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75 Years of Chromatography

A Historical Dialogue

L. S. ETTRE *and* A. ZLATKIS (Editors).

Journal of Chromatography Library - Volume 17

On the occasion of the 75th anniversary of the invention of chromatography, this book compiles the personal stories of 59 pioneers of the various chromatographic techniques (including five Nobel Prize laureates). In their contributions to this volume, these pioneers review the events which influenced them to enter the field; explain the background of their inventions; summarize their activities and results during their professional lives; and discuss their interactions with other scientists and other disciplines.

This book is more than a nostalgic recollection of the past for those who have been in chromatography for some time. It also provides, for the younger generation of chromatographers, a unique record of how present-day knowledge was accumulated.
The final chapter is devoted to "Those who are no longer with us".

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(*Nobel Prize laureates)

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CHROMBIO. 372

PROFILING OF UREMIC SERUM BY HIGH-RESOLUTION GAS CHROMATOGRAPHY—ELECTRON-IMPACT, CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A fast and reliable procedure for gas chromatographic profiling of components in ultrafiltrated uremic serum has been developed, using glass capillary columns. Sample pretreatment consists of ultrafiltration, evaporation and silylation. Some twenty components are identified by electron-impact and chemical ionization mass spectrometry. A comparison is made between profiles of sera from a series of uremic patients, before and after hemodialysis, and from non-uremic sera. Significant differences are found between these profiles. A "dialysis ratio" is introduced as a parameter for the removal of retained components by hemodialysis treatment.

INTRODUCTION

Patients with endstage renal failure have to be submitted to regular treatment with an artificial "kidney". These patients show a complex of clinical symptoms, usually called "the uremic syndrome", or "uremia". Many of these symptoms are related to a disturbance in the homeostatic or regenerative function of the kidney, which results in retention of metabolic products and in disorders of hormonal and metabolic function.

There are indications that retained components can act as cell toxins or as inhibitors of enzyme action. The identity of these components is still subject to discussion. In recent years several authors have mentioned the importance of compounds of medium molecular weight [1—3].

Others re-emphasized the role of components of lower molecular weight, such as methylguanidine, guanidino acids, amines, phenolic acids, polyols, inositol and other compounds [4—9]. Although experiments were carried out to test the various hypotheses, no definitive conclusions could be drawn [10].

Therefore it is difficult to improve "artificial kidney" strategies and equipment in a planned and efficient way. For this purpose, it is necessary to develop analytical techniques which give data on the effectiveness of the treatment. Moreover, analytical information can lead to clinical tests on the toxicological behaviour of certain compounds, and could contribute to a better understanding of biochemical and physiological processes in uremia. Several profiling techniques have been applied in the analysis of body fluids of uremic patients. These profiling techniques give information for a whole range of compounds. Dzúrik et al. [11], Chang [12], Gordon et al. [3], Cueille [13] and others tried to characterize uremic plasma by gel permeation chromatography, especially with reference to "middle molecules". Senftleber et al. [14] and Veening [15] analysed serum and hemodialysis fluid with reversed-phase liquid chromatography. Mikkers et al. [16] reported on an isotachophoretic profiling technique which gives information on ionic compounds with low as well as high molecular weight. Masimore et al. [17] examined volatile components in hemodialysis fluid by gas chromatography—mass spectrometry (GC—MS) using packed columns. Bultitude and Newham [18] applied the same technique in the analysis of uremic serum, also using packed columns. In the latter, a laborious and time-consuming sample pretreatment procedure of several days, including fractionation by gel permeation chromatography and freeze-drying, was used. In this report a reliable GC-profiling technique, using glass capillary columns, and a fast pretreatment procedure is described.

EXPERIMENTAL

Samples, reagents and materials

Blood samples of ten uremic patients, before and after hemodialysis on polyacrylonitrile RP6 (Rhone-Poulenc, Paris, France) and cuprophane GM (Gambro-Major, Lund, Sweden) membranes, were obtained from the Nephrological Division of the University Hospital of Ghent (Belgium). After centrifugation, serum samples were stored at −18° until used. A pool of serum from non-uremic persons was prepared. Removal of high molecular weight substances was carried out by ultrafiltration on Amicon (Lexington, Mass., U.S.A.) XM 50 membranes. Chemical derivatization was performed in borosilicate reaction vessels (Hewlett-Packard, Avondale, Pa., U.S.A.) with bistrimethylsilyltrifluoroacetamide (BSTFA) from Pierce (Rockford, Ill., U.S.A.). Straight-chain C13- and C22-hydrocarbons [(from Phillips Petroleum (Bartlesville, Okla., U.S.A.) and Applied Science Labs. (State College, Pa., U.S.A.)] were used as internal standards. A standard solution was prepared by dissolving 21 mg of C13 and 4.9 mg of C22 in 50 ml *n*-hexane. Reagent gas for chemical ionization (CI) was isobutane (CH 35) from l'Air Liquide (Paris, France).

Apparatus

Pressure-ultrafiltration was carried out in a microcell [19]. For GC separations a Perkin-Elmer F-30 instrument was used. The standard sample introduction system was replaced by a moving-needle injector [20]. Average carrier gas velocity (helium) was 28 cm/sec. An oven temperature programme was used starting with an isothermal period of 2 min at 110°, an increase of 5°/min to 200° and remaining isothermic at 200 ° for 35 min. Injection and detection temperatures were maintained at 250°. Glass capillary columns (47 m), deactivated [21] with Carbowax 20M and coated with SE-30 (layer thickness 0.2 μm) were prepared by the static coating procedure of Rutten and Rijks [22]. The flame ionization detector signal was recorded on two traces because of large concentration differences for different compounds (2 mV and 50 mV full scale corresponding to $4 \cdot 10^{-12}$ A and $2 \cdot 10^{-10}$ A respectively). Mass spectra were obtained with a 4000 GC—MS system from Finnigan (Sunnyvale, Calif., U.S.A.) coupled to a D 116E minicomputer (Digital Computer Controls). A platinum—iridium capillary was used as a GC—MS interface. In MS analysis the electron energy was 70 eV in both the electron-impact (EI) and the CI mode. Source temperature was 250° under EI and 220° under CI, sensitivity 10^{-8} and 10^{-9} A/V, multiplier voltage 1675 V, and reagent gas pressure 13 Pa (0.1 Torr.) in chemical ionization.

Procedure

Serum samples are pressure-ultrafiltrated under nitrogen to remove high molecular weight components such as proteins (cut-off at 50,000). Aliquots of 250 μl of the ultrafiltrated material are evaporated to dryness under a nitrogen stream in a sandbath at 70°. The dried samples are derivatized to enable GC separation and are allowed to react with 250 μl of BSTFA reagent at 80° for 2 h. After dilution with 250 μl *n*-hexane, aliquots of 50 μl of the standard solution (C13 and C22 in hexane) are added.

Samples of 0.5 μl are applied to the tip of the moving needle. After 90 sec, during which solvent, volatile reaction products and unreacted BSTFA are allowed to evaporate, the sample is injected.

RESULTS AND DISCUSSION

Reliability of the method

As a retention parameter the relative retention is used. In our experiments this parameter appeared to be more reliable than the retention index. The reproducibility of the temperature programme was tested by injecting the same sample six times within a short period. Five peaks (peak numbers 35, 49, 67, 75, 78 in Fig. 1) throughout the whole temperature range show coefficients of variation for the relative retention (with respect to C22) which are less than 0.4%. This demonstrates that the reproducibility of the temperature programme is good.

The same peaks were tested in chromatograms that were recorded in the course of two months. Coefficients of variation between 0.2 and 2% for different peaks represent long-term changes in column performance and carrier gas flow. This result is satisfactory, however, the maximum value of 2% can

cause difficulties in distinguishing one peak from another for certain components (e.g. peaks 34 and 35 in Fig. 1).

Peak height is used as a quantitative measure. For near-baseline peaks or unresolved peaks this gives better results than peak area measurement. Reproducibility of the normalized peak heights, from four injections, is measured for 15 major peaks in the chromatograms. The coefficients of variation of different peaks were between 2 and 9%. The influence of the sample pretreatment on peak height variation was studied by derivatizing an ultrafiltrated serum sample four times. Each sample was then injected four times. A Student's *t*-test (on the mean) and a *F*-test (on variance ratio) were applied to peak heights "within and between" samples. This led to the conclusion that variance due to sample pretreatment does not differ from variance from the analysis step (95% probability level).

Application to a series of uremic patients

Predialysis and postdialysis serum samples were submitted to the described procedure. Typical gas chromatograms are shown in Fig. 1. Differences in

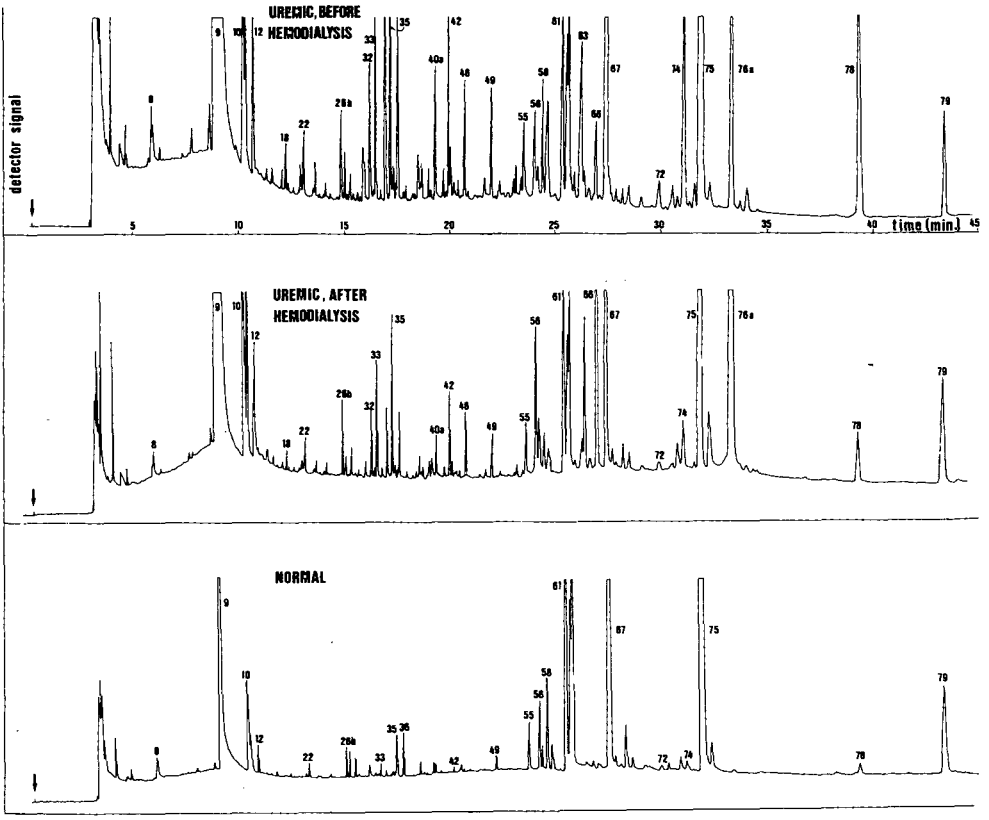

Fig. 1. Gas chromatographic profiles of ultrafiltrated serum from an uremic patient, before and after hemodialysis treatment, and from a pool of non-uremic sera. Glass capillary column coated with SE-30. The 2-mV trace corresponding to a signal of $4 \cdot 10^{-12}$ A f.s.d is shown.

TABLE I

CLASSIFICATION ACCORDING TO DIALYSIS RATIO

Group	$\overline{D}_x$	Number of peaks	Major GC peaks
1	$0.6 \leqslant D < 1.4$	8	22, 27, 28, 67, 75
2	$D \geqslant 1.4$	36	18, 24, 26, 26b, 30, 32, 33, 34, 35, 36, 40a, 42, 43, 46, 49, 53, 54, 54a, 58, 62a, 72, 73, 74, 78
3	$D < 0.6$	1	56
4	variating	13	23, 44, 55, 61, 62, 63, 66, 76a

concentrations of various components are very obvious. All ten patients showed similar profiles, however, individual deviations, both qualitative and quantitative, did occur. For quantification of the effect of hemodialysis treatment a "dialysis ratio" (D) is defined in the following way:

$$D_x = \frac{H'_{x,\ \text{before}}}{H'_{x,\ \text{after}}}$$

where H'_x is the normalized peak height for component x.

This dialysis ratio (D) was determined for some 70 peaks in the chromatograms of the ten patients. From these data an average D ($\overline{D}$) was calculated for each compound. Then the components were classified into four groups, according to their $\overline{D}$. The maximal error in D can be calculated from that in peak height. A maximal variation coefficient of 9% in peak height was found. This leads to a maximum value of approximately 20% for the variation coefficient (V.C.) of D. On this basis all components with D-values between 1—0.4 and 1+0.4 were considered to be unaffected by hemodialysis treatment ($D \pm 2$ V.C.). These components are classified in group 1 (see Table I). Group 2 represents components that show a decreased concentration as a result of dialysis treatment ($D \geqslant 1.4$). Only very few components showed higher concentrations after hemodialysis, and are placed in group 3 ($D < 0.6$). Group 4 represents components that show a great variation in D in the samples of different patients. The table demonstrates that group 2 components are very well removed during dialysis, and that concentrations of group 3 components appear to be raised as a result of the treatment. From peak heights and D-values for samples of different patients it is concluded that the higher the concentrations, the higher the dialysis ratio. The ten patients show substantial differences in "overall" concentrations. Some of them have postdialysis profiles that approach those for non-uremic sera.

The underlined peak numbers in Table I refer to compounds that were identified by GC—MS and from GC retention data. Table II lists these compounds.

TABLE II

IDENTIFIED COMPONENTS IN UREMIC SERUM

Peak number	Relative retention	Compound*	Dialysis ratio ($\bar{D}$)	Group
9	0.153	urea	1.40	2
10	0.178	phosphoric acid	2.40	2
11	0.183	glycerol	—	—
18	0.228	tartronic acid (tent.)	2.71	2
22	0.251	threonine	1.05	1
26b	0.296	homoserine (tent.)	1.47	2
30	0.322	Δ-pyrrolidone-5-carboxylic acid	2.70	2
32	0.327	threitol (tent.)	2.30	2
33	0.336	erythritol	2.10	2
35	0.352	erythronic acid	3.27	2
40a	0.403	tartaric acid	3.42	2
42	0.417	2-deoxy-erythropentonic acid	1.96	2
49	0.469	arabinitol	2.93	2
54/54a	0.498/0.504	hydroxy or dicarboxylic acids	2.98/3.24	2/2
55	0.507	arabinonic acid	—	4
56	0.520	citric acid	0.57	3
58	0.530	fructose	1.61	2
61	0.553	galactose	—	4
63	0.574	3-deoxy-arabinohexonic acid (tent.)	—	4
66	0.591	glucono-1,4-lactone	—	4
67	0.605	α-D-glucose	1.25	1
72/73	0.665/0.682	mannitol and/or glucitol	6.09/3.72	2/2
74	0.695	isomer of myo-inositol (tent.)	3.52	2
75	0.716	β-D-glucose	1.28	1
76a	0.751	mannonic or gluconic acid	—	4
78	0.900	myo-inositol	7.00	2

*Tent. = tentatively identified.

Identification by mass spectrometry

Many carbohydrate-related trimethylsilyl derivatives have EI mass spectra that look very similar. Although different classes of these compounds (e.g. aldoses, aldonic acids and polyols) demonstrate some characteristic fragment or rearrangement ions, no molecular ions are found [23—26]. Because of this similarity, reference spectra from different origin were used [24, 25, 27]. Moreover extra information on molecular weight was obtained for some components by recording CI mass spectra. Although polyols and aldonic acids showed molecular ions in these spectra, aldoses did not. Differences between EI and CI spectra for several classes of compounds will be discussed in a separate publication. Table III shows the highest mass ions in EI and CI (isobutane) spectra of some components.

Peak 18 (Fig. 1, Table II) must be a hydroxy acid or a dicarboxylic acid with molecular weight of 336. Its spectrum (EI) shows an abundant peak at *m/z* 292, which probably results from a McLafferty-type rearrangement of a trimethylsilyl group [28]. The *m/z* 292 ion is the highest mass ion in the EI spectrum. The CI spectrum shows a peak at *m/z* 337, which is probably the $(M+1)^+$ molecular ion. Peak 18 is therefore tentatively identified as tartronic

TABLE III
COMPARISON OF EI AND CI SPECTRA

Peak number	Compound name	Mol. weight	Highest mass ions in	
			EI (70 eV)	CI (70 eV, 13 Pa)
33	erythritol-4TMS	410	320, 307, 293, 277	411, 321, 305, 293
35	erythronic acid-4TMS	424	409, 379, 319, 292	425, 409, 335, 307
40a	tartaric acid-4TMS	438	423, 333, 305, 292	439, 423, 321, 292
42	2-deoxyerythropentonic acid-4TMS	438	348, 335, 333, 321	439, 423, 349, 333
49	arabinitol-5TMS	512	320, 319, 317, 307	513, 333, 307, 303
56	citric acid-4TMS	480	465, 375, 363, 347	481, 465, 363, 319
78	inositol-6TMS	612	507, 432, 393, 367	613, 433, 393, 367

acid (hydroxymalonic acid). The spectra of peaks 54 and 54a also show ions at *m/z* 292, but no molecular weight information is available. Hippuric acid, which can not be derivatized in a reproducible way, eluted in a few chromatograms simultaneously with arabinonic acid (peak 55). Peak 74 shows an EI spectrum that is similar to the spectrum of myo-inositol (peak 78). Moreover peak heights of peaks 74 and 78 seem to be related to each other, so it is to be concluded that it is an isomer of myo-inositol.

The components at peak numbers 22, 26b, and 30 were included in Table II at the last moment. Obviously they are related to amino acid metabolism. Peak 30 was identified as Δ-pyrrolidone-5-carboxylic acid. This is a product of an intramolecular peptide bonding (cyclisation) in glutamic acid. It is not known whether this compound is really present in uremic serum in this quantity or is formed from glutamic acid in the derivatization step [29].

CONCLUSIONS

A reproducible and reliable GC method for profiling of uremic serum has been described. Profiles from pre- and postdialysis serum show that hemodialysis treatment results in a significant decrease of the concentration of many components. However, it is observed that different components are not removed to the same extent. Therefore a component-specific parameter, the dialysis ratio, is introduced. It could be seen that different patients showed substantial differences in "overall" concentration. Some patients showed postdialysis profiles that seemed "worse" than predialysis profiles of other patients. Some postdialysis profiles had "overall" concentrations comparable to those for non-uremic serum. Components that are detected by this method are related to carbohydrate metabolism, such as aldoses, aldonic acids and polyols. Also other organic acids and some nitrogen containing compounds are detected. The toxicological behaviour of these components is not yet well understood [4].

In order to include other classes of compounds it is necessary to apply several techniques simultaneously. The isotachophoteric profiling technique developed in this laboratory by Mikkers et al. [16], is very suitable for profiling of ionic substances in serum.

In the described GC procedure sample pretreatment consisted of ultra-

filtration, evaporation and silylation. Total analysis time is only 6 h including sample pretreatment. Bultitude and Newham [18] reported on a laborious pretreatment procedure of several days including fractionation by gel permeation chromatography. Despite the fact that no such fraction technique was applied in our GC method, the same range of compounds (and some more) are detected.

The use of glass capillary columns gives more detailed information than earlier investigations using packed columns.

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CHROMBIO. 364

PERIODATE OXIDATION ANALYSIS OF CARBOHYDRATES

XIII*. SIMULTANEOUS GAS CHROMATOGRAPHIC DETERMINATION OF THE ALDEHYDES IN THE PERIODATE OXIDATION PRODUCTS OF NON-DIALYZABLE URINARY CARBOHYDRATE MATERIALS AS DIETHYL DITHIOACETALS

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SUMMARY

The aldehydes in the periodate oxidation products of non-dialyzable urinary carbohydrate materials were determined simultaneously by gas chromatography of their diethyl dithioacetal derivatives. The yields of aldehydes for normal male urine varied considerably among subjects, but their molar ratios were almost constant. The average values for glyceraldehyde/glyoxal and lactaldehyde/glyoxal molar ratios were 0.41 and 0.27, respectively. The average amount of L-fucose at the non-reducing terminals of carbohydrate chains, as estimated from the yield of lactaldehyde, was about 5 mg/day.

INTRODUCTION

There are a number of diseases associated with the abnormal accumulation of complex carbohydrate materials in urine. Fucosidosis [1] and mannosidosis [2] are the most well-known cases which are characterized by excretion of L-fucose- and D-mannose-rich oligosaccharides due to an inheritable deficiency of α-fucosidase and α-mannosidase, respectively. Aspartylglycosaminuria [3] is noted for an unusual accumulation of N-acetylglucosaminoaspartate induced by lack of the enzyme which catalyzes cleavage of the sugar—amino acid bond. Abnormal accumulation of carbohydrate materials is also found in Hurler's syndrome [4], G_{M1} gangliosidosis [5], and I-cell mucolipidosis [6]. The

*Part XII see ref. 8.

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increased amounts of urinary carbohydrate materials in these metabolic disorders is so drastic that they can be easily characterized by conventional determination of monosaccharides. However, we can expect that there might be many other cases in which deviation of the amounts of constituent monosaccharides from normal values is not so great, but significant change of carbohydrate structure is still involved, including monosaccharide sequence and linkage type.

Recent advancements in mechanistic studies of biosynthesis and metabolism of carbohydrate materials suggests that detailed analysis of these materials might provide information reflecting the activity of carbohydrate transferases and hydrases in tissues and body fluids, and hence serve for diagnosis of the physical state. From such viewpoints our project is concerned with elucidating the fine structures of macromolecular carbohydrate materials in body fluids to correlate their variation to disease states.

We have already published a new convenient method [7, 8] for the simultaneous determination of conjugated aldehydes in the periodate oxidation products of carbohydrate materials. This paper aims to establish the conditions for the determination of aldehydes formed by periodate oxidation of non-dialyzable fractions of human urine.

EXPERIMENTAL

Chemicals

All chemicals were of the highest grade commercially available. D-Xylitol (internal standard) was also obtained from a commercial source, and dried in vacuo for 24 h over phosphorus pentoxide. Pyridine was dehydrated by heating it under reflux with barium oxide, and distilled before use.

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a hydrogen flame ionization detector. A glass column (2 m × 3 mm I.D.) packed with 3% silicone OV-1 on Chromosorb W AW DMCS (80–100 mesh) was used at 170°, and the carrier gas (nitrogen) was regulated at a flow-rate of 50 ml/min throughout the work. Peaks were integrated by a Shimadzu Chromatopak E1A integrator.

Urine samples

These were obtained from male volunteers. For the study of daily variation each sample was used immediately after collection, but for other assays samples were pooled in a refrigerator before 24-h composites were obtained.

Pre-treatment of urine samples

A 10-ml portion of each sample was dialyzed in a Visking tube against tap water for 24 h, except for the samples for the study of dialysis efficiency. The latter samples were dialyzed under the conditions indicated in Table II. The non-dialyzable fraction was concentrated below 40°, and the volume was adjusted to 1.00 ml.

Procedure for the assay of aldehydes

The procedure was essentially the same as that described in the previous paper [8]. A 0.1 *M* sodium metaperiodate solution (100 μl) contained in a small sample tube (5 cm × 5 mm I.D.) was evaporated in vacuo in a desiccator containing sodium hydroxide. To the residue was added an aliquot of the concentrate of the non-dialyzable fraction of urine. The volume of the aliquot added corresponded to that resulting from 0.1% of a 24-h composite sample. Water was added to make the total volume to 200 μl, and the mixture was heated for 3 h at 50° in the dark. The reaction mixture was cooled on an ice-bath, and 0.1 *M* silver nitrate solution (120 μl) was added with vigorous shaking. For the study of the oxidation course, an aliquot of 10% of the reaction mixture obtained from the ten-fold scale reactants was removed after appropriate reaction time, and worked up in the same manner. The mixture was evaporated in vacuo in a desiccator, and to the residue was added a 2:1 (v/v) mixture (20 μl) of ethanethiol and trifluoroacetic acid. The whole was shaken gently for 10 min, closed tightly with a polyethylene stopper. Then a 0.02 *M* solution of D-xylitol (internal standard) in pyridine (50 μl) was added, followed by hexamethyldisilazane (100 μl) and trimethylchlorosilane (50 μl), and the mixture was incubated for 30 min at 50° with occasional shaking. The mixture was centrifuged, and a 1-μl sample of the supernatant was injected into the gas chromatography column. The retention time and molar response factor of each aldehyde derivative, both relative to D-xylitol trimethylsilylate, were, in the following order: glyoxal, 1.49, 0.52; glyceraldehyde, 0.68, 0.64; lactaldehyde, 0.29, 0.51; erythrose, 1.81, 0.77; hydroxymalonaldehyde, 3.12, 0.75.

RESULTS AND DISCUSSION

The aldehydes in the periodate oxidation products were determined by a modification [8] of the procedure [7] established in this laboratory. All kinds of aldehyde except for glyoxal were quantitatively derived to diethyl dithioacetal trimethylsilylates by a simple procedure of treating oxidized samples with a mixture of ethanethiol and trifluoroacetic acid, followed by hexamethyldisilazane and trimethylchlorosilane. Glyoxal was converted into its bis(dithioacetal). Gas chromatography of the derivatized products made it possible to determine all the aldehydes simultaneously. The accompanying periodate and iodate ions in the oxidation reaction mixtures interfered with the determination of aldehydes, but they were conveniently removed by addition of silver nitrate.

Fig. 1 depicts a typical gas chromatogram of dithioacetal derivatives obtained from the non-dialyzable fraction of human urine. There are three major peaks of lactaldehyde (peak 1), glyceraldehyde (peak 2), and glyoxal (peak 4) derivatives, together with minor peaks of erythrose (peak 5) and hydroxymalonaldehyde (peak 6) derivatives. Lactaldehyde is exclusively derived from the methylpentose at the non-reducing terminal. Since L-fucose is the sole methylpentose constituent found in urinary carbohydrate materials, the amount of lactaldehyde corresponds to that of the terminal L-fucose. Glyceraldehyde arises mainly from the hexose residues at the non-reducing ends. The non-reducing hexose residues substituted at C-2, C-6, and both C-2

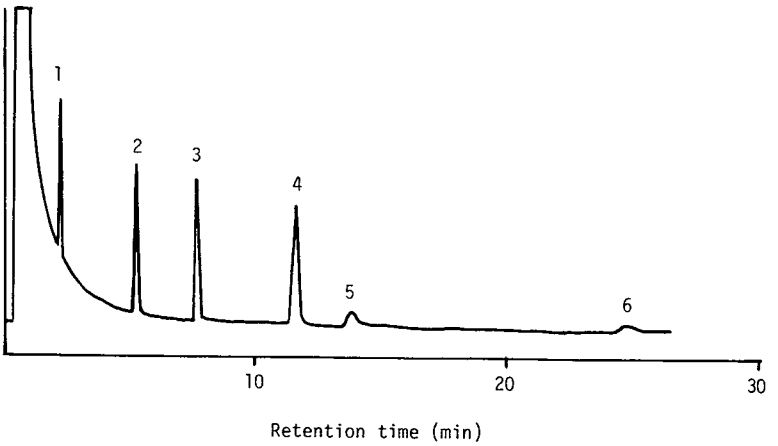

Fig. 1. Gas chromatographic separation of aldehydes in the periodate oxidation products of non-dialyzable urinary carbohydrate materials as diethyl dithioacetal derivatives. Peak assignment: 1, Lactaldehyde; 2, glyceraldehyde; 3, D-xylitol (internal standard); 4, glyoxal; 5, erythrose; 6, hydroxymalonaldehyde.

and C-6 are also possible sources of glyceraldehyde. The D-xylose residue, in which the C-4 hydroxyl group is substituted but the C-2 and C-3 hydroxyl groups are free, can also produce glyceraldehyde, but this portion of glyceraldehyde is small considering the content of D-xylose. Other pentoses can be disregarded since they are not usually present in urinary carbohydrate materials. Glyoxal is a universal aldehyde liberated from the C-1—C-2 part of every non-reducing monosaccharide residue whose C-2 and C-3 hydroxyl groups are not substituted. Erythrose and hydroxymalonaldehyde are unique components formed, respectively, from the C-3—C-4—C-5—C-6 part of the non-reducing hexose residues, in which the C-4 hydroxyl group is substituted but the C-2 and C-3 hydroxyl groups are unsubstituted, and from the C-1—C-2—C-3 part of the non-reducing hexose residues whose C-2 hydroxyl group is occupied but whose C-3 and C-4 hydroxyl groups are free. Oxidizable N-acylhexosamine residues may form dialdehydes, but the component aldehydes are not detected in the gas chromatogram, as they are not derivatized to dithioacetal derivatives under the conditions used for the present assay [9].

Fig. 2 shows the course of oxidation, as observed from the yields of main aldehydes. It also includes the changes in their molar ratios. The yields of all the aldehydes were rapidly increased in the initial 30 min when the non-dialyzable fraction was oxidized at 50°. The rate of increase slowed down thereafter for all the aldehydes. It is noticed that both the lactaldehyde/glyoxal and glyceraldehyde/glyoxal molar ratios remained almost constant throughout oxidation for at least 6 h.

Table I gives the precision data obtained for the products of 3-h oxidation. It is indicated that each aldehyde could be determined with high reproducibility. Efficiency of urine dialysis should not be ignored, since incomplete removal of dialyzable substances caused underestimation of aldehydes for an unknown reason, as seen from Table II. Dialysis should be performed against tap water for at least 24 h. Partial enzymic hydrolysis of some monosaccharide residues

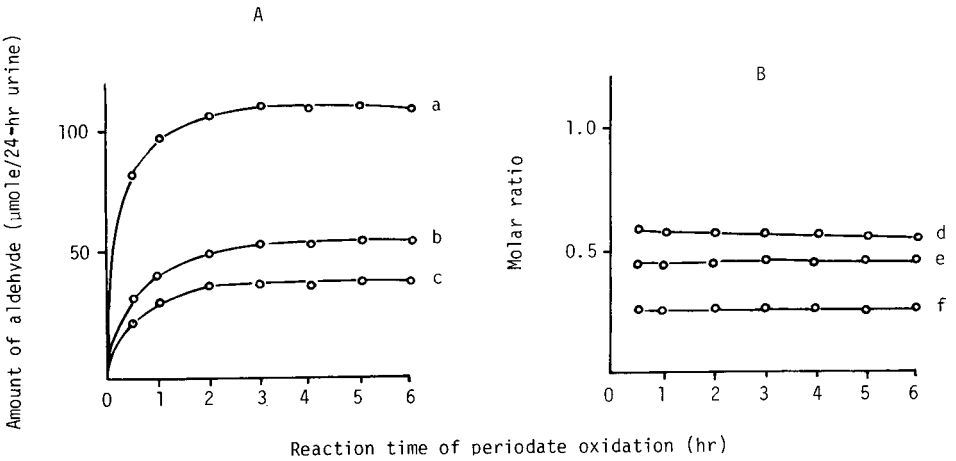

Fig. 2. Course of periodate oxidation of the non-dialyzable fraction of urine, as observed by the yields of aldehydes (A) and their molar ratios (B). a, Glyoxal; b, glyceraldehyde; c, lactaldehyde; d, lactaldehyde/glyceraldehyde; e, glyceraldehyde/glyoxal; f, lactaldehyde/glyoxal.

TABLE I

PRECISION OF THE DETERMINATION OF MAIN ALDEHYDES IN PERIODATE OXIDATION PRODUCTS OF NON-DIALYZABLE URINARY CARBOHYDRATE MATERIALS

No.	Aldehyde* found (μmole/24-h urine)			Molar ratio		
	GlycerA	LactA	GO	GlycerA / GO	LactA / GO	LactA / GlycerA
1	45	31	118	0.38	0.26	0.69
2	47	29	111	0.42	0.26	0.62
3	43	30	107	0.40	0.28	0.70
4	42	29	102	0.41	0.28	0.69
5	48	29	112	0.43	0.26	0.60
6	43	28	103	0.42	0.27	0.65
7	50	30	115	0.43	0.26	0.60
Average	45	29	110	0.41	0.27	0.65
CV (%)	7.1	3.3	5.5	4.4	3.5	6.8

*GlycerA, glyceraldehyde; LactA, lactaldehyde; GO, glyoxal.

during dialysis may be possible, but this is unlikely to be the case from comparing the amounts of aldehydes for 3-day dialysis in pooled water with 1-day dialysis against tap water. The difference in dialysis time did not influence the yield of aldehyde.

Table III shows the distribution of the main aldehydes for several normal male subjects. The yields of aldehydes varied considerably among subjects, but the molar ratios of lactaldehyde/glyoxal and glyceraldehyde/glyoxal were almost constant, giving average values of 0.41 and 0.27, respectively. The lactaldehyde/glyceraldehyde molar ratio was distributed over a wider range

TABLE II

EFFECT OF DIALYSIS EFFICIENCY ON THE DETERMINATION OF ALDEHYDES

Mode of dialysis	Duration of dialysis (days)	Aldehyde* found (μmole/24-h urine)			Molar ratio		
		GlycerA	LactA	GO	GlycerA / GO	LactA / GO	LactA / GlycerA
Pooled water** (1.1 ml of urine)	1	11	19	67	0.16	0.28	1.73
	2	35	25	91	0.38	0.27	0.71
	3	39	25	94	0.41	0.27	0.64
Tap water (10 l/h)	1	39	26	96	0.41	0.27	0.67
	2	41	25	98	0.42	0.26	0.61

*Abbreviations as in Table I.
**Pooled water was changed every day.

TABLE III

DISTRIBUTION OF MAIN ALDEHYDES FOR NORMAL MALE URINE

Subject No.	Age	Blood group	Aldehyde* found (μmole/24-h urine)			Molar ratio			Amount of L-fucose (mg/day)
			GlycerA	LactA	GO	GlycerA / GO	LactA / GO	LactA / GlycerA	
1	26	A	29	19	71	0.41	0.27	0.66	3.1
2	30	A	48	28	120	0.40	0.23	0.58	4.6
3	42	A	32	30	94	0.34	0.32	0.94	4.9
4	11	A	67	39	150	0.45	0.26	0.58	6.4
5	22	B	43	23	93	0.46	0.25	0.53	3.8
6	22	O	38	23	90	0.42	0.26	0.61	3.8
7	7	O	46	35	112	0.41	0.31	0.76	5.7
8	21	AB	66	37	160	0.41	0.23	0.56	6.1
Average			46	29	111	0.41	0.27	0.65	4.8
CV (%)			30	25	28	9	13	21	25

*Abbreviations as in Table I.

centering at 0.65. Relatively higher values were observed for subjects 3 and 7, who belong to the same family. It is also noticeable that the type of blood group did not significantly affect the yield of aldehyde.

