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# QUANTITATIVE GAS CHROMATOGRAPHIC DETERMINATION OF TWO OXIDIZED METABOLITES OF THE DIURETIC MEFRUSIDE IN HUMAN URINE, PLASMA AND RED BLOOD CELLS

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## SUMMARY

A gas chromatographic method is reported for the quantitative analysis of two metabolites of mefruside, viz., 5-oxo-mefruside (mefruside lactone) and its hydroxy-carboxylic acid analogue in human body fluids. Use was made of extractive methylation as the derivatization technique, and quantitation was achieved, with a suitable internal standard, by means of a nitrogen-sensitive detector.

Because the two metabolites are linked chemically through a lactone—open acid equilibrium, interconversion prior to their separation had to be avoided. A pH partitioning study was performed to find optimal separation conditions. The lactone could be extracted quantitatively at pH 7.4, without any trace of co-extracted hydroxy acid. The latter was extracted either at pH 2 directly (in the case of plasma and urine), or after conversion to the lactone at pH 7.4 (in the case of red cells or whole blood). Concentrations down to 25 ng per sample of both compounds could be analysed with a standard deviation of 5%.

The two metabolites of mefruside equilibrated instantaneously between red cells and plasma in vitro. At 37°, the red cell/plasma concentration ratio was 20 for the lactone, but only 0.1 for the open acid compound. 5-Oxo-mefruside was able to displace mefruside from its red blood cell binding sites in vitro.

## INTRODUCTION

Recently a few methods for the selective determination of the diuretic mefruside in body fluids have been published [1, 2]. After administration of mefruside to humans less than 1% of the dose was recovered in the urine as the unchanged compound [3]. It seemed logical, therefore, to search for metabolites of this drug in man.

In the rat, two urinary metabolites, which were formed by oxidation of the

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C-5 atom of the tetrahydrofuran ring of mefruside, constituted together ca. 35% of the dose [4]. These metabolites appeared to be related through a lactone—hydroxy acid equilibrium (Fig. 1). The reaction was shifted completely to the lactone side under acidic conditions (e.g. pH 2) and to the open acid side at alkaline pH (e.g. pH 11), the rates of ring closure and hydrolysis being pH-dependent [5, 6]. In aqueous buffers of pH 7—8, interconversion of both compounds was unmeasurably slow, but it became highly accelerated by the addition of rat plasma or rat liver homogenates. Also, rapid turnover of the two metabolites has been observed in vivo, in dogs [6].

Our attention was drawn to the above metabolites in particular because both substances had been reported to bring about the same diuretic effect as the parent drug after intravenous administration to rats [7]. This raised the possibility that the two compounds would be determinants of drug action also in the human situation. The aim of the present investigations was therefore to develop an assay for these mefruside metabolites and to demonstrate their formation after administration of mefruside to man. Due to their particular chemical constitution, care had to be taken to avoid unwanted interconversion of the compounds in vitro prior to their separation.

## MATERIALS AND METHODS

### *Drugs*

5-Oxo-mefruside, 4-chloro-$N^1$-methyl-$N^1$-(tetrahydro-2-methyl-5-oxo-2-furanyl)methyl-1,3-benzenedisulfonamide (Fig. 1), and the internal standard, 4-chloro-$N^1$-methyl-$N^1$-(3-methoxypropyl)-1,3-benzenedisulfonamide,

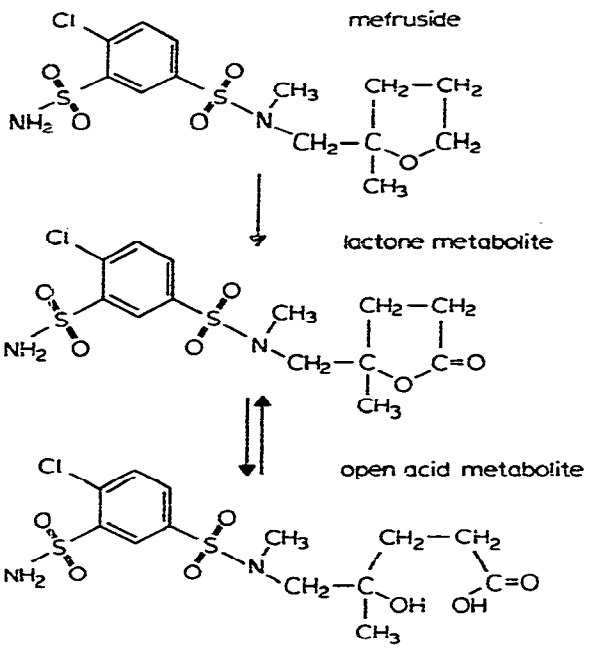


Fig. 1. Structural formulae of mefruside, 5-oxo-mefruside and its hydroxycarboxylic acid analogue.

