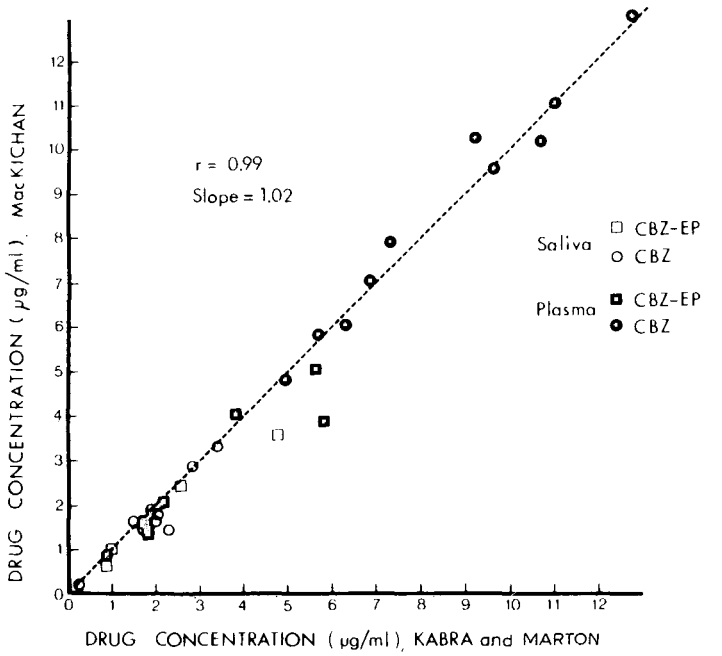

Fig. 2. Concentrations of CBZ and CBZ-EP in plasma and saliva of epileptic patients as measured by the present HPLC method and by the HPLC method of Kabra and Marton [12].

compound were noted with the use of the author's HPLC assay. Concentrations of CBZ and CBZ-EP ranged, respectively, from 0.2 to 3.3 and 0 to 2.4 μg/ml in saliva and from 4.8 to 13 and 0.4 to 4 μg/ml in plasma. The graph demonstrates an excellent correlation between concentrations of both compounds as determined by the two methods, with a correlation coefficient of 0.99 and a slope of unity.

Fig. 3 shows results of the analyses of CBZ and CBZ-EP in plasma and CBZ in saliva from a subject given a single 400-mg dose of CBZ. A peak CBZ concentration of 5 μg/ml in plasma was reached in approximately 8 h, while the peak CBZ-EP concentration was approximately 0.7 μg/ml. Saliva and free plasma concentrations of CBZ averaged 24% and 25% of total plasma CBZ concentrations, respectively. Although concentrations of CBZ-EP were detected in plasma following a single dose, sensitivity of the assay did not allow quantitation in saliva or protein binding determinations. The decline of CBZ and CBZ-EP in plasma and of CBZ in saliva were log-linear demonstrating similar half-lives of 30, 33 and 29 h, respectively.

DISCUSSION

The method presented here is rapid, reproducible, specific and sensitive enough to allow simultaneous quantitation of both CBZ and CBZ-EP in plasma and saliva for routine monitoring of CBZ therapy. In addition, the double internal standardization approach, similar to that used by Greenblatt [13] for

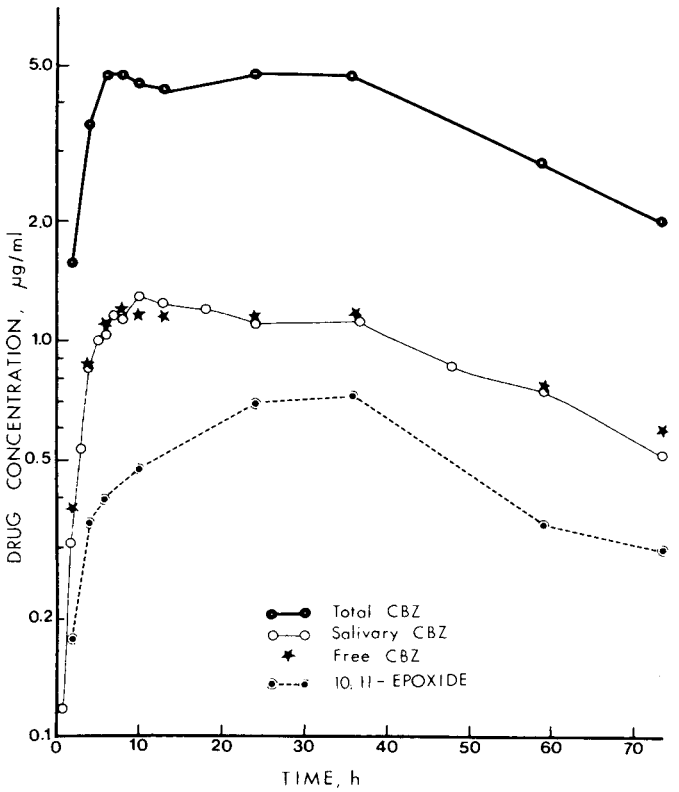

Fig. 3. Concentrations of CBZ and CBZ-EP in plasma and saliva during 72 h after a single 400-mg oral dose of CBZ given to a normal volunteer.

diazepam, greatly facilitates single-dose disposition studies of CBZ in humans. Relatively high concentrations of the parent compound, CBZ, are quantitated with use of a relatively large amount of one internal standard, ND, while the low concentrations of the epoxide metabolite are more accurately quantitated with use of a smaller amount of the second internal standard, LOR. In studies involving chronic dosing of CBZ, when concentrations of the metabolite approach those of the parent compound, both internal standards are not necessary. The potential problem of drug interference with one internal standard can be circumvented, however, by use of the other.

Due to its simplicity and rapidity, enzyme-mediated immunoassay may be the most common method of routine clinical CBZ analysis [8]. This method has the disadvantages of non-specificity and inability to quantitate the epoxide metabolite, making it unsuitable for most research studies of CBZ metabolism and disposition [8].

In addition to the capability for simultaneous analysis of CBZ and its active metabolite, our HPLC method shares with most GLC methods [6, 7] the advantages of high specificity and sensitivity. Both of these compounds, however, have been shown to decompose at GLC temperatures [14]. As a result, lengthy and tedious sample preparation procedures such as derivatization are often necessary. Liquid chromatography, in addition to

simplicity of instrumentation and sample preparation, has the advantage of allowing separation of thermally labile compounds at ambient temperature.

Early HPLC methods for simultaneous analysis of CBZ and CBZ-EP [9–11] have suffered from relative insensitivity to the epoxide metabolite. In order to reliably quantitate this compound, these methods require either extraction of large volumes of plasma and/or alteration of detector sensitivity during the chromatography step. In addition, some HPLC methods [9, 10] require the use of more lengthy extraction procedures to eliminate interferences from concomitantly administered drugs. With our method, the desired sensitivity is achieved for both CBZ and CBZ-EP using only 0.5 ml of plasma or saliva and a one-step extraction procedure, with minimal interference from other anticonvulsant drugs.

The results of our single-dose CBZ study are in excellent agreement with observations of other investigators. As compared to the peak CBZ concentration of 5 μg/ml attained in our study, Levy et al. [15] using a GLC technique, measured peak CBZ concentrations of 3.3—5.4 μg/ml in serum of normal subjects following a single 400-mg CBZ dose. Using HPLC, Eichelbaum et al. [4] observed average peak concentrations of 2 μg/ml and 0.1 μg/ml for CBZ and CBZ-EP, respectively, in the plasma of normal subjects following single 200-mg doses. The length of time (8 h) required to reach the peak plasma CBZ concentration in this study was within the range of 2—18 h reported by other investigators [15, 16] and has been explained by dissolution-rate limited absorption of the tablet secondary to the low water solubility of this drug [15].

As compared to the 30-h CBZ elimination half-life reported in this study, half-lives ranging from 24 to 55 h have been reported in single-dose studies by investigators using different analytical techniques [4, 15, 17]. In addition, the parallel decline of the plasma concentration–time profiles for CBZ and its epoxide metabolite observed in our study is also consistent with previous reports [4, 5]. The explanation for this lies in the fact that the half-life of CBZ-EP is much shorter than that of the parent drug [18]. Because the metabolite cannot be removed any faster than it is produced, an apparent half-life equal to that of the parent drug is observed.

Analysis of CBZ in saliva can provide a convenient and non-invasive alternative to the determination of CBZ in plasma. In this study, saliva CBZ concentrations in the volunteer subject paralleled plasma concentrations with a mean saliva:plasma ratio of 0.24. This is in good agreement with recent studies [19, 20] in which the saliva:plasma ratios ranged from 0.23 to 0.37 over similar plasma concentration ranges. It is therefore feasible that analysis of salivary samples can provide estimates of CBZ concentrations in plasma.

A more important advantage to analysis of drugs in saliva is that it often reflects the unbound, pharmacodynamically active fraction of drug in plasma. This is especially important for drugs that are highly or variably protein bound. We found that free plasma CBZ concentrations in our single subject were equivalent to salivary concentrations, and averaged 25% of total plasma concentrations. Using ultrafiltration techniques, Hooper et al. [21] reported a high degree of interindividual variability in the plasma protein binding of CBZ; free plasma CBZ concentrations ranged from 10 to 30% (mean 18%) in normal

subjects, and from 8 to 60% (mean 27%) in epileptic patients. Because of this variability in the free fraction of CBZ, interpretation of a therapeutic range of CBZ based on total plasma concentrations is difficult and may be facilitated by establishment of a therapeutic range for CBZ in saliva.

Assay sensitivity did not allow analysis of the epoxide metabolite in the saliva of the volunteer subject following a single dose of CBZ in this study. In contrast to a recent report [5], sensitivity of our HPLC method did allow quantitation of this metabolite in the saliva of epileptic patients who took CBZ chronically, as shown in Fig. 3. Although there was almost a four-fold difference between mean concentrations of parent drug and metabolite in plasma (8.5 and 2.4 μg/ml, respectively), the concentrations of CBZ and CBZ-EP in saliva were similar with average values of 1.7 and 1.2 μg/ml, respectively. Assuming that saliva is a reflection of free CBZ-EP in plasma, this apparent discrepancy can be explained by the fact that CBZ-EP is only 45–50% protein bound as compared to 75% for CBZ [18]. It is clear that further studies are necessary to evaluate the contribution of CBZ-EP to total anticonvulsant activity as well as the relationships between salivary, free and total plasma CBZ-EP concentrations in patients. Use of this sensitive and specific HPLC method for simultaneous analysis of CBZ and CBZ-EP in saliva may prove to be a more rational and convenient method for monitoring CBZ therapy in epileptic patients.

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Biomedical Applications

CHROMBIO. 476

RAPID DETERMINATION OF INDOMETHACIN AND SALICYLIC ACID IN SERUM BY MEANS OF REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the quantitative analysis of indomethacin and salicylic acid in blood serum and urine by high-performance liquid chromatography is described. A C_{18}-bonded silica was used as the stationary phase and mixtures of ethanol, *n*-butanol and aqueous buffer as the mobile phase. Before injection the serum is deproteinized and extracted in one step.

The recovery of the extraction was found to be 88% and 77% for indomethacin and salicylic acid, respectively. The relative standard deviations of the analysis for 0.5 μg indomethacin and 5 μg salicylic acid per ml serum were 3.6% and 3.2%, respectively. The detection limits for indomethacin and salicylic acid were 2 ng. This corresponds for both substances to 0.1 μg/ml serum for an injection volume of 100 μl.

The method enables simultaneous determination of possibly formed metabolites. A number of concurrently administered drugs do not interfere with the analysis. The interactive effects of co-medication of indomethacin and salicylic acid on the serum concentration of indomethacin is demonstrated by measuring the pharmacokinetic curves.

INTRODUCTION

Indomethacin [1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-indole-3-acetic acid], an anti-inflammatory drug, is frequently used in combination with salicylate in the treatment of rheumatoid arthritis [1]. It is well known that indomethacin rapidly metabolizes in the body and that some of the metabolites lack anti-inflammatory activity [2—8]. However, conflicting results have appeared in the literature about the influence of salicylates on the plasma level and metabolism of indomethacin [9—14].

In order to investigate this effect, a rapid and selective method for the simultaneous determination of indomethacin, its metabolites and salicylic acid in serum is required. Until now, extraction procedures combined with radioactive [3—5, 10] or fluorimetric [11—13] techniques have been applied.

Although these methods are quite sensitive, they do not discriminate between the drug, its metabolites [3, 10—13] and, in the case of fluorimetry, salicylate [15] and other administered drugs [3].

A number of chromatographic methods has proved to be successful [2, 6, 14, 16—19]. In order to determine indomethacin by means of gas chromatography (GC), derivatization is required [6, 14, 16—18]. This step introduces additional uncertainties in the quantitative analysis. Anion-exchange chromatography has been used for the separation of indomethacin, its metabolites and salicylic acid prior to radioactive quantitation [7—9]. However, the limit of determination of this method did not meet the requirements for pharmacokinetic studies. Recently, thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) procedures for the analysis of indomethacin have been reported [19—21]. However, these methods do not allow the simultaneous determination of metabolites and salicylic acid.

In the present paper we describe a method for the quantitative determination of indomethacin, its major metabolites and salicylic acid by means of HPLC with UV detection. The method has been successfully applied to a pharmacokinetic study of the influence of salicylate on the plasma level and metabolism of indomethacin.

EXPERIMENTAL

Apparatus

The HPLC experiments were carried out on a high-pressure liquid chromatograph (Siemens SP 200, Siemens, Karlsruhe, G.F.R.) using UV detection at 235 nm (Pye-Unicam LC-UV, Philips, Eindhoven, The Netherlands) a high-pressure sampling valve (Valco CV-6-UHP a, Valco Instruments, Houston, Texas, U.S.A.) with a 100-μl loop. a stainless-steel column (250 mm × 4.6 mm I.D.) and a linear potentiometric recorder (Servogor 542, Goerz, Vienna, Austria).

The acidic medium requires that all connections are made of stainless-steel 316 capillary tubing and stainless-steel Swagelok couplings. For the extractions a Vortex mixer (Scientific Industries, Springfield, N.Y., U.S.A.) was used. The experiments were carried out at room temperature.

Materials

In all experiments double-distilled water was used. A commercially available HPLC column containing an alkyl-modified material (Zorbax ODS, 5 μm, Dupont Instruments, Newton, Conn., U.S.A.) was used. All chemicals were of analytical reagent grade.

Procedures

The HPLC column was washed successively with 75% (w/v) ethanol and the appropriate eluent (both 100 times the column volume). Blood samples were drawn by venipuncture and centrifuged (10 min, 300 *g*). The blood serum was deep-frozen until assay.

For deproteinization, 0.3 ml serum was mixed with 1 ml 0.3 *M* perchloric acid of pH 0.7 in a glass-stoppered centrifuge tube (25 ml). After 10 min, 4 ml

dichloromethane was added and the aqueous phase was extracted by mixing 1 min on a vibration mixer. After centrifugation (15 min, 300 *g*), the aqueous phase was removed. A 3-ml portion of the organic phase was transferred to another centrifuge tube and evaporated until dryness under a stream of nitrogen.

The same extraction procedure was followed for urine starting with 1.3 ml urine (acidified to pH 1 with 70% (w/v) perchloric acid). In order to hydrolyze the glucuronides 0.65 ml urine (pH 5) was mixed with 0.63 ml 0.1 *M* acetate buffer (pH 5), containing 10^4 U β-glucuronidase per ml. After incubation at 37° during 1 h, 0.02 ml 70% (w/v) perchloric acid was added before extraction at pH 1.

The residues were dissolved in 1 ml of the eluent and centrifuged (1 min, 300 *g*). The supernatant was injected by means of a sample loop of 100 μl volume. To avoid memory effects the sample loop was rinsed with water after each injection. To prevent contamination the glassware used in contact with dichloromethane was rinsed with methanol and dried with nitrogen.

Standard solutions of indomethacin in 50% (w/v) ethanol were found to be stable for several weeks. In contradiction to earlier reports [17], in our experiments no loss of indomethacin and salicylic acid from frozen serum and urine samples was found during one month sample storage.

RESULTS AND DISCUSSION

In order to investigate the interactive effects of co-medication of indomethacin and salicylic acid, knowledge about their metabolic pathways is required. Fig. 1 shows which metablites and conjugates of indomethacin and salicylic acid have been found in serum and urine [2, 4, 22]. The conjugates mainly occur in urine [3, 5].

For the simultaneous determination of indomethacin, its main metabolites and salicylic acid in serum, the applicability of an alkyl-modified silica in combination with mixtures of alcohols and aqueous buffer was investigated.

In order to determine optimal chromatographic conditions the influence of the type and concentration of the organic modifier and pH on the retention of the compounds in question was investigated.

The results of these experiments showed that the logarithm of the capacity ratio of all compounds changes linearly with the concentration of *n*-propanol as is commonly observed in reversed-phase systems. However, it was found that the stability of the phase system decreased at increasing concentration of *n*-propanol.

For that reason also *n*-butanol was tested. When *n*-butanol was used as modifier at a concentration as large as possible with respect to solubility [6% (w/v) *n*-butanol], the capacity ratio of indomethacin was too large to measure. To increase the concentration of *n*-butanol, mixtures of *n*-butanol, ethanol and aqueous buffer were used as the eluent. Using these eluents a remarkable improvement of the column stability was observed. Moreover, the viscosity of the eluent was much lower than with *n*-propanol, as was noticed previously [23].

The dependence of the capacity ratios on the concentration of *n*-butanol at a

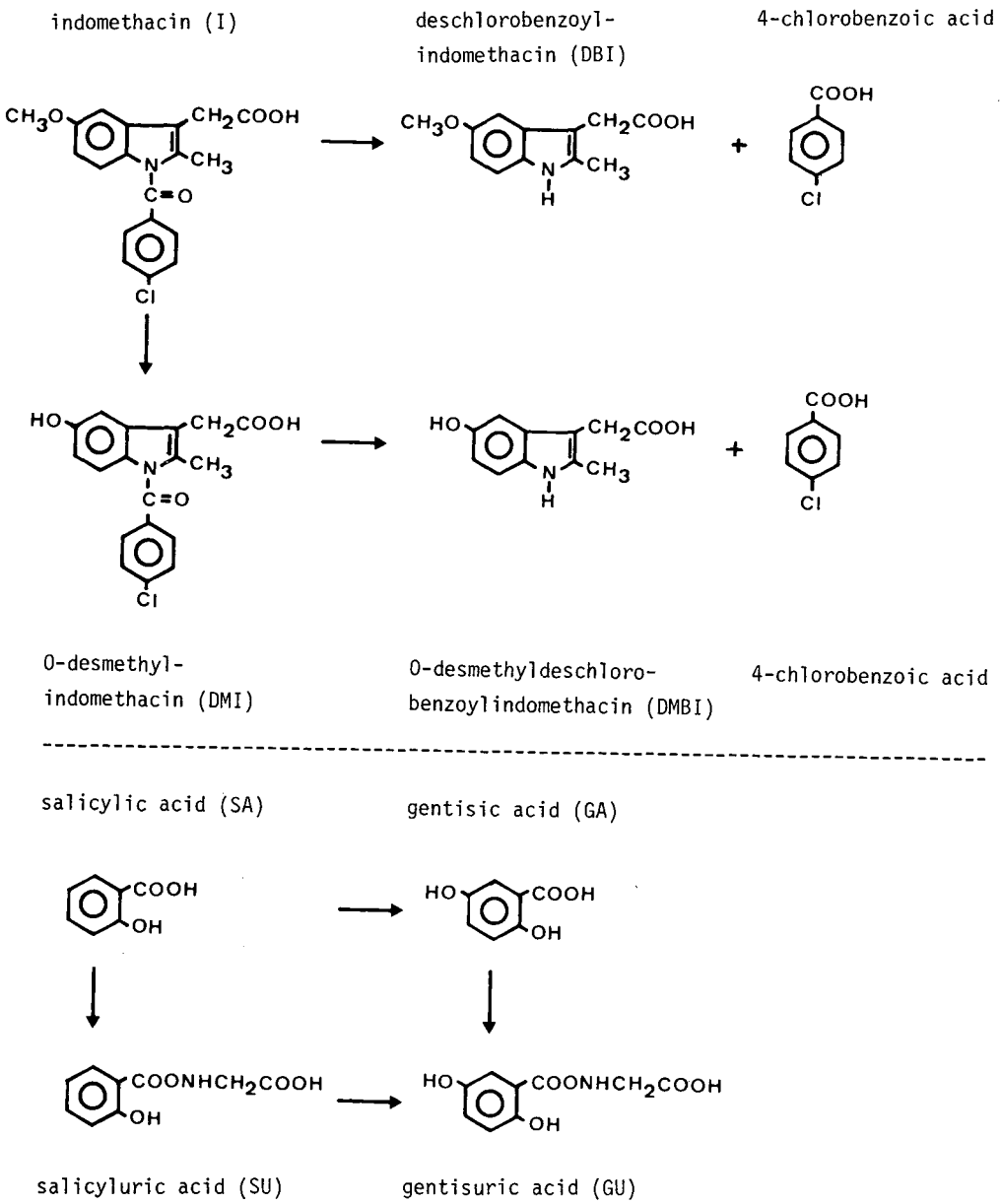

Fig. 1. Metabolic pathways of indomethacin and salicylic acid. All compounds form conjugates with glucuronic acid at the —COOH or the —OH group (RCOOH → $RCOOC_6H_9O_6$; R'OH → $R'OC_6H_9O_6$).

fixed concentration of 15% (w/v) ethanol is shown in Table I.

The plot of the logarithm of the capacity ratios versus the concentration of *n*-butanol now deviates from linearity. This is probably due to a change in the amount of ethanol, adsorbed at the column, with the concentration of *n*-butanol in the eluent (e.g. mixed adsorbed phase).

TABLE I

INFLUENCE OF THE CONCENTRATION OF *n*-BUTANOL IN THE ELUENT ON THE CAPACITY RATIO (k_i') AT A FIXED CONCENTRATION OF ETHANOL

The eluent consisted of 7—15% (w/v) *n*-butanol—15% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate, pH 2.3.

Compound	k_i'			
	n-Butanol concentration (%, w/v)			
	7	8	10	15
2,5-Dihydroxybenzoic acid	0.80	0.77	0.68	0.61
5-Methoxy-2-methyl-indole-3-acetic acid	0.96	0.86	0.73	0.64
Salicylic acid	3.39	2.91	2.19	1.27
4-Chlorobenzoic acid	6.59	5.20	3.45	1.63
Indomethacin	12.30	11.12	6.11	2.08

In all further experiments the eluent consisted of mixtures of *n*-butanol, ethanol and aqueous buffer. For these eluents the effect of pH was investigated with respect to the separation of indomethacin, salicylic acid and their metabolites from drugs, which generally occur in aspirin tablet formulations. The results of these experiments are shown in Table II.

TABLE II

INFLUENCE OF pH ON THE CAPACITY RATIO (k_i')

The eluent consisted of 13% (w/v) *n*-butanol—13% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate, pH 2—6.5.

Compound	k_i'			
	pH			
	2	3.5	5.0	6.5
5-Hydroxy-2-methyl-indole-3-acetic acid	0.61	0.58	0.27	0.02
4-Hydroxyacetanilide	0.64	0.71	0.72	0.70
Caffeine	0.85	0.93	0.93	0.90
2,5-Dihydroxybenzoic acid	1.21	0.86	0.22	0.15
Salicylamide	1.23	1.35	1.36	1.33
Acetylsalicylic acid	1.24	1.28	0.54	0.18
5-Methoxy-2-methyl-indole-3-acetic acid	1.60	1.52	1.04	0.20
Phenacetin	1.71	1.86	1.86	1.82
Salicylic acid	2.67	1.99	0.47	0.30
O-Desmethylindomethacin	3.18	3.35	2.30	0.88
4-Chlorobenzoic acid	3.52	3.65	2.00	0.60
Indomethacin	5.18	5.45	3.96	1.32

It can be seen that the separation between the relevant compounds is optimal in the pH range 2—4. In Fig. 2 the separation of test mixtures of a number of anti-inflammatory drugs is shown at different pH values. As shown in Fig. 3 salicylic acid is well resolved from indomethacin and its metabolites at pH 3.45.

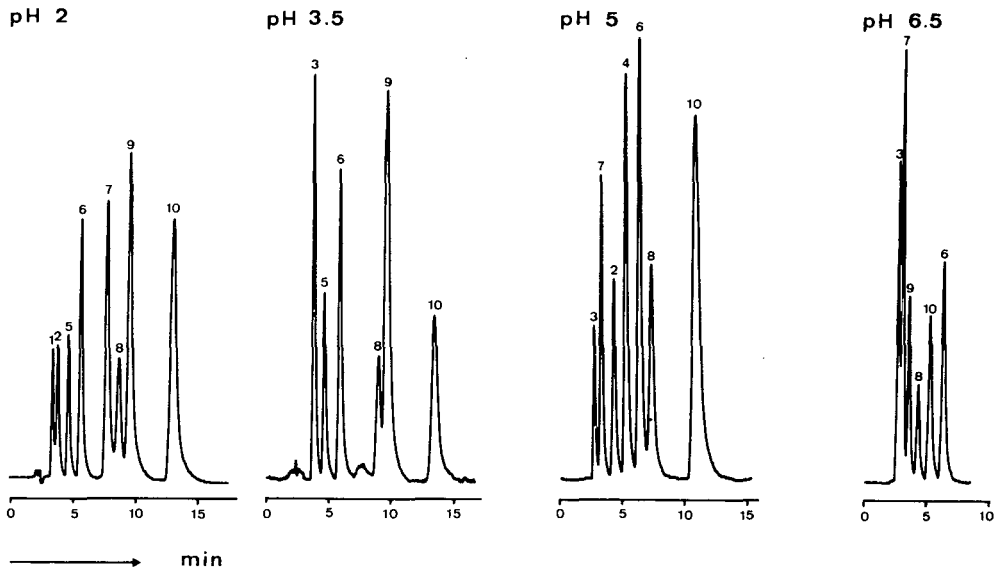

Fig. 2. The effect of pH on the separation of anti-inflammatory drugs. Eluent, 13% (w/v) *n*-butanol—13% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate; pH 2—6.5, column, 250 mm × 4.6 mm I.D.; injection volume, 100 µl; wavelength 235 nm. Peaks: 1, 4-hydroxyacetanilide; 2, caffeine; 3, 2,5-dihydroxybenzoic acid; 4, salicylamide; 5, acetylsalicylic acid; 6, phenacetin; 7, salicylic acid; 8, O-desmethylindomethacin; 9, 4-chlorobenzoic acid; 10, indomethacin.

Quantitative analysis

Precision. The linearity of the chromatographic method was tested by injecting a volume of 100 µl of solutions of indomethacin (0.1—2.5 µg/ml) and salicylic acid (1—25 µg/ml). A correlation coefficient of 0.9999 was found for the linear relationship between peak height and amount of indomethacin and salicylic acid. The relative standard deviation for replicate analyses of indomethacin was 1.3% at 1 µg/ml (n = 10) and of salicylic acid 1.5% at 10 µg/ml (n = 10).

The detection limit, defined as three times the standard deviation of the noise (about $3 \cdot 10^{-5}$ a.u.) was determined to be 2 ng for both indomethacin and salicylic acid.

Recovery and reproducibility. Using a procedure from the literature [24], the deproteinized serum can be injected directly onto the column. However, compared with the recovery of salicylic acid (90%) for indomethacin a rather low recovery was found (50%). This must be attributed to the higher degree of adsorption of indomethacin to the serum proteins in an aqueous perchloric acid solution of pH < 1 [25, 26].

It was found that the recovery of indomethacin can be improved when the serum was deproteinized with perchloric acid and extracted with dichloromethane in one step, according to a method reported in the literature [27].

To determine the recovery and reproducibility of this method, known amounts of indomethacin and salicylic acid were added to blank serum before

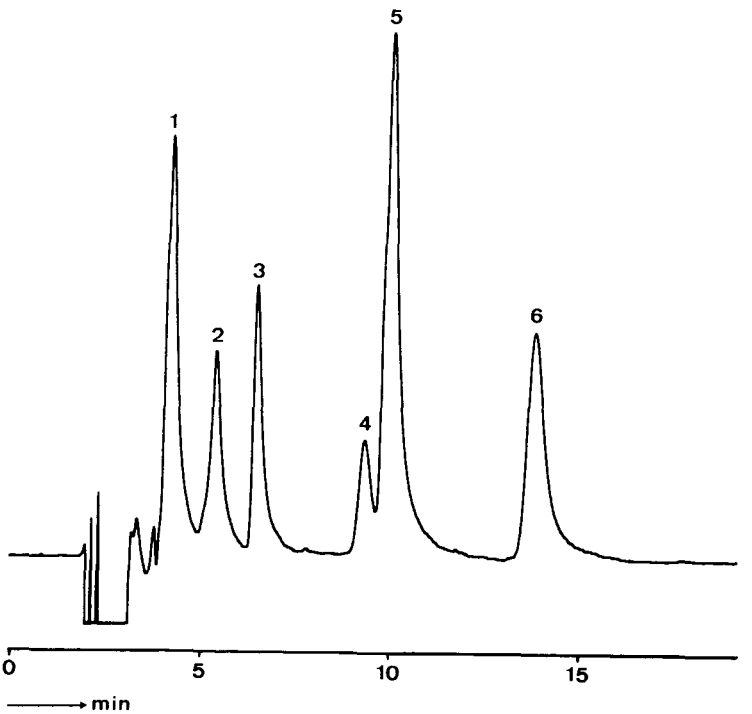

Fig. 3. The separation of a test mixture of salicylic acid, indomethacin and its metabolites. Eluent, 13% (w/v) *n*-butanol—13% (w/v) ethanol—0.08 *M* perchloric acid—0.05 *M* phosphate; pH 3.45; column, 250 mm × 4.6 mm I.D.; injection volume, 100 µl; wavelength, 235 nm; for abbreviations see Fig. 1. Peaks: 1, DMBI; 2, DBI; 3, SA; 4, DMI; 5, 4-chlorobenzoic acid; 6, indomethacin.

extraction. The extracts were analyzed by HPLC. The recovery from serum was found to be 87.5 ± 3.6% for indomethacin measured within the range 0.5—10 µg/ml serum and 76.6 ± 3.2% for salicylic acid within the range 5—100 µg/ml serum.

For aqeous solutions (acidified to pH 1 with perchloric acid) a recovery of 95.0 ± 2.0% was found for indomethacin and 80.0 ± 2.0% for salicylic acid. These results show that only a minor part of the compounds remained adsorbed at the serum proteins.

Pharmacokinetic study

The effect of concurrent administration of salicylic acid and indomethacin on their concentrations in serum was investigated by measuring a pharmacokinetic curve. Drugs, which generally occur in aspirin tablet formulations (see Table II), do not interfere with the analysis of indomethacin, salicylic acid and their metabolites at an eluent composition being selected on the basis of Tables I and II. A number of drugs commonly administered to rheumatic patients such as prednisone, glibenclamide, pentazocine and diclofenac do not interfere with the analysis.

Blood samples were taken from three patients (1 male, 2 female) with rheumatoid arthritis being treated with indomethacin and salicylic acid according to the medication scheme shown in Table III. Every fourth day of

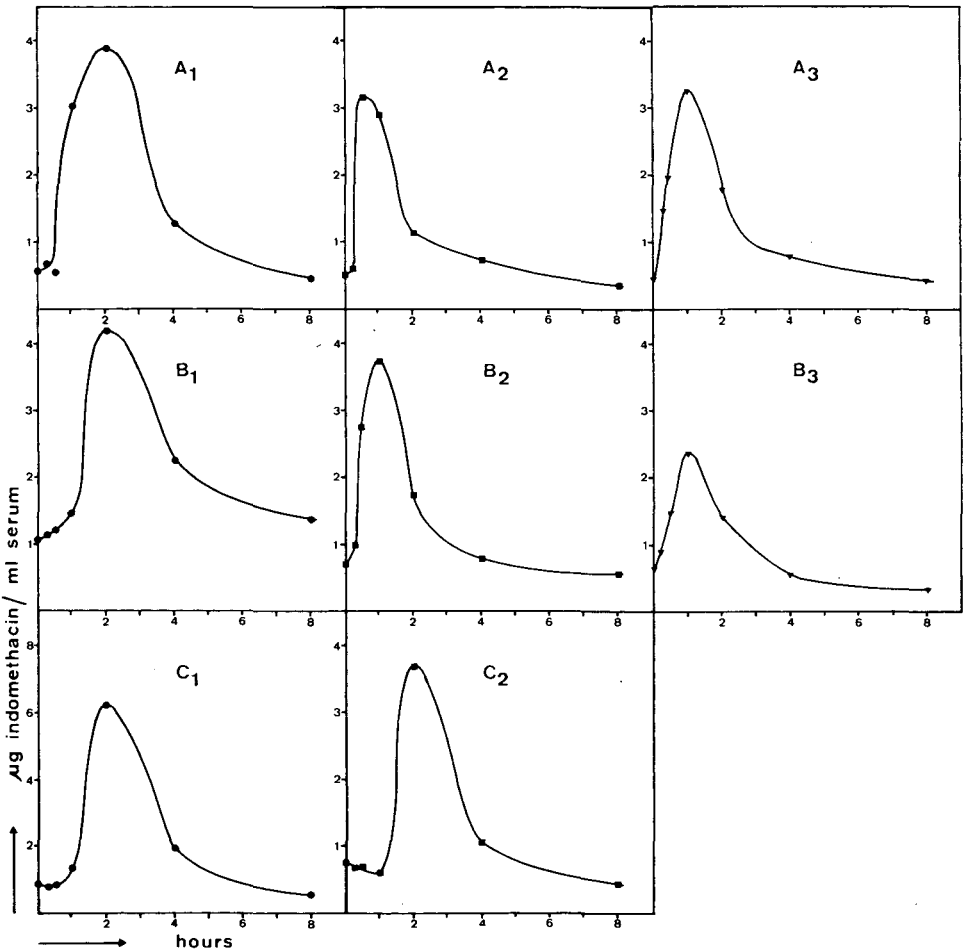

Fig. 4. Pharmacokinetic curves of indomethacin in the presence and in the absence of concurrently administered salicylic acid for a trial of three rheumatic patients. See Table III for medication scheme; simultaneously administered drugs: patient 1 (female), prednisone; patient 2 (male), nihil; patient 3 (female), prednisone, glibenclamide, pentazocine.

a series A, B and C blood samples were taken at time intervals of 1/4, 1/2, 1, 2, 4 and 8 h after the administration of the morning dose at 8.00 a.m. During this 8-h period the urine was collected to determine the total amount of the drugs, their metabolites and their glucuronides (see Fig. 1).

The pharmacokinetic curves of indomethacin and salicylic acid are shown in Figs. 4 and 5. The results for indomethacin show some changes in the area under the curves and the half-time and height of the maximum serum concentration.

Series A can be compared to series B (e.g. in the absence and in the presence of concurrently administrated salicylic acid). For patients 1 and 2 an increase of the area under the pharmacokinetic curve and of the maximum serum concentration of indomethacin was found going from A to B, and for patient 3 a decrease was found. For patients 1 and 3 the half-time remained 2 and 1 h,

TABLE III

MEDICATION SCHEME TO STUDY THE EFFECT OF CONCURRENT ADMINISTRATION OF INDOMETHACIN AND SALICYLIC ACID

Series	Day	Drugs*
A	1	50 mg indomethacin
	2	
	3	
	4**	
B	1	50 mg indomethacin +
	2	1 g sodium salicylate
	3	
	4**	
C	1	50 mg indomethacin
	2	
	3	
	4**	

*Simultaneously administered orally, three times daily at 8.00 a.m., 4.00 p.m. and 12.00 p.m. 50 mg indomethacin in capsule and 10 ml buffered medicine containing 1 g sodium salicylate.

**After the morning dose of 8.00 a.m. blood samples were taken at 8.15, 8.30, 9.00, 10.00, 12.00 a.m. and 4.00 p.m.

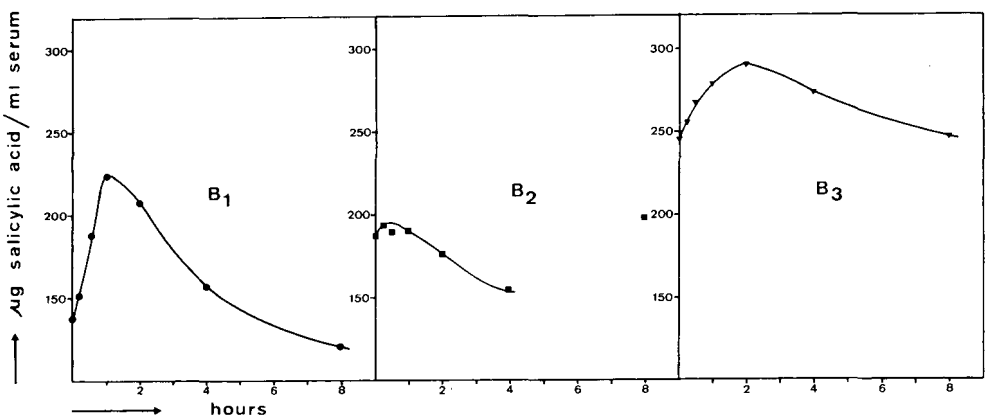

Fig. 5. Pharmacokinetic curves of salicylic acid for series B of the medication scheme.

respectively, while for patient 2 a shift from 0.5 to 1 h was found.

Comparing series C (e.g. in the absence of salicylic acid) to series A and B, the same curves were expected as for series A. However, for patients 1 and 2 the area and maximum increased again compared to series B, while for patient 2 a further shift of the half-time was found. For patient 3 the medication had to be stopped because of side effects.

The differences between the results for each individual patient are also illustrated by the pharmacokinetic curves of salicylic acid (see Fig. 5). It should be noticed that no salicylic acid was found in the serum samples of series C_1 and C_2.

The metabolites of indomethacin such as DMI, DBI, DMBI and 4-chloro-

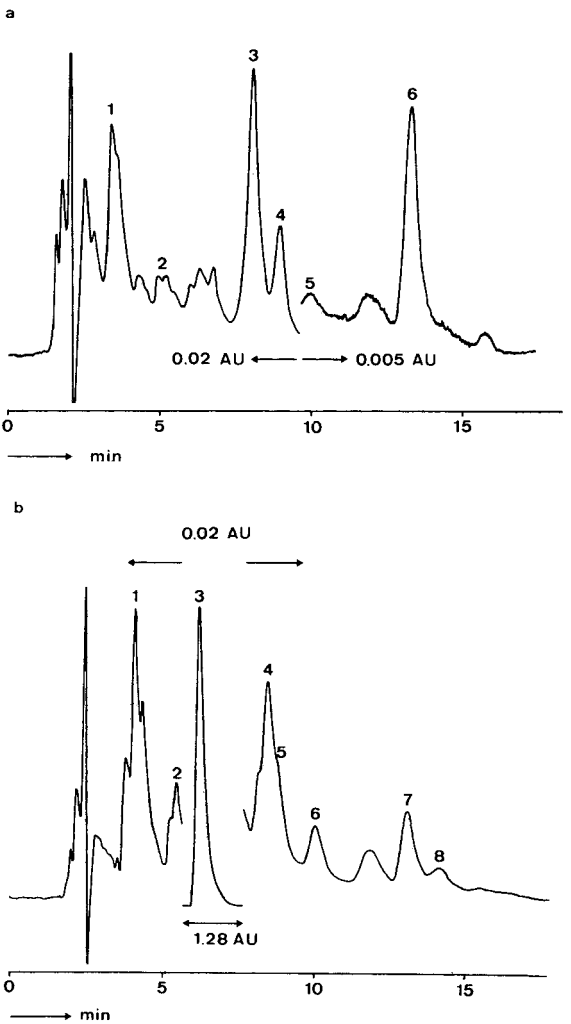

Fig. 6. Chromatograms of extracted serum, taken after oral administration of indomethacin in the presence and in the absence of salicylic acid. Conditions as in Fig. 3. (a) Series C_1 at 8.00 a.m.; peaks: 1, DMBI; 2, DBI; 3, serum background; 4, DMI; 5, 4-chlorobenzoic acid; 6, I. (b) Series B_3 at 10.00 a.m.; peaks: 1, DMBI; 2, DBI; 3, SA; 4, serum background; 5, DMI; 6, 4-chlorobenzoic acid; 7, I; 8, glibenclamide.

benzoic acid can be determined simultaneously with indomethacin and salicylic acid, as shown in Fig. 6a and b. However, the analytical results for the quantitative determination of metabolites in serum were for indistinct reasons less reproducible than for indomethacin and salicylic acid.