Periodate oxidizes a monosaccharide residue to yield a pair of hydroxyaldehyde (glycolaldehyde, glyceraldehyde, lactaldehyde, or erythrose) and dicarbonyl (glyoxal or hydroxymalonaldehyde) compounds in equimolar amounts. Since glycolaldehyde was not detected and the amounts of erythrose and hydroxymalonaldehyde were very small, the difference between the amount of glyoxal and the total amount of glyceraldehyde plus lactaldehyde is approximately equal to the total amount of the monosaccharide residues that are attached by unoxidizable monosaccharide residues but which possess free hydroxyl groups at C-2 and C-3. These structures will give rise to glyoxal but no unsubstituted hydroxyaldehyde. The proportion of the total amount of such monosaccharide residues relative to that of all monosaccharide residues cleaved amounted to $1 - (0.41 + 0.27) = 0.32$, which is approximately one third. The low yields of erythrose imply that there were only small amounts of hexose residues whose C-4 hydroxyl group is substituted but whose C-2 and C-3 hydroxyl groups are unoccupied. Similarly, the poor yields of hydroxymalonaldehyde indicate a

TABLE IV

DAILY VARIATION OF ALDEHYDES

Fraction No.	Sampling time	Volume (ml)	Aldehyde* found						Molar ratio		
			nmole/ml of urine			μmole/fraction			GlycerA / GO	LactA / GO	LactA / GlycerA
			GycerA	LactA	GO	GlycerA	LactA	GO			
1	11 a.m.	200	89	55	212	17.7	11.0	42.3	0.42	0.26	0.62
2	3 p.m.	120	48	28	110	5.8	3.3	13.2	0.44	0.25	0.58
3	7 p.m.	195	62	36	128	12.1	7.0	25.0	0.48	0.28	0.58
4	11 p.m.	135	55	31	124	7.4	4.2	16.8	0.44	0.25	0.56
Composite		650	67	39	150	43.6	25.4	97.5	0.45	0.26	0.58

*Abbreviations as in Table I.

low abundance of hexose residues having substituted C-2 and free C-3 and C-4 hydroxyl groups.

The data in Table III also allow the estimation of the amount of L-fucose located at the non-reducing terminal. The average value calculated from the amount of lactaldehyde was approximately 5 mg/day. This value was slightly lower than that obtained by a colorimetric procedure [10] (about 7.5 mg/day). The dithioacetal method tends to underestimate aldehydes, especially for small amounts of samples; the losses of both glyoxal and glyceraldehyde for 10^{-8} mole of methyl α-D-glucopyranoside were approximately 25% [8]. On the other hand, the colorimetric procedure is presumed to overestimate them due to interaction with concomitant hexoses and amino sugars.

Table IV gives the aldehyde distribution for urine samples collected from a healthy young man at various times in 24 h. Although the yields of aldehydes per unit volume, i.e. the concentrations of carbohydrate materials, varied among samples, all the molar ratios were again approximately equal independent of collecting time. The non-dialyzable fraction of urine contains a variety of carbohydrate materials including oligosaccharides, acid mucopolysaccharides, and glycoproteins. The constancy of the molar ratios of aldehydes suggests that the mixture ratios of these materials are almost unchanged during 24 h.

The present study of the products of periodate oxidation provides information on the structures of urinary carbohydrate materials. Our program is now to obtain data for pathologically abnormal conditions by this procedure.

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Biomedical Applications

CHROMBIO. 361

COMPARISON OF DIFFERENT HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEMS FOR THE PURIFICATION OF ADRENAL AND GONADAL STEROIDS PRIOR TO IMMUNOASSAY

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SUMMARY

The high-performance liquid chromatography of nineteen hormonal steroids with special respect to its suitability for routine purification of these steroids from crude, organic extracts of biological fluids prior to final quantitation by immunoassay has been studied. In all systems the gradient elution technique was applied. Separation of steroids has been investigated using different stationary phases chemically coated with non-polar, hydroxyl, NO_2 and CN groups. Reproducibility of retention times was studied on a stationary phase coated with hydroxyl groups (DIOL column) using different organic eluents. Coefficients of variation range from 0.76 to 8.16%. Reproducibility was shown to be unequivocally better in the gradient part than in the isocratic part of the chromatographic run. In contrast to the other steroids, 18-hydroxylated steroids were more or less unstable in certain systems studied. As to resolution and reproducibility, the DIOL column run with an *n*-hexane—dioxane gradient has been shown to be superior to the other systems studied.

INTRODUCTION

The direct and specific measurement of hormonal steroids in biological fluids by immunoassay only is feasible if the steroid occurs in relatively high amounts, such as cortisol in serum [1, 2], or if highly specific antisera are available [3]. However, for most steroid determinations — especially for those of urinary free steroids — these prerequisites are not valid and purification steps are necessary prior to the final immunological quantitation [4] if the estimation is to be specific. Similarly, if several steroids have to be determined simultaneously in one sample, a separation of these steroids is obligatory [5—7]. Numerous methods of purification or separation including nearly all known techniques of chromatography have been reported in the literature hitherto. All these procedures, however, are rather time-consuming and scarcely fit to be

TABLE I

THE SYSTEMS OF COLUMN PACKINGS AND SOLVENTS EXAMINED IN THE PRESENT STUDY

System	Trademark of column	Origin	Chemically bonded group	Mean diameter of particles (μm)	Internal diameter of column (mm)	Components of solvents	
						A	B
1	RP-18	Hewlett-Packard, Böblingen, G.F.R.	Octadecylsilyl	10	4	Water	Methanol
2	DIOL	Merck	OH	10	3	*n*-Hexane	*n*-Hexane—isopropanol (70:30)
3	DIOL	Merck	OH	10	3	*n*-Hexane	*n*-Hexane—dioxane (50:50)
4	DIOL	Merck	OH	10	3	*n*-Hexane	CH_2Cl_2
5	DIOL	Merck	OH	5	4	*n*-Hexane	*n*-Hexane—isopropanol (70:30)
6	Nucleosil NO_2	Chrompack, Middelburg, The Netherlands	NO_2	5	4.6	*n*-Hexane	*n*-Hexane—ethanol (50:50)
7	Nucleosil CN	Chrompack	CN	5	4.6	*n*-Hexane	*n*-Hexane—isopropanol (70:30)

automated for use in the routine laboratory. The rapid development in the field of high-performance liquid chromatography (HPLC) seems to establish this technique as a version of chromatography suitable for complete automation [8]. Separation of steroids by HPLC meanwhile has been documented by several authors using isocratic systems [9—12] or using gradient elution with a non-polar reversed-phase system [13].

The purpose of the present study was to examine the separation of nineteen physiologically important steroids by HPLC using different polar bonded phases and different eluents with special respect to the suitability of these systems for a practicable purification of the steroids prior to immunoassay.

MATERIALS AND METHODS

A Hewlett Packard high-performance liquid chromatograph (Model 1084A), equipped with a fixed UV detector at 254 nm, two solvent and two pump systems, a variable volume (10—250 μl) injector, and a plot/print terminal was used throughout the study. All operating parameters of the chromatograph, such as flow-rate, temperature of solvents and column room, composition of the solvents as well as print or plot specifications, were regulated and controlled by microprocessors. In addition, changes of these parameters during a run, for example changing the solvent composition during gradient elution, were time programmable.

The eluents were fractionated using a time-regulated sample collector (LKB, Ultrorac®, Type 7000). ^{3}H radioactivity was measured in a liquid scintillation spectrometer (Packard Instruments, Model 2480) using Biofluor® (New England Nuclear) as scintillation cocktail.

Packing material

Stationary phases chemically bonded to silica gel as supporting material were examined exclusively in this study. The length of all columns was 25 cm. Trade names, origins of the various columns, chemically bonded groups, mean particle diameters, internal diameter of columns and the various eluents are listed in Table I.

Chemicals

n-Hexane, propan-2-ol, dioxane and dichloromethane were of p.a. grade and purchased from Merck (Darmstadt, G.F.R.). Solvents were degassed under vacuum immediately before use. Water was deionized and glass-distilled. Extrelut® for extraction of steroids from serum or urine samples was purchased from Merck. Radioactive steroids were from New England Nuclear (Dreieichenhain, Frankfurt/Main, G.F.R.) and non-radioactive steroids from Steraloids (Pawling, N.J., U.S.A.) except 18-OH-deoxycorticosterone and 18-OH-corticosterone, which were from Makor Chemicals Ltd. (Jerusalem, Israel). The purity of steroids absorbing at 254 nm was checked by thin-layer chromatography (system: chloroform—methanol, 9:1, v/v). The trivial names and abbreviations used throughout are listed in Table II.

TABLE II

TRIVIAL NAMES AND ABBREVIATIONS

Trivial name	Abbreviation	Peak number in figures	Chemical name
Progesterone	P	(1)	Pregn-4-ene-3,20-dione
Androstenedione	AD	(2)	Androst-4-ene-3,17-dione
Pregnenolone	PL	(3)	3β-Hydroxypregn-5-en-20-one
5α-Dihydrotestosterone	α-DHT	(4)	17β-Hydroxy-5α-androstan-3-one
Dehydroepiandrosterone	DHEA	(5)	3β-Hydroxyandrost-5-en-17-one
Deoxycorticosterone	DOC	(6)	21-Hydroxypregn-4-ene-3,20-dione
Testosterone	T	(7)	17β-Hydroxyandrost-4-en-3-one
Estrone	E_1	(8)	3-Hydroxyestra-1,3,5(10)-trien-17-one
17-OH-Progesterone	17-OH P	(9)	17α-Hydroxypregn-4-ene-3,20-dione
17-OH-Pregnenolone	17-OHPL	(10)	17α, 3β-Dihydroxypregn-5-en-20-one
Estradiol	E_2	(11)	Estra-1,3,5(10)-triene-3,17β-diol
11-Deoxycortisol	S	(12)	21,17α-Dihydroxypregn-4-ene-3,20-dione
18-OH-Deoxycorticosterone	18-OH-DOC	(13)	18,21-Dihydroxypregn-4-ene-3,20-dione
Corticosterone	B	(14)	11β,21-Dihydroxypregn-4-ene-3,20-dione
Cortisone	E	(15)	17α,21-Dihydroxypregn-4-ene-3,11,20-trione
Aldosterone	Aldo	(16)	11β,21-Dihydroxy-18-al-pregn-4-ene-3,20-dione
Estriol	E_3	(17)	Estra-1,3,5(10)-triene-3,16α,17β-triol
Cortisol	F	(18)	11β,17α,21-Trihydroxypregn-4-ene-3,20-dione
18-OH-Corticosterone	18-OH-B	(19)	11β,18,21-Trihydroxypregn-4-ene-3,20-dione

Chromatographic procedure

Appropriate amounts of ethanolic solutions of steroids were evaporated to dryness in a stream of nitrogen. The residues were redissolved in the eluents exhibiting the initial composition of the gradient elution patterns, thus yielding concentrations of 500 ng of each steroid per 100 μl of eluent. In all systems studied, 100 μl of the steroid mixture were transferred to glass microvials and injected with the variable-volume injector. If not otherwise stated the temperature of the column was 30°. UV-absorbing steroids were detected at 254 nm. In all chromatograms described, attenuation was 64×10^{-4} a.u./cm. Radioactive steroids were located by liquid scintillation counting of 0.5-ml eluate fractions. Gradients were run linearly with intermittent isocratic periods. In each system studied, gradient profiles were varied until the best separation of the steroid compounds in the corresponding system was achieved. The final gradient was held at 100% of solvent B in order to eliminate polar components in the case of serum or urine extracts.

Extraction procedure

Samples of urine or serum were extracted using a solid phase technique [14].

Procedure 1. Two millilitres of urine diluted 1 to 20 with water were pipetted onto 1.2 g of Extrelut and pre-extracted with 20 ml of carbon tetrachloride followed by 20 ml of dichloromethane.

Procedure 2. Two millilitres of urine or serum diluted with one part of water were extracted correspondingly with 20 ml of ether.

The organic extracts were evaporated to dryness and redissolved in the eluent for chromatography.

RESULTS

Separation on different stationary phases

In Fig. 1 the chromatogram of eleven steroids absorbing at 254 nm on a reversed-phase system (see system 1 in Table I) is shown. The band widths of all steroids range from 0.6 ml for P to 1.3 ml for F. Peaks are broader at the end than at the beginning of an isocratic part of the gradient profile. While a distinct resolution between progesterone derivatives differing in one hydroxyl group is obvious, adequate separation of isomeric steroids is scarcely achieved; for example, separation of the double-hydroxylated progesterone derivatives S, 18-OH-DOC and B. The retention time is longer for steroids containing an hydroxyl group than for the analogous steroid with a C=O group (see 18-OH-B and Aldo or AD and T).

The chromatogram of adrenal and gonadal steroids on a stationary phase chemically coated with hydroxyl groups (DIOL column) is shown in Fig. 2 (system 2 in Table I). Steroids without absorbance at 254 nm were localized by radiofractionation. The elution pattern of steroids is nearly inverse to that in system 1. Band widths of absorbing steroids are comparable to those measured in system 1.

Similarly, separation of isomeric steroids is moderate (S and 18-OH-DOC or F and 18-OH-B). However, this system enables the distinct resolution of at least nine individual steroids, while the other steroids may be separated in terms of steroid groups.

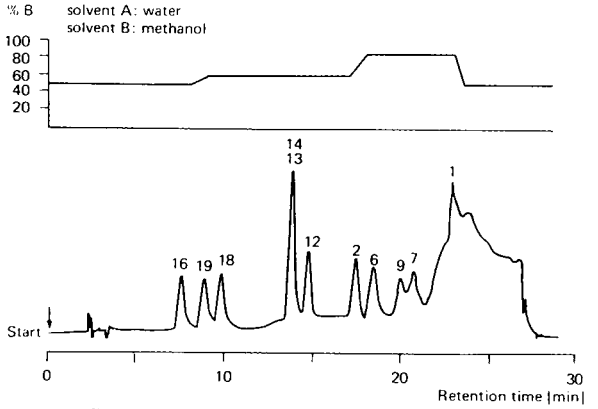

Fig. 1. Separation of adrenal steroids on a reversed-phase system using a water—methanol gradient (system 1 in Table I) and UV detection. For numbers of peaks, see Table II.

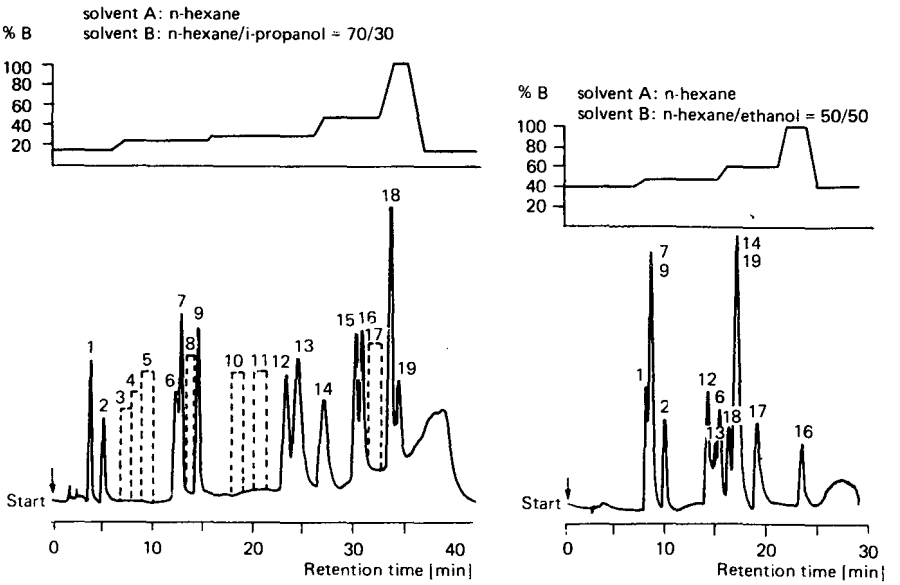

Fig. 2. Separation of adrenal and gonadal steroids on a DIOL column using an *n*-hexane—isopropanol gradient (system 2 in Table I). Steroids were detected by UV absorption or radioactivity.

Fig. 3. Separation of adrenal steroids on an NO_2 column using an *n*-hexane—methanol gradient (system 6 in Table I) and UV detection.

The elution pattern of steroids chromatographed on a stationary phase chemically coated with NO_2 groups (system 6 in Table I) is shown in Fig. 3. In this system, ethanol was used as polar component of the gradient mixture. In contrast to systems 1 and 2, resolution of the steroids differing in hydroxyl groups was rather impaired (P and 17-OH-P, DOC and 18-OH-DOC, B and 18-OH-B). However, a stronger affinity and selectivity of this polar phase to steroids differing in CO groups is apparent if the retention times of the steroids T and AD, as well as of E and Aldo, are considered.

In Fig. 4 the chromatogram of the steroids on a polar stationary phase coated with CN groups (system 7 in Table I) is demonstrated. Adequate resolution of steroids is nearly absent in this system. If comparing the retention times of 18-OH-DOC and DOC, or those of 18-OH-B and B, this system seems to operate — at least partially — like the reversed-phase system (system 1).

Influence of temperature and diameter of particles on separation

The influence of column temperature on the resolution of steroids is shown by the chromatogram in Fig. 5. The system is identical to system 2 except that the temperature of the column room was raised to 40°. By comparing the elution profiles of Figs. 2 and 5, it becomes evident that resolution of isomeric steroids is significantly impaired by increasing the temperature.

Fig. 6 shows a chromatogram of the DIOL column using particles of 5 μm diameter instead of 10 μm (system 5 in Table I). Band widths range from 0.6 to 1 ml. Resolution of the individual steroids is significantly superior to that

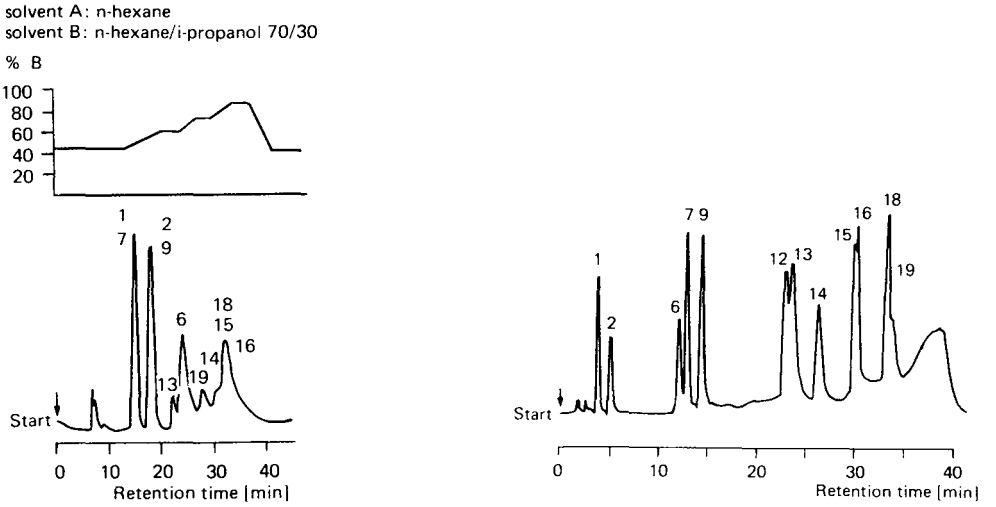

Fig. 4. Separation of adrenal steroids on a CN column using an *n*-hexane—isopropanol gradient (system 7 in Table I) and UV detection.

Fig. 5. Separation of adrenal steroids on a DIOL column using an *n*-hexane—isopropanol gradient (system 2 in Table I) and UV detection. Temperature of column was 40° instead of 30° (see Fig. 2).

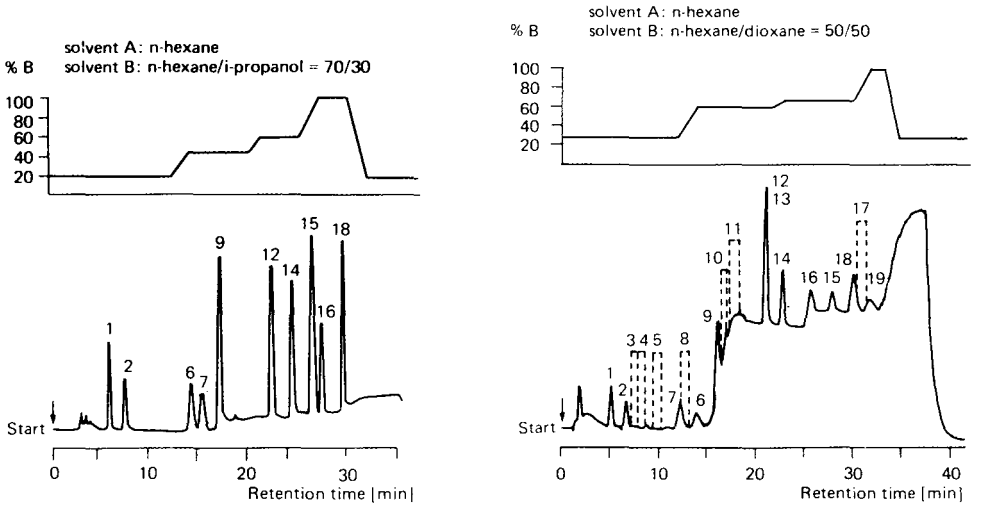

Fig. 6. Separation of adrenal steroids on a DIOL column using an *n*-hexane—isopropanol gradient (system 5 in Table I) and UV detection. Diameter of particles was 5 μm instead of 10 μm.

Fig. 7. Separation of adrenal and gonadal steroids on a DIOL column using an *n*-hexane—dioxane gradient (system 3 in Table I). Steroids were detected by UV absorption or radioactivity.

with system 2. However, 18-hydroxylated steroids such as 18-OH-DOC and 18-OH B, were obviously damaged on this column packing, as even 5-μg amounts of these steroids remained undetectable in terms of discrete peaks.

Influence of solvents on separation

Fig. 7 shows the chromatogram of a DIOL column using dioxane as polar component of the eluent (system 3 in Table I). Due to the relatively high absorbance of dioxane itself, the baseline changes markedly with the increasing gradient. On the other hand, it is well demonstrated by this profile that the maximum gradient of 100% is completely abolished if the column is washed with the initial composition of the gradient for at least 5 min. As to the resolution of individual steroids, it is obvious that this system provides excellent separation of all the steroids studied. Apart from PL and DHT, and 18-OH-DOC and S, all steroids are separated distinctly and no steroid was damaged.

If dichloromethane is used as polar component of the gradient mixture (system 4 in Table I) an elution pattern as shown in Fig. 8 can be observed. Apart from the reversal behaviour of the steroids DHT and PL, DOC and DHEA, or B and 18-OH-DOC, no marked differences are apparent compared with the chromatogram obtained using system 2. As with system 4, the 18-hydroxylated steroid 18-OH-B was not detectable as an absorbing fraction in this system.

Reproducibility of retention times

A high degree of reproducibility of retention times is one of the most important requirements if steroid amounts not detectable by absorbance have to be fractionated prior to immunoassay. This parameter was studied in detail for systems 2—4 (Figs. 2, 7 and 8) by evaluating several chromatographic runs each followed immediately by the start of the next one, thus simulating conditions of automatic analyses of series of samples. The results are listed in Table III. Highest values of absolute standard deviations were 1 min in system

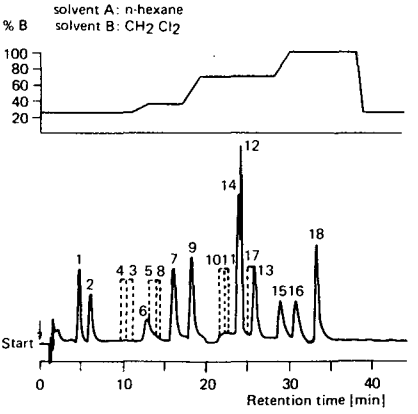

Fig. 8. Separation of adrenal and gonadal steroids on a DIOL column using an *n*-hexane—dichloromethane gradient (system 4 in Table I). Steroids were detected by UV absorption or radioactivity.

2, 1.01 min in system 4 and only 0.49 min in system 3. Coefficients of variation ranged from 6% in system 2, to 8.16% in system 4 and to 3.96% in system 3. A more precise analysis of the data reveals that in all systems reproducibility of retention times in terms of coefficient of variation is relatively low in the initial part of the run and is continuously impaired during the isocratic parts of the gradient.

Reproducibility of the retention times in the chromatography of organic extracts of urine was studied with the dichloromethane extracts of ten different samples of urine, which were diluted 1:20 with water (see extraction procedure 1 in the experimental part) and to which 1 μg cortisol had been added (Fig. 9). It must be pointed out that the gradient profile chosen provided the elution of cortisol at the end of the slowly increasing linear gradient of the run. The resulting parameters of reproducibility were mean retention time $\overline{x}$ = 7.55 min, S.D. = 0.03 min, CV = 0.4%; and the range 7.52—7.61 min.

Extracts of serum and urine

Chromatograms representative of the HPLC of ether extracts of urine and serum (see extraction procedure 2 in the experimental part) are shown in Fig. 10. Comparison with the chromatogram of the mixture of pure steroids (each peak corresponded to about 500 ng of steroid) documents quite well that apart from cortisol in the serum chromatogram (about 80 ng) all other steroids

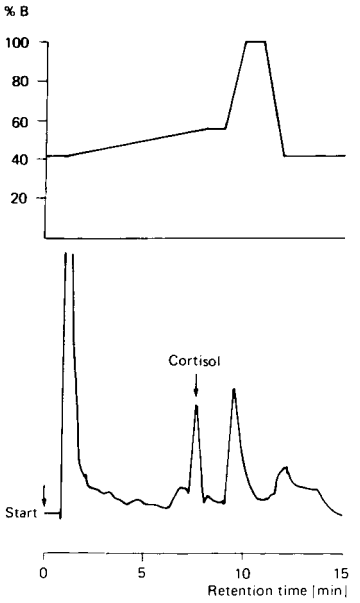

Fig. 9. Chromatogram of a dichloromethane extract of 100 μl urine diluted 1:20 with water. Cortisol (1 μg) had been added to the sample. Conditions of chromatography were similar to those in system 2 (see Table I).

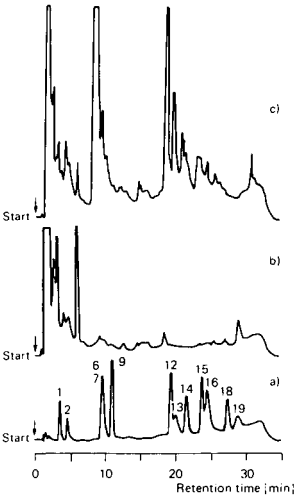

Fig. 10. Representative chromatograms of ether extracts of 2 ml urine (c) and 1 ml serum (b) compared with that of a mixture of pure steroids (a). The amount of each steroid was about 500 ng in chromatogram a. The DIOL column, an *n*-hexane—isopropanol gradient (system 2 in Table I) and UV detection were used.

TABLE III

REPRODUCIBILITY OF RETENTION TIMES OF STEROID HORMONES SEPARATED BY
See systems 2—4 in Table I.

Steroid	System 2 (n = 18)			
	$\bar{x}$ (min)	S.D. (min)	CV (%)	Range (min)
Progesterone	3.28	0.06	1.88	3.16—3.39
Androstedione	4.11	0.10	2.60	3.88—4.29
Deoxycorticosterone	8.02	0.31	3.96	7.33—8.60
Testosterone	8.44	0.40	4.75	7.89—9.20
17-OH-Progesterone	10.49	0.69	6.63	9.36—11.58
Deoxycortisol	18.96	0.66	3.51	17.98—19.92
18-OH-DOC	19.88	0.59	3.00	19.12—20.77
Corticosterone	21.98	0.70	3.20	21.13—22.96
Cortisone	25.29	0.88	3.49	24.29—26.75
Aldosterone	26.52	1.00	3.78	25.14—28.02
Cortisol	31.55	0.35	1.12	31.12—32.11
18-OH-Corticosterone	32.09	0.37	1.15	31.64—32.67

exhibiting rather low physiological concentrations in these biological fluids are not detectable by absorbance if 1—2-ml samples are assayed. Furthermore, the peak areas of the individual steroids are overlapped — this is especially pronounced in the chromatogram of the urine sample — by unspecific components exhibiting much stronger absorbance than the steroids of interest.

DISCUSSION

The HPLC of steroid hormones recently reviewed by Fitzpatrick [15] has been intensively investigated during the last five years and has been shown to represent an excellent technique for the separation of steroid mixtures. However, photometric quantitation in addition to separation of steroid hormones from urine or serum samples by HPLC is limited to steroids occurring in relatively high amounts in biological fluids, such as cortisol in serum [16, 17] and estriol in urine [18]. Due to their low physiological concentrations and to the strong interference of unspecifically absorbing compounds, as well illustrated in the chromatograms of Fig. 10, other steroids of biological interest will hardly be assayable by HPLC alone [15]. On the other hand, the immunoassay technique widely used for steroid estimation lacks sufficient specificity in many cases [19]. Thus, one may conclude that HPLC and immunoassay in conjunction represents quite an attractive means of determining single and particularly multiple steroids in small samples of biological fluids [20].

The suitability of HPLC in this field depends on several criteria:

(1) adequate separation of steroids,

(2) good reproducibility of retention times and independence of chromatographic behaviour from individual sample background,

System 3 (n = 18)				System 4 (n = 15)			
$\bar{x}$ (min)	S.D. (min)	CV (%)	Range (min)	$\bar{x}$ (min)	S.D. (min)	CV (%)	Range (min)
4.36	0.08	1.92	4.26—4.63	4.79	0.36	7.67	4.30—5.40
5.52	0.11	2.13	5.43—5.92	6.07	0.46	7.66	5.47—6.98
10.88	0.38	3.50	10.35—11.95	12.49	1.01	8.16	11.42—14.80
9.44	0.31	3.31	9.03—10.43	15.79	0.58	3.69	15.12—17.05
12.49	0.49	3.96	11.71—13.92	17.93	0.59	3.33	17.26—19.27
19.59	0.19	0.99	19.25—19.97	23.91	0.18	0.76	23.48—24.16
19.59	0.19	0.99	19.25—19.97	25.61	0.25	0.97	24.85—25.94
21.10	0.19	0.94	20.73—21.51	23.61	0.17	0.74	23.23—23.85
26.06	0.20	0.79	25.68—26.45	28.92	0.37	1.29	27.74—29.35
23.68	0.22	0.95	23.24—24.12	30.56	0.39	1.30	29.62—31.13
28.12	0.32	1.14	27.66—28.97	33.22	0.33	0.99	32.07—33.53
29.50	0.29	1.00	29.04—30.06	—	—	—	—

(3) easy evaporation of the eluent,

(4) complete elimination of polar or non-polar steroids from the column after each run and rapid reequilibration,

(5) automation of sample loading and of fractionation.

(1) As regards the systems studied, adequate separation of steroids is achieved by the reversed-phase system (system 1) and by the DIOL systems. The quality of the reversed-phase system measured in this study confirms the findings reported by other authors [11, 13]. The good suitability of the DIOL systems for steroid separation has not been reported hitherto. This finding, however, is not astonishing, as the properties of this stationary phase are comparable to those of the widely used Celite columns which were introduced by Abraham and Odell several years ago [21] and have been shown to provide excellent separations of steroids [19]. Of the eluents studied, the *n*-hexane—dioxane mixture provides the best resolution (Fig. 7). An equivalent level of separation is achieved by the isopropanol system if particles of 5 μm in diameter are used (Fig. 6). However, this system is disqualified as 18-hydroxylated steroids are damaged. A possible explanation for this latter effect may be the instability of these steroids on silica gels as observed in thin-layer chromatography [22].

(2) The best reproducibility of retention times measured in this study is provided by system 7. However, the problem of reproducibility is obviously independent of the nature of the eluent, which is illustrated by comparing the chromatogram obtained using system 7 with that using the DIOL system (Fig. 9). The quality of reproducibility seems to be better if steroids are eluted by a gradient (see reproducibility of cortisol in Fig. 9). Impaired reproducibilities of the initial period of each run (see Table III) should generally be improvable if the steroid of interest is eluted during a gradient part of a chromatographic

run. No, or negligible, influence of the individual sample background could be demonstrated in the chromatography of the extracts of diluted urine samples (Fig. 9).

(3) As eluted fractions after chromatography have to be evaporated to dryness prior to immunological quantitation, the volatile nature of the eluents used in systems 2—7 makes the polar-coated stationary phases unequivocally superior to the reversed-phase system which requires time-consuming evaporation of aqueous solutions.

(4) Application of the gradient technique also carries the advantage that polar components of the sample to be chromatographed are easily eliminated from the polar-coated column by increasing the gradient to maximum polarity for a sufficient time, as shown in Fig. 10. As documented by the rather good reproducibility of the initial peaks of each run, sufficient reequilibration of the columns coated with polar groups is already achieved after 5 min.

(5) The problem of automation is of a technical nature and is already or will be solved by manufacturers of HPLC equipment in the near future.

In conclusion the findings of the present study demonstrate quite well that combination of HPLC using stationary phases chemically coated with hydroxyl groups, gradient elution with organic eluents and automated equipment represents a suitable tool for the routine separation of steroid hormones prior to immunoassay.

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CHROMBIO. 375

DETERMINATION OF CORTISOL IN HUMAN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the measurement of cortisol in human plasma using 45% aqueous methanol eluent on a 120 mm × 4.5 mm I.D. Hypersil octadecylsilane column with UV detection at 239 nm after a simple dichloromethane extraction and evaporation with a prednisone internal standard.

The sample preparation time and chromatography time are each about 15 min and linear correlations have been obtained with plasma samples assayed by the Mattingly fluorimetric technique and a commercial-kit competitive protein binding method. Concentrations down to 30 nmol/l may be measured and the method can be used when fluorimetry is invalidated by interference, particularly from spironolactone.

INTRODUCTION

The application of high-performance liquid chromatography (HPLC) for the determination of plasma cortisol concentrations using normal-phase systems has been described by Trefz et al. [1] using silica columns and by Van den Berg et al. [2] with Nucleosil-NO_2 columns. Schwedt et al. [3] studied both normal- and reversed-phase systems for determining urinary free cortisol but favoured normal-phase adsorption chromatography. The simplicity and versatility of the reversed-phase system aqueous methanol with octadecylsilane (ODS)-silica makes this the most likely system to be available in clinical chemistry laboratories at present, and this paper describes its application to the measurement of plasma cortisol when the commonly used fluorimetric technique described by Mattingly [4] is invalidated by interfering substances, particularly spironolactone.

MATERIALS AND METHODS

Equipment

An Applied Chromatography Systems (Model 750/03) reciprocating pump was used with detection at 239 nm by a Cecil CE 212 variable wavelength

UV monitor fitted with an 8-μl chromatography cell. A 120 mm × 4.5 mm I.D. stainless-steel column was packed by a slurry technique with Hypersil ODS (Shandon, London, Great Britain). Injection was performed with a "Specac" loop injector fitted with a 72-μl loop.

Reagents

Dichloromethane and methanol, both HPLC grade, were obtained from Rathburn Chemicals, Walkerburn, Great Britain. The sodium hydroxide used was 0.25 *M* in water.

Standard cortisol was obtained from Sigma (St. Louis, Mo., U.S.A.). A stock solution of 18.1 mg/l (50 μmol/l) in ethanol was diluted 1:100 to a working standard of 181 μg/l (500 nmol/l) with 3% bovine serum albumin in water. Prednisone (Koch-Light, Colnbrook, Great Britain) was used as internal standard at a concentration of 2 mg/l (5.89 μmol/l) in 10% aqueous ethanol. The competitive protein binding (CPB) "Cortipac" Kits (Radiochemical Centre, Amersham, Great Britain) were used.

Procedure

1 ml plasma, 100 μl of internal standard (corresponding to 200 ng prednisone), 100 μl 0.25 *M* sodium hydroxide and 7 ml dichloromethane were vortexed for 30 sec in a stoppered tube and then centrifuged for approximately 5 min at 2000 r.p.m. (approximately 1300 *g*). A 5-ml aliquot of the organic layer was evaporated to dryness with a stream of air in a water bath at 40°. The residue was dissolved in 100 μl eluent, 72 μl of which was injected. The eluent system was 45% methanol in water at a flow-rate of 1 ml/min corresponding to a pressure drop of 100 bars. The ratios of peak heights of cortisol and prednisone in chromatograms from serum samples were compared with that obtained when 1 ml working standard (equivalent to 500 nmol/l cortisol) was similarly processed.

