Journal of Chromatography, 273 (1983)335—345
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1549

COMPARISON OF TWO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF OXMETIDINE AND ITS METABOLITES IN PLASMA, BILE AND URINE SAMPLES

R.M. LEE* and R.D. McDOWALL

Department of Metabolic Biochemistry, Smith Kline and French Research Ltd., The Frythe, Welwyn, Hertfordshire (Great Britain)

(First received May 13th, 1982; revised manuscript received October 15th, 1982)

SUMMARY

Two high-performance liquid chromatographic methods for the assay of oxmetidine are described: both utilize the same liquid extraction from plasma, urine and bile samples. A normal-phase technique is considered most suitable for the analysis of plasma extracts and a reversed-phase method is preferred for the assay of excretory fluids such as urine and bile which will contain polar metabolites in detectable quantity as well as unchanged oxmetidine.

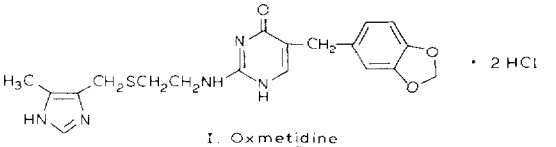
The methods are sensitive enough to follow the kinetic changes in concentration for up to 8 h after the administration of recommended therapeutic doses. Both methods can be automated in respect of the high-performance liquid chromatograph and the samples can be stored for several weeks at —20°C without prejudicing the accuracy of the analysis.

INTRODUCTION

Oxmeditine (SK&F 92994, Fig. 1) is a potent antagonist of the action of histamine at H_2-receptors [1]. It has been tested in animal models and in human volunteer studies [2] during which correlations between the pharmacodynamics and the plasma kinetics have been sought.

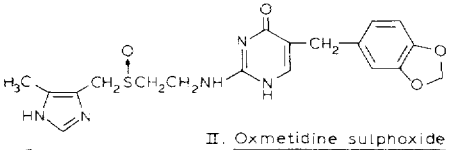
In order to follow the pharmacokinetics of absorption and elimination, high-performance liquid chromatographic (HPLC) methods of determining plasma concentrations of oxmetidine were investigated. The first was a simple modification of the normal-phase adsorption chromatography used extensively for cimetidine analysis [3], the second was a reversed-phase technique, which was developed with a view to carrying out the rapid assay of urine samples for oxmetidine and metabolites without the delay normally associated with a normal-phase technique. This delay is engendered by the need to allow polar materials, e.g. creatinine and the metabolites of oxmetidine which absorb at

0378-4347/83/0000—0000/$03.00



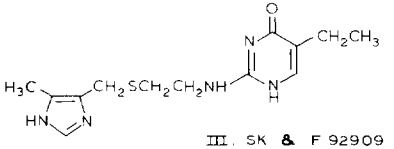
I. Oxmetidine

2-[2-(5-methyl-4-imidazolylmethythio)ethylamino]-5-(3,4-methylenedioxybenzyl)-4-pyrimidone diHCl



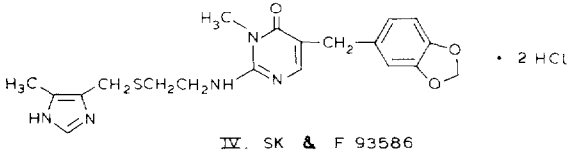
II. Oxmetidine sulphoxide

2-[2-(5-methyl-4-imidazolylmethylsulphinyl)ethylamino]-5-(3,4-methylenedioxybenzyl)-4-pyrimidone



III. SK & F 92909

2-[2-(5-methyl-4-imidazolylmethylthio)ethylamino]-5-ethyl-4-pyrimidone



IV. SK & F 93586

2-[2-(5-methyl-4-imidazolylmethylthio)-ethylamino]-1-methyl-5-(3,4-methylenedioxybenzyl)-6-pyrimidone diHCl

Fig. 1. Formulae of oxmetidine, its sulphoxide, and internal standards.

about 230 nm, to elute from a silica adsorption column. Such polar substances are not present in interfering amounts in plasma samples, but are a source of considerable delay in the assay of urine and bile, for which a reversed-phase column should be more suitable.