The collected urine samples were tested for the presence of the drugs, their metabolites and their glucuronides. As shown in Fig. 7a and b, a considerable concentration of metabolites was found in urine. From the peak heights before and after treatment of the urine with β-glucuronidase the concentrations of the free compounds and their glucuronides were calculated. As shown in Table IV, the relative abundance depends on the kind of compound.

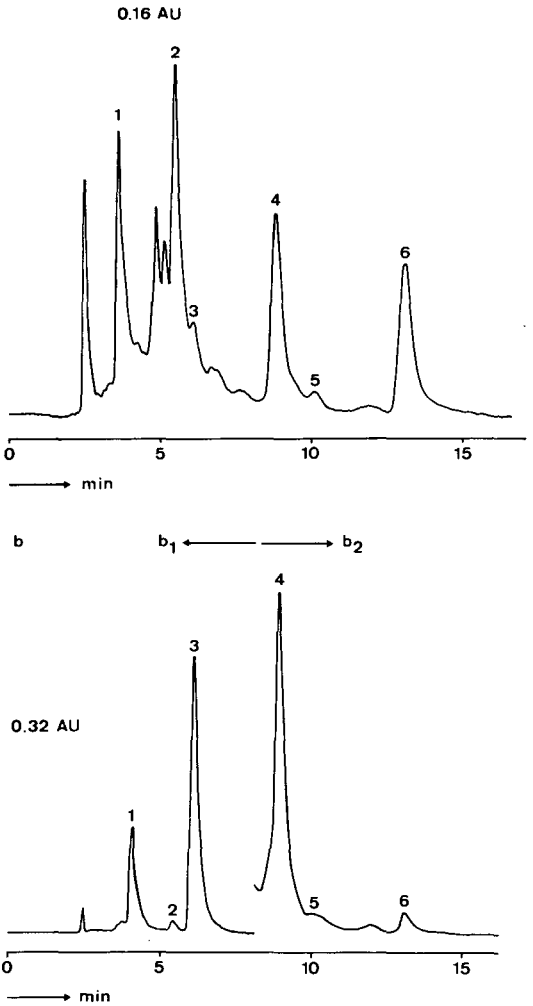

Fig. 7. Chromatograms of extracted urine, collected during the 8-h period of a pharmacokinetic curve in the presence and in the absence of salicylic acid. Conditions as in Fig. 3, (a) Series A_3 ; peaks: 1, DMBI; 2, DBI; 3, pentazocine; 4, DMI; 5, 4-chlorobenzoic acid; 6, I. (b) Series B_3 ; for part b_1 of the chromatogram the urine extract of part b_2 was 50 times diluted with the eluent; peaks: 1, SU; 2, DBI; 3, SA; 4, DMI; 5, 4-chlorobenzoic acid; 6, I.

TABLE IV

THE FREE COMPOUNDS AND THEIR GLUCURONIDES IN URINE

Compound	Glucuronide	Ratio
$\text{Indomethacin}_{\text{free}}$	$\text{Indomethacin}_{\text{gluc}}$	1:3
$\text{Salicylic acid}_{\text{free}}$	$\text{Salicylic acid}_{\text{gluc}}$	1:0.7
DMI_{free}	DMI_{gluc}	1:10
DBI_{free}	DBI_{gluc}	1:2

CONCLUSIONS

Only a small volume of blood serum is required to determine low concentrations of indomethacin and salicylic acid by means of a rapid extraction procedure and subsequent analysis by HPLC. Metabolites can be determined simultaneously.

The method is simple and reliable. The time needed for one analysis is about one hour. The stability of the chromatographic phase system is remarkably high: no change was noticed after injection of 2000 extracts of serum. Therefore, the method is suitable for routine analysis.

The results of the present pharmacokinetic study do not solve the clinical issue of a possible interaction of indomethacin and salicylic acid. Future research will be devoted to this subject for a larger group of rheumatic patients.

ACKNOWLEDGEMENTS

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DETERMINATION OF NALIDIXIC ACID AND ITS TWO MAJOR METABOLITES IN HUMAN PLASMA AND URINE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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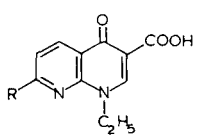
SUMMARY

This paper describes a precise and sensitive method for analysis of nalidixic acid and its two major metabolites in plasma and urine following the oral administration of a therapeutic dose in humans. After addition of an internal standard (oxolinic acid), 1-ml samples of plasma or urine are extracted at acidic pH with chloroform. The extracts are purified by re-extraction with sodium hydroxide solution and then chloroform. The final extracts are evaporated to dryness, reconstituted in mobile phase and injected into a high-performance liquid chromatograph equipped with RP-8 column and UV detector operating at 254 nm. The limit of sensitivity of the method is lower than 0.5 μg/ml of plasma or urine for each compound. The applicability of the method to pharmacokinetic studies of nalidixic acid in humans is demonstrated.

INTRODUCTION

Nalidixic acid, 1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid (NA) is an antibacterial agent [1,2] which is widely used in the treatment of urinary tract infections.

Contrarily to its metabolism which has been widely studied [3], the pharmacokinetics of NA in man are poorly described. This is probably due to the lack of an analytical method allowing to measure precisely NA concentrations in biological fluids separately from its two major metabolites, 7-hydroxynalidixic acid (HNA) and 7-carboxynalidixic acid (CNA).



R = CH_3 NA
= CH_2OH HNA
= COOH CNA

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Most procedures used for quantitation of NA are based on its fluorescence [2,4]. These techniques, which need large plasma volumes (1—3 ml), are non-specific towards the metabolites and give relatively high blank values and non-linear calibration curves.

A high-performance liquid chromatographic (HPLC) method using an ion-exchange column has been recently described [5], in which no internal standard was used, and the major metabolite in plasma, HNA, could not be determined as it was not separated from the solvent peak in the system used.

Two other HPLC methods have been developed. The first [6] was devoted to the determination of CNA only, and the second [7] to NA exclusively. On the other hand, two gas chromatographic methods have also been established, but their application was limited to the determination of NA alone in tablets [8] or in plasma [9].

In the present work, we describe a HPLC method which allows a precise and sensitive analysis of plasma and urine samples for NA, HNA and CNA simultaneously, using a standard reversed-phase column and, as internal standard, oxolinic acid (OXA), a compound whose structure is near to NA. An example of a pharmacokinetic study demonstrates its utility for the analysis of the three compounds in biological fluids.

EXPERIMENTAL

Apparatus

A Hewlett-Packard Model 1081B high-performance liquid chromatograph was used, equipped with a Hewlett-Packard Model 1036A UV detector operating at 254 nm, and a RP-8 prepacked reversed-phase column, particle size 10 μm, (Hewlett-Packard), (250 × 4.6 mm I.D.).

Chemicals and reagents

NA (Negram), HNA and CNA were generously supplied by the Sterling-Winthrop Research Institute (Rensselaer, N.Y., U.S.A.) and OXA was a gift from Substantia Laboratories (Courbevoie, France). Methanol and chloroform (Merck, Darmstadt, G.F.R.) were distilled before use. Ion-exchange resin Dowex 1-X4, 200—400 mesh, Cl^- form was purchased from Roth (Karlsruhe, G.F.R.). All other reagents were of analytical grade (Merck).

The mobile phase consisted of 55% methanol and 45% phosphate buffer containing 7.5 g/l KH_2PO_4 and 2.5 g/l Na_2HPO_4 (adjusted to pH 8.2 with 1 *N* sodium hydroxide). N,N,N-Trimethylcetylammonium bromide (2 g/l) (Merck) was added to the mixture.

Stock solutions of internal standard (OXA) and the mixture of NA, HNA and CNA were prepared by dissolving 1 mg/ml of each compound in 0.03 *N* sodium hydroxide.

Working solutions were prepared by diluting the stock solutions in 0.03 *N* sodium hydroxide to obtain various concentrations of the three compounds studied, between 1 and 50 μg/ml for plasma and between 10 and 200 μg/ml for urine, and a fixed concentration of OXA, 10 and 50 μg/ml for plasma and urine, respectively.

Correlation curves were obtained after adding 1 ml of each of these diluted solutions to 1 ml of corresponding biological fluid.

Procedures

Plasma extraction. To 1 ml of human plasma in a 15-ml glass centrifuge tube, were added 1 ml of the internal standard solution (10 μg/ml), 0.5 ml of 1 *N* hydrochloric acid and 8 ml of freshly distilled chloroform. The tube was stoppered and placed on a rotating shaker (60 rpm) for 15 min. After centrifugation (15 min at 1200 *g*) 6.5 ml of the organic layer were transferred to another tube and extracted with 6 ml of 0.2 *N* sodium hydroxide for 15 min. Following centrifugation (10 min), 5 ml of the aqueous phase were transferred to a new tube containing 2 ml of 1 *N* hydrochloric acid and the mixture was extracted with 6 ml of chloroform for 15 min. After centrifugation (10 min), 5 ml of the organic phase were transferred to a 10 ml conical tube and evaporated to dryness under a stream of nitrogen on a 45° water-bath. The residue was dissolved in 0.25—5 ml of mobile phase and 20 μl of this solution were injected onto the column by injection loop at a flow-rate of 0.6 ml/min under a pressure of about 30 bars at room temperature.

Urine extraction. To 1 ml of human urine in a 15-ml glass centrifuge tube, were added 1 ml of the internal standard solution (50 μg/ml), 0.5 ml of 1 *N* hydrochloric acid and 8 ml of freshly distilled chloroform. The tube was stoppered and placed on a rotating shaker (60 rpm) for 15 min. After centrifugation (10 min at 1200 *g*) 7 ml of the organic phase were transferred to another tube and extracted with 6 ml of 0.2 *N* sodium hydroxide for 15 min. Following centrifugation (10 min), 5 ml of the alkaline aqueous phase were loaded on a short column (4 mm I.D.) packed with a glass wool plug and with ion-exchange resin Dowex 1-X4 (1 cm). The complete outflow was followed by two bidistilled water washings of 2 ml each. The elution of the four compounds was performed by washing the column twice with 2.5 ml of 1 *N* hydrochloric acid. The eluate was extracted with 6 ml of chloroform for 15 min. After centrifugation (10 min), 5 ml of the organic phase were transferred into a 10-ml conical tube and evaporated to dryness under a stream of nitrogen on a 45° water-bath. The residue was redissolved in 0.5—10 ml of mobile phase and this solution injected into the HPLC system.

RESULTS AND DISCUSSION

Specificity

Fig. 1A shows that NA is clearly separated from its two metabolites. Under the prescribed conditions, the retention times were 7.0, 8.8, 13.8 and 15.4 min for HNA, OXA, CNA and NA, respectively.

Because nalidixic acid and related compounds gave very strongly tailing peaks in most chromatographic systems, the paired-ion technique has been used. This procedure allowed the best separation. Symmetrical chromatographic peaks were obtained if the degree of ion-pair dissociation was kept constant. This was achieved when a high and constant concentration of the counter-ion was maintained in the mobile phase. Such a situation can be obtained by adding a cation to the aqueous mobile phase, which brings about a

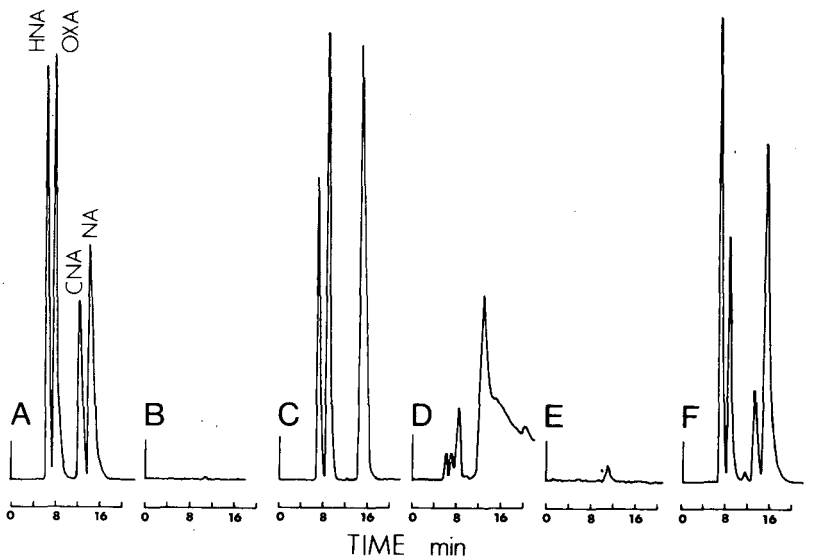


Fig. 1. Chromatograms of (A) a mixture of HNA, OXA, CNA and NA (50 ng of each compound injected); (B) a blank plasma extract; (C) plasma extract of a subject given an oral dose of nalidixic acid (HNA, OXA and NA; CNA is not present in plasma); a blank urine extract before (D) and after (E) the use of the ion-exchange resin; (F) urine extract of a subject given an oral dose of nalidixic acid (HNA, OXA, CNA and NA).

suitable ion-pair extraction of the counter-ion into the stationary phase [9,10]. Valid results were obtained with N,N,N-trimethylcetylammonium bromide at a concentration of 2 g/l in the mobile phase.

Plasma components did not interfere (Fig. 1B and C), but in the case of urine, it appeared necessary to clear the extracts from endogenous substances which gave a strong UV absorption. For this purpose, several resins were tested, and Dowex 1-X4 (strongly basic anion exchanger) was chosen because it gave the best recovery and cleaning. The residual peak observed in control urine has a retention time different from that of CNA (Fig. 1D, E and F).

Linearity and sensitivity

As shown in Fig. 2, the calibration graphs plotted as the peak height ratios of NA, HNA and CNA to the internal standard against the concentrations of each compound were straight lines. They were calculated by the least squares method and their equations are listed in Table I.

The concentration ranges studied in plasma (1—50 μg/ml) and urine (10—200 μg/ml) were chosen to correspond to the expected concentrations in biological fluids after a single therapeutic administration of NA in humans.

The detection limit, defined on the basis of the amount of compound injected which caused an absorption two times greater than the standard deviation of baseline noise, was found to be at 0.9, 2.1 and 1.8 ng, that is 10, 60 and 15 ng/ml of plasma for HNA, CNA and NA, respectively. In urine, the detection limit was higher than that observed in plasma, since endogenous

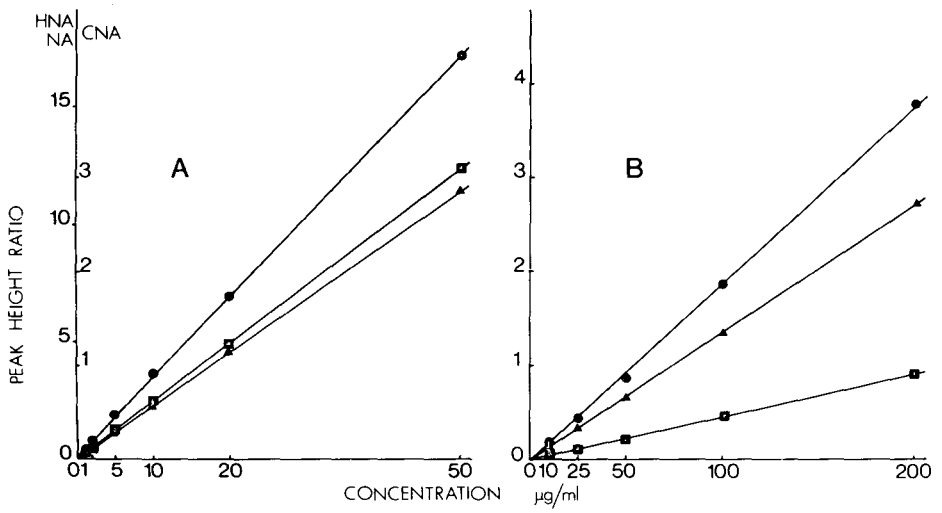


Fig. 2. Extracted standard curves of (A) plasma and (B) urine spiked with HNA (•), CNA (▪) and NA (▲); each point represents the average of six determinations.

substances increased the background noise, and found to be at 80, 420 and 130 ng/ml for HNA, CNA and NA, respectively.

Reproducibility and recovery

In order to check the reproducibility of the extraction procedures used, various spiked plasmas and urines were repeatedly analysed. Table II shows the results obtained for several concentrations of HNA, CNA and NA. The overall mean coefficients of variation were ± 2.6%, ± 4.8% and ± 2.7% for HNA, CNA and NA, respectively.

TABLE I

LINEAR STANDARD CURVES DETERMINATION

Biological fluid	Compound	No. of points	Slope ± S.E.	Intercept ± S.E.	r^2
Plasma	HNA	6	0.340 ± 0.074	0.098 ± 0.0072	0.9987
	CNA	6	0.061 ± 0.007	0.011 ± 0.0007	0.9996
	NA	6	0.227 ± 0.026	0.049 ± 0.0026	0.9996
Urine	HNA	5	0.019 ± 0.003	− 0.042 ± 0.0003	0.9993
	CNA	5	0.0046 ± 0.0005	− 0.010 ± 0.00005	0.9997
	NA	5	0.014 ± 0.0012	− 0.004 ± 0.0001	0.9998

TABLE II

REPRODUCIBILITY OF THE ASSAY IN HUMAN PLASMA AND URINE

Compound	Biological fluid (1 ml)	Amount of compound added (μg)	Average of 6 assays (μg)	Coefficient of variation (%)
HNA	Plasma	1	0.99	2.5
		2	2.04	2.3
		5	5.13	2.9
	Urine	10	10.2	3.1
		25	24.8	2.0
		50	50.5	2.7
CNA	Plasma	1	0.93	8.8
		2	1.97	7.2
		5	5.12	3.9
	Urine	10	9.69	4.4
		25	25.9	1.0
		50	49.7	3.7
NA	Plasma	1	0.97	3.7
		2	1.92	3.6
		5	5.04	0.6
	Urine	10	10.4	4.7
		25	24.3	1.8
		50	49.6	1.8

TABLE III

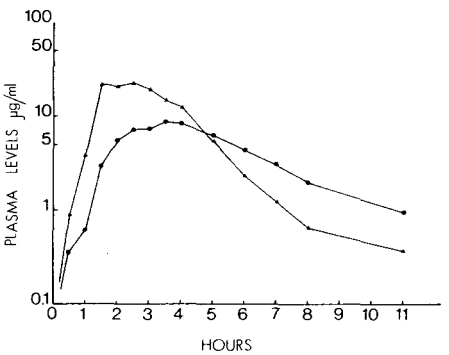
RECOVERY OF HNA, CNA AND NA FROM PLASMA AND URINE

Biological fluid	Compound	Added (μg/ml)	Found (μg/ml)	Recovery (%)	Mean ± S.E.
Plasma	HNA	2	1.64	82.0	83.4
		5	4.14	82.8	±1.8
		10	8.55	85.5	
	CNA	2	0.65	32.5	33.3
		5	1.69	33.8	±0.7
		10	3.37	33.7	
	NA	2	1.89	94.5	97.9
		5	4.96	99.2	±3.0
		10	10.02	100.2	
Urine	HNA	25	18.8	75.2	75.3
		50	38.2	76.4	±1.1
		100	74.3	74.3	
	CNA	25	7.8	31.2	30.3
		50	15.0	30.0	±0.8
		100	29.8	29.8	
	NA	25	22.9	91.6	93.1
		50	46.9	93.8	±1.3
		100	93.8	93.8	

The peak heights measured after extraction were compared to those obtained after injecting precise volumes of a solution of known concentration onto the HPLC column by means of the injection loop. The results, listed in Table III, show that HNA and NA were correctly extracted, whereas only 30% of CNA was recovered. This poor recovery for CNA was due to its low solubility in chloroform. For the three compounds, the recoveries observed were similar for plasma and urine, indicating that the use of ion-exchange resin in the urine procedure did not alter the extraction phase. It must be noted that the internal standard was extracted with a recovery of 97.5% and 95.5% for plasma and urine, respectively.

Kinetic studies

The method was applied to pharmacokinetic studies of NA in humans. Figs. 3 and 4 show the log plasma levels and cumulative urinary excretion curves versus time after administration of a single oral dose of 1 g of NA to a healthy volunteer.



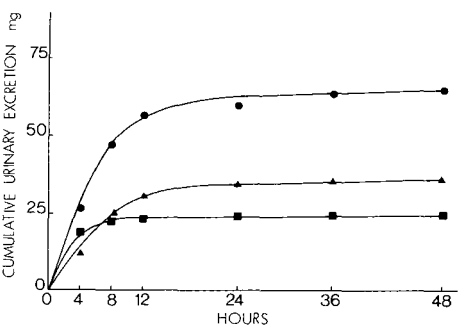


Fig. 3. Plasma level time curve obtained for NA (▲) and HNA (●) following a single oral administration of 1 g of nalidixic acid to a healthy volunteer (CNA was present in urine only).

Fig. 4. Cumulative urinary excretion of non-conjugated NA (▲), HNA (●) and CNA (■) observed after a single oral administration of 1 g of nalidixic acid to a healthy volunteer.

It must be noted that CNA could only be detected in urines. In plasma, NA concentration reached a maximum (25 µg/ml) within 2 h and then decreased rapidly to 0.2 µg/ml 11 h following the administration. Metabolite HNA exhibited a similar evolution but at lower levels than NA, and after a delay due to its formation from the parent drug. The cumulative urinary excretion curves for the compounds indicated that nalidixic acid was mainly eliminated as HNA and CNA.

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DETERMINATION OF DISULFIRAM AND METABOLITES FROM BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the determination of disulfiram, diethyldithiocarbamate, diethyldithiocarbamate methyl ester, carbon disulfide, and diethylamine from a single sample of plasma or urine. The analytical procedure is based on a quantitative stepwise extraction of disulfiram and diethyldithiocarbamate methyl ester, or the conversion of diethyldithiocarbamic acid, carbon disulfide and diethylamine to diethyldithiocarbamate methyl ester for chromatographical determination. The procedure is specific, precise and simple. The application of the analytical methods developed for the determination of disulfiram and the various metabolites in plasma from mice given disulfiram intraperitoneally or humans given Antabuse orally is illustrated.

INTRODUCTION

Disulfiram (tetraethylthiuram disulfide, Antabuse®) (DSF) has been used in the treatment of alcoholism since its introduction by Hald et al. [1]. Even though DSF has been used in alcoholics for approximately thirty years, little is known about its absorption and elimination characteristics. The main reason for this paucity of information appears to be due to the lack of an analytical method suitable for use in humans which can determine DSF and its metabolites in biological fluids.

A number of different analytical methods for the determination of disulfiram and its metabolites have been employed. These include spectrophotometry [2—8], polarography [9—13], proton magnetic resonance [14] and gas chromatography [15—17]. The use of radioactive disulfiram in man and animals also has been employed to study the metabolites and excretion characteristics of DSF [18—21]. Although the various methods employed are suitable, none of these appear to combine the criteria of convenience, speed, and with some of the methods, sensitivity. Furthermore, the use of radioactive DSF

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limits the usefulness of this method in studies of a patient population. The studies to be reported here describe a high-performance liquid chromatographic (HPLC) method which appears to fulfil many of the desired criteria.

EXPERIMENTAL

Chemicals

DSF was supplied by Ayerst Laboratories (New York., U.S.A.). Diethyldithiocarbamate (DDTC) was purchased from Sigma (St. Louis, Mo., U.S.A.), and diethyldithiocarbamate methyl ester (DDTC-Me) was prepared in our laboratory [21]. All solvents were of analytical grade and used without further purification. The heptane must be of fluorescence grade. Solvents were tested for purity by evaporating aliquots equal in volume to those used in the extraction procedure, and chromatographing them in the liquid chromatograph at the highest sensitivity.

Apparatus

A Perkin-Elmer (Norwalk, Conn., U.S.A.) Series 2 high-performance liquid chromatograph equipped with a 10 μm silica A column (25 cm $\times$ 0.26 cm) and a Model 440 absorbance detector at 254 nm (Waters Assoc., Milford, Mass., U.S.A.) were used in these studies. (Note: more recently a 5 μm, 25 cm Spherisorb silica column from Laboratory Data Control (Riviera Beach, Fla., U.S.A.) was used which improved peak sharpness and retention times.) The mobile phase employed was a mixture of heptane—tetrahydrofuran—methanol (97.6:2.2:0.2) with a flow-rate of 1.2 ml/min.

Preparation of standards

The internal standard employed was ethyl-*p*-nitrobenzoate. The internal standard solution was prepared by adding ethyl-*p*-nitrobenzoate to analytical grade ethanol, and the mixture then added to chloroform to make a final concentration of either 0.2 μg/ml or 1 μg/ml depending upon need.

Stock solutions of DSF, DDTC, DDTC-Me, diethylamine (DEA) and carbon disulfide (CS_2) in methanol were prepared at concentrations of 1 mg/ml. Dilutions when necessary were made to bring the final volume added to the sample to less than or equal to 20 μl/ml. Standards added to plasma, urine, or buffer were in a range from 0.5 to 2 μg/ml. The standards were extracted as described below.

Extraction procedure

The extraction solution used was 0.01 *M* EDTA in 1.0% sodium chloride, adjusted to pH 8.5 by the addition of sodium hydroxide. Into a 15-ml screw-top tube, 1 ml of either plasma, urine or buffer, 2 ml of 0.01 *M* EDTA solution, and 5 ml of the chloroform solution containing the internal standard were added. The tubes were sealed and shaken on a Labquake Shaker (Lab Industries, Berkeley, Calif., U.S.A.) for 10 min, after which they were centrifuged at 1765 *g* (Dynac Centrifuge, Model 0101) for 10 min. Because there exists a potential for acid-catalyzed decomposition of DDTC, urine cannot be collected and stored. Therefore, 0.5 ml of 0.5 *N* sodium hydroxide must be

added to the urine sample. A 2.5-ml aliquot of the aqueous layer containing the DDTC, DEA and CS_2 was transferred to a second 15-ml screw-top tube. The chloroform from the organic phase was evaporated at room temperature under a stream of nitrogen to a final volume of approximately 50 μl. This was then injected into the liquid chromatograph and the concentration of DSF and DDTC-Me determined by comparison of their peak heights to those obtained using standard solutions. To the 2.5 ml aqueous phase, 150 μl of methyl iodide were added to convert the DDTC present to DDTC-Me. This mixture was then vortexed for 30 sec and left to stand at room temperature for 15 min, after which 5 ml of the chloroform extraction solution containing the internal standard were added. The tube was shaken for 10 min, and then centrifuged for 10 min at 1765 *g*.

The upper aqueous phase from the mixture was now separated and 1-ml aliquots placed into two 15-ml screw-top tubes (tubes A and B). The organic phase from this mixture was removed, placed in a conical centrifuge tube, and the chloroform evaporated to a final volume of 50 μl. This volume was injected into the chromatograph and the concentration of DDTC-Me determined. To tube A, 100 μl of CS_2 were added and left to stand for 10 min. This converted the DEA to DDTC. After 10 min, 100 μl of methyl iodide were added to methylate the DDTC. To tube B, 100 μl of DEA were added which converted the CS_2 to DDTC, and left to stand for 10 min; then 100 μl of methyl iodide were added. After the addition of the methyl iodide, both tubes A and B were stoppered, vortexed for 30 sec, and left to stand at room temperature for 15 min. This completed the methylation of DDTC to methyl ester.

At the end of the 15-min period, 5 ml of the chloroform extraction solution containing the internal standard were added to both tubes A and B, the tubes shaken for 10 min, and then centrifuged at 1765 *g* for 10 min. The chloroform layer from each tube was pipetted into two separate glass conical centrifuge tubes, and the chloroform evaporated under nitrogen at room temperature to a final volume of 50 μl. At this time, 10 μl of the final 50-μl volume was removed from each glass conical centrifuge tube, injected into the chromatograph, and DDTC-Me from each tube determined. The DDTC-Me peak height obtained was compared to the standard curve and represented the amount of DEA and CS_2 extracted from tubes A and B, respectively.

Standard curves

Standard curves were prepared by adding known amounts of DSF, DDTC-Me, DDTC, CS_2 and DEA to buffer, plasma or urine. Analysis for each was then carried out as described. The peak height ratios of DSF and DSF-Me to the internal standard were calculated and plotted against known concentrations. The peak height ratios of unknown samples were calculated in the same manner and compared to the standard curves.

Recovery

Recovery studies were carried out by the separate addition of 0.5 μg of DSF, DDTC, DDTC-Me or DEA and 5 μl of CS_2 to 1 ml of either physiological phosphate buffer, plasma or urine. DSF and the various metabolites were then extracted as described and the percentage recovery determined by comparison with a standard concentration of drug entity.

In vivo studies

Mice. Male mice (HA/ICR, Sprague—Dawley, Madison, Wisc., U.S.A.) weighing 30—35 g were given DSF 200 mg/kg intraperitoneally (i.p.). The DSF was solubilized with polysorbate 80, and then 1% methylcellulose was added to give a final preparation for injection. A volume of 0.1 ml of DSF suspension per 10 g of animal weight (200 mg/kg) was administered i.p. Mice were sacrificed by decapitation at 5, 10, 20 and 30 min after DSF administration. Blood was collected, and 50 μl of 1 *M* sodium citrate solution were added to prevent coagulation. After centrifugation at 1765 *g* for 10 min, 100—500 μl of plasma were taken and extracted as described above.

Human. Male alcoholic volunteers were given 500 mg of Antabuse at 8:00 a.m. after an overnight fast. An indwelling venous catheter was placed in the subject's arm and 4-ml blood samples were drawn into vacutainer tubes at various times after dosing. Plasma samples were then analyzed for DSF and the various metabolites.

RESULTS

A scheme illustrating the various steps in the extraction procedure is outlined in Fig. 1. In Fig. 2, a typical separation of DSF, DDTC-Me and internal standard in the mobile phase extracted from plasma and urine is shown. Fig. 2 also includes chromatograms for both plasma and urine blanks extracted in the same manner as plasma and urine containing DSF and DDTC-Me. No interference by the plasma and urine peaks with those of DSF and DDTC-Me was found. The solvent peaks shown in the blank samples are due to the increased sensitivity used for these injections. The retention times for DSF, DDTC-Me and the internal standard were 7.4, 3.1 and 4.3 min, respectively, for the mobile phase and flow-rate used.

The standard curve obtained for DSF and DDTC-Me in plasma is illustrated

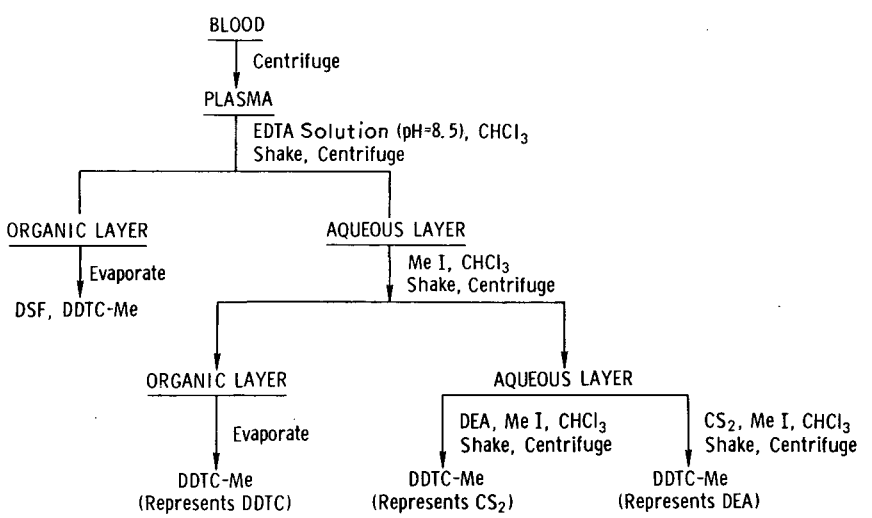

Fig. 1. Extraction scheme for disulfiram and metabolites. For extraction from urine or buffer, begin at plasma step.

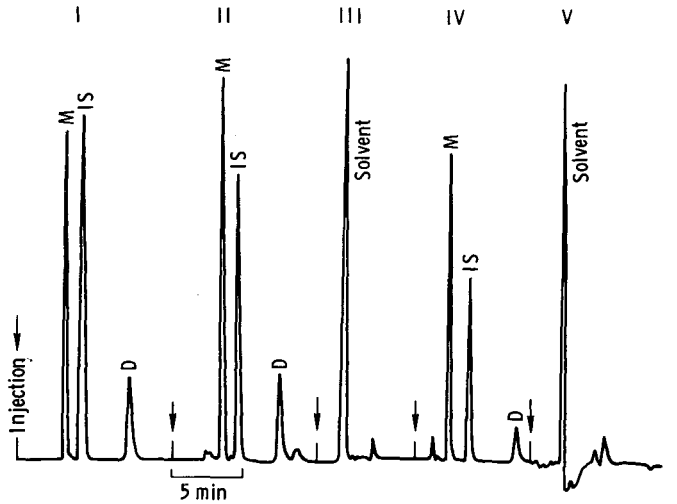

Fig. 2. HPLC chromatograms of DSF (D), DDTC-Me (M) and internal standard (IS) in mobile phase (I), plasma (II), and urine (IV). Plasma blank (III) and urine blank (V) are also shown. Points of injection are indicated by arrows. See text for column description, eluent, flow-rate and retention times.

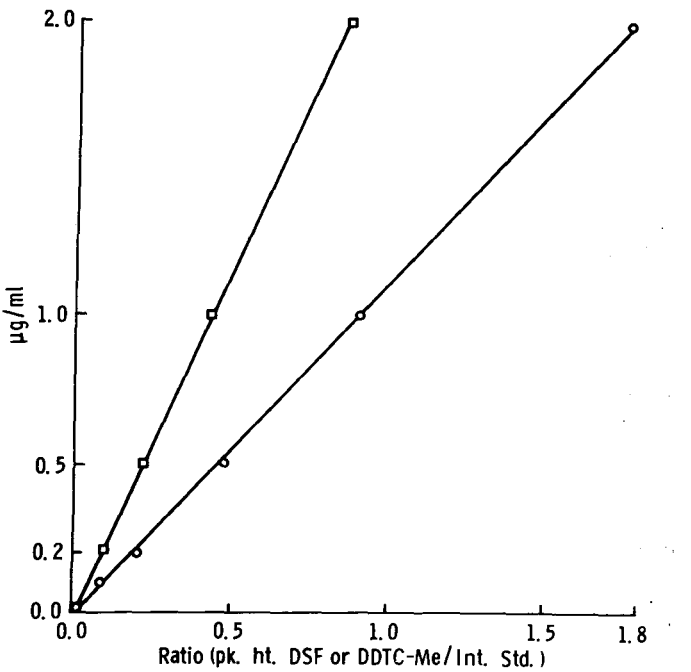

Fig. 3. Standard curves prepared by plotting concentration of DSF (□) or DDTC-Me (○) extracted from plasma against the peak height ratio of DSF or DDTC-Me/internal standard.

in Fig. 3. DSF and DDTC-Me were added to plasma in concentrations of 0.2—2.0 μg/ml and the samples extracted as described. The amount (μg/ml) of drug added was plotted against the ratio of the peak height obtained for the drug to the peak height of the internal standard. Good linearity was found with correlation coefficients of 0.999 and 0.995 for DSF and DDTC-Me, respectively. Standard curves for DDTC, CS_2 and DEA showed correlation coefficients of 0.995 or better. The minimum amount of DSF and DDTC-Me that could be detected was 5.0 ng and 2.5 ng, respectively.

The recoveries of DSF, DDTC, DDTC-Me, DEA and CS_2 from buffer, plasma and urine are given in Table I. Almost complete recovery was obtained when DSF and the various metabolites were added to phosphate buffer. When added to either plasma or urine, however, recovery of DSF and metabolites was not complete. Recoveries for DDTC and DDTC-Me from plasma and urine were reasonably good, even though only 71% of added DDTC could be recovered from urine. The values obtained are the mean of ten determinations, except for the DDTC study which is the mean of nine determinations. The percentage recovery was not influenced by changes in the concentration of DSF or the various metabolites in the concentration range studied. This is suggested from the good linearity of the standard curves shown in Fig. 3. Various experiments during the course of these studies verified this result.

The accuracy and reproducibility of the analytical method developed is illustrated in Table II. After the addition of 0.5 μg/ml of DSF, DDTC, DDTC-Me, DEA and 5.0 μg/ml of CS_2 to plasma, the amount added compared favor-

TABLE I

PERCENTAGE RECOVERY

Values are the average percentage recovery of ten determinations ± S.E. except for the DDTC samples which are the average of nine determinations.

	DSF	DDTC	DDTC-Me	DEA	CS_2
Plasma	51 ± 3.8	85 ± 10.1	91 ± 3.1	52 ± 5.6	48.7 ± 10.5
Urine	96.2 ± 11.1	71.2 ± 9.2	92.5 ± 2.5	45.2 ± 5.5	55.4 ± 3.8
Buffer	98.4 ± 4.7	94.9 ± 7.0	96.9 ± 4.4	85.8 ± 5.2	97.4 ± 8.2

TABLE II

REPRODUCIBILITY OF EXTRACTION

Values represent the average of ten determinations ± S.E., except for DDTC which is the average of nine determinations.

Drug entity	Amount added (μg/ml)	Amount found (μg/ml)
DSF	0.5	0.5 ± 0.04
DDTC	0.5	0.5 ± 0.11
DDTC-Me	0.5	0.5 ± 0.07
DEA	0.5	0.48 ± 0.18
CS_2	5.0	4.8 ± 0.38

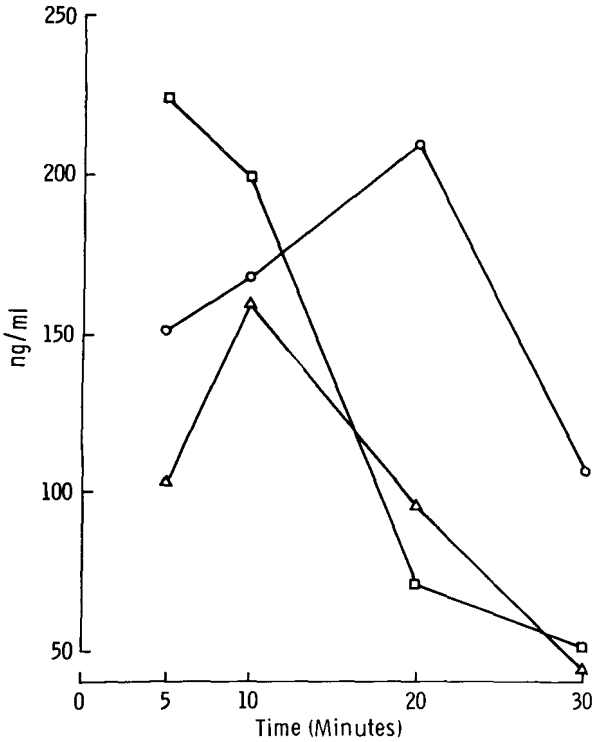

Fig. 4. Plasma concentration of DSF (□), DDTC-Me (○) and DDTC (△) as a function of time after i.p. injection of DSF (200 mg/kg) to mice.

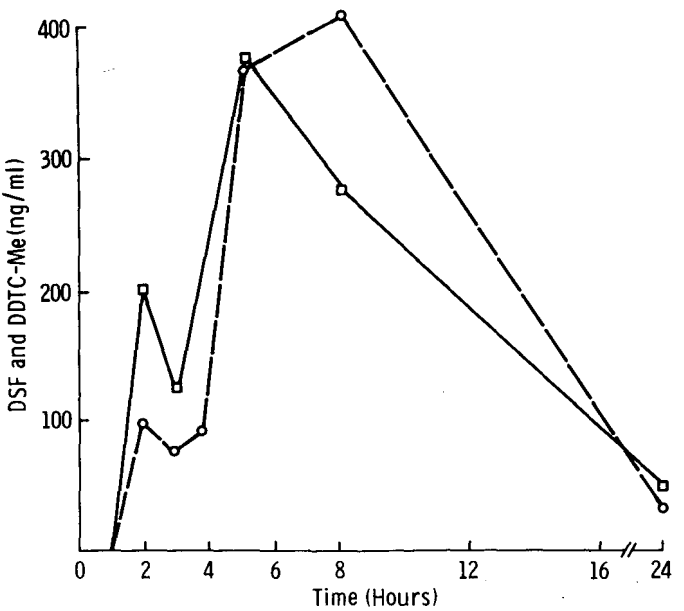

Fig. 5. Plasma DSF (□) and DDTC-Me (○) as a function of time after a single dose of 500 mg of DSF (Antabuse) to an alcoholic volunteer.

ably to that found after subsequent extraction and correction for protein binding.