RESULTS

Fig. 1 shows a separation of six steroids chromatographed in this system. Cortisol is well separated from prednisone, corticosterone, dexamethasone, 11-deoxycortisol, and 17-hydroxyprogesterone but not from prednisolone. Spironolactone eluted more than 30 min after cortisol (Fig. 2). The steroid retention data are shown in Table I. A chromatogram of a normal plasma is shown in Fig. 3.

The recovery of prednisone and cortisol from a pooled plasma sample was calculated by chromatographing extracts before and after adding known amounts of prednisone and cortisol. There was a constant recovery of 86.1% for cortisol and 80.0% for prednisone. Recovery from water was found to be about 10% greater and therefore it was necessary to prepare the standard cortisol solution in a protein matrix; 3% bovine serum albumin (BSA) was sufficient and convenient. Vortex mixing for 30 sec did not produce emulsions; it was compared with extraction by gentle rotation for 15 min and found to be equally efficient.

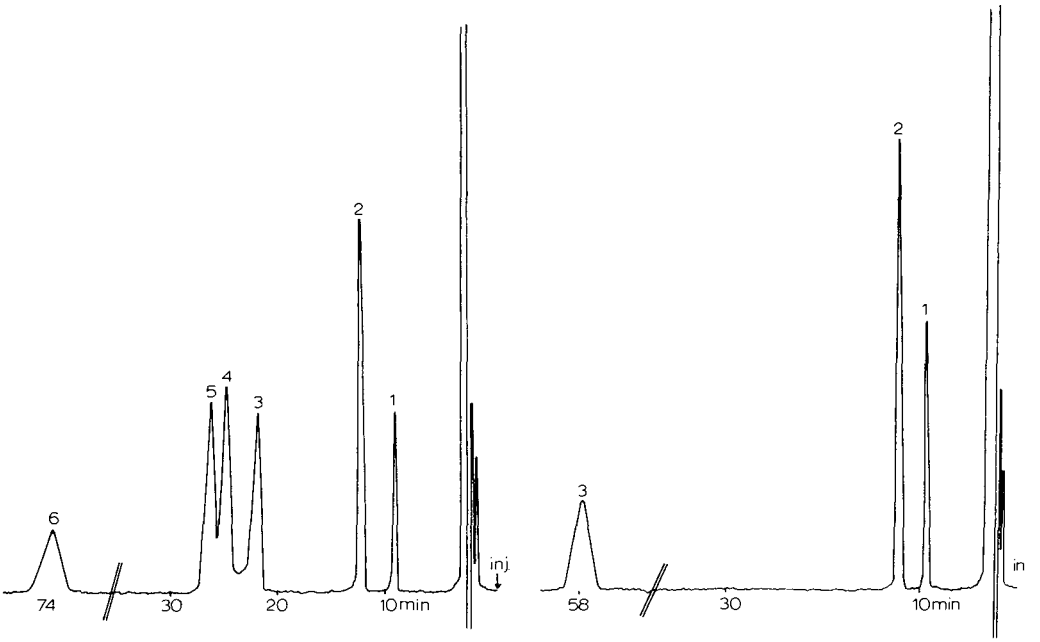

Fig. 1. Separation of a standard steroid mixture. Peaks: 1 = prednisone; 2 = cortisol; 3 = corticosterone; 4 = dexamethasone; 5 = 11-deoxycortisol; 6 = 17-hydroxyprogesterone. Column, Hypersil ODS 120 × 4.5 mm I.D.; eluent, methanol—water (45:55); flow-rate, 1 ml/min; sample injection, 72-μl sample loop. Detection at 239 nm.

Fig. 2. Separation of spironolactone from cortisol. Peaks: 1 = prednisone; 2 = cortisol; 3 = spironolactone. Chromatography as in Fig. 1.

Standard solutions in 3% BSA were assayed in duplicate over the range 62—2000 nmol/l; the response was linear; the average standard deviation of the duplicates over the whole range was 6.76 nmol/l (CV 4.4%). A blank extract of the 3% BSA showed no significant interference (Fig. 4). Six replicate analyses of the same sample in a single run gave values 511 ± 10.6 (S.D.) nmol/l

TABLE I

STEROID RETENTION DATA FOR FIG. 1

Compound	Capacity factor (k')	Retention relative to cortisol
Prednisone	7.7	0.72
Cortisol	10.7	1.00
Prednisolone	11.0	1.03
Corticosterone	19.7	1.85
Dexamethasone	22.0	2.06
11-Deoxycortisol	23.7	2.23
Spironolactone	46.0	4.35
17-Hydroxyprogesterone	68.0	6.10

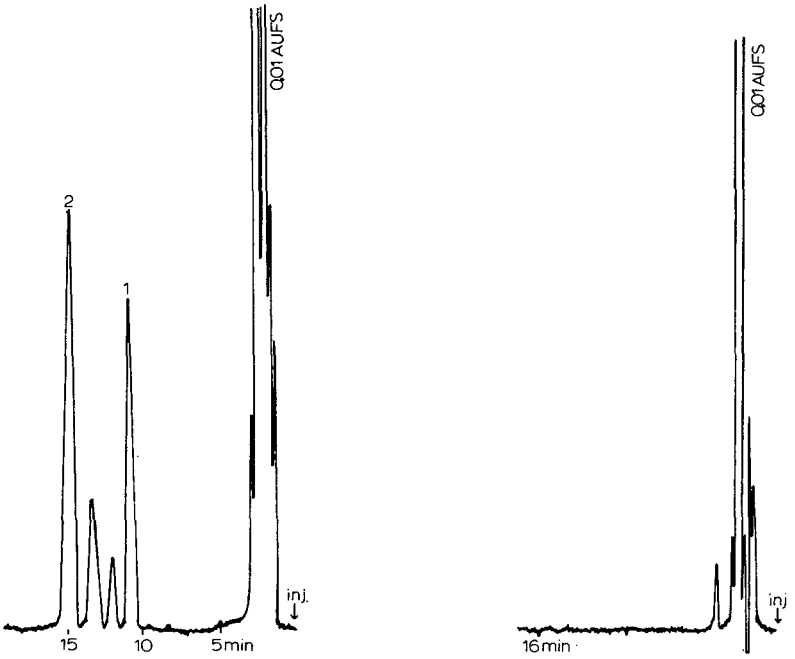

Fig. 3. Chromatography of a normal plasma extract. Peaks: 1 = prednisone; 2 = cortisol. Chromatography as in Fig. 1.

Fig. 4. Blank extraction of 3% bovine serum albumin.

(CV 2.1%). The same sample analysed on five different days gave a between batch CV of 3.6%.

In 41 random laboratory samples the results obtained by HPLC were compared with those obtained by the Mattingly method; the correlation is shown in Fig. 5 (r = 0.8792). A competitive protein binding method was compared for 17 samples and this correlation is shown in Fig. 6 (r = 0.8918).

A sample can be prepared for chromatography in 20 min; uncontaminated samples can be injected at 20-min intervals.

DISCUSSION

Fluorimetric procedures are commonly employed for routine cortisol analysis but they lack specificity and the administration of fluorogenic drugs, particularly spironolactone, invalidates the results. Competitive protein binding has improved specificity but several naturally occurring steroids possess a high affinity for the corticosteroid binding globulin and may disturb the determination in newborns, pregnancy and the adrenogenital syndrome. Radioimmunoassay procedures have similar problems.

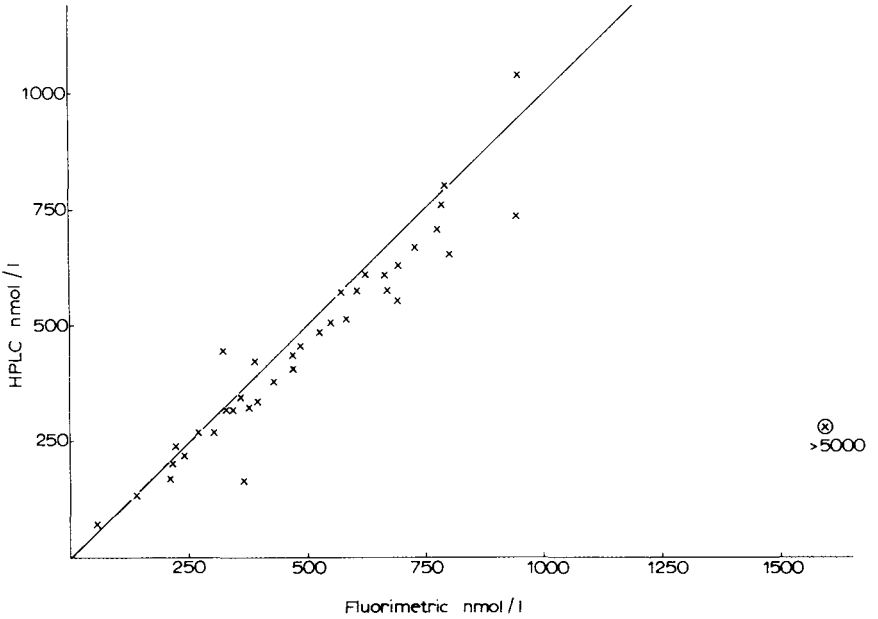

Fig. 5. Scattergraph of HPLC method vs. fluorimetry (Mattingly). The line shown is $x = y$. $r = 0.8792$; $y = 0.88\ x + 18$ (where y = HPLC). ⊗ = plasma from patient receiving spironolactone.

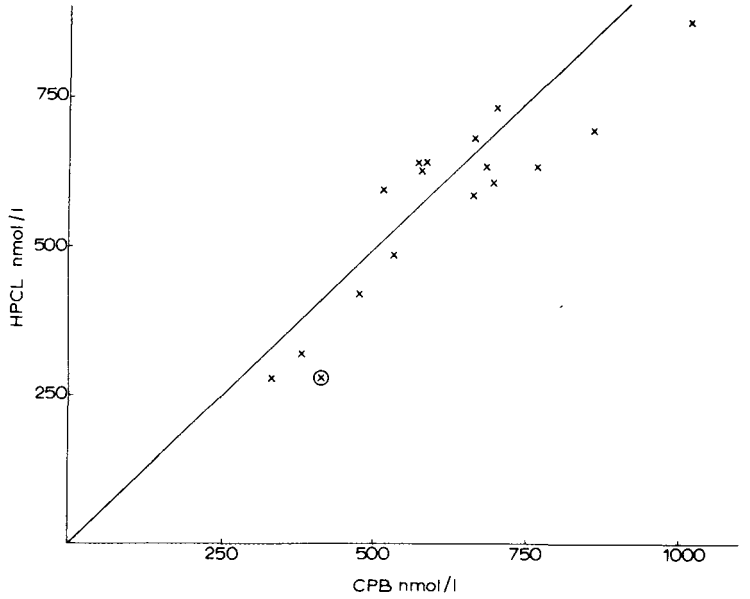

Fig. 6. Scattergraph of HPLC method vs. CPB (Cortipac). The line shown is $x = y$. $r = 0.8918$; $y = 0.835\ x + 57.6$ (where y = HPLC). ⊗ = plasma from patient receiving spironolactone.

The method described in this paper allows the specific determination of cortisol in plasma when other methods are invalidated. An example of a patient on spironolactone is shown in Figs. 5 and 6 and HPLC has been used in this laboratory for the determination of cortisol on several patients receiving this drug. The good correlations shown in Figs. 5 and 6 on patients receiving a variety of hospital prescriptions indicates that other commonly used drugs do not interfere with the HPLC procedure.

The simple reversed-phase procedure described is one likely to be available in any laboratory practising HPLC and is cheap and easy to run and maintain. In particular, it avoids the commonly encountered frustrations of normal-phase systems which use fully or partly water saturated solvents. It has proved robust in use with fast analysis time and no significant problems of late eluting peaks. It is possible to perform at least 20 analyses by this method in one day and so it may be used for a small routine workload, except for patient receiving large doses of prednisone or prednisolone, when the steroid therapy would require to be substituted. In patients receiving small doses the usual request is for the short-term synacthen response which could be readily measured.

ACKNOWLEDGEMENT

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CHROMBIO. 363

DETERMINATION OF 5-DIMETHYLAMINONAPHTHALENE-1-SULFONYL DERIVATIVES OF URINARY POLYAMINES BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and specific method for the determination of diamines and polyamines by ion-pair high-performance liquid chromatography is described. The 5-dimethylamino-naphthalene-1-sulfonyl derivatives of putrescine, 1,6-diaminohexane, spermidine and spermine are separated on a μBondapak C_{18} reversed-phase column with 1-heptanesulfonic acid and acetonitrile as the mobile phase. All compounds are eluted within 30 min using a programmed solvent gradient system. The method has a lower detection limit of 1 pmole on column.

Because of the simplicity of the method, its application provides a better means for closely monitoring patients undergoing treatment for various types of genito-urinary neoplastic diseases.

INTRODUCTION

A recent review of the literature has indicated that many new analytical procedures have been developed during the last five years for measuring the concentration of diamines and polyamines in various types of physiological fluids [1—4]. However, much of the work reported in the literature uses techniques of previously developed methods. The combined use of high-performance liquid chromatography (HPLC) and fluorometry [5—7] has contributed much to these recent developments. At the same time, the original work of Seiler and Wiechmann [8], who used 5-dimethylaminonaphthalene-1-sulfonyl chloride to derivatize di- and polyamines, has improved significantly upon the sensitivity of this method.

Because of the refinements in the reported procedures, an increased interest

has developed among researchers to apply these new methods to problems pertaining to various diseases states involving polyamines.

In this report, we describe a relatively simple and specific ion-pair HPLC method for quantifying 5-dimethylaminonaphthalene-1-sulfonyl derivatives of diamines and polyamines in derivatized urine specimens. The purpose of this study is to offer to the clinician, an extremely sensitive method which may be used as a tumor marker technique in detecting and closely monitoring patients with various types of genito-urinary neoplastic diseases.

MATERIALS* AND METHODS

A Waters Model APC/GPC-204 liquid chromatograph was used throughout this study. The system consisted of two Model 6000A high-pressure pumps, a Model 660 solvent programmer, and U6K loop injector. An Aminco Fluoro-Colorimeter equipped with a 365-nm excitation and a 510-nm emission filter was employed for fluorometric detection. A Houston Instrument Omni-Scribe A5000 dual pen recorder and a Columbia Scientific Industries Supergrator-3 integrator recorded and integrated all peak areas. Spectroquality acetonitrile (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.) mixed with PIC B-7 reagent (1-heptanesulfonic acid, Waters Assoc., Milford, Mass., U.S.A.) was used in a gradient mode for separating the various polyamines. Standard solutions of each compound were prepared by using 99% putrescine, 99% spermidine, 97% spermine and 98% 1,6-diaminohexane (Aldrich, Milwaukee, Wisc., U.S.A.). Dansyl chloride (5-dimethylaminonaphthalene-1-sulfonyl chloride, 100 mg/ml, Pierce, Rockford, Ill., U.S.A.) was used to derivatize the urinary polyamines.

PROCEDURES

Dansylated polyamines were prepared as follows: Two hundred microliters of hydrolyzed urine, prepared as described below is pipetted into 13 mm × 100 mm silanized glass tubes. Twenty microliters of a 100 n*M*/ml solution of 1,6-diaminohexane (internal standard), 280 μl of 0.5 *M* carbonate buffer (pH 9.2), 100 mg of anhydrous potassium carbonate, and 500 μl of 10 mg/ml of dansyl chloride in acetone were added and thoroughly mixed. The tubes were sealed with parafilm and the samples were incubated in the dark at 54° for 60 min. At the end of the incubation time, the reacted samples were allowed to cool to room temperature. The dansylated polyamines were extracted into 1 ml of ethyl acetate. The samples were thoroughly mixed. Five μl of the ethyl acetate extract containing the dansylated polyamines were injected onto the column for analysis.

A prepacked 300 × 3.9 mm I.D. μBondapak C_{18} column was employed to chromatograph the dansylated polyamines. μBondapak C_{18} is a 10-μm particle size packing material, which is designed for both analytical and semi-pre-

*The manufacturers' names and products are given as scientific information only and do not constitute an endorsement by the United States Government.

parative separations. The mobile phase consisted of 0.02 *M* solution of 1-heptanesulfonic acid combined with acetonitrile. The PIC B-7 reagent was prepared by mixing 40 ml of the pre-package reagent with 460 ml of glass distilled water. The pH of the solution was 3.40. A concave gradient (curve No. 8, solvent programmer) was used to elute the various dansylated polyamines from the column. Curve No. 8 may be produced in any dual pumping chromatographic system by using the below formulae.

$$\% \text{ flow from pump B} = (FC - IC)\left(\frac{t}{T}\right)^m + IC \quad (1)$$

$$\% \text{ flow from pump A} = 100\% - \% \text{ flow from pump B} \quad (2)$$

where: FC = final concentration; IC = initial concentration; t = time into the run; T = time for the total gradient run; m = 3.00.

Gradient parameters were 50% acetonitrile and 50% 1-heptanesulfonic acid at zero time. Upon injection, the acetonitrile was increased from 50% to 80% within a 20-min period. Total analysis time was 30 min. Flow-rate for the dual pumping system was 2 ml/min. Column pressures ranged between 1200 and 1500 p.s.i. All separations were performed at ambient temperatures. 1,6-Diaminohexane was used as an internal standard. Each specimen was run in duplicate to ensure reproducibility. Peak areas were measured by an on-line computing integrator. The detection limit of the method was 1 pmole on column with a signal-to-noise ratio of 3 to 1.

SAMPLES

Urine specimens collected from 20 normal subjects and 85 patients undergoing therapy for a variety of urologic malignancies were used for this study. One-milliliter volumes of urine were mixed with an equal volume of concentrated hydrochloric acid and incubated at 100° for 14 h. Analyses were performed immediately or several days later.

RESULTS AND DISCUSSION

The importance of early detection in various types of neoplasm is difficult to overemphasize. The constant search by investigators to develop a simple and specific test to reveal the presence of neoplastic diseases, long before the clinical symptoms become apparent has always been a major goal of the modern clinician. It is the purpose of this report to investigate the usefulness of the urinary polyamines for developing such a test.

An ion-pair reversed-phase HPLC procedure was developed to separate polyamines in urine specimens from normal subjects and patients with known malignancies. From a series of standard solutions and experimental samples, the application of the new method is demonstrated by the chromatograms depicted below. Figs. 1 and 2 represent the separation of a standard solution containing the dansylated derivatives of putrescine, spermidine, and spermine. 1,6-Diaminohexane was incorporated into both the standards and experimental samples for normalizing the values obtained for each separation. The chroma-

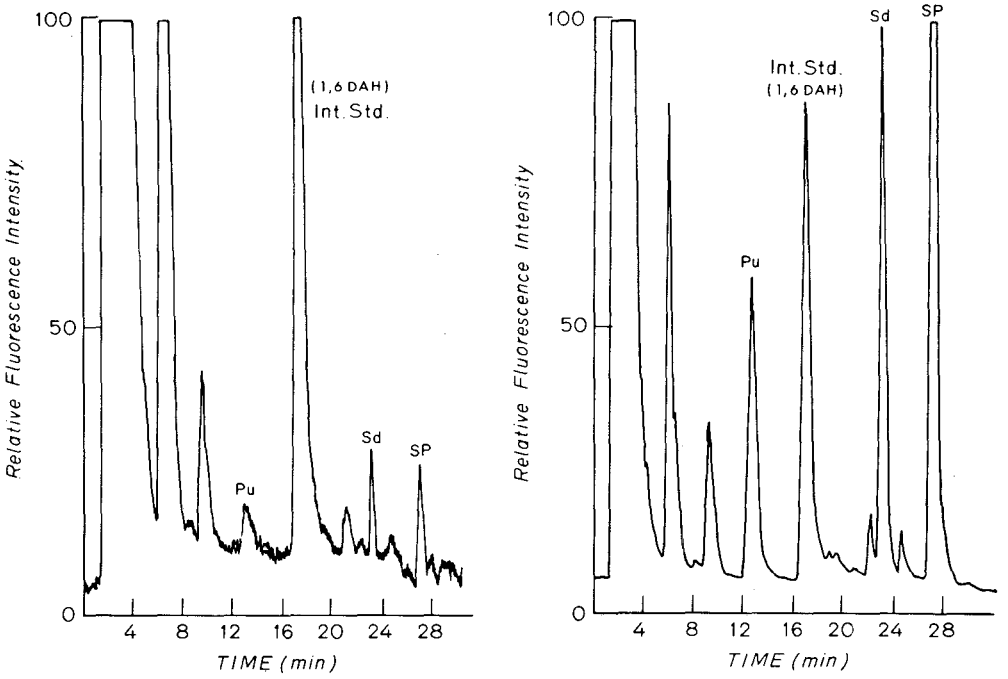

Fig. 1. Separation of a standard solution containing 1.10 pmole of putrescine (Pu), spermidine (Sd), and spermine (Sp). Column: 30 cm × 3.9 mm μBondapak C_{18}. Mobile phase: gradient mode 50% acetonitrile—50% 1-heptanesulfonic acid (zero time) 50%—80% acetonitrile (20 min), 2 ml/min. Internal standard: 1,6-diaminohexane (1,6DAH). Meter multiplier: 0.1.

Fig. 2. Chromatogram of a 100-pmole sample of polyamine standard detected at 365-nm excitation and 510-nm emission. Meter multiplier: 0.1. Abbreviations as in Fig. 1.

togram shown in Fig. 1 represents the lower detection limit of the method (1.10 pmole). A higher concentration (100 pmole) of the standard solution was also applied to the column to determine the optimum operational range of the method (Fig. 2). Linearity was observed for all concentrations of polyamines used in this study (25 pmole—1 nmole). The correlation coefficients for putrescine, spermidine, and spermine were 0.923, 0.961 and 0.942, respectively.

Based on these initial separations, urine specimens with polyamines values between 25–100 pmole were also analyzed. Figs. 3 and 4 are chromatograms showing the separation of dansylated polyamines prepared from the urine of normal and abnormal subjects. The normal urine samples were analyzed to establish baseline values for putrescine, spermidine, and spermine. From the results obtained, a 2—3-fold increase was noted between the control group and the experimental group. The results are shown in Table I.

In the patients with known carcinomas of the kidney, prostate, bladder and testis, a two-fold increase in the mean values of putrescine and spermidine were noted. At the same time, no appreciable differences were seen between the spermine values of the two groups. Sanford et al. [9] have suggested that a significant increase in the concentration of putrescine and spermidine in

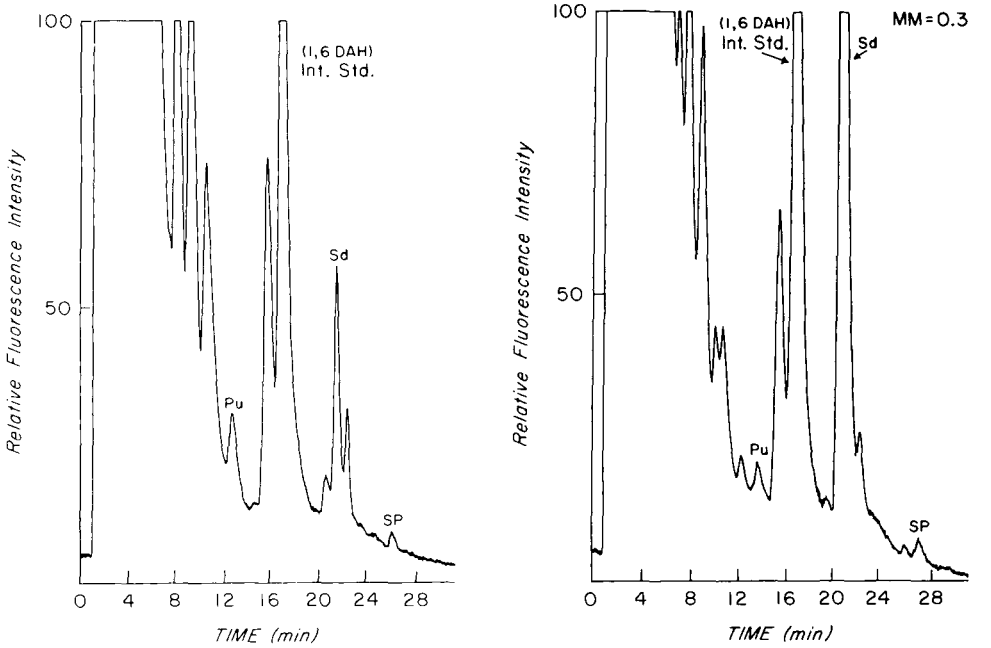

Fig. 3. Chromatogram showing a dansylated urinary polyamine specimen from a normal subject containing 1,6DAH (internal standard). Sample volume: 5 μl. Meter multiplier: 0.3. Column temperature: ambient conditions. Abbreviations as in Fig. 1.

Fig. 4. Chromatogram of a dansylated urine specimen from a carcinoma patient. Meter multiplier: 0.3. Abbreviations as in Fig. 1.

patients with genito-urinary diseases from that of the normal subject is a reliable indicator for use as a tumor marker.

Our HPLC technique is capable of accomplishing the parameters prescribed for making a distinction between normal subjects and patient with known genito-urinary carcinomas. Therefore, the use of this new analytical procedure holds great promise as a tumor marker technique. It achieves the degree of sensitivity and specificity desired in polyamine profiling which has been unattainable in earlier methodologies.

TABLE I

URINARY POLYAMINES VALUES (mg/24 h) OF 20 NORMAL SUBJECTS (NS) VERSUS 85 GENITO-URINARY CARCINOMA PATIENTS (CP)

	Putrescine		Spermidine		Spermine	
	Range	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.
NS	0.22—3.18	0.98 ± 0.49	0.26—18.66	4.57 ± 1.02	U.D.*—0.75	0.31 ± 0.09
CP	0.25—5.38	2.09 ± 0.70	0.45—47.96	9.24 ± 2.98	U.D.*—1.47	0.23 ± 0.13

*U.D. = Undetectable.

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DETERMINATION OF DOPA, DOPAMINE, DOPAC, EPINEPHRINE, NOREPINEPHRINE, α-MONOFLUOROMETHYLDOPA AND α-DIFLUOROMETHYLDOPA IN VARIOUS TISSUES OF MICE AND RATS USING REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION*

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SUMMARY

A method for the determination of catecholic amino acids and amines by reversed-phase ion-pair high-performance liquid chromatography with electrochemical detection has been developed. By using octanesulfonic acid for ion pairing and by optimising ionic strength, pH and methanol concentration of the mobile phase, separation was achieved of 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), epinephrine (EPI), and dopamine (DA). α-Difluoromethyldopa (DFMD) and α-monofluoromethyldopa (MFMD), two potent enzyme-activated irreversible inhibitors of aromatic amino acid decarboxylase were also separated from the natural catechols. Concentrations of catechols and inhibitors were measured in brains, hearts and kidneys of mice treated with small repeated doses of MFMD. The method has also been applied to the determination of catechols in other organs such as prostates and seminal vesicles of rats and in smaller tissues like mesenteric arteries. A semi-automated procedure making use of an automatic sample processor and a digital integrator permitted the analysis of as many as sixty samples per day.

INTRODUCTION

Recently it has been shown that α-difluoromethyldopa (DFMD) and monofluoromethyldopa (MFMD) are potent enzyme-activated inhibitors of aromatic L-α-amino acid decarboxylase (AADC; E.C. 4.1.1.26) in vitro and in vivo [1—3]. Biochemical studies of these inhibitors called for a sensitive and simple method for the determination of the inhibitors and of 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine (NE), epinephrine (EPI) and dopamine (DA). A great variety of methods exist for the determination of natural catechols in tissues and body fluids. The widely used fluorometric method [4] lacks specificity while the radioenzy-

*A preliminary report of these investigations has been presented at the *6th International Symposium, Biomedical Applications of Chromatography, Hluboká, Czechoslovakia, May 21—24, 1978.*

matic methods [5, 6], although very sensitive, are not suited for the determination of the inhibitors, DFMD and MFMD. Gas—liquid chromatography [7, 8] allows the study of a great number of metabolites but requires derivatization before analysis. Liquid chromatography with pre-column derivatization with fluorescamine [9], *o*-phthaldialdehyde [10] or dansyl chloride [11], coupled with fluorometric detection has been employed for the analysis of catecholamines. Separation on a cation-exchange column followed by reaction with *o*-phthaldialdehyde has been used for the analysis of catechols in biological samples [12]. Derivatization, besides being time consuming in the case of the pre-column reactions, cannot be readily applied to compounds such as DFMD and MFMD in which the nucleophilicity of the carboxylic acid and the nitrogen groups has been greatly reduced. Therefore, high-performance liquid chromatography (HPLC) with electrochemical detection [13—15] which combines sensitivity with efficiency seemed the method of choice to measure concentrations of the inhibitors (DFMD and MFMD) and of the catechols in the same sample.

Reversed-phase ion-pair chromatography or soap chromatography [16] combines the advantages of reversed-phase and ion-exchange chromatography. By using octanesulfonic acid (OSA) as anionic modifier and by varying methanol concentration, ionic strength and pH of the mobile phase and the concentration of the anionic modifier, we established the optimal conditions for the separation of the catechols and of the inhibitors MFMD and DFMD. The consequences of inhibition of AADC by MFMD, the more potent inhibitor of AADC, on catechol levels in brain, heart and kidney of mice are reported In addition, catechol levels in prostates, seminal vesicles and mesenteric arteries of untreated rats have been measured.

MATERIALS AND METHODS

Chemicals

The catechol standards norepinephrine·HCl (NE), epinephrine (EPI) , dopamine·HCl (DA), 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxyphenylglycol (DOPEG), and α-methyldopa (α-MeDOPA) were purchased from Sigma (St. Louis, Mo., U.S.A.) and 3,4-dihydroxybenzylamine·HBr from Aldrich (Beerse, Belgium). D,L-α-Difluoromethyldopa (DFMD; RMI 71801) and D,L-α-monofluoromethyldopa (MFMD; RMI 71963) were synthesized in our Centre. Heptanesulfonic acid and octanesulfonic acid sodium salt were obtained from Eastman Kodak (Rochester, N.Y., U.S.A.) and ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) from Carl Roth (Karlsruhe, G.F.R.). All other reagents were reagent grade from E. Merck (Darmstadt, G.F.R.).

Animals

Male CD1 albino mice (20—25 g) and male Sprague Dawley rats (200—300 g) from Charles River (Saint Aubin les Elbeuf, France) were used throughout these studies.

Chromatography

The high-performance liquid chromatograph consisted of a model 6000A

solvent delivery system, a U6K injector for the early experiments and a μBondapak C_{18} column (10-μm particle size range, 30 cm × 3.9 mm I.D.) all from Waters Assoc. (Milford, Mass., U.S.A.). For some experiments a LiChrosorb RP-18 column (10-μm particle size, 25 cm × 4.6 mm I.D.) from H. Knauer (Oberursel, G.F.R.) was used. An automatic sample injection system WISP model 710 from Waters Assoc. was used for the majority of the measurements. The pre-column (7 cm × 2 mm I.D.) filled with Partisil-10 ODS from Whatman (Clifton, N.J., U.S.A.) was refilled every week and was used to protect the main column. The detector was a Model LC-15 electrochemical detector from Bioanalytical Systems (West Lafayette, Ind., U.S.A.). The electrode was a wax-impregnated carbon paste. Its potential was maintained at + 0.9 V versus a silver—silver chloride reference electrode. The signal was recorded on a Kontron W+W recorder (Zürich, Switzerland) and the peak areas were determined with an Autolab System I integrator from Spectra Physics (Santa Clara, Calif., U.S.A.). The flow-rate was usually 1 ml/min.

Solvents

Water with resistivity greater than 10 $M\Omega \cdot cm^{-1}$ was obtained from a Millipore "Milli Q" system. Methanol, spectroscopic grade, was from E. Merck. The buffer consisted of 2 volumes of 0.02 *M* citric acid and 1 volume of 0.02 *M* Na_2HPO_4. The mobile phase was a 85:15 (v/v) mixture of this buffer with methanol (the amount of methanol depending on the capacity of the column) and made 2.5×10^{-3} *M* with sodium octylsulfonate and 5×10^{-5} *M* with Na_2EDTA. The pH was then adjusted to the desired value with concentrated phosphoric acid, usually to 3.25—3.35. The solvent mixture was filtered under vacuum through a 0.45-μm Millipore HA type filter before use.

Standards

Catechol stock solutions were prepared in 5×10^{-2} *M* $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ and 0.05% (w/v) Na_2EDTA at a concentration of 1 mg catechol per 100 ml. They were freshly prepared every 10 days and stored at 5°. Adequate standard solutions were obtained by diluting these stock solutions 50 times with 0.05 *N* $HClO_4$. Volumes of 25 μl or 50 μl were injected either with a Hamilton microsyringe or with the automatic sample injector.

Sample preparation

Extraction of the catechols followed a known procedure [17]. The tissues were homogenized in 2 or 4 ml (depending on the size of the tissues) of 0.4 *M* $HClO_4$ containing 0.05% (w/v) Na_2EDTA and 0.1% (w/v) $Na_2S_2O_5$, and α-MeDOPA (between 100 and 250 ng/ml depending on the sizes of the samples) as the internal standard. After centrifugation, the supernatant was added to 100 mg or 300 mg alumina and buffered to pH 8.0—8.4 with 0.1 *M* tricine containing 2.5% (w/v) Na_2EDTA and 2.1% (w/v) sodium hydroxide. The vials were shaken for 10 min on a reciprocal shaker, the supernatant removed and the alumina washed three times with 10 ml of water. Finally the catechols were eluted from the alumina by agitation for 10 min with 1 ml of 0.05 *M* $HClO_4$ containing 0.1% (w/v) $Na_2S_2O_5$ and 0.025% (w/v) Na_2EDTA. The supernatant was filtered off and either stored at —30° for later determination or transferred into minivials (250-μl size) of the automatic injector.

Recovery of the extraction procedure

Two series of experiments were conducted in order to determine the recovery of the various catechols from tissues. Known amounts of the catechols were dissolved in the 0.4 *M* $HClO_4$ solution and these solutions were used for tissue homogenization. In the first experiment, 2 ml of the solution were added to 100 mg of alumina and taken through the whole adsorption and extraction procedure outlined in the previous section. In a second experiment, 2 ml of the same solution were added to brains or hearts of mice, homogenized and extracted via the same procedure. The levels of catechols thus found were compared to the levels obtained in control brains or hearts extracted with perchloric acid. The recoveries from tissues were calculated from the difference between the values, with and without added standards.

RESULTS AND DISCUSSION

Determination of the chromatographic conditions

The goal of this study was to achieve after alumina adsorption the separation of all the catechols expected to be present in tissues. Apart from the inhibitors, DOPA, DOPAC, NE, EPI, DA and DOPEG are the most abundant. In order to make the method quantitative and reproducible, an internal standard, carried through the extraction procedure, was included. In similar studies α-MeDOPA and 3,4-dihydroxybenzylamine [18, 19] have been used. We chose α-MeDOPA because of its structural analogy with the inhibitors.