EXPERIMENTAL

All chemicals in this study were analytical grade with the following exceptions: 1-octanol (Koch-Light, Colnbrook, Great Britain, puriss), methanol and acetonitrile (Rathburn, Walkerburn, Great Britain, HPLC grade), 0.88 sp. gr. ammonium hydroxide solution (May and Baker, Dagenham, Great Britain, reagent grade), camphorsulphonic acid (Aldrich, Milwaukee, WI, U.S.A., reagent grade), β-D-glucuronidase EC 3.2.1.31 (Sigma, St. Louis, MO, U.S.A., type B3) from bovine liver. Samples of water were purified by deionisation, then distilled in an all-glass apparatus and stored in glass containers.

All solvents and solutions for HPLC were filtered through either 0.45-μm membrane filters (Millipore, Bedford, MA, U.S.A., type HA) for aqueous solutions or 0.5-μm membrane filters (Millipore type FH) for organic solvents.

A solution of carbonate buffer (1 mol/l; pH 9) was prepared by a modification of the method used by Delory and King [4]: to 5 l of sodium bicarbonate (1 mol/l) was added sufficient sodium carbonate (1 mol/l) to adjust the pH value of the solution to 9.0.

The solution of acetate buffer (0.1 mol/l; pH 5.0) was prepared by the addition of 322 ml sodium acetate solution to 678 ml acetic acid (0.1 mol/l).

A stock solution of oxmetidine was prepared by weighing 10 mg base equivalent of the dihydrochloride salt, dissolving it in approximately 0.5 ml water and making up to a 100-ml volume with ethanol. Solutions of oxmetidine sulphoxide (II, Fig. 1) and the two internal standards SK&F 92909 (III, Fig. 1) and SK&F 93586 (IV, Fig. 1) were similarly prepared by weighing 5, 40 and 100 mg base equivalent respectively, and dissolving each in separate aliquots of water and ethanol as above. All stock ethanolic solutions were stored at −20° C and found to be stable for at least three months under these conditions.

Working solutions of the respective internal standards were prepared fresh daily by diluting the stock solutions 100-fold with carbonate buffer (1.0 mol/l; pH 9).

Human plasma for preparation of standards was obtained by centrifugation (at 4° C) of the blood from volunteers and stored in 20-ml sterivials at −20° C until thawed for use. The polypropylene centrifuge tubes (12 ml) and stoppers used for sample extraction were obtained from Henleys Medical Supplies, London, Great Britain, (type 300 PP and 301 PT, respectively).

Preparation and storage of plasma samples

Blood from patients and volunteers, who had received oxmetidine, was taken by syringe into tubes containing lithium heparin as an anticoagulant. Each tube was mixed gently for a few minutes and then centrifuged to separate the plasma, which was transferred to plain tubes and stored at −20° C as soon as possible after the separation. Bile and urine samples were frozen in plain tubes as soon as possible after collection, and stored at −20° C until analysed.

Analysis of plasma

To a 2-ml plasma sample in a polypropylene centrifuge tube (12 ml) was added 1 ml of carbonate buffer (1 mol/l; pH 9), containing 4 μg base equivalent SK&F 92909 or 10 μg base equivalent of SK&F 93586 as an internal standard. If the volume of the sample was less than 2 ml then sufficient 0.9% sodium chloride solution was added to adjust the volume to the required amount.

A 5-ml volume of 1-octanol was added to the samples and the tubes were stoppered and rotated for 15 min on a blood-cell suspension mixer. The organic layer was separated from the aqueous phase by centrifugation at 1500 *g* (5 min) and 4—4.5 ml of the octanol layer were transferred to a second polypropylene centrifuge tube containing 3 ml 0.02 *M* hydrochloric acid.

The same rotary mixing and centrifugation technique was used to re-extract the compound into the acid and to separate the phases. The octanol layer was removed by aspiration and 2.5 ml of the acid was transferred to a clean polypropylene tube; 250 μl acetonitrile was added and mixed before saturating the whole with solid potassium carbonate (c5g). This had the effect of salting out the acetonitrile into a discrete layer, which was removed, after centrifuga-

tion (1500 *g* for 5 min at 4°C) and stored at −20°C to await HPLC separation and analysis.