The plasma concentration profile for DSF, DDTC-Me and DDTC as a function of time after the i.p. administration of 200 mg/kg of DSF to mice is shown in Fig. 4. DSF is detected in plasma 5 min after its administration. Plasma DDTC and DDTC-Me appear to peak 10 and 20 min, respectively, after DSF administration. In Figs. 5 and 6 the plasma concentrations of DSF, DDTC, DDTC-Me, CS_2 and DEA are shown in an alcoholic volunteer at various times after a single dose of orally administered Antabuse. As can be seen, both nanogram and microgram quantities of the drug entities can be found.

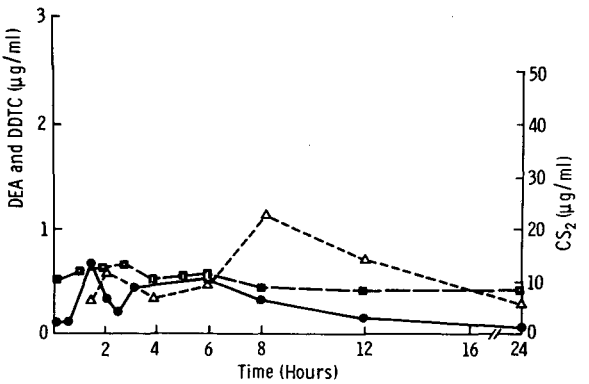

Fig. 6. Plasma concentrations of DEA (●), DDTC (△) and CS_2 (■) as a function of time after a single dose of 500 mg of DSF (Antabuse) to an alcoholic volunteer.

DISCUSSION

The sharpness and symmetry of the peaks shown in Fig. 2 and the linearity of the standard curve in Fig. 3 justify the use of peak height measurements to determine drug concentration. Furthermore, neither plasma nor urine contained endogenous substances which interfered with the HPLC method as the blanks did not show interfering peaks (Fig. 2). In addition, the internal standard employed could be added directly at the extraction step and then extracted along with the other compounds of interest.

The standard curve shown in Fig. 3 was prepared by adding known concentrations of DSF and DDTC-Me to human plasma, and then extracting the drug as previously described. The standard curve gave good linearity in the concentration range 0.2—2.0 μg/ml, with correlation coefficients equal to or greater than 0.995. Intercepts of 15 ng or less were calculated from least-squares regression analysis; this represents an error of 7.5%. The minimum amounts that could be detected in the mobile phase were 2.5 ng for DDTC-Me and 5.0 ng for DSF.

Recovery of DSF and other metabolites added to phosphate buffer was complete (Table I). However, DSF, DEA and CS_2, when added to plasma, and the addition of DEA and CS_2 to urine, showed lower recoveries. It is possible that protein binding may contribute to the lower DSF recovery. This is suggested from preliminary studies where approximately 50% of the DSF

was found bound to bovine serum albumin (unpublished results). This degree of binding to albumin was not found with either DDTC or DDTC-Me. This seems to correlate with the recovery studies for these metabolites as shown in Table I. The lower recoveries of DEA and CS_2 do not appear to be due to volatility, and the reason for this binding at this time is uncertain.

The usefulness of the HPLC method is illustrated in Fig. 4, where the concentration—time profile of DSF, DDTC and DDTC-Me as a function of time after DSF administration to mice is shown. In these experiments, CS_2 and DEA determinations were not made. However, these have been carried out in preliminary studies in alcoholics, and their concentrations can be readily determined. The data illustrated in Fig. 4 show that as the plasma concentration of DSF falls, DDTC increases. The observation that peak plasma levels for DDTC-Me occur after peak levels of DDTC is not unreasonable as methylation of DDTC must first occur. Further discussion concerned with DSF distribution, metabolism and excretion appears elsewhere [22]. In Fig. 5, DSF and DDTC-Me increased gradually after Antabuse, falling to negligible levels after 24 h in this volunteer. Larger quantities of CS_2, DEA and DDTC are observed in plasma (Fig. 6). The plasma levels of DSF found in mice (Fig. 4) and alcoholic volunteers (Fig. 5) appear to be similar, even though the mice received a 30-fold greater dose of DSF. This anomaly is due to the presence of the methylcellulose—polysorbate 80 vehicle which has subsequently been shown to hinder absorption of DSF. In more recent studies with a saline—methylcellulose vehicle in rats this problem has been eliminated.

In developing this HPLC method the effect of pH on extraction was investigated. When the pH was decreased, decomposition of DDTC to CS_2 and DEA occurred. It was found that optimal extraction and minimal decomposition of metabolites occurred at a pH of 8.5.

The accuracy of the analytical method described was tested by adding DSF and its metabolites to plasma (Table II). After extraction, the peak heights of the test samples were compared with those of known standards. The DSF values were corrected for protein binding, while the DEA and CS_2 were corrected for conversion to DDTC-Me which was found to be approximately 95% efficient. The greater variability for CS_2 and DEA may be due to the fact that three extraction steps are required.

The newly developed HPLC method determines DSF and its metabolites with less sample manipulation than most other methods. The method is more sensitive than the colorimetric methods and simpler than those methods requiring the conversion of DDTC to CS_2 and correlation with DSF concentrations. Because DSF can be measured directly, there are fewer problems with the interpretation of the data. Furthermore, because of the sensitivity of the analytical method, only small samples need be used. This has proved advantageous in clinical studies where multiple samples of 1 ml of plasma have been used.

In conclusion, the HPLC method described fulfils most of the desired criteria. The method is rapid, reproducible, accurate and has the sensitivity needed to determine low concentrations of DSF and its metabolites in biological fluids and tissue. Also, because this is a non-radioactive method, it lends itself to various types of clinical studies.

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF SULFINPYRAZONE AND ITS METABOLITES IN BIOLOGICAL FLUIDS

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SUMMARY

A rapid, sensitive, and specific high-performance liquid chromatographic method is described for the quantitative analysis of sulfinpyrazone and its sulfone and *p*-hydroxy metabolites in plasma and urine. The method uses two different procedures for sample preparation: (1) a rapid and convenient procedure using a single extraction with 1-chlorobutane and subsequent back-extraction into sodium hydroxide solution for the analysis of sulfinpyrazone and its sulfone metabolite, and (2) a more time consuming procedure using triple extraction with ethylene dichloride, a buffer wash, and back extraction into the base for the additional analysis of the *p*-hydroxy metabolite. The lower limit of sensitivity for sulfinpyrazone is 50 ng/ml. Concentrations of sulfinpyrazone between 0.05 to 0.1 and 50 μg/ml were measured with an average coefficient of variation of 3.9%, ranging from 1.5 to 6.1%.

INTRODUCTION

Several methods have been reported for the analysis of the antiplatelet and uricosuric agent sulfinpyrazone in biological fluids (Table I). The original spectrophotometric assay [1, 2] involves solvent extraction from acidified plasma or urine with ethylene dichloride, a buffer wash, back extraction into sodium hydroxide solution, and subsequent measurement of the ultraviolet absorbance at 260 nm. This method has been found to suffer from both the lack of sensitivity and specificity inherent in spectrophotometric assays [3].

Three high-performance liquid chromatographic (HPLC) methods and one gas—liquid chromatographic (GLC) method have been described. Two HPLC methods have obvious disadvantages, one using radiolabelled sulfinpyrazone as an internal standard requiring the collection of the effluent solvent mixture and subsequent liquid scintillation counting [4], the other not having any internal

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TABLE I

COMPARISON OF METHODS FOR ANALYSIS OF SULFINPYRAZONE IN BIOLOGICAL FLUIDS

References	Analytical method	Sample	Sensitivity (μg/ml)	Reproducibility (coefficient of variation)	Comments
1,2,3	Spectro-photometric	Plasma, urine	Not given	Not given	Lacks specificity and sensitivity
4	HPLC	Serum	3 μg/ml	Not given	Uses radiolabelled internal standard
5	HPLC	Plasma, urine	0.2 μg/ml	3.8% (replicates of 0.2—80 μg/ml from plasma and urine)	No internal standard. Separate metabolites
6	HPLC	Plasma, urine	2 μg/ml	4.3% (calibration curves of 2—100 μg/ml from plasma)	Simple two-step extraction
7	GLC	Plasma	Not given	Not given (correlation coefficient 0.99 for calibration curve)	Several transfer or extraction steps. Derivatization. Time consuming
This paper	HPLC	Plasma, urine	0.05 μg/ml	3.9% (calibration curves of 0.1—50 μg/sample from plasma and urine)	Simple two-step extraction (or triple extraction). Measures simultaneously sulfinpyrazone and metabolites

standard [5]. The third HPLC method uses warfarin as an internal standard and is simple and specific but lacks sensitivity [6]. These methods have used 1-chlorobutane [4, 6] or a mixture of 1-chlorobutane and ethylene dichloride [5] as the extraction solvent, and an evaporation step [4], direct injection of the organic solvent [5], or back-extraction into sodium hydroxide solution [6]. The absorbance has been measured at 254 nm [4, 5] or 275 nm [6]. The GLC method is time consuming and involves several transfer or extraction steps, the use of benzene as the extraction solvent, evaporation, and derivatization before chromatography [7].

Sulfinpyrazone is both metabolized in the liver and excreted unchanged in the urine. The metabolites are *p*-hydroxy-sulfinpyrazone, 4-hydroxy-sulfinpyrazone, a sulfone metabolite of sulfinpyrazone, and glucuronides of sulfinpyrazone and the sulfone metabolite [8, 9]. Although one of the HPLC methods has demonstrated that the non-glucuronated metabolites are separated from sulfinpyrazone [5], no attempt has been made to develop an HPLC assay which measures sulfinpyrazone and its metabolites. This is an important consideration, however, for clinical pharmacological studies on sulfinpyrazone, since it has been suggested that its antiplatelet effect may involve a metabolite [10]. This paper describes a simple, specific, sensitive, and accurate HPLC assay for the simultaneous analysis of sulfinpyrazone and two of its metabolites in plasma and urine.

EXPERIMENTAL

Reagents and materials

Sulfinpyrazone (1,2-diphenyl-4-[2-(phenylsulfinyl)ethyl]-3,5-pyrazolidinedione) and its metabolites, *p*-hydroxy-sulfinpyrazone (G 32642), 4-hydroxy-sulfinpyrazone (GP 52097), and sulfone metabolite of sulfinpyrazone (G 31442), were a gift from Ciba-Geigy (Basle, Switzerland). The internal standard, clofibrinic acid [2-(4-chlorophenoxy)-2-methylpropionic acid], was obtained from Sigma (St. Louis, Mo., U.S.A.). The acetonitrile was of "distilled in glass" quality and was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All glassware used during sample preparation was silanized. The silanizing agent, Prosil-28, was obtained from PCR Research Chemicals (Gainesville, Fla., U.S.A.). All other solvents and reagents were of analytical grade.

Sample preparation

A schematic representation of the procedure for the analysis of sulfinpyrazone and its sulfone metabolite is shown in Fig. 1. Plasma (1 ml) or urine (1 ml) is placed in a PTFE-lined screw-capped culture tube, and 60 μl of inter-

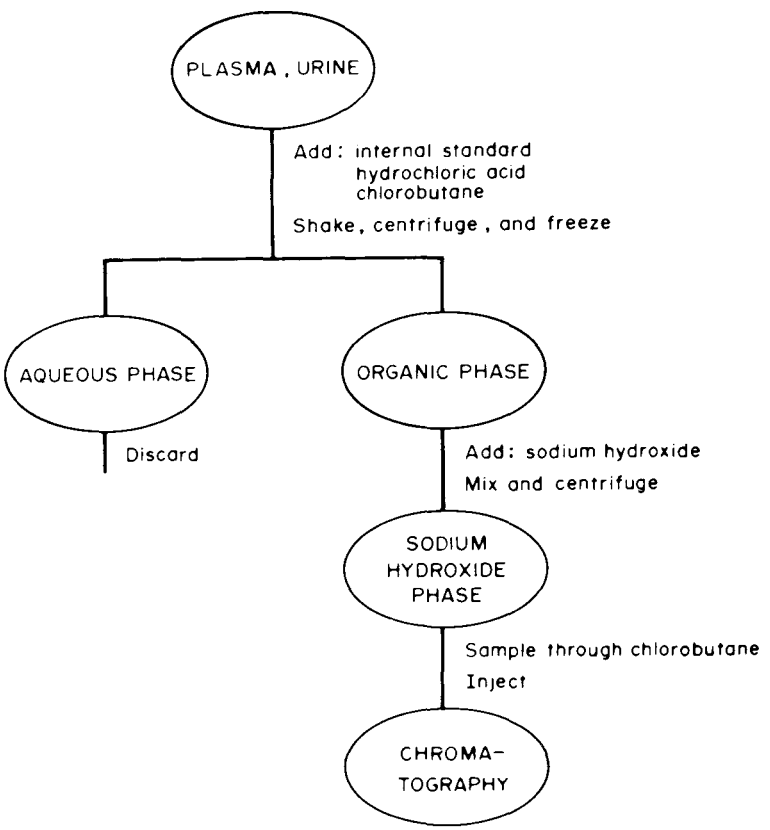

Fig. 1. Flow diagram of the sample preparation for the HPLC analysis of sulfinpyrazone and its sulfone metabolite.

nal standard solution (containing 60 μg of clofibrinic acid), 2 ml of 1 *N* HCl and 1 ml of 1-chlorobutane are added. The samples are extracted by mixing (using a Labquake automatic shaker) for 10 min, followed by centrifugation at 1000 *g* for 10 min to separate the aqueous and organic phases. The lower aqueous phase is frozen by immersing the tube in a dry ice—acetone bath, and the organic phase is poured into another tube, which has an elongated cone (capacity approximately 100 μl). Then 200 μl of 0.1 *N* NaOH are added, and the mixture is agitated in a Vortex mixer for 30 sec. After brief centrifugation, 100 μl of the aqueous phase are sampled from the elongated cone and injected into the chromatograph. When smaller volumes of plasma are used (0.1 and 0.5 ml), the same procedure is followed with two minor modifications, i.e., the volume of 1 *N* HCl added is reduced such that it remains twice that of the plasma sample, and the amount of internal standard added is reduced to 40 μg for 0.5 ml sample and to 20 μg for 0.1 ml sample.

For the additional analysis of *p*-hydroxy-sulfinpyrazone, 1 ml of plasma or urine is extracted three times with 1 ml of ethylene dichloride following the addition of the internal standard and hydrochloric acid solutions. The combined organic phase is washed by mixing with 1 ml McIlvaine's citric acid—phosphate buffer (citric acid 0.1 *M*, disodium phosphate 0.2 *M*; pH 5.0) for 5 min prior to back-extraction into the sodium hydroxide solution.

Chromatography

The high-performance liquid chromatograph consisted of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000A high-pressure solvent delivery system which was equipped with a Model U6K injector and fitted with a Waters μBondapak C_{18} reversed-phase column (30 × 0.39 cm I.D.; particle size 10 μm). The absorbance was measured at 270 nm, with a 0.1 a.u.f.s. deflection, using a Waters Model 450 variable-wavelength detector. The mobile phase was the same as used in a recently published method [6], i.e., 0.1 *M* ammonium acetate in acetonitrile—water (30:70), adjusted to pH 5.0 with acetic acid. The flow-rate of the solvent mixture was 1 ml/min with a column input pressure of 55 atm (800 p.s.i.). Chromatograms were recorded on a Linear Instruments (Irvine, Calif., U.S.A.) Model 585 dual-pen recorder.

Calibration and accuracy

Calibration curves were constructed by adding known amounts of sulfinpyrazone and internal standard to control plasma or urine, and plotting the peak height ratio of sulfinpyrazone to internal standard against the amount of sulfinpyrazone added. In order to calibrate the method and determine its accuracy for each series of unknown samples, standards of 0.1, 0.5, 5, 10, 25, and 50 μg of sulfinpyrazone (in 100 μl of distilled water) were added to the control samples, which were assayed concurrently with the unknown samples. The mean normalized peak height ratios were used to calculate the amount of sulfinpyrazone in unknown samples, and the standard deviation of the normalized peak height ratios was used to determine the accuracy of the method over the range of sulfinpyrazone standards employed. The reproducibility of the method was also studied by submitting five replicate plasma samples containing 0.5, 5, 10, 25, and 50 μg of sulfinpyrazone to the entire procedure. Likewise, calibration

curves were constructed for the sulfone metabolite by adding standards of 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 μg to the same control samples. Replicate samples were also analyzed for the sulfone metabolite at these same concentrations. Calibration curves were also constructed for *p*-hydroxy-sulfinpyrazone by adding standards of 0.05, 0.1, 0.5, 1.0, 2.5, and 5.0 μg to control samples and using the ethylene dichloride extraction. The effect of sample size on the method was investigated by constructing additional calibration curves using 0.1 and 0.5 ml of plasma.

To estimate the recoveries for the method, the peak heights of analyzed samples containing known amounts of sulfinpyrazone, its metabolites, and the internal standard were compared to the respective peak heights obtained by injecting equal amounts directly into the chromatograph.

Application of the method to measure plasma concentrations

A healthy male volunteer received a single oral dose of 200 mg of sulfinpyrazone (one capsule, Anturane®, Ciba-Geigy). Samples of venous blood were collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 7, 8 and 24 h after administration. The blood was collected in silanized culture tubes and anticoagulated with heparin (10 units of sodium heparin per ml of blood). After centrifugation, the plasma was transferred to glass vials and stored at −40° until analyzed.

RESULTS AND DISCUSSION

Since sulfinpyrazone is metabolized in the liver to three known metabolites, i.e., sulfone, *p*-hydroxy, and 4-hydroxy metabolites (and glucuronated products of sulfinpyrazone and its sulfone metabolite) [8, 9], and a metabolite may be involved in its antiplatelet effect [10], we were interested in developing an HPLC assay which would simultaneously measure sulfinpyrazone and its metabolites in biological fluids for subsequent clinical pharmacological studies. Preliminary experiments showed that sulfinpyrazone, and the sulfone and *p*-hydroxy metabolites all had absorption peaks between 255 and 270 nm. The 4-hydroxy metabolite, however, does not appreciably absorb ultraviolet light in this range. Since the latter is found in minimal concentrations in plasma, accounts for only less than 1% of the eliminated drug in man, and is therefore clearly the least important of the metabolites, its analysis was not pursued.

Although sulfinpyrazone has an absorption peak at 260 nm [1], the absorbance is measured at 270 nm in this method. This is because the internal standard, clofibrinic acid, has an absorption peak at about 280 nm (its major absorption peak, however, is at 226 nm). A compromise is therefore made between these two absorption peaks.

The reported methods for the quantitative analysis of sulfinpyrazone in biological fluids have used as the extraction solvent either ethylene dichloride [1, 2], 1-chlorobutane [4, 6], or a mixture of these two solvents [5], while one method uses benzene [7]. Preliminary experiments demonstrated two significant differences between ethylene dichloride and 1-chlorobutane as extraction solvents, i.e., 1-chlorobutane does not appreciably extract *p*-hydroxy-sulfinpyrazone from acidified plasma while ethylene dichloride does, and ethylene dichloride has to be washed with buffer before subsequent back-extraction into

the base, which 1-chlorobutane does not, to eliminate interfering peaks. This therefore resulted in the development of two different procedures for sample preparation: (1) a rapid and convenient procedure using a single extraction with 1-chlorobutane and subsequent back-extraction into sodium hydroxide solution for the analysis of sulfinpyrazone and its sulfone metabolite, and (2) a more time consuming procedure using triple extraction with ethylene dichloride and a buffer wash, when it is also desired to determine the concentrations of the *p*-hydroxy metabolite. The total recoveries of the three compounds using the former procedure range between 50 and 65%, while the total recoveries of the four compounds using the latter procedure range between 70 and 80%. It should be noted that the injection of the small volume of base (i.e., 100 μl) onto the column did not result in premature deterioration of the column.

Figs. 2 and 3 show chromatograms for blank plasma and plasma containing added amounts of the four compounds of interest using the two different procedures for sample preparation; Fig. 2 shows chromatograms following the

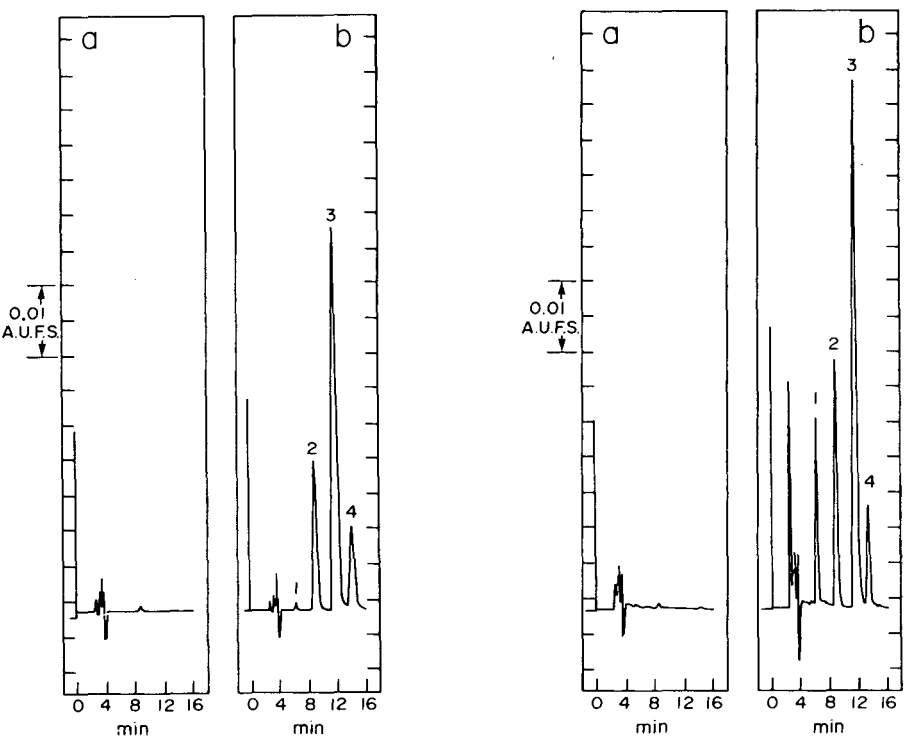

Fig. 2. Chromatograms of (a) control plasma and (b) plasma containing added amounts of (1) *p*-hydroxy-sulfinpyrazone, (2) the internal standard (clofibrinic acid), (3) sulfinpyrazone, and (4) the sulfone metabolite of sulfinpyrazone following the sample preparation using the single extraction with 1-chlorobutane. For visual clarity, only one tracing of the dual pen recording is shown.

Fig. 3. Chromatograms of (a) control plasma and (b) plasma containing the same added compounds and in the same amounts as in Fig. 2 following the sample preparation using the triple extraction with ethylene dichloride.

single extraction with 1-chlorobutane, Fig. 3 shows chromatograms following the triple extraction with ethylene dichloride and the buffer wash. Note the markedly increased recovery of *p*-hydroxy-sulfinpyrazone, as well as of the other compounds on the latter figure.

Estimates of accuracy for the method are shown in Table II. The average normalized peak height ratio for sulfinpyrazone obtained from calibration curves from plasma (different volumes) and urine had a mean coefficient of variation of 3.9% for a total of 14 such calibration curves using different volumes of plasma. This estimate of accuracy covers the entire range of the assay procedure, from 50 ng to 50 μg of sulfinpyrazone per sample. Calibration curves for the sulfone metabolite (n=12) and the *p*-hydroxy metabolite (n=2) from plasma and urine had mean coefficients of variation of 5.9% and 7.1%, respectively. These estimates for the metabolites cover the concentration range of 0.05 or 0.1 to 5.0 μg/ml. Reproducibility studies on replicates of sulfinpyrazone and its sulfone metabolite provided similar estimates of accuracy of the method (Table II). It should be noted that both procedures of sample preparation yield similar estimates of accuracy. As it may be necessary to use variable volumes of plasma for sulfinpyrazone measurement, the effects of plasma volume on the method (see Experimental) were examined as judged by the

TABLE II

ESTIMATES OF ACCURACY OF THE METHOD FOR DETERMINING SULFINPYRAZONE AND TWO OF ITS METABOLITES IN BIOLOGICAL FLUIDS

Biological fluid (volume)	Concentration range, μg/ml	Average mean normalized peak height ratio (number of studies)	Average coefficient of variation, % (range)
Sulfinpyrazone			
Calibration curve data			
Plasma (1.0 ml)	0.1—50	0.270 (7)	4.3 (1.5—6.1)
Plasma (0.5 ml)	0.1—50	0.174 (2)	3.9 (3.8—4.0)
Plasma (0.1 ml)	0.5—50	0.092 (2)	3.7 (3.4—4.0)
Plasma* (1.0 ml)	0.1—50	0.250 (1)	1.9
Urine (1.0 ml)	0.1—50	0.221 (2)	3.3 (2.1—4.5)
Reproducibility at a given concentration			
Plasma (1.0 ml)	0.5—50	0.265 (5)	3.8 (1.8—5.9)
Sulfone metabolite			
Calibration curve data			
Plasma (1.0 ml)	0.05—5.0	0.235 (7)	6.6 (5.1—9.4)
Plasma (0.5 ml)	0.05—5.0	0.152 (2)	5.3 (4.8—5.8)
Plasma* (1.0 ml)	0.1 —5.0	0.160 (1)	4.8
Urine (1.0 ml)	0.05—5.0	0.181 (2)	4.8 (3.8—5.8)
Reproducibility at a given concentration			
Plasma (1.0 ml)	0.1—5.0	0.225 (5)	5.0 (1.2—7.2)
p-Hydroxy-sulfinpyrazone			
Calibration curve data			
Plasma* (1.0 ml)	0.1—5.0	0.273 (2)	7.1 (5.6—8.6)

*Using the triple extraction with ethylene dichloride and a buffer wash.

peak height ratio, which has to be corrected for the different amounts of internal standard added, and the coefficient of variation; these estimates are independent of the volume of plasma used between 0.1 and 1.0 ml.

Application of the method to the determination of sulfinpyrazone and its sulfone metabolite in plasma from a healthy male volunteer is demonstrated in Fig. 4. It can be seen that the concentrations of the sulfone metabolite are approximately one-tenth of those of sulfinpyrazone and that they appear to fall parallel to those of the parent drug. It should be noted that no detectable concentrations of *p*-hydroxy-sulfinpyrazone were observed (following the triple extraction with ethylene dichloride).

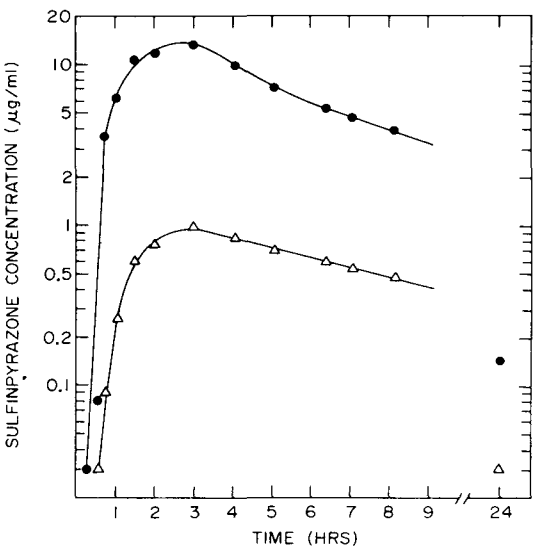

Fig. 4. Semi-logarithmic plot of plasma concentrations of sulfinpyrazone (•) and its sulfone metabolite (△) in a healthy male subject after a single oral dose of 200 mg of sulfinpyrazone.

The method described here for the quantitative determination of sulfinpyrazone and its sulfone metabolite, using the single extraction with 1-chlorobutane, is simple and rapid. By using the techniques described, 20—30 samples can easily be assayed in a day. When it is also desired to determine the concentrations of *p*-hydroxy-sulfinpyrazone, the method using the triple extraction with ethylene dichloride and the buffer wash is used. Such sample preparation, although more time consuming, allows the simultaneous determination of sulfinpyrazone, and its sulfone and *p*-hydroxy metabolites.

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SELECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAYS FOR HYDRALAZINE AND ITS METABOLITES IN PLASMA OF MAN

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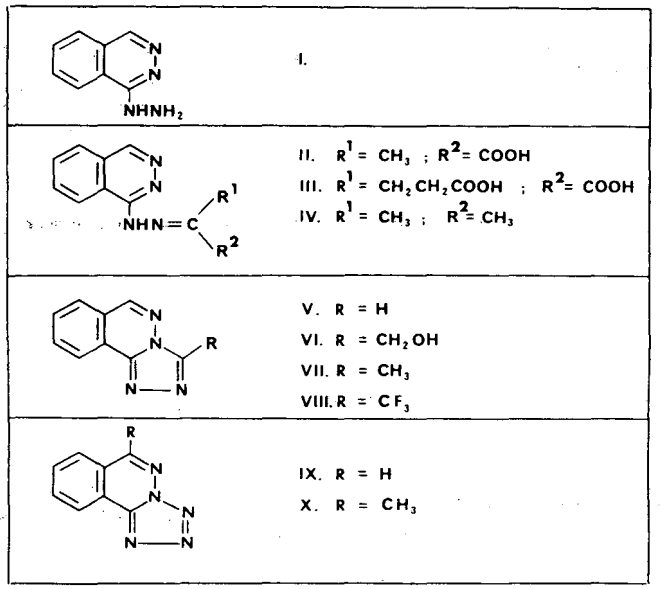
SUMMARY

Selective high-performance liquid chromatographic assays for hydralazine (I), hydralazine pyruvic acid hydrazone (II) and the acetylation metabolites, namely *s*-triazolo[3,4-*a*]-phthalazine (V) and 3-hydroxymethyl (VI) and 3-methyl-*s*-triazolo[3,4-*a*]phthalazine (VII) in human plasma were developed. Utilizing the fluorescence of these compounds or their derivatives the limits of detection could be extended down to 5 nmole/l (1 ng/ml) for I, 1 nmole/l (0.2 ng/ml) for II and 0.5 nmole/l (0.1 ng/ml) for V—VII. The intra-assay coefficients of variation for the assays ranged from 2 to 7% over the concentration range 5.0 to 0.05 μmole/l and the inter-assay variability in the slope of the standard curves ranged from 4 to 8%. An improved method for measuring the sum of I plus all its hydrazones (apparent I) was also developed. On addition of I to fresh plasma at 37°, half the added I was converted to II within 15 min and there was no detectable level of I, 2 h after the addition. The plasma level—time course of I, and its metabolites in a healthy volunteer (slow acetylator) following separate oral and intravenous administrations of I indicated that I contributed only a small fraction (4.3 and 4.7% respectively) to the area under the plasma level—time curve of apparent hydralazine.

INTRODUCTION

With the "comeback" of hydralazine (I) [1] there has been renewed interest in the measurement of plasma levels of the drug and its metabolites in man. Consequently, several plasma assay methods have been reported [2—4] together with determined pharmacokinetic parameters [5—10]. However, it has been convincingly demonstrated that the available assay methods lack specificity for unmetabolized hydralazine [11—13]. Acid labile hydrazones are converted back to hydralazine by the acidic conditions of the derivatization procedures and it is an "apparent hydralazine" level which is obtained. Two modifications of the original gas—liquid chromatographic assay have been reported



[13, 14] and one of these [13] still lacked sufficient specificity [15] for accurate determinations of I. Many of the pharmacokinetic parameters determined using the non-specific hydralazine assays are therefore misleading.

We have shown that the major hydrazone present in plasma is hydralazine pyruvic acid hydrazone (II) [12], formed by chemical reaction between hydralazine and endogenous pyruvic acid and accounts for more than 90% of apparent hydralazine levels at steady-state. Other hydrazones (III and IV) have also been identified in plasma but not quantitated [16].

In the present report specific high-performance liquid chromatographic (HPLC) assays for the parent drug (I), its metabolites (II, V—VII) and apparent hydralazine in plasma are described. I and II were measured after conversion to the fluorescent tetrazolo- and triazolo[3,4-*a*]phthalazine derivatives, respectively, and V—VII detected using their native fluorescence.

MATERIALS AND METHODS

Reagents and materials

All reagents were analytical grade and aqueous solutions were prepared using glass-distilled water. Specially purified acetonitrile (Unichrom from Ajax Chemicals, Melbourne, Australia) was used for HPLC. The synthesis of the hydralazine metabolites and 3-trifluoromethyl-*s*-triazolo[3,4-α]phthalazine (VIII) has been described previously [12]. 4-Methylhydralazine was generously donated by Ciba-Geigy, Basle, Switzerland.

Standards

Stock solutions of hydralazine hydrochloride (I, 50 μmole/l) and the internal standard, 4-methylhydralazine (16.7 μmole/l) were prepared in 0.01 *N* aqueous hydrochloric acid and stored in glass at 4°. These solutions were prepared fresh

weekly. Solutions of the hydrazones (II, III and IV) were prepared in 0.01 *N* aqueous sodium hydroxide and prepared fresh prior to each assay run or study. A stock solution containing the metabolites V, VI and VII (5.0 μmole/l each) was prepared in 0.01 *N* hydrochloric acid and was stable for several months at 4°. The internal standard for the assay of the metabolites V—VII, 3-trifluoromethyl-*s*-triazolo[3,4-*a*]phthalazine (VIII) was prepared in 0.01 *N* hydrochloric acid (5 μmole/l) and was also stable for several months at 4°.

Plasma standards of I, II and V—VII were prepared using the above solutions at the time of each assay run. In the case of I and II, derivatization was performed immediately after the addition of the standard and internal standard solutions to plasma to avoid losses due to reaction with endogenous keto-acids present in plasma.

In all cases, peak area ratios of the drug and metabolites to the internal standard were determined for plasma standards and unknowns and quantification performed by reading unknown values from a plotted standard curve.

Blood collection and plasma treatment

Special procedures were necessary and were adopted when plasma concentrations of circulating levels of hydralazine were measured. Venous blood samples (8—12 ml) were drawn quickly into plastic syringes and immediately transferred to ice-cold polypropylene tubes containing 125 I.U. of lithium heparin. The blood was then aliquoted into polypropylene conical centrifuge tubes (TC-10 centrifuge tube from Medical Plastics, Melbourne, Australia) and centrifuged at 8000 *g* in an Eppendorf Model 5412 centrifuge for 30 sec. The plasma was immediately transferred to a polystyrene tube kept in ice and two 1-ml aliquots put into separate glass tubes each containing 75 μl of 50% aqueous sodium nitrite. These samples were then immediately treated in the way described for the assay of I and II respectively. The entire procedure from the time of drawing the blood to the time of derivatization should be performed in less than 5 min. The remainder of the plasma was stored at −20° and assayed for apparent hydralazine and metabolites V—VII within 24 h.

High-performance liquid chromatography

The chromatograph used (Spectra-Physics Model SP 8000) was equipped with a ternary solvent system, helium degass and automatic data reduction facilities. A 10-μm alkyl phenyl reversed-phase column (μBondapak/phenyl from Waters Assoc., Milford, Mass., U.S.A.) was used at a column temperature of 50°. The mobile phase was 1.5 m*M* aqueous phosphoric acid—acetonitrile (either 85 : 15 or 80 : 20) at a flow-rate of 2 ml/min and all solvent lines from the column to the detector were carefully thermally insulated. The column effluent was monitored using a fluorescence detector (Schoeffel, Model 970) at an excitation wavelength of 250 nm with an emission cut-off filter allowing 90% transmission at 360 nm. The fluorimeter sensitivity setting was 3.5, range 0.1 μA full-scale and time constant 4.0 sec. Samples were injected automatically using an autosampler (Spectra-Physics Model 8010). All files for the operation of the chromatograph and for the processing of raw data for each assay were stored on disc (Spectra-Physics Model 8040) and recalled as required.

HPLC assay of I

To one of the tubes containing plasma and sodium nitrite described above, was added the internal standard solution (4-methylhydralazine, 150 μl of 16.7 μmole/l) and 2 ml of 0.02 *N* aqueous hydrochloric acid to give a final pH of 5.5. The mixture was then briefly vortexed and allowed to stand for exactly 10 min at 20 ± 1°. The entire procedure from the time of drawing the blood to the time of addition of the acid must be performed in less than 5 min. To the acidic mixture was then added 1 ml of an aqueous 1 *N* sodium hydroxide—0.6 *M* sodium tetraborate solution (pH 10) followed by chloroform (Nanograde from Mallinckrodt, St. Louis, Mo., U.S.A.). Extraction was carried out by shaking at 110 rpm for 5 min. The phases were separated by centrifugation (10 min at 1100 *g*) and the aqueous layer removed by vacuum aspiration. The organic layer was poured into culture tubes (diSPo tubes from Scientific Products, McGaw Park, Ill., U.S.A.) (75 × 12 mm) and evaporated under a stream of pure nitrogen at 45°. The residue was reconstituted in 0.5 ml of mobile phase consisting of 1.5 m*M* aqueous phosphoric acid—acetonitrile (85 : 15) and 50 μl injected into the chromatograph.

HPLC assay of II

The method employed for the assay of metabolite II was identical to that described for hydralazine itself except that plasma standards and samples containing II were treated with 0.1 *N* hydrochloric acid rather than 0.02 *N* providing a final pH of 3.9. Again the entire procedure from the time of drawing the blood to the time of acid treatment should be performed in less than 5 min. The level determined by this procedure included the acetylated material VII which was quantitated separately. The level of II was then determined by subtraction.

HPLC assay of V, VI and VII

To 1 ml of heparinized plasma in a glass tube was added the internal standard solution (VIII, 150 μl of 5.0 μmole/l) followed by 1 ml of an aqueous 1 *N* sodium hydroxide—0.6 *M* sodium tetraborate solution (pH 10). Five ml of chloroform were added and the mixture shaken at 100 rpm for 5 min. The phases were separated by centrifugation (10 min at 1100 *g*) and the aqueous layer removed by vacuum aspiration. The organic layer was poured into culture tubes and evaporated as before. The residue was dissolved in 0.5 ml of mobile phase consisting of 1.5 m*M* aqueous phosphoric acid—acetonitrile (80 : 20) and 50 μl injected into the chromatograph.

HPLC assay of apparent hydralazine

The method was a modification of the method of Jack et al. [4]. To 1 ml of heparinized plasma was added the internal standard solution (4-methylhydralazine, 150 μl of 16.7 μmole/l) followed by 2 ml of 2 *N* hydrochloric acid and 200 μl of 50% aqueous sodium nitrite solution. The mixture was vortexed and allowed to stand at 20 ± 1° for 15 min. An amount of 2.5 ml of 4 *N* aqueous sodium hydroxide was then added followed by 5 ml of chloroform. Extraction was carried out by shaking at 100 rpm for 5 min and the phases were separated by centrifugation (10 min at 1100 *g*). The aqueous layer was removed by

vacuum aspiration and the organic layer poured into culture tubes and evaporated as before. The residue was reconstituted in 1 ml of mobile phase [1.5 m*M* aqueous phosphoric acid—acetonitrile (85 : 15)] and 50 μl injected into the chromatograph.

Reproducibility and recovery

The intra-assay reproducibilities for the hydralazine and metabolites assays were determined by assaying five replicate plasma samples containing added amounts of drug and metabolites at concentrations ranging from 0.05—5.0 μmole/l.

Inter-assay reproducibility was not determined directly because of the rapid reaction of the parent drug with α-keto acids in plasma which precluded storing a batch of frozen samples and assaying one within each assay run. However the variation in the slopes of the standard curves for each assay was determined for five consecutive runs on different days. Recoveries of the methods were determined by injecting known amounts of IX and V—VII into the chromatograph and comparing the peak areas with those obtained for plasma standards of known concentration.

Specificity of the HPLC assays

The hydralazine hydrazones (II—IV) were added to plasma to give a final concentration of 5.0 μmole/l and assayed for hydralazine by the method described above. If conversion of the hydrazones to I occurred, the percentage converted was determined in each case. The hydralazine metabolites (V—VII) and a number of relevant and representative fluorescent drugs and their metabolites propranolol, 4-hydroxypropranolol, N-desisopropylpropranolol, quinidine, dihydroquinidine, 3-hydroxyquinidine, imipramine and desipramine were added to plasma and subjected to the conditions of the hydralazine and hydralazine pyruvic acid hydrazone assays. If a peak was obtained the retention time was recorded. Similarly, the hydrazones II—IV and propranolol, quinidine, imipramine and their metabolites were added to plasma and checked for interference in the assay for V—VII.