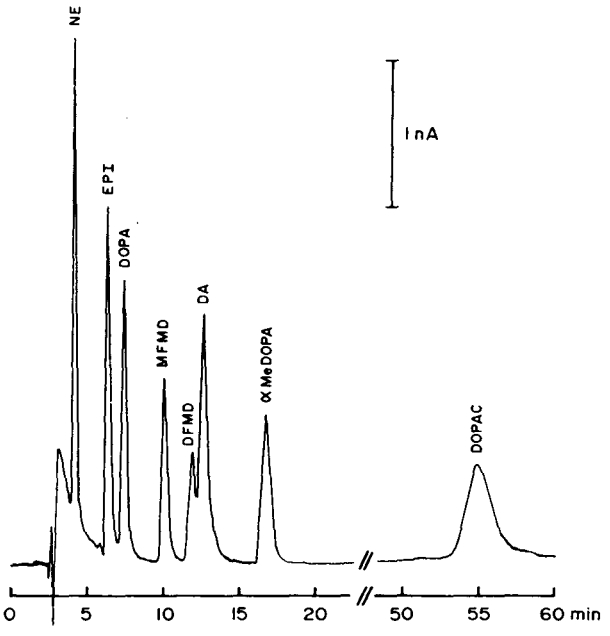

Fig. 1. Chromatogram of a standard mixture of 5 ng of each catechol. Column, LiChrosorb RP-18, 10 μm; mobile phase, 2:1 (v/v) mixture of citric acid (0.05 *M*) and Na_2HPO_4 (0.05 *M*) with EDTA (5×10^{-5} *M*); pH = 3.24; sample volume, 25 μl; flow-rate, 1 ml/min; temperature, ambient; electrode potential, + 0.9 V vs. Ag/AgCl reference electrode.

As was expected, the separation of catecholic compounds on reversed-phase packed columns depended strongly on the methanol composition, the ionic strength and the pH of the mobile phase. Fig. 1 shows the chromatogram obtained with a 2:1 (v/v) mixture of 0.05 *M* citric acid and 0.05 *M* Na_2HPO_4 (pH 3.24). These conditions are similar to those already published [18] for the separation of NE and DA. A clean separation of all the catechols of interest, with the exception of DFMD and DA, was obtained. This method has two major drawbacks: the excessive retention times and the elution of NE close to the elution front. It had been shown previously that by adding an anionic detergent [16] the separation pattern of the catechols can be drastically changed [20, 21]. Fig. 2 shows the chromatogram obtained by using the same buffer at lower ionic strength but after addition of heptanesulfonic acid (3.5×10^{-3} *M*) and 14% methanol. The desired separation of all the compounds has now been achieved, but the retention times especially for DA are still unsatisfactory. By using OSA at a lower concentration (2.5×10^{-3} *M*) fairly similar results are obtained, as is shown in Fig. 3. 3,4-Dihydroxyphenylglycol (DOPEG), which does not appear on this chromatogram, is eluted very close to the elution front and does not interfere with the other catechols of interest. In order to obtain the required separation, the ionic strength of the buffer, the concentration of the anionic detergent and especially the pH, were optimised. Fig. 4 shows the variation of the retention times of the cate-

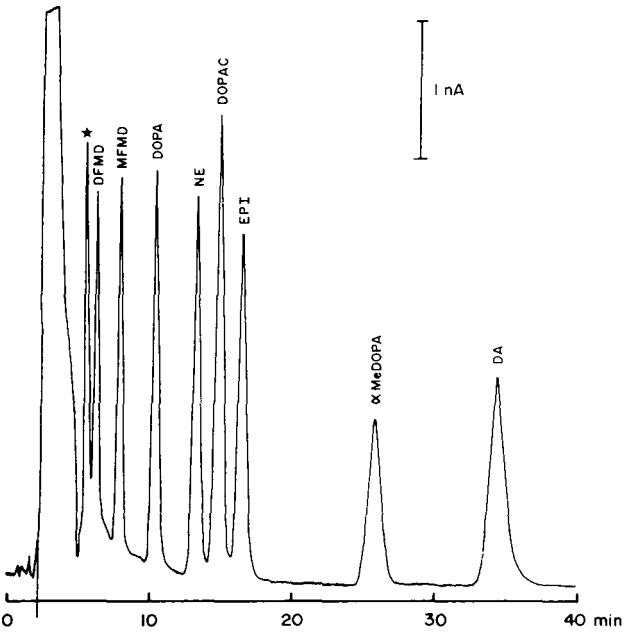

Fig. 2. Chromatogram of the same standard solution as in Fig. 1. Column LiChrosorb RP-18, 10 μm; mobile phase, 86:14 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol with heptanesulfonic acid (3.5×10^{-3} *M*) and EDTA (5×10^{-5} *M*); pH = 3.35; otherwise the conditions are the same as described in the legend to Fig. 1; ★, unknown peak deriving from the eluent.

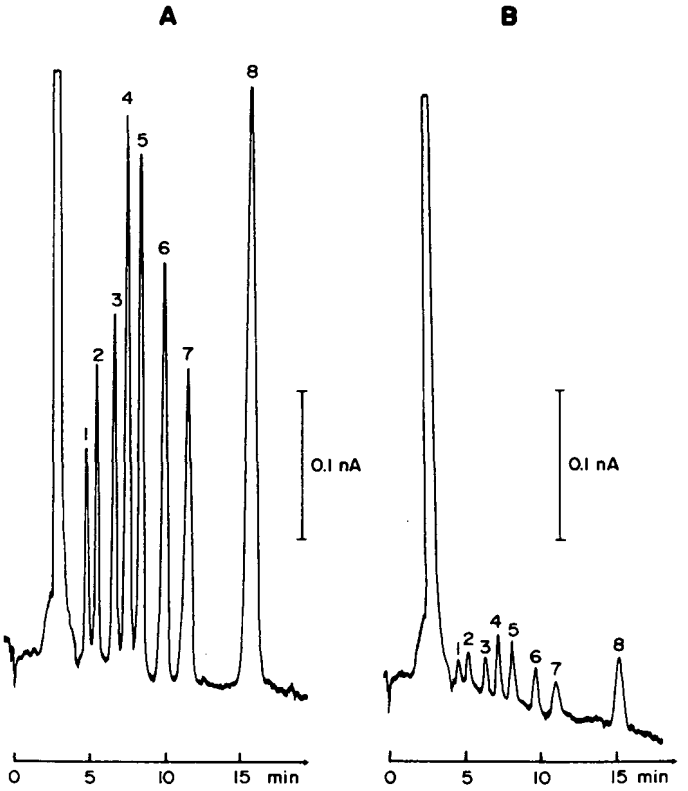

Fig. 3. Typical chromatograms showing the sensitivity of the method for standard solutions of catechols. Peaks: 1, DFMD; 2, MFMD; 3, DOPA; 4, DOPAC; 5, NE; 6, EPI; 7, α-MeDOPA 8, DA. Chromatographic conditions: column, μBondapak C_{18}; mobile phase, 87:13 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol with octanesulfonic acid (2.5×10^{-3} *M*) and EDTA (5×10^{-5} *M*); pH = 3.22; flow-rate, 1 ml/min; electrode potential, + 0.9 V vs. Ag/AgCl reference electrode; temperature, ambient. Trace A corresponds to 0.3 ng of 1, 2, 3 and 4; 0.35 ng of 5 and 6; 0.53 ng of 7 and 8. Trace B corresponds to 30 pg of 1, 2, 3 and 4; 35 pg of 5 and 6; 53 pg of 7 and 8.

chols with the pH of the eluent, when ionic strength, concentration of OSA and percentage of methanol are held constant. Two general statements can be made. Firstly, the retention times of the amines are independent of the pH of the eluent. This can be predicted from the fact that in the pH range of 2.4—3.5 the amino groups are completely protonated. Secondly, the retention times of the amino acids, especially those of DOPA and α-MeDOPA strongly increase as the pH decreases. This can be explained by the fact that ion-pair formation increases as the protonation of the carboxylic group increases (i.e. as the pH decreases) [22]. The pK_a values for DOPA (2.30 and 8.70 for the COOH and NH_2 groups respectively) [23], α-MeDOPA (2.4 and 8.8), DFMD (<1.7 and 7.05) and MFMD (1.7 and 8.10) [24] are in good agreement with the observed retention times. For MFMD and DFMD, the carboxylic groups remain mostly in the COO^- form and thus diminish the "amount" of ion-pair formation by repulsion with the negatively charged anionic detergent.

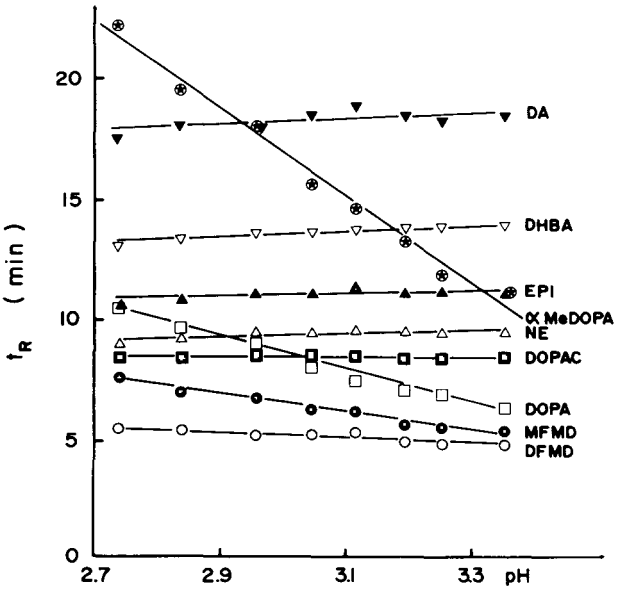

Fig. 4. Relationship between the retention times t_R and the pH of the mobile phase. Column, μBondapak C_{18} ; mobile phase, 85:15 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol, with octanesulfonic acid (2.3×10^{-3} *M*); pH adjusted to the different values with phosphoric acid or sodium hydroxide; flow-rate, 1 ml/min; temperature: ambient; electrode potential: + 0.9 V vs. Ag/AgCl reference electrode.

These results show that different chromatographic conditions can lead to similar separations. Different anionic compounds, detergents or simple anions [25, 26] will give similar results with a different order of elution for the different catechols. A further consequence is that one can compensate for the loss of resolution of a column by decreasing the methanol concentration, by increasing the amount of the OSA or by changing both and adjusting the pH. In this case peak broadening occurs.

All chromatograms shown above were obtained after addition to the eluent of a small amount of Na_2EDTA (5×10^{-5} *M*) which strongly complexes metal ions, significantly improves the stability of the baseline and prevents the occurrence of spurious peaks due to contamination by metal ions [27—29]. Fig. 3 shows typical chromatograms obtained in the conditions outlined above with 300—500 pg in A and 30—50 pg in B of each catechol in a standard mixture. This sensitivity has been obtained by controlling carefully the eluent (addition of Na_2EDTA) and by applying to the wax-impregnated carbon paste electrode a working potential of + 0.9 V versus a Ag/AgCl reference electrode.

The routine assays were conducted under operating conditions (eluent composition) similar to those of Fig. 3, but at a lower sensitivity similar to that of Figs. 1 and 2. For most of the samples, an automatic sample injector and a digital integrator were used. On a 24 h per day working basis, as many as sixty samples could be assayed. The main problems were rapid loss of resolution for some columns and variation of the sensitivity of the carbon paste electrode.

However, as mentioned above, the loss of resolution can be partly overcome by adjusting the chromatographic parameters. Into some columns as many as 5000 samples could be injected without sizable loss of capacity or resolution. The carbon paste electrode can be easily repacked and reconditioned overnight.

For several reasons, especially changes of temperature and eluent composition, variations in sensitivity are observed. Therefore calibration has to be repeated at regular time intervals. The use of internal standards is necessary.

Calibration and recovery experiments

Various volumes of freshly prepared standard solutions were injected. Peak height measurements and peak areas calculated by the integrator were found to be linear for all the constituents in the range of 0.2 to 20 ng.

Several recovery experiments with 500-ng amounts of the catechols with and without tissues were conducted as described in the methods section. The results for brains are presented in Table I. Other experiments with 100 and 250 ng of each catechol gave similar results. The recoveries expressed as the ratio of recovery of α-MeDOPA and of the other catechols, in presence and in absence of tissues, remained constant with percentage variations ranging from 1.1 for DA to 6.6 for DOPAC as calculated from the differences of the ratios of Table I. The reproducibility expressed as the relative standard deviations (coefficient of variation) of the recoveries in absence of tissues varied from 3.5% for DOPA to 8.7% for DOPAC. As expected the acids such as DOPAC and the amino acids gave lower recoveries than the corresponding amines. These results are comparable to published values [26, 29—31]. α-MeDOPA appeared well-suited as an internal standard. Similar recovery experiments with hearts showed analogous results but with somewhat greater variations in the recovery, presumably due to the fact that the hearts were not as completely homogenized as the brains.

TABLE I

RECOVERY OF CATECHOLS FROM ALUMINA IN ABSENCE AND PRESENCE OF BRAIN TISSUE

	In absence of tissues*		In presence of tissues**	
	Recovery (%) ± S.D., n = 5	Mean ratio to α-MeDOPA	Recovery (%) ± S.D., n = 10	Mean ratio to α-MeDOPA
MFMD	57.5 ± 2.7	1.18	49.1 ± 3.1	1.21
DOPA	62.8 ± 2.2	1.08	51.6 ± 3.6	1.15
DOPAC	47.6 ± 4.1	1.42	39.2 ± 5.1	1.52
NE	77.9 ± 6.8	0.87	65.1 ± 7.3	0.91
EPI	76.7 ± 3.6	0.88	66.1 ± 3.5	0.90
α-MeDOPA	67.8 ± 5.7	1	59.5 ± 5.0	1
DA	74.2 ± 5.3	0.91	64.3 ± 9	0.92

*Values are the means of five determinations with 500 ng of each catechol.

**Values are the means of ten determinations with 500 ng of each catechol added to whole brain of mice and with ten control brains extracted as outlined in the text.

Calculation of the results

For most of the experiments, 100 ng (for mesenteric artery), 250 ng (for half brain and heart), 500 ng (for whole brain, heart, adrenal, kidney and prostate) of α-MeDOPA were added to the perchloric acid used for the homogenization of the tissues. To take into account the possible variation in sensitivity, one or two injections of the standard (same volumes were used for the unknown samples) were made after every sixth sample. From their peak heights or peak areas, the response factors were calculated for each constituent and updated after every sixth sample. With these response factors, the concentrations of the unknown catechols were calculated, then divided by the recovery of α-MeDOPA and multiplied with the ratio of recovery given in Table I to obtain the final amounts of catechols in ng or in ng per g of tissue weight. With this procedure, changes in sensitivity and variations in recovery from one sample to another were taken into account.

Applications of the method to different tissues

Catechols in brain, heart and kidney of mice after treatment with MFMD. Fig. 5 shows the chromatograms of brains of mice injected every 12 h for 3 days with 5 mg MFMD/kg (B) or with 25 mg/kg (C) by the intraperitoneal (i.p.) route. MFMD is not detectable in chromatogram B, but the DOPA peak is increased and the NE and DA peaks are decreased in comparison to the control. In chromatogram C, MFMD is clearly detectable and DOPA is markedly increased whereas the concentrations of DOPAC, NE and DA are

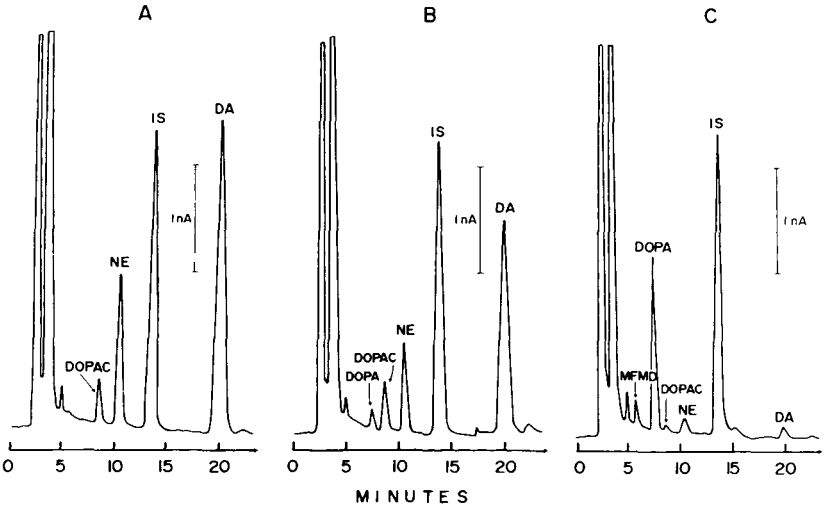

Fig. 5. Chromatograms of catechols from brains of mice, injected every 12 h for 3 days with saline (A), with 5 mg MFMD/kg/injection (B) or with 25 mg MFMD/kg/injection (C). α-MeDOPA as internal standard (IS). Chromatographic conditions: column, μBondapak C_{18}; mobile phase, 87:13 (v/v) mixture of the citrate—phosphate buffer (0.02 *M*) and methanol with octanesulfonic acid (2.5×10^{-3} *M*) and EDTA (5×10^{-5} *M*); pH = 3.25; flow-rate, 1 ml/min; electrode potential, + 0.9 V vs. Ag/AgCl reference electrode; temperature, ambient.

considerably reduced. Fig. 6 shows the effect of four different doses of MFMD on MFMD, DOPA, DOPAC, NE and DA. The results are expressed in ng/g wet tissue ± S.E.M. after correction for recovery. The results clearly indicate the potency of MFMD as an AADC inhibitor in mouse brain. The levels of NE and DA found in the control animals are in good agreement with published values [32].

Similar effects were obtained for heart, kidney and adrenals. The catechol levels found in heart and kidney at the two highest doses (6 × 5 and 6 × 25 mg/kg) are presented in Table II. At these doses, MFMD can be easily determined, the concentrations of NE and, to a lesser extent, DA decrease and there is a significant increase in DOPA.

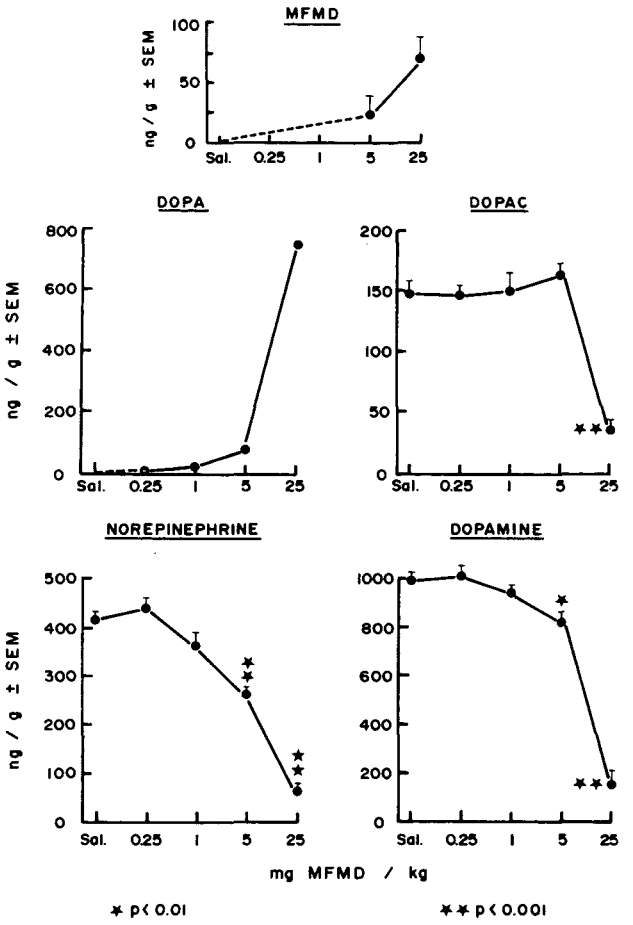

Fig. 6. Effect of repeated administration of small doses of MFMD on DOPA, DOPAC, NE, DA and MFMD content of mouse brain. Groups of 5 mice were injected i.p. every 12 h for 3 days with increasing doses of MFMD. They were sacrificed 6 h after the last injection. Five mice injected with saline served as controls. Half brains were homogenized in 0.4 *M* $HClO_4$ and processed as described in the experimental procedure. Values are expressed in ng/g wet weight ± S.E.M. * $p < 0.01$ and **, $p < 0.001$, Student's *t*-test.

TABLE II

CATECHOL LEVELS IN HEART AND KIDNEY OF MICE AFTER TREATMENT WITH MFMD

Values are given in ng per g wet weight ± S.E.M. (n = 5). Statistically significant differences compared to controls as determined by Student's t-test are shown by (*) $p < 0.05$, (**) $p < 0.005$, (***) $p < 0.001$, NS = not significant. Mice were injected every 12 h for 3 days via the i.p. route. Control animals received an equivalent volume of distilled water. Animals were sacrificed 6 h after the last dose.

	MFMD	DOPA	NE	EPI	DA
Heart					
Control	—	32 ± 14	650 ± 48	—	30 ± 6
6 × 5 mg/kg	30 ± 3	115 ± 21 (*)	400 ± 23 (**)	—	⩽15 ± 5 (*)
6 × 25 mg/kg	142 ± 40	248 ± 23 (***)	103 ± 23 (***)	—	⩽20 ± 10 (NS)
Kidney					
Control	—	⩽2.5 ± 2.5	300 ± 22	20 ± 17	13.3 ± 4.9
6 × 5 mg/kg	21 ± 5	181 ± 27 (***)	201 ± 32 (*)	61 ± 40 (NS)	11.5 ± 4.0 (NS)
6 × 25 mg/kg	193 ± 66	207 ± 23 (***)	70 ± 14 (***)	11 ± 6.5 (NS)	4.9 ± 0.5 (*)

Catechol determination in ventral prostate of rats. For the determination of catechols in the ventral prostate of male adult rats, two different methods were used: Method A followed essentially the experimental procedure outlined previously in the text by using 2 ml of 0.4 M $HClO_4$ (with $Na_2S_2O_5$, EDTA and 500 ng of α-MeDOPA as internal standard) for the tissue homogenization, then adsorption on 100 mg of alumina and finally extraction with 1 ml 0.05 M $HClO_4$. Method B used 2 ml of 5% trichloroacetic acid (with $Na_2S_2O_5$, EDTA and α-MeDOPA) for tissue homogenization. Table III presents the results expressed in ng/g tissue ± S.E.M. The mean tissue weight was 591 ± 25 mg (n = 9) in method A and 556 ± 25 mg (n = 16) in method B. The values found for NE are in good agreement with published values [33] obtained with a fluorometric method, but the values found for DA are much lower than the values reported. The HPLC method of catechol determination in prostate allowed us to study the effect on catechol levels of a combined treatment with DFMD and DOPA as compared to treatment with DOPA and DFMD alone [34].

Catechol concentrations in seminal vesicles. Seminal vesicles of rats were homogenized using the same methods A and B as were used for the prostates. The mean tissue weight after removing secretions was 169 ± 9 mg (n = 9) in method A and 167 ± 7 (n = 10) in method B. The results presented in Table IV agree well with published values for NE [35]. There are no significant differences between the values found by the two methods.

TABLE III

CATECHOL LEVELS IN RAT VENTRAL PROSTATE AND SEMINAL VESICLE

	DOPA		DOPAC		NE		DA	
	Method A*	Method B**	Method A	Method B	Method A	Method B	Method A	Method B
Prostate*** ng/g wet weight ± S.E.M.	⩽10 ± 5	⩽10 ± 5	<5	<5	666 ± 56	637 ± 20	43 ± 6	30 ± 3
Seminal vesicle† ng/g wet weight ± SEM	<5	<5	5 ± 5	19 ± 5	1752 ± 90	1795 ± 76	167 ± 13	152 ± 11

*Tissues were homogenized in 2 ml of 0.4 *M* $HClO_4$ with 500 ng of α-MeDOPA as internal standard. After adsorption on alumina the catechols were extracted with 1 ml of 0.05 *M* $HClO_4$ and processed as described in the text.

**Tissues were homogenized in 5% (w/v) trichloroacetic acid and then processed as described in the first footnote.

***Values are the means of $n = 9$ prostates for method A and $n = 16$ for method B.

†Values are the means of $n = 9$ seminal vesicles for method A and $n = 10$ for method B.

Determination of catechols in small tissue samples: application of the method to mesenteric arteries. For small tissue samples, such as the mesenteric arteries, portal veins and brain areas the procedure outlined in the experimental part has been scaled down. 0.5 ml of 0.4 *M* $HClO_4$ containing 100 ng of α-MeDOPA as internal standard (with $Na_2S_2O_5$ and EDTA) were used for the tissue homogenization (for about 40 mg of tissue). Adsorption was done on 25 mg of alumina and back extraction with 100 μl of 0.1 *M* $HClO_4$. Aliquots (50 μl) of this extract were injected and the ionic strength of the buffer used for the preparation of the eluent was increased to 0.035 *M* in order to avoid changes in baseline and retention times. Table IV shows the results obtained with mesenteric arteries. Their mean weight was 40 ± 4 mg ($n = 9$). This allowed us to study the effect of MFMD treatment on the catecholamine levels in mesenteric arteries and in portal veins [36].

General remarks. From the above examples, it is apparent that the homogenization and extraction procedure has to be adapted to the tissue size of each organ. An internal standard is essential for verification of the reproducibility of the extraction procedure within an experiment and also allows the

TABLE IV

CATECHOL LEVELS IN MESENTERIC ARTERIES

	DOPA	DOPAC	NE	DA
ng per organ ± S.E.M. ($n = 9$)	7.6 ± 3	10.4 ± 1.3	58.3 ± 4.6	6.4 ± 1.2
ng per g wet weight ± S.E.M. ($n = 9$)	183 ± 66	279 ± 42	1600 ± 240	169 ± 97

comparison of values from experiments performed at different times. For example, for mouse brain, the NE and DA values found over a five month period remained constant, 406 ± 6 and 1012 ± 16 ng/g, respectively (mean ± S.E.M., $n = 74$).

By scaling down the volumes used for the homogenization and extraction procedure, the catechol concentrations in small tissue samples can be easily measured. By working at a higher sensitivity such as in chromatogram B of Fig. 3 and by carefully controlling the temperature, 50—100 pg of catechols may be measured with a signal-to-noise ratio greater than 5. This sensitivity is adequate for the measurements of catechols in biological fluids, such as serum or plasma [26, 30, 31].

By using an automatic injector and a digital peak integrator fifty to sixty samples could be analyzed per day.

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Biomedical Applications

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SIMPLIFIED DETERMINATION OF LORAZEPAM AND OXAZEPAM IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Lorazepam and oxazepam in plasma and urine were measued by gas chromatography—mass spectrometry. Oxazepam was used as an internal standard in the assay of lorazepam and vice versa. After removal of interfering substances with *n*-hexane, the drugs were extracted with benzene and converted to N_1,O_3-bistrimethylsilyl derivatives. Glucuronide forms of the drugs were extracted after hydrolysis with β-glucuronidase. A common fragment ion at *m/e* 429 was used to monitor the two drugs. The sensitivity was 2 ng/ml for both drugs, which was sufficient to determine plasma and urine concentrations after therapeutic doses to humans.

INTRODUCTION

Lorazepam and oxazepam are the 1,4-benzodiazepine class of tranquillizers bearing a hydroxy group at the C-3 position of the benzodiazepine ring. Several methods of determining these drugs in biological fluids have been reported and applied in clinical studies [1—7]. However, when we were faced with the necessity of measuring lorazepam in plasma in response to the request by clinicians, it was considered desirable to develop a simpler, less time-consuming assay to deal with a large number of samples. This paper describes a simplified method of determining lorazepam and oxazepam in plasma and urine. The method involves solvent extraction of the sample and gas chromatography—mass spectrometry of the silylated extract.

EXPERIMENTAL

Chemicals

Lorazepam and oxazepam tablets were obtained commercially (Wypax®, 0.5-mg tablet, Yamanouchi Pharmaceutical, Tokyo, Japan; Hilong®, 10-mg tablet, Banyu Pharmaceutical, Tokyo, Japan). Lorazepam standard was kindly supplied by Wyeth Japan Corporation (Tokyo, Japan). Oxazepam standard was obtained by extraction from commercial tablets with acetone and recrystallization from ethyl acetate–*n*-hexane; m.p. 200.5° (lit. 200–205° [8]); anal. calcd. for $C_{15}H_{11}ClN_2O_2$: C, 62.8, H, 3.87%; found: C, 62.2, H, 3.88%. Bis(trimethylsilyl)acetamide (BSA) and bovine liver β-glucuronidase (13,000 Fishman units per ml) were products of Tokyo Kasei Kogyo (Tokyo, Japan) and Tokyo Zoki (Tokyo, Japan), respectively. Other reagents used were commercial preparations and of analytical grade.

Gas chromatography–mass spectrometry

An Hitachi RMU-6MG combined gas chromatograph–mass spectrometer fitted with an accelerating voltage alternator was employed. Separation was accomplished using a glass column (1 m × 3 mm I.D.) packed with 3% OV-1 on Chromosorb W AW DMCS (80–100 mesh). The column temperature was maintained isothermally at 210°, while the flash and separator temperatures were held at 270°. The flow-rate of carrier gas helium was 30 ml/min. The ionization potential and trap current were 20 eV and 80 μA, respectively. The entrance and collector slits of the mass spectrometer were adjusted to 0.4 mm. The multiplier voltage supply was set at 1.2–2.0 kV.

Determination of unchanged drug in plasma and urine

To each plasma or urine sample (1 ml) either oxazepam (25 ng) or lorazepam (150 ng) was added as an internal standard. After addition of hydrochloric acid (0.1 *M*, 2 ml), the plasma sample was washed with *n*-hexane (4 ml). The aqueous layer was then saturated with sodium bicarbonate and washed again with *n*-hexane (4 ml). Urine sample was directly washed with *n*-hexane (4 ml) twice. Each aqueous layer was then extracted with benzene (4 ml). The benzene layer was evaporated to dryness under reduced pressure and a 40% solution of BSA in ethyl acetate (50 μl) was added to the residue. The mixture was kept in a water-bath at 60° for 30 min. After cooling, an aliquot (2 μl) was injected into the column for gas chromatography–mass spectrometry. A fragment ion at *m/e* 429 was used to monitor both lorazepam and oxazepam. The amount of the drug in each sample was calculated by measuring the peak height ratio and referring to the standard curve. A standard curve was prepared by subjecting the control plasma or urine samples, to which known amounts of the drugs had been added, to the above procedure. The peak height ratio of the drug and internal standard was plotted against the concentration of the corresponding drug.

Determination of glucuronide conjugate in plasma and urine

To each plasma or urine sample (1 ml) oxazepam (25 ng) or lorazepam (150 ng) was added as an internal standard. After addition of acetate buffer (0.5 *M*,

2 ml, pH 5.0) and β-glucuronidase (1000 U), the mixture was incubated at 37° for 24 h. The plasma mixture was then acidified with hydrochloric acid (1 *M*, 0.2 ml) and washed with *n*-hexane (4 ml). The aqueous layer was saturated with sodium bicarbonate and washed again with *n*-hexane (4 ml). Urine mixture was washed twice with *n*-hexane (4 ml). The subsequent procedure was followed as described for the unchanged drug. The quantity of the glucuronide conjugate was calculated by subtracting the drug concentration determined before hydrolysis from that after hydrolysis.

Extraction recoveries

Control plasma samples (1 ml) containing lorazepam (30 ng) or oxazepam (300 ng) were carried through the above procedure without addition of the internal standard. Oxazepam (25 ng) or lorazepam (150 ng), dissolved in benzene, was added to the benzene extract and the benzene solution was evaporated to dryness under reduced pressure. The subsequent procedure was carried out as described above. Recoveries were calculated by comparing the peak height ratios with those obtained when each compound and internal standard, dissolved in benzene, were processed without the extraction procedure.

Human studies

After overnight fasting, three male subjects received 1.5 mg of lorazepam as three 0.5-mg tablets. Another three subjects received a 10-mg tablet of oxazepam. Blood samples were obtained from the antecubital vein with heparinized syringes and centrifuged at 980 *g* for 15 min. Urine samples were collected for 24 h. The plasma and urine samples were stored at −20° until required for assay.

RESULTS AND DISCUSSION

1,4-Benzodiazepine drugs usually contain electronegative groups so that analysis can be done by electron-capture gas chromatography [9]. Earlier methods depended on the acid-catalyzed hydrolysis of the 1,4-benzodiazepine nucleus to the corresponding aminobenzophenone derivative before chromatography [10]. Knowles and co-workers [1, 2] applied this principle to lorazepam and oxazepam and established an assay method which was sensitive enough to determine drug levels as low as 10 ng/ml (lorazepam) or 20 ng/ml (oxazepam). Plasma concentrations of these drugs in man have been determined by this method. Determination of lorazepam and oxazepam as benzophenones is not necessarily free from drawbacks. Theoretically, the parent drug and certain metabolites [11, 12] may be hydrolyzed to the same benzophenone, which impairs the specificity of the assay. Practically, the lengthy hydrolysis procedure is inconvenient for analyzing a large number of samples.

More recently, methods have been developed in which the intact benzodiazepine molecules are directly subjected to gas chromatography after suitable clean-up [13, 14]. This modification greatly simplified the previous method which required hydrolysis. Lorazepam and oxazepam presented a special

problem in the application of this modification because of the presence of a hydroxyl group at the C-3 of the benzodiazepine nucleus. They undergo thermal rearrangement to quinazoline derivatives in the gas chromatographic column with the result that the reproducibility of the assay is not good [4]. Two approaches have been made to solve this problem. One is an attempt to make the rearrangement quantitative by selecting the solvent in which the drug is injected into the column. De Groot et al. [4] used toluene for this purpose and determined without hydrolysis the lorazepam level in human plasma after an overdose. The sensitivity, however, seemed insufficient to measure drug concentrations occurring after therapeutic doses, although recently Greenblatt et al. [7] have reported better sensitivity with practically the same method. The other approach is to derivatize these drugs into forms that are unable to rearrange. De Silva et al. [15], in their comprehensive and informative study, worked out several general methods for determining a wide range of 1,4-benzodiazepines including lorazepam and oxazepam. These two drugs were determined apparently as their monotrimethylsilyl (TMS) derivatives. Still, the procedure seemed to require considerable sample purification, consisting of extraction and back extraction at different pH's before derivatization. Vessman et al. [6] derivatized oxazepam into its dimethyl form by the procedure known as extractive alkylation and established a method which is capable of determining oxazepam levels as low as 1 ng/ml. The method reported in this paper falls into the same category as these two methods in that the drugs are derivatized to avoid thermal rearrangement. The characteristic of the present method lies in its greater simplicity, as compared with the above methods, without sacrificing sensitivity.