In addition to the test samples, plasma samples containing known quantities of oxmetidine were carried through the procedure under the same conditions; 12 tubes containing 2 ml blank plasma to which had been added, in duplicate, 0, 1, 2, 4, 8 and 16 μg base equivalent oxmetidine were used, and from the assay of these a calibration curve was constructed.

Analysis of urine and bile

The procedure for the extraction of oxmetidine from urine and bile was exactly as described for the extraction from plasma. However, the urine of subjects receiving oxmetidine has been shown to contain the sulphoxide metabolite and glucuronide conjugates of both oxmetidine and its sulphoxide [5].

The unconjugated sulphoxide has been shown to be extracted under the conditions employed but the glucuronides were not; thus, an estimate of the concentrations of these conjugates in urine and bile was dependent upon their hydrolysis to the aglycones and re-assay of the samples to give a total for oxmetidine and sulphoxide which included the glucuronide-conjugated material.

This hydrolysis was achieved by incubating 1-ml samples of urine or bile with β-glucuronidase (2 mg) for 24 h at ambient temperature. The sample was then increased to 2 ml by the addition of 1 ml of 0.9% sodium chloride solution, and the internal standard was added in carbonate buffer as previously described. The rest of the extraction procedure was as given for plasma samples.

For urine and bile samples calibration curves were constructed for oxmetidine sulphoxide as well as for oxmetidine. To each 1 ml of urine or bile were added 0, 1, 5, 10 or 20 μg of oxmetidine and 0, 0.5, 2.5, 5 or 10 μg of oxmetidine sulphoxide; these spiked samples were extracted along with the test samples for each batch of samples assayed.

HPLC equipment

The chromatograph had the following components: the pump was a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A or equivalent, the injectors used were Rheodyne 7125 valve type (50 μl loop) or WISP (Waters Assoc.) automatic. The columns used were a normal-phase column, Ultrasphere-Si, particle size 5 μm, 250 × 4.6 mm I.D. and a reversed-phase column, Ultrasphere-ODS, particle size 5 μm, 150 × 4.6 mm I.D. The Perkin-Elmer LC100 column oven was set at 25°C for normal-phase working, and 75°C for reversed-phase working. The detectors, a Perkin-Elmer LC55B (226 nm) or a LC 75 (226 nm), were set to give 0.04 or 0.08 absorbance units full scale (a.u.f.s.).

HPLC solvent systems

Normal phase. For the normal-phase work with the Ultrasphere-Si 5-μm column the solvent was a mixture of acetonitrile—methanol—water—ammonium hydroxide (sp. gr. 0.88) (200:40:10:1.5, v/v).

The acetonitrile, methanol and water were mixed and degassed at reduced

pressure before the addition of the ammonium hydroxide. This solvent was then passed through the column for 1 h, with recycling, to equilibrate the system before commencing the assays. If an automatic injector is to be used, this solvent is usable for up to 48 h after equilibration provided that ammonia loss is reduced by capping the reservoir.

Reversed phase. The solvent for the reversed-phase system was a mixture of acetonitrile—0.02 *M* camphorsulphonic acid solution (30:70, v/v). The latter was prepared by dissolving 1.16 g of camphorsulphonic acid in 175 ml of distilled water and filtering the solution through a 0.45-μm Millipore HA filter.

The solvent mixture was degassed at reduced pressure and then pumped through the system for about 30 min in order to equilibrate the system at 75°C. During the preparation of this solvent, the column was back-flushed with filtered methanol at room temperature, since this was found to lengthen the working life of the column.