identical retention time to the derivative of the latter. This was not unexpected because both compounds pass through an alkaline layer (0.1 *M* NaOH) prior to methylation, so that the lactone is transformed completely to the free acid form, and a common species is generated. A discussion on the structure of this derivative during GC will be given in the Results section below.

*pH-dependent distribution between aqueous and organic phases*

*Lactone partitioning.* Into glass tubes containing 2 ml of aqueous buffers varying from pH 2 to pH 10 [citrate—phosphate—borate (0.1 *M*), according to Teorell and Stenhagen], were pipetted 10-ml portions of a 0.5 μg/ml solution of 5-oxo-mefruside in freshly distilled diethyl ether. The tubes were well-closed and vigorously shaken for 5 min. After brief centrifugation, as much as possible of the diethyl ether layer was transferred to another tube. The samples containing the buffers of highest pH were handled first in order to minimize lactone hydrolysis. At pH 11, the half-life of lactone hydrolysis is 27 min [6], which implies that after 5 min in such solution 88% of the drug is still in the lactone form, i.e. a loss of 12%. Because the highest pH we employed was a full pH unit lower (pH 10), we estimated a loss of less than 5% at that pH, which should have an insignificant influence on the shape of the distribution curve.

*Hydroxy acid partitioning.* Aliquots (0.1 ml) of a 50 μg/ml solution of the hydroxy acid analogue of 5-oxo-mefruside in 0.1 *M* NaOH were added to 1.9-ml portions of buffer at pH 2—10 (same buffer as described above). After mixing, 10-ml portions of freshly distilled diethyl ether were added and the tubes were vigorously shaken for 5 min. After short centrifugation (1 min), as much as possible of the organic layers was transferred into new tubes. For samples containing the buffers of lowest pH this separation was performed at once in order to minimize lactone formation. The half-life of ring closure at pH 2 is 3.3 h [6], which means that 93.3% of drug is still in the free acid form after 5 min at this pH, so that the observed distribution at the lowest pH values, too, should be a true reflection of the lipophilicity of the pure open acid species.

*Measurement of extraction recovery.* To calibrate the above partitioning studies, known amounts of lactone (0.1—0.5 μg) and free acid (0.05—5 μg) were dissolved in diethyl ether (10 ml) and 0.1 *M* NaOH (2 ml), respectively. The sodium hydroxide layers were used directly; the diethyl ether layers were extracted first with 2 ml of 0.1 *M* NaOH for 5 min, and then used in the extractive methylation, together with internal standard (5 μg in 0.1 ml of 0.1 *M* NaOH). The percentage extraction at each pH was calculated by comparing the ratios of the peak areas of drugs and internal standard.

*Procedure for the extraction of 5-oxo-mefruside and hydroxy acid analogue from biological samples*

In order to determine 5-oxo-mefruside, 2 ml of plasma, 0.5—1 ml of packed red cells (1—2 ml of whole blood), or 1 ml of urine, were extracted at pH 7.4 twice with 10 ml of diethyl ether under the same conditions, including addition of internal standard, as described for mefruside [1].

After this extraction of 5-oxo-mefruside, the same biological samples served to determine the hydroxy acid species. Plasma (2 ml) or urine (0.25—1 ml) was adjusted to pH 2 with a few drops of 2 *M* aqueous HCl and extracted, after renewed addition of internal standard, twice with 10 ml of diethyl ether for 5 min. Red blood cell or whole blood samples, however, extracted at pH 2, yielded a very high background signal in their gas chromatograms and in addition unknown interfering peaks. Therefore, the hydroxy acid in these samples was converted into the lactone form by leaving the tubes at pH 2 for 24 h or longer. Subsequently, the samples were adjusted again to pH 7.4 with solid sodium bicarbonate (100—200 mg/sample); internal standard and buffer at pH 7.4 were added to give a final volume of 2.5 ml and the lactone was extracted in the same way as described at the beginning of this section. Previously, we had verified that ring closure was complete under these conditions, by comparison with known amounts of 5-oxo-mefruside. (Moreover, in assaying urine concentrations of the hydroxy acid, the same quantitative results were found from the lactone conversion procedure as from direct extraction at pH 2.)