Plasma samples were washed with *n*-hexane at an acid pH and, after neutralization, again washed with *n*-hexane, while urine samples were washed directly with *n*-hexane. This procedure removed interfering endogenous materials without loss of the drugs. Subsequently, the drugs were extracted with benzene. The extraction recoveries of lorazepam and oxazepam added to plasma at a concentration of 30 ng/ml and 300 ng/ml were 87.8 ± 0.6% and 76.8 ± 1.8% (mean ± S.E.M., n = 6), respectively. The drugs extracted were derivatized into the N_1,O_3-bis-TMS forms on treatment with BSA and subjected to gas chromatography—mass spectrometry. The introduction of two TMS groups under the conditions employed was confirmed from the mass spectra of the two derivatives (Fig. 1), which showed corresponding molecular ions at *m/e* 464 (lorazepam) and *m/e* 430 (oxazepam), respectively. Both spectra had an intense fragment ion at *m/e* 429, which was used to monitor the two drugs. The bis-TMS derivatives were preferred over the O_3-mono-TMS derivatives because of less adsorption onto the column and less tailing of the chromatographic peaks. The chromatograms obtained from human plasma samples are shown in Fig. 2. The peaks of oxazepam and lorazepam appeared at retention times of 1.7 min and 2.7 min, respectively, and were well separated from endogenous peaks. Two endogenous peaks were observed in drug-free control plasma at 3.3 min and 11.0 min, the latter being identified as cholesterol from its mass spectrum. When the plasma samples were directly extracted with benzene, additional endogenous peaks appeared within 3 min and disturbed

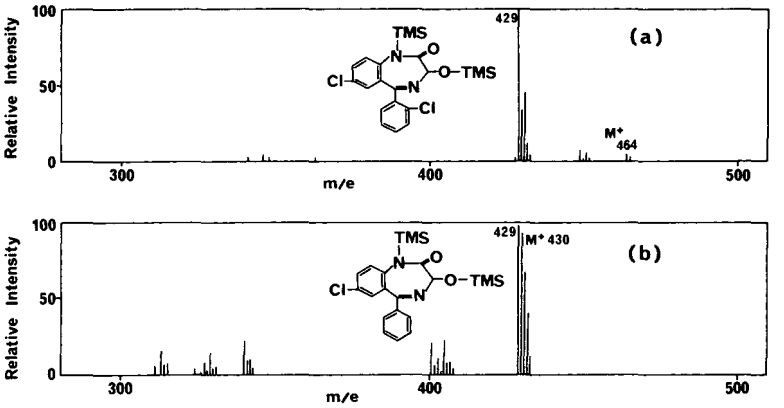

Fig. 1. Mass spectra of TMS derivatives of lorazepam (a) and oxazepam (b).

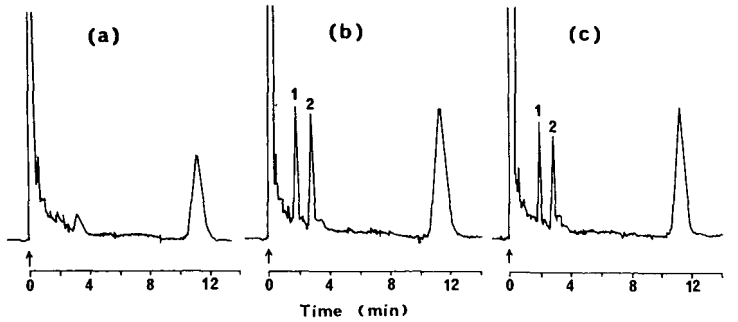

Fig. 2. Chromatograms of lorazepam (2) and internal standard (1, oxazepam) extracted from plasma: (a) control plasma; (b) control plasma to which lorazepam (25 ng/ml) and internal standard (25 ng/ml) were added; (c) plasma obtained 2 h after administration of lorazepam (1.5 mg) to a subject.

the assay. Washing with *n*-hexane removed these interfering materials and also reduced the cholesterol peak significantly, so that continuous assay of the samples was done readily. The chromatograms obtained from urine samples did not show endogenous peaks regardless of simplified washing procedure. Standard curves prepared for determining plasma levels of the drugs are shown in Fig. 3. Similar standard curves were obtained for urine samples. The lowest level of the two drugs measurable was 2 ng/ml for both plasma and urine.

Free and glucuronide forms of lorazepam were measured by the present method after three volunteer subjects had received an oral dose of 1.5 mg. The results are illustrated in Fig. 4 and Table I. Average plasma concentrations of the free drug reached a peak level of 27.7 ng/ml at 2 h and declined with a half-life of about 11 h. The concentration of the conjugated drug in plasma peaked at 3 h and was lower than that of the free drug. Urinary excretion was mostly in the form of the conjugate, which amounted to 69% of the dose in 24 h. The unchanged drug accounted for less than 1% of the dose. These data generally agreed with those reported previously [7].

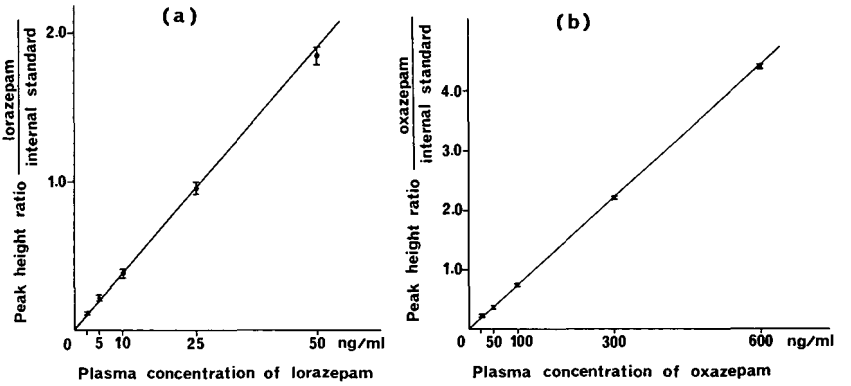

Fig. 3. Standard curves of lorazepam (a) and oxazepam (b) (mean ± S.E.M., n = 3).

Similarly, plasma and urine levels of oxazepam were measured after an oral dose of 10 mg was given to another three subjects. The maximum plasma concentration of the free drug was 210.8 ng/ml, which was reached at 3 h. Elimination half-life was about 4 h. The concentration of conjugated oxazepam in plasma was lower than that of the free drug until the 5th hour (Fig. 5). As with lorazepam, the majority of the dose was excreted in urine as the conjugate (Table I). These studies demonstrated the usefulness of the present method in monitoring the lorazepam and oxazepam levels in clinical practice.

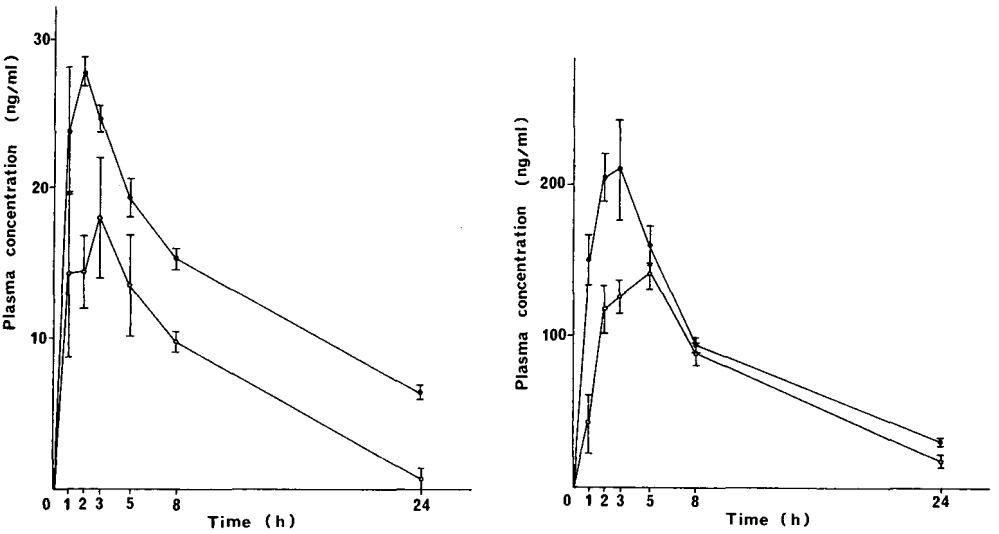

Fig. 4. Plasma concentration of unchanged drug (•) and conjugated drug (○) after oral administration of 1.5 mg lorazepam to three subjects (mean ± S.E.M.).

Fig. 5. Plasma concentration of unchanged drug (•) and conjugated drug (○) after oral administration of 10 mg oxazepam to three subjects (mean ± S.E.M.).

TABLE I

URINARY EXCRETION OF UNCHANGED AND CONJUGATED DRUG AFTER ORAL ADMINISTRATION OF 1.5 mg LORAZEPAM AND 10 mg OXAZEPAM TO THREE SUBJECTS

Time (h)	Recoveries of dose (mean ± S.E.M. %)		
	Drug	Lorazepam	Oxazepam
0—8	Unchanged	0.10 ± 0.02	5.2 ± 0.5
	Conjugated	26.1 ± 3.6	41.7 ± 9.0
0—24	Unchanged	0.59 ± 0.24	7.7 ± 0.5
	Conjugated	68.3 ± 5.2	67.3 ± 1.7

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CHROMBIO. 369

QUANTITATIVE ANALYSIS OF VOLATILE HALOTHANE METABOLITES IN BIOLOGICAL TISSUES BY GAS CHROMATOGRAPHY

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SUMMARY

A simple and sensitive gas chromatographic method for the determination of 2-chloro-1,1-difluoroethylene (CDE) and 2-chloro-1,1,1-trifluoroethane (CTE), two highly volatile metabolites of halothane, in blood, liver and isolated hepatic microsomes is described. The entire head-space in equilibrium with a known volume or weight of the sample is injected into the gas chromatograph equipped with a flame ionization detector. Quantification is accomplished with standards prepared by fortifying blank samples with known concentrations of CDE and CTE which are treated under the same conditions as the samples. Detection limits for CDE and CTE were 2 pmole/ml in blood and 10 pmole/g in liver and the mean relative standard deviations are no greater than ± 6% except for CTE in hepatic microsomes (± 9%). A preliminary study of blood CDE and CTE levels in humans anesthetized with halothane is reported.

INTRODUCTION

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a volatile anesthetic widely used for clinical applications. Traditionally halothane was considered to be an inert anesthetic, but since 1964 it has been shown to undergo biotransformation to trifluoroacetic acid, N-trifluoracetyl-2-ethanolamide, N-acetyl-S-(2-bromo-2-chloro-1,1-difluoroethyl)-L-cysteine, inorganic chloride, fluoride and bromide [1—4]. Recently, two volatile metabolites of halothane, 2-chloro-1,1,1-trifluoroethane (CTE) and 2-chloro-1,1-difluoroethylene (CDE), have been identified in the expired air of halothane anesthetized rabbits [5] and man [6]. These two metabolites could be related to the observed hepatotoxicity of halothane which occurs under hypoxic conditions [7].

Presently, methods for the isolation and quantification of these two highly volatile low molecular weight metabolites in biological tissues are non-existent. The boiling points for both compounds are extremely low (CTE + 6.9°, CDE −17.7°). This property offers the possibility of quantification by head-space gas chromatographic analysis. This principle has been applied to the analysis of

ethanol [8] and inhalation anesthetics [9] in blood. This paper presents a simple and sensitive method for the determination of CTE and CDE in hepatic microsomes, blood and liver in which quantification is accomplished by a head-space technique utilizing gas chromatography with flame ionization detection. The head-space technique does not require extraction, separation or other isolation steps which would cause extensive losses of these volatile substances.

EXPERIMENTAL

Materials

Halothane (Ayerst Laboratories, New York, N.Y., U.S.A.), CTE and CDE (PCR Research Chemicals, Gainesville, Fla., U.S.A.) were determined by gas chromatography to be 99+% pure. Pesticide quality N,N-dimethylformamide (DMF) and *n*-heptane (Matheson, Coleman and Bell, Norwood, Ohio, U.S.A.) were used without further purification. Sampling vessels were 1- and 5-ml screw cap microreaction vials with PTFE lined rubber septums (Supelco, Bellefonte, Pa., U.S.A.). Gas tight, push-button valve Precision Sampling syringes (5 ml) were obtained from Alltech, Arlington Heights, Ill., U.S.A.

Biological samples

Immediately after exposure of male rats to halothane (1% for 2 h in air) blood samples were removed from the tail vein with a syringe coated with heparin solution and 200-μl aliquots were transferred to 1-ml septum vials kept on ice. In certain cases, animals were sacrificed and blood was obtained in a similar manner from the inferior vena cava. From these same animals the liver was removed and a 2.0-g portion was transferred to a Dounce homogenizing tube containing 6.0 ml of cold 50 m*M* Tris—1.15% potassium chloride buffer (pH 7.4). After homogenization, 200 μl of the homogenate were transferred to a 1-ml septum vial kept on ice.

Microsomes were isolated from livers of rats not exposed to halothane by differential centrifugation according to the method described by Sipes et al. [10]. Aliquots of microsomal suspensions (2 ml) were placed into 5-ml vials and mixed with 0.5 ml of a NADPH generating system [10] to give a protein concentration of 5 mg/ml. The vials were then purged with nitrogen, tightly capped and allowed to equilibrate to 37° for 10 min. The reaction was then initiated by the addition of 18.8 μmoles of liquid halothane (2 μl). After a 30-min incubation at 37° with constant shaking, the reaction was terminated by addition of 20 μl of 6 *N* hydrochloric acid. Blood samples were stored at 3° and liver and microsomal samples were frozen at —76° until analyzed.

Gas chromatography

Gas chromatographic analysis was performed on a Varian 1440 gas chromatograph equipped with a flame ionization detector (FID) and a Varian CDS 101 integrator. The integrator was programmed to measure separated peak areas by the tangent baseline method. Separation was attained with a 1.8 m × 2 mm I.D. stainless-steel column packed with Porapak Q, 100—120 mesh. The injection port was maintained at 180°, the column at 150°, and the detector at 240°. Flow-rates were 30 ml/min for the nitrogen carrier gas, 300 ml/min for

air, and 30 ml/min for hydrogen. The electrometer was set at range 10^{-12} and attenuation 2. The identity of volatile metabolites which were detected by this gas chromatographic system were confirmed on a 3300 Finnigan gas chromatograph—mass spectrometer equipped with a Finnigan 6110 data system.

Analytical procedure

Prior to analysis the samples were equilibrated at room temperature for 1 h, vortexed for 20 sec and placed on a block heater at 37° for a minimum of 20 min. The needle of a gas-tight syringe was inserted through the septum of the vial into the vapor phase, without touching the liquid. The valve was opened, and the plunger was drawn slowly to the 5.0-ml mark of the syringe over a time period of 15—20 sec. Before removing the syringe from the vial, the push-button syringe valve was closed. The head-space sample was then compressed by depressing the plunger until the syringe pressure approximately equaled the chromatograph inlet pressure (35 p.s.i.). Injection of the compressed head-space sample into the column was performed by inserting the syringe needle into the injection port, opening the syringe valve, and then depressing the syringe plunger in a rapid, smooth motion. Each chromatographic determination, including the elution of halothane, required 20—25 min. The concentrations of CTE and CDE in microsomal, blood, and liver samples were determined with the aid of standard calibration curves.

CTE and CDE standards

Separate stock solutions of CTE and CDE were prepared by slowly bubbling the respective gas into 5.0 ml of dimethylformamide (DMF), which is an excellent low-volatility carrier for these compounds, in screw capped glass vials and their concentrations were determined by weight difference. Serial dilutions of the stock solutions with additional DMF were made (0.0048 to 6.8 μmoles/ml) and 2.0—5.0 μl of these solutions were added to septum vials containing 200 μl of blood, 200 μl of liver homogenate, or 2.5 ml of microsomal incubation media.

These standards were freshly prepared for each phase of the study, and were treated in the same manner as the biological samples. The integrator peak areas per syringe of head-space vapor were plotted against the corresponding standard concentrations of these metabolites. At least three standards in duplicate were prepared that encompassed the integrator peak areas of the particular biological samples that were assayed. Blank samples containing only DMF did not interfere with the gas chromatographic analysis of CTE and CDE. In addition, CTE and CDE standards were prepared in *n*-heptane in a similar manner as the DMF standards for recovery studies.

Statistics

Linear regression analysis, sample concentration and relative standard deviation computations were performed on a programmable Wang 500 calculator (Wang Instruments).

RESULTS AND DISSCUSSION

The high volatilities of CTE and CDE in aqueous solutions allow for the success of head-space analysis, although an optimum equilibration temperature (37°) and equilibration time (20 min) are necessary for reproducible chromatographic peaks. The FID was chosen for quantitative analysis since the sensitivity of the tritium electron capture detector was much lower. In addition, the excellent linear range (10^7) of the FID allowed for determination of the parent halothane as well as its metabolites per injection of head-space gas.

Sample chromatogram

Typical gas chromatograms of the direct injection of the head-space from rat blood containing CDE, CTE and halothane and from blood from a non-halothane anesthetized rat show adequate resolution on Porapak Q from normal blood constituents (Fig. 1). Chromatograms of liver homogenates and microsomal suspensions yielded very similar separation and resolution of CDE and CTE. In Fig. 1, the peaks with retention times of 2.2 and 3.4 min corresponded to those of authentic CDE and CTE, respectively, and represented blood concentrations of 2.1 and 8.4 nmole/ml. In addition, mass spectrometry confirmed these peaks to be CDE and CTE. The first two peaks eluting before CDE were air and water. At low concentrations of CDE (< 0.1 nmole/ml) the peak was reduced to a shoulder of the water peak, thus the integrator was programmed to measure peak areas by the tangent baseline method to avoid integration of part of the water. Mass spectrometric analysis of the peak that elutes after CTE was identified as acetone. From mass spectral analysis, the unknown peak that eluted prior to halothane appears to be an alcohol. This chromatographic system is also capable of resolving trifluoroethanol and 2-bromo-2-chloro-1,1-di-

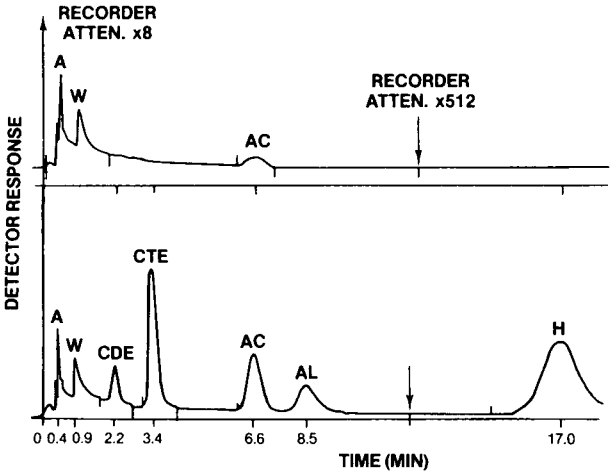

Fig. 1. Lower: typical gas chromatogram of vial head-space gas of 200 μl of inferior vena cava blood from a rat anesthetized with 1% halothane for 2 h. Upper: blank; identical conditions as in lower chromatogram, except with blood from an untreated rat. Peaks: A = air, W = water; CDE = 2-chloro-1,1-difluoroethylene (2.1 nmole/ml); CTE = 2-chloro-1,1,1-trifluoroethane (8.4 nmole/ml); AC = acetone; AL = alcohol; H = halothane.

fluoroethylene, other possible halothane metabolites, which were not found in any of the biological tissues investigated.

Linearity

Typical calibration curves of CDE and CTE described in the procedure are shown in Figs. 2 and 3. A linear relationship between detector response of the head-space gases and concentration of the gases in the liquid phase exists within the concentration range studied. Correlation coefficients were greater than 0.999. The applicability of the excellent wide linear range obtained is illustrated in Fig. 3. The standard concentrations of CDE and CTE (Fig. 3) reflect the experimental range of peripheral venous blood concentrations in rats and humans exposed to halothane, while the concentrations given in Fig. 2 represent

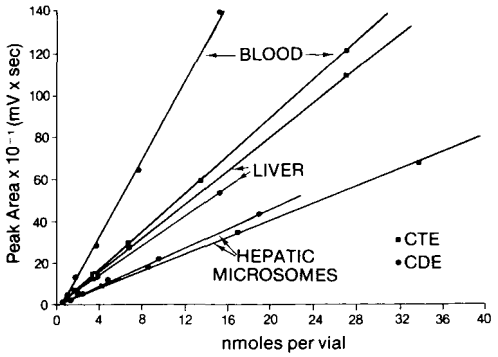

Fig. 2. Gas chromatographic calibration curves for the determination of CDE and CTE in central venous blood, liver homogenate and hepatic microsomal suspension. Detector response is measured as the integrator peak area per syringe of head-space gas plotted against the total vial content of CDE or CTE. Each point represents the mean of 3 replicate standards.

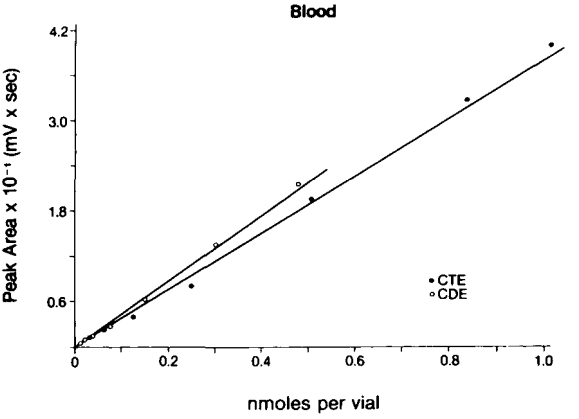

Fig. 3. Gas chromatographic calibration curves for the determination of CDE and CTE in peripheral venous blood. Conditions same as Fig. 2. Note that concentration ranges of CDE and CTE in central venous blood are approximately 50 and 30 times greater respectively than the corresponding concentration ranges in peripheral venous blood. Each point represents the mean of 4 replicate standards.

the range for central venous blood and liver of rats exposed to halothane and in vitro hepatic microsomal incubations with halothane. This represents an overall linear range of approximately 10^3, well within the acceptable linear range of the FID (10^7).

Precision

The precision of the method was determined by comparing the integrator peak areas of replicate control samples of blood, liver homogenate, or microsomal suspension fortified with known concentrations of CDE and CTE. The results presented in Tables I and II show that the mean relative standard deviations did not exceed 6% except for CTE in microsomal suspension (9%).

Recovery

The amounts of CDE and CTE recovered from the various biological samples are presented in Tables I and II. Depending on the sample up to 84% of CDE and 60% of CTE could be recovered by single sampling of the head-space. The percent recovered was calculated from standards prepared in heptane which were injected directly onto the gas chromatographic column. The higher recovery of CDE over CTE obtained from all biological samples probably reflects the greater volatility of CDE.

In order to determine the total recovery of the method, repeated injections were made of the head-space of microsome standards into the gas chromatograph until the detector gave no further response to CDE and CTE. Between each injection, the vials were allowed to re-equilibrate before additional sampling of the head-space. The total amount in each vial was computed by comparing the sum of the integrator peak areas with the peak areas obtained from

TABLE I

RECOVERY AND PRECISION OF THE GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF CDE IN BIOLOGICAL TISSUES

Replicate samples	CDE added (nmoles)	CDE recovered (nmoles)	Recovery (%)	Mean peak area (mV × sec)	Rel. S.D. (%)
Blood	0.0095	0.0079	83	0.40	7.5
(4)	0.15	0.12	80	6.2	6.0
	0.95	0.84	88	43	1.7
			Mean 84±4 S.D.		Mean 5.1
Liver	1.9	1.2	63	62	1.4
(3)	3.8	2.7	71	136	2.8
	15	10	67	533	1.2
			Mean 67±4 S.D.		Mean 1.8
Microsome	1.2	0.46	38	24	3.1
(3)	4.8	2.3	48	119	5.2
	19	8.3	44	426	10.3
			Mean 43±5 S.D.		Mean 6.2

TABLE II

RECOVERY AND PRECISION OF THE GAS CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF CTE IN BIOLOGICAL TISSUES

Replicate samples	CTE added (nmoles)	CTE recovered (nmoles)	Recovery (%)	Mean peak area (mV × sec)	Rel. S.D. (%)
Blood	0.032	0.018	56	1.2	6.8
(4)	0.51	0.31	61	19	7.2
	1.7	1.05	62	65	5.5
			Mean 60±3 S.D.		Mean 6.5
Liver	0.84	0.49	58	30	1.8
(3)	6.8	4.5	66	276	7.2
	27	18	67	1083	6.6
			Mean 64±5 S.D.		Mean 5.2
Microsome	2.1	0.74	35	45	13.0
(3)	8.4	2.9	35	179	6.1
	34	11	32	665	8.0
			Mean 34±2 S.D.		Mean 9.0

CDE and CTE heptane standards. The data presented in Table III for microsomes show high recovery of CDE and CTE except at very low concentrations. The low recoveries at lower concentrations may be partly due to the measurement of smaller peak areas, adsorption of the compounds within the system, or partitioning of the compounds in the PTFE parts of the sampling syringe. Similar results were obtained with blood and liver homogenate standards. In any event, the single sampling of the head-space gas appears to be reasonably linear and precise for the accurate determination of the CDE and CTE levels in biological tissues.

TABLE III

TOTAL RECOVERY OF CDE AND CTE FROM HEPATIC MICROSOMAL SUSPENSION BY REPEATED GAS CHROMATOGRAPHIC HEAD-SPACE ANALYSIS

Added (nmoles)		Recovered (nmoles)		Recovery* (%)	
CDE	CTE	CDE	CTE	CDE	CTE
0.24	0.42	0.12	0.35	50	83
1.4	2.5	1.1	2.2	79	88
5.7	10	5.0	9.8	88	98
23	40	23	40	100	100

*Each recovery is the mean value of 2 replicate standards.

Sample reproducibility

Five replicate blood samples taken from a rat exposed to halothane were analyzed and found to have a relative standard deviation (S.D.) of the peak areas of 4% for CDE and 8% for CTE, indicating good reproducibility. The relative S.D. of the weights of the five aliquots of blood was 4%, suggesting that the variability in the peak areas is partly due to the variability in the blood weights.

Effect of protein-lipid content of sample

The volatility and detection of CTE and CDE is dependent on the composition of the aqueous phase, that is the concentration of protein and lipid in the sample. Calibration curves of CTE with two different dilutions of liver homogenate are shown in Fig. 4. The decreased recovery with increased concentration of liver indicates that the liquid to gas partition coefficient is highly dependent on the protein-lipid content in the sample. The solubility dependence of anesthetic agents on the composition of blood [11—13] and tissue homogenates [11] has been well documented.

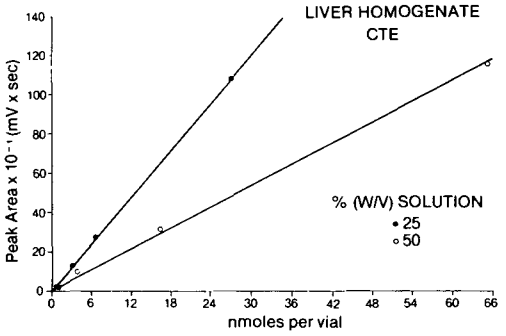

Fig. 4. Gas chromatographic calibration curves of CTE in liver homogenate as a function of homogenate composition. Conditions same as Fig. 2. Each point represents the mean of 3 replicate standards.

Detection limits

Detection limits were approximately 2 pmole/ml for CDE and 2.5 pmole/ml for CTE in blood, and 15 pmole/g for CDE and 10 pmole/g for CTE in liver under the conditions used. Incubation of hepatic microsomal suspension for 30 min containing 2 μl of halothane (18.8 μmole) and 5.0 mg/ml of microsomal protein, yielded detection limits of approximately 0.1 pmole/mg protein for CDE and CTE.

Applications

The assay procedure described above has been applied to the study of the metabolism of halothane in the hypoxic rat model [14]. It has also been used to study the reductive metabolism of halothane by isolated microsomal systems under anaerobic conditions [14].

The applicability of the head-space method for the determination of the vol-

atile metabolites of halothane in man, was tested by drawing venous blood samples from a patient during and after halothane anesthesia. Blood concentration—time curves of CDE and CTE on a semi-logarithmic scale are shown in Fig. 5. There was a rapid increase in the production of CDE and CTE during anesthesia and the levels peaked at the end of anesthesia. The half-lives of elimination of CDE and CTE were estimated to be 1¼ h and 3¼ h respectively. Since CDE and CTE are reductive metabolites of halothane and reductive metabolism of halothane preceeds its toxicity this assay method could be used as a clinical monitor for possible development of halothane hepatotoxicity in patients undergoing surgery in conjunction with drug pretreatment.

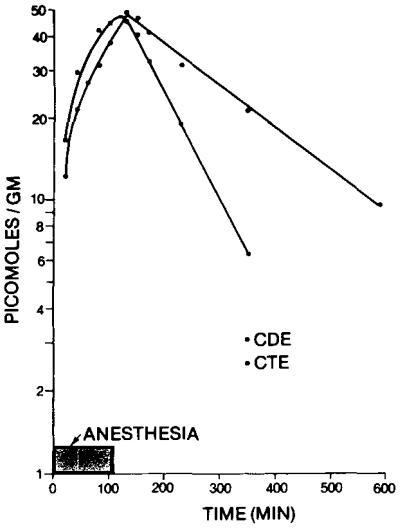

Fig. 5. Peripheral venous blood concentration vs. time curves for CDE (▪) and CTE (•) during and after anesthesia with 1.5% halothane for 110 min in a human patient undergoing surgery. Note that metabolite levels rose rapidly during anesthesia and peaked at the end of anesthesia. Half-lives of elimination were $t_{½}$ CDE = 1¼ h and $t_{½}$ CTE = 3¼ h.

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DETERMINATION OF TRIMETHOPRIM IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid, sensitive, and specific high-performance liquid chromatographic assay was developed for the determination of trimethoprim in blood, plasma, and urine using normal-phase (adsorption) chromatography on a microparticulate silica column and UV monitoring at 280 nm. Trimethoprim is selectively extracted from the biological sample matrix at alkaline pH with chloroform, providing nearly quantitative extraction (>95%) and a sensitivity limit of 0.01 to 0.02 μg/ml blood or plasma, without interference from sulfonamides.

INTRODUCTION

Trimethoprim (TMP, Fig. 1) is a synthetic antibacterial, marketed in combination with sulfamethoxazole (SMZ)* and used extensively in the treatment of a variety of infections in man [1—3].

Analytical methods for the quantitation of trimethoprim in biological fluids have included microbiological assay [2], spectrofluorometry [4, 5], differential pulse polarography [6], gas—liquid chromatography [7], thin-layer chromatography [8], and high-performance liquid chromatography (HPLC) [9—11]. Many of these procedures involve tedious sample preparation steps and have sensitivity limits of about 0.1 μg TMP per ml of plasma or blood.

Following a single therapeutic dose of TMP in 24 normal subjects (400 mg TMP in combination with 2.0 g SMZ), the reported mean blood levels ranged from 3.21 μg TMP per ml at 2 h to 1.12 μg TMP per ml at 24 h [12]. These concentrations are easily determined by the existing methodology [2, 4—11]. However, in various clinical situations, such as limited sample size (<1.0 ml plasma) from pediatric patients, low single oral doses of TMP (100 mg or

*Bactrim ™ (Roche Laboratories Division, Hoffmann-La Roche), and Septra™ (Burroughs Welcome); trimethoprim—sulfamethoxazole (1:5).

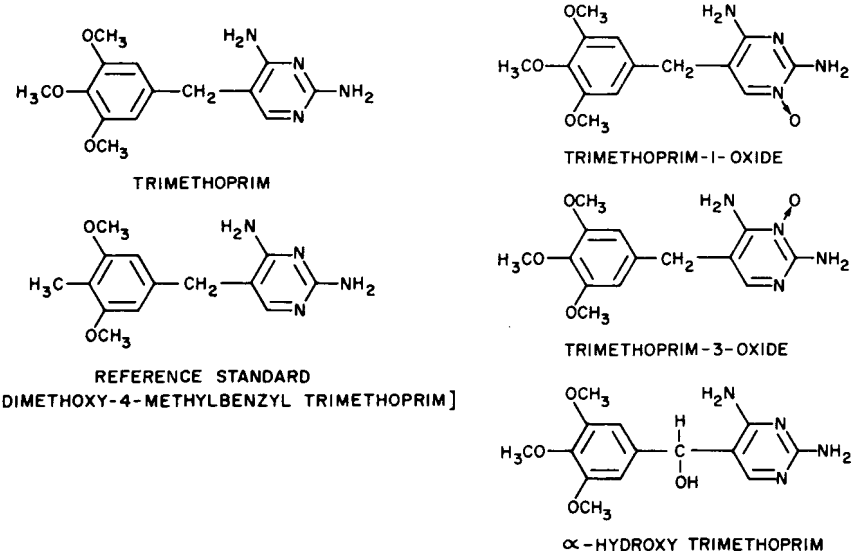


Fig. 1. Structure of trimethoprim, trimethoprim metabolites and the reference standard.

less), and in bioequivalency and pharmacokinetic studies, many of these methods are not sufficiently sensitive (0.01 μg/ml) for the reliable determination of a plasma level—time curve from 0 to 48 h. An HPLC method that is specific, precise, rapid, simple to perform, and with a sensitivity limit of 0.01 to 0.02 μg/ml (using a 2-ml blood or plasma specimen) has been developed. The assay utilizes adsorption (normal-phase) chromatography with isocratic elution at ambient temperature and measurement of the UV absorbance of the eluant at 280 nm. Quantitation is performed using the peak height ratio of TMP to a reference standard, the 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)-pyrimidine analog of TMP. The assay can also be applied to the measurement of the urinary excretion of TMP.

MATERIALS AND METHODS

Reagents

Reagent grade chloroform (a) for the extraction solvent was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). Methanol and chloroform (b), which was used for the chromatography solvent, preserved in 1% ethanol, were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). 0.2 *N* aqueous sodium carbonate and 0.01 *N* aqueous sulfuric acid were prepared. Concentrated ammonium hydroxide (Mallinckrodt, St. Louis, Mo., U.S.A.) was 28—30%, sp. gr. 0.90.

The mobile phase was composed of chloroform (b) and a mixture of methanol—water—ammonium hydroxide (150:9:1, v/v) in a 500:25 (v/v) ratio. Prepare fresh for each chromatographic run, and vacuum degas for approximately 5 min with ultrasonic vibrating.

Apparatus

A Waters Assoc. high-performance liquid chromatographic system consisting of the following components: Model 6000A solvent delivery system, Model U6K sample injector, and a Model 440 UV detector operated at 0.01 or 0.02 a.u.f.s. with a 280-nm wavelength kit (Waters Assoc., Milford, Mass., U.S.A.), in conjunction with a 10-mV recorder (Model 56 Perkin-Elmer) and a 10-μm silica gel column (μPorasil, Waters Assoc.), 30 cm × 3.9 mm I.D., was used for chromatographic analysis. The mobile phase flow-rate was 1.5 ml/min at a pressure of 500 p.s.i.

Under these conditions, the reference standard and TMP have retention times (t_R) of approximately 4.8 and 5.3 min, respectively, and 100 ng of the reference standard and TMP give about 50% full-scale response at 0.02 a.u.f.s.

Standard solutions (aqueous) for preparation of blood, plasma, and urine standards

TMP. A stock solution of 100 μg/ml was prepared by accurately weighing 10.0 mg of pure material (pharmaceutical grade, >99% purity) and dissolving in 100 ml of 0.01 *N* sulfuric acid in a volumetric flask. A 1:10 dilution of the stock solution in distilled water yielded a working standard solution of 10 μg TMP per ml.

Reference standard

A TMP analog, 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)-pyrimidine (Fig. 1), was used as the reference standard. A stock solution of 100 μg/ml in 0.01 *N* sulfuric acid was prepared, followed by a 1:10 dilution of the stock solution in distilled water to yield a 10 μg/ml working standard solution.