Stability of derivatized plasma samples to storage

I and II were added to plasma to provide a concentration of 5.0 μmole/l each and 1-ml aliquots placed in polystyrene tubes containing 75 μl of 50% aqueous sodium nitrite. The internal standard, followed by 2 ml of 0.1 *N* hydrochloric acid were then added and the tubes allowed to stand at 20 ± 1° for 10 min. An amount of 1.0 ml of 1 *N* aqueous sodium hydroxide—0.6 *M* sodium tetraborate (pH 10) was then added to all tubes and of these, five were immediately extracted with chloroform in glass tubes and assayed for I and II. Of the remaining tubes, five were left at 20 ± 1° and extracted 24 h later. Another five were stored at 4° for 4 h before extraction and assay. The remainder were frozen and stored at −20°. A number of these tubes were thawed 24 h, 1 week and 2 weeks after the initial treatment and assayed as before.

Reaction of I in plasma in vitro at 37°

Fresh venous blood from a non-medicated normal volunteer was heparinized

and centrifuged immediately. To the plasma was added hydralazine to provide a final concentration of 1.1 μmole/l and the mixture maintained at 37° in a water bath. Samples (1 ml) were taken at times 5, 10, 20, 30, 40, 60, 90 and 120 min after the addition and assayed for I, II and apparent hydralazine by the methods described. Measurement of I was also performed using 0.1 *N* hydrochloric acid treatment in order to compare with the results obtained using 0.02 *N* acid.

Pharmacokinetic studies

A healthy volunteer (87 kg) was acetylator phenotyped [17] and given an oral (1 mg/kg) and an intravenous (0.375 mg/kg) dose of hydralazine on separate occasions three weeks apart. The approximate oral dose (correct to the nearest 5 mg) was made up of a suitable combination of whole or fractions of 50- and 25-mg Apresoline® tablets and was administered with 150 ml of water. Blood samples were drawn at times 0, 10, 20 min and 0.5, 0.75, 1.0, 1.25, 1.5, 2, 2.5, 3, 4, 6, 8 and 10 h after the dose. The exact intravenous dose (Apresoline® for Injection) was diluted in 20 ml of sterile water for injection and administered as an infusion over 5 min. Blood samples were drawn from an ante cubital vein at times 0, 5, 10, 15, 20, 25 min and 0.5, 0.75, 1.0, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 7 and 10 h after commencement of the infusion. All blood samples were treated in the manner described earlier and assayed for I, II and V—VII.

RESULTS

HPLC assays for hydralazine and metabolites

Chromatograms obtained for the assays of I, II and V—VII and apparent hydralazine are shown in Figs. 1—3. The intra-assay coefficients of variation for the assays are shown in Table I, variations in the slope of the standard curves in Table II and recoveries for each of the assays in Table III. The limits of detection (determined at peak height twice noise) were 5 nmole/l (1 ng/ml) and 1 nmole/l (0.2 ng/ml) for I and II respectively and 0.5 nmole/l (0.1 ng/ml) for metabolites V—VII.

TABLE I

INTRA-ASSAY REPRODUCIBILITY FOR THE ASSAYS OF HYDRALAZINE AND METABOLITES

Drug/metabolite	Coefficient of variation (%) at concentrations* (μmole/l)						
	5.0	2.5	1.25	0.5	0.25	0.125	0.05
I	2	3	3.5	3.5	–	–	4
II	3	3	3	3.5	–	–	7
V	–	–	–	3	2	3	3
VI	–	–	–	2	4	3	2
VII	–	–	–	3	2	3	2

*Five replicate determinations at each concentration.

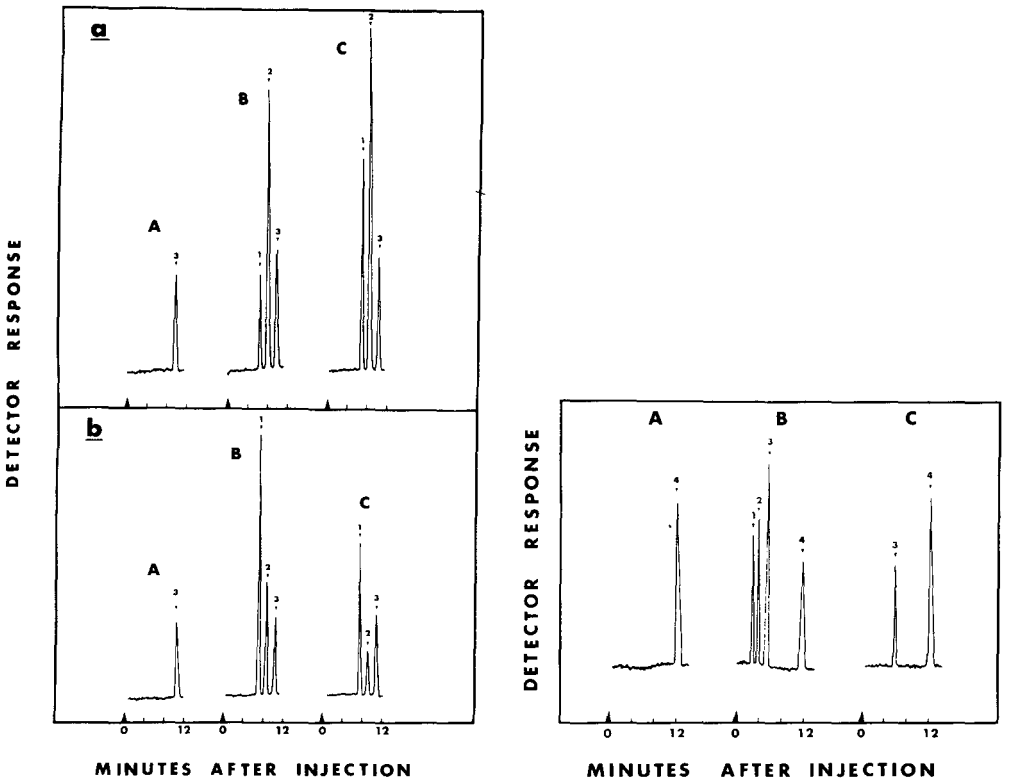


Fig. 1. Chromatograms obtained for the assay of I and II at (a) pH 3.9 and (b) pH 5.5. A = blank plasma; B = plasma standard containing I and II (1.25 and 5.0 μmole/l for a and b respectively); C = plasma from a volunteer following an intravenous dose of hydralazine (0.375 mg/kg) and containing I (2.9 μmole/l) and II (1.4 μmole/l). Peaks: 1 = IX, derivative of I; 2 = VII, derivative of II; 3 = X, derivative of the internal standard.

Fig. 2. Chromatograms obtained for the assay of the hydralazine metabolites V—VII. A = blank plasma; B = plasma standard containing 0.25 μmole/l each of V, VI and VII; C = plasma from a volunteer following an oral dose of hydralazine (1 mg/kg) and containing VII. Peaks: 1 = VI; 2 = V; 3 = VII; 4 = internal standard VIII.

TABLE II

VARIATION IN THE SLOPE OF THE STANDARD CURVES FOR THE HYDRALAZINE AND METABOLITE ASSAYS

Drug/metabolite	Coefficient of variation* (%)
I	8
II	8
V	5
VI	6
VII	6
Apparent hydralazine	4

*Determined from five consecutive standard curves on different days for each assay.

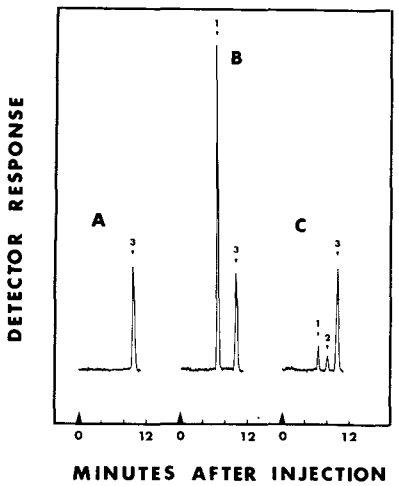


Fig. 3. Chromatograms obtained for the assay of apparent hydralazine, i.e. hydralazine plus hydrazones. A = blank plasma; B = plasma standard containing 5.0 μmole/l of I; C = plasma from a volunteer following an oral dose of hydralazine (1 mg/kg) and containing 0.34 μmole/l of apparent hydralazine. Peaks: 1 = IX; 2 = VII; 3 = X.

TABLE III

RECOVERIES FOR THE HYDRALAZINE AND METABOLITE ASSAYS

Drug/metabolite	Concentration range (μmole/l)	Recovery (%)
I	1.25 — 5.0	75
II	1.25 — 5.0	88
V	0.125— 0.5	98
VI	0.125— 0.5	60
VII	0.125— 0.5	98
Apparent hydralazine*	1.25 —10.0	86

*Recovery of I or II added to plasma.

Nitrous acid generated using 0.02 *N* hydrochloric acid (pH 5.5) converted more than 75% of the hydralazine in plasma to the derivative IX but only partly converted II to VII (17% recovery). Nitrous acid generated using 0.1 *N* hydrochloric acid (pH 3.9) resulted in almost 90% conversion of II to VII and after subtraction of the endogenous plasma level of VII allowed accurate quantitation of II. However, reduced accuracy can be expected if levels of VII are much greater than II. The conversion of II to VII was confirmed by gas chromatography—mass spectrometry where the spectrum obtained for the product was identical to that of an authentic sample of VII. Although no conversion of II to IX occurred, the stronger acid conditions (pH 3.9) converted 70% of III and 5% of IV added to plasma (5.0 μmole/l) to IX and therefore interfered in the determination of the parent drug. Separate conditions were therefore required for the estimation of I and II, that is, 0.02 *N* and 0.1 *N* hydrochloric acid providing pH 5.5 and 3.9 respectively. Metabolite III was not cyclized with weak nitrous acid to a triazolo[3,4-*a*]phthalazine in the same way as metab-

olite II. The peak obtained for III in the hydralazine assay was characterized by its retention time and spectral properties which were identical to those of IX.

The use of 4-methylhydralazine pyruvic acid hydrazone [12] as internal standard for the quantification of metabolite II rather than 4-methylhydralazine had no advantages in terms of accuracy or precision and resulted in considerably longer assay time due to the longer retention time of its derivative.

The use of excess strong acid (2 *N*) in the apparent hydralazine assay resulted in complete conversion of the hydrazones II—IV to the hydralazine derivative (IX) which was quantitated. This assay therefore provided an estimate of the hydralazine plus hydrazones level. If a smaller volume or more dilute acid was used relative to the plasma volume, some conversion of II to VII occurred and the total or apparent level was under-estimated.

The chromatographic and fluorimetric conditions for the assay of I, II and apparent hydralazine were identical and for the assay of metabolites V—VII, only a slight change in the mobile phase, aqueous phosphoric acid—acetonitrile from 85 : 15 to 80 : 20 was required for the chromatography. Using pre-programmed chromatographic conditions the injection and quantitation of large numbers of samples containing I, II and V—VII was carried out automatically overnight.

Specificity of the HPLC assays

Using 0.02 *N* hydrochloric acid, there was no interference by the hydralazine hydrazones (II and IV) in the hydralazine assay. The α-ketoglutaric acid hydrazone (III) was converted to the hydralazine derivative to the extent of 10%, however since plasma levels of this metabolite appear to be less than 10% of the apparent hydralazine level this represents an insignificant interference. The retention times of the other known fluorescent metabolites of hydralazine

TABLE IV

RETENTION TIMES (sec)

Derivative/metabolite	Mobile phase (1.5 m*M* phosphoric acid—acetonitrile)	
	85 : 15	80 : 20
Metabolite VI	230	160
3-Hydroxyquinidine	230	160
Metabolite V	310	230
4-Hydroxypropranolol	350	270
Tetrazolo[3,4-*a*]phthalazine (IX)*	400	–
N-Desisopropylpropranolol	430	310
Quinidine	430	310
Metabolite VII	490	320
Dihydroquinidine	530	350
6-Methyltetrazolo[3,4-*a*]phthalazine (X)**	600	–
Propranolol	920	620
3-Trifluoromethyl-*s*-triazolo[3,4-*a*]-phthalazine***	–	680
Desipramine	–	1450
Imipramine	–	1650

*Hydralazine derivative from nitrous acid treatment.

**4-Methylhydralazine derivative from nitrous acid treatment.

***Internal standard for the assay of metabolites V—VII.

and propranolol and quinidine and their metabolites in the hydralazine and hydralazine pyruvic acid hydrazone assays are shown in Table IV. Quinidine eluted between IX and VII and dihydroquinidine eluted between VII and X. If present in high levels, quinidine and its metabolites could therefore interfere in the assays for I and II. In the assay for the metabolites, 3-hydroxyquinidine had a retention time similar to VI and interfered in the determination of this metabolite. Imipramine and desipramine eluted after the internal standard in all assays.

Stability of derivatized samples to storage

As a result of the rapid disappearance of hydralazine in freshly drawn blood samples, derivatization had to be performed within 5 min of collection and the actual time elapsed noted. However, after nitrous acid derivatization and basification plasma samples could be stored for considerable lengths of time in polystyrene tubes at a variety of temperatures without a reduction in recovery (Table V).

TABLE V

STABILITY OF DERIVATIZED PLASMA SAMPLES TO STORAGE IN THE ASSAYS OF I AND II

The added amount was 5.0 μmole/l for I and II.

Storage conditions	Level obtained (μmole/l)	
	I	II
Immediate assay	5.1 ± 0.1*	5.1 ± 0.2*
24 h at 20°	5.2 ± 0.3*	5.0 ± 0.2*
4 h at 4°	5.1 ± 0.2*	5.0**
24 h at −20°	5.1 ± 0.1*	5.2 ± 0.5*
1 week at −20°	4.9**	5.1**
2 weeks at −20°	5.0**	5.3**

*Five determinations.
**Single determinations.

Reaction of I in plasma in vitro at 37°

Hydralazine (I) reacted rapidly with endogenous pyruvic acid in plasma at 37° to form II and as the level of I fell, there was a corresponding rise in the level of II (Fig. 4). The sum of the levels of I and II at any time was not significantly different from the apparent hydralazine level measured simultaneously and none of the metabolites V—VII were formed. Levels of I determined specifically at pH 5.5 declined to 0 μmole/l in 2 h, however estimations of the hydralazine level at pH 3.9 plateaued at 0.06 μmole/l probably due to interference by a small level of hydrazone III which was converted to IX. At sampling times up to 1 h there were no significant differences between the levels of I measured using the different acid strengths.

Pharmacokinetic studies

The plasma level time courses of I and its metabolites following separate oral and intravenous administration of the drug to a fasting healthy volunteer are shown in Fig. 5a and b respectively. The areas under the concentration—time

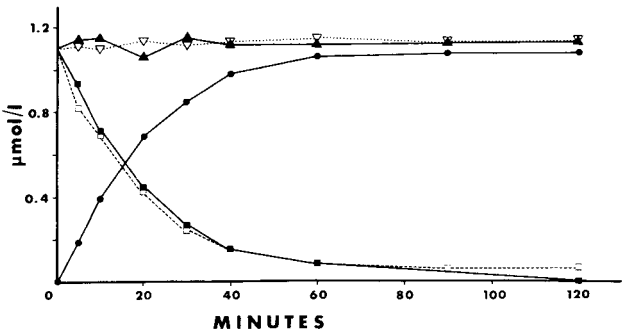


Fig. 4. Reaction of I (1.1 µmole/l) in fresh plasma at 37°. □——□ = I measured at pH 3.9; ■——■ = I measured at pH 5.5; ●——● = II; ▲——▲ = apparent hydralazine; ▽ ... ▽ = I + II.

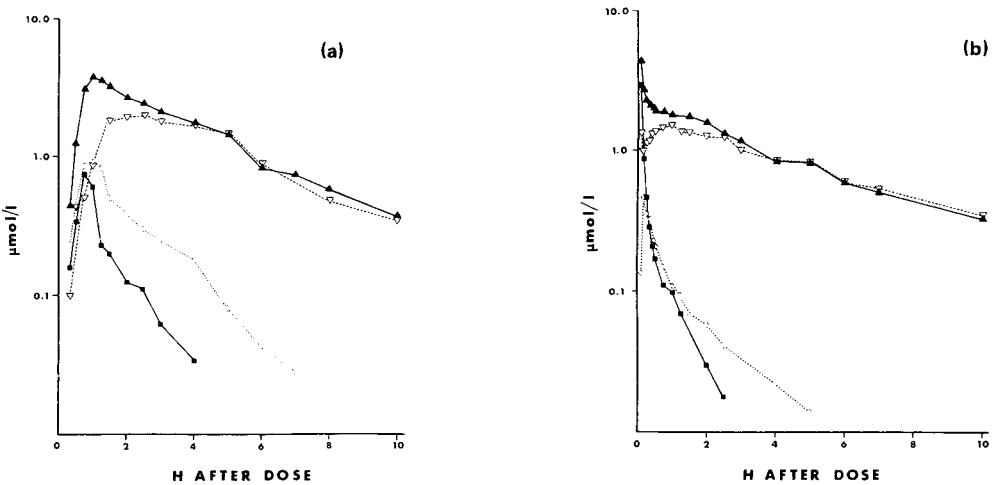


Fig. 5. Plasma level—time course of hydralazine and metabolites following an oral dose of hydralazine (1.0 mg/kg) to a healthy volunteer (slow acetylator) (a) and following an intravenous infusion (over 5 min) of hydralazine (0.375 mg/kg) to the same volunteer (b). ■——■ = I; ▽- - -▽ = II; ◇ ... ◇ = VII; ▲——▲ = apparent hydralazine.

curve (AUC) for each component are summarized in Table VI. The subject was a slow acetylator of sulfamethazine.

Following oral administration, the AUC for I constituted only 4.3% of the apparent hydralazine AUC. A very rapid disappearance of I in plasma was observed with no detectable level being found 5 h after the dose. The major component of the apparent hydralazine present was II which accounted for 100% of the apparent hydralazine at times beyond 4 or 5 h after the dose. The only triazolo[3,4-*a*]phthalazine metabolites observed were VI and VII with only traces of the former.

Following intravenous administration of hydralazine, the levels of I declined rapidly paralleling the in vitro loss of I at 37° in fresh plasma. Again the major component of the apparent hydralazine present was II and at times greater than 4 h after the dose constituted 100% of the apparent hydralazine level. Metab-

TABLE VI

AREAS UNDER CURVE (AUC) FOR HYDRALAZINE AND METABOLITES FOLLOWING ORAL (1 mg/kg) AND INTRAVENOUS ADMINISTRATION (0.375 mg/kg) TO A HEALTHY VOLUNTEER

Drug/metabolite	AUC (μmole/l/h)	
	Oral	Intravenous
Apparent hydralazine	17.8	11.51
I	0.77	0.54
II	11.30	9.96
VI	0.24	0
VII	1.87	0.65

olite VII was the only triazolophthalazine metabolite observed after intravenous administration of the drug. In both the oral and intravenous dose studies, the levels of metabolite II slowly increased and then declined with a half-life of 4.0 and 3.0 h respectively. In the intravenous study, the levels of apparent hydralazine did not decline in a simple mono- or bi-exponential fashion but showed fluctuations.

DISCUSSION

Selective and convenient HPLC assays for hydralazine (I), its pyruvate hydrazone (II) and metabolites (V—VII) in plasma were developed. I was quantitated using 4-methylhydralazine as internal standard after selective conversion to the respective tetrazolo[3,4-*a*]phthalazines (IX and X) using dilute nitrous acid at final pH 5.5. II was converted to VII in high yield with nitrous acid at pH 3.9 and was quantitated after subtraction of the endogenous plasma level of VII. The native fluorescence of tetrazolo[3,4-*a*]phthalazine (IX) and V—VII allowed the sensitive detection of very low plasma levels after oral and intravenous dosage. Other assay methods for I were investigated without success. For example, the addition of propionic anhydride to buffered plasma under a variety of conditions only partly converted I to 3-ethyl-*s*-triazolo[3,4-*a*]phthalazine (<25%).

For the quantification of apparent hydralazine levels, that is, hydralazine plus all hydrazones, strongly acidic conditions were employed. This procedure was similar to that published [4] with the exception that excess acid (relative to the plasma volume) was used to ensure that complete conversion of the hydrazones to hydralazine was accomplished. Measurement of the released hydralazine was carried out by conversion to IX followed by HPLC with fluorescence detection and using 4-methylhydralazine as the internal standard. Published methods [4, 13] have often under-estimated the apparent hydralazine level due to incomplete conversion of the hydrazones. For example, only 50% recovery of II was observed using the method described by Zak et al. [13], the remainder probably being converted to VII.

The experiment we reported in an earlier publication [12] in which I was added to fresh plasma and the levels of I, II and apparent hydralazine were measured, was repeated at 37° using the simplified methods described presently. The reaction was noticeably faster at 37° and after 15 min half the hydralazine was converted to II with no measurable level of hydralazine in plasma after 2 h.

The very rapid reaction of hydralazine with pyruvic acid in plasma indicates the necessity to derivatize samples immediately after drawing the blood. This was accomplished by preparing tubes containing the required amount of sodium nitrite, rapidly centrifuging blood (30 sec) and immediately adding the internal standard and acid. Derivatization occurred very rapidly trapping hydralazine as the stable tetrazolo[3,4-*a*]phthalazine derivative (IX) and II as derivative VII. Following basification, the samples were stable to storage for long periods.

In the case of the metabolites V, VI and VII and apparent hydralazine it was adequate to freeze the plasma samples immediately (−20°) and assay them the next day. No significant decrease in the level of apparent hydralazine occurred over 24 h at −20°. Previous observations that the apparent hydralazine level declines slowly even at −20° may be misleading due to the incomplete conversion of II using the methods published.

In the pharmacokinetic studies hydralazine constituted only a small fraction of the AUC of apparent hydralazine with hydrazone II accounting for all the apparent hydralazine 4 h after the oral and intravenous doses. This result is consistent with our earlier observation that more than 90% of the apparent hydralazine in plasma from patients taking oral hydralazine at steady-state is present as the pyruvate hydrazone. At times immediately following the doses there was a fraction of the apparent hydralazine which was not hydralazine or its pyruvate hydrazone. Using the combined levels of I and 70% of III obtained at pH 3.9 a significant fraction of this material was estimated to be the α-ketoglutaric acid hydrazone III.

Acetylation has been described as the major route of hydralazine elimination [5] and it is therefore important in pharmacokinetic studies to measure metabolites V—VII all of which are derived from acetylation. Wagner et al. [18] have reported that the major urinary metabolite of hydralazine in man is conjugated VI. Only small levels of unconjugated metabolite VI were observed in plasma and attempts to measure levels of conjugated material were seriously hampered by interference from relatively large amounts of fluorescence material present in several different commercial preparations of the mixed glucuronidase—sulphatase enzymes [19].

Recently published work [20, 21] has confirmed our observation [11] that the pyruvic acid hydrazone of hydralazine is inactive when administered intravenously to animals, however, the activities of this metabolite and the other hydralazine metabolites still await investigation in man.

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ANALYSIS OF METHOTREXATE BY ISOTACHOPHORESIS

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SUMMARY

This paper shows that the anion of methotrexate (MTX) can be readily separated and quantified by isotachophoresis. An extraction method for MTX is also presented, appropriate for isotachophoretic studies. The extraction of MTX is based on the complexation and precipitation of MTX with metal ions. The recovery of MTX from plasma is about 75%.

INTRODUCTION

Isotachophoresis is a technique appropriate for analysing ionic compounds such as drugs and drug metabolites. This paper describes a method to determine methotrexate (MTX): the isotachophoretic analysis of this compound is compared with an already existing analytical method based on an enzyme reaction.

The pK values of MTX are 3.36 (α-carboxyl); 4.70 (γ-carboxyl) and 5.71 (N-1) [1]; hence at pH 8.4 MTX has a negative charge.

The compound is only slightly soluble in water at pH 7, and more soluble at lower or higher pH. MTX is almost insoluble in many organic solvents, pyridine and dimethylformamide being the exceptions.

In isotachophoresis the separation time depends among other things on the concentrations of the ionic species; ions with both high concentrations and mobilities require an increase in the time needed for separation. Thus the chloride ion prevents a rapid analysis for MTX in plasma. Plasma samples can be pretreated by various methods to eliminate chloride, several procedures having been tried by ourselves. Neither the use of ion-exchange resins nor the use of electro-dialysis proved to be useful, because of inadequate recovery or too great a complexity. In the course of our experiments it was found MTX precipitates with certain metal ions, and an isolation procedure was based on this finding.

MATERIALS AND METHODS

Apparatus

Isotachophoretic experiments were performed in an apparatus provided with both UV absorption and conductivity detection, as described by Everaerts et al. [2]*. The separation capillary was approx. 200 mm × 0.2 mm I.D. The electric current was stabilized at 17.5 μA. The electrolyte system used in the isotachophoretic experiments is specified in Table I.

TABLE I

OPERATIONAL SYSTEM FOR MTX ANALYSIS

Parameter	Electrolyte	
	Leading	Terminator
Anion	Cl^-	histidine
Concentration	0.008 *M*	0.008 *M*
Counter ion	Tris	Tris
pH	8.4	9.5
Additive	0.05% Mowiol	
Solvent	water	water

The enzymatic assay of MTX was carried out according to the method of Overdijk et al. [3]. Spectrophotometric determinations were performed at 305 nm, using a Cecil CE 505 double beam instrument.

Radioactivity was measured in an Isocap 300 (Searle). As counting solution, Dimilume (Packard) was used.

Reagents

Reagents used were of analytical grade. Water was purified by Millipore ultrafiltration or was of double distilled quality. Mowiol 8-88 was donated by Hoechst-Holland (Amsterdam, The Netherlands) and purified by ion-exchange (Merck V, Merck, Darmstadt, G.F.R.) chromatography. A commercial preparation of methotrexate (82% MTX) from Lederle (Haarlem, The Netherlands) and a 95% pure preparation kindly supplied by them, were used. [3′-5′,9(*n*)-^{3}H]Methotrexate (spec.act. 250 mCi/mmol) was obtained from The Radiochemical Center (Amersham, Great Britain).

Isolation procedure

An isolation procedure consisting of three steps was used.

(1) Plasma (0.4 ml) and 0.1 ml dimethylformamide were pipetted into a centrifuge tube. After mixing, 0.4 ml trichloroacetic acid (10%, w/v) and 0.4 ml 1 *N* $AgNO_3$ were added. The contents of the tube were mixed in an ultrasonic bath and thereafter centrifuged for 10 min at 1500 *g*. The supernatant was poured off into a second tube, and the extraction was repeated twice, using 0.2 ml dimethylformamide and 0.3 ml trichloroacetic acid.

*The instrument was assembled by the technical staff of the laboratory, in collaboration with the Department of Analytical Instrumentation, Technische Hogeschool Eindhoven. Information about this aspect can be obtained from the authors.

(2) To the combined supernatants 3 ml 1 *N* mercaptoethanesulfonic acid—Tris mixture (pH 6.4) was added and after centrifuging (10 min, 1500 *g*) the supernatant was discarded.

(3) The precipitate was washed with 3 ml water, dried under nitrogen at room temperature and dissolved in 80 μl 1 *N* Tris. A 1-μl aliquot was used for isotachophoresis.

RESULTS AND DISCUSSION

Figs. 1 and 2 show isotachopherograms of 95% pure MTX and the commercial product (82% MTX). The differences between the two are due to impurities and decomposition products in the commercial preparation.

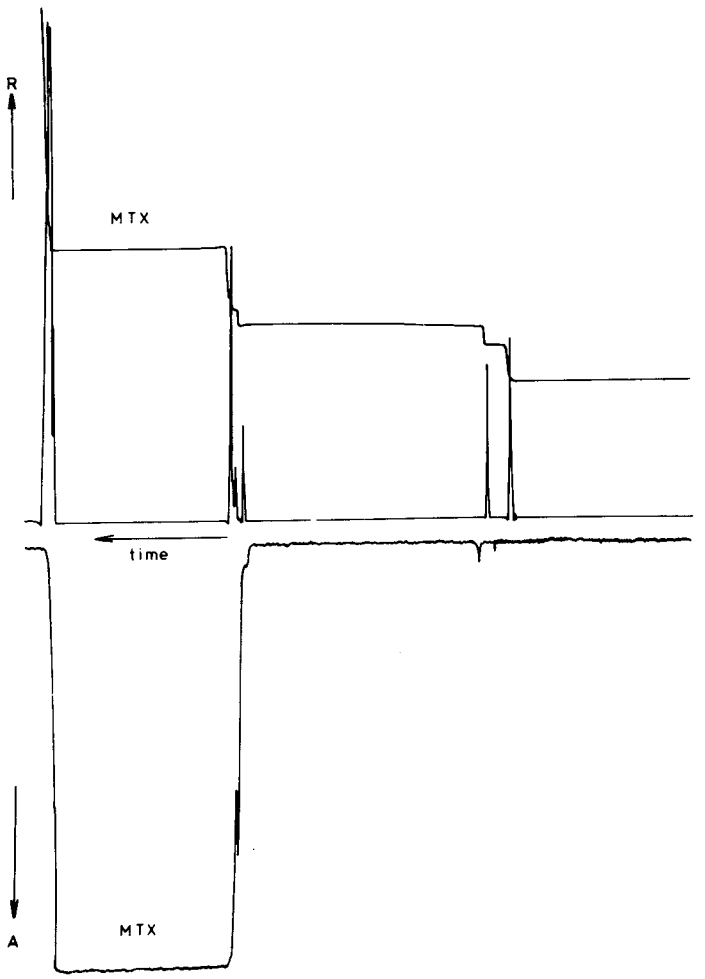

Fig. 1. Isotachopherogram of 1 μg 95% pure MTX. The nature of the anions in the zones is indicated by their resistance level (R) or UV absorption (A). The zone length indicates the quantity of anion passing the detector. Also shown is the differential signal of the conductometer, facilitating the determination of zone length.

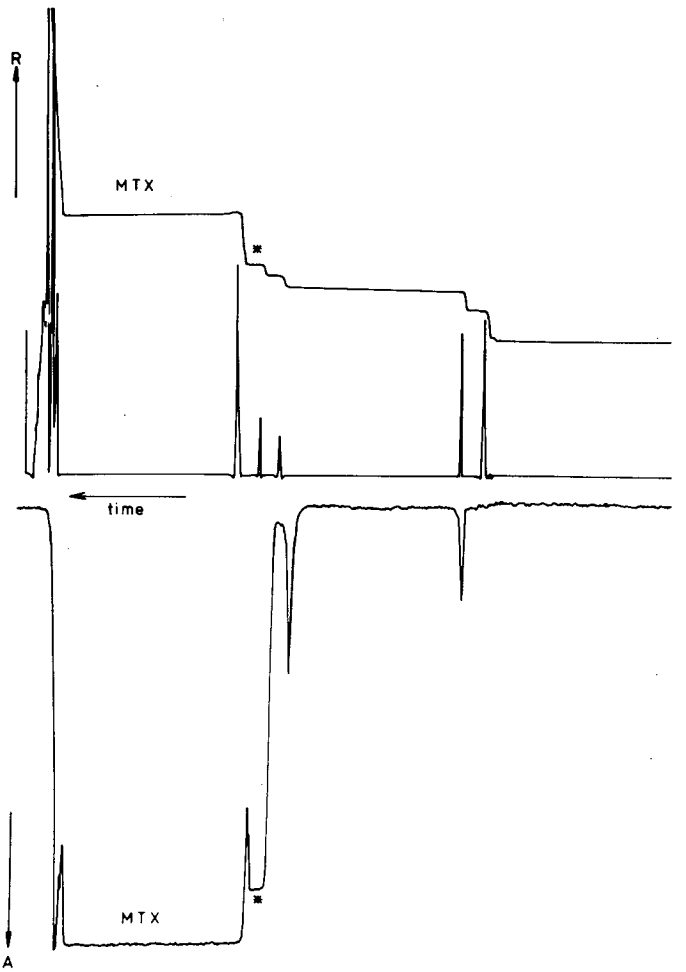

Fig. 2. Isotachopherogram of 1 μg MTX (82%) of commercial grade, containing impurities. The impurity marked * has isotachophoretic properties almost the same as those of folic acid. Recording speed 100 mm/min. Analysis time approx. 18 min.

A calibration curve for the commercial MTX preparation is obtained by injecting known amounts of MTX onto the column. The results are shown in Fig. 3.

To analyse plasma samples containing MTX, it is necessary to obtain protein- and chloride-free extracts. In the first isolation step, proteins are eliminated by the addition of trichloroacetic acid, and chloride ions are precipitated by $AgNO_3$ at pH 2. MTX forms complexes with Co^{3+}, Al^{3+}, Hg^{+}, Tl^{+} and Ag^{+}, and other metal ions. The solubility of the Ag—MTX complex depends on the pH. Addition of $AgNO_3$ at a pH of about 2 results in a soluble Ag—MTX complex. In the second step this Ag—MTX complex is precipitated by elevating the pH to about 6. The supernatant then contains a negligible quantity of MTX (Fig. 4).

In the third step the Ag—MTX precipitate is rinsed, dried and redissolved in Tris buffer; the isotachopherogram of 1 μl of such an extract of human plasma is shown in Fig. 5.

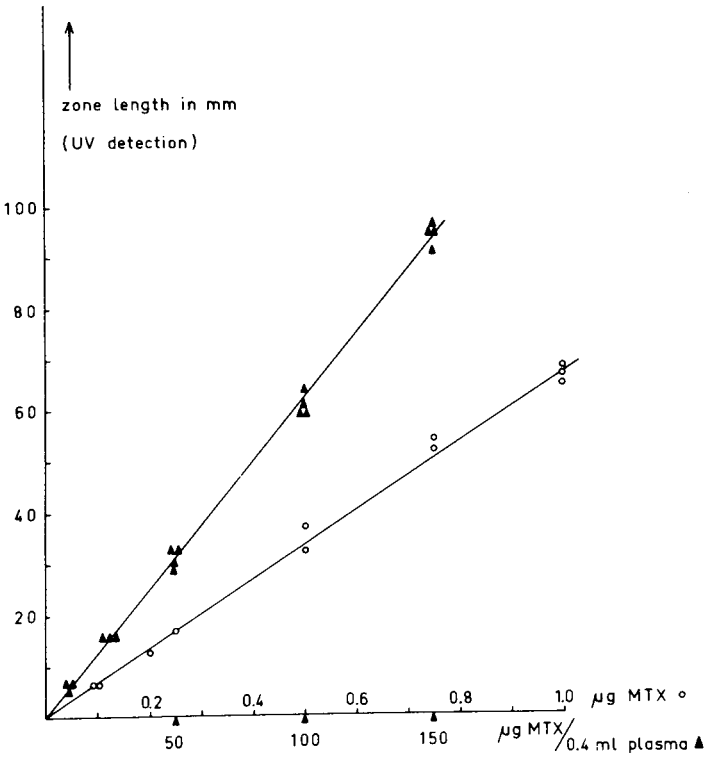

Fig. 3. Standard curves of commercial grade MTX, measured directly (○) and added to plasma and measured after isolation (▲).

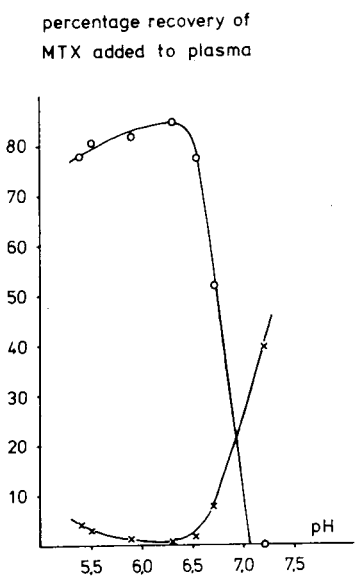

Fig. 4. Quantity of MTX, recovered in the supernatant of step 2 of the isolation procedure (×) and in the final extract of step 3 (○) at different pH values, as measured by spectrophotometry.

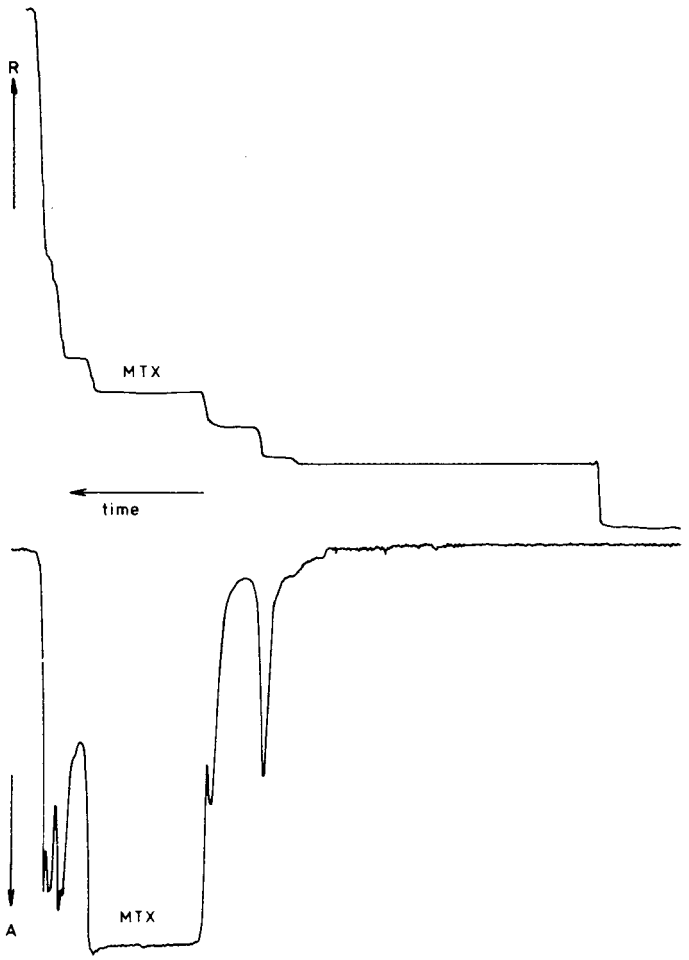

Fig. 5. Isotachopherogram of 1 μl of an extract of patient plasma. Recording speed 100 mm/min. Analysis time approx. 20 min.

A calibration curve of MTX added to plasma and isolated as described is depicted in Fig. 3. The recovery of these samples was 75% with a standard deviation of 3.2. The lower limit in this assay is approx. 10 μg per 0.4 ml plasma. The isolation procedure for MTX was also checked by adding quantities of labeled MTX to plasma. Table II shows the losses in the different steps of the procedure.

The overall recovery is in agreement with the recoveries shown in Fig. 4, measured spectrophotometrically. The recovery is twice as high as in a previously described isolation method of MTX [4].

The isotachophoretic method was compared with the enzymatic assay of MTX. Table III shows the results. In these runs the isotachophoretic assay is more reproducible. At the moment, the minimum quantity of MTX which is detectable does not cover all the requirements of clinical practice. However, by means of coupled columns [5] and by mixed zone isotachophoresis [6], now under study, the detection limits will greatly diminish.

TABLE II

RECOVERY OF ^{3}H-MTX FROM PLASMA

	Radioactivity (%)	S.D. (%)	*n*
Recovery in extraction step 1	93	3.2	6
Waste in extraction step 2; MES—Tris buffer	10	0.5	4
Waste in extraction step 3; 3 ml wash water	1	0.3	6
Final recovery	84	2.0	4

TABLE III

COMPARISON OF THE ISOTACHOPHORETIC AND ENZYMATIC ASSAY METHODS

Patients were administerd high doses of MTX. In both methods duplos were obtained on differend days using different standard curves.

Patient No.	MTX concentration in plasma (μg/ml)			
	Isotachophoresis		Assay with folic acid reductase	
1	40	43	24	20
2	124	121	242	139
3	514	506	424	345
4	462	473	282	311
5	68	80	65	80
6	276	244	247	276

Analysis by isotachophoresis is based on well defined physico-chemical parameters for separation and detection, and thus has a high degree of specificity. This is illustrated by Fig. 6. An MTX extract was dried under nitrogen at 70° in an acidic medium. The isotachopherogram shows evidence of significant decomposition.