Flow-rates and retention times

With the Ultrasphere-Si 5-μm column, a flow-rate of 1.8 ml min^{-1} was used to give retention times of 3 min for oxmetidine and 4 min for the internal standard, SK&F 92909. In this system, oxmetidine sulphoxide was retained for about 10 min and creatinine for about 6 min. Using the Ultrasphere-ODS column and a flow-rate of 2.0 ml min^{-1}, oxmetidine sulphoxide was retained for 2.1 min, oxmetidine for 2.6 min and the internal standard (SK&F 93586) for 3.2 min; creatinine was not seen in a urine chromatogram, and, when deliberately added to an extract, creatinine enhanced the absorption associated with the solvent front.

Quantitation of chromatograms

The area under each peak was determined by an integrator (Perkin-Elmer Sigma 10, or Laboratory Data Control 308) connected to the UV detector. The ratio of the area of the unknowns to that of the internal standard was then used to calculate the concentration of the unknown by extrapolation from a two-variable linear regression, which was obtained from the corresponding ratios for known amounts of oxmetidine or oxmetidine sulphoxide added to pre-dose samples of plasma, bile or urine.

Studies with [^{14}C]*oxmetidine*

The efficiency of the extraction procedure was tested by spiking samples of plasma and urine with ^{14}C-labelled oxmetidine (17.1 Ci/mg; 99% radiochemically pure), and extracting these in the manner described.

The radioactivity in the recovered acetonitrile was compared to that in the spike by adding each of them to 5 ml of Picofluor scintillant and counting the resulting scintillations in a Denley Mark III spectrometer.

RESULTS AND DISCUSSION

Recovery of oxmetidine from plasma and urine

The recovery of ^{14}C added to plasma and urine samples as [^{14}C]oxmetidine was used as a measure of the availability of oxmetidine for assay, and the results are shown in Table I. For samples of plasma containing 1—7.5 μg of oxmetidine the average recovery was 61.8%; for samples of urine containing 1—20 μg (17—340 nCi of ^{14}C) the average recovery was 60.6%. With regard to the volumes of octanol and acid harvested the theoretical possible recovery was 75%.

TABLE I

RECOVERY OF RADIOACTIVITY FROM PLASMA AND URINE CONTAINING ADDED [^{14}C]OXMETIDINE

C.V. = coefficient of variation.

Fluid	*Oxmetidine* in sample ($mg\ l^{-1}$)	*Original dpm* in the spike, mean ± C.V. (%)	*Recovered dpm in* acetonitrile layer ($n = 20$), mean ± C.V. (%)	Mean recovery (%)
Plasma	1.0	42314 ± 1.7	25383 ± 10.9	60.0
	2.0	85623 ± 2.0	48081 ± 18	56.1
	5.0	200123 ± 7.0	121198 ± 11.4	60.6
	10.0	417410 ± 1.6	265899 ± 11.5	63.7
	15.0	612980 ± 1.7	420141 ± 8.0	68.5
Urine	1.0	50168 ± 6	29029 ± 16	57.9
	5.0	219907 ± 0.8	134954 ± 7.0	61.4
	10.0	429763 ± 1.0	252135 ± 1.5	58.7
	15.0	642319 ± 1.0	368336 ± 9.0	57.3

The variability in recovery between samples (expressed by the coefficient of variation in Table I) would be offset by the presence of an internal standard, which would be expected to undergo the same variability. Thus about 60% of the oxmetidine in biological fluids was available for the chromatographic separation and quantification by UV absorption.

Chromatographic separation

Normal phase. A typical normal-phase chromatogram of oxmetidine and the internal standard, SK&F 92909, extracted from a 2-ml plasma sample is shown in Fig. 2 together with the appropriate controls.

To test the precision and accuracy of the method ten spiked samples for each of six concentrations of oxmetidine were extracted on three separate occasions. The results of these assays are shown in Table II as concentrations calculated from average peak heights for each concentration together with the variability for precision (shown as coefficient of variation) and accuracy (as percentage error). At all concentrations between 0.1 and 7.5 mg/l the method can be seen to be accurate and precise enough for use in the assay of samples from clinical trials and other studies.

