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Note

Improved assay procedure for oxmetidine and its metabolites in plasma, urine and bile samples

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In recently published methods for the assay of oxmetidine (2-[2-(5-methyl-4-imidazolylmethylthio)ethylamino]-5-(3,4-methylenedioxybenzyl)-4-pyrimidone dihydrochloride; SK&F 92994) in various biological fluids [1], the plasma extracts were analysed by a simple modification of the normal phase high-performance liquid chromatographic (HPLC) assay used for cimetidine [2]. The presence of the more polar oxmetidine sulphoxide in extracts of bile and urine prompted the development of a reversed-phase HPLC system [1], for the determination of unchanged drug and metabolites in these fluids.

Recently, however, the manufacturers of the preferred reversed-phase column (Altex Ultrasphere ODS) have changed the method of end capping the octadecylsilane (ODS) packing material. Using the new columns with the original solvent system resulted in broad peaks with little resolution between metabolite, oxmetidine and internal standard. This necessitated the search for a modified solvent system that could be used with the new columns for the analysis of urine and bile samples.

This paper describes the new solvent system, the different internal standard and the modified extraction procedure, which are now considered appropriate for the assay of oxmetidine and its sulphoxide in extracts of urine and bile. The new method may also be used in place of the normal-phase chromatography for extracts of plasma [1].

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used in this study were analytical grade with the following

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exceptions: 1-octanol was puriss (Koch-Light, Colnbrook, U.K.); methanol, water and acetonitrile were HPLC grade (Rathburn, Walkerburn, U.K.); 1-pentanesulphonic acid sodium salt was reagent grade (Kodak, Rochester, NY, (U.S.A.). The solutions of 1 mol l^{-1} carbonate buffer (pH 9.0) and 0.1 mol l^{-1} acetate buffer (pH 5.0) were prepared as described previously [1]. It was necessary to filter the carbonate buffer through 0.45-μm filters in order to reduce the solvent front absorption and baseline shift on the chromatogram. Control human plasma and biological samples for analysis were prepared and stored as previously described [1].

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 solvents or 0.5-μm membrane filters (Millipore, Type FH) for organic solvents.

The stock solutions of oxmetidine for the plasma and urine assay were prepared by weighing 5.91 and 11.83 mg of the dihydrochloride salt (equivalent to 5 and 10 mg of base), and dissolving same in approximately 0.5 ml water before making the solution to 100 ml volume with methanol. Solutions of oxmetidine sulphoxide and the internal standard (SK&F 93586, 2-[2-(5-methyl-4-imidazolyl-methylthio)-ethylamino]-1-methyl-5-(3,4-methylenedioxybenzyl)-6-pyrimidone dihydrochloride) were similarly prepared by weighing 5.87 and 23.53 mg of the dihydrochloride salt (equivalent to 5 and 20 mg of the respective bases) and dissolving each in appropriate volumes of water and methanol as described. All stock methanolic solutions were stored at $-20°C$ and found to be stable for at least three months under these conditions.

The polypropylene centrifuge tubes (12 ml) and stoppers used for sample extraction were obtained from Henleys Medical Supplies, London, U.K. (Type 300PP and 301PT, respectively).

Extraction procedures for plasma, urine and bile

The plasma extraction was essentially the same as previously reported [1] except that the internal standard SK&F 92909 must be replaced with SK&F 93586. This latter compound (2 μg in 50 μl of methanol) was added to plasma samples before the addition of 1 ml of carbonate buffer to adjust the pH to 9.0. In the final salting out phase 250 μl ethanol replaced the equivalent volume of acetonitrile to make the sample injections compatible with the new solvent system.

The extraction of oxmetidine and its sulphoxide from bile and urine was as described by Lee and McDowall [1] except that an equivalent volume of ethanol was substituted for acetonitrile during the salting out process.

Chromatographic operating conditions

The chromatograph consisted of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.). The sample extract was introduced into the system via either a Rheodyne Model 7125 valve injector (Berkeley, CA, U.S.A.) or an automatic injector (Model WISP, Waters Assoc.). Sample extracts that were injected by autosampler were held in spring-loaded microinserts (Type 3-CV, Chromacol, London, U.K.) within 4-ml vials with self-sealing septa (Cat. Nos.

by a stainless-steel column 150 mm × 4.6 mm I.D. packed with 5-μm Ultrasphere ODS (Altex Scientific, Berkeley, CA, U.S.A.) and the column eluent was monitored by a Model 441 fixed-wavelength detector fitted with a cadmium lamp and 229-nm filter (Waters Assoc.). A variable-wavelength detector set at 226 nm and 0.04—0.08 absorbance units full scale was also used but proved to be less sensitive. The signal from the detector was fed into a Model 301 integrator (Laboratory Data Control, Stone, U.K.).

The solvent system was a mixture of water—methanol—acetonitrile (45:44:11, v/v) containing 0.095 mol l^{-1} pentanesulphonic acid and prepared as follows: 17.33 g pentanesulphonic acid (sodium salt) was dissolved in 450 ml distilled water, and the pH of the solution was adjusted to 3.0 with 10 mol l^{-1} sulphuric acid; 440 ml methanol and 110 ml acetonitrile were added and dissolved air was removed by the application of reduced pressure. The column was equilibrated by passing solvent through it for approximately 1 h before commencing the analysis. On completion of analysis it is recommended that the column be flushed with filtered methanol for 1—2 h.

At a flow-rate of 1.5 ml min^{-1} the approximate retention times of oxmetidine sulphoxide, oxmetidine and SK&F 93586 (the internal standard) were 4.8, 5.8 and 6.8 min, respectively.

Samples of up to 10 μl of the ethanol extracts were injected onto the chromatograph to obtain separations of the three peaks of interest. The injection of more than 20 μl ethanol often resulted in loss of resolution.

Quantification

The area under each peak was determined by an integrator connected to the UV detector. Peak height measurements can also be used. The ratios of the areas or heights of peaks assigned to oxmetidine and oxmetidine sulphoxide to that of the internal standard in the plasma, bile or urine samples were then used to calculate the concentrations of these compounds, using calibration curves obtained from the corresponding ratios for standards containing known amounts of oxmetidine or oxmetidine sulphoxide.

RESULTS AND DISCUSSION

Recovery of oxmetidine from plasma and urine

The recovery of oxmetidine from plasma and urine samples has been published previously [1]. The substitution of ethanol for acetonitrile did not affect these values and the results of the previous study remain valid.

Selectivity

Typical chromatograms of oxmetidine, its sulphoxide and the internal standard (SK&F 93586) following the injection of a solution of pure standards and extracts of plasma, urine and bile are presented in Figs. 1—4, respectively. These chromatograms were produced by the injection of up to 10 μl of sample extract onto the column; volumes greater than 20 μl ethanol tended to produce asymmetric peaks and poor resolution.

Under normal in vivo conditions oxmetidine sulphoxide is cleared rapidly from the plasma and not usually observed in chromatograms; however, during

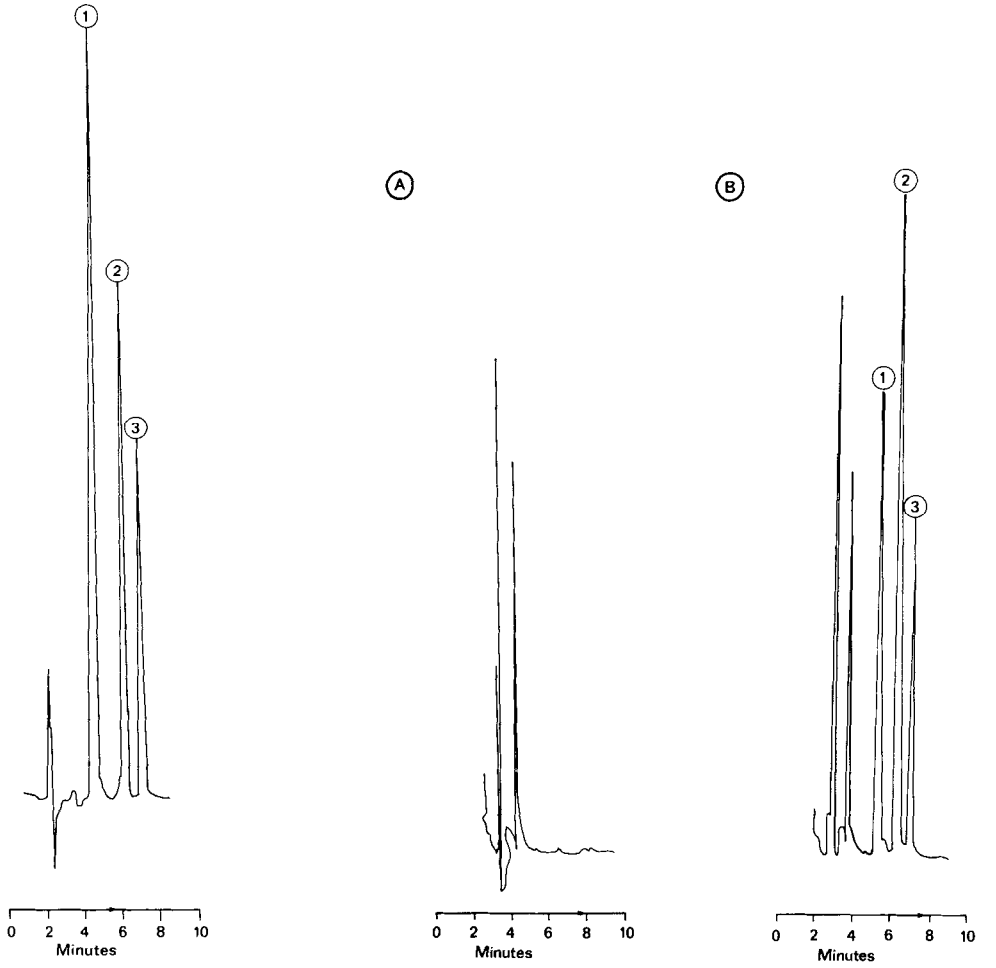

Fig. 1. Reversed-phase chromatogram of pure standards. Peaks: 1 = oxmetidine sulphoxide SK&F 93154; 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586.

Fig. 2. Reversed-phase chromatograms of human plasma extracts. A = Extracted blank plasma; B = extracted sample plasma. Peaks: 1 = oxmetidine sulphoxide SK&F 93154, 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586.

renal insufficiency when clearance is slowed, the metabolite may be present in measureable amounts and this technique can be used to quantify it.

No unwanted peaks with relevant retention times (up to 15 min) were observed for extracts of plasma, urine and bile samples with the exception of a peak with a retention time of approximately 5.1 min found in dog bile extracts. This peak was resolved from oxmetidine sulfoxide and oxmetidine but, at low concentrations of drug and metabolite, affected the quantification of both these compounds (Fig. 4).

Precision and accuracy of the assay

The precision and accuracy of this technique for plasma samples are presented in Table I; the mean concentrations calculated from ten individual

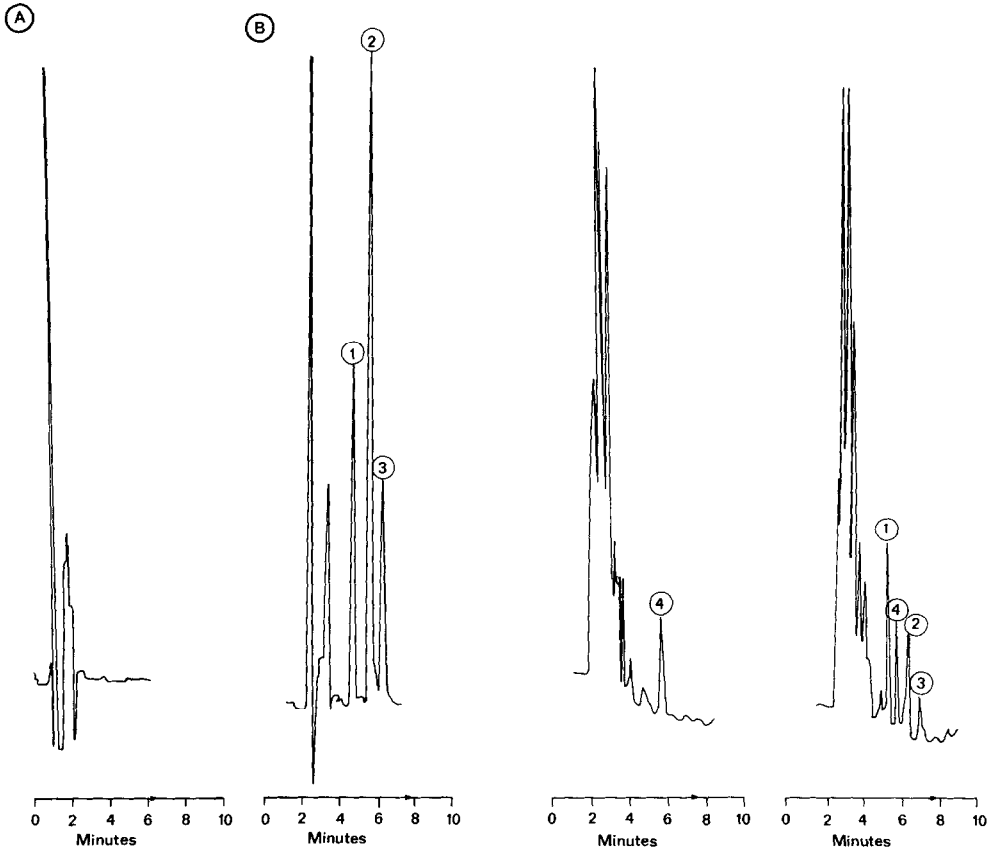

Fig. 3. Reversed-phase chromatograms of human urine extracts. A = Extracted blank urine; B = extracted sample urine. Peaks: 1 = oxmetidine sulphoxide SK&F 93154; 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586.

Fig. 4. Reversed-phase chromatograms of dog bile extracts. Peaks: 1 = oxmetidine sulphoxide SK&F 93154; 2 = oxmetidine SK&F 92994; 3 = internal standard SK&F 93586; 4 = endogenous peak.

TABLE I

PRECISION AND ACCURACY OF THE MODIFIED REVERSED-PHASE HPLC ASSAY OF OXMETIDINE IN HUMAN PLASMA (n = 10)

Oxmetidine concentration ($mg\ l^{-1}$)	Concentration calculated from peak area ratios (mean ± S.D.) ($mg\ l^{-1}$)	Coefficient of variation (%)	Bias* (%)
0.25	0.29 ± 0.04	13.8	17.6
0.50	0.55 ± 0.04	7.3	10.0
2.50	2.53 ± 0.07	2.8	−1.2
5.00	5.02 ± 0.06	1.2	0.4
10.00	9.96 ± 0.15	1.5	−0.4

*Bias calculated as $\frac{(\text{mean calculated concentration} - \text{actual concentration}) \times 100}{\text{actual concentration}}$.

TABLE II

PRECISION AND ACCURACY OF THE MODIFIED REVERSED-PHASE HPLC ASSAY OF OXMETIDINE IN HUMAN URINE

Concentration ($mg\ l^{-1}$)	Concentration calculated from peak area ratios (mean ± S.D.) ($mg\ l^{-1}$)	n^*	Coefficient of variation (%)	Bias (%)
Oxmetidine				
0.5	0.55 ± 0.08	9	14.5	10.0
5.0	4.92 ± 0.18	10	3.7	−1.6
10.0	10.44 ± 0.23	10	2.2	4.4
Oxmetidine sulphoxide				
0.25	0.23 ± 0.06	9	26.1	−8.0
2.5	2.49 ± 0.26	9	10.4	−0.6
5.0	5.03 ± 0.27	9	5.4	0.6

*n = Number of samples assayed.

TABLE III

PRECISION AND ACCURACY OF THE MODIFIED REVERSED-PHASE HPLC ASSAY OF OXMETIDINE IN DOG BILE

Concentration ($mg\ l^{-1}$)	Concentration calculated from peak area ratios (mean ± S.D.) ($mg\ l^{-1}$)	n^*	Coefficient of variation (%)	Bias (%)
Oxmetidine				
0.5	0.62 ± 0.32	5	51.6	24.0
5.0	5.60 ± 0.42	10	7.5	12.6
10.0	11.40 ± 0.65	10	5.7	14.0
Oxmetidine sulphoxide				
0.25	0.38 ± 0.07	10	18.4	51.6
2.5	2.61 ± 0.08	10	3.1	4.4
5.0	5.66 ± 0.08	10	1.4	13.2

*n = Number of samples assayed.

assays of each of five spiked concentrations, are given together with estimates of the precision and accuracy. The precision, as measured by the coefficient of variation (C.V.), was between 1.2 and 7.3% over the concentration range 10.0—0.5 $mg\ l^{-1}$; this was similar to that found with the original normal-phase method. At a concentration of 0.25 $mg\ l^{-1}$ the C.V. was 13.8% which was slightly higher than that of the normal-phase plasma assay (10% C.V. at 0.1 $mg\ l^{-1}$).

The accuracy of the assay as measured by percent bias was very good between 10.0 and 2.5 $mg\ l^{-1}$ (−1.2 to 0.4%), however, at the lower concentrations (0.25 and 0.5 $mg\ l^{-1}$) the bias was 17.6% and 10%, respectively.

The validity of the assay for both oxmetidine and oxmetidine sulphoxide in urine is presented in Table II. The results were essentially similar to those published previously [1].

Table III presents the corresponding precision and accuracy data for oxmetidine and its sulphoxide metabolite calculated from assays of spiked control dog bile. Quantification of the two compounds was complicated by the presence of an endogenous peak, which eluted from the column between oxmetidine sulphoxide and oxmetidine. The coefficient of variation for the assay of bile, for both the drug and metabolite, was greater than for the corresponding concentrations in the urinary assay but still acceptable. At concentrations below 1 mg l^{-1} the assay for both compounds in bile was less reliable and subject to larger variation than in urine.

Comparison of normal phase and reversed-phase HPLC assays for the determination of oxmetidine in plasma

In order that continuity of information is maintained it is essential to show that the results obtained before and after any modification to an analytical

TABLE IV

INDIVIDUAL OXMETIDINE CONCENTRATIONS IN SAME PLASMA SAMPLE DETERMINED BY NORMAL-PHASE AND REVERSED-PHASE ASSAYS

Sample No.	Concentration of oxmetidine (mg l^{-1})		
	Actual concentration	Calculated by normal-phase assay	Calculated by reversed-phase assay
1	0.50	0.55	0.53
2		0.54	0.51
3		0.54	0.53
4		0 55	0 53
5		0 38	0 51
6	1.00	1 02	1.03
7		1.01	1.01
8		0.98	0.99
9		1.02	0.99
10		1 01	0.97
11	4.00	4.04	4.09
12		4 04	4.03
13		4.03	4.01
14		N.R.*	4.02
15		4 00	4.02
16	8.00	8.24	7.98
17		6.88	8.09
18		7.96	7.80
19		8.07	7 93
20		7.93	7.81

*N.R = no result.

TABLE V

MEAN CONCENTRATION, BIAS, COEFFICIENT OF VARIATION OF OXMETIDINE CONCENTRATIONS DETERMINED IN PLASMA BY NORMAL-PHASE AND REVERSED-PHASE ASSAYS

Actual concentration		Oxmetidine concentration (mg l^{-1})	
		Normal-phase assay	Reversed-phase assay
0.5	Mean	0.51	0.52
	± S.D. (n)*	0 07 (5)	0.01 (5)
	C.V. (%)**	13.7	1.9
	Bias (%)***	2	4
1.0	Mean	1.01	1.00
	± S D. (n)	0.02 (5)	0.02 (5)
	C.V. (%)	2.0	2
	Bias (%)	1	nil
4.0	Mean	4.03	4.03
	± S.D. (n)	0.02 (4)	0.03 (5)
	C.V (%)	0.5	0 7
	Bias (%)	0.7	0.7
8.0	Mean	7.82	7.92
	± S D. (n)	0.54 (5)	0.12 (5)
	C.V. (%)	6.9	1.5
	Bias (%)	−2.3	−1

*n = Number of assays.

**$\text{C.V (\%)} = \dfrac{\text{S.D} \times 100}{\text{mean}}$

***$\text{Bias (\%)} = \dfrac{\text{mean calculated concentration} - \text{actual concentration}}{\text{actual concentration}}$.

procedure are comparable. Thus, replicate assays were performed on the same plasma samples by the original normal-phase assay and the new reversed-phase assay (Tables IV and V).

The results show good agreement between the two procedures at all four concentrations; however, at 0.5 and 8.0 mg l^{-1} the reversed-phase assay was more precise (as measured by the coefficient of variation) than the normal-phase assay. The accuracy of the two assays at all concentrations was similar. Thus the reversed-phase assay may be used in confidence in place of the normal-phase assay.

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REFERENCES