HPLC standards (external standards)

A stock solution of TMP, 100 μg/ml in chloroform (b), and a stock solution of the reference standard, 100 μg/ml in chloroform (b) were prepared. A series of standard solutions are then prepared by diluting aliquots of the stock solutions to 10 ml in volumetric flasks with chloroform (b) as shown below for use in establishing a calibration curve from 0.020 to 0.200 μg of TMP containing 0.100 μg of reference standard per 10 μl injected:

Solution	TMP (stock) (ml)	Conc. TMP (μg/ml)	Conc. TMP (μg/10 μl)	Reference standard (stock) (ml)
1	0.2	2	0.020	1.0
2	0.5	5	0.050	1.0
3	1.0	10	0.100	1.0
4	2.0	20	0.200	1.0

Plasma or blood sample preparation

Into 50-ml glass-stoppered round bottom centrifuge tubes, 1.0 μg of the reference standard (100-μl aliquot of working standard solution in 0.01 *N* sulfuric acid) was added. A 2.0-ml aliquot (or less) of plasma or blood was taken for each unknown and added to the reference standard followed by 5 ml of 0.2 *N* sodium carbonate solution. The samples were mixed well on a

Vortex mixer, and then 12 ml of chloroform was added to each tube using a Repipet (Lab Industries, Berkeley, Calif., U.S.A.). Internal standards of 0.2, 0.5, 1.0, and 2.0 μg of TMP were included, by the addition of 20-, 50-, 100-, and 200-μl aliquots, respectively, of the 10 μg/ml working standard solution of TMP in 0.01 *N* sulfuric acid along with 1.0 μg of reference standard (100 μl of the working standard solution in 0.01 *N* sulfuric acid) to separate 2-ml specimens of control plasma or blood. A control plasma, blood or urine sample was also included without addition of the internal standard to determine if interfering endogenous substances were present in the extracts.

All samples were stoppered with PTFE No. 16 stoppers and shaken for 10 min on a reciprocating shaker at a slow to moderate speed. The samples were then centrifuged for 10 min at 1000 *g* at 5 to 10° in a refrigerated centrifuge

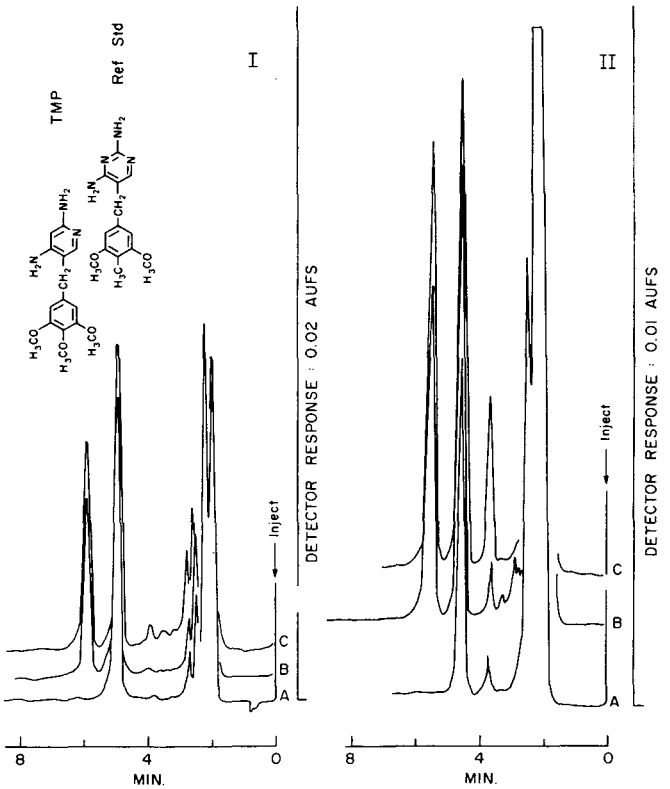


Fig. 2. Chromatogram I: Extracts of: (A) control human blood extract with reference standard (1.0 μg added), 10 μl/100 μl injected; (B) control human blood extract with 1.0 μg TMP and 1.0 μg reference standard added, 10 μl/100 μl injected; and (C) 8-h blood sample extract following administration of 160 mg TMP orally, with 1.0 μg reference standard added, 10 μl/100 μl injected. Chromatogram II: Extracts of: (A) control human plasma extract with reference standard (1.0 μg added), 10 μl/100 μl injected; (B) control human plasma extract with 1.0 μg TMP and 1.0 μg reference standard added, 10 μl/100 μl injected; and (C) 8-h plasma sample extract following administration of 100 mg TMP orally, with 1.0 μg reference standard added, 10 μl/100 μl injected.

(Model PR-J with a No. 253 horizontal head; Damon/IEC Division, Needham, Mass., U.S.A.). The aqueous layer was aspirated and discarded and a 9—10 ml aliquot of chloroform was carefully removed from each sample and transferred to a clean 15-ml conical centrifuge tube. The chloroform was evaporated to dryness under a stream of nitrogen in a N-EVAP assembly (Organomation Assoc., Worcester, Mass., U.S.A.) at 50°. The residues were dissolved in 100 μl (or less) of mobile phase and 10 μl routinely injected for HPLC analysis. Typical chromatograms of plasma and blood extracts are shown in Fig. 2.

Urine sample preparation

For urine specimens, the 24-h and 48-h fractions were diluted 1:10 with water prior to analysis (usually there is sufficient intact drug excreted up to 48 h to permit this dilution and still be able to quantitate the levels). Urine fractions collected beyond 48 h were not diluted. A 1.0-ml aliquot of diluted or undiluted urine specimen was taken for analysis and treated exactly in the manner described for plasma and blood with the exception of the addition of 2 μg of reference standard (200 μl of the working standard solution in 0.01 *N* sulfuric acid) to the unknowns. Internal standards of 1, 2, 5, and 10 μg of TMP per ml (addition of 0.1, 0.2, 0.5, and 1.0 ml working standard solution in 0.01 *N* sulfuric acid, respectively, to diluted or undiluted control urine) and 2 μg of reference standard (200 μl of the working standard solution in 0.01 *N* sulfuric acid) added to each tube were included with each set of unknowns. Typical chromatograms of urine extracts are shown in Fig. 3.

Calculations

The concentrations of TMP in the unknown plasma, blood or urine samples were determined by a computer program using a least-squares best fit straight line established from the experimentally determined peak height ratio of TMP to the reference standard in the internal standards and the unknowns. The equation of a typical calibration curve from 0.1 to 5.0 μg/ml was $y = 0.343\ x + 0.0803$ and the coefficient of correlation was 0.9993.

Recovery and sensitivity limits

The recovery of TMP was found to be 95 ± 5% S.D. over the range 0.1 to 10 μg/ml plasma, blood, and urine (using 2 ml of plasma, blood, and 1.0 ml of undiluted or diluted urine and the injection of 10 μl from 100 μl of the reconstituted residues). The sensitivity limit can be increased to 0.01 μg of TMP per ml of plasma or blood by the injection of a 10-μl aliquot from 25 μl of a reconstituted extract, operating the detector at an attenuation of 0.005 a.u.f.s.

Specificity of the method

The method was found to be specific for TMP, without interference from endogenous plasma or blood extracted materials or three known metabolites of TMP (Fig. 1). Authentic standards of three identified metabolites of TMP in plasma, the N-1-oxide, N-3-oxide, and the α-hydroxy metabolite were chromatographed to determine their retention times (t_R) under the conditions used for the analysis of TMP. The retention times of these metabolites and two sulfonamides are presented in Table I. It appeared that the N-1-oxide of

TMP, if present in the extracts would interfere with the quantitation by affecting the peak height of the reference standard. However, when a series of blood extracts from a subject who had received 400 mg TMP were prepared without addition of the reference standard, no indication of the presence of the N-1-

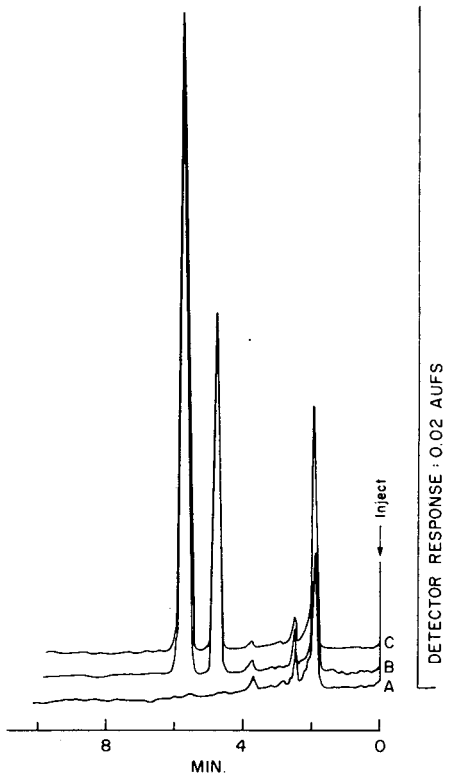


Fig. 3. Chromatograms of (A) control human urine extract (24-h collection) diluted 1:10 prior to extraction; (B) control urine extract with 5.0 μg TMP and 2.0 μg reference standard, 10 μl/200 μl injected; and (C) human urine sample extract from 0—24-h collection following a 160-mg single oral dose of TMP diluted 1:10 prior to extraction, 10 μl/200 μl injected.

TABLE I

RETENTION TIMES OF TMP AND RELATED COMPOUNDS BY HPLC

Compound	t_R (min)
TMP	5.6
Reference standard	4.73
N-1-oxide of TMP	4.80
N-3-oxide of TMP	29
α-Hydroxy-TMP	18
SMZ	7.1
N_4-Acetyl-SMZ	27

oxide was observed in the chromatograms of specimens taken from 1 h to 24 h following TMP administration.

Interference in the quantitation of the urine extracts as a result of the presence of the N-1-oxide metabolite was not encountered, although levels of that metabolite have been reported following oral administration of TMP in man [9, 13]. In these reports only about 2.1% of the administered dose was excreted as the N-1-oxide in 24 h, and in addition, authentic standards of the N-1-oxide are poorly recovered under the extraction conditions of this assay (<50%). Therefore, the method presented here is specific for TMP using the reference standard as described.

SMZ and the N4-acetyl SMZ metabolite do not interfere with the analysis of TMP since they are not extractable from blood, plasma or urine at pH 11 into chloroform, and in addition, they are chromatographically separated in this system (Table I). Possible interference from other classes of compounds was not evaluated in this system.

Application of the method to biological specimens

One normal adult volunteer was administered two different formulations of Bactrim™ (TMP, 160 mg and SMZ, 800 mg) on separate occasions. Oxalated whole blood specimens were collected prior to dosing, and at 1, 2, 3, 4, 6, 8, 12, and 24 h following dosing. In addition, urine specimens were collected prior to dosing and at 0 to 24, 24 to 48, and 48 to 72 h following dosing. The period between administration of the two formulations was two weeks to ensure a sufficient drug washout period. The blood and urine specimens were assayed by the HPLC method described and also assayed by a previously reported spectrofluorometric method [5]. The data obtained for these analyses are given in Tables II and III. The coefficients of correlation for the two sets of blood level data were 0.957 and 0.994, respectively. The coefficients of correlation for the two sets of urine data in Table III were 0.999 and 0.988, respectively.

TABLE II

COMPARISON OF TMP BLOOD LEVELS (μg/ml) IN ONE SUBJECT FOLLOWING ORAL ADMINISTRATION OF TWO BACTRIM FORMULATIONS* BY SPECTROFLUOROMETRIC AND HPLC METHODS

Time (h)	Formulation A		Formulation B	
	Fluorometric	HPLC (UV)	Fluorometric	HPLC (UV)
0	0	0	0	0
1	0.75	0.69	0.71	0.71
2	1.12	1.11	1.72	1.64
3	1.45	1.26	1.69	1.74
4	1.29	1.34	1.60	1.65
6	1.18	1.10	1.44	1.47
8	1.00	1.12	1.26	1.27
12	0.91	0.89	0.89	1.00
24	0.33	0.47	0.39	0.41

*160 mg TMP and 800 mg SMZ administered.

TABLE III

COMPARISON OF TMP URINARY EXCRETION LEVELS (mg) IN ONE SUBJECT FOLLOWING ORAL ADMINISTRATION OF BACTRIM FORMULATIONS* BY SPECTROFLUOROMETRIC AND HPLC METHODS

Time period (h)	Formulation A		Formulation B	
	Fluorometric	HPLC (UV)	Fluorometric	HPLC (UV)
Control	0	0	0	0
0—24	56.4	51.3	63.0	55.0
24—48	20.7	19.8	28.2	31.7
48—72	2.4	1.7	3.4	3.0
cumulative 0—72	79.5	72.8	94.6	89.7
% of administered dose	49.7%	45.5%	59.1%	56.1%

*160 mg TMP and 800 mg SMZ administered.

Another study involving administration of 100-mg TMP formulations to a volunteer and collection of plasma specimens prior to administration, and at 0.5, 1, 2, 3, 4, 8, 12, 24, and 48 h following dosing, was analyzed using the HPLC method. The data for that subject are shown in Table IV. The HPLC method was successfully used to quantitate TMP plasma levels as low as 0.01 μg/ml and demonstrates the utility of the method on a routine basis.

TABLE IV

TMP PLASMA LEVELS (μg/ml) IN ONE SUBJECT FOLLOWING ORAL ADMINISTRATION OF TWO TMP FORMULATIONS (100 mg TMP) BY HPLC ASSAY

Time (h)	Formulation	
	A	B
0	0	0
0.5	0.91	0.57
1	0.84	0.86
2	0.79	0.92
3	0.77	0.80
4	0.69	0.64
8	0.51	0.55
12	0.32	0.36
24	0.10	0.10
48	0.02	0.01

RESULTS AND DISCUSSION

A rapid, sensitive, and specific HPLC assay was developed for the determination of TMP in blood, plasma, and urine using normal-phase (adsorption)

chromatography on a microparticulate silica column. TMP is selectively extracted from the biological sample matrix at alkaline pH into chloroform, providing nearly quantitative extraction (>95%) and a sensitivity limit of 0.01 to 0.02 μg/ml of blood or plasma.

The UV absorption spectrum for TMP in the HPLC mobile phase used is shown in Fig. 4. The absorbance maximum is at 290 nm, and is sufficiently sensitive to allow for detection of nanogram quantities of TMP in that mobile phase at 280 nm using a fixed-wavelength detector. TMP, being a weak difunctional base with a pK_a of 7.2, required a basic modifier in an organic solvent to reduce its elution time from a silica column. The mobile phase used in the development of this method is a modification of a system reported by Bye and Brown [10]. The use of small percentages (<1%) of ammonium hydroxide and distilled water in a chloroform—methanol mobile phase on a silica column was found to be effective in reducing the elution time of TMP, without resorting to high percentages of polar organic solvents (e.g., methanol) or organic amines (e.g., diethanolamine), which could result in column deterioration or reduction in column life. This HPLC mobile phase has been used daily for six months, without any signs of deterioration of column performance. Typical chromatograms of blood and plasma extracts are shown in Fig. 2, and under these conditions of analysis, the efficiency of the column was calculated to be approximately 20,000 plates per meter. After each use of the column with this mobile phase, chloroform is pumped through the column to remove

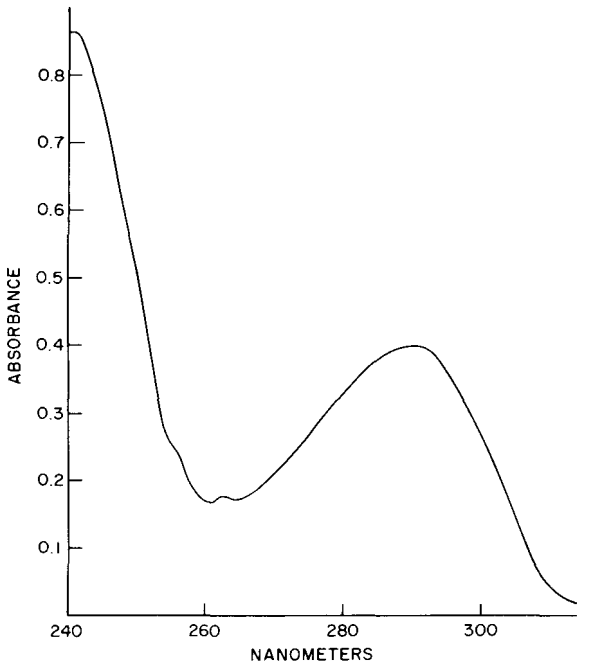


Fig. 4. UV absorption spectrum of TMP (10 μg/ml) in the HPLC mobile phase [500 ml chloroform + 25 ml of a mixture of methanol—water—ammonium hydroxide (150:9:1)]. The reference cell contained the mobile phase.

the trace ammonium hydroxide and water and avoid column degradation by long-term exposure at basic pH.

A TMP analog, 2,4-diamino-5-(3,5-dimethoxy-4-methylbenzyl)-pyrimidine was chosen as the reference standard based on its similar extractability and chromatographic behavior enabling a short total time of analysis (<10 min) between injections. The endogenous co-extracted biological material is eluted primarily in the void volume of the column, and no other interfering substances were observed in the measurement of TMP and the reference standard.

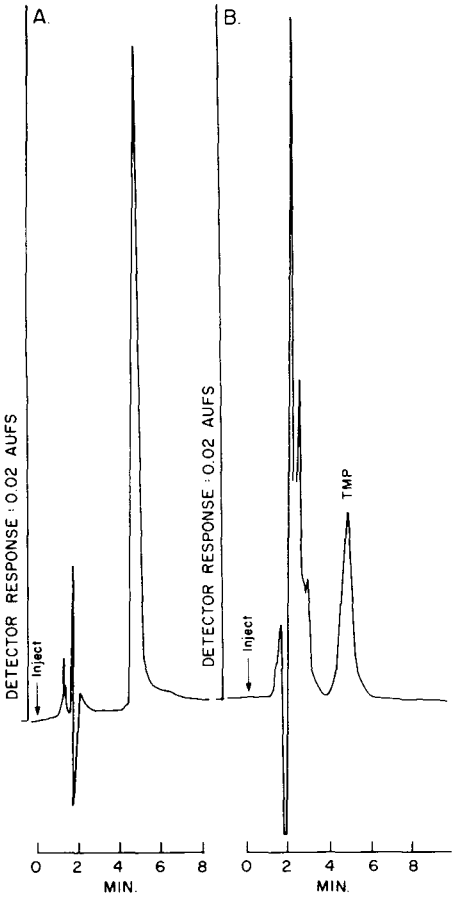
Further interest in enhancing the sensitivity of TMP detection in biological fluids, led to the investigation of UV detection of the compound below 254 nm, using a variable-wavelength detector*. It was observed that the UV absorbance of TMP increases markedly below 250 nm (see Fig. 4); however, the HPLC mobile phase described could not be effectively used below 254 nm, since the UV cut-off of chloroform is 245 nm. Although modification of the mobile phase to substitute chloroform with a more suitable UV-transparent solvent (e.g., hexane) is possible, such an approach was not undertaken.

However, reversed-phase chromatography was investigated as an alternative to the normal-phase separation, since such separations for TMP have been reported in the literature [9, 11, 14]. A rapid separation of TMP on a μBondapak C_{18} column (Waters Assoc.) was accomplished (Fig. 5) by modification of an acetonitrile—phosphate buffer (pH 6) system [14]. The conditions for the reversed-phase chromatography were: mobile phase, 20% acetonitrile (Burdick & Jackson Labs.) in pH 6 phosphate buffer (0.05 *M* prepared as a mixture of KH_2PO_4 and K_2HPO_4), flow-rate, 2.0 ml/min at a pressure of 1500 p.s.i. Although the absolute sensitivity of TMP at 225 nm was 3.7 times that at 254 nm, and increased to 4.25 times at 210 nm, the additional sensitivity could not be fully exploited, due to the necessity of dissolving biological extracts in a minimum of 750 μl of a 50% acetonitrile—pH 6 buffer solution. Thus, as seen in Fig. 5, a typical extract required 750 μl of solvent to completely dissolve the residue of the plasma extract and the injection of a 50-μl aliquot to attain a sensitivity comparable to normal-phase separation, thus offering no advantage over that system.

The intrinsic fluorescence reported for TMP in solution [15] was confirmed in the HPLC mobile phase used in the method described. A scan of the corrected fluorescence spectrum using a Farrand MK-1 spectrofluorometer (Farrand Optical Co., Valhalla, N.Y., U.S.A.) showed an emission maximum at 335 nm with excitation at 285 nm. The effluent from the UV detector was connected in series to a dual monochromator spectrofluorometer** equipped with a HPLC flow cell (20 μl; Perkin-Elmer Part No. 010-0137) for a comparison of the sensitivity limits using both modes of detection. A typical chromatogram of the fluorescence detection mode for a pure standard and a blood sample

*Model 785; Micromeritics, Norcross, Ga., U.S.A.

**Model 204 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, Conn., U.S.A.). The instrumental parameters were as follows: a 150-W xenon lamp light source, xenon lamp power supply and a R212 photomultiplier were used. The sensitivity controls were set at 11 and the selector set at 10. Excitation monochromator set at 290 nm, with emission monochromator set at 340 nm.



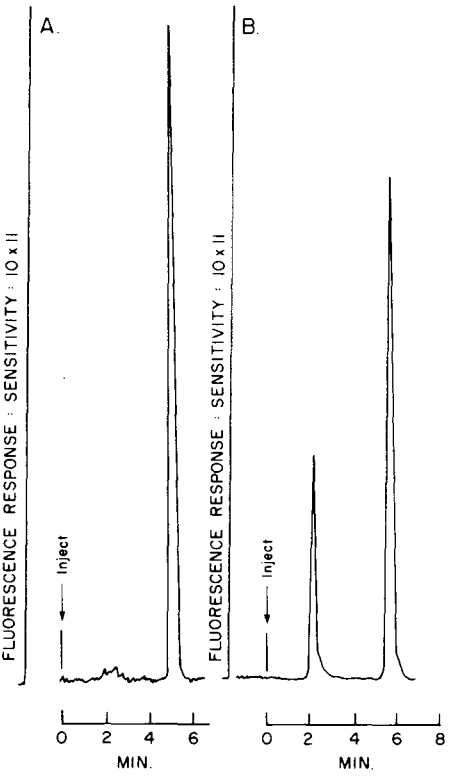


Fig. 5. Chromatogram of a reversed-phase separation on μBondapak C_{18} column using a variable-wavelength UV detector at 225 nm of (A) 100 ng TMP standard and (B) control plasma extract containing 1.0 μg TMP, 50 μl/750 μl injected.

Fig. 6. Chromatograms using fluorescence detection at 290 nm (activation) and 340 nm (emission) of (A) 100 ng TMP standard and (B) control blood extract containing 1.0 μg TMP, 10 μl/100 μl injected.

are shown in Fig. 6A and B. A 100-ng TMP standard gave nearly full scale response with the fluorescence detector operating at a signal-to-noise ratio of 2:1, which is no more sensitive than the UV detector at 280 nm, using an attenuation of 0.005 a.u.f.s. However, the potential advantage of the fluorescence mode is in those instances where additional specificity is required, (e.g., coadministration of other drugs).

In conclusion, the HPLC analysis of TMP in biological fluids is a viable technique that represents a significant improvement over many of the previously used procedures. The method is simple, rapid, sensitive, reproducible, readily automatable, and has been used on a routine basis for many months without

any column degradation. It was used to quantitate TMP levels following low single oral doses and in cases of limited sample size.

A comparison of adsorption vs. reversed-phase chromatography and the use of variable-wavelength UV vs. fluorescence detection yielded no advantage in sensitivity. Furthermore, the reversed-phase mode introduced problems due to poor solubility of the lipophilic sample residue of the chloroform extract which are not encountered using adsorption mode for chromatographic analysis.

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Note

Quantitative analysis of vitamin K_1 and vitamin K_1 2,3-epoxide in plasma by electron-capture gas—liquid chromatography

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Studies on the disposition of vitamin K_1 in man and animals have so far been performed using radioactive tracer techniques [1—5]. This was necessary since specific and sensitive analytical methods for the determination of vitamin K_1 and its epoxide metabolite, including gas—liquid chromatography (GLC) [6, 7] and high-performance liquid chromatography [8], were lacking. We now describe a GLC method which permits studies on the disposition of vitamin K_1 following therapeutic doses.

EXPERIMENTAL

Reagents

All solvents were pro analysi grade (Merck, Darmstadt, G.F.R.) and were used without further purification. Vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), vitamin $K_{2(20)}$ (menaquinone-4), and the commercially available Konakion® drops (20 mg vitamin K_1 per ml) and Marcumar® tablets (3 mg racemic phenprocoumon) were kind gifts from Hoffmann-La Roche, Grenzach-Wyhlen, G.F.R. Vitamin K_1 2,3-epoxide (2-methyl-3-phytyl-1,4-naphthoquinone 2,3-oxide) was synthesized from vitamin K_1 by the method of Tishler et al. [9].

Preparation of samples

Plasma samples of 0.2—0.8 ml were placed into 15-ml glass tubes. Ninety microlitres of an ethanolic solution of vitamin $K_{2(20)}$ (1.5 μg/ml, internal standard), 2 ml of double-distilled water, and 10 ml of *n*-hexane—absolute ethanol (1:1) were added. The tubes were fitted with PTFE-lined screw-caps and extracted for 30 min on a rotary mixer at 25 rpm. After centrifugation the upper hexane layer was removed, placed into a pointed glass tube, and evaporated to dryness under a stream of nitrogen. The residue was redissolved

in 10—25 μl of *n*-hexane and 1—2 μl were injected into the gas chromatograph. All glassware and stoppers were rinsed before use with *n*-hexane. Care was taken to protect the samples from light.

Gas—liquid chromatography

A DANI gas chromatograph (Model 3600) equipped with an electron-capture detector (radioactive source ^{63}Ni, 10 mCi, operated in the pulse mode with modulated frequency) was used. The column was a silanized O-shaped pyrex glass tube, 190 cm × 2.2 mm I.D., packed with 3% OV-17 on 90—100 mesh Anakrom Q (NEN Chemicals, Dreieichenhain, G.F.R.). Operating conditions: column oven temperature, 302°; injection port temperature, 315°; detector temperature, 305°. Oxygen-free nitrogen was used as carrier gas at a flow-rate of 80 ml/min. The column was conditioned for 24 h at 315° before use.

Calibration

Peak height ratios were calculated by dividing the height of the vitamin K_1 or vitamin K_1 2,3-epoxide peak by the height of the internal standard (vitamin $K_{2(20)}$). Standard curves of vitamin K_1 and vitamine K_1 2,3-epoxide, prepared by adding known amounts (0.005, 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4 μg) to 1 ml of pooled human plasma, were run with each set of determinations. The peak height ratios were plotted against the concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide, and the least-squares regression lines were calculated. The concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide in the unknown plasma samples were derived from the regression equation obtained for the standard curves.

Experiments in humans

Vitamin K_1 was administered orally in a dose of 20 mg as Konakion drops in 100 ml tap water to a healthy male subject (26 years, 75 kg). The subject fasted for 8 h before drug administration and food was not allowed until 3 h after drug administration. Three weeks after the end of this study the subject received an oral dose of 20 mg vitamin K_1. In addition phenprocoumon (0.4 mg/kg) was administered orally (Marcumar tablets) 8 h prior to the administration of vitamin K_1. Blood samples of 5 ml were drawn frequently for up to 72 h from a peripheral vein. The blood samples were heparinized and centrifuged. The plasma samples were kept frozen at —20° and protected from light.

A patient who suffered from acute pancreatitis (male, 58 years, 94 kg) received first an intravenous dose of 5 mg vitamin K_1 (0.5 ml Konakion injection solution) and 14 days later, when the acute phase of the disease was successfully treated and he was allowed to eat, an oral dose of 10 mg vitamin K_1 (Konakion drops) in about 50 ml tap water was administered.

Another patient (female, 58 years, 85 kg) who was submitted to the hospital because of signs of subarachnoid haemorrhage due to phenprocoumon overdosage (Quick value of 9% at the time of admission) received 20 mg vitamin K_1 (2 ml of Konakion, injection solution) intravenously. Eight hours later a blood sample was drawn and analyzed for vitamin K_1 and vitamin K_1 2,3-epoxide.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms obtained from blank plasma (Fig. 1A) and from plasma to which vitamin K_1, vitamin K_1 2,3-epoxide and menaquinone-4 (internal standard) were added (Fig. 1B). All three substances can be separated sufficiently under the experimental conditions described above. Retention times relative to the internal standard were 0.66 and 0.58 for vitamin K_1 and vitamin K_1 2,3-epoxide, respectively. No endogenous material present in the plasma of ten untreated subjects was found to interfere with the signal of either substance. However, a small signal with a somewhat shorter retention time than the epoxide metabolite (0.56 relative to the internal standard) was always present when aliquots from Konakion injection solutions or Konakion drops were extracted. Since this unknown material regularly amounted to 3—5% of the vitamin K_1 signal, the peak heights of vitamin K_1 2,3-epoxide could be corrected.

The calibration curves obtained for vitamin K_1 and vitamin K_1 2,3-epoxide are shown in Fig. 2. These curves were linear up to 0.4 μg/ml plasma for both compounds when 0.8 ml plasma was extracted. The lower limit of detection was 3 ng/ml plasma for both substances.

The precision of the method is shown in Table I. There was good agreement between added and recovered vitamin K_1 and vitamin K_1 2,3-epoxide at the two plasma concentrations studied. Moreover, day-to-day variations in the slopes of the calibration curves were small (coefficient of variation was below 10% within a time period of two months). The final recoveries for vitamin K_1, vitamin K_1 2,3-epoxide, and vitamin $K_{2(20)}$ (menaquinone-4) ranged from 65 to 105%. These variations, however, do not affect the accuracy of the method,

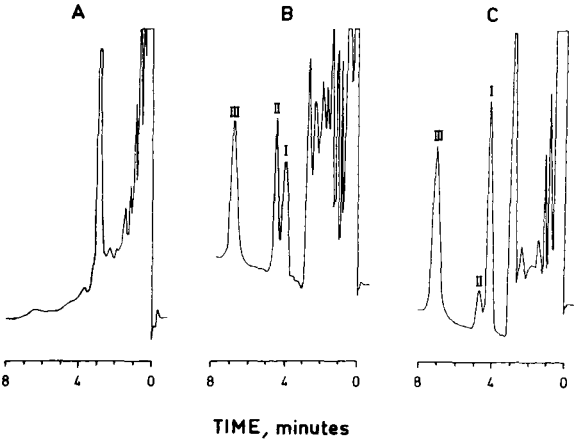


Fig. 1. Gas—liquid chromatograms of vitamin K_1 2,3-epoxide (I), vitamin K_1 (II) and vitamin $K_{2(20)}$ (III). (A) Blank extract of 0.8 ml human plasma. (B) Extract of 0.8 ml human plasma to which vitamin K_1 (0.1 μg/ml) and vitamin K_1 2,3-epoxide (0.1 μg/ml) and 0.169 μg/ml vitamin $K_{2(20)}$ were added. (C) Extract of 0.8 ml human plasma obtained from a patient who took an overdose of phenprocoumon. This patient was treated with a dose of 20 mg vitamin K_1 intravenously; 8 h later a plasma sample was obtained, spiked with vitamin $K_{2(20)}$ as internal standard and assayed.

since the ratios between the internal standard to vitamin K_1 and to vitamin K_1 2,3-epoxide remained constant (coefficient of variation below 3%, n = 20).

Examples of the application of the method are shown in Figs. 1, 3 and 4. The plasma concentration—time curves of vitamin K_1 and vitamin K_1 2,3-

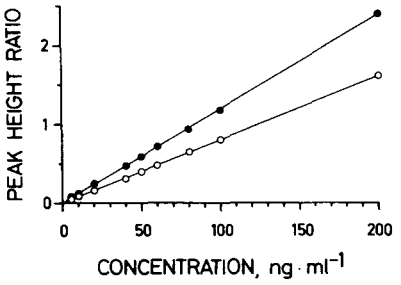
TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ANALYTICAL METHOD

Concentration (μg/ml)

Vitamin K_1 added to plasma		Vitamin K_1 found		Vitamin K_1 epoxide added to plasma		Vitamin K_1 epoxide found	
1	2	1	2	3	4	3	4
0.04	0.2	0.045	0.190	0.04	0.1	0.038	0.101
0.04	0.2	0.040	0.193	0.04	0.1	0.036	0.103
0.04	0.2	0.040	0.188	0.04	0.1	0.042	0.099
0.04	0.2	0.041	0.196	0.04	0.1	0.038	0.101
Mean ± S.D.							
0.04	0.2	0.042 ± 0.002	0.192 ± 0.004	0.04	0.1	0.039 ± 0.003	0.101 ± 0.001

epoxide in the healthy male subject who received an oral dose of 20 mg vitamin K_1 8 h after an oral dose of 30 mg phenprocoumon are shown in Fig. 3. After the 20-mg oral dose plasma levels of the vitamin rose sharply at about 3 h after administration and peak plasma levels were reached at about 4 h. Thereafter, plasma levels declined at least bi-exponentially with time. The shape of the curve is similar to those obtained with [^{3}H]vitamin K_1 [1]. Moreover, the



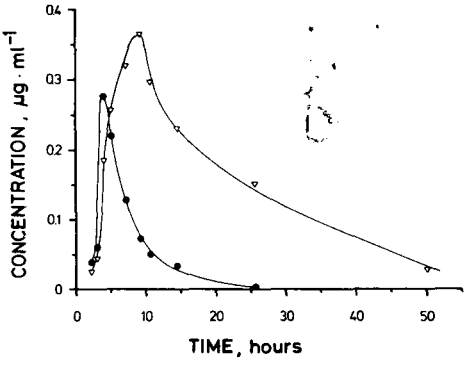


Fig. 2. Calibration graphs for vitamin K_1 (•) and vitamin K_1 2,3-epoxide (○) obtained from human plasma to which different concentrations of the compounds were added. Samples of 0.8 ml plasma were assayed. Peak height ratio = height of vitamin K_1 (or vitamin K_1 2,3-epoxide)/height of vitamin $K_{2(20)}$.

Fig. 3. Plasma concentration—time curves of vitamin K_1 (•) and vitamin K_1 2,3-epoxide (▽) in a healthy male subject who received a single oral dose of 20 mg vitamin K_1 8 h after a single oral dose of 30 mg phenprocoumon.

epoxide metabolite accumulates in the plasma and its concentrations even exceeded those of the native vitamin. This metabolite could not be detected in the study performed three weeks earlier where this subject received the same oral dose of vitamin K_1 only. In this study the magnitude of the area under the plasma concentration—time curve of vitamin K_1 was similar to that shown in Fig. 3. The accumulation of the epoxide metabolite in the plasma following pretreatment with phenprocoumon most likely reflects interruption of the vitamin K-epoxide cycle due to inhibition of the epoxide reductase by phenprocoumon [10, 11].

The plasma concentration—time curve of vitamin K_1 following intravenous (5 mg) and oral (10 mg) administration of the vitamin in the patient suffering from pancreatitis is shown in Fig. 4. Obviously, the systemic availability of vitamin K_1 following oral administration was very small (about 15%) when dose-corrected areas were used. This probably reflects an impaired absorption of the vitamin due to the underlying disease [1].

No quantifiable amounts of vitamin K_1 2,3-epoxide could be detected in our studies following administration of single oral and intravenous doses of vitamin K_1. However, in the presence of phenprocoumon large amounts of the epoxide metabolite appeared in the plasma. This was seen in the healthy subject treated additionally with phenprocoumon (Fig. 3) and also in the patient intoxicated with phenprocoumon (see Fig. 1C).

Vitamin $K_{2\,(20)}$ (menaquinone-4), which was used as the internal standard in our method, was not detected in the plasma of untreated, vitamin K_1 treated, and vitamin K_1 and phenprocoumon treated subjects.