Parallel to each series of biological samples, two or three standards of 5-oxo-mefruside and its hydroxy acid counterpart, added to a corresponding volume of buffer at pH 7.4, were extracted at pH 7.4 or pH 2, derivatized and submitted to GC, in order to check the overall procedure. For this calibration, known amounts covering the concentration range of the unknown samples were taken, for example, 0.5, 1 and 2.5 μg of 5-oxo-mefruside in the urine assay.

*Sampling of blood and urine, in vitro distribution between plasma and red cells*

The method of collection of venous blood from human subjects was the same as described earlier [1]. Briefly, 7-ml heparinized blood samples were centrifuged at 1500 *g* for 3 min at room temperature, immediately after vein puncture, and the plasma was rapidly separated. Because the equilibration of mefruside between plasma and red cells was unmeasurably rapid, and the position of the final equilibrium temperature-dependent, the decrease in blood temperature inherent in routine treatment of blood samples (vein puncture, centrifugation) caused an altered red blood cell/plasma concentration ratio of this drug [1]. A similar phenomenon might be possible for 5-oxo-mefruside and its hydroxy acid counterpart. Therefore, partitioning of these two compounds between plasma and erythrocytes was studied in fresh human blood, according to the methods published previously [1]. Thus, the rate of distribution at 37°, and the difference between plasma concentrations present at in vivo temperature (37°) and those found after routine treatment of the blood (27.5°) were determined. A complicating factor arose from interconversion of the lactone and the open acid in incubations of fresh human plasma. Although different in extent for each plasma sample, hydrolysis of the lactone could account for as much as 25% of the added amount, but ring closure of the hydroxy acid was always less than 5% (at plasma concentrations of 40 μg/ml). This pattern was qualitatively in agreement with observations on the interconversion of the two species in rat plasma [6]. However, because interconversion in human plasma appeared to be finished after 15—30 min, we pre-

incubated the two substances for 30 min in plasma at 37° prior to the red blood cell partitioning experiments, and measured the concentrations of the drug under study in both plasma and red cells. The concentrations used in the present in vitro studies are indicated in the Results section.

Urine from human experiments was adjusted to pH 8 with a few drops of 5 *M* NaOH, prior to extraction. Care was taken to mix the contents of the tube vigorously during pH adjustment, in order to prevent hydrolysis of 5-oxo-mefruside.

*Comparison of presumed metabolite with synthetic 5-oxo-mefruside by gas chromatography—mass spectrometry*

Electron-impact mass spectra of the methylated derivatives of synthetic 5-oxo-mefruside and mefruside were compared with that of the suspected metabolite by means of a LKB 9000 gas chromatograph—mass spectrometer combination (LKB, Bromma, Sweden), at an accelerating potential of 20 eV, trap current of 60 μA and ion-source temperature at 260°. For GC separation a glass column (1.5 m × 3 mm I.D.) was used, packed with 3% OV-101 on Gas-Chrom Q, 100—120 mesh (Applied Science Labs., State College, Pa., U.S.A.), at an oven temperature of 255°. The retention times of the methylated derivatives of internal standard, mefruside, and 5-oxo-mefruside were 8.5 min, 13 min and 18 min, respectively.

## RESULTS AND DISCUSSION

*Drug partitioning*

The distribution of 5-oxo-mefruside and its open-chain analogue between organic phase and aqueous buffer of varying pH is shown in Fig. 2. The plots indicate that the lactone can be recovered in the ether layer by a single extraction already with a 87% yield at pH 2—8.5, similarly to extraction of mefruside, whereas the carboxylic acid can only be obtained in high yield (76%) below pH 3, and is not extracted at all above pH 7. This latter detail was checked by separate extractions at pH 7.4 (not shown here), using as aqueous phases buffer alone, and buffered plasma, red cells and urine. These experiments confirmed that the quantity of the open acid compound, recovered in the organic phase by two subsequent extractions with diethyl ether at this pH, was not detectable in our GC assay, viz. <10 ng of a total amount of 5 μg. This means that the percentage of hydroxy acid extracted at pH 7.4 is even lower than 0.2%. In this way, the two structural analogues were separated prior to the subsequent steps of the analysis.