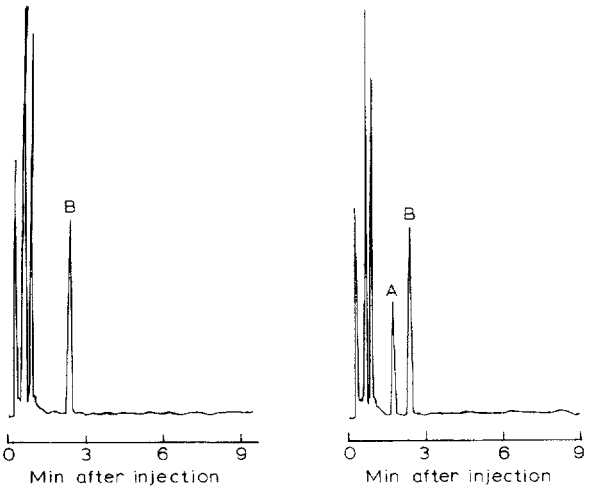


Fig. 2. Normal-phase chromatograms of extracted human plasma before (left) and after (right) the administration of oxmetidine. Peaks: A = oxmetidine, B = internal standard (SK&F 92909).

TABLE II

PRECISION AND ACCURACY OF THE NORMAL PHASE HPLC ASSAY OF OXMETIDINE IN PLASMA

Amount added to sample (μg per 2 ml)	Average concentrations (μg per 2 ml) calculated from peak heights		
	Mean ± S.D.	C.V. (%)	Error (%)
0.2	0.20 ± 0.02	10.0	Nil
1.0	1.02 ± 0.08	7.8	2.0
2.0	1.98 ± 0.06	3.1	2.0
5.0	4.94 ± 0.14	2.8	1.0
10.0	9.96 ± 0.30	3.0	0.4
15.0	15.08 ± 0.60	3.9	0.5

An example of such an assay is shown in Fig. 2 and indicates that extracted plasma samples contain only oxmetidine in detectable quantities, there being no peaks with longer retention times than the internal standard (4 min), which would have been observed if polar metabolites such as the sulphoxide had been present.

However, when urine samples were extracted and assayed by this technique (Fig. 3) two peaks with relatively long retention times were seen, corresponding to peaks obtained when authentic oxmetidine sulphoxide and creatinine were injected into the chromatograph. Because of the time required to clear these substances from the column, a reversed-phase HPLC technique was considered to be more suitable for urine sample assay.

Reversed phase. A typical reversed-phase chromatogram of oxmetidine, its sulphoxide and the internal standard, SK&F 93586, extracted from a spiked 2-ml urine sample is shown in Fig. 4, together with the appropriate controls.

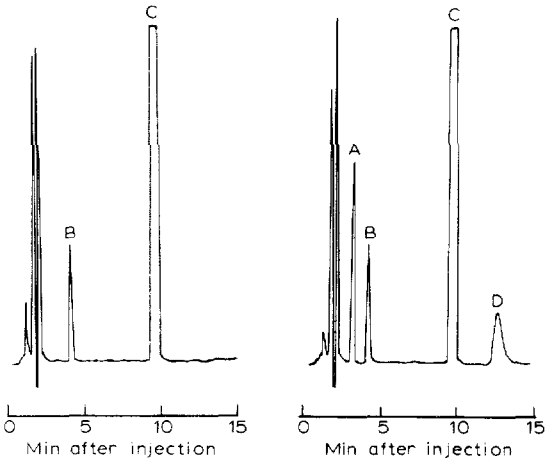


Fig. 3. Normal-phase chromatograms of extracted human urine before (left) and after (right) the administration of oxmetidine. Peaks: A = oxmetidine, B = internal standard (SK&F 92909), C = creatinine, D = oxmetidine sulphoxide.