The analytical method described may overcome some of the limitations connected with the use of radioactive material. The sensitivity of this method is sufficient to detect with accuracy vitamin K_1 plasma concentrations produced

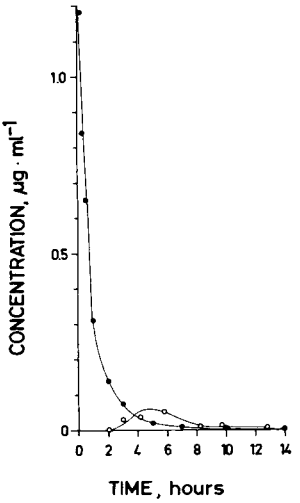


Fig. 4. Plasma concentration—time curves of vitamin K_1 following an intravenous dose of 5 mg (•) and following an oral dose of 10 mg (○) vitamin K_1 to a subject who suffered from pancreatitis.

by therapeutic doses of this vitamin. Moreover, it is possible to follow in the plasma the formation of vitamin K_1 2,3-epoxide in anticoagulant treated subjects, thus permitting studies in man on the effect of these drugs on vitamin K_1 metabolism.

ACKNOWLEDGEMENTS

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Biomedical Applications

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Note

Simple rapid method for the separation and quantitative analysis of carbohydrates in biological fluids

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The concentrations of several mono- and disaccharides in body fluids may be changed in some anomalies of carbohydrate metabolism; therefore the quantitative estimation of sugars in biological fluids could be of great clinical importance.

Thin-layer chromatography (TLC) is the method used most often for quantitative sugar analysis [1—9]. The screening method of Kraffczyk et al. [10] is widely used in clinical laboratories, but it does not suit requirements for quantitative results. All other standard methods have a few major problems: interference by normally encountered urinary substances, time-consuming pre-treatment, insufficient separation or a long development time.

The present method eliminates all these disadvantages and allows the identification and estimation of most of the commonly occurring sugars in biological fluids by means of one-dimensional TLC.

MATERIALS AND METHODS

TLC plates, 20 × 20 cm aluminium-backed silica gel 60 plates were from Merck (Merck No. 5553, Darmstadt, G.F.R.). Diphenylamine, aniline, acetone, chloroform, ethanol and orthophosphoric acid (85%) were of analytical grade and supplied by Merck. Charcoal, Darco G 60, was supplied by Fluka, Buchs, Switzerland.

Sugar stock solutions were prepared by dissolving 500 mg of each sugar (sugar standards, collection A and B, Merck No. 8005 and No. 8002) in 100 ml of 20% ethanol. Working standards ranging from 10 mg/100 ml to 500 mg/100 ml were prepared by diluting a stock solution of each sugar with 20% ethanol.

The chromatogram developing solvent was acetone—chloroform—water (85:10:5). The detection reagent was prepared by dissolving 2 g of diphenylamine and 2 ml aniline in 100 ml of acetone and adding 15 ml of 85% orthophosphoric acid. This was made up fresh daily.

Preparation of samples

Prior to chromatography all urine samples were tested with Benedict's reagent to ensure that the concentration was not higher than 500 mg/100 ml. To 10 ml of the sample 1 ml of ethanol is added and the mixture is treated with about 100 mg of charcoal. Finally the mixture is filtered and the filtrate is ready for application.

Plasma has to be filtered on a Sartorius SM-11310 filtration membrane (Sartorius, Göttingen, G.F.R.), and is then ready for application.

Cerebrospinal fluid can be applied directly to the TLC plate.

Application and development

Two to ten microlitres of the sample are applied to the plate as 0.5-cm stripes using a Hamilton microsyringe. The distance between two samples is 1 cm and the application line is placed 2 cm from the edge of the plate. A stream of warm air is used during the application.

Chromatographic development is performed in a vapour-saturated chromatographic tank at room temperature. The plate is developed twice in order to obtain a better separation. After each development the plate is dried in the oven for 2 min at 110°. Running time is 70 min and solvent migration is 17.5 cm.

Detection

The dried plates are sprayed with diphenylamine—aniline—phosphoric acid reagent, and heated in the oven for 20 min at 110°. The sugars are estimated by scanning the plates in a spectrodensitometer KM-3 (Carl Zeiss, Oberkochen, G.F.R.). If not scanned immediately, the plates should be stored in the dark at 4° for a maximum of 5 days.

RESULTS AND DISCUSSION

Aluminium-backed silica gel plates were found to yield the best separation of sugars (Fig. 1). The carbohydrates appear well defined as coloured spots, being very compact. Because of different absorption maxima each sugar has to be measured separately, but this handicap can be minimalised by rectangular measurement.

R_F values, colour, absorption maxima, sensitivity and normal values are summarised in Table I.

The precision of the method was estimated from 22 measurements of the same standard mixture over a period of several weeks, averaging 91.1 mg/100

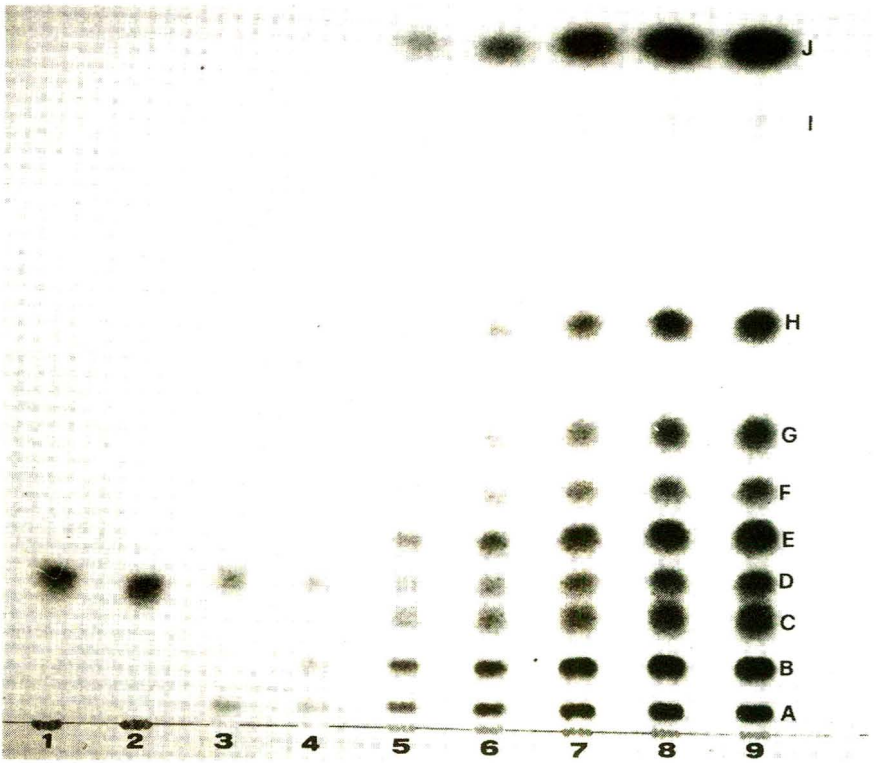

Fig. 1. Chromatogram showing TLC separation of sugars from normal plasma and urine (1, 4), plasma and urine from a patient with diabetes mellitus (2, 3) and sugar standards (5—9) as follows: A, lactose; B, saccharose; C, galactose; D, glucose; E, fructose; F, sorbose; G, arabinose; H, xylose; I, rhamnose; and J, 2-deoxy-D-ribose.

TABLE I

R_F VALUES, COLOUR, ABSORPTION MAXIMA, SENSITIVITY AND NORMAL VALUES OF THE CARBOHYDRATES STUDIED

Sugar	Colour	R_F × 100	Absorption maxima (nm)	Sensitivity limit (mg/100 ml)	Normal values	
					Urine (mg/24 h)	Plasma (mg/100 ml)
Lactose	Blue	4	600	5	0—100	—
Saccharose	Grey—violet	12	540	5	0—20	—
Galactose	Blue	16	650	10	0—20	—
Glucose	Blue	22	650	10	15—150	70—110
Fructose	Orange—red	27	530	5	—	—
Sorbose	Light-brown	32	550	10	—	—
Arabinose	Blue	36	650	10	—	—
Xylose	Blue	48	650	5	10—100	—
Rhamnose	Yellow—green	63	370	15	—	—
2-Deoxy-D-ribose	Red	71	520	5	—	—

ml (S.D. 4.4), with a coefficient of variation of 4.8%. Relationship of peak height to concentration is linear up to 500 mg/100 ml and the sensitivity limit is 5 mg/100 ml.

It has been recognized only recently that normal urine contains many sugars in very low concentration, the clinical significance of which has not yet been established [11]. The results of our method of carbohydrate analysis of normal human urine and plasma (Table I) are identical with those published previously [12, 13].

With a growing interest in carbohydrate metabolism in diagnosing disorders involving carbohydrates, there is an increased need for a more sensitive and simple assay method. We have developed a simple TLC method for carbohydrates, after investigating various steps in the analysis, which is sufficiently sensitive and reproducible for clinical purposes as well as for research work. Specifically, this method can be used as a diagnostic aid in most known clinical syndromes, such as diabetes mellitus, glycosuria, galactosuria, ribosuria, lactose intolerance, fructosuria, fructose intolerance, essential and alimentary pentosuria.

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Biomedical Applications

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Note

Micromethod for the gas chromatographic determination of morpholine in biological tissues and fluids

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Morpholine, tetrahydro-2H-1,4-oxazine, is widely used as a corrosion inhibitor, a neutralizer, and a scrubbing agent. Derivatives of morpholine are also used in plasticizers, synthetic lubricants, emulsifiers, and antioxidants. These are also used by pharmaceutical industries. Morpholine, under physiological conditions, can form N-nitrosomorpholine [1,2], a potent carcinogen [3—6]. Morpholine reacts readily even with the low levels of nitrite in saliva [2], to form N-nitrosomorpholine. Because morpholine can readily form N-nitrosomorpholine, it may present a potential hazard to those handling it in large quantities.

In order to evaluate the potential hazard and to study the metabolism of morpholine, a method had to be devised to isolate and determine morpholine levels in tissue and biological fluids. There is no known reference to this procedure in the literature. This paper, therefore, presents a chromatographic method by which microgram levels of morpholine in blood, urine, and biological tissue can be accurately measured.

EXPERIMENTAL

Reagents

Morpholine was obtained from Fisher Scientific (Fairlawn, N.J., U.S.A.). An aqueous stock morpholine solution of 1 mg/ml was used to prepare plasma concentrations of 5, 10, 20, 50, 100, 150, and 400 μg morpholine per ml of plasma. A second aqueous stock solution of 10 mg/ml morpholine was used for injecting liver or lung tissue and for adding to urine samples to achieve concentrations ranging from 25 to 200 μg of morpholine per g of tissue or 0.125 to 2 mg of morpholine per ml of urine. The above blood, urine and tissue concentrations of morpholine were used for recovery studies.

Picric acid, 2,4,6-trinitrophenol, was purchased from Fisher Scientific. A 0.5 *M* solution of picric acid in methanol was used for acidification of samples. All other chemicals used were of reagent grade.

Procedures

Five volumes of methanol were added to a 0.5—5 ml sample of plasma or urine to precipitate the protein and extract the morpholine. The glass stoppered tubes containing the samples in methanol were centrifuged and the supernatant transferred to a round-bottom flask. Tissues were homogenized in a glass homogenizer with 10 volumes of methanol and extracted five times with 10 volumes of methanol (v/w). These extracts were combined and transferred to a round-bottom flask. Extracts from blood, urine and tissues were thereafter processed in a similar manner. These extracts were acidified with picric acid to pH 2–2.5, as determined with pH indicator paper, and then evaporated under vacuum in a rotary evaporator. To the dry residue was added a mixture of diethyl ether and 3 *N* sulfuric acid (4:1) and this was transferred to a centrifuge tube. The amount of sulfuric acid in the mixture of diethyl ether, on a molar basis, was three times the amount of picric acid added previously for acidification. The diethyl ether phase containing the picric acid was discarded. The aqueous phase containing the morpholine was re-extracted twice with diethyl ether to eliminate any picric acid remaining from the first extraction.

The solution containing the morpholine was neutralized with calcium carbonate. The precipitate was washed twice with water. The original supernatant and the washings were combined. Five microliters of the combined extracts were used for injection in the gas chromatograph.

Gas chromatography

A Tracor gas chromatograph, series MT200, equipped with a flame ionization detector, was used in all experiments. A glass column (1.83 m × 4 mm) packed with Chromosorb 103 (100—120 mesh) (Johns-Manville, Denver, Colo., U.S.A.) was found to be most suitable. The glass column was silylated before being packed by filling it with a solution of 5% dichlorodimethylsilane (DCDMS) in toluene. The solution was allowed to stand inside the column for at least 30 min, then the column was rinsed successively with toluene and methanol and dried with a stream of nitrogen.

To pack the column, one end of it was connected to the household vacuum and the other end was connected to a funnel containing the packing material. During the packing, the column was gently tapped. Before the column was used, it was preconditioned at 230° overnight. The following gas chromatographic parameters were used: column temperature, 145° isothermal; detector temperature, 240°; injector temperature, 210°; helium flow-rate at the detector point, 20 ml/min; input attenuator, 1; output attenuator, 1; recorder chart speed, 10 mm/min. The detector had a hydrogen flow-rate of 40 ml/min and an air flow-rate of 325 ml/min. The retention time of morpholine, under these conditions, was 23 min.

RESULTS AND DISCUSSION

Fig. 1A illustrates a chromatogram of blood plasma without morpholine, and Fig. 1B illustrates a chromatogram of blood plasma in which morpholine was added. Fig. 1C is a chromatogram of a urine sample and Fig. 1D is a urine sample spiked with morpholine. The samples containing morpholine and the samples which didn't contain morpholine were treated the same way following the procedure described under Experimental. These figures illustrate the separation of morpholine from other plasma or urine components which could have been co-extracted with morpholine.

Known amounts of morpholine were added to blood samples and their morpholine content was determined following the method described in this paper. A typical standard curve of the above determinations is shown in Fig. 2. The procedure was found to yield linear results from 0 to 200 μg/ml of plasma.

The recovery of morpholine was calculated by comparing the chromatographic peak heights of samples spiked with known amounts of morpholine before being processed, with those of morpholine standards. Differences in concentration of morpholine among the biological fluids and tissues (after inhalation of morpholine vapors) were taken into consideration when the range of morpholine levels was chosen during the recovery studies. The recovery of morpholine from plasma, urine, and liver tissue is shown in Table I.

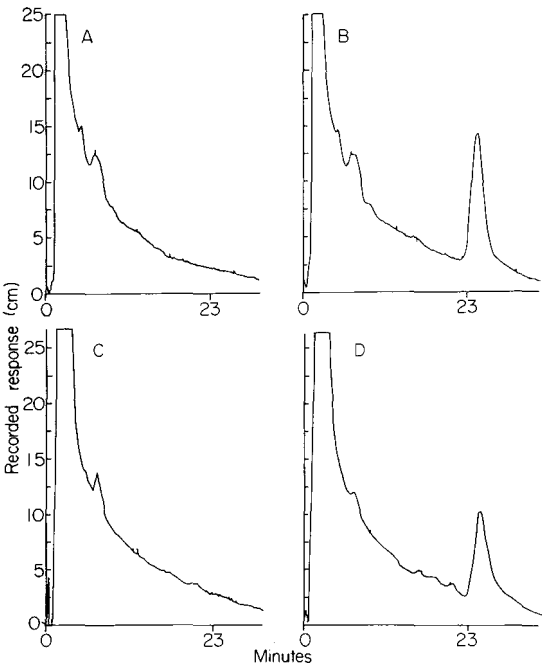

Fig. 1. Gas chromatograms of (A) blood plasma blank; (B) blood plasma with morpholine; (C) urine blank; (D) urine with morpholine. All samples were treated as described in Procedures.

TABLE I

RECOVERY OF ADDED MORPHOLINE FROM PLASMA, URINE AND LIVER TISSUE

Biological fluid or tissue	Amount (μg) of morpholine added per g of tissue or ml of body fluid	Recovery (%)	Standard deviation	Number of replicates
Plasma	5	62.8	3.5	6
	10	70.2	3.6	4
	20	63.5	6.2	6
	50	69.7	7.9	8
	100	63.2	4.2	4
	150	63.0	2.0	4
	400	61.4	2.3	4
Urine	125	55.5	3.5	5
	250	58.1	1.4	4
	500	58.0	1.4	4
	1500	58.5	2.1	4
	2000	57.1	1.6	4
Tissue	25	62.2	4.9	4
	50	67.5	6.4	2
	100	64.5	4.9	2
	150	67.7	5.7	4
	200	60.2	4.5	4

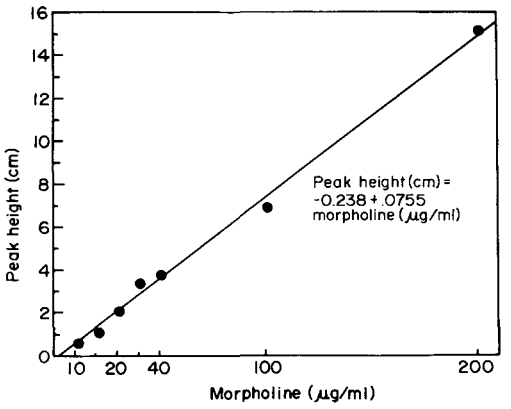

Fig. 2. Standard curve for recovered morpholine.

The recovery of morpholine was: 61% to 70% for plasma, 55% to 58% for urine and 60% to 67.5% for liver tissue.

The precision of the method was checked by using multiple replicates at various concentrations and calculating the standard deviation. The standard deviation ranged from 2 to 8% recoveries.

Results from inhalation experiments using the described method are shown in Table II.

TABLE II

DISTRIBUTION OF INHALED MORPHOLINE IN NZW FEMALE RABBIT TISSUES

Animals were exposed to morpholine vapors (250 ppm for 5 h) by nose exposure. At the end of exposure period the animals were sacrificed and their tissue analyzed for morpholine concentration as described in the text.

Tissue	Morpholine concentration* (μg/g or ml)
Liver	40.6 ± 9.0
Kidney	118.2 ± 6.3
Perirenal fat	10.1 ± 1.8
Lung	40.0 ± 3.9
Spleen	43.9 ± 6.9
Brain	41.9 ± 16.0
Adrenal glands	3.8 ± 0.4
Skeletal muscle	17.8 ± 10
Ovaries	11.2 ± 3.5
Feces	34.7 ± 3.8
Bile	34.0 ± 0.81
Urine	324 ± 86
Blood	20.7 ± 1.30

*Mean ± standard error of the mean (3 experiments).

With this micromethod, plasma, urine, and tissues from animals exposed to morpholine can easily be analyzed with accuracy and precision.

ACKNOWLEDGEMENTS

The author thanks Drs. E.A. Zeller and J.R. Hass for their fruitful suggestions during the development of the method and Ms. J. Schroeder and Mr. W. Gibson for their technical assistance.

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Note

Gas–liquid chromatographic determination of clobazam and N-desmethyl-clobazam in plasma

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Clobazam is a newly developed benzodiazepine with the nitrogen atoms of the heterocyclic ring in the 1,5- instead of the 1,4-position as in the best known benzodiazepines. The pharmacological properties [1—3] and clinical effects [4—6] have been reported and the compound is currently used as an anxiolytic agent.

Metabolic studies after administration of ^{14}C-labeled clobazam to man, rat, monkey and dog [7] show that the compound was rapidly and extensively metabolized. Although the structure of several metabolites has not yet been identified, one important pathway noted in all the species examined was N-desmethylation, with the formation of N-desmethyl-clobazam. As a basis for future examination of the possible pharmacological significance of N-desmethyl-clobazam, since the N-desmethyl metabolites of other compounds of the benzodiazepine series have been seen to be active [8—12], we have developed a gas—liquid chromatographic (GLC) method for simultaneous determination of clobazam and N-desmethyl-clobazam in plasma samples. The procedure is specific and sensitive; plasma level curves of both compounds after administration of clobazam to guinea pigs, have been plotted.

MATERIALS AND METHODS

Standard and reagents

Clobazam and N-desmethyl-clobazam were obtained from Hoechst (Frankfurt, G.F.R.). Diazepam (kindly supplied by Ravizza, Muggio, Italy) was used as an internal marker. Other reagents were acetone (Carlo Erba, Milan, Italy) and benzene (Pestanal grade, Hoechst).

Apparatus

A Carlo Erba Fractovap 2150 gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The chromatographic column was a glass tube (1 m × 4 mm I.D.) packed with 80—100 mesh Chromosorb G AW DMCS with 5% OV-25 (Supelco, Bellefonte, Pa., U.S.A.) as the liquid phase. The column temperature was 290°, the detector temperature 300° and injector port temperature 320°. The carrier gas was nitrogen at a flow-rate of 60 ml/min.

For mass spectrometry (MS) a mass spectrometer combined with a gas chromatograph (LKB 9000) was used under the following conditions: energy of the ionization beam 70 eV; ion source temperature 250°, accelerating voltage 3.5 kV and trap current 100 μA. The gas chromatograph was operated under the same conditions as above.

Standard external calibration curves

Clobazam and N-desmethyl-clobazam were dissolved in acetone (1 μg/ml) and combined aliquots of the compounds were evaporated to dryness. The dry residues were dissolved in 100 μl of acetone containing diazepam (0.25 μg/μl) as a marker, and 1—2 μl were injected into the gas chromatographic column. The ratio of the peak areas of the compounds to that of the internal marker were linear in the range from 0.1 to 2 μg per injection.

Extraction procedure

To 0.1—0.5 ml of heparin-treated plasma 2.4—2 ml of 0.5 *M* phosphate buffer (pH 9.5) were added and the samples were extracted twice for 15 min with 5 ml of benzene. After centrifugation the benzene extracts were combined and evaporated to dryness in vacuo. The dry residue was dissolved in 100—500 μl of acetone containing diazepam (0.25 μg/μl) and 1—2 μl were injected into the gas chromatographic column.

Drug-free plasma samples with known amounts of clobazam and N-desmethyl-clobazam (10—200 ng) were analyzed concurrently with each set of unknown samples. Concentrations of both compounds in the unknown samples were obtained from the ratio of the peak areas obtained to the internal standard curves. In these experimental conditions the minimum detectable amounts were 0.1 ng per injection.

Animal studies

Male albino guinea pigs (Pelizzari, Cremona, Italy) weighing about 250 g were injected intraperitoneally (i.p.) with clobazam (10 mg/kg) and killed at various times after drug administration.

Blood samples were collected in heparinized tubes, centrifuged, and plasma was analyzed as described above.

RESULTS AND DISCUSSION

The procedure described permits rapid and specific determination of clobazam and N-desmethyl-clobazam in plasma. Extraction with benzene results in a clear extract which can be injected directly into the gas chromatographic

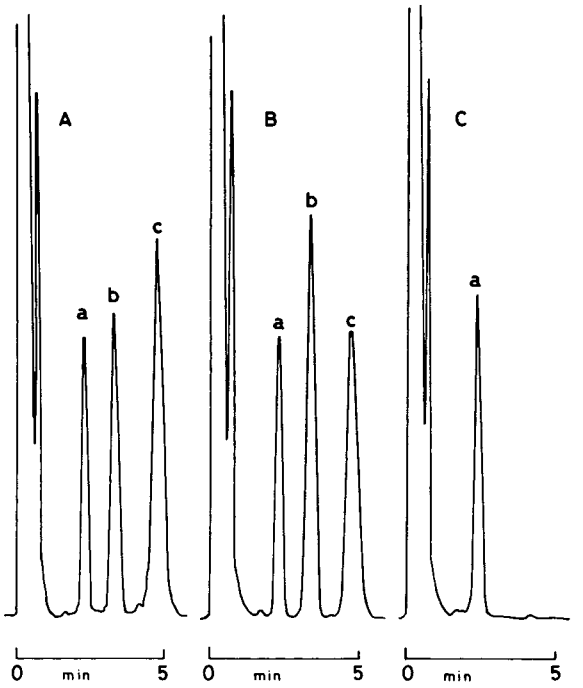

Fig. 1. Gas chromatograms of plasma extracts from guinea pigs injected with clobazam (A), from plasma to which 100 ng of both compounds were added (B) and from drug-free plasma (C). Peaks: (a) diazepam; (b) clobazam; (c) N-desmethyl-clobazam.

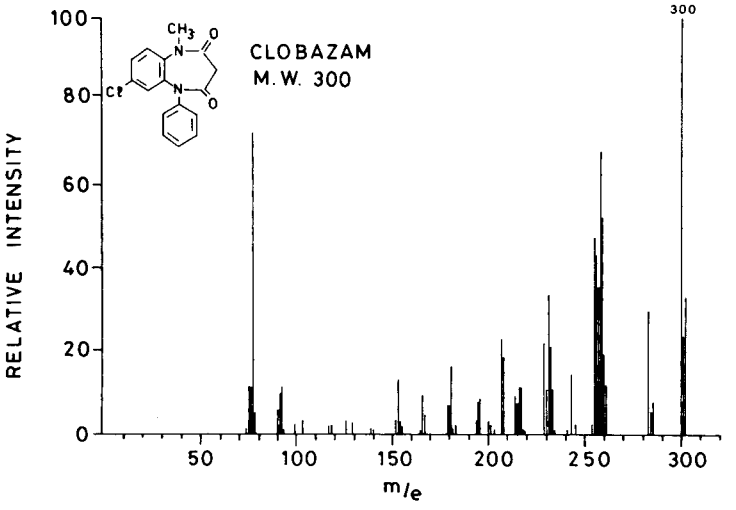

Fig. 2. Mass spectrum of clobazam.

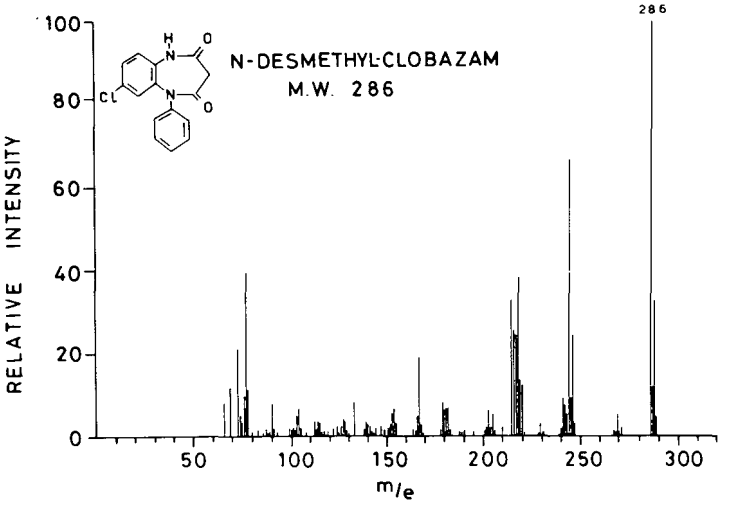

Fig. 3. Mass spectrum of N-desmethyl-clobazam.

column without further purification. In preliminary studies to choose the most suitable stationary phase for the determination of clobazam and its metabolite, 5% OV-25 on Chromosorb G AW DMCS gave the best results.

Fig. 1 presents typical gas chromatograms of extracts from plasma of guinea pigs treated with clobazam (10 mg/kg, i.p.), from plasma to which 100 ng of both compounds were added and from drug-free plasma. Retention times were 3.3 min for clobazam and 4.7 min for N-desmethyl-clobazam. Specificity of the analysis was confirmed when unknown plasma samples from guinea pigs given clobazam (10 mg/kg, i.p.) were analyzed by combined GLC—MS. The mass spectra obtained from the analysis of the GLC peaks (Figs. 2 and 3) were identical to those after injection of authentic compounds. A summary of the recovery results during kinetics studies in guinea pigs is presented in Table I. Clobazam is extracted reproducibly over the range of 10—200 ng,

TABLE I

RECOVERY OF CLOBAZAM AND N-DESMETHYL-CLOBAZAM FROM PLASMA

Each value is the mean of eight determinations.

Amount added (ng)	Clobazam		N-Desmethyl-clobazam	
	Amount found (ng ± S.D.)	Recovery (% ± S.D.)	Amount found (ng ± S.D.)	Recovery (% ± S.D.)
10	9.4 ± 0.6	94 ± 0.6	9.0 ± 0.7	90 ± 7.5
25	24.0 ± 1.4	95 ± 5.6	23.0 ± 2.2	92 ± 9.0
50	47.1 ± 2.0	94 ± 4.0	45.8 ± 2.8	92 ± 5.7
100	97.8 ± 4.4	98 ± 4.4	91.0 ± 4.4	92 ± 4.4
200	195.4 ± 4.7	98 ± 5.6	187.5 ± 11.5	94 ± 8.8

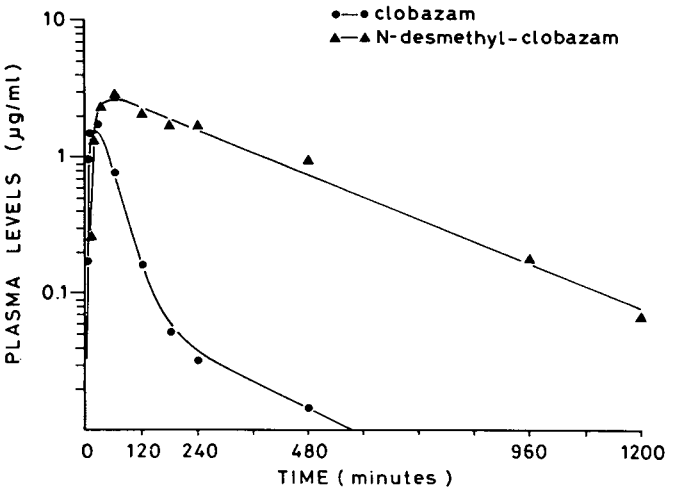

Fig. 4. Plasma level—time curves of clobazam and N-desmethyl-clobazam after intraperitoneal injection of clobazam (10 mg/kg) to guinea pigs. Each point is the mean value for six animals.

with recovery between 94–98% with a coefficient of variation (C.V.) between 4.26–6.32%. N-Desmethyl-clobazam (10–200 ng) was extracted reproducibly between 90—94% with a C.V. of 4.8–9.8%.

The validity of the analytical procedure was demonstrated by studying the plasma curves of both compounds in guinea pigs (Fig. 4) given clobazam (10 mg/kg, i.p.). Clobazam was rapidly adsorbed, rising to plasma peak concentrations between 15 and 30 min.

From the peak the plasma concentrations showed a biphasic decline with an initial phase lasting for the first 120 min followed by a second slower phase. The half-life of the β-phase was 187 min. N-Desmethyl-clobazam was detected 5 min after administration of clobazam reaching peak concentrations at 60 min. The half-life of the metabolite was 225 min.

In conclusion the specificity and sensitivity of this procedure appear to be satisfactory for pharmacokinetics studies with clobazam. Findings in this laboratory indicate that the method can be extended to other animal species and to various organs after tissue homogenization.

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Note

Improved method for the electron-capture gas chromatographic determination of trichloroacetic acid in human serum

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Trichloroacetic acid is one of the main metabolites of chloral hydrate and trichloroethylene. It is assumed that this compound may be highly cardiotoxic in man [1]. In several cases of chloral hydrate intoxications, multiple multifocal ventricular extrasystoles have been described. In order to obtain a relationship between the clinical state of an intoxicated patient and the serum concentration of trichloroacetic acid, it is necessary to evaluate an adequate method for determining this metabolite.

Several analytical methods have been reported which can be divided into procedures [2, 3] based on the colorimetric determination according to Fujiwara, and gas chromatographic methods, performed either on a strongly polar phase of the underivatized trichloroacetic acid after extraction [4, 5] or on a non-polar phase after extraction and derivatization [6–8]. The colorimetric methods lack specificity and sensitivity when applied to biological fluids. The reported gas chromatographic methods do not give satisfactory separations between the solvent, water and trichloroacetic acid, or they show tailing of the trichloroacetic acid peaks. Also, various head space techniques for the analysis of trichloroacetic acid after derivatization or decarboxylation have been described [9—12], but these methods are time consuming and inaccurate. Using the above mentioned procedures no reproducible results could be obtained. The purpose of this investigation was to develop a specific and highly sensitive procedure for the determination of trichloroacetic acid in human serum.

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EXPERIMENTAL

Instrumentation

A Tracor Model 550 gas chromatograph equipped with a pulsed mode ^{63}Ni electron-capture detector and a Tekman Model TE 200 1-mV recorder were used. Analyses were performed on a glass column (1.8 m × 4 mm I.D.) packed with 3% OV-17 on Gas-Chrom Q (80—100 mesh). Pre-purified nitrogen was used as the carrier gas at a flow-rate of 35 ml/min. The temperature of the column was maintained at 80°, the injection port at 150° and the detector at 260°.

Procedure

A 0.10-ml serum sample was diluted to 5.0 ml with distilled water using a dispenser/diluter into a disposable glass tube. After mixing for 30 sec on a whirl mixer, 0.20 ml were pipetted into a 10-ml extraction tube. To this solution, 0.2 ml 3 *M* sulphuric acid and 4.0 ml of a solution containing 4 μg/ml *o*-dichlorobenzene in toluene as the internal standard were added. This solution was mixed for 15 min and then centrifuged at 2500 *g* for 10 min.

2.00 ml of the toluene phase were transferred to another extraction tube and 0.5 ml of the methylating agent (14% boron trifluoride in methanol; Sigma, St. Louis, Mo., U.S.A.) was added. The tube was mixed for 3 min and then heated at 80° for 90 min. After cooling the mixture to room temperature, 1.0 ml of distilled water was added to remove excess derivatization reagent. The tube was centrifuged at 3000 rpm for 5 min and 1 μl of the toluene phase was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Chromatograms of serum samples (Fig. 1) demonstrate that no interfering peaks occur. The retention time of trichloroacetic acid methyl ester is 92 sec. The standard curve, prepared by adding known amounts of trichloroacetic acid to blank human serum was linear over the range of 20—200 mg/l ($r = 0.98$, slope = 3.92×10^{-3}, y-intercept = 0.15 mg/l). The inter-assay (day-to-day) variation for the method was investigated by replicate analyses ($n = 5$) of spiked serum samples ranging from 25 -220 mg/l, and showed a coefficient of variation ranging from 10—5% for the respective concentrations. The recovery of various amounts of trichloroacetic acid (20—200 mg/l) added to serum, varied from 90—97%. Using the described method, the lowest trichloroacetic acid serum concentration which can be measured is about 10 mg/l. The amounts of trichloroacetic acid which were found after the intake of 10—20 g chloral hydrate in three patients ranged from 265—120 mg/l over the first 72 h.

Chloral hydrate and trichloroethanol did not interfere in the described gas chromatographic procedure.

Summarizing, it can be concluded that a sensitive, specific and reliable method, which is suitable for routine analysis has been developed to determine trichloroacetic acid in human serum.

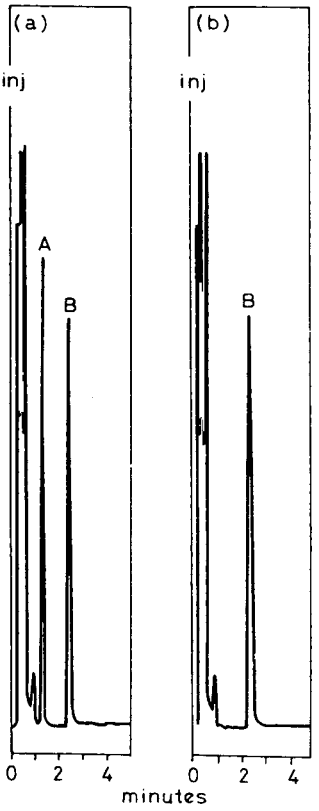

Fig. 1. Gas chromatograms of a serum sample containing 200 mg/l trichloroacetic acid (a) and a blank serum sample (b). Peaks A and B represent 0.5 ng trichloroacetic acid and 4 ng *o*-dichlorobenzene (internal standard), respectively.