*Gas chromatographic determination of 5-oxo-mefruside and its open acid counterpart in plasma, red blood cells and urine*

Calibration graphs, prepared by adding known amounts of the lactone and the open acid to blank human plasma, urine and red cells, and plotting, after GC analysis, peak area ratios of drugs and internal standard against concentration, were linear and passed through the origin. The standard deviation of the whole procedure, determined from repeated assay of the same samples was ca. 5% ($n$ = 10) for both compounds at concentrations between 0.05 and 10 μg/

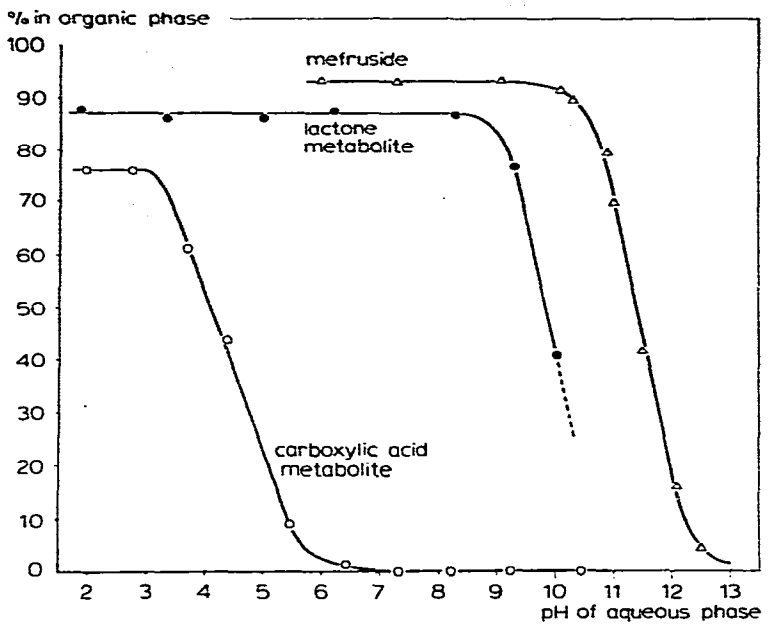


**Fig. 2. Plots showing the percentage of extraction of mefruside, 5-oxo-mefruside and the hydroxy acid metabolite against the pH of the aqueous phase (diethyl ether, water, phase volume ratio: $V_{org}/V_{aq} = 5$).**

sample. The lowest concentration which could accurately be analysed (i.e. with the above standard deviation) was approximately 25 ng/sample, whereas amounts down to 5—10 ng in biological extracts could still be detected. The recovery of 5-oxo-mefruside by two extractions with diethyl ether at pH 7.4 was 98.5 ± 1% (mean ± S.D., $n = 4$) in the concentration range investigated, independent of the choice of buffer alone, plasma, red cells or urine as the aqueous phase. The corresponding figure for extraction of the hydroxy acid analogue at pH 2 was 94 ± 2% (mean ± S.D., $n = 4$).

*Identification of mefruside metabolites in biological samples*

The gas chromatograms obtained by analysis of urine from human subjects who had ingested an oral dose of mefruside (25 or 50 mg) as described elsewhere [3], showed a large peak eluting after that of mefruside itself, with a retention time of 6.3 min (Fig. 3), which was the same as that of the methylated derivative of synthetically prepared 5-oxo-mefruside. The peak was present already after extraction of urine at pH 7.4, but became larger when urine had been extracted at pH 2. This extra amount could also be recovered by extraction at pH 7.4 if the urine had previously been allowed to stand at pH 2 for several hours.

The electron-impact mass spectrum of the presumed metabolite, extracted from human urine and separated by gas chromatography, was identical with that of the pure reference compound. The mass spectra of the methyl derivatives of mefruside and 5-oxo-mefruside are shown in Fig. 4. The fragmentation of both compounds is consistent with the scheme of Fig. 5. After being