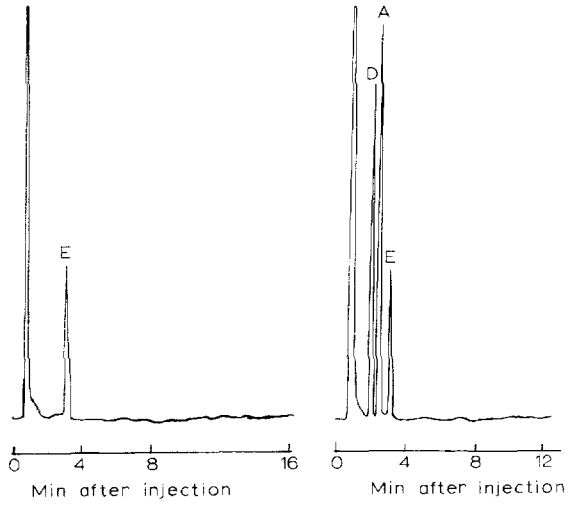


Fig. 4. Reversed-phase chromatograms of extracted human urine before (left) and after (right) the administration of oxmetidine. Peaks: A = oxmetidine, E = internal standard (SK&F 93586), D = oxmetidine sulphoxide.

The precision and accuracy of this technique was tested for four concentrations of oxmetidine and four concentrations of oxmetidine sulphoxide in urine. The results in Table III are concentrations calculated from the average peak areas for each concentration, together with the variation to be expected for precision and accuracy.

Precision for oxmetidine and for the sulphoxide in urine was good except at the lowest concentrations and accuracy was also less at low concentrations. Nevertheless, at concentrations likely to be encountered in urine samples from patients, the assay was sufficiently accurate and precise.

TABLE III

PRECISION AND ACCURACY OF THE DETERMINATION OF OXMETIDINE AND ITS SULPHOXIDE METABOLITE IN URINE BY REVERSED-PHASE HPLC

Concentration (mg/l)	Concentrations calculated from peak areas			
	Mean ± S.D.	n^*	C.V. (%)	Error (%)
Oxmetidine				
1.0	1.05 ± 0.13	30	12.4	4.8
5.0	4.84 ± 0.20	30	4.2	3.3
10.0	9.98 ± 0.38	29	3.8	0.2
20.0	20.93 ± 0.82	30	3.9	4.7
Oxmetidine sulphoxide				
0.5	0.62 ± 0.09	10	13.3	24.0
2.5	2.43 ± 0.19	30	7.9	3.0
5.0	5.03 ± 0.36	29	7.2	0.7
10.0	10.84 ± 1.00	30	9.2	8.4

*n = Number of samples assayed.

The typical chromatogram (Fig. 4), obtained during the assay of urine from volunteers receiving oxmetidine, indicates that extracted urine samples contain oxmetidine and oxmetidine sulphoxide in addition to the added internal standard. No other peaks were observed, so creatinine was added to the extract and the chromatography repeated, resulting in an enhancement of the absorption ascribed to the solvent front. The retention times for oxmetidine sulphoxide, oxmetidine and SK&F 93586 were 2.1, 2.6 and 3.2 min, respectively, and the assay was completed within 4 min, permitting an assay rate of 15 samples per hour manually or by automatic injector.

No peaks with long retention times (up to 20 min) were observed for extracts of urine and bile samples known to contain a glucuronide conjugate of oxmetidine, and it is concluded that this metabolite was not extracted from the samples. However, after treatment with glucuronidase, the concentration of oxmetidine in some urine and bile extracts increased, as also did the concentration of sulphoxide.

This reversed-phase method was also tried for the assay of plasma extracts, but was found to be unsuitable because of indigenous material which was extracted and co-chromatographed with the desired peaks. Thus, it was concluded that the reversed-phase technique should be reserved for urine and bile samples, and that plasma analysis of unchanged oxmetidine was best achieved by normal-phase chromatography. The stability of oxmetidine in the different biological fluids was, therefore, investigated using the technique appropriate to the sample.

Stability of oxmetidine in plasma

Short term stability at ambient temperatures is shown in Table IV and indicates that, at 0.5 and 2.5 mg l^{-1}, the concentration of oxmetidine in plasma apparently increased by 16—20% over 6 h. Although the samples were in

stoppered tubes, this increase may have been due to concentration by evaporation, so exposure to ambient temperature should be avoided as much as possible. Longer term stability at −20°C is also shown in Table IV and indicates that plasma samples may be stored at this temperature for 12 weeks without significant change in the value of the oxmetidine concentrations, although at 12 weeks the variation in the assay had increased.