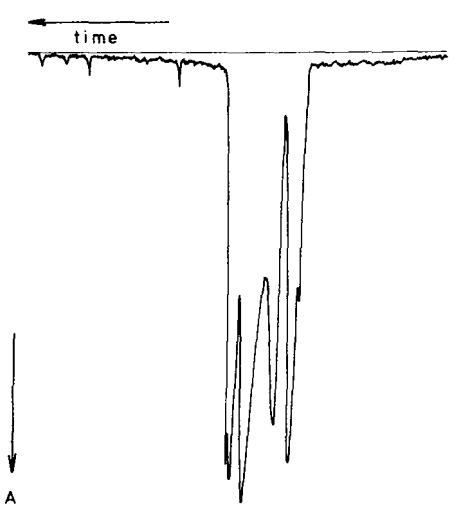

Fig. 6. Isotachopherogram of an extract of MTX, dried under nitrogen at 70° in an acidic medium. The 1-μl sample injected should have contained about 1 μg MTX. UV signal only is shown.

The advantages of isotachophoresis over many of the other analytical techniques are the simplicity of the instrumentation and instrument handling; great versatility (columns can be used immediately after filling with leading electrolyte; no column packing or equilibration) and the absence of band-broadening during analysis. We observed that several cytostatic drugs could be readily separated in an isotachophoretic system. Hence, it can be anticipated that isotachophoretic assay procedures for other drugs and metabolites will follow.

ACKNOWLEDGEMENTS

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Note

Method for the measurement of hydroxylamine in colonic fluid using derivatisation and gas chromatography

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Man consumes considerable amounts of nitrates and nitrites, a proportion of which are food preservatives [1]. Metabolism of nitrates and nitrites by colonic bacteria in man can produce carcinogens such as N-nitroso derivatives of amines [2]. Bacteria furthermore are able to reduce nitrate to ammonia by assimilatory reduction, in the process of which hydroxylamine is formed [3].

$$NO_3^- \xrightarrow[\text{reductase}]{2e^-} NO_2^- \xrightarrow[\text{reductase}]{2e^-} \begin{bmatrix} NOH \\ (?) \end{bmatrix} \xrightarrow[\text{reductase}]{2e^-} \underset{\text{(hydroxylamine)}}{NH_2OH} \xrightarrow[\text{reductase}]{2e^-} NH_3$$

Hydroxylamine is a mitogen [4] and a powerful metabolic inhibitor [5] the harmful metabolic effects of which have been established on isolated suspensions of intestinal epithelial cells [6].

Whether nitrites used for preservatives are harmful in the form of hydroxylamine, is presently controversial because no specific method of measuring hydroxylamine in biological samples is at present available. Numerous non-specific methods have been described [7]. The present report concerns a method of measuring hydroxylamine in stool water obtained in vivo by dialysis of the colonic contents of man. The method entails gas chromatography with a combined and synchronous use of nitrogen and flame ionisation detectors.

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EXPERIMENTAL

Materials

Hydroxylamine hydrochloride, acetone oxime, hydrazine hydrate, methanol and acetone were obtained from BDH (Poole, Great Britain).

Apparatus

A Pye 104 gas chromatograph fitted with a flame ionisation detector and a nitrogen detector was used. The outlet of the column was split 1 : 1 to each detector. The glass column (1.5 m × 4 mm I.D.) was packed with Pennwalt 223 on 80—100 mesh Gas-Chrom R (Applied Science Labs., State College, Pa., U.S.A.) at 150° with the injection port at 160°. The carrier gas was nitrogen at 60 ml/min.

If hydrazine is present (see Discussion below) a 1.5 m × 4 mm I.D. glass column packed with Carbowax 20M on Diatomite C AW DMCS (60—80 mesh) at 120° with the injection port at 150° is used.

For the gas chromatography—mass spectrometry analysis a Pye 104 fitted with the Pennwalt column was interfaced to a VG Micromass Q9K quadrupole mass spectrometer via a glass jet separator.

Procedure

The faecal dialysate was collected as described by Wrong et al. [8]. In principle this is an in vivo dialysis of stool. All water-soluble metabolites of bacteria are drawn into the dialysis tubing (Visking dialysis tubing 1/4 in., Scientific Instruments, London, Great Britain) which is filled with dextran (molecular weight 40,000). The contents of the dialysis tubing were stored in the deep freeze and then analysed as indicated.

Initially, to check the reaction, hydroxylamine in the form of the hydrochloride was dissolved in 10 parts water to 1 part methanol—acetone (1 : 1). Acetone oxime dissolved in water was used for identification and to check the recovery values.

Subsequently hydroxylamine was added to the dialysate in the range 0.1 mg to 1 g/l with acetone added in slight excess of the stoichiometric requirements. The calibration was found to be linear within this range. Recovery values were greater than 90%.

DISCUSSION

Hydroxylamine, because of its reactive and labile nature, will not chromatograph intact. It reacts with ketones to give oximes:

$$R_1R_2CO + NH_2OH \longrightarrow \left[R_1R_2C\begin{matrix}OH\\NHOH\end{matrix}\right] \xrightarrow{-H_2O} R_1R_2C{=}NOH$$

The oximes are well defined crystalline solids and the yields are good.

Using the above procedure quantitative yields of acetone oxime were obtained. Fig. 1 shows the response on each detector to the acetone oxime produced. Substitution of methyl ethyl ketone for acetone produced 2-butanone

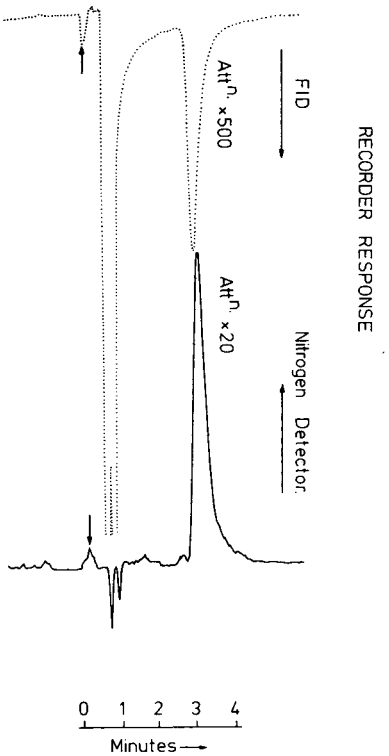

Fig. 1. Hydroxylamine hydrochloride in aqueous solution with added methanol—acetone (1 : 1) injected on to Pennwalt 223 at 150° showing response on each detector to acetone oxime produced (0.05 g/l hydroxylamine).

oxime (methyl ethyl ketoxime) with a retention time of 5 min. This reaction would be useful if a substance such as an amine which eluted at the same time as acetone oxime is present in the sample. Large quantities of primary and secondary amines are present in colonic dialysate [9] but they, as well as nitrites, nitrates and ammonia, do not interfere with the detection of acetone oxime.

When hydrazine in the form of hydrazine hydride was added to the original hydroxylamine hydrochloride solution and immediately injected on to the column a compound eluting at the same time as acetone oxime (3 min) was observed, and identified by mass spectrometry as acetone hydrazone, together with a peak for acetone azine which had a retention time of 4.3 min under the conditions used. After several hours the interfering compound had reduced to less than 10% and the yield of acetone azine was within 90% of the expected value. This behaviour is similar to that reported by Selim and Warner [10].

If hydrazine is present the Carbowax 20M column may be used. Under the conditions stated the interfering peak elutes at 0.9 min, not completely resolved from acetone azine (1.1 min). These elute ahead of acetone oxime which has a retention time of 2.6 min.

The use of the nitrogen detector only confirms the presence of the nitrogen containing compound but does not give an increase in sensitivity against the flame ionisation detector for acetone oxime. If there is an excess of solvent

then the acetone oxime lies on the trailing edge of the solvent peak and the baseline has not quite returned to normal in the case of the flame ionisation detector. Due to the small response for the solvent on the nitrogen detector (the response is in fact negative) the problem does not occur and in such cases the use of the nitrogen detector for quantitation is to be prefered.

RESULTS AND CONCLUSION

No hydroxylamine was detected in the five samples of dialysate that were examined. However the method as far as ascertainable is specific for hydroxylamine even in the presence of numerous nitrogen-containing and other volatile compounds. Addition of hydroxylamine hydrochloride to colonic dialysate is measureable within the range 0.1 mg/l to 1 g/l without interference of such chemical groups. Measurement of hydroxylamine in man on various nitrate diets and in subjects with damaged intestinal mucosa (ulcerative colitis) is now feasible.

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Note

Simplified liquid chromatographic—electrochemical determination of norepinephrine and dopamine in rat brain

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Recently developed liquid chromatographic—electrochemical (LC—EC) methods [1—5] for the determination of norepinephrine (NE) and/or dopamine (DA) in brain tissue have provided advantages in speed, sensitivity, and cost. However, all of the methods still employ a preliminary purification step. We have found that the compounds can be determined in rat brain by the direct injection of the supernatant obtained after sonication and centrifugation of the tissue.

EXPERIMENTAL

Materials

The LC—EC system consisted of an Altex 110A pump, a Rheodyne 70-10 injection valve, and a stainless steel 500 × 1.0 mm column dry-packed with pellicular Vydac SC cation-exchange resin (Rainin Instrument Co., Brighton, Mass., U.S.A.). A Model LC-4 electrochemical controller was used with a CP-S carbon paste electrode (Bioanalytical Systems, West Lafayette, Ind., U.S.A.). The potential was set at +0.5 V with respect to a Ag/AgCl reference electrode. A citrate—acetate buffer solvent system [2] was delivered at a flow-rate of 1.0 ml/min.

Method

Weighed whole rat brains (1—2 g) were placed in polycarbonate centrifuge tubes containing 4.0 ml of 0.1 *M* $HClO_4$ (with 400 μl of 1 *M* $NaHSO_3$ per liter). After the addition of 500 ng of dihydroxybenzylamine (DHBA) (5.0 μl of 10.0 mg DHBA per 100 ml of 0.1 *M* $HClO_4$), the brain was sonicated at a medium setting for two 30-sec periods using a Branson Polytron sonicator (Branson Sonic Power Co., Danbury, Conn., U.S.A.). After adding 0.5 ml of 3.4 *M* $HClO_4$ and vortex mixing, the samples were centrifuged at 10,000 *g* for 10 min

and a portion of the supernatant stored in a small polyethylene tube. The catecholamines (NE and DA) were determined by injecting 20 μl of the supernatant into the LC—EC system. The NE and DA peak heights were ratioed to the DHBA peak height, and the concentrations (ng/g brain) calculated knowing the relative response of the standards, the amount of DHBA added, and the brain weight. When determining NE and DA in brain punches and areas [6] weighing 2—10 mg each, the tissue was sonicated in 200 μl of 0.1 M $HClO_4$ after the addition of 10 ng of DHBA, and then centrifuged and determined as above.

RESULTS AND DISCUSSION

A chromatogram of catecholamine standards and two different rat brain samples is shown in Fig. 1. Up to 36 samples can be easily analyzed in 8 h. The standards and samples were determined with typical coefficients of variation (C.V.) of less than 5% and with absolute detection limits of ca. 10—20 pg. The internal standard (DHBA) was well recovered [74.9 ± 12% (mean ± S.D.), n = 110] from whole brains. Mean recoveries of DHBA from particular brain areas (whole areas and punches) ranged from 84 to 99% with C.V. values of 4—9%. Split samples which were analyzed using both this method and a procedure with an alumina absorption step [2] showed excellent agreement ($r > 0.99$). The low oxidation potential and the cation-exchange resin combined to give sufficient selectivity for injection of the unpurified and unconcentrated sonicate. When compared to a recent reversed-phase LC—EC method [1] a saving in time in the sample preparation and chromatography steps is apparent. We are

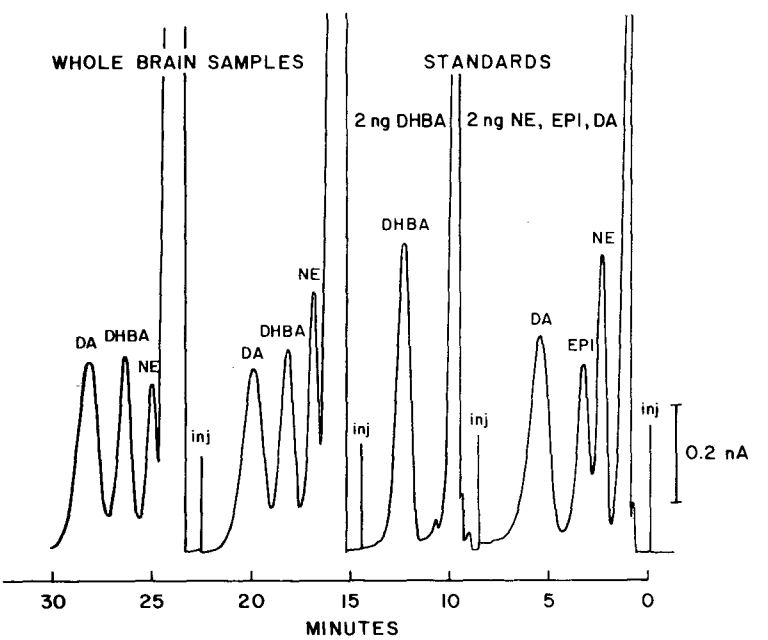

Fig. 1. Chromatogram of catecholamine standards and two different rat brain samples (ca. 1.4 g), all run at 2 nA full scale. Dihydroxybenzylamine (DHBA) was added to the brains as an internal standard (500 ng/brain). DA, dopamine; NE, norepinephrine; EPI, epinephrine.

presently developing methods involving the direct injection of the same supernatant into a reversed-phase LC—EC/fluorimetric system [7] in order to determine a variety of indolic and catechol metabolites.

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Note

High-performance liquid chromatographic determination of 5-hydroxyindole-3-acetic acid in urine using Sephadex G-10 for isolation

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It is well known that certain carcinoid tumors may be characterized by a release of serotonin (5-hydroxytryptamine, 5-HT), and that urine from patients with metastatic carcinoids contains abnormally high amounts of 5-hydroxyindole-3-acetic acid (5-HIAA), the main metabolite of 5-HT. Some of the methods to measure 5-HIAA in urine have been discussed by the author [1] who also presented a simple, sensitive and selective two-step chromatographic procedure. In that work, dinitrophenyl-coupled, thiolated Sephadex G-25 (DNP-S-Seph.G-25) was synthesized and used for the chromatographic isolation in combination with quantitative analysis by reversed-phase high-performance liquid chromatography (HPLC), using UV-absorbance detection. The present paper reports a further simplification utilizing Sephadex G-10 instead of the synthesized adsorbent. The new method was used to investigate the 5-HIAA excretion in urine from patients with carcinoid syndrome as well as from healthy individuals.

EXPERIMENTAL

Apparatus

The chromatographic isolation equipment [1] was modified as follows. The pump was replaced by a 13-canal micro-pump, MP 13 A (Ismatec, Zurich, Switzerland), with Technicon SMA flow rated pump tubes, flow-rate 0.05 ml/min (Technicon, Tarrytown, N.Y., U.S.A.). As isolation columns (4.5 cm × 4 mm I.D.) pipettes were utilized as before but were cut off leaving 12 cm and marked at a height of 4.5 cm. For a separate study (Table I), 30 cm × 4 mm I.D. columns were made from intact pipettes, marked at a height of 30 cm. Except for this study the fraction collector was omitted. The previously described [1] HPLC—UV equipment was used without any modification.

Reagents

See ref. 1, Reagents, for (1) buffer, (2) mobile phase and (3) a stock solution of 5-HIAA (100 μg/ml) in the buffer, checked and stored as described (diluting to 25 μg/ml resulted in A_{280} = 0.710 S.D. ± 0.015, n = 5, for each of the two lots); prepare the working standards 25, 12.5 and 6.25 μg/ml by serial dilution from stock solution as previously described; (4) pack the 4.5 cm × 4 mm I.D. columns with Sephadex G-10 (Pharmacia, Uppsala, Sweden) and equilibrate with about 20 ml buffer as in ref. 1, Preparation of isolation columns. Store the packed columns tightly capped at 5°, without any preservative in the buffer; (5) pack as described in (4) the 30 cm × 4 mm I.D. columns with the respective gel Sephadex G-10, G-15, G-25 and LH-20 (Pharmacia), Biogel P-2 (Bio-Rad Labs., Richmond, Calif., U.S.A.), and DNP-S-Seph.G-25 used in the earlier [1] work (for a modified synthesis, see pp. 54–55 in ref. 2), and then equilibrate each packing with about 40 ml of buffer (store the 30-cm columns as described for the 4.5-cm ones); (6) other reagents: blue silica gel (Grace, Homburg, G.F.R.), 4-hydroxy-3-methoxymandelic acid (VMA), 4-hydroxy-3-methoxyphenylacetic acid (HVA), tryptophan (Trp), tryptamine (T) and indole-3-acetic acid (IAA) (Sigma, St. Louis, Mo., U.S.A.).

Procedure

Calibration process. Place a set of one to three columns, 4.5 cm × 4 mm I.D., packed with Sephadex G-10 and equilibrated, over one to three sets of 15 small test tubes, marked for 1.0 ml. Suck off the buffer above the bed with a disposable Pasteur pipette, add 500 μl of a 25 μg/ml standard solution with a capillary pipette and allow to drain completely into the gel. Wash by means of 2 or 3 drops of the buffer and allow to drain. Refill the empty space with the buffer, connect the columns with the pump (flow-rate 3 ml/h), and collect 1-ml fractions. Monitor the absorbance of the individual fraction at 280 nm. Inject solution from each of the found UV-positive fractions, Nos. 7–12, and from both fractions beyond, Nos. 6 and 13, onto the reversed-phase column (PXS 1025, Partisil-10 ODS). Note the presence of 5-HIAA on the basis of the retention time by comparison with a directly injected standard, and establish the range of positive fractions.

Sample preparation. Ensure non-intake of banana or pineapple within 24 h before and during any urine collecting [3]. Collect and store 24-h urine specimens as described in ref. 1. For morning urine (voided after fasting overnight), note the volume, keep 9.7 ml and mix with 0.3 ml of glacial acetic acid, filter, and store as 24-h specimens; analyze at once or within 2 weeks.

Determination of 5-HIAA in urine. Carry out the determination as described for the calibration above with following modifications. Place a set of one to ten calibrated 4.5 cm × 4 mm I.D. columns over one to ten sets of two 15-ml Präzision test tubes (Scherf, Ostheim, G.F.R.), graduated for 10 ml. Run the samples (500 μl) on tinfoil-wrapped isolation columns. Collect 6 ml and discard. Collect 7 ml in the second test tube (tinfoil-wrapped), and keep for separation and quantitative determination on the HPLC—UV system. Prior to injection, check the system by direct injection of a 5-HIAA standard onto the ODS column, conditioned with the mobile-phase eluent (average peak height for a 25 μg/ml standard at 0.16 a.u.f.s. is 92 ± 2 mm). Obtain the 5-

HIAA concentration via a calibration curve (peak height in mm versus concentration in μg/ml), constructed by means of the standard solutions 25, 12.5 and 6.25 μg/ml, run in the same way as the samples. Concentrations greater than 25 μg/ml will require dilution of the urine samples. For quantities less than 1 μg/ml, concentrate as follows: pour 2 ml of the second fraction (7 ml) into a test tube (4 cm × 15 mm I.D.), placed in a desiccator, evaporate in vacuum at room temperature in the presence of blue silica gel, dissolve the residue quantitatively by washing the tube walls with 0.4 ml of the buffer, and inject at once onto the ODS column.

After using, regenerate the G-10 columns by eluting with about 20 ml buffer. Flush the ODS column daily with methanol, and store in methanol at 5°.

RESULTS AND DISCUSSION

A study on isolation ability of some available gels

To ascertain whether DNP-S-Seph.G-25 [1] could be replaced with a commercially available gel, a comparison between 30 cm × 4 mm I.D. columns packed with various gels (see Reagents) was performed. The elution positions of 5-HIAA were established in the same chromatographic conditions (Table I). By using Sephadex G-10, a threefold retention was attained; the retention was also found to be greater on G-15 (about 50%), while no difference appeared on LH-20. A comparison of the elution position of 5-HIAA with a screening of UV-positive urine compounds on G-10 (Fig. 1) showed that G-10 is the gel of choice for isolation of 5-HIAA from urine.

In order to obtain additional information on the isolation ability of the above packings, the retention behaviour of the aromatic urine metabolites VMA, HVA, Trp, T, 5-HT and IAA was studied. Table I shows how the reten-

TABLE I

RETENTION OF SOME AROMATIC COMPOUNDS, RUN ON SOME UNCHARGED HYDROPHILIC* GELS

Eluted compound	Elution range on respective gel, as Nos. of UV-positive fractions**					
	G-10	G-15	G-25	LH-20	Biogel P-2	DNP-S-Seph.G-25
VMA	6—10	5—6	4—5	4—6	4—5	5—6
HVA	10—14	7—9	7—8	8—10	4—5	8—9
Trp	7—9	6—8	3—5	4—5	4—5	7—9
T	6—10	5—7	3—6	3—4	4—5	8—10
5-HT	9—13	8—11	3—6	5—6	4—5	11—15
IAA	44—56***		5—8	15—22		18—22
5-HIAA	52—64***	21—29	7—10	18—23	7—10	18—24

*LH-20 and DNP-S-Seph.G-25 are also partly hydrophobic.

**250 μl of a 40 μg/ml solution of respective compound (IAA concentration unknown) chromatographed on a 30 cm × 4 mm I.D. column in 0.1 *M* ammonium formate buffer, pH 3 (flow-rate 3 ml/h). Fractions of 1 ml collected and monitored at 280 nm.

***If run in 0.1 *M* ammonium formate buffer, pH 8, IAA was eluted in Nos. 5—7 and 5-HIAA in Nos. 7—9.

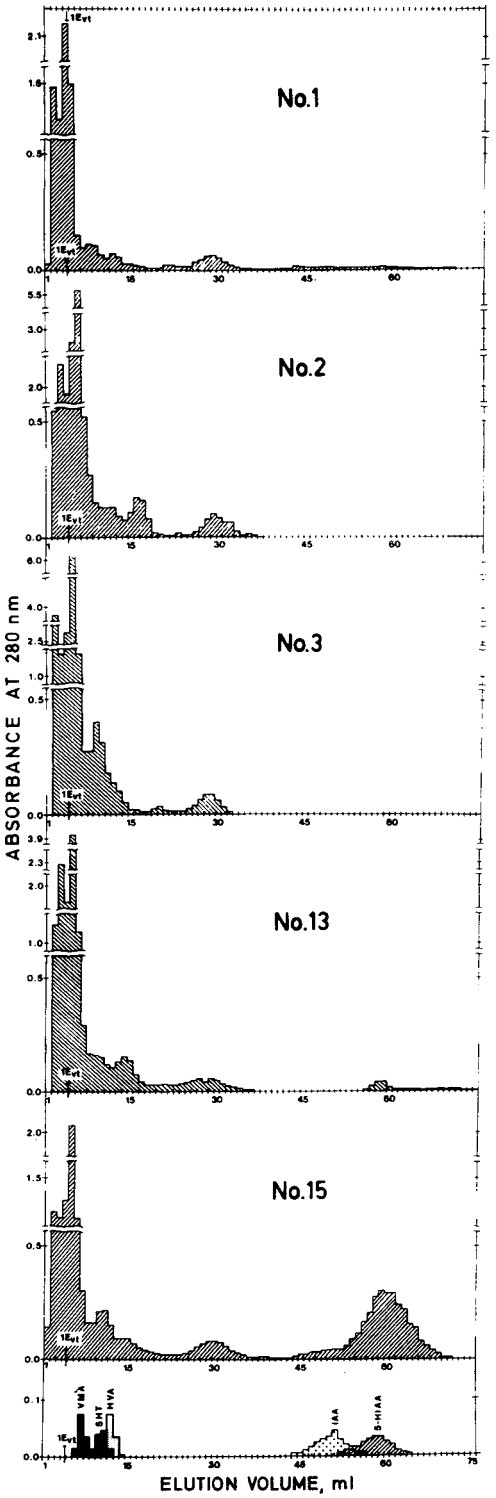

Fig. 1. Sephadex G-10 chromatograms of some aromatic compounds (bottom) and of 24-h urine specimens from carcinoid patients (subject Nos. 13 and 15) and from healthy individuals (subject Nos. 1—3). Conditions as in Table I, second footnote.

tion on the investigated G-gels varies with the amount of gel accessible to the solute: the tighter the cross-linking, the greater retention. Run on G-10, IAA was retarded to the same magnitude as 5-HIAA. The other compounds showed moderate adsorption as reported for aromatic amino acids on G-10 [4] and on G-25 [5]. The occurrence of a ring-substituted hydroxyl group was found to enhance adsorption sufficiently to allow separation of 5-HT from T and 5-HIAA from IAA with little overlapping. On the other hand, the replacement of a hydrogen on the α-carbon atom by a hydroxyl group was found to produce the opposite effect, since VMA, which elutes faster, could be fully separated from HVA. The elution behaviour on Biogel P-2 proved similar to that on Sephadex G-25 for most of the investigated compounds. However, a comparison with G-10 or G-15 could not be carried through, owing to the lack of gels with a tighter cross-linking than P-2 in the Biogel P series.

The retardation of 5-HIAA and IAA at pH 3 on G-10 was substantially reduced at pH 8 (Table I) and was almost doubled with 1 *M* sodium chloride in the buffer (unpublished results), pointing to hydrophobic interactions.

From the study one may conclude that Sephadex G-10 is the most suitable for isolation of the metabolites 5-HIAA, IAA, HVA and 5-HT. The isolation of metabolites from urine, such as Trp, T and VMA, which are least retarded on G-10, probably require cross-linked gels still tighter than G-10. Alternatively, G-10 covalently bounded to hydrophobic ligands, such as aliphatic chains or as aromatics containing more than one ring, may also be suitable.

5-HIAA analysis

The present method offers advantages over the previous one [1]. Besides eliminating the DNP-S-Seph.G-25 synthesis, an enhanced sensitivity is attained (Fig. 2) since the stronger adsorption allows use of shorter columns with less total bed volume (0.56 ml) and application of a double sample volume (0.50 ml). The specificity [1] is secured still more on G-10, as the isolation of 5-HIAA from the other UV-positive endogenous urine metabolites (Fig. 1) is

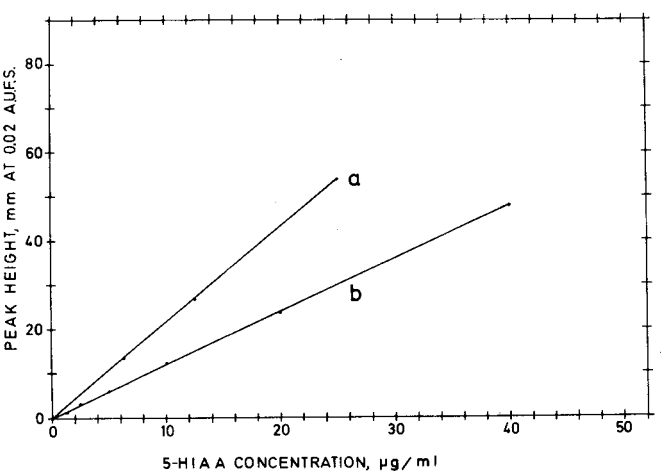

Fig. 2. 5-HIAA calibration curve of (a) the new and (b) the previous method.

TABLE II

DETERMINATION OF 5-HIAA IN URINE COLLECTED FROM HEALTHY INDIVIDUALS AND FROM PATIENTS WITH CARCINOID SYNDROME

No.*	Sex	Age	Weight (kg)	Urine (ml/24 h)	5-HIAA concentration in urine**			
					Previous method [1]		Present method	
					μg/ml	mg/24 h	μg/ml	mg/24 h
1	female	50	55	1440	3.3	4.6	3.3	4.6
2	male	62	79	1100	4.6	4.9	4.7	5.0
				1050	4.4	4.5	4.2	4.3
				690	6.7	4.5	6.6	4.4
3	female	18	63	550	7.0	3.7	7.2	3.8
6***	female	56	79	780	7.1	5.4	6.9	5.2
9	male	48	110	2060	2.8	5.6	2.8	5.6
16	female	55	67	1230			3.4	4.0
				300 §			2.3	
17	female	58	70	975			2.6	2.4
18	male	63	67	1375			3.8	5.1
19***	female	23	60	1070			4.0	4.2
20***	female	55	65	1320			3.8	4.9
12	female	62		1060			40.0	41.1
15	male	60		1340	439.6	571.5	446.4	580.3
				350 §			315.4	
21	male	56		500 §			24.7	

* Numero series started in ref. 1; from former subjects new samples collected at later occasions; No. 16, first new subject; No. 12, 15 and 21, patients under medical treatment.

** Corrected for acetic acid.

*** Took daily medicine containing levothyroxinnatrium.

§ Morning urine.

improved. The results, obtained by analyzing the same samples according to both methods, were almost identical (Table II). Fig. 3 illustrates typical HPLC chromatograms obtained by the new method. Table II shows the normal urinary 5-HIAA level, ranging from 2.4 to 5.6 mg per 24 h, found in specimens from ten healthy subjects, as well as the values in urine from some carcinoid

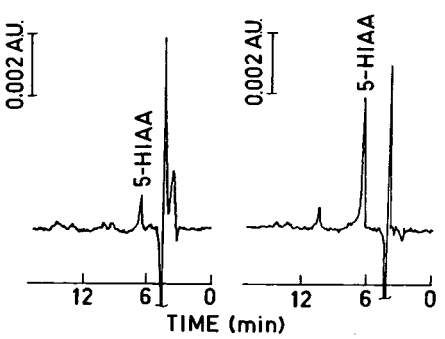

Fig. 3. Typical HPLC chromatograms of 5-HIAA in urine, obtained by the present method. A 24-h urine specimen from the healthy subject No. 2, containing 6.6 μg/ml (left), and a morning urine specimen from the carcinoid patient No. 21, containing 24.7 μg/ml (right).

TABLE III

TYPICAL ELUTION PROFILES OF 5-HIAA ON SEPHADEX G-10 COLUMNS OF DIFFERENT HEIGHT

500 μl of a 25 μg/ml standard solution chromatographed as in Calibration process, but injections onto ODS from each 1-ml fraction; operating as in Apparatus.

Column dimensions (cm × mm I.D.)	G-10, lot No.	Peak height (mm), measured in 1-ml fractions														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
4.5 × 4	2042	0	0	0	0	0	0	16	39	94	96	66	34	6	0	0
5.0 × 4	2042	0	0	0	0	0	0	0	3	17	67	115	100	40	15	3
4.5 × 4	6789	0	0	0	0	0	0	15	43	86	105	75	33	7	0	0

patients. The recovery of added 5-HIAA (6.25, 12.5 and 25 μg, respectively, per ml urine) was found to be 99.9% (R.S.D. = 0.5%; $n = 3 \times 5$). For quantitative performance on the small G-10 columns it is necessary to maintain the calibrated elution range intact: any drying phenomenon or any air bubble, appearing on storing, necessitates repacking and recalibration. Concerning columns of this small size, a few mm divergence in bed height causes a range displacement of one or two ml (Table III), followed by 5-HIAA losses of up to 5% and should be avoided by checking the height prior to the start and by readjusting.

Some of the patient specimens contained only low amounts of 5-HIAA, even if significantly exceeding the upper limit of the normal level, but the samples in question were collected after medical treatment (see also ref. 1). In general, patients with carcinoid syndrome were found to excrete extremely high amounts of this metabolite (ref. 1 and refs. 6—8 therein). However, such patient specimens, when available for analysis, are commonly collected from subjects taken for medical investigation in already advanced stages of the disease. Thus the possibility of detecting the tumor in its early stages calls for chemical detection in connection with healthy controls of groups in risk zones. The presented method may provide a suitable tool for these purposes as well.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Natural Science Research Council (grant Nos. K-0220-045 and 3953-100). The author is indebted to Professor Carl-Henrik de Verdier for being permitted to use HPLC—UV equipment and patient specimens at the Department of Clinical Chemistry, University Hospital, Uppsala.

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Note

Quantitation of ibuprofen in biological fluids by gas chromatography—mass spectrometry

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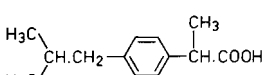
The binding of drugs to serum and tissue proteins is known to be an important determinant of their disposition kinetics and pharmacodynamics [1—5]. Consequently, there is an increasing awareness of the need to measure free drug concentrations in patient serum. This provides a more reliable approach to dosage adjustment and subsequent modulation of clinical effect [6—8]. If a drug is strongly and extensively (say >99%) bound to serum proteins and the total drug concentration achieved is in the low $\mu g\ ml^{-1}$ range, then the assay of the free drug requires extremely sensitive and precise techniques. This is particularly so where the amount of biological material available for assay is small, for example, serum ultrafiltrate and synovial fluid.



I

Ibuprofen (I), reported in 1967 [9], is widely used in the treatment of rheumatoid arthritis and osteoarthroses. In vitro binding of the drug to whole human plasma [10] and purified albumins [11] has been determined to be in excess of 99% by scintillation counting of [^{14}C]ibuprofen, a technique unsuitable for in vivo estimations in man.

Kaiser and Vangiessen [12] have reported a gas—liquid chromatographic (GLC) determination of the drug in plasma, by extraction into benzene and subsequent analysis of the methyl ester derivative (MeIb). Also, Hoffman [13] has developed a simpler GLC assay for the underivatized drug utilizing a 5% FFAP stationary phase on Gas-Chrom W HP, 80—100 mesh. Whilst both assays show equal and adequate sensitivity (lower limit of detection, 0.5 μg

ml^{-1} plasma) for the determination of total serum ibuprofen in the normal therapeutic concentration range (approx. 20 $\mu g\ ml^{-1}$) they are grossly inadequate for the measurement of the much lower corresponding free drug concentrations.

We have developed and report here a method for the assay of ibuprofen in biological fluids which provides a 500-fold improvement in sensitivity with greater precision than previously published assays. This assay has been used successfully to determine free concentrations of ibuprofen in whole serum and synovial fluid samples, taken simultaneously from rheumatoid patients, as part of a study to examine distribution of the drug between these two regions. Details of this clinical study will be published elsewhere.

EXPERIMENTAL

Materials

Ibuprofen, 2-(4-isobutylphenyl)propionic acid, was a gift from The Boots Pure Drug Co. (Nottingham, Great Britain). Fresh batches of distilled ethereal diazomethane were prepared immediately before use from N-methyl-N-nitroso-*p*-toluene sulphonamide (Merck-Schuchardt, Munich, G.F.R.). Deuterated ibuprofen for use as the internal standard was synthesized as described below. All solvents were redistilled before use.

Internal standard

To a vigorously stirred mixture of ibuprofen (20 mg), dichloromethane (5 ml) and aluminium chloride (60 mg), ^2H_2O (0.04 ml) was added dropwise over 5 min. The reaction mixture was stirred for 30 min at room temperature and then poured onto ice (^2H_2O). The product was extracted with diethyl ether (2 × 5 ml) and the combined extracts washed with water (5 ml), dried (Na_2SO_4) and evaporated to yield pale brown crystals of deuterated ibuprofen. This material was redissolved in dichloromethane and the aluminium chloride/^2H_2O exchange reaction was repeated.

A portion of the product from the second exchange reaction was methylated with diazomethane and analysis by charge exchange mass spectrometry showed the deuterium content of the ibuprofen to be: d_0 (0.5%), d_1 (6.5%), d_2 (26.4%) d_3 (38.5%), d_4 (25.2%) and d_5 (2.9%). The mass spectrum also showed that deuterium incorporation had taken place almost exclusively on the aromatic ring.

Gas chromatographic—mass spectrometric (GC—MS) analysis of the methylated product, under the conditions given below, showed the presence of two isomeric compounds. The major isomer (retention time 1.75 min) was shown to be the methyl ester of deuterated ibuprofen and the minor isomer (retention time 1.4 min) was the methyl ester of 2-(4-*sec.*-butylphenyl)-propionic acid. These two isomers may be easily distinguished by their charge exchange mass spectra as the characteristic benzylic cleavage of the alkyl side-chain produces a different product in each case (Fig. 1). Since the two isomers were well resolved by the GC column no attempt was made to remove the minor isomeric impurity.

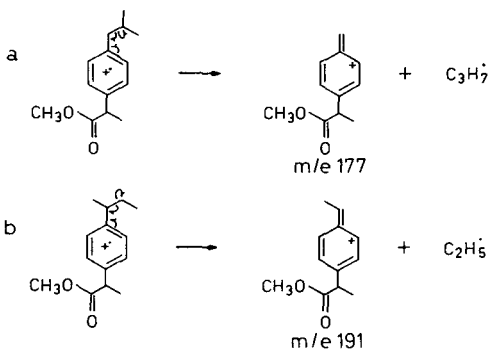


Fig. 1. Charge exchange-induced benzylic cleavage of the methyl esters of (a) 2-(4-isobutylphenyl)propionic acid and (b) 2-(4-*sec.*-butylphenyl)propionic acid.

Gas chromatography—mass spectrometry

GC—MS analyses were carried out on a Finnigan 3200 chemical ionization mass spectrometer interfaced to a Finnigan 9500 gas chromatograph. The glass GLC column (0.91 m × 2 mm I.D.) was packed with 3% OV-17 on Chromosorb W AW DMCS, 120—140 mesh. The column was operated at 150° and the injection port and GC—MS interface were maintained at 260°. Methane was used as the GC carrier gas and chemical ionization reactant gas. A flow-rate of 20 ml min^{-1} generated an ion source pressure of 130 Pa. Charge exchange mass spectra were generated by using helium in place of methane (flow-rate 20 ml min^{-1}, source pressure 55 Pa). An electron beam energy of 110 eV was used to generate chemical ionization mass spectra. The ion source and analyser regions of the mass spectrometer were operated at 60—100°. A Finnigan 6110 interactive data system was used to control the mass spectrometer during selected ion monitoring and to calculate the heights of peaks in selected ion chromatograms.

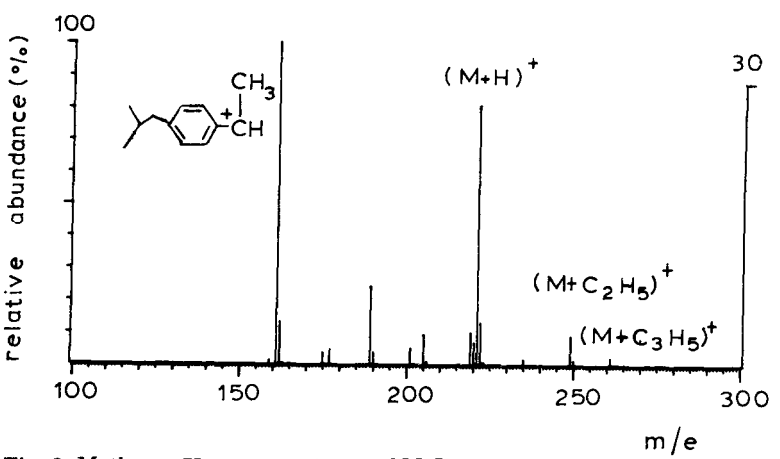


Fig. 2. Methane CI mass spectrum of MeIb.

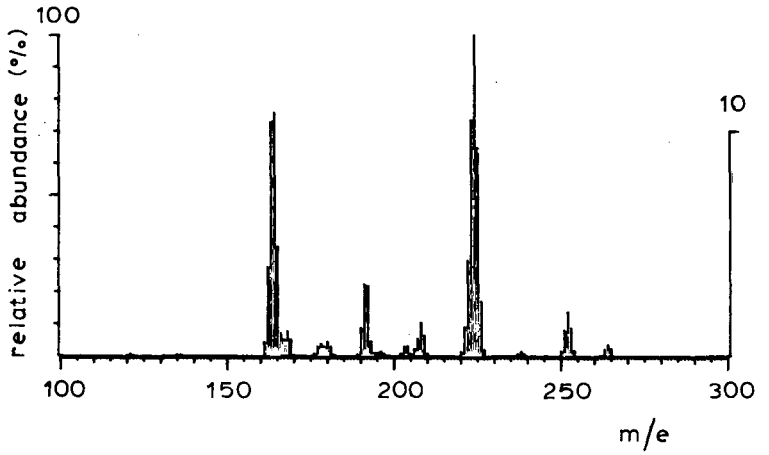


Fig. 3. Methane CI mass spectrum of deuterated MeIb.