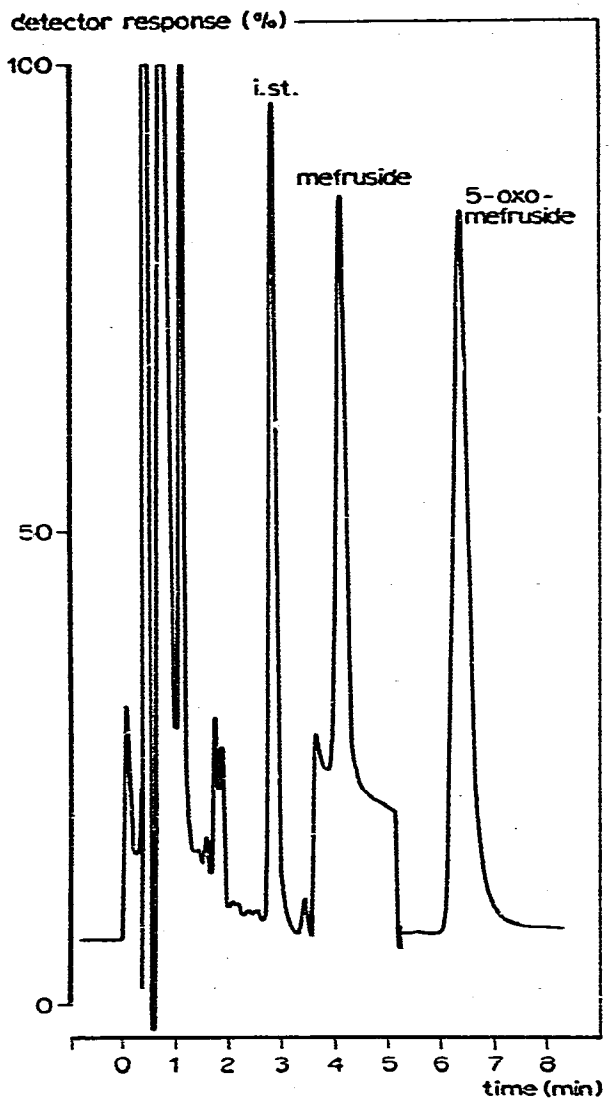


Fig. 3. Typical gas chromatogram showing the presence of mefruside (0.15 μg/ml) and 5-oxo-mefruside (2.7 μg/ml) in human urine, analysed 5 h after intake of a 50-mg dose of mefruside by a normal human subject (i.st. = internal standard).

split off, the tetrahydrofuran ring of both mefruside and its oxidized metabolite appears to be relatively stable, as the *m/e* 85 and 99 fragments are very abundant. It can be seen that a number of peaks in the upper panel are shifted to the right with respect to the mefruside spectrum with 14 *m/e* units (namely 99 vs. 85, 142 vs. 128, and 381 vs. 367), which differences are clearly attributable to the replacement of two hydrogen atoms by oxygen at the C-5 atom of the tetrahydrofuran moiety. Furthermore, the fragmentation pattern of the two compounds is closely comparable with that of a structural analogue with a straight chain (III in Fig. 5), which is used as the internal standard in the GC assay.