TABLE IV

ASSAY OF OXMETIDINE IN PLASMA AFTER STORAGE AT AMBIENT TEMPERATURE OR AT LOW TEMPERATURE

Time after addition of oxmetidine to plasma	Storage temperature	Assay	
		0.5 mg l^{-1}	2.5 mg $^{-1}$
0 h	Ambient	0.49 ± 0.03	2.47 ± 0.07
2 h	Ambient	0.51 ± 0.01	2.68 ± 0.08
4 h	Ambient	0.53 ± 0.03	2.62 ± 0.04
6 h	Ambient	0.59 ± 0.02	2.90 ± 0.04
1 week	−20°C	0.51 ± 0.02	2.54 ± 0.03
2 weeks	−20°C	0.56 ± 0.03	2.58 ± 0.06
3 weeks	−20°C	0.48 ± 0.02	2.57 ± 0.08
5 weeks	−20°C	0.53 ± 0.02	2.59 ± 0.08
6 weeks	−20°C	0.53 ± 0.02	2.52 ± 0.06
12 weeks	−20°C	0.49 ± 0.05	2.62 ± 0.16

TABLE V

ASSAY OF OXMETIDINE IN URINE AFTER STORAGE AT AMBIENT TEMPERATURE OR AT LOW TEMPERATURE

Time after addition of oxmetidine to urine	Storage temperature	Assay	
		10 mg l^{-1}	20 mg l^{-1}
0 h	Ambient	10.04 ± 0.40	19.84 ± 0.41
6 h	Ambient	9.69 ± 0.21	19.81 ± 0.53
18 h	Ambient	9.44 ± 0.28	19.42 ± 0.22
24 h	Ambient	9.29 ± 0.27	18.52 ± 0.70
1 week	−20°C	9.59 ± 0.26	18.97 ± 0.53
3 weeks	−20°C	9.79 ± 0.19	19.28 ± 0.54
5 weeks	−20°C	9.75 ± 0.17	19.94 ± 0.27
6 weeks	−20°C	9.58 ± 0.23	18.97 ± 0.30
8 weeks	−20°C	9.49 ± 0.70	19.43 ± 1.20
14 weeks	−20°C	9.05 ± 0.24	17.51 ± 0.27

Stability of oxmetidine in urine

Urine samples did not lose significant amounts of oxmetidine when stored at room temperature for 6 h. However, by the following day a reduction in concentration of about 8% had occurred (Table V). At concentrations generally found in urine (10—20 mg l^{-1}) oxmetidine was stable for up to eight weeks

after the addition of the drug, when the samples were kept at −20°C. However at fourteen weeks of storage at low temperature, about 10% of the oxmetidine was no longer available, so that urine samples should be assayed within eight weeks of collection (Table V).

Oxmetidine has been assayed in plasma samples taken from studies designed to investigate the kinetics of the compound in man and experimental animals. In these studies, plasma concentrations ranging from 50 ng/ml to 50 μg/ml have been encountered.

Extraction and analysis of urine and bile samples have demonstrated that both of these fluids may contain unchanged oxmetidine and/or oxmetidine conjugated with glucuronic acid, and that the major unconjugated metabolite is the sulphoxide shown in Fig. 1.

ACKNOWLEDGEMENTS

The authors acknowledge the skills of Dr. T.H. Brown and Dr. G.R. White in the Synthesis of Compounds II—IV.

REFERENCES

1 R.C. Blakemore, T.H. Brown, G.J. Durant, J.C. Emmett, C.R. Ganellin, M.E. Parsons and A.C. Rasmussen, Brit. J. Pharmacol., 70 (1980) 105.
2 J.G. Mills, M.A. Melvin, R. Griffiths, R.H. Hunt, W. Burland and G.J. Milton-Thompson, Gut, 21 (1980) A462.
3 R.M. Lee and P.M. Osborne, J. Chromatogr., 146 (1978) 354—360.
4 G.E. Delory and E.J. King, Biochem. J., 39 (1945) 245—247.
5 R.M. Lee, unpublished results.