Ibuprofen was analysed by monitoring the major (M+1) ion peak of its methyl ester derivative at *m/e* 221 (Fig. 2) and the (M+1) ion peak of the [$^2H_3$]ibuprofen internal standard at *m/e* 224 (Fig. 3).

Extraction procedure

Blood and synovial fluid, from rheumatoid patients on steady state ibuprofen therapy, were collected into 10-ml plain tubes. Clotted blood was centrifuged at 1000 *g* and ambient temperature for 15 min. An aliquot of recovered serum was equilibrated at 37° and ultrafiltered through a Pellicon PTGC series membrane, retention molecular weight 10,000 (Millipore, Bedford, Mass, U.S.A.), and approx. 0.2 ml of ultrafiltrate collected for subsequent free drug determination. Whole serum, serum ultrafiltrate and synovial fluid were stored at −20° until required for assay. The biological fluid (0.2 ml, made up to this volume if necessary with water) to be assayed was placed in a centrifuge tube and 0.2 ml of deuterated ibuprofen in 0.01 *M* NaOH (equivalent to either 50 or 1000 ng [$^2H_3$]ibuprofen) was added. The solution was acidified with 1 *M* HCl (0.5 ml), mixed thoroughly and extracted with diethyl ether (3 × 5 ml). The organic phase was concentrated on a water bath at 40° to approximately 10 μl. The extract was methylated with freshly prepared diazomethane (0.4 ml) in diethyl ether and the reaction allowed to go to completion (30 min). The sample volume was reduced to <10 μl on the water bath and the whole sample injected into the GC—MS system.

Calibration standards

Two standard curves were constructed to cover the drug concentration ranges expected in the samples (1—250 ng and 250—2000 ng). Aliquots of methanol containing known amounts of ibuprofen were evaporated to dryness in centrifuge tubes using nitrogen gas and 0.2 ml blank serum added with thorough mixing. These were extracted as described above, together with a blank serum sample to detect any interfering peaks. New standard curves were

obtained on each day samples were to be assayed. Estimates of precision at high and low drug concentration levels were obtained by replicate extraction and assay of both 500 and 1 ng calibration standards.

RESULTS AND DISCUSSION

Peak shapes in the selected ion chromatograms were good with little tailing (Fig. 4a) and therefore the more easily measured peak height ratio of MeIb/[$^2H_3$]MeIb was used for calibration. It is generally considered that the preparation of standard solutions is associated with much greater error than the actual mass spectrometric determinations [14]. For this reason the regression lines were calculated by minimization of the sum of squared deviations of the standards from the fitted line rather than the peak height ratios.

Calibration curve data for the lower range (1—250 ng) show excellent linearity (equation of regression line $y = mx + c$, where $m = 54.19$, $c = -7.41$ and $r = 0.9999$) and 1 ng of MeIb could be detected with a coefficient of variation (C.V.) of 6.8% ($n = 6$). Data for the upper range (250—2000 ng) were equally good ($m = 1055$, $c = -86$ and $r = 0.9998$), and 500 ng of MeIb were detected with a C.V. of 1.43% ($n = 6$).

The non-zero intercepts are a result of the presence of an ion of low abundance at *m/e* 221 in the chemical ionization (CI) mass spectrum of deuterated MeIb (Fig. 3). In this spectrum *m/e* 221 accounts for approximately 3% of the total abundance of the isotopic cluster of ions between *m/e* 221 and 227, a figure significantly higher than the abundance of the d_0-ibuprofen calculated from the charge exchange mass spectrum. The difference arises because the CI mass spectrum contains $(M - H)^+$ and $M^{+\cdot}$ ions in addition to the $(M+H)^+$ ion (Fig. 2). Therefore, in the CI spectrum of deuterated MeIb the

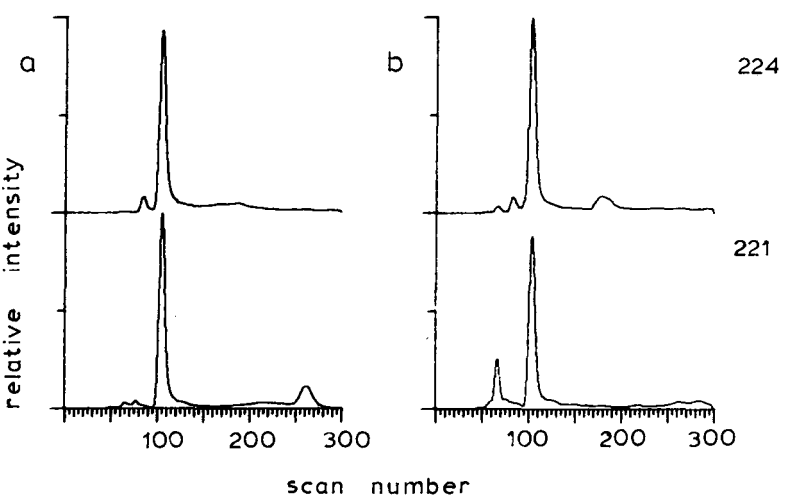


Fig. 4. Selected ion chromatograms *m/e* 221 (MeIb) and 224 ([$^2H_3$]MeIb). (a) Calibration standard (52.6 ng, ibuprofen) extracted from serum; (b) patient (B.C.) administered 400 mg ibuprofen three times daily, for two days prior to sample collection. Sample calculated to contain 42 ng ibuprofen in 130 μl serum ultrafiltrate.

$(M+H)^+$ ion at *m/e* 223 will be accompanied by a $(M-H)^+$ ion at *m/e* 221 and the $(M+H)^+$ ion at *m/e* 222 will be accompanied by an $M^{+\cdot}$ ion also at *m/e* 221. The situation is further complicated since the molecule contains both hydrogen and deuterium atoms, there is the possibility that the $(M+H)^+$ ion at *m/e* 224 will be accompanied by an $(M-D)^+$ ion again at *m/e* 221. However, as cleavage of C—H bonds is considerably more facile than cleavage of C—D bonds, $(M-D)^+$ ions will probably be of much lower abundance than $(M-H)^+$ ions.

This method has been successfully used to assay free concentrations of ibuprofen in whole serum and synovial fluid. Fig. 4b is a typical selected ion chromatogram obtained for unbound ibuprofen in whole serum, obtained from a rheumatoid patient (B.C.) on steady state therapy, and represents 42 ng of drug in 130 μl of serum ultrafiltrate.

The estimated detection limit for this assay is in the region of 200 pg and results from increasing interference of foreign peaks rather than from lack of GC—MS sensitivity. Experiments with standard aqueous solutions indicated that ibuprofen can be detected at the 50-pg level (signal-to-noise ratio >10:1) and a more sophisticated extraction technique may make detection at this level possible for serum extracts.

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Note

Mexiletine analysis in blood and plasma using gas chromatography and nitrogen-selective detection

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A number of gas chromatographic methods have been described for the analysis of mexiletine in biological fluids [1—8]. These methods require the derivatization of mexiletine prior to chromatography [1—4, 6, 7], the use of comparatively large sample volumes, typically 2 ml of plasma [1, 2, 4, 6], or electron-capture detection [3, 6, 7]. We describe a method for the analysis of mexiletine without derivatization, which requires small sample volumes, is more sensitive and has comparable reproducibility to previously reported methods.

EXPERIMENTAL

Materials

Mexiletine [1-(2,6-dimethylphenoxy)-2-aminopropane] and the internal standard 1-(2,4-dimethylphenoxy)-2-aminopropane, were supplied by Boehringer Ingelheim, Sydney, Australia. Diethyl ether and dichloromethane were of analytical grade (Merck, Darmstadt, G.F.R.). All other reagents were of reagent grade.

Sample preparation

Plasma or blood, 0.05—1.0 ml, is placed in a 15-ml capacity PTFE-lined screw cap culture tube, with 1250 ng or 250 ng of internal standard contained in 250 μl of water. Diethyl ether (5 ml) and 0.25 ml of 1 *N* sodium hydroxide are added and the sample extracted using a vortex mixer for 1 min. The organic and aqueous phases are separated by centrifugation for 5 min and the lower aqueous phase frozen in a dry-ice—acetone bath. The upper organic phase is

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poured into a second tube which has an elongated cone at its base of approximately 50 μl capacity. Dilute sulphuric acid (0.5 ml of 0.2 *M*) is added to the tube which is mixed with a vortex mixer (30 sec), the phases are again separated, frozen and the diethyl ether discarded. The tubes are placed in a vortex evaporator (Buchler Instruments, Fort Lee, N.J., U.S.A.) and evaporated at reduced pressure and room temperature for 5 min to remove traces of diethyl ether. Sodium hydroxide solution (0.25 ml of 1 *N*) and 50 μl of dichloromethane are added to the tubes which are mixed for 30 sec and centrifuged. Aliquots (1 μl) of the dichloromethane phase, sampled through the aqueous phase, are injected into the gas chromatograph.

Chromatographic conditions

A Hewlett-Packard Model 5730A gas chromatograph fitted with a nitrogen/phosphorus-selective flame ionisation detector was used for the analysis. A 1.8 m × 2 mm I.D. glass column packed with 1.5% Carbowax 20M and 5% KOH on Supelcoport 80—100 mesh was used for the separation. The injector port, oven and detector were maintained at 200°, 130° and 250° respectively. The flow-rates of the nitrogen carrier gas, hydrogen and air, were 30 ml/min, 3 ml/min and 50 ml/min respectively. Prior to use, the column was conditioned with the above carrier gas flow-rate at 220° for 16 h. Chromatograms were recorded on a Hewlett-Packard Model 7123A recorder.

Calibration and accuracy

The method was calibrated by adding known amounts of mexiletine and internal standard, each contained in 100 μl of aqueous solution, to plasma or blood which was then analysed. Calibration curves were established covering two ranges. The higher range was calibrated with samples containing 50, 100, 250, 500, 1000 and 2000 ng of mexiletine and used 1250 ng of internal standard. The lower range was calibrated with samples containing 5, 10, 20, 50, 75 and 100 ng of mexiletine and used 250 ng of internal standard.

A normalised peak height ratio was determined by dividing the peak height ratio of mexiletine to internal standard by the amount of mexiletine in each standard. The mean normalised peak height ratio was used to calculate the amount of mexiletine in unknown samples and the coefficient of variation was used to establish the reproducibility of the method over the entire range of each calibration curve.

RESULTS AND DISCUSSION

Under the chromatographic conditions described, the retention time of mexiletine was 3.5 min and that of the internal standard 4.75 min (Fig. 1A). Control plasma or blood samples did not contain peaks that interfered with that of mexiletine or internal standard (Fig. 1B). No peaks were observed which interfered with the peaks due to mexiletine or the internal standard when samples of lignocaine, procainamide, N-acetylprocainamide, tocainide, disopyramide or propranolol were injected into the gas chromatograph.

A total of 18 calibration curves in the range of 50—2000 ng were prepared from plasma (1 ml) over a period of approximately three months. The average

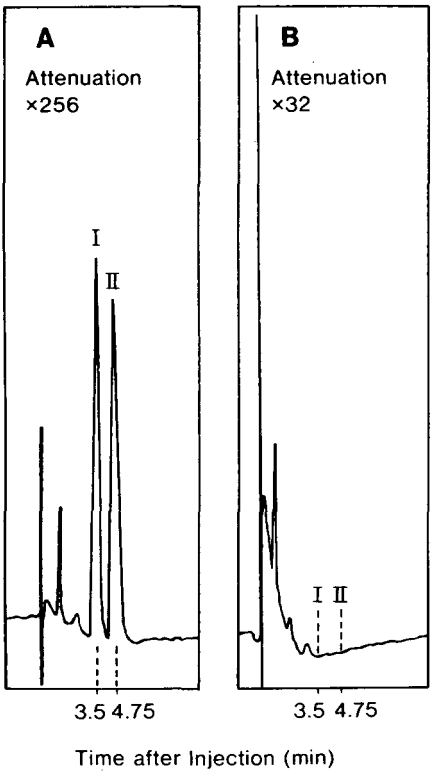

Fig. 1. Chromatograms of (A) a plasma sample (1 ml) containing 850 ng of mexiletine (I) and 1250 ng of internal standard (II); (B) a control plasma sample, I and II indicating the times that would correspond to mexiletine (3.5 min) and internal standard (4.75 min) peaks. Note that the sensitivity in B is 8 times that shown in A.

coefficient of variation of the normalised peak height ratio was 5.86%, the lowest value being 3.4% and the highest 9%. The calibration curves were linear, the mean correlation coefficient of the 18 curves being 0.997. The method was also calibrated in the range of 5—100 ng of mexiletine using 1-ml samples of plasma. The coefficient of variation of the normalised peak height ratio in this range was 4.9%. Calibration curves from whole blood samples had similar slopes and reproducibility to those from plasma. Given the ability of the method to accurately measure 5 ng of mexiletine, this would enable the bottom of the therapeutic range (500 ng/ml) to be measured with 25 μl of plasma.

Some analytical characteristics of previously reported methods for the analysis of mexiletine are compared with the present method in Table I. Willox and Singh [3] and Perchalski et al. [7] have described methods using polyfluoroalkylamide derivatives of mexiletine which give sensitivities comparable to that reported in the present method. All other methods report calibration curves with the lowest concentration an order of magnitude above that used with the present method. The reproducibility of the present method is comparable to or better than that of other methods in Table I. The choice of a suitable stationary phase results in symmetrical peaks (Fig. 1A) without derivatization. Similar-

TABLE I

COMPARISON OF THE ANALYTICAL CHARACTERISTICS OF METHODS FOR MEXILETINE IN BIOLOGICAL FLUIDS

Biological fluid	Sample size (ml)	Derivative	Detector*	Reproducibility (C.V. %)**	Calibration range (ng/ml)	Reference
Plasma Urine	2	butyramide	NPD	3.4	250—2500	1
Plasma	2	acetamide	FID	–	–	2
Blood Plasma Urine	1	hepta-fluoro-butyramide	EC	***	20—2000	3
Plasma	2	acetamide	FID	–	100—?	4
Plasma	1	underivat-ised	NPD	8.7	100—?	5
Plasma	2	hepta-fluoro-butyramide	EC	7.25	200—1000	6
Plasma	2	pentafluoro-propion-amide/tri-fluoroacet-amide	EC/ FID	6	7—2000	7
Blood Plasma	0.2	underivat-ised	FID	4.3	500—16,000	8
Blood Plasma	0.05—1.0	underivat-ised	NPD	5.8	5—2000	This paper

*NPD = nitrogen/phosphorus-selective detector; FID = flame ionisation detector; EC = electron-capture detector.
**C.V. = coefficient of variation.
***$r = 0.99$.

ly the use of nitrogen-selective detection, in addition to producing good sensitivity, enhances the selectivity of the method, compared to those analyses using flame ionization or electron-capture detection.

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Note

Specific and sensitive method for the determination of aspirin and salicylic acid in plasma using reversed-phase high-performance liquid chromatography

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(First received June 26th, 1979; revised manuscript received October 22nd, 1979)

Assays of aspirin (ASA) and salicylic acid (SA) by gas—liquid chromatography [1, 2] have been reported. However, time consuming chemical derivatizations, such as silylation, were necessary. The compounds have also been analysed by high-performance liquid chromatography (HPLC) [3—6]. One method [3] dealt with analyses of ASA and SA in pharmaceutical formulations and two other methods [4, 5] measured just SA and salicyluric acid (SU) in biological fluids. Only one method [6] determined simultaneously ASA, SA and SU in body fluids, but it required a mixture of solvents for extraction including benzene which is toxic.

This paper describes a rapid determination of ASA and SA in human plasma. One simple, non-toxic extraction solvent is used and a small plasma sample is needed.

EXPERIMENTAL

Materials

Methanol (HPLC grade) and chloroform (Distal Reagent) were obtained from Fisons (Loughborough, Great Britain); aspirin and salicylic acid from Sigma (London, Great Britain); and the internal standard, 3,4-dimethylbenzoic acid from Aldrich (Gillingham, Great Britain). Deionised water was further purified for HPLC by passing through two mini-filters (Whatman, Maidstone, Great Britain), firstly grade 80 (8 μm) and secondly grade 10 (0.9 μm).

A 0.072% acid solution (w/v, pH 2) was prepared by diluting 14.4 ml of a 5% orthophosphoric acid (w/v, pH 1) to 1 l with filtered deionized water.

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Instrumentation

Chromatography was performed on a Model SP8000 microprocessor-controlled high-performance liquid chromatograph with in-built data system (Spectra-Physics, St. Albans, Great Britain) equipped with the Model SP770 variable-wavelength detector (Spectra-Physics) set at 234 nm. An injection loop of 10 μl was used. The chromatographic column was a stainless-steel tube (250 × 4.6 mm I.D.) pre-packed with LiChrosorb RP-18 10 μm (Owens Polyscience, Macclesfield, Great Britain). The mobile phase consisted of methanol—0.072% orthophosphoric acid (55:45, v/v) and the flow-rate was 1.5 ml/min at 40°. The working pump pressure was approximately 94 bars (9.4 MN/m^2).

The ratios of the areas relating to the peaks of the drug or metabolite standard to that of the added internal standard peak were plotted against the known concentrations to provide standard curves. The best straight line was calculated using the least square linear regression method (Texas Instruments SR-51-II calculator).

Collection and storage of samples

The blood samples were collected into lithium heparin plastic tubes (Searle Diagnostic, High Wycombe, Great Britain) containing aqueous potassium fluoride solution (50 μl, 50% w/v) and kept in an ice bath. The samples were then centrifuged at 2°. The plasma was separated and analysed immediately or deep frozen (−20°) until analysis.

Sample preparation

A suitable range of concentrations for ASA (0.5—20 μg/ml) and for SA (0.5—100 μg/ml) were prepared by adding to human plasma known amounts of the two compounds dissolved in acetonitrile (1 mg/ml).

Aliquots of plasma (200 μl) were pipetted into 20-ml (16 mm O.D.) extraction tubes (Sovirel, Levalloix-Perret, France) and followed by the addition of 300 μl of a 10 μg/ml solution of 3,4-dimethylbenzoic acid in acetonitrile, 1 ml of 5% orthophosphoric acid (w/v, pH 1) and 10 ml of chloroform. After screw-capping, the tube contents were mixed on a reciprocating table (25 oscillations per min) at room temperature for 30 min and then centrifuged at 1000 *g* for 10 min. The lower organic layer (9 ml) was transferred into a 10 ml BC24/C14T conical centrifuge tube (Quickfit & Quartz, Corning, Stone, Great Britain) and evaporated to dryness under nitrogen in an ice water bath. The dry residues were dissolved in 200 μl of the mobile phase used and 10 μl of this was injected into the chromatograph.

Validation

Reproducibility of the assay was determined by carrying out six replicate analyses of each plasma standard with ASA concentrations at 1 and 20 μg/ml and SA concentrations at 2 and 100 μg/ml.

RESULTS

Typical chromatograms are shown in Fig. 1. The retention times of ASA, SA and 3,4-dimethylbenzoic acid were 165, 246 and 433 sec respectively. No other interfering peaks were observed.

The calibration curves for ASA and SA were linear ($y = 0.0360x - 0.0057$, and $y = 0.4307x + 0.0108$, respectively).

The coefficients of variation determined from peak area ratios of the compound to the added internal standard in plasma were less than 7% at all concentrations investigated. The results for the method precision and reproducibility are summarised in Table I.

The recovery of ASA and SA was determined by comparing the peak areas from extracted and non-extracted standards and found to be > 95% for ASA and 70% for SA.

A plasma profile from a healthy female volunteer who had taken 600 mg soluble ASA orally is given in Fig. 2. ASA was rapidly absorbed and eliminated, and its concentration at 2 h was below 0.5 μg/ml. However, the metabolite (SA) did not reach a peak level until 70—90 min after ASA dosing and was detectable at 24 h.

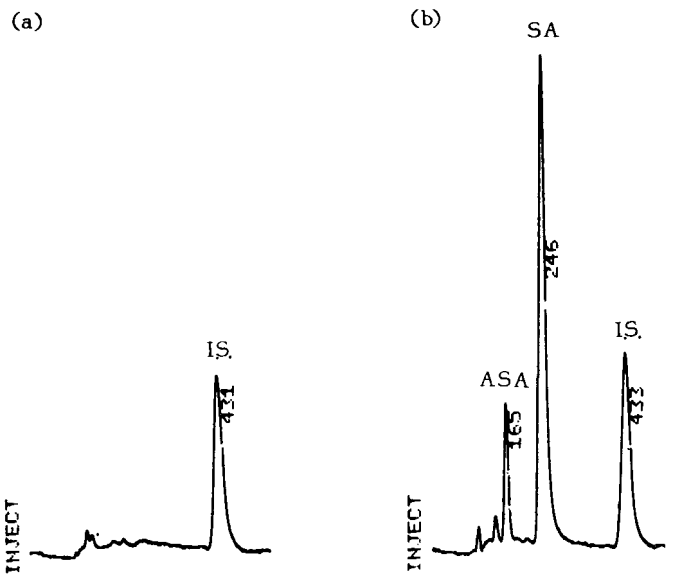

Fig. 1. HPLC determination of aspirin (ASA) and salicylic acid (SA) in plasma. (a) Blank plasma containing internal standard (I.S.) only. (b) Plasma sample containing aspirin (ASA, 9.5 μg/ml), salicylic acid (SA, 36.3 μg/ml) and 3,4-dimethylbenzoic acid (I.S., 10 μg/ml).

TABLE I

METHOD PRECISION AND REPRODUCIBILITY

Compound	Concentration in plasma (μg/ml)	n	Ratio of peak area of compound / peak area of I.S.*			C.V. (%)
			Mean	±S.D.	Range	
Aspirin	1	6	0.033	0.0021	0.030—0.035	6.4
	20	6	0.718	0.0125	0.697—0.730	1.7
Salicylic acid	2	6	0.091	0.0022	0.088—0.094	2.4
	100	3	4.306	0.1684	4.127—4.461	3.9

*I.S. = Internal standard.

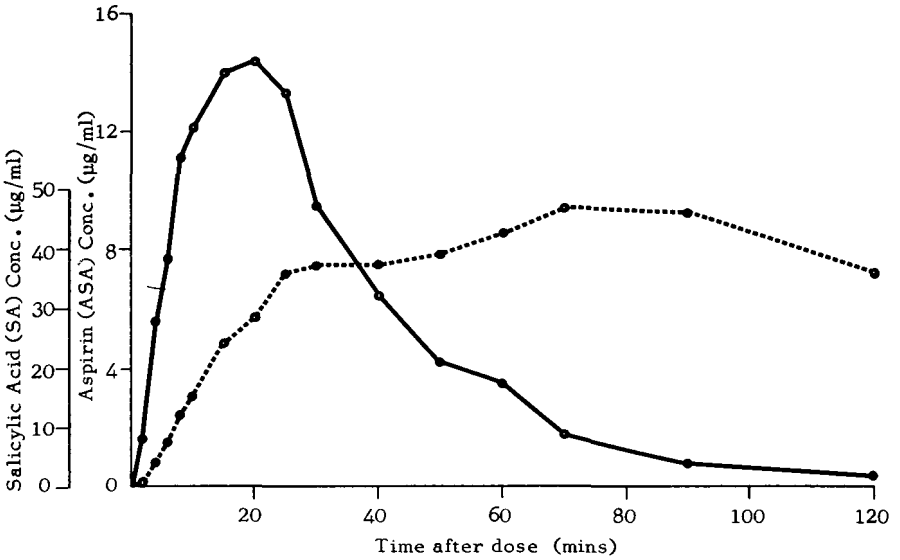

Fig. 2. Typical plasma profile of aspirin (ASA) (—) and salicylic acid (SA) (---) from a healthy fasting female after taking 600 mg of aspirin (2 soluble 300-mg tablets) orally.

DISCUSSION

The maximum concentration of ASA (14.4 μg/ml) and SA (46.8 μg/ml) observed after an oral dose of 600 mg soluble ASA were in the expected range [7, 10]. The plasma half life of ASA (15.5 min) calculated from the results agreed with the value found by Rance et al. [2].

For accurate determinations of ASA and SA, two important precautions, often ignored in some published methods [3, 5] were necessary. Firstly, the blood samples must be collected into fluoride treated tubes on ice to prevent ASA hydrolysis in human blood and plasma [11]. Hydrolysis was demonstrated in two studies [12, 13]. Secondly, the extraction solvent must be evaporated in an ice water bath to prevent underestimations of SA due to the loss through sublimation as has been reported [3].

No method published, to our knowledge, takes account of both precautions together. Only one other HPLC method [6] has been described and the chromatographic conditions are similar to ours. However, no precautions were taken to prevent ASA hydrolysis; the internal standard coeluted with an endogenous peak in dosed human plasma samples and the method was validated for spiked human and dosed rabbit plasma samples only.

The presently described method has adequate sensitivity and precision for monitoring ASA in the presence of a wide range of concentrations of SA. The detection limit for either compound is 0.5 μg/ml. No interference from the other materials present in human plasma was observed. Hence this would be useful in studies concerned with the bioavailability and pharmacokinetics of ASA and its major metabolite, SA, in man.

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Note

Reversed-phase high-performance liquid chromatographic analysis of methotrexate and 7-hydroxymethotrexate in serum

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High dose methotrexate (MTX) followed by citrovorum factor as a biochemical rescue has been widely used in the treatment of a variety of human cancers [1—5]. Because of the inherent risk of toxicity from this regimen [6—8], patient management requires the monitoring of serum MTX to allow identification of patients with high, potentially toxic, MTX concentrations and/or delayed MTX elimination. While the pharmacokinetic behavior of MTX has been reported to be quite different at high dose showing biexponential [9, 10] plasma disappearance up to 72 h post dose compared to a triexponential pattern at lower doses [11], this may be attributable to methodologic differences. In addition, the presence of metabolites, particularly 7-hydroxymethotrexate (7-OH-MTX), appears to be significant [12, 13] at high dose after it had been previously thought to be unimportant at low doses [14].

While there are several analytical methods in the literature for plasma methotrexate including enzyme inhibition [15, 16], protein binding [17], radioimmunoassay [18, 19] and fluorescence [20, 21], none of these employ separation steps capable of resolving or quantitating 7-OH-MTX. Since most were developed prior to a recognition of significant metabolite formation in humans the absolute specificity is not known. A high-performance liquid chromatographic (HPLC) assay for MTX using fluorescence detection has been reported [22]; however, this procedure involves oxidation of MTX to a fluorescent species prior to chromatography and does not differentiate 7-OH-

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MTX. Our laboratory first reported an HPLC assay capable of quantitating both MTX and 7-OH-MTX following high dose MTX therapy using a strong anion-exchange column [23]. In the present paper we report a reversed-phase chromatographic procedure with increased sensitivity and markedly improved column life, which is applicable to the measurement of MTX and 7-OH-MTX in plasma samples from patients receiving MTX therapy in both conventional and high dose therapeutic regimens.

EXPERIMENTAL

Non-formulated (USP) and formulated MTX were obtained from Lederle Labs. (Pearl River, N.Y., U.S.A.). The purity of the former was found to be 98.5% and the latter 95% by this HPLC method. 7-OH-MTX was isolated from rabbit liver homogenate as described previously [23] and an authentic specimen was kindly supplied by Dr. David Johns of the National Cancer Institute (Bethesda, Md. U.S.A.). *p*-Aminoacetophenone as the internal standard was obtained from Aldrich (Milwaukee, Wisc., U.S.A.) and was reported >99% pure. All were used without further purification. All other chemicals were of analytical reagent grade and all solvents were HPLC quality grade. Distilled water was purified by passing it through a reverse osmosis four filter system (Millipore, Bedford, Mass., U.S.A.). Standard solutions of MTX and 7-OH-MTX were prepared in purified water at concentrations of 1 mg/ml. Internal standard solutions were prepared in methanol at a 1.0 mg/ml concentration. All standards were refrigerated at 4° and have been found to be stable over several months.

Serum samples were either from patients receiving MTX therapy under protocol or were normal human sera spiked with MTX and/or 7-OH-MTX. The analytical procedure involved the addition of 25 μl of a 0.01 mg/ml solution of *p*-aminoacetophenone as the internal standard and 1.5 ml of 1.0 *N* $HClO_4$ to a 1.0-ml serum standard or patient sample. The denatured protein was separated by centrifugation at 1650 *g* for 10 min and to the clear supernatant was added 5 g of solid $(NH_4)_2SO_4$ and 2.0 ml of ethylacetate—isopropanol (10:1). This was shaken for 20 min on a table top shaker at high speed and the organic layer was transferred to a small disposable tube (12 × 75 mm) and evaporated in a 60° sand bath under a stream of nitrogen. The residue was reconstituted in 100 μl of 0.005 *M* K_2HPO_4, vortexed and 10—50 μl injected into the chromatograph. Standard curves were generated over the range 0.1—8.0 μg/ml ($2.2 \cdot 10^{-7}$—$1.8 \cdot 10^{-5}$ *M*). MTX concentrations were determined by calculating peak height ratios of drug:internal standard. Generally 7-OH-MTX was calculated similarly, in terms of MTX equivalents due to the scarcity of pure standard of the metabolite.

A Waters Assoc. ALC Model 202 liquid chromatograph (Milford, Mass., U.S.A.) equipped with a U6K injector and a Model 440 ultraviolet detector fitted with a 313 nm filter was used for the analyses. Chromatography was performed on a 25 cm × 4.1 mm I.D. stainless steel column packed with either RP-8, 10 μm particle size (Altex, Costa Mesa, Calif. U.S.A.) or RP-8, 7 μm particle size (Knauer, Western Analytical Products, Yorba Linda, Calif., U.S.A.). The mobile phase consisted of 0.1 *M* phosphate buffer (pH 6.8)—

methanol (85:15) at a flow-rate of 1.5 ml/min and a pressure of 1500 p.s.i. The separation was run at ambient temperature.

RESULTS AND DISCUSSION

Typical chromatograms for a patient sample and a serum blank are shown in Fig. 1. Retention times for MTX, 7-OH-MTX and internal standard were 6.6, 7.4 and 8.8 min, respectively under the analytical conditions described using the 10-μm RP-8 column. Peak shape was generally symmetical and peak height ratios were used to calculate MTX and 7-OH-MTX concentrations. Standard curves from spiked pooled serum were linear in the range of 0.1—8 μg/ml ($2.2 \cdot 10^{-7}$ *M*—$1.86 \cdot 10^{-5}$ *M*) for MTX and 7-OH-MTX. The lower limit of sensitivity from a 1.0-ml specimen with a 25-μl injection volume was 50 ng/ml ($1.1 \cdot 10^{-7}$ *M*) on the 10-μm column. Recovery for MTX was 46% and for 7-OH-MTX was 26% based upon injection of unextracted standards. Typical variation in the duplicate analysis of specimens run from the same standard curve was found to be of the order 5—10% and the relative standard deviation of a 2.0 μg/ml spiked MTX plasma sample run repeatedly was 7.4%, which was typical of the between-run variation observed over this concentration range.

The present method was compared to the HPLC strong anion-exchange method published previously [23] by analyzing duplicate patient samples by both procedures using independently prepared standard curves. In general the agreement was quite good for both MTX and 7-OH-MTX as illustrated by the representative comparison for 5 patient specimens in Table I. The present procedure using a 10-μm column is about 2—3-fold more sensitive than the anion-exchange procedure due mainly to improved peak shape and resolution from baseline components. The utilization of the 7-μm average particle diameter RP-8 column further improved peak shape and resolution and resulted in a minimum detectable MTX concentration of 15 ng/ml ($3.3 \cdot 10^{-8}$ *M*). The retention times were slightly longer under these conditions. This increment of improved sensitivity also makes it possible to detect serum MTX and 7-OH-MTX levels following both high dose and conventional dose therapy.

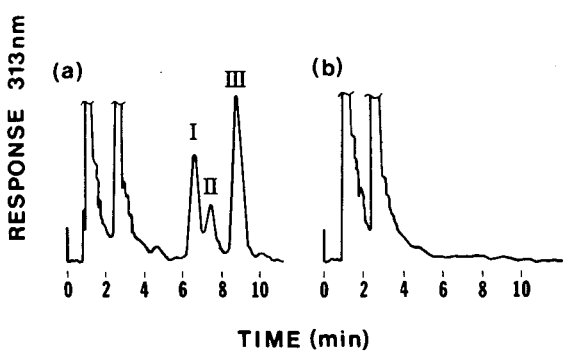

Fig. 1. HPLC chromatograms from (a) a patient receiving high dose MTX intravenous infusion over 6 h and (b) individual patient plasma blanks at 0.01 a.u.f.s. Column was 10 μm RP-8 and conditions are described in the text. I = MTX; II = 7-OH-MTX; III = internal standard.

TABLE I

COMPARISON OF REVERSED-PHASE (RP-8) AND STRONG ANION-EXCHANGE (SAX) HPLC ANALYSIS OF PATIENT METHOTREXATE SAMPLES*

Doses ranged from 2—8 g/m² body surface area administered as a 6-h continuous intravenous infusion.

Patient	MTX concentration (*M*)		7-OH-MTX concentration (*M*)	
	RP-8	SAX	RP-8	SAX
1	$7.0 \cdot 10^{-6}$	$9.8 \cdot 10^{-6}$	$3.9 \cdot 10^{-6}$	$6.6 \cdot 10^{-6}$
2	$3.0 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$	ND**	ND**
3	$7.6 \cdot 10^{-7}$	$6.8 \cdot 10^{-7}$	$1.5 \cdot 10^{-6}$	$1.2 \cdot 10^{-6}$
4	$2.0 \cdot 10^{-7}$	$4.0 \cdot 10^{-7}$	$9.0 \cdot 10^{-8}$	ND**
5	$2.6 \cdot 10^{-7}$	$3.0 \cdot 10^{-7}$	$6.7 \cdot 10^{-6}$	$8.8 \cdot 10^{-6}$

*See ref. 23.

**ND represents concentrations less than $1.0 \cdot 10^{-7}$ *M* for the SAX method and $7.5 \cdot 10^{-8}$ *M* for the RP-8 method.

In addition to improved sensitivity, the reversed-phase procedure appears to be more reproducible and durable than the ion-exchange procedure over time. The anion-exchange column utilized in the previous assay [23] did not withstand the required pH 7.4 buffer mobile phase over long periods of time and an increased pressure and decreased resolution was noted after 100—400 injections on most columns. Attempted clean-up or separation procedures did not reverse this and a costly column inventory was required. Under the described analytical conditions, the present reversed-phase columns appear stable and remain functional after more than 2000 injections with no appreciable loss of resolution or increase in pressure. In addition, the presence of perchlorate as an ion pair with MTX and 7-OH-MTX, and the difficulty associated with any clean-up extraction steps with amphoteric molecules like the folates, makes the use of the more stable reversed-phase system much more appealing than the less stable ion-exchange systems.

Due to the scarcity of authentic 7-OH-MTX, metabolite levels were generally expressed from a MTX standard curve in terms of MTX equivalents. In one series of samples a 7-OH-MTX standard curve was run and the resultant 7-OH-MTX levels were compared to those calculated from the MTX standard curves. The results indicated that actual 7-OH-MTX levels were four times greater. Generally 7-OH-MTX levels were insignificant at early times (up to 10 h) then increased and were equal to or greater than those of MTX after 24 h. The plasma time course of drug and metabolite for a typical patient is illustrated in Fig. 2. Due to the probable higher levels as described above, it is clear that 7-OH-MTX may be as important or more important in the clinical response and toxicity seen in high dose therapy. These data coupled with the recent suggestion that 7-OH-MTX may be implicated in renal toxicity in the rhesus monkey [12] suggests that the plasma levels of both MTX and 7-OH-MTX be monitored in managing high dose MTX patients. The sensitivity of the present procedure is adequate for the measurement of MTX serum levels with most protocols [10, 12, 24] requiring monitoring until the MTX level drops below

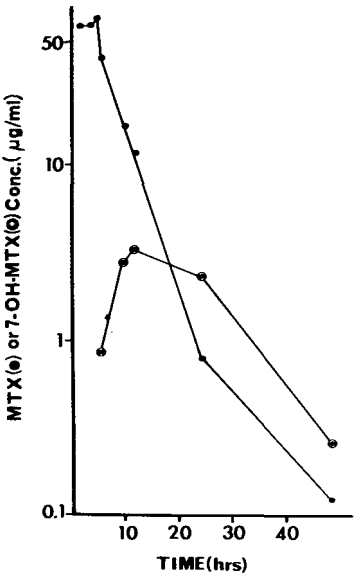

Fig. 2. Plasma time course for MTX (●) and 7-OH-MTX (◉) in a typical patient receiving 2.5 g MTX by intravenous infusion over 4 h.

$1 \cdot 10^{-7}$ *M*. In addition, while many of the previously published non-chromatographic methods are suitable for detection of parent MTX, none of these methods allow direct measurement of metabolite levels.

ACKNOWLEDGEMENT

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Note

High-performance liquid chromatographic analysis of verapamil

II. Simultaneous quantitation of verapamil and its active metabolite, norverapamil

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Several analytical procedures have been published recently for measuring the concentration of verapamil in plasma and urine [1—3]. These reports discuss the separation of verapamil from its N-dealkylated metabolites (Fig. 1). However, no procedure has been reported for measuring the N-demethylated metabolite, norverapamil which has been reported to accumulate in the plasma of patients after oral administration [4]. This metabolite apparently does not accumulate significantly in the plasma of patients receiving single intravenous doses of verapamil, however it does accumulate to concentrations equal to or greater than those of verapamil during oral administration. Norverapamil has been reported to be about 20% as potent a vasodilator as verapamil when administered intra-arterially to dogs, but appears to have no significant effect on AV nodal conduction [5].

While previously developed assay procedures for verapamil are very useful for measuring concentrations of the drug in biological fluids following short-term or acute administration, it remains to be seen if these procedures provide adequate separation of verapamil from norverapamil. Due to the close structural similarity between verapamil and norverapamil and the high concentrations of the metabolite observed in plasma of patients on chronic oral therapy, it is difficult to adequately separate these two compounds by our previously reported high-performance liquid chromatographic (HPLC) procedure [3], without substantially slowing down the chromatography. This modification would greatly restrict the number of samples which could be analyzed daily and a loss of sensitivity would also occur. Therefore an alternative chromatographic procedure was developed to facilitate separation and simultaneous quantitation of

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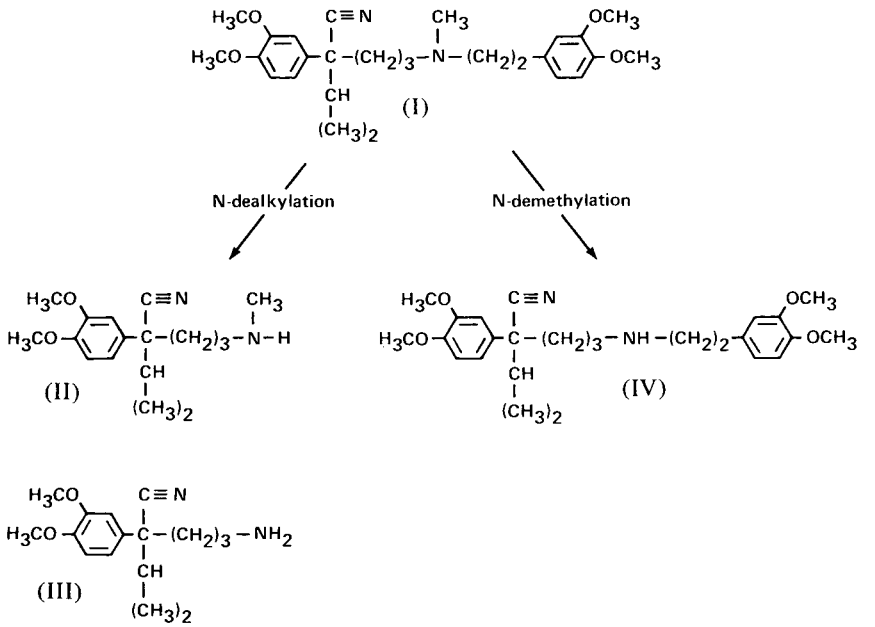

Fig. 1. Metabolic scheme for verapamil (I). The N-demethylated compound (IV) is the major active metabolite, norverapamil.

verapamil and norverapamil in human plasma. The method described here is a reversed-phase HPLC procedure which has a lower limit of sensitivity of 3 ng/ml for both verapamil and norverapamil when analyzed simultaneously.