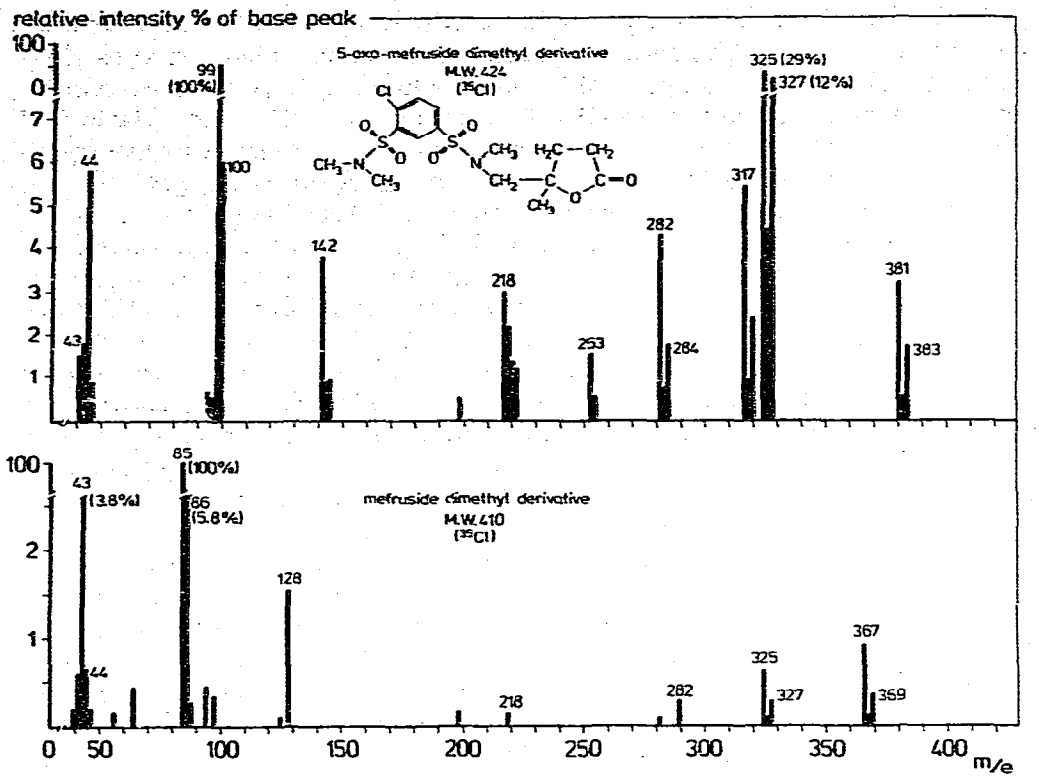


Fig. 4. Electron-impact mass spectra (20 eV) of the methylated derivatives of 5-oxo-mefruside and mefruside, after GC separation.

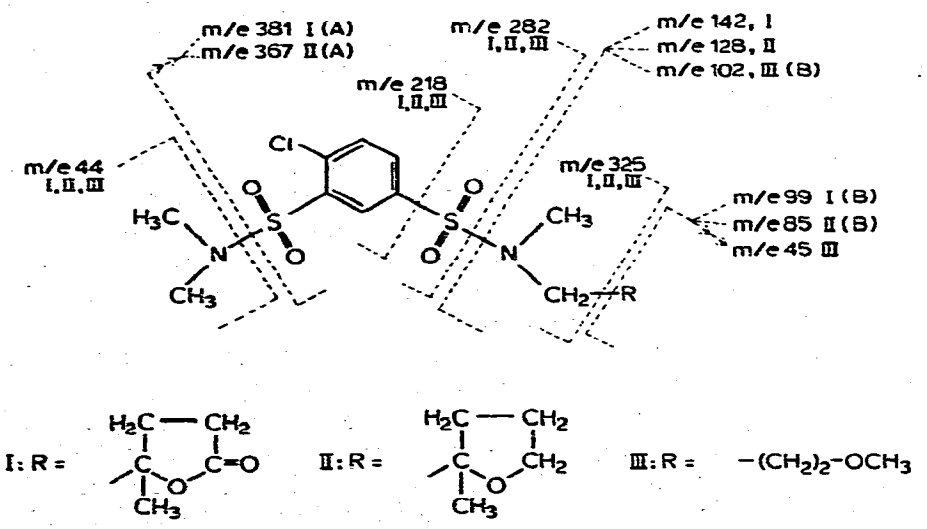


Fig. 5. Common mass fragmentations of the dimethylated derivatives of 5-oxo-mefruside (I, M.W. = 424, $^{35}Cl$), mefruside (II, M.W. = 410, $^{35}Cl$), and a straight-chain analogue (III, M.W. = 384, $^{35}Cl$). (A) Presumably formed after H shift from *m/e* 44 fragment. (B) Base peak.

Only the lactone form of both occurring oxidized metabolites of mefruside has been drawn in Fig. 4. The thermodynamic equilibrium between these two species lies completely at the open-acid side at alkaline pH [6]. There can be no doubt, therefore, that any derivative formed by extractive methylation of either the lactone or the open acid as starting substance, existed in the open-chain form exclusively prior to injection into the gas chromatograph. Whether two, three or even four methyl groups have been introduced at that stage is not known. There is, however, besides the excellent agreement of the mass fragmentation pattern and molecular formula depicted in Fig. 4, another argument to favour the view that *during gas chromatography* the methylated derivative possesses the structure shown. It is well-known that, on heating, γ-hydroxycarboxylic acids and their ester (and even ether) derivatives are readily converted to the corresponding lactones [9]. Operated at a temperature of 280°, the injection port of the gas chromatograph must amply have provided such a condition. It must be concluded, therefore, that both metabolites of mefruside (i.e. the lactone and the open acid) are chromatographed as the same methylated derivative, with the formula depicted in Fig. 4.