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Note

Comparison of gas–liquid chromatography with nitrogen–phosphorus selective detection and high-performance liquid chromatography methods for caffeine determination in plasma and tissues

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Highly reliable analytical methods for the determination of caffeine in various biological materials are required to provide data needed to elucidate the role of caffeine in clinical therapeutics and toxicology [1—6]. Gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC) have been used extensively in our and in other laboratories [7—12]. This report describes experience gained with a programme of quality control which enabled us to check the precision and the comparability of the two techniques.

MATERIALS AND METHODS

Standard and reagents

Anhydrous caffeine and theophylline monohydrate were obtained from Carlo Erba, Milan, Italy. Hexane, chloroform, methanol and acetonitrile (Lichrosolv, Merck, Darmstadt, G.F.R.) were UV grade. Tetrahexylammonium hydroxide was purchased from Hässle, Göteborg, Sweden. Standard solution: caffeine was dissolved in methanol (100 μg/ml) and stored at +4°.

The theophylline-1-propyl derivative, the internal standard, was prepared as follows. Theophylline (50—100 μg) was dissolved in 2 ml of 0.1 *N* sodium hydroxide and 0.5 ml of a solution of 0.1 *N* tetrahexylammonium hydroxide in 0.1 *N* sodium hydroxide. Three ml of methylene chloride–1-iodopropane (5:1) were added to the aqueous phase and the samples were horizontally shaken in glass tubes for 40 min in a thermostatic water bath at 60°. After centrifugation the organic layer was transferred into another glass tube and dried under nitrogen. Two ml of hexane were added to the dry residue, left for 10 min in an ultrasonic tube and dried. The dry residue was dissolved in hexane in order to reach a concentration of theophylline derivative of 10 μg/ml (stability at 4° up to 4 weeks).

This internal standard was chosen because of its retention time for both methods, its chemical similarity with caffeine and its difference from possibly interfering substances.

Gas chromatograph—nitrogen—phosphorus selective detector

A Carlo Erba Fractovap Model 2003 gas chromatograph equipped with a nitrogen—phosphorus selective detector with potassium chloride as alkaline ion source was used. The column was a glass tube (3 m × 3 mm I.D.) packed with Gas-Chrom Q (100—120 mesh) coated with 5% SE-30 (Carlo Erba) and conditioned for 24 h at 290°. Operating conditions were as follows: helium flow-rate (carrier), 40 ml/min; hydrogen flow-rate, 35 ml/min; air flow-rate, 300 ml/min; injection port temperature 275°; and column temperature 270°.

High-performance liquid chromatograph

A Perkin Elmer series 2/2 liquid chromatograph equipped with a Model LC 55 detector, and a reversed-phase column (0.25 m × 2.6 mm) packed with 10 μm (average particle diameter) octadecylsilica (ODS-SIL-X-1; Perkin Elmer, Norwalk, Conn., U.S.A.) was used. The column was eluted with a mixture of 750 ml of twice-distilled water and 250 ml of acetonitrile. The flow-rate was 1.2 ml/min. The detector was used at 273 nm.

Extraction

A 0.1—1.0-ml sample of plasma or brain homogenate (1:10 w/v of distilled water) is brought to pH 7.0 with 1.9—1.0 ml of $Na_2B_4O_7 \cdot 10\ H_2O$, 0.08 *M* buffer. The samples are shaken for 20 min with 5 ml of chloroform in glass test tubes containing 0.05 ml of the internal standard solution.

After centrifugation for 10 min at 1200 *g*, 4 ml of the chloroform mixture are transferred into another test tube and evaporated to dryness under a gentle stream of nitrogen in a thermostatic water bath at 40°. The residue is dissolved in 50 μl of methanol and 1—2 μl of this solution are injected into the gas chromatograph.

The calibration curve is prepared with increasing amounts, 5—60 μl, of the methanol solution of caffeine. To the dry residue, 1 ml of blank plasma or blank brain homogenate is added, and the samples are then processed as above. After gas chromatographic determination the methanol solvent is evaporated. The residue is dissolved in 100 μl of the chromatographic mobile phase (see above) and 10 μl of each sample are injected into the liquid chromatograph. The average recovery from plasma was 92% and from brain 86%.

Experimental design

Brain and plasma samples which during a toxicological study gave caffeine levels of 4.7—5.3 μg/ml (or μg/g) and of 47—53 μg/ml (or μg/g) were pooled to provide the material for the quality control. Each pool of brain and plasma was divided into five specimens. The four blocks of samples thus obtained were analysed over a period of three months for a total of five replicates for each brain sample and six for each plasma sample. The volumes extracted were the following: plasma: 0.1 ml (low concentration), 0.05 (high concentration); brian: 1.0 ml (low concentration), 0.5 (high concentration). A calibra-

tion curve (0.5, 1.0, 2.0, 3.0, 4.0, 6.0 μg of caffeine) was made for each run of plasma and brain homogenate samples. Extractions and measurements were carried out by a team of three operators.

Statistical analysis

The precision of the two methods was calculated from the coefficient of variation (CV%) (standard deviation/mean·100). GLC—nitrogen—phosphorus selective detection (NPSD) and HPLC methods were first compared according to Westgard and Hunt [13] by linear regression analysis, and then more correctly by the method of Bartlett [14] applied to the natural logarithms of measured concentrations [15, 16].

RESULTS

Fig. 1 shows typical GLC—NPSD and HPLC chromatograms. The overall sensitivity of the methods was 0.25 μg/ml; using more than 1 ml of plasma lower concentrations can be measured, also increasing the volume of the extractive solvent. Internal and external calibration curves (Fig. 2) passed through the origin and were linear from 0.25 to 2 μg for GLC—NPSD and from 0.25 to more than 20 μg for HPLC. Caffeine concentrations exceeding the range of linearity of the GLC calibration curve were obtained from graphical interpolation. Table I shows coefficients of variation given by the two methods. Coefficients of variation for plasma and brain did not differ significantly, but they were higher with the GLC method at the lower concentration.

The GLC—NPSD method was chosen as reference (for the linear regression analysis on natural values) since it was employed for two years in this laboratory before the introduction of the HPLC technique. This first approach gave:

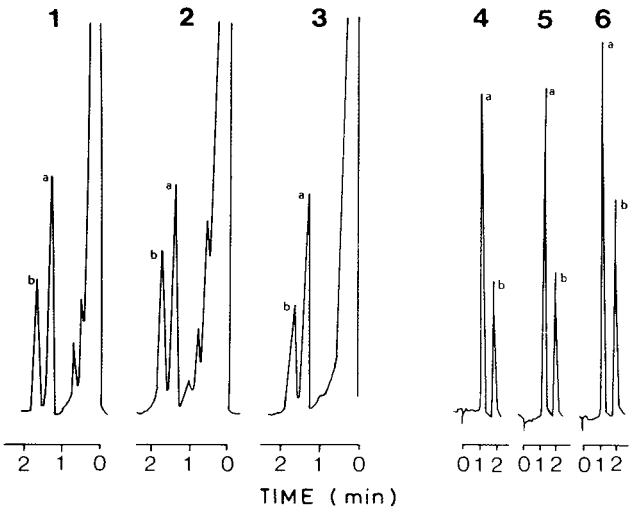

Fig. 1. Gas chromatograms of 1: plasma extract sample; 2: brain extract sample; 3: external sample. HPLC chromatograms of 4: external sample; 5: brain extract sample; 6: plasma extract sample. Peaks: a = caffeine; b = theophylline-1-propyl derivative.

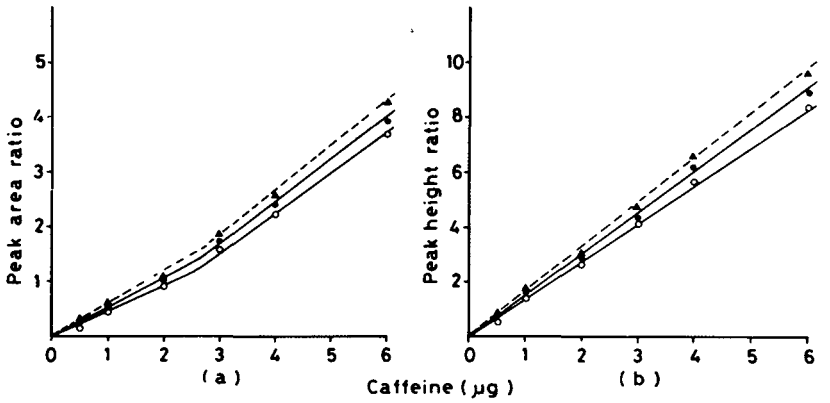

Fig. 2. Standard calibration curves for caffeine by (a) GLC—NPSD and (b) HPLC methods. ▲-----▲, External standard; ●——● , internal standard, from plasma; ○——○, from brain. Propyl-theophylline was added just before injecting the sample, in order to estimate absolute recovery.

TABLE I

PRECISION OF CAFFEINE ASSAY METHODS IN PLASMA AND BRAIN

	Plasma				Brain			
	GLC—NPSD		HPLC		GLC—NPSD		HPLC	
	Low	High	Low	High	Low	High	Low	High
X	4.8	44.0	4.8	41.2	5.0	45.5	5.2	46.2
S.D.	0.7	4.5	0.5	4.1	0.9	3.5	0.5	4.3
CV	14.6	10.2	10.4	10.0	18.0	7.7	9.6	9.3
n	30	30	30	30	25	25	25	25

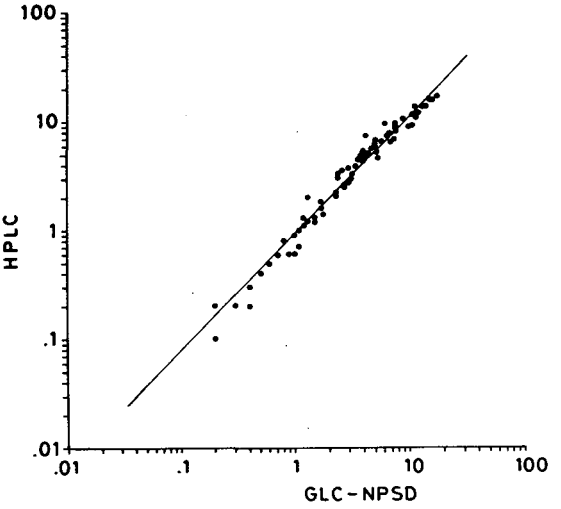

Fig. 3. Comparison between GLC—NPSD and HPLC assays of 88 plasma rat samples.

$r = 0.982$ ($p \ll 0.01$) and $F = 2233$ ($p \ll 0.01$) for a line with a slope of 1.04 and an intercept of 0.209, not significantly different from zero.

As the true caffeine concentrations were not available the accuracy of the reference method could not be established, and since both methods did not show a constant variance over the entire concentration range (constant CV, see Table I) we applied the Bartlett approach to the ln-transformed data. This more correct approach gave $r = 0.988$ and $F = 3533$, both highly significant, for a line with a slope of 1.13 (see also Fig. 3).

If we assume a concentration of 10 μg/ml (chosen as representative of the data in Fig. 3) for the GLC—NPSD method, the corresponding value found using HPLC is 10.76 ± 1.48 μg/ml.

DISCUSSION

In this laboratory these two methods responded as reported in the original papers [9, 10], except that the HPLC detector gave a linear response over a broader range of concentrations than the NPSD detector. From the present study both methods appeared to be sufficiently reliable, although lower concentrations were better assayed with HPLC, as shown by the lower coefficient of variation at the low concentration (Table I).

Toxicological studies deal with very low concentrations of caffeine in humans and with doses and concentrations in animals of up to a hundred times greater [17]. Because of its greater precision over a wider range of concentrations and because it measures caffeine metabolites as well, HPLC appears to be the method of choice.

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Biomedical Applications

CHROMBIO. 373

Note

Estimation of chlorambucil, phenyl acetic mustard and prednimustine in human plasma by high-performance liquid chromatography

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(Received April 12th, 1979)

Chlorambucil {4[4-bis(2-chloroethyl) aminophenyl]butyric acid} is an alkylating agent used in the treatment of human cancer, particularly chronic lymphocytic leukaemia (CLL) [1], Hodgkin's disease [2] and carcinoma of the ovary [3]. Prednimustine {pregna-1,4-diene-11β,17α,21-triol-3,20-dione, 21-(4[4-bis(2-chloroethyl)aminophenyl]butyric acid)}, the prednisolone ester of chlorambucil, has recently been undergoing clinical trial for the treatment of various malignancies including CLL [4].

The estimation of alkylating agents in biological fluids has previously been accomplished by a variety of techniques. These include colorimetry [5], mass spectrometry [6], radiolabelling [7] and gas—liquid chromatography [8]. However, these methods are too complex and time-consuming to allow of their routine use in the clinical laboratory. Recently high-performance liquid chromatography (HPLC) has been employed in the estimation of melphalan {4[bis (2-chloroethyl)amino]-L-phenylalanine}, a chemically related alkylating agent [9, 10]. In the present study a rapid simple HPLC method is reported for the simultaneous estimation of chlorambucil, prednimustine and the chlorambucil metabolite, phenyl acetic mustard, {2[4-bis(2-chloroethyl)-aminophenyl]acetic acid} [11] in human plasma. The HPLC method described has facilitated a study of the fate of chlorambucil and prednimustine in man, the results of which have previously been reported in a preliminary form [12].

EXPERIMENTAL

Apparatus

HPLC separations were carried out on a Waters Assoc. Model ALC/GPC

204 chromatograph (Waters Assoc., Milford, Mass., U.S.A.). This consisted of two Model 6000A solvent pumps, a Model 660 solvent programmer, a Model U6K injector, a Model 440 UV detector, and a Model 450 variable-wavelength detector fitted with a stop/flow spectrum facility. Signals from the detector were recorded on a three-pen Model B-381H Rikadenki recorder (Rikadenki Kogyo, Tokyo, Japan). All separations were accomplished on a Waters Assoc. μBondapak C_{18} column operating in reversed-phase mode. Solvents were filtered and degassed prior to use on a 0.45-μm Millipore filter (Millipore, Bedford, Mass., U.S.A.). Sample injections were made with a 100-μl Pressure Lok Syringe (Precision Sampling, Baton Rouge, La., U.S.A.).

Materials

Glass double distilled water was used in all experiments. Ethyl acetate (Koch-Light, Colnbrook, Great Britain), methanol (James Burroughs, London, Great Britain), acetic acid, sodium sulphate and citric acid (BDH Chemicals, Poole, Great Britain) were all analytical grade. Chlorambucil was a gift from the Wellcome Foundation, Beckenham, Great Britain, and prednimustine was a gift from AB Leo, Helsingborg, Sweden. Phenyl acetic mustard was synthesised by Professor Walter Ross [13] at the Institute of Cancer Research, London, Great Britain.

Patient treatment

Previously untreated patients with Hodgkin's disease requiring chemotherapy were studied immediately before the start of conventional therapy [2]. Following the insertion of an indwelling intravenous cannula patients received either 10 mg of chlorambucil or 20 mg prednimustine, orally.

Extraction procedure

Blood samples were taken into heparinised tubes and centrifuged at 600 *g* for 10 min at 4°. Duplicate 1-ml aliquots of plasma were removed and placed in 15-ml conical glass centrifuge tubes. Two milliliters of ethyl acetate were added to each tube, a fine emulsion was produced by vigorous agitation on a "Whirlimixer", and the tube was immersed in a methanol—carbon dioxide bath at ca. −68°. When completely frozen, the tubes were centrifuged at 600 *g* for 10 min at 4°, during which time the aqueous phase thawed, allowing the organic layer to be removed subsequently. This procedure was repeated with a further 2 ml of ethyl acetate and the pooled ethyl acetate extracts were dried over anhydrous sodium sulphate for 1 h at room temperature. Two milliliters of the dried ethyl acetate extract were removed and evaporated to dryness in a stream of nitrogen at 45°. The residues were redissolved in 100 μl of ethyl acetate and stored in sealed vials at 4° prior to assay.

HPLC analysis

Fifty microliters of the ethyl acetate concentrate (representing 0.25 ml plasma) were injected. Samples were eluted by running a linear gradient from methanol—0.175 *M* acetic acid (60:40, v/v) to 100% methanol over 10 min, commencing at injection time. A flow-rate of 2 ml/min was used on all occasions. The absorbances at 254 nm and 280 nm of the eluate were recorded

simultaneously (0.02 or 0.05 a.u.f.s., chart speed 2 cm/min). At the end of each separation the column was returned to the starting solvent over a 5-min linear gradient. The area under each peak was determined by Xeroxing, cutting out and weighing the relevant peaks. Quantitation was then achieved by comparison with the areas obtained following the injection of reference compounds.

RESULTS AND DISCUSSION

Quantitative aspects of the method

A typical separation of chlorambucil, phenyl acetic mustard and prednimustine is illustrated in Fig. 1. Model 440 UV detector response was linear over the range 5—1000 ng for chlorambucil, phenyl acetic mustard and prednimustine, with a correlation coefficient (r) = 0.999 in each case. The recoveries of chlorambucil, phenyl acetic mustard and prednimustine, from plasma, were investigated by the addition of these compounds to 1-ml aliquots of plasma from healthy human donors. Compounds were added in 10 μl of methanol so as to produce plasma concentrations ranging from 0.05 μM to 10 μM. Samples were assayed in quadruplicate and analysed immediately.

Chlorambucil recovery was linear over the range 0.05—10 μM (r = 0.988) with an extraction efficiency of 77.5%. Prednimustine recovery was linear over the range 0.05—10 μM (r = 0.997) with an extraction efficiency of 95.4%. Phenyl acetic mustard recovery, however, was only linear over the range 0.5—10 μM (r = 0.948) with an extraction efficiency of 32.0%. The poor extraction efficiency of phenyl acetic mustard probably contributed to its higher limit of detection. Attempts to improve the extraction efficiency of phenyl acetic mustard were made by reducing the plasma pH to 3.0 with 1 M citric acid. However this resulted in irreversible protein precipitation during the

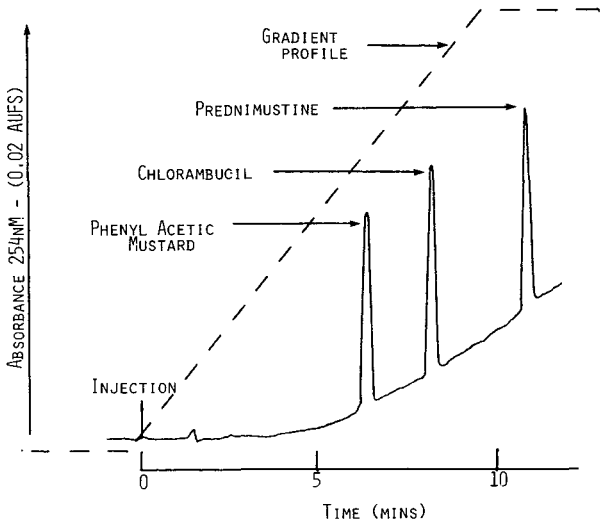

Fig. 1. HPLC separation of phenyl acetic mustard, chlorambucil and prednimustine.

first extraction which prevented the estimation of drug concentrations lower than 3 μM. Hence in all experiments the pH of the plasma samples was left unchanged and relevant calibration factors were used in the calculation. Storage of plasma samples for three weeks at −28° prior to analysis did not affect recovery.

Pharmacokinetic studies

Chlorambucil. The pharmacokinetics of chlorambucil following a 10-mg oral dose were investigated in six patients. Peak levels of chlorambucil were detected 30 min or 1 h after administration, subsequently decaying with a $t_{½}$ of 1.7 h (Fig. 2). Examples of the chromatograms obtained before and 2 h after the oral administration of 10 mg of chlorambucil are shown in Fig. 3. In addition to the chlorambucil peak, a peak with a retention time similar to that of phenyl acetic mustard was detected in the plasma extracts of three patients 2 h after oral administration of 10 mg chlorambucil. The identities of the chlorambucil and phenyl acetic mustard peaks in plasma extracts were verified by retention time, by λ_{max} determinations from stop/flow spectra (Table I), and in two patients by mass spectrometry. Samples for mass spectrometry were obtained by peak collection from the effluent of the HPLC, methylated, and the mass spectra obtained compared with those produced by

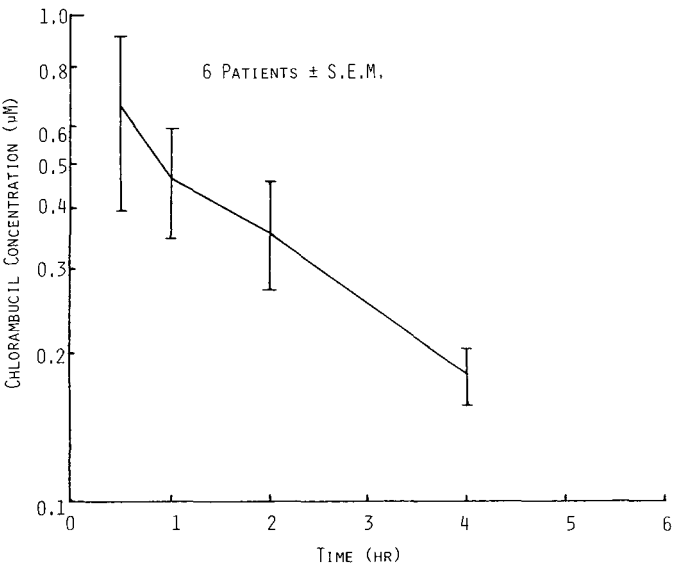

Fig. 2. Concentration of chlorambucil in human plasma following a 10-mg oral dose.

synthetic standards. Hence it has been demonstrated that chlorambucil is metabolised to phenyl acetic mustard in man. However, insufficient assay sensitivity precluded a pharmacokinetic study of this metabolite.

Prednimustine. Twenty milligrams of prednimustine were given orally to six patients and blood samples taken at 0.5, 1, 2, 4 and 6 h and thereafter at six-hourly intervals up to 48 h.

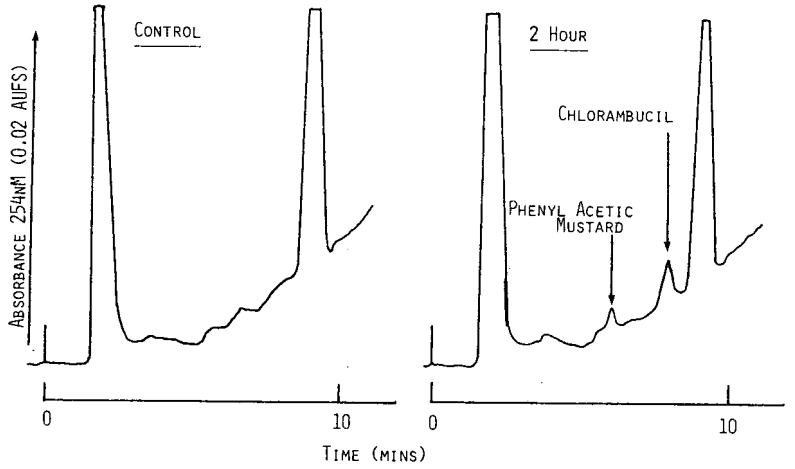

Fig. 3. Chromatograms of extracts from plasma obtained before and 2 h after a 10-mg oral dose of chlorambucil.

TABLE I

PLASMA COMPONENT IDENTIFICATION BY HPLC

	Chlorambucil		Phenyl acetic mustard	
	Standard	Plasma component	Standard	Plasma component
Retention time (min)	7.83 ± 0.07*	7.88 ± 0.12*	5.95 ± 0.07*	6.01 ± 0.09*
λ_{max} (nm)	254—256	254—256	256—259	257—260

*Mean of 6 determinations ± S.E.M.

At no time could prednimustine, chlorambucil or phenyl acetic mustard be detected in the plasma. In all patients it had previously been demonstrated that chlorambucil was absorbed following oral administration. Hence these results indicate that the bioavailability of orally administered prednimustine is lower than that of chlorambucil.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the assistance of Dr. A.H. Calvert in obtaining clinical specimens and Dr. T.J. McElwain for permission to study patients contributing to these studies. We also wish to thank Dr. M. Jarman and Mr. M. Baker for the mass spectrometry studies, and Drs. R. Wilkinson and A. McLean for much helpful advice and discussion. This work was supported by a grant from the Cancer Research Campaign.

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JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

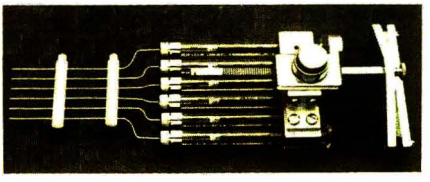
NEWS SECTION

APPARATUS

N-1296

NEW MULTIPLE DELIVERY DISPENSER

Originally designed for serotyping in homotransplantation, the new Hamilton-Terasaki dispenser has wide application wherever there is a need for accurate multiple sample delivery. The new dispenser delivers six identical microsamples



at one time. The volumes dispensed range from 0.5 to 10 μl. In the design Hamilton's accurate gas and liquid tight syringes are used. Plungers and barrels are interchangeable. With the dispenser removable needles are supplied, which are easier to clean. All parts of the Hamilton-Terasaki dispenser are field replaceable.

N-1301

BIOCHEMICAL RESEARCH AND CONTROL PRODUCTS CATALOG

The Brinkmann Instruments catalog BR 360 "Products for Bio-Chemical Research and Control", features a comprehensive listing of many products for use in the laboratory, such as chromatography apparatus and accessories, and electrophoresis equipment.

N-1291

A NEW NAME

Gelman Sciences, Inc. has become the new name for the Gelman Instrument Company, its name for more than the last 20 years. Gelman Sciences, Inc. manufactures membrane filtration equipment for air and water pollution monitoring, pharmaceutical and electronic processing equipment for use in hospital, clinical and pathological laboratories. The firm has operations in the U.S.A., Canada, the U.K., Italy, France and Australia.

N-1273

NEW PHOTOMETER FOR AMINO ACID ANALYSIS

A data sheet for the Model D-550 dual-channel ratio photometer, a high-performance detector for amino acid analysis, is available from the Dionex Corporation (formerly Durrum Instrument Corporation). The data sheet pictures the photometer, lists its features and describes the principles of operation. A functional diagram illustrates the photometer and its relationship to an amino acid analyzer. Also included is a table of applications. A linear absorbance range up to 3.5 a.u. and accurate quantitation from 20 pmoles to more than 50 nmoles is claimed.

CHEMICALS

N-1314

PHARMACIA CATALOG 1979

Pharmacia Fine Chemicals AB has sent us their 1979 general catalog, describing the Pharmacia products for chromatography, electrophoresis and cell studies. New in this catalog are the products Sephacryl S-300 Superfine for faster gel filtration; Sephasorb HP Ultrafine, a new adsorbent for liquid chromatography; Lentil Lectin-Sepharose 4B for purification of membrane glycoproteins; Cytodex 1, the microcarrier for high yield cell culture; and Pharmalite, a range of new ampholytes for isoelectric focusing.

N-1248

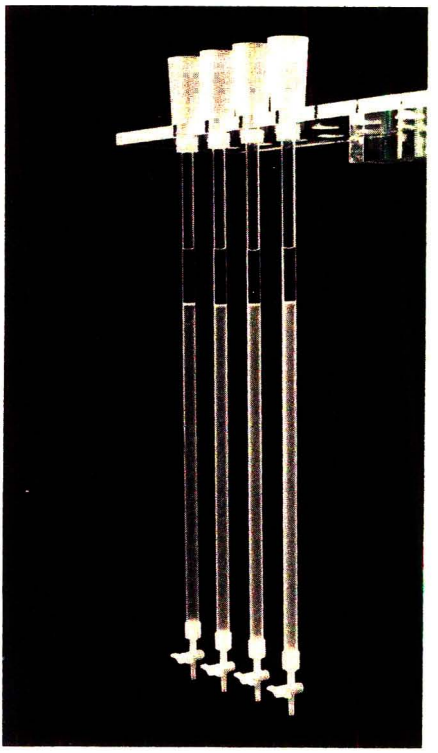
BIO-RADIATIONS

The 26th issue of Bio-Radiations, a publication of Bio-Rad Laboratories, contains informative articles, reference lists, a list of free reprints and a description of new products in the field of DNA sequencing, RIA, immunoadsorption, enzyme purification, SDS electrophoresis and gradient gels. Among the new products are a slab cell, super slab, for DNA sequencing and a high-capacity, activated affinity chromatography support, Affi-Gel-10.

N-1250

CEA BUFFER EXCHANGE COLUMNS

Isolab introduces a newly developed simplified column chromatography procedure to replace the tedious dialysis step in the CEA-Roche™ assay and reduce the time required for buffer exchange from 15 hours to 20 minutes. In



the Roche procedure a dialysis of a perchloric acid CEA extract against four changes of water and one change of ammonium acetate buffer is recommended. The Isolab column requires twenty minutes to accomplish the exchange. The other steps in the CEA-Roche procedure are strictly followed. The columns are prefilled and pre-equilibrated for immediate use. They can be regenerated and reused.

N-1286

NEW GEL FILTRATION MEDIUM

Pharmacia Fine Chemicals introduces a new gel filtration medium, Sephacryl S-300 Superfine. The new medium is fast and easy to pack and gives a good resolution over the protein molecular weight range of 10,000 to 1.5 million. Furthermore, the company provides new molecular weight standards for gel filtration of proteins.

N-1311

ELECTROPHORESIS CALIBRATION KITS

A new booklet, of the same title, was edited by Pharmacia Fine Chemicals. It describes two new kits for the determination of molecular weights of proteins by polyacrylamide gel electrophoresis. The Pharmacia high molecular weight (HMW) and low molecular weight (LMW) electrophoresis calibration kits consist of ten vials containing a lyophilized mixture of highly purified protein standards.

N-1280

ONE-STEP CHROMATOGRAPHY FOR PROTEASE-FREE IgG

An IgG fraction free of proteolytic activity can be obtained in one step by chromatography of whole serum on a new affinity chromatographic material available from Bio-Rad Laboratories. The yield of active antibody is comparable to that obtained using conventional DEAE cellulose techniques. Antibody preparations obtained on the new Bio-Rad DEAE Affi-Gel Blue can be stored without deterioration caused by conversion of plasminogen to active protease, and can be used in immunoassays and in the preparation of labelled antibodies.

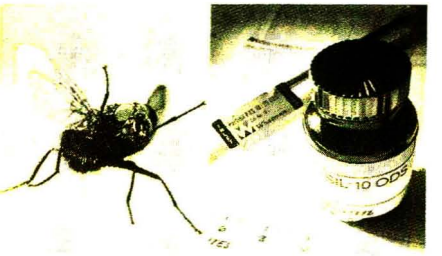
The new material also provides improved fractionation of other serum proteins. DEAE Affi-Gel Blue is an affinity chromatography matrix made by coupling Cibacron Blue F3GA to specially prepared DEAE Bio-Gel A.

PROCEDURES

N-1316

ANALYSIS OF PESTICIDES

"Analysis of pesticides by Whatman HPLC" is a Whatman publication (8122 PA L) and a guide to column packing and solvent mixtures required for optimum separation of specific compounds, together with particular chromatograms obtained in a number of separations. A list of references is also provided.



N-1261

DIONEX DIALOGUE NO. 6

The latest 4-page issue of Dialogue announces the change in name of the Durrum Corporation in Dionex Corporation. Three applications of biological ion chromatography are described: analyses of organic acids; phosphate and sulfate in human serum of kidney dialysis patients; and the analysis of organic and inorganic ions in dairy products. In addition, details are given of an automatic system for biological ion chromatography, the AutoIon^{TM}. Also described is a new line of elution buffers for amino acid analysis, the Hi-Phi eluents, complementing the fluorescence detection system of the Model D-500 amino acid analyzer.

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-1317

BULLETIN ON VALPROIC ACID ANALYSIS

A technical bulletin "Analysis of Valproic Acid", has been released by Supelco. This bulletin discusses the use of SP-1000, a terephthalic acid modified Carbowax, in analyzing valproic acid and other antiepileptic drugs. Valproic acid in concentrations from 2 to 100 μg/ml of serum may be chromatographed without derivatization using a 3-foot column packed with 10% SP-1000 on 80–100 Supelcoport. Other antiepileptic drugs do not interfere with the isothermal analysis of valproic acid on this column. Details are given in the Supelco Bulletin 778.

NEW BOOKS

Enzyme-activated irreversible inhibitors (Proc. Int. Symp. Substrate-Induced Irreversible Inhibition of Enzymes, Strasbourg, July 24–25, 1978), edited by N. Seiler, M.J. Jung and J. Koch-Weser, Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford, 1978, VIII + 359 pp., price Dfl. 110.00, US$ 49.00, ISBN 0-444-80080-8.

Amino acid determination – Methods and techniques, edited by S. Blackburn, Marcel Dekker, New York, Basel, 2nd (revised) ed., 1978, XII + 368 pp., price SFr. 82.00, ISBN 0-8247-6349-1.

Cimetidine, edited by W. Creutzfeldt, Excerpta Medica, Amsterdam, New York, 1978, 328 pp., price Dfl. 122.00, US$ 54.25, ISBN 0-444-90048-9.

Chromatography of synthetic and biological polymers, Vol. 1, Column packings, GPC, GF and gradient elution, edited by R. Epton, Ellis Horwood (Wiley), Chichester, 1978, IX + 368 pp., price £ 18.00, ISBN 0-85312-069-2.

Chromatography of synthetic and biological polymers, Vol. 2, Hydrophobic, ion-exchange and affinity methods, edited by R. Epton, Ellis Horwood (Wiley), Chichester, 1978, IX + 353 pp., price £ 18.50, ISBN 0-85312-073-0.

Drug metabolism in man, edited by J.W. Gorrod and A.H. Beckett, Taylor & Francis, London, 1978, 284 pp., price £ 12.00, ISBN 0-85066-104-8.

Blood drugs and other analytical challenges, edited by E. Reid, Ellis Horwood (Wiley), Chichester, 1978, XI + 355 pp., price £ 19.50, ISBN 0-85312-124-9.

Biological and biomedical applications of isoelectric focusing, edited by N. Catsimpoolas and J. Drysdale, Plenum, New York, London, 1977, XV + 351 pp., price US$ 39.00, ISBN 0-306-34603-6.

Biological/biomedical applications of liquid chromatography, edited by G.L. Hawk, Marcel Dekker, New York, Basel, 1979, XVII + 736 pp., price US$ 45.00, ISBN 0-8247-6784-5.

Progress in clinical pharmacy (Proc. Eur. Symp., Mainz, November 2–4, 1978), edited by D. Schaaf and E. van der Kleijn, Elsevier/North-Holland, Amsterdam, Oxford, New York, 1979, X + 274 pp., price Dfl. 90.00, US$ 44.00, ISBN 0-444-80122-7.

Frontiers in bioorganic chemistry and molecular biology, edited by Yu.A. Ovchinnikov and M.N. Kolosov, Elsevier/North-Holland Biomedical Press, Amsterdam, Oxford, New York, 1979, XII + 233 pp., price Dfl. 120.00, US $ 58.50, ISBN 0-444-80072-7.

Analysis of drugs and metabolites by gas chromatography–mass spectrometry, Vol. 6, Cardiovascular, antihypertensive, hypoglycemic, and thyroid-related agents, by B.J. Gudzinowicz and M.J. Gudzinowicz, Marcel Dekker, New York, Basel, 1979, XI + 446 pp., price Sfr. 106.00, ISBN 0-8247-6757-8.

Electrophoresis '78 (Proc. Int. Conf. Electrophoresis, Cambridge, Mass., April 19–21, 1978), edited by N. Catsimpoolas, Elsevier/North-Holland Biomedical Press, New York, Amsterdam, Oxford, 1978, XI + 443 pp., price Dfl. 117.00, US $ 52.00, ISBN 0-444-00294-4.

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 authors' 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.

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