EXPERIMENTAL

Verapamil, the two N-dealkylated metabolites and the internal standard (α-isopropyl-α-[(N-methyl-N-homoveratryl)-β-aminoethyl]-3,4-dimethoxyphenylacetonitrile hydrochloride) were obtained as HCl salts from Knoll Pharmaceutical Company (Whippany, N.J., U.S.A.) and norverapamil HCl was supplied by Knoll AG (Ludwigshafen, G.F.R.). Glass distilled acetonitrile was purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All other chemicals and solvents were of reagent grade.

The extraction procedure and instrumentation as well as internal standard remain the same as previously reported [3]. Changes have been made in the chromatography to facilitate the separation of verapamil and norverapamil within reasonable time limits. The column used was a 10-μm particle size μBondapak C_{18} (30 cm × 3.9 mm I.D.) (Waters Assoc., Milford, Mass., U.S.A.). The mobile phase consisted of acetonitrile—dilute H_2SO_4 (29:71). The dilute acid (ca. 0.004 *N*) was prepared by adjusting the pH of distilled water to 2.4 with dropwise addition of concentrated H_2SO_4. The flow-rate of mobile solvent was 150 ml/h which developed a precolumn pressure of 108 atm (1500 p.s.i.).

A blinded study was carried out with plasma samples to which were added known concentrations of verapamil, norverapamil, and the two N-dealkylated metabolites. They were analyzed for verapamil and norverapamil concentra-

tions by the method reported here without knowledge of the actual concentrations. The samples were initally spiked in the laboratories of Knoll AG. The results were then compared with the actual values.

RESULTS AND DISCUSSION

The retention times for verapamil, the internal standard, norverapamil and the two N-dealkylated metabolites are listed in Table I. With the conditions used, the two N-dealkylated metabolites were not completely separated from one another. These could be resolved if one either decreased the percentage of the acetonitrile in the eluting solvent or slowed the flow-rate considerably. These changes would result in much longer retention times with subsequent loss of sensitivity for verapamil. Since these two N-dealkylated metabolites are of very low pharmacological activity, compared to verapamil, their quantitation was not considered important.

TABLE I

RETENTION TIMES OF VERAPAMIL AND METABOLITES

Compound	Retention time (min)
Metabolite III	3.2
Metabolite II	3.5
Internal standard	10.5
Norverapamil	11.7
Verapamil	13.0

Chromatograms of an extracted blank plasma sample (A), injected standard solutions (B), and an extracted plasma sample from a patient on long-term oral verapamil therapy (C) are shown in Fig. 2. The peak in the patient sample (C) which eluted just prior to the internal standard did not correspond to any of the three metabolites being measured or the other medications which the patient was concurrently receiving and was not identified. This peak has not been observed in plasma samples from other patients. The concentrations of verapamil and norverapamil in the patient sample were 350 ng/ml and 245 ng/ml, respectively. This patient was taking 80 mg of verapamil HCl four times daily and the sample was drawn just after administration of a morning dose. Other patient samples which have been analyzed have had concentrations of norverapamil which were in some cases greater than those of verapamil.

Standard curves were prepared by adding known amounts of verapamil, norverapamil and 25 ng of internal standard to blank plasma, analyzing the samples, and determining the verapamil and norverapamil to internal standard peak height ratios. The curves were linear from 2 to 500 ng/ml for both verapamil and metabolite. A standard curve for both compounds over a range of 0 to 115 ng/ml is shown in Fig. 3. At a given concentration, the peak height of verapamil was greater than norverapamil, despite the fact that the latter eluted from the column first. This suggests that verapamil is more fluorescent than the metabolite at the wavelengths used. The slopes of the standard curves are 0.356 and 0.296 for verapamil and norverapamil, respectively.

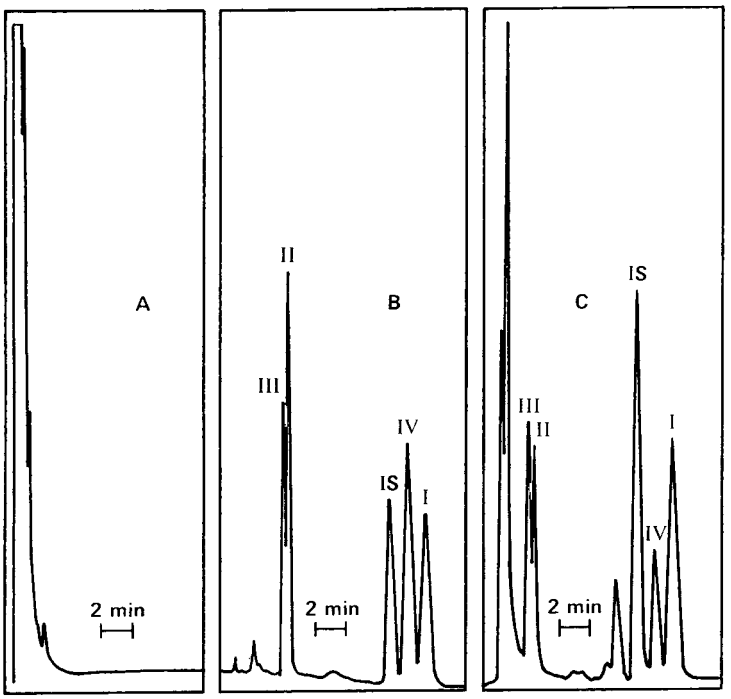

Fig. 2. Chromatograms of an extracted blank plasma sample (A), a mixture of verapamil (I), norverapamil (IV), internal standard (IS), and the two N-dealkylated metabolites (II, III) (B), and an extracted plasma sample from a patient receiving chronic oral verapamil (C).

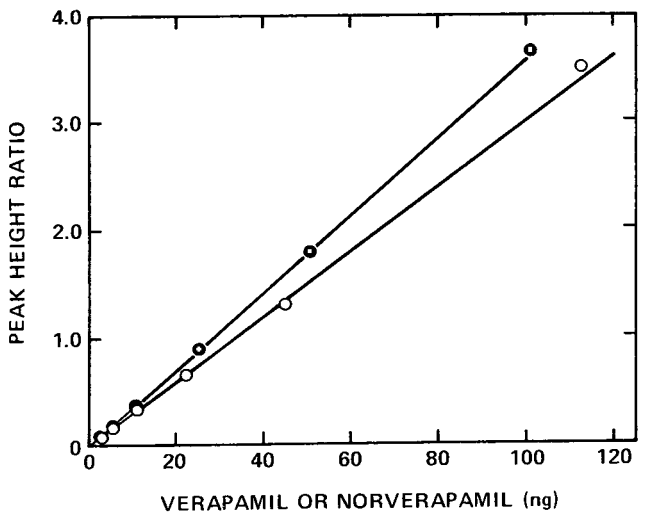

Fig. 3. Calibration curves for verapamil (•) and norverapamil (○). The coefficients of variation for the normalized peak height ratios were 3.0% and 3.7% for verapamil and norverapamil, reespectively.

The reproducibility of the system was evaluated by analyzing five plasma samples to which were added 25 ng of verapamil and 22 ng of norverapamil. The coefficient of variation for the verapamil samples was 5.1% and for the norverapamil samples 3.5%. These values are similar to the coefficient of variation observed with the previously reported procedure [3]. To facilitate analysis of samples of high concentration without preparing a high-range standard curve, the influence of varied volume size was evaluated. Ten ng of verapamil and 11 ng of norverapamil were added to varied volumes of blank plasma ranging from 100 μl to 1 ml. Internal standard was added and the samples analyzed. The coefficients of variation for verapamil and norverapamil were 3.1% and 5.1%, respectively. The extraction efficiency was determined for norverapamil by comparing the peak heights of chromatograms from extracted and directly injected samples of the metabolite. The extraction efficiency was greater than 60%.

The results of the comparison samples which were analyzed by the HPLC procedure reported here are summarized in Table II. Verapamil and norverapamil were analyzed simultaneously by the HPLC procedure. The samples containing the low verapamil concentrations contained the lower norverapamil concentrations and in similar manner the medium- and high-range verapamil samples contained the medium- and high-range norverapamil concentrations, respectively. The deviations of the results are expressed as percent of the spiked value. The overall percent deviation for the 17 verapamil and norverapamil analysis were 4.6 and 9.8, respectively. Correlations for the two methods were good with $r = 0.99$ for both compounds.

TABLE II

RESULTS OF BLINDED ANALYSIS STUDY

Range (ng/ml)	*N*	Average percent deviation
Verapamil		
1—10	6	4.7
10—100	5	6.1
100—500	6	3.2
Norverapamil		
1—20	6	22.1
50—200	5	6.6
200—1000	6	0.1

Interference from other drugs was evaluated by adding various drugs to plasma in quantities representative of therapeutic or higher concentrations. These were then extracted and injected into the chromatograph. The following drugs were evaluated: digoxin, theophylline, disopyramide, propranolol, quinidine, procainamide, chlorthiazide, prazosin, furosemide, and hydralazine. None of these compounds interfered with the analysis of verapamil or norverapamil.

The method reported here differs from our previously reported procedure in that this method allows the simultaneous quantitation of verapamil and its major active metabolite, norverapamil. The previous method is sufficient for analysis of samples from patients receiving single intravenous bolus doses where

the metabolite does not accumulate to sufficient concentrations to interfere with the assay. These samples could also be analyzed at a more rapid rate with the present method by increasing the percent of acetonitrile in the mobile phase. The method reported here, however, provides a better separation of verapamil from norverapamil which is sufficient to allow quantitation of both species.

ACKNOWLEDGEMENT

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CHROMBIO. 497

Note

Comparison of high-performance liquid chromatography with an enzyme multiplied immunoassay technique for the analysis of serum procainamide and N-acetylprocainamide

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The use of procainamide (PA) for the control of cardiac arrhythmias is well established [1]. The major metabolite, N-acetylprocainamide (NAPA) has been found to be equally effective against cardiac arrhythmias and often exceeds the concentration of the parent compound in serum [2]. Therapeutic drug level monitoring of PA and NAPA in serum is recommended because the therapeutic range is narrow (4—8 mg/l), toxicity above these levels can be serious, and individual differences in absorption, distribution, and elimination make serum drug concentration of PA a better guide for clinical effectiveness than total dose [3].

A homogeneous enzyme immunoassay, the Enzyme Multiplied Immunoassay Technique (EMIT^{R}), for the assay of serum PA and NAPA, has been developed recently by the Syva Corporation (Palo Alto, Calif., U.S.A.) [4, 5]. While analysis of PA and NAPA by EMIT requires a separate assay for each drug, both tests are rapid and require essentially no sample preparation.

High-performance liquid chromatography (HPLC) for the simultaneous determination of serum PA and NAPA is also a relatively recent analytical technique [6—9]. Simultaneous determination by HPLC of serum PA and NAPA involves simple organic extraction and reversed-phase chromatography and has been shown to be sensitive, specific and suitable for routine clinical use.

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A comparison of these two procedures in a clinical setting would be useful to laboratories considering the future use of either of these methods. This communication reports the results of a study in which serum samples, obtained from patients in a coronary intensive care unit who were receiving a number of drugs in addition to PA, were assayed by both EMIT and HPLC for serum PA and NAPA.

MATERIALS AND METHODS

Samples

A total of 79 blood samples was obtained from patients in the Cardiac Care Unit in the University of California, Davis, Medical Center (Sacramento, Calif., U.S.A.). All patients had cardiac arrhythmias requiring therapy by pharmacological agents. PA was administered in doses ranging from 3.0 to 6.0 g/day orally (p.o.) and from 1 to 5 mg/min intravenously (i.v.). Blood samples were obtained at various times after drug administration to obtain as broad a range of drug concentrations as possible. These included pre-drug (PA) control samples.

A variety of other drugs were used in the clinical management of these critically ill patients; these included digoxin, diazepam, phenobarbital, furosemide, dopamine, norepinephrine, nitroprusside, nitroglycerine, phenytoin, penicillin, gentamicin and cephalosporin. In addition, all patients had received lidocaine prior to receiving procainamide and some had received quinidine prior to lidocaine.

Blood samples were obtained by venipuncture, centrifuged at 380 *g* for 10 min and the serum removed and frozen at -20° until assayed. To minimize bias, the order of the samples was randomized prior to analysis and the analyst was unaware of the results of one method while performing the other.

EMIT assay

All components for analysis, including standards, controls and equipment were part of the assay system supplied by Syva for evaluation of the procedure for PA and NAPA analysis. The immunoassay procedure was performed according to the protocol provided by Syva which has been reported previously in abstract form [4, 5]. The procedure employed a Model 300-N Gilford microsample spectrophotometer with an automatic sampling system (Gilford Instrument Labs., Oberlin, Ohio, U.S.A.). The spectrophotometer was coupled to a Model 2400 printer-calculator (Cavro Scientific Instruments, Los Altos, Calif., U.S.A.). Samples were dispensed and diluted with a Model 1500 pipettor-dilutor (Cavro Scientific Instruments). Briefly, the procedure consisted of taking a 50-μl sample, diluting with buffer, adding antibody and drug-labeled glucose-6-phosphate dehydrogenase (G6PDH), then measuring spectrophotometric absorbance changes over 45 sec. This assay can be done quite rapidly because no sample preparation is required. Once calibration curves for both PA and NAPA are constructed, the analysis of a single sample for both compounds can be accomplished in 10 min.

Specific antisera were obtained from sheep previously immunized with derivatized procainamide covalently linked to a protein carrier. Drug-labeled enzyme was prepared by linking derivatized procainamide to G6PDH. When

antibody binds to procainamide-labeled G6PDH, the activity of the enzyme is reduced. Free drug in the test sample decreases this antibody-induced inactivation of the enzyme. The subsequent increase in enzyme activity is directly related to the amount of procainamide. Spectrophotometric absorbance changes (340 nm) reflecting the conversion of NAD to NADH are measured as the analytical endpoint. The range of drug concentrations which may be measured by this assay is 1.0—16 mg/l for both PA and NAPA. Evaluation of the assay system by the manufacturer showed that concentrations of NAPA greater than 40 mg/l must be present in the sample to produce interference in the EMIT PA assay; PA will not cross-react with the EMIT NAPA assay until levels greater than 100 mg/l are present [10]. Other compounds of similar chemical or pharmacological properties were tested and found not to cross-react with either the PA or NAPA reagents at concentrations less than 100 mg/l.

Calibration curves were constructed by analyzing duplicate samples containing PA and NAPA at concentrations of 1, 2, 4, 8 or 16 mg/l and plotting the data points on graph paper supplied by the manufacturer and which has been specially matched with the reagents. Data points were plotted as change in absorbance versus log drug concentration. Curves were linear over the range of concentrations measured and all data points were within the 95% confidence interval for the regression line. Within-run precision for the EMIT assays was determined by analyzing 20 samples of two sera which had been fortified with 4 mg/l PA or 4 mg/l NAPA. The mean value for PA was 4.05 mg/l, ± 0.20 S.D. and a coefficient of variation (C.V.) of 5%. For NAPA the mean value was 3.8 mg/l, ± 0.08 S.D. and a C.V. of 2%. Day-to-day precision was somewhat less as the coefficients of variation for PA and NAPA increased to 11% and 5% respectively.

HPLC assay

Assay by HPLC involved double extraction of serum samples with ethyl acetate, the use of an internal standard, and reversed-phase liquid chromatography.

Chemicals and reagents

All reagents were of analytical or reagent grade. Procainamide HCl was obtained from K & K Labs. (Plainview, N.Y., U.S.A.). N-Acetyl-procainamide and the internal standard, *p*-amino-N-(2-dipropylaminoethyl)benzamide HCl (the dipropyl analog of PA) were obtained from E.R. Squibb and Sons (Princeton, N.J., U.S.A.). All calculations of PA and NAPA amounts were in terms of free base. Working solutions of PA and NAPA and the internal standard were made in distilled water and stored at 4°.

Ethyl acetate used in the extraction was glass-distilled in our laboratory. All glassware used in the assay was silanized with Siliclad^{R} (Clay-Adams, Parsippany, N.J., U.S.A.), a water-soluble silanizing agent.

Sample preparation

Ethyl acetate extraction of plasma samples was performed as follows. To a 60 × 125 mm PTFE-lined, screw cap culture tube, was added 0.5 ml plasma or serum, 0.5 ml internal standard (24 mg/l), 0.1 ml 2 *N* NaOH, and 3.0 ml ethyl

acetate. The sample was vortexed 30 sec and centrifuged for 5 min at 360 *g*. The organic (upper) phase was pipetted into another 16 × 125 mm culture tube, and the aqueous phase was reextracted with another 3.0 ml of ethyl acetate. The organic phase from this second extraction was then combined with that from the first. One drop of concentrated HCl was added and the mixture vortexed 5 sec. Samples were placed in a water bath (40°), and the solvent evaporated to dryness under a gentle stream of nitrogen. The residue was frozen until ready for assay or was reconstituted immediately by adding 0.5 ml mobile phase. The reconstituted sample was allowed to equilibrate at room temperature for 30 min followed by vortexing 5 sec. A 50-μl aliquot was injected into the liquid chromatograph for analysis.

Liquid chromatography

A Waters Model 6000A high pressure liquid chromatograph was used equipped with a Model U6K loop injector and a Model 440 fixed wavelength (254 nm) ultraviolet absorbance detector (Waters Assoc., Milford, Mass., U.S.A.). A 30 cm × 3.9 mm I.D. μBondapak C_{18} reversed-phase column (Waters Assoc.) was used. In addition, a 0.5-μm HPLC in-line filter (Alltech Assoc., Arlington Heights, Ill., U.S.A.) and a 8.0 × 0.6 cm guard column packed with Co:Pell^R ODS pellicular packing (Whatman, Clifton, N.J., U.S.A.) were fitted between the injector and the analytical column to prolong the analytical column life.

The mobile phase was acetonitrile in distilled water (100 ml/l) with acetic acid (40 ml/l) and sodium acetate (4 g/l). The flow-rate was 2 ml/min at an approximate pressure of 2000 p.s.i. The mobile phase was filtered through a 0.45-μm filter before use.

Retention times for PA, NAPA and the internal standard were 3.3 min, 4.8 min and 7.6 min, respectively. The total chromatographic analysis time was approximately 12 min for the three compounds. The ratios of the area under the peaks for standards and unknown to peak area of the internal standard were used for quantitation. Minimum detectable amount, defined as that amount of drug producing a detector response twice that of background noise, was 4 ng (20-μl samples of 0.2 mg drug/l) for both PA and NAPA.

Calibration curves were constructed by extracting and analyzing pooled normal human sera which contained PA and NAPA at concentrations of 2, 5, 7, 10 or 15 mg/l and fitting the data points by linear regression. Curves were linear over this range of concentration with correlation coefficients typically being $\geqslant$ 0.950 for both drugs. Within-run precision for the HPLC method was determined by the analysis of five samples fortified at 2 mg/l PA and NAPA and five samples fortified at 10 mg/l PA and NAPA. At the 2 mg/l concentration the coefficients of variation for PA and NAPA were 4% and 6% respectively and at the 10 mg/l concentration the coefficients of variation for PA and NAPA were 4% and 1% respectively. Day-to-day precision was somewhat less as the coefficients of variation for PA and NAPA at 2 mg/l increased to 11% and 7% respectively.

RESULTS AND DISCUSSION

Figs. 1 and 2 are scattergrams which compare values obtained by HPLC and EMIT. The results from the two methods for both PA and NAPA were in close agreement. A statistical comparison of these data by least squares analysis and linear regression shows that for PA, the correlation coefficient was 0.895, and the least square values of slope and intercept were 1.110 and −0.400, respectively. The correlation coefficient for N-acetylprocainamide was 0.964, and the slope and intercept values were 0.942 and 0.248, respectively.

The extraction procedure used for HPLC assay was a modification of several published methods [6—9]. Both *n*-propanol—chloroform (1:9) and methylene chloride were tried as the extracting solvent for PA and NAPA, according to the procedures of Carr et al. [6] and Rocco et al. [7], and rejected in favor of ethyl acetate. Extraction with ethyl acetate gave a more consistent recovery because it did not form emulsions. In addition, ethyl acetate is the top layer in a solvent—water mixture and can be transferred more rapidly and with greater precision than can methylene chloride or chloroform, which are more dense than water. Two extractions with ethyl acetate increased both recovery and precision (C.V.) by 5% over one extraction for PA and increased recovery, but not precision, of NAPA by 27%.

PA has been found to be non-specifically bound to plasma proteins [11] and absorbed on glassware [6, 12] which has resulted in poor extractability and non-linear calibration curves. When silanized glassware or polypropylene tubes

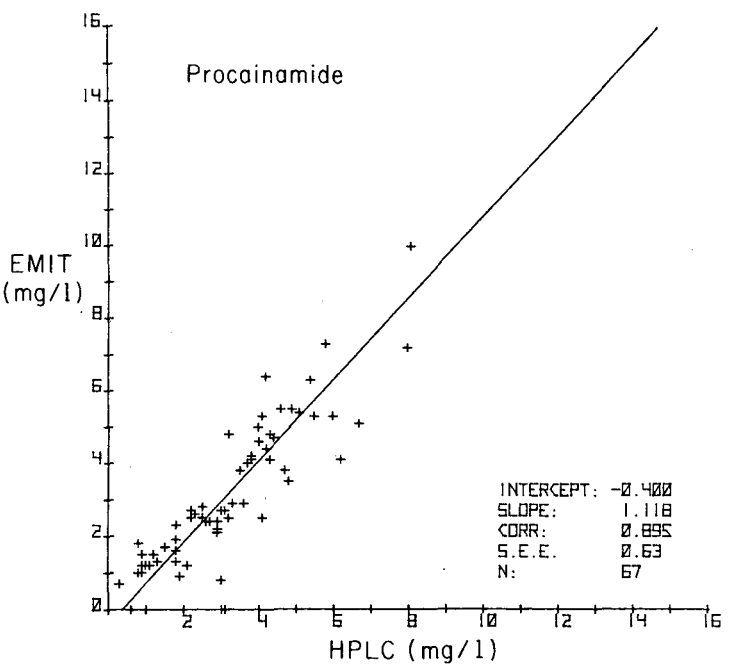

Fig. 1. Scattergram comparing serum procainamide concentrations determined by EMIT with those determined by HPLC.

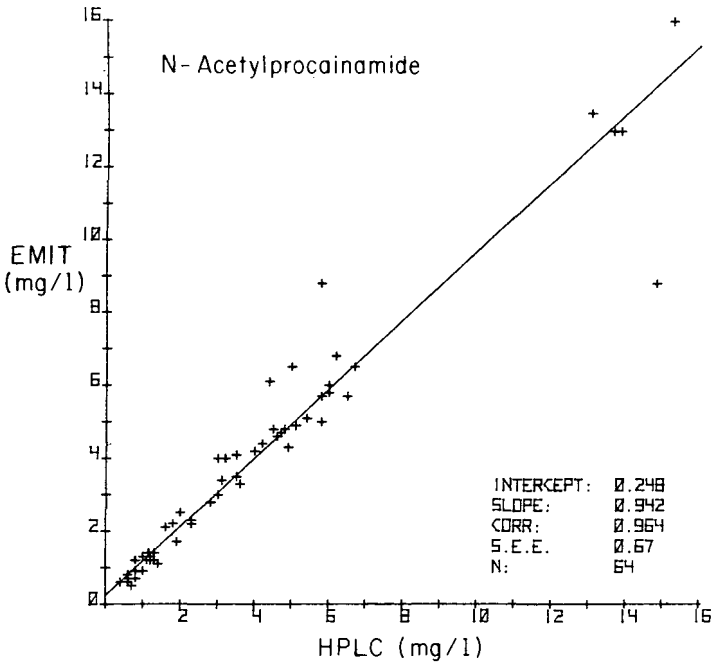

Fig. 2. Scattergram comparing serum N-acetylprocainamide concentrations determined by EMIT with those determined by HPLC.

were used for extraction and concentration, this problem was overcome. In the present study, silanizing all glassware prior to use increased recovery of PA from 58 to 85% and precision (C.V.) from 9 to 5% (10 samples fortified at 5 mg/l). For NAPA, recovery and precision remained unchanged at 96% and 3% (C.V.) respectively. Although recovery of PA can be increased by the use of appropriate solvents and silanizing all glassware, nonspecific binding may still be a potential source of error.

The acetonitrile, sodium acetate-buffered eluting solvent was chosen as the mobile phase for the PA and NAPA HPLC assay because it gave sharp, symmetrical peaks with good separation of PA, NAPA and the internal standard. Methanol in water, which was used as the mobile phase by Rocco et al. [7] gave poorer resolution and broader peaks over the range of methanol:water ratios evaluated.

This study attempted to rigorously challenge the specificity of the two assay methods since all samples were obtained from patients who were receiving an average of six other drugs in addition to PA. In fact, all patients received lidocaine prior to PA and most had received quinidine. There was no apparent interference by any of these other medications in either assay as evidenced by the absence of artifacts in the pre-drug (PA) control samples. The somewhat lower correlation between the two methods for PA may be due to (1) the known incomplete and somewhat variable recovery of PA from plasma which may result in an error in the HPLC procedure or (2) the presence of heme pig-

ments, lipids or bilirubin in the patient sera which is a known source of error in any enzyme multiplied immunoassay technique [13].

In conclusion, in the clinical setting in which a number of pharmacological agents are being administered concomitantly with PA, both the HPLC assay and the EMIT assay described herein were shown to be sensitive and specific techniques for the therapeutic drug level monitoring of PA and the active metabolite NAPA. The HPLC assay has the advantage of allowing simultaneous determination of both PA and NAPA but has the disadvantage of requiring prior extraction with organic solvent. EMIT has the advantage of being a simpler, more rapid assay but requires separate tests for PA and NAPA.

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The Syva Corporation provided the equipment and furnished all reagents used in the EMIT^{R} assays.

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Note

Rapid and sensitive determination of chlorthalidone in blood, plasma and urine of man using high-performance liquid chromatography

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Chlorthalidone is a diuretic drug widely used in anti-hypertensive therapy [1]. This sulphonamide-like diuretic differs chemically from the thiazides by the nature of the heterocyclic ring, although its pharmacological action is indistinguishable from that of the thiazides. The drug is administered orally, in a dose of 25—100 mg daily and is claimed to have a prolonged action [2—5], mainly due to the long biological half-life of the compound (30—80 h) [6—9].

The volume of distribution of chlorthalidone appears to be relatively large, 200—400 l [9], resulting in maximum plasma concentrations of 100—400 ng/ml. However, this compound reaches 10—30 times greater concentrations in red blood cells [7—10]. For the determination of the low concentrations in plasma only a limited number of methods are available. A spectrophotometric method for the determination of chlorthalidone in whole blood and urine has been published by Tweeddale and Ogilvie [6], permitting the determination of a minimum concentration of 1 mg/ml. Gas—liquid chromatographic (GLC) methods have also been published [11—13]; most of these methods, based on the conversion of chlorthalidone to its tetramethyl derivative by extractive alkylation [11, 12], are still quite laborious and may pose considerable methodological problems for a large series of samples. A sensitive and selective

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direct GLC method has been developed by Fleuren and Van Rossum [13]; although plasma concentrations as little as 10 ng/ml can be measured, this method still requires 1 ml of plasma, which can only be obtained by venipuncture. For kinetic studies in volunteers, large series are required, which can easily be obtained and analysed by the proposed method.

In an earlier high-performance liquid chromatography (HPLC) paper from our group, dealing with the determination of diazoxide [14], the separation characteristics of chlorthalidone were mentioned. However, the method was still not sensitive enough for the routine determination of chlorthalidone in kinetic studies. Therefore, a new HPLC method for the analysis of chlorthalidone in plasma, urine and whole blood was developed. Analytical details and some results of the pharmacokinetics of chlorthalidone in healthy volunteers are presented in this paper.

MATERIALS AND METHODS

Apparatus

A Spectra Physics 3500B high-performance liquid chromatograph was used, equipped with a variable wavelength spectrophotometric detector (Model SP 770). A stainless-steel column (15 cm × 4.6 mm I.D.) was packed with LiChrosorb RP-18, particle size 5 μm, obtained from Chrompack (Middelburg, The Netherlands). An injection loop of 100 μl was used. Detection of chlorthalidone was effected at 226 nm. The detection limit is 30 ng/ml.

Solvents

The solvent used was a mixture of 0.01 *M* sodium acetate in water and acetonitrile (400:100, v/v) and the flow-rate was 1.6 ml/min, at a pressure of 165 atm.

Drugs

Chlorthalidone was obtained from Ciba-Geigy (Arnhem, The Netherlands) and probenecid from Sigma (Brunschwig Chemicals, Amsterdam, The Netherlands).

Subjects

Two healthy, caucasian subjects, both employees of the Department of Clinical Pharmacy, Nijmegen, participated in this study. Chlorthalidone was administered orally either as a dose of 100 mg as Hygroton® tablets (Ciba-Geigy) or as an experimental generic preparation (Pharmachemie, Haarlem, The Netherlands).

Blood samples of 0.7 ml were collected at scheduled time intervals by fingertip puncture (Microlance No. 433, Becton-Dickinson). An amount of 0.1 ml of blood was used for the determination of the whole blood chlorthalidone concentration. The remaining 0.6 ml was centrifuged at 2600 *g* for 5 min and the plasma was immediately separated from the red blood cells. Spontaneously voided urine was collected over a period of 70 h. The pH of the urine was not affected.

Sample preparation

Whole blood. Whole blood (0.1 ml) is mixed with 0.4 ml of 0.33 *N* perchloric acid at a temperature of 4° in a vortex mixer. Deproteinization and haemolysis are complete after standing for 5 min. The mixture is centrifuged for 5 min at 2600 *g* in a Heraus Christ centrifuge. A 100-μl aliquot of the clear supernatant is injected onto the column.

Plasma. A 0.2-ml aliquot of a potassium dihydrogen phosphate solution in water (0.067 *M*) containing probenecid (1 mg/l) as internal standard, was added to 0.2 ml of plasma and 1 ml of diethyl ether, and was mixed for 1 min in a vortex mixer. The mixture is then centrifuged in another tube and evaporated to dryness. The residue is dissolved in 0.15 ml of eluent and 0.1 ml injected onto the column.

Urine. Urine (10 μl) is added to 0.2 ml of the eluent and 0.1 ml of this mixture is injected directly onto the column.

Recovery

The recovery of chlorthalidone added to human whole blood in the concentration range of 1—10 μg/ml was found to be 47.6 ± 2.7% (S.D.) and in urine 100 ± 2% (S.D.). The recovery of the chlorthalidone extracted from plasma in the concentration range of 50—500 ng/ml was found to be 70 ± 3% (S.D.). The calibration curves were linear over the concentration ranges measured, and the sensitivity limit for chlorthalidone was 30 ng/ml.

RESULTS

Fig. 1 shows high-performance liquid chromatograms of whole blood, plasma and urine samples obtained from a volunteer after the intake of 100 mg chlorthalidone. As shown in the blank samples no interfering peaks are found either in direct injections (urine) or after deproteinization (whole blood), or extraction (plasma).

Fig. 2 shows the concentration—time profiles of whole blood and plasma and the renal excretion rate—time profile of chlorthalidone in a volunteer after an oral dose of 100 mg. The calculated pharmacokinetic parameters of the volunteers are summarized in Table I. The plasma half-life time of elimination in this study varies from 26.5—42 h, whereas the half-life time measured in whole blood appeared to be somewhat longer (33—57 h).

About 40% of chlorthalidone is excreted in urine unchanged, although big variations are found. The renal excretion rate hardly appeared to be affected by urinary flow or urinary pH, but was strongly influenced by the plasma concentration. The relationship between the renal excretion rate and the plasma concentration (the proportionality factor being the renal clearance) clearly shows a biphasic character. The renal clearance of chlorthalidone was much higher (161 ml/min) during the absorption and distribution phase of the drug in the body than during the elimination phase (59 ml/min) (Fig. 3).

The concentration—time courses of chlorthalidone in plasma and whole blood are not synchronous. A linear relation between blood and plasma concentration was found ($r = 0.929$) after absorption and distribution processes were complete. The uptake of chlorthalidone in the red blood cells was slower than the drug supply after absorption, resulting in altering whole blood:plasma

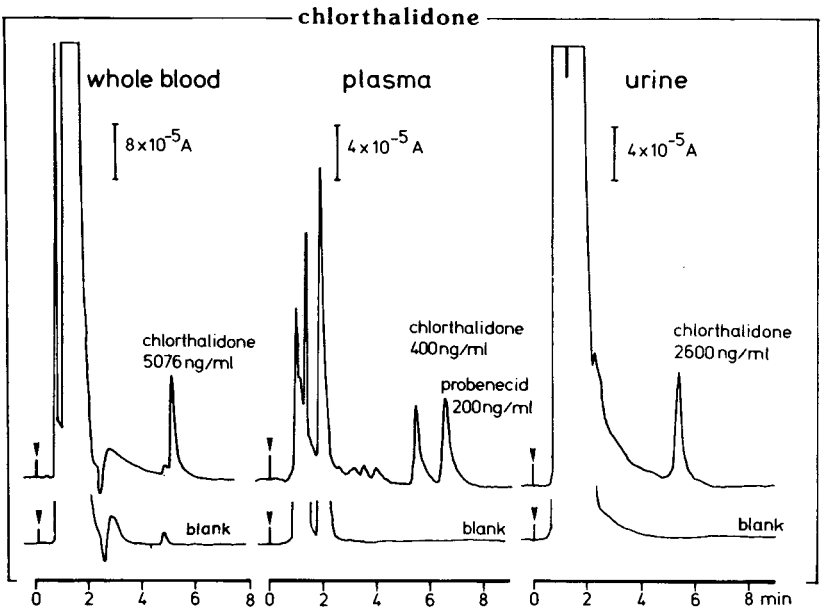

Fig. 1. HPLC chromatograms of chlorthalidone in whole blood, plasma and urine samples obtained from volunteers after the intake of 100 mg chlorthalidone and their respective blanks, sampled prior to drug intake.

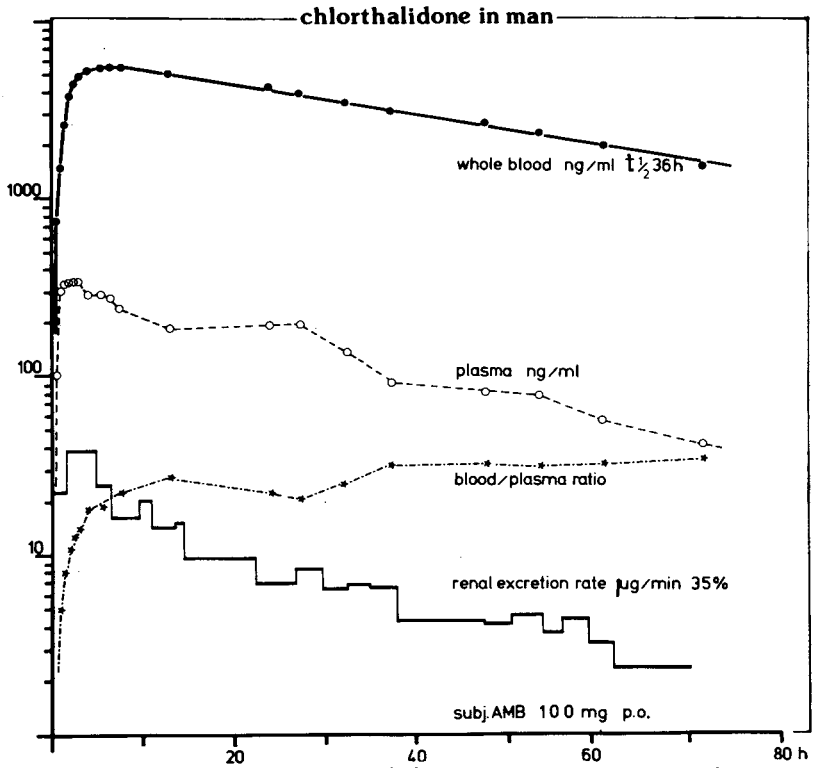

Fig. 2. Pharmacokinetics of chlorthalidone in man after an oral dose of 100 mg. The plasma elimination curve is much lower than the whole blood elimination curve, due to the selective uptake of chlorthalidone in red blood cells.

TABLE I

SOME PHARMACOKINETIC PARAMETERS OF CHLORTHALIDONE AFTER ORAL ADMINISTRATION OF 100 mg TO VOLUNTEERS

Subject	Body weight (kg)	Plasma $t_{½}$ (h)	Whole blood $t_{½}$ (h)	Concentration ratio whole blood:plasma	Percentage excreted in urine* (%)	Renal clearance** (ml/min)
JED	60	26.5	—***	—***	50.9	67.5 ± 16.8
		26.5	36	28.3 ± 5.4	42.7	60.6 ± 35.4
		30.5	34	26.1 ± 3.0	44.9	54.0 ± 15.3
		28.5	33	39.8 ± 8.4	38.3	62.4 ± 15.3
AMB	57	40.0	—***	—***	23.4	67.5 ± 11.8
		29.0	36	20.5 ± 5.2	34.8	65.1 ± 22.3
		42.0	57	25.9 ± 5.5	27.2	51.9 ± 13.7
		40.0	33	26.6 ± 4.6	19.2	39.0 ± 14.3

*Percentage of the dose excreted in urine as the unchanged drug during 70 h (ca. 2 × $t_{½}$).
**Calculated from plasma concentrations and renal excretion rates during the elimination phase.
***Not measured.

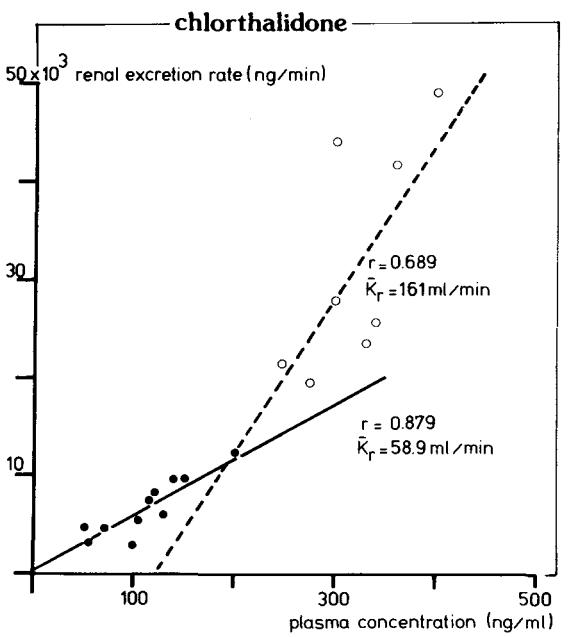

Fig. 3. The relationship between the renal excretion rate and the plasma concentration, the proportionality factor being the renal clearance. The renal clearance of chlorthalidone is much higher during the absorption and distribution phase of the drug than during the elimination phase.

concentration ratios during the first 8 h after drug intake (Fig. 4). Chlorthalidone could not be measured in saliva in this experiment, because the concentrations appeared to be below the detection limit (30 ng/ml).

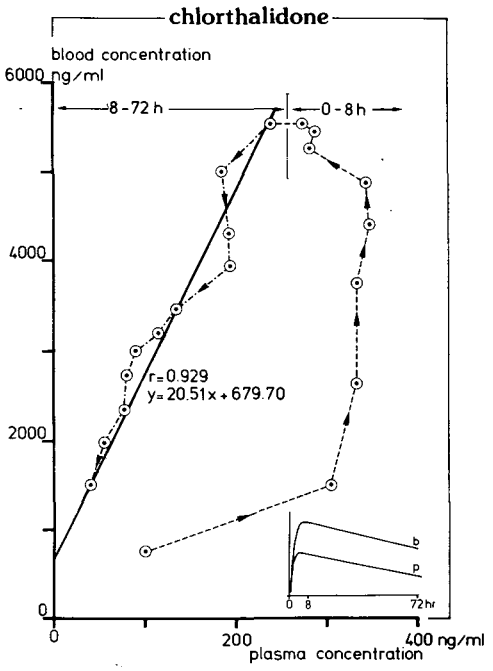

Fig. 4. The relationship between whole blood and plasma concentrations of chlorthalidone. The uptake of chlorthalidone in the blood cells is slower than the drug supply after absorption, resulting in changing blood:plasma concentration ratios during the first 8 h after drug intake.

DISCUSSION

The HPLC method described in this paper permits the determination of chlorthalidone in biological fluids at concentrations as low as 30 ng/ml. The method has advantages over the spectrophotometric method of Tweeddale and Ogilvie [6] with respect to sensitivity. The published GLC methods [11—13] are sensitive, but are mainly based on derivatization, with the exception of the method of Fleuren and Van Rossum [13], which still needs 1.0 ml of plasma to attain a detection limit of 10 ng/ml. These volumes can only be obtained from venipuncture, which may pose practical problems when large sample series are required for kinetic studies. With our method large numbers of samples can easily be obtained by fingertip puncture and the concentration of chlorthalidone can be measured in plasma, whole blood and urine.