Duhm et al. [4] have proposed N-demethylation of mefruside or its metabolites in the rat to account for a small fraction of dose, leading to carbon dioxide in expired air after administration of the methyl-$^{14}$C-labelled drug. The methylation which is employed in the present assay could have masked possible biological formation of $N^1$-demethylated 5-oxo-mefruside or its hydroxy acid congener. The methylated product of this metabolite would be indistinguishable from that originating from 5-oxo-mefruside (or open acid analogue) itself. We were already able to exclude the presence of detectable amounts of demethylated mefruside in human body fluids by the use of propyl iodide as the derivatizing agent [1]. In an analogous way, we now carried out propylation of both urine, plasma and red cell extracts from several human subjects, and did not find a trace of a substance with a GC retention time other than that belonging to the propylated derivative of 5-oxo-mefruside itself. In this way, the selectivity of the assay for 5-oxo-mefruside and its open acid counterpart was considered to be affirmed.

*In vitro distribution of mefruside metabolites between plasma and erythrocytes*

5-Oxo-mefruside and its hydroxy acid analogue equilibrated instantaneously between plasma and red cells of human blood. Thus, no difference was observed in the red cell/plasma concentration ratios at 37°, whether the blood had been centrifuged immediately after mixing red cells with plasma, or at 1.5, 5, 15 or 45 min after the start of the incubation.

The lactone and the open acid differed greatly in their extent of red cell partitioning. While the lactone reached concentrations in erythrocytes which were ca. twenty times higher than those in plasma, the hydroxycarboxylic acid concentrations in red cells were only one-tenth of those in plasma, at a whole blood concentration of 8 μg/ml for both experiments. For this reason, only the red cell uptake of the lactone was subjected to further investigation. The red cell/plasma concentration ratio appeared to be constant in the concentration range used, 2—15 μg/ml whole blood, and mean ratios of 18 and 21 were found from incubations at 37° in blood from two human subjects.

When blood incubated with the lactone at 37° was centrifuged at room temperature for 3 min (resulting in a blood temperature of 27.5° [1]), the plasma concentrations of the drug were 96.5 ± 1.5% (mean ± S.D., $n = 6$) of the values obtained when the whole procedure was carried out at 37°. It was concluded therefore that plasma concentrations of 5-oxo-mefruside, found after immediate centrifugation of blood following vein puncture, differ by only ca. 3.5% from the concentrations actually present at in vivo temperature.

Because both 5-oxo-mefruside and mefruside [1] are rapidly taken up by red blood cells, we were interested to see if the binding of the metabolite would influence that of the parent drug. Fig. 6 shows that the erythrocyte/plasma concentration ratio of mefruside, incubated in whole human blood, decreases with increasing concentration of 5-oxo-mefruside. Although the interaction seems of little importance at whole blood concentrations of the lactone below 1 μg/ml, the effect visible at higher concentrations might also play a role in vivo. Some evidence for displacement of mefruside from its red cell binding sites in vivo can be taken from a recent kinetic study with mefruside in human subjects [3].

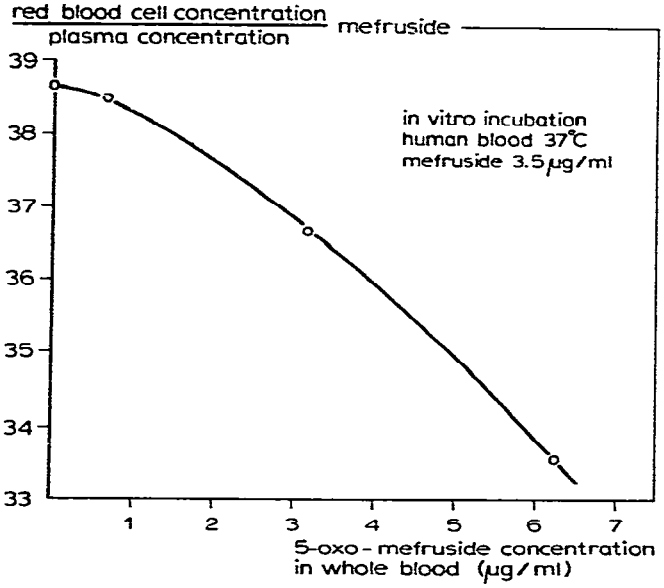


Fig. 6. Graph indicating displacement of mefruside from its red blood cell binding sites by 5-oxo-mefruside in vitro (not included in this figure is a red cell/plasma concentration ratio of 9.4, measured at a lactone concentration of 30 μg/ml in the same blood).

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