Journal of Chromatography, 251 (1982) 215–224
Chromatographic Reviews
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,508

USE OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY FOR THE STUDY OF THE METABOLISM OF RANITIDINE IN MAN

L. E. MARTIN*, JANET OXFORD and R. J. N. TANNER
Biochemical Pharmacology Department, Glaxo Group Research Limited, Ware, Herts. (Great Britain)

CONTENTS

1. Introduction . . . 215
2. Experimental . . . 216
2.1. Reagents and materials . . . 216
2.2. High-performance liquid chromatography . . . 216
2.3. HPLC–MS interface system . . . 216
2.4. Mass spectrometry . . . 216
2.5. Qualitative HPLC–MS analyses . . . 217
2.5.1. Standard solution of ranitidine and metabolites . . . 217
2.5.2. Identification of ranitidine and metabolites in urine . . . 217
2.6. Quantitative HPLC–MS analysis . . . 217
2.6.1. Preparation and analysis of calibration standards . . . 217
2.6.2. Determination of ranitidine in human urine . . . 219
3. Results . . . 219
3.1. Qualitative HPLC–MS analyses . . . 219
3.1.1. Identification of ranitidine and metabolites in urine . . . 219
3.2. Quantitative HPLC–MS analyses . . . 219
3.2.1. Determination of ranitidine in urine . . . 219
4. Discussion . . . 222
5. Conclusion . . . 224
6. Summary . . . 224
References . . . 224

1. INTRODUCTION

Gas chromatography–mass spectrometry (GC–MS) is an established technique for identifying and measuring low concentrations of drugs and metabolites in biological fluids. For quantitative analysis of drugs either a stable isotope-labelled analogue or a homologue which can yield an ion of the same m/z value as the compound is used as an internal standard.

Prior to analysis by GC–MS the analyte has to be extracted into an organic solvent, and in the case of a compound which is not amenable to GC a suitable volatile derivative has to be formed. High-performance liquid chromatography (HPLC) has assumed an increasing role in the analysis of drugs and metabolites in biological fluids. This is because it offers the following advantages over GC: (a) samples of biological fluids can be injected directly on to either a pre-column, or the analytical column; (b) the technique will handle thermolabile and involatile compounds.

0378-4355/82/0000–0000/$02.75

One limitation to the application of HPLC, in the determination of drugs in biological fluids, has been the availability of suitable sensitive and specific detector systems for compounds which do not absorb strongly in the UV or the visible region, cannot be made to fluoresce, and cannot be detected by electrochemical methods.

The mass spectrometer is a sensitive and specific universal detector, which if used to analyse the eluate from an HPLC column could overcome this limitation. We have described the use of on-line HPLC–MS to identify ranitidine, the anti-ulcer drug, and three metabolites, ranitidine-N-oxide, ranitidine-S-oxide and desmethylranitidine in urine from rabbits given oral doses of 100 mg of [^{14}C]ranitidine per kilogram of body weight[1].

In this paper further applications of HPLC–MS are described for the identification of ranitidine and three metabolites in a methanol extract of urine from a subject given an oral dose of 250 mg (*ca.* 3 mg/kg) of ranitidine, and the on-line determination of ranitidine in the urine of a patient given an intravenous dose of 50 mg of ranitidine. A selected-ion monitoring (SIM) technique[2] was used for this analysis and [$^{2}H_3$]-ranitidine was the internal standard.

2. EXPERIMENTAL

2.1. *Reagents and materials*

All reagents were of analytical grade. The solvents used for HPLC were degassed by sonication before use. Ranitidine hydrochloride, ranitidine-N-oxide, ranitidine-S-oxide and desmethylranitidine hydrochloride were synthesised in the Chemistry Division of Glaxo Group Research, Ware, Great Britain. Tris-deuterated ranitidine was synthesised by Mr. S. Young of the Chemical Development Section.

2.2. *High-performance liquid chromatography*

A Pye LC-XPD (Pye Unicam, Cambridge, Great Britain) was used to pump the mobile phase, methanol–propan-2-ol–5 *M* ammonium acetate (50:50:1), through a Rheodyne Model 7125 (Pye Unicam) sample injection valve. This was connected to a stainless-steel column, 100 × 5 mm I.D. (Shandon Southern Products, Runcorn, Great Britain) packed with Spherisorb S5 CN (Phase Separations, Clwyd, Great Britain). A Pye LC3 variable-wavelength UV detector (Pye Unicam) set at 320 nm was used to monitor the eluate. UV chromatograms were recorded on a Rikadenki DB6 pen recorder (Mitsui Machinery Sales