Preliminary pharmacokinetic results as shown in this paper (Fig. 2, Table I) are in good agreement with earlier reports [6—10, 15, 16]. Chlorthalidone is excreted mainly unchanged in urine. In this study about 40% of the administered dose could be traced in 72 h ($2 \times t_{1/2}$) with wide inter- and intra-individual variations (Table I). These differences are reported earlier in literature [8—10, 15, 16] and are attributed to variations in bioavailability, which is relatively poor for chlorthalidone [8, 9]; non-linear binding of this drug to red blood cells [10, 15] and dose-dependent urinary excretion [16].

Some of these phenomena are shown in Figs. 3 and 4.

Detailed results of the bioavailability of some chlorthalidone preparations and the clinical implications will be the subject of further publication.

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Note

Determination of a new mucolytic drug Adamexina in biological liquids by photodensitometry

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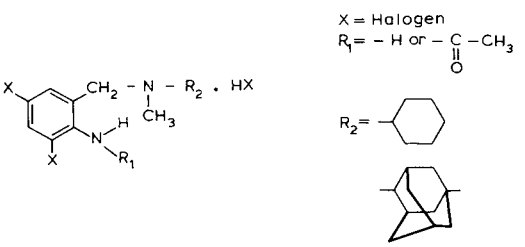
The main clinical and pharmacological action of mucolytic drugs is that of modifying the bronchial secretion, lowering its viscosity and secretolytic action, increasing the fluidity of sputum and facilitating its expulsion, while lowering the coughing reflexes. The drug typifying this class of compounds is Bromexine, N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)ammonium chloride, the properties of which have been described by several authors [1—8]. Investigations oriented towards obtaining a product with a wider field of action have resulted in the synthesis of Adamexina®**, in which the cyclohexyl ring of Bromexine is replaced by adamantane, which lends to the new mucolytic molecule its sympathicomimetic and antiviral properties [9—14].



Pharmacokinetic studies on Bromexine agree in pointing out the impossibility of determing levels in blood and urine by chemical methods, due to the low values found, so that some methods are proposed, based upon a labelled molecule, thus differentiating the intact drug from the several

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**Ferrer's Synthesis and Patent.

metabolites by chromatographic separation. Preliminary tests carried out with Adamexina show that the levels in plasma will also be low, probably due to the binding of the drug with blood cells and to its affinity with several organs and tissues as described for Bromexine [15, 16].

Attempts at conducting pharmacokinetic studies in human patients have resulted in the need for developing and improving the usual physico-chemical methods in spite of the above mentioned difficulties. Thus the drug is isolated from the remaining plasma components by a suitable extractive and chromatographic system, and then determined.

Adamexina shows UV absorption at 288 nm in ethanol, however $E_{1\ cm}^{1\%} = 11.8$ is too low to make direct quantitative determinations. On the other hand, due to the molecular structure, formation of a fluorescent derivative is not easily attained and native fluorescence is zero. Separation by gas chromatography can be carried out in a silanized glass column packed with 3% OV-17 on Chromosorb W HP (100—120 mesh). However the maximum sensitivity obtained is only 50 ng because of adsorption problems making the quantitative analysis difficult.

A method yielding better results was that proposed by Haefelfinger [17, 18]. This method is based on the introduction of nitro groups into the aromatic ring followed by reduction to form the primary amine, diazotization and coupling, either forming a coloured compound or reacting the amine formed with fluorescamine to yield a fluorescent compound. All these reactions are carried out in situ on silica gel chromatoplates.

EXPERIMENTAL

Reagents

Anhydrous sodium sulphate, concentrated hydrochloric acid, 1 *N* potassium hydroxide, absolute ethanol, chloroform and diethyl ether were analytical grade.

Nitrating mixture. To 40 ml of methanol is carefully added a mixture of 30 ml 65% nitric acid and 10 ml 95—97% sulphuric acid, with continuous cooling. The mixture of nitric and sulphuric acids is very carefully prepared and under very intense cooling. This mixture is stable for several weeks.

Reducing titanium chloride solution. To 20 ml of methanol, 4 ml of titanium(III) chloride solution (15% of the titanium salt in 4% hydrochloric acid) are added. This solution is not stable for more than one hour.

Sodium nitrite solution. Sodium nitrite (400 mg) is dissolved in 20 ml of 1 *N* hydrochloric acid. It is prepared just before use.

N-(1-Naphthyl)ethylenediamine solution. N-(1-Naphthyl)ethylenediamine dihydrochloride (1 g) is dissolved in 100 ml methanol. Although this solution is stable for several days, it is preferable to prepare it just before use.

Standards

Adamexina (prepared by our Department of Synthesis) is dissolved in absolute ethanol, in various concentrations.

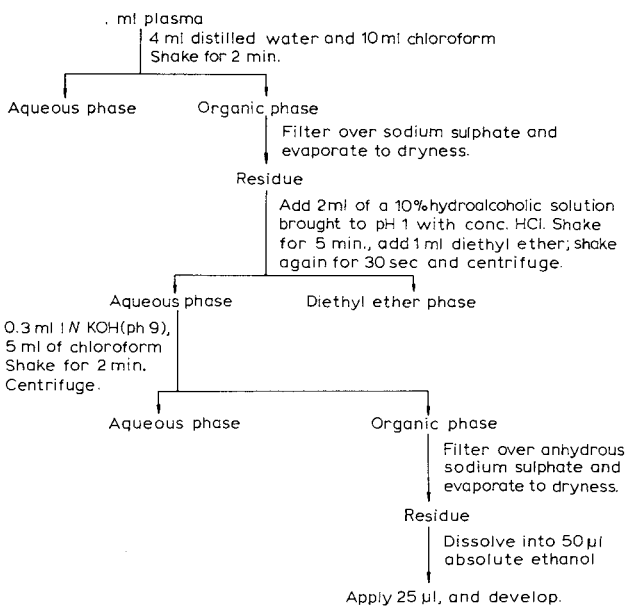
Thin-layer chromatography

Commercially available silica gel G plates (20 × 20 cm, 0.25 mm thick, Merck, Darmstadt, G.F.R.) without fluorescence are used. The tanks were obtained from Desaga (Heidelberg, G.F.R.). Micropipettes (20 or 25 µl) for application of solutions onto the plates were obtained from Pedersen (Copenhagen, Denmark).

Apparatus

In order to quantify the spots in situ a Zeiss PMQ II spectrophotodensitometer was used.

Extraction



Chromatographic conditions

Stationary phase: silica gel G activated for 45 min at 110°. Mobile phase: ethyl acetate—acetic acid—water (60:15:15). Elution height: 10 cm. Elution time: 1 h 15 min. R_F: 0.6–0.7. Chamber: not saturated. Elution temperature: room temperature (20–25°).

Detection

The detection consists of four steps:

(1) Nitration. Spray with sulphonitric mixture. Place the plate in a lidded stainless-steel box, which is kept in an oven at 115° for 15 min. Subsequently, it is cooled.

(2) Reduction. Spray with titanium chloride solution in methanol. Put in an oven at 115° for 30 min. Remove the plate and allow to cool.

(3) Diazotization. Spray with sodium nitrite dissolved in an acid medium. Then dry thoroughly with cold air.

(4) Coupling. Spray with N-(1-naphthyl)ethylenediamine solution in methanol. Then dry for 1 min under warm air. Slightly diffused, violet-rose spots appear.

Instrumental parameters

Apparatus: Zeiss PMQ II spectrophotodensitometer. Reading form: remission. λ_{max}: 510 nm. Slit: variable, according to concentration. Register speed: 50 mm/min. Scanning speed: 50 mm/min.

Calculations

The area unit means (Am) obtained on reading the spots in both directions (from the elution front and perpendicular to it) and integration of the respective chromatographic peaks are used for standards in order to draw the corresponding calibration straight line, for each plate. Usually [19—25], the $Am^2/100$ ratio and the quantity applied (μg) give the best coefficients of correlation and therefore are often used.

As readings are not referred to an absolute blank but to the plate blank, the straight lines do not pass through the origin. Nevertheless this is not important if working within the experimental range of the straight line and the line is not extrapolated. The obtained values, corrected with the dilution factors, will give the actual plasma concentration expressed in μg/ml.

RESULTS AND DISCUSSION

Extraction

The deproteinization of plasma has been attempted with the usual reagents, such as trichloroacetic acid and mixtures of sodium tungstate—maleic acid. In each case, although the precipitation is abundant, the precipitating agent reacts with Adamexina or, at best, secondary reactions take place with some of the other plasma components, giving a sequence of chromatographic spots which interfere with the estimation of the Adamexina.

Precipitation with organic solvents has been the only viable way tried with any success. With this in view, the most suitable solvent was chosen, through the calculation of the partition coefficients (Table I). Chloroform, because of the greater solubility of the drug at any pH, and diethyl ether, because of the slight solubility of the drug at acid pH, were the most advantageous solvents for the extraction and washing of the extracts, respectively.

Formation of derivatives

The procedures for obtaining derivatives in situ have been considered, and the following reactions appeared to be very sensitive:

$$\text{Adamexina} \xrightarrow{HNO_2} \text{Nitrous deriv.} \xrightarrow{\text{soft } H_2} \text{Hydrazine} \xrightarrow{\text{Fluorescamine}} \rightarrow \text{Fluorescent derivative [26]}$$

The reaction takes place, but it has little sensitivity. Detection limit is 250 ng/ml.

TABLE I

CALCULATION OF THE PARTITION COEFFICIENTS OF ADAMEXINA FOR DIFFERENT pH VALUES

For a concentration of 25 mg in 100 ml of a 10% hydroalcoholic solution. Extraction is made with equal volumes.

Solvent	pH = 5.7 (water)		K_p	pH = 8 (NH_4OH)		K_p	pH = 3 (acetic acid)		K_p
	Aqueous phase	Organic phase		Aqueous phase	Organic phase		Aqueous phase	Organic phase	
Chloroform	0.085	0.385	77	0.020	0.380	19	0.040	0.385	9.1
Carbon tetrachloride	0.155	0.200	1.3	0.175	0.290	1.7	0.255	0.100	0.4
Diethyl ether	0.150	0.170	1.1	0.185	0.130	0.7	0.250	0.060	0.3
Benzene	0.140	0.215	1.5	0.145	0.210	1.5	0.230	0.115	0.5
Methylene chloride	0.020	0.395	19.8	0.040	0.365	9.1	0.070	0.320	4.6

Adamexina $\xrightarrow{\text{Hydrolysis}}$ Aromatic primary amine $\begin{cases} \text{a) Diazotization and Coupling} \rightarrow \text{Coloured derivative} \\ \text{b) Fluorescamine} \rightarrow \text{Fluorescent derivative} \end{cases}$

There are some difficulties in effecting the initial hydrolysis in a quantitative manner. Recovery is 60–70%.

Adamexina $\xrightarrow{\text{Nitration}}$ Nitrated deriv. $\xrightarrow{\text{soft } H_2}$ Aromatic primary amine $\begin{cases} \text{a) Diazotization and coupling} \longrightarrow \text{Coloured derivative} \\ \text{b) Fluorescamine} \longrightarrow \text{Fluorescent derivative} \end{cases}$

(a) The formation of a coloured derivative, although requiring several successive treatments, does not present prohibitive complications. The optimal conditions have to be established by experiment, by trying different reagent concentrations, as well as times and temperatures for each of the steps of the reaction. In this respect, the colour of the background of the plate plays a decisive role. When the reaction rate is increased, especially the nitration and the reduction, the background colour rises rapidly.

Under the final conditions, the violet-rose colour of the spot, as well as the

TABLE II

EXAMPLES OF CALIBRATION STRAIGHT LINES FOR DIFFERENT CHROMATOPLATES

Plate	Quantity applied (μg)	$Am^2/100$
1*	1	100
		109
	0.5	52
		57
	0.1	11
		15
2**	1	105
		114
	0.5	58
		56
	0.1	18
		17
3***	2	424
		394
	1	181
		182
	0.5	56
		48

*$r = 0.99; y = 2.90 + 101.6x$.
**$r = 0.99; y = 6.75 + 102.3x$.
***$r = 0.99; y = 61.75 + 236.5x$.

yellowish white hue of the background of the plate remain stabilized for at least 5 h; this time can be increased up to 24 h by keeping the plate protected from light. The sensitivity limit, in this case, is 50 ng, which is sufficient for our purpose. The reaction can also be carried out with Bromexine or with compounds having similar structures, although in this specific case the sensitivity is only 250 ng, a limit which can doubtlessly be improved by a specific study of every step of the reaction.

The response of the blank plasma is nil in human plasma and in rat plasma when it is fresh, and there appears only a very slight spot at the same R_F as Adamexina when these plasma samples have been kept for some days in a freezer; but even at worst the interference would only represent 20 ng, that is to say, out of our sensitivity limits.

(b) Owing to the positive evolution of the above test, it has not been deemed necessary to consider this possibility.

Calibration curve

Due to the multiple factors involving the chromatographic process, especially in the development step, it is necessary to draw a calibration straight line independently for each chromatoplate in order to attain a fair adjustment. Several examples are shown in Table II.

TABLE III

VALUES OF THE PERCENTAGES OF RECOVERY IN PLASMA

The range of concentration was 0.5—4 μg/ml equivalent to 0.25—2 μg applied onto the plate. Mean percentage = 82.4%; S.D. = 6.78; n = 20; variation coefficient (P = 95%) = 3.2%. Straight line parameters: $r = 0.9902$; $y = 0.0517 + 0.7421x$.

Quantity applied (μg)	Recovery (%)
2	75
	85
	70
	70
	83
1	78
	75
	80
	85
0.5	85
	92
	85
	85
	78
	80
	92
	88
0.25	90
	92
	80

Recovery and confidence limits

The mean percentage of recovery in plasma is 82.4% with a range of concentrations between 0.25 and 2 μg applied on the plate.

The statistical data needed to establish the precision of the method and to calculate the corresponding calibration straight line are given in Table III.

In conclusion, the method is suitable for the estimation of levels of Adamexina and of other 2-amino-3,5-dibromobenzyl derivatives in plasma; the pharmacokinetics of such drugs in man or in some animal species, as well as the bioavailability of the various pharmaceutical preparations, will be studied further.

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Biomedical Applications

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Note

Quantitative Bestimmung von Diclofenac-Natrium aus Plasma durch Absorptionsmessung mit Hilfe der direkten Auswertung von Dünnschichtchromatogrammen*

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(Eingegangen am 21. September 1979)

Diclofenac-Natrium (Voltaren®), das Natriumsalz der *o*-(2,6-Dichlorphenyl)-aminophenylessigsäure, hat in letzter Zeit grosse Bedeutung in der Rheumatherapie erlangt.

In der Literatur wurden bisher drei Verfahren zur quantitativen Bestimmung dieses Wirkstoffes in biologischem Material beschrieben. Stierlin und Mitarbeiter [1—4] führten die Messungen mit radioaktiv markierter Substanz durch. Da dabei jedoch auch radioaktiv markierte Abbauprodukte mitbestimmt werden und bei pharmakokinetischen Untersuchungen in grösserem Rahmen eine quantitative Erfassung nach Einnahme des handelsüblichen Präparates erforderlich ist, eignet sich dieses Verfahren nicht für Reihenuntersuchungen.

Geiger et al. [5] entwickelten 1975 ein empfindliches gaschromatographisches Verfahren, das später von mehreren Arbeitsgruppen [1, 2, 4, 6, 7] angewandt wurde. Der Nachteil der Methode besteht in einer komplizierten, langwierigen 4-stufigen Extraktion und Derivatisierung der Substanz vor ihrer Bestimmung.

Von Brombacher et al. [8] wurde 1977 ein vereinfachtes Extraktionsverfahren für die Gaschromatographie angegeben; insgesamt bleibt aber die Methode wegen der erforderlichen Derivatisierung noch immer arbeits- und zeitaufwendig. Als Nachweisgrenze werden 100 ng/ml angegeben.

Wir versuchten daher, eine dünnschichtchromatographische Methode zu entwickeln, die bei guter Spezifität und Genauigkeit eine rasche und einfache Erfassung von Diclofenac aus biologischem Material ermöglicht.

*Teilergebnisse der Dissertation A. Schumacher, in Vorbereitung.
**An den die Korrespondenz zu richten ist.

METHODIK

1.0 ml Plasma wird mit 0.1 ml 3 *N* HCl angesäuert und in eine Spritze mit Luerlock-Anschluss aufgezogen. Die Spritze wird anschliessend auf eine SEP-PAK C_{18}-Kartusche (Waters Assoc., Königstein, B.R.D.) aufgesteckt und das Plasma durch die Kartusche langsam hindurchgedrückt. Mit 5.0 ml Wasser werden hydrophile und anschliessend mit einer Mischung von Äthanol—Wasser (35:65, v/v) lipophilere Plasmabestandteile ausgewaschen. Mit 2.0 ml Methanol p.A. wird anschliessend die Substanz aus der Kartusche eluiert, wobei die ersten 0.4 ml, die als Mischfraktion noch keine Substanz enthalten, verworfen werden.

Die Methanolfraktion wird bei 80° unter Einblasen von Stickstoff zur Trockne eingeengt, der Rückstand wird anschliessend in 100 µl Essigsäureäthylester aufgenommen. Davon werden 40 µl strichförmig mit einem Linomaten III (Camag, Muttenz, Schweiz) auf HPTLC-Fertigplatten Kieselgel-60 F_{254} mit Konzentrierungszone (Merck, Darmstadt, B.R.D.) 10 × 20 cm aufgetragen. Die Strichbreite beträgt 5 mm. Bei einem 1-cm-Abstand der Punkte voneinander können 15 Proben und drei Standards auf eine Platte aufgetragen werden.

Standards werden durch Zusatz von Diclofenac-Natrium zu gepooltem Plasma hergestellt. Dazu werden 10.0 mg Substanz in 100.0 ml Aceton gelöst und 0.8 ml dieser Lösung nochmals auf 10.0 ml verdünnt, entsprechend einer Konzentration von 80.0 µg Diclofenac-Natrium pro 10.0 ml Aceton. 5.0 ml dieser Lösung werden durch Einblasen von Stickstoff bis zur Trockne eingeengt, dann werden 50.0 ml gepooltes Plasma zugegeben. Dadurch ergibt sich eine Konzentration von 800.0 ng Diclofenac-Natrium pro 1.0 ml Plasma.

Nach dem Trocknen der Proben auf der Platte erfolgt die Chromatographie in dem Fliessmittel Dichlormethan—Methanol—Tetrahydrofuran (85:15:0.5), wobei die Substanz bei einer Laufstrecke von 7 cm einen hR_F-Wert von 64 besitzt.

Nach der Chromatographie wird die Platte für 10—15 min unter einer UV-Lampe (254 nm) zur Minderung der Untergrundabsorption der Platte bestrahlt und anschliessend direkt mit dem Chromatogramm-Spektralphotometer

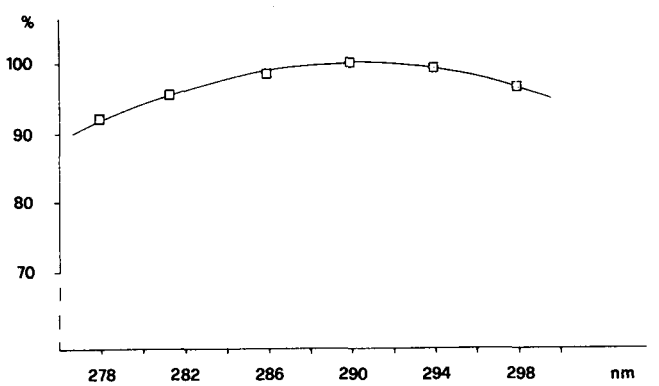

Fig. 1. Absorptionsspektrum in Remission von Diclofenac nach Chromatographie auf HPTLC-Fertigplatten Kieselgel-60 F_{254} mit Konzentrierungszone (Merck) 10 × 20 cm mit Dichlormethan—Methanol—Tetrahydrofuran (85:15:0.5).

KM 3 der Firma Zeiss ausgewertet. Gemessen wird im Absorptionsmaximum auf der Platte von 290 nm (Fig. 1) unter Verwendung einer Deuteriumlampe. Messanordnung: Remission Monochromator-Probe; Spaltgrösse: 1 × 3.5 mm.

Die Absorptions-Ortskurven werden durch einen Perkin-Elmer-Recorder 56 aufgezeichnet, wobei die Eingangsspannung am Schreiber entsprechend der Konzentration pro Fleck von 1 bis 0.2 V verändert werden kann. Die Tischgeschwindigkeit beträgt 20 mm/min, der Schreibervorschub 120 mm/min. Die Auswertung erfolgt über die Flächen unter den Absorptions—Ortskurven.

Zur Bestimmung der Linearität zwischen den Flächen unter den Absorptions—Ortskurven und den aufgetragenen Substanzmengen wurden Testplasmen mit 125.0, 250.0, 500.0, 1000.0, 1500.0, 2000.0 und 2500.0 ng Diclofenac-Natrium pro ml verwendet. Zur Ermittlung der Präzision des Verfahrens wurden, entsprechend der nach der therapeutischen Dosis von 50 mg im biologischen Material zu erwartenden Konzentrationen, pro ml Testplasma 1500.0, 750.0 und 200.0 ng Diclofenac-Natrium zugesetzt.

Die Untersuchungen auf Richtigkeit des Verfahrens wurden mit Lösungen von Diclofenac in Aceton durchgeführt.

ERGEBNISSE UND DISKUSSION

Die Eichkurven gehen durch den 0-Punkt. Linearität zwischen den Flächen unter den Absorptions—Ortskurven und den aufgetragenen Substanzmengen besteht von 0 bis 800 ng Diclofenac pro Fleck (≙ 0—2.0 µg Diclofenac-Natrium pro 1.0 ml Plasma). Bestimmbar sind Mengen < 100 ng pro ml Plasma.

Der mittlere lineare Regressionskoeffizient beträgt 0.996. Zur Erstellung der Eichgeraden genügt daher jeweils ein einziger Kurvenpunkt, der zur Erhöhung der Genauigkeit aus drei Messwerten ermittelt wird.

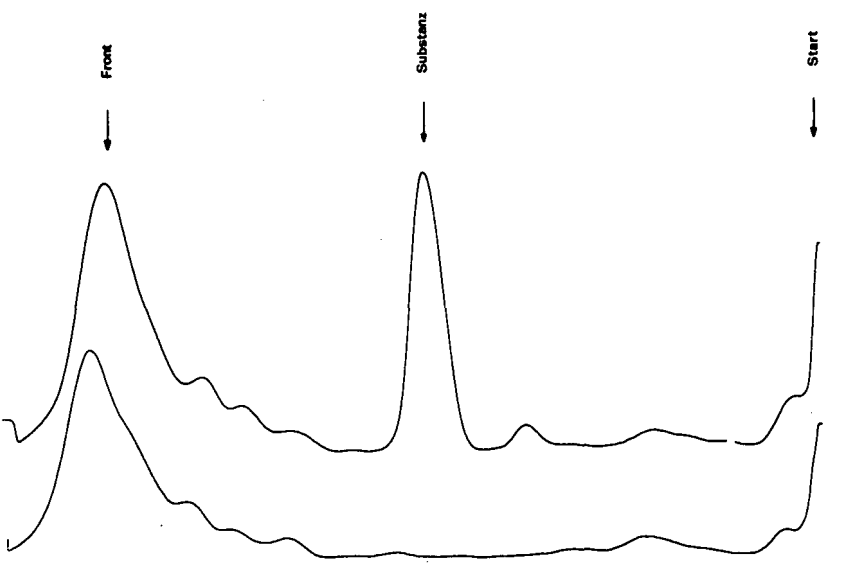

Fig. 2. Absorptions—Ortskurven der Chromatogramme eines Plasmas ohne Diclofenac-Natrium und eines Probanden-Plasmas nach oraler Gabe von 50 mg Voltaren® zur Zeit des maximalen Blutspiegels.

Bei einer Konzentration von 1500.0 ng Diclofenac-Natrium pro ml Testplasma betrug die relative Standardabweichung 3.5%, bei 750.0 ng 4% und bei 200.0 ng 6% ($N = 8$).

Die Wiederfindungsraten betrugen, unabhängig von den Konzentrationen der Proben an Diclofenac-Natrium, 75%.

Zur Überprüfung der Methode wurde Versuchspersonen eine einmalige Dosis von 50 mg Diclofenac-Natrium oral verabreicht. Die Ergebnisse stimmen weitgehend mit den durch gaschromatographische Analysen enthaltenen Daten überein. In Fig. 2 ist die Absorptions—Ortskurve eines Chromatogramms eines Probanden-Plasmas zur Zeit des maximalen Blutspiegels dargestellt und als Vergleich dazu die Absorptions—Ortskurve des Leerwertes, der vor Einnahme der Substanz abgenommen wurde.

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Journal of Chromatography, 181 (1980) 516—517
Biomedical Applications

CHROMBIO. 463

Book Review

Biological/biomedical applications of liquid chromatography, edited by G.L. Hawk, Marcel Dekker, New York, 1979, XVII + 736 pp., ISBN 0-8247-6784-5, price US$ 45.00.

The general interest in biomedical applications of chromatography results in organizing many symposia devoted to this topic. Some of these meetings that have been held over the last few years, were later published in book form. The first monograph "High Pressure Liquid Chromatography in Clinical Chemistry", edited by P.F. Dixon et al. that appeared in 1976 is now balanced by its American counterpart "Biological/Biomedical Applications of Liquid Chromatography", edited by G.L. Hawk. This book represents 36 selected papers from the symposium held in Boston, October 13—14, 1978. Similarly to numerous other books, the misleading title of liquid chromatography is used though the topic discussed is solely liquid column chromatography.

Published papers can be categorized into three groups of about equal size:

(1) Biomedical applications (referring to papers about lipids, prostaglandins, porphyrins, steroids, inhibitors of hormone activity, nucleotides, catecholamines, vitamin D and haemoglobins).

(2) Diverse biological and biochemical applications (such as fatty acids, mycolic acids, peptides, structure of proteins, kinetics of enzymatic reactions, nucleic acids, pteridines and nitroso compounds).

(3) Drug monitoring and pharmacokinetic studies (including anticancer compounds, antibiotics, radiopharmaceuticals, procainamide, xanthine alkaloids and anticonvulsants).

In between these mainly primary papers, there are four reviews scattered throughout this book. The volume is introduced by J.L. Waters' "Liquid chromatography, past, present and future". Especially in an introductory chapter devoted to the history of chromatography, there should not have been such errors as dating the discovery of paper chromatography in 1952 (instead of 1943), partition chromatography in 1942 (instead of 1941), etc. The discovery of thin-layer chromatography is usually ascribed to Ismailov and Shraiber. If the author is of another opinion, he should prove this by adding an appropriate reference. Two other reviews are devoted to drug monitoring. The first of these is an overview by Pippenger (with 13 references). The second is that of Haggerty where the reader can find a small chapter about profiling body fluids (though it is not dealt with profiling in the very

sense of the word). Prough and Dunn present a review about the application of high-performance liquid chromatography in following various metabolic reactions.

Any publication which, at the beginning of the rapid development in a certain area, shows the possibility of the application of a new technique, is certainly welcomed and adds to the progress of the area in question. It is, however, impossible to omit some of the drawbacks of the Hawk's book which, in general, one can find in any symposia proceedings: besides the good and very good papers, there were other papers that were much lower in quality. The main drawback in comparison with papers published in journals is in the lack of the experimental details. The published papers are not arranged according to the topics and the style, including literature data, is not quite uniform. Finally, the gap between the symposium meeting and the appearance of the Proceedings is quite long. On the other hand, a meticulously worked out subject and author index makes the book reasonably organized. The price of the book is adequate to the size.

Prague (Czechoslovakia) K. MACEK

Author Index

Subject Index

Vol. 181, No. 3–4

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

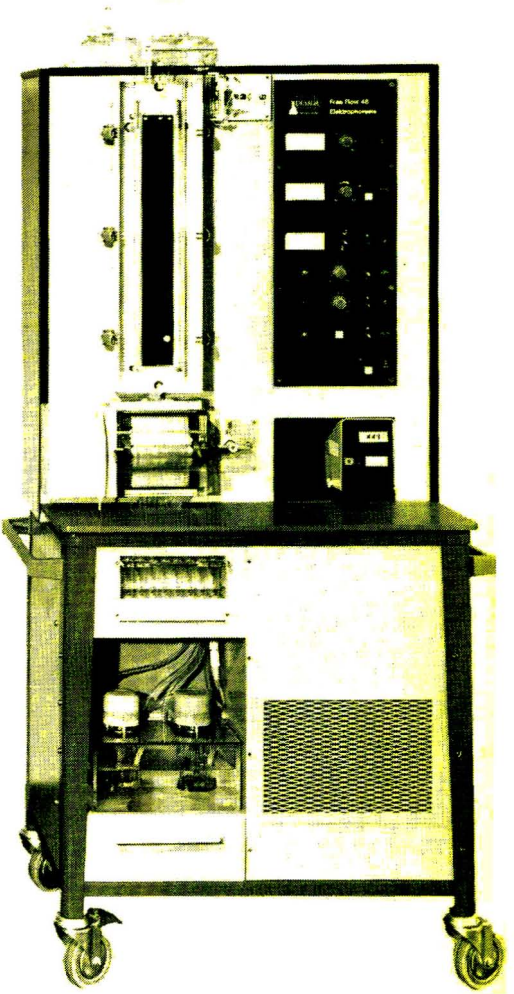
NEWS SECTION

APPARATUS

N-1340

ELECTROPHORETIC SEPARATOR

Desaga have introduced their Model FF-48 preparative scale free-flow electrophoretic separator for use in many areas of biological research. The FF-48 can be used for isolation and separation of leucocytes, separation and characterization of immunocompetent cells, clarification of immunological processes on a cellular level, enrichment and isolation of cell organelles, isolation of enzymes, selection of mutants, and experiments with bacteria, proteins, proteides and mucopolysaccharides. The separating chamber of the FF-48 consists of two glass plates mounted parallel to each other at a distance of 0.5 mm. The sample is continuously injected into a buffer film which is drawn through the chamber by a 48-channel pump that causes the fractions to be collected in 48 test-tubes. The total width of the 0.5 mm thick buffer film is 70 mm. The FF-48 is uniformly cooled and the built-in d.c. power supply has ratings of 1200 V, 250 mA and a maximum output of 150 W.

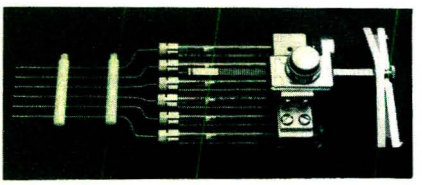


For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-135

CONVERSION KIT FOR DISPENSERS

Laboratories using the Hamilton Terasaki dispensers containing six fitted syringes for precision multiple sample dispensing can convert to removable needles and interchangeable syringe parts with a kit from the Hamilton Company. The conversion kit contains all components necessary to convert the dispenser, and is available in a choice of five dispensing volumes ranging from 0.5 to 10 µl. The adaptation of the Terasaki dispenser can be done in the laboratory.



N-1349

IMPROVED TUBE GEL CHAMBER

A redesigned tube gel chamber for electrophoresis has been introduced by the Research Products Division of Miles Laboratories. The new Canalco Tube Gel Chamber is suitable for electrophoresis in uniform or gradient gels, isoelectric focusing, and isotachophoresis. The tube gel chamber has an 18-tube capacity, accommodates 5-mm and 6-mm gels, and has an adjustable-height upper buffer vessel. At maximum height, the upper vessel accepts 200-mm gel tubes. The lower vessel of the new chamber is a glass water jacket that offers more than 1000 cm^2 of cooling surface. Except for the borosilicate glass water jacket and the platinum electrodes, the new Canalco chamber is made of acrylic plastic.

N-1354

MICROLAB M

The Microlab M from Hamilton is a versatile instrument designed for dispensing and dilution applications in the laboratory. The unit incorporates a stepper motor with a synchronized motor-driven valve block and is computer compatible via a RS 232 C interface. The Microlab M can accommodate all standard Hamilton syringes from 50-µl to 25-ml capacity. The instrument is controlled by an external control unit with a keyboard and display. A total of 99 programs including 384 individual volume steps or parenthesis functions can be stored. The Microlab M can be used as a reagent diluter, a reagent dispenser or as a transfer pipette in radioimmunoassay applications, enzymology, haematology, serology and other fields.

N-1347

BRINKMANN PRODUCTS FOR BIO-CHEMICAL RESEARCH & CONTROL

The 1979 catalog "Brinkmann Products for Bio-Chemical Research & Control" gives a complete survey of the various fields of chemical analysis in which the company is active. The first 10 pages of the catalog give complete information on the Brinkmann products for TLC, HPLC and isoelectric focusing and electrophoresis systems. Other parts of the catalog describe fraction collectors and sample concentrators.

CHEMICALS

N-1378

BIO-RAD CATALOG

Many tools for electrophoresis, chromatography and immunochemistry are featured in Bio-Rad Laboratories' catalog of materials, equipment and systems for analysis of biomolecules. New in this catalog are complete systems for electrofocusing, DNA sequencing techniques with the "Super Slab" electrophoresis cell, new columns for HPLC, and a system for cell surface labeling with solid-phase antibodies. The 140-page catalog is packed with applications data, product specifications, ordering information and tips on how to use the techniques to best advantage.

N-1385

BIO-RADIATIONS 30

New materials, equipment and techniques for chromatography, electrophoresis and cell culture are introduced in the 30th issue of Bio-Radiations from Bio-Rad Labs. In this issue a new analytical electrofocusing system is described. This system adds considerably to the power and the versatility of this protein separation technique. Further, there is an article on five ways to take advantage of hydroxylapatite chromatography for separating and concentrating proteins. Finally, a low-cost HPLC reversed-phase column is described.

N-1345

LABORATORY CATALOGUE 1979

800 PPC is the reference on Whatman LabSales' new 19-page catalogue of products for laboratory filtration and paper chromatography. In addition the catalogue describes extraction thimbles, pH indicator papers, lens cleaning tissue and disposable phase separators.

N-1377

AFFINITY CHROMATOGRAPHY GUIDE AND SEPARATION NEWS

"Affinity Chromatography, guide to methods and applications" is a brochure from Pharmacia Fine Chemicals. The brochure contains an introduction to the separation technique itself and gives a systematic approach to the choice of the systems for an actual analysis.

"Separation News" is a periodical available from the same manufacturer on separation techniques in the field of the life sciences. The fourth 1979 issue features articles on gel filtration work, hydrophobic interaction chromatography, practical notes on immunoelectrophoresis, and abstracts from leading articles in the field of the life sciences.

N-1369

AFFINITY CHROMATOGRAPHY PRODUCT

Pierce Chemical Company announces the introduction of Immobilized Lima Bean Trypsin Inhibitor (LTI), a powerful tool in removal and purification of trypsin, chymotrypsin and elastase. The immobilized preparation contains 4 mg of LTI per ml of settled gel. Immobilized LTI binds 2–3 mg of trypsin and 3–4 mg of chymotrypsin per ml of settled gel and can be regenerated by washing with 0.1 *M* acetic acid, pH 3.0.

N-1343

IMMOBILIZED SUGARS

A new line of affinity adsorbents for isolation and purification of lectins, carbohydrate binding proteins, toxins and glycosidases are now available from the Pierce Chemical Company. The adsorbents, Selectins, are currently offered as immobilized N-acetyl-D-glucosamine, lactose, maltose, melibiose, N-acetyl-D-galactosamine, D-galactosamine, L-fucose and D-mannose. The Selectins from Pierce are referenced with preparative applications, binding capacities and ligand-leash structure. Each Selectin is supplied as a 50% aqueous slurry.

PROCEDURES

N-1387

HPLC NEWSLETTER

In the "Liquid and Thin Layer Chromatography Notes", available from the Schoeffel Instruments Division of Kratos, Inc., HPLC applications are described. The first (biomedical) issue gives details on two applications in the biomedical field. The first report deals with the use of stopped-flow excitation wavelength scanning in the fluorescence analysis of catecholamines. The second report describes the separation and UV detection of amino acids and peptides at 200 nm. Other features included in the newsletter are a bibliography update section which provides several references of interest to the biomedical/biochemical chromatographer; and an instrument corner which details in brief three new instrumentation developments by the company.

N-1331

SEPARATION NEWS 1979, No. 2

The second 1979 issue of the Pharmacia journal "Separation News" contains articles about special gels for difficult separations in the gel filtration technique, about the temperature conditions for isoelectric focusing, and about cell separation by affinity chromatography. Furthermore the journal gives a book review and a series of abstracts of important papers in the field of biology and biochemistry.

N-1355

SEPARATION NEWS 1979, No. 3

The third 1979 issue of the Pharmacia journal "Separation News" contains articles about procedures for separation of some plasma proteins and membrane glycoproteins by affinity chromatography; methods for using immunoadsorbents. Furthermore the journal gives a series of abstracts of important papers in the field of immunochemistry, clinical chemistry, biology and biochemistry.

NEW BOOKS

Protides of the biological fluids, Colloquium 27, edited by H. Peeters, Pergamon, Oxford, Elmsford, N.Y., 1979, 880 pp., 471 figs., price US$ 96.00, £ 44.00, ISBN 0-08-024933-7.

Detection and measurement of free Ca^{2+} in cells, edited by C.C. Ashley and A.K. Campbell, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, IX + 461 pp., price Dfl. 135.00, US$ 65.75, ISBN 0-444-80185-5.

Butterworths Medical Dictionary, by M. Critchley, Butterworths, Sevenoaks, 2nd ed., 1980, 1974 pp., price £ 15.00, US$ 33.75, ISBN 0-407-00193-X.

Medical microbiological techniques, by F.J. Baker and M.R. Breach, Butterworths, Sevenoaks, 1980, *ca.* 400 pp., price *ca.* £ 14.00, *ca.* US$ 31.50, ISBN 0-407-00099-2.

Fundamentals of enzyme kinetics, by A. Cornish-Bowden, Butterworths, Sevenoaks, 1979, 244 pp., price £ 8.50, US$ 19.25, ISBN 0-408-10617-4.

Analytical procedures for therapeutic drug monitoring and emergency toxicology, by R.C. Baselt, Wiley, Chichester, New York, 1980, *ca.* 350 pp., price *ca.* US$ 35.00, £ 21.40.

Biological/biomedical applications of liquid chromatography II, edited by G.L. Hawk, Marcel Dekker, New York, Basel, 1979, XIII + 504 pp., price SFr. 100.00, ISBN 0-8247-6915-5.

Recent developments in mass spectrometry in biochemistry and medicine, Vol. 2, edited by A. Frigerio, Plenum, New York, London, 1979, X + 492 pp., price US$ 45.00, ISBN 0-306-40294-7.

Chromium in nutrition and metabolism, edited by D. Shapcott and J. Hubert, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, 1979, VIII + 264 pp., price Dfl. 90.00, US$ 44.00, ISBN 0-444-80188-X.

GENERAL INFORMATION

(A leaflet *Instructions to Authors* can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For reviews, see page 2 of cover under Submission of Papers.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.*, Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc. should be on separate sheets.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet*. If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts*. Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

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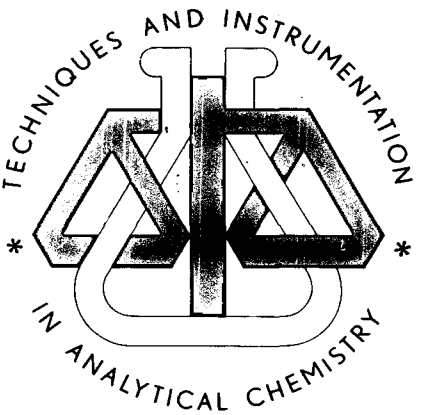
Evaluation and Optimization of Laboratory Methods and Analytical Procedures



A Survey of Statistical and Mathematical Techniques

D.L. MASSART, A. DIJKSTRA *and* L. KAUFMAN.

with contributions by S. Wold, B. Vandeginste *and* Y. Michotte

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This book provides detailed treatment, in a single volume, of formal methods for optimization in analytical chemistry. It is a comprehensive and practical handbook which no analytical laboratory will want to be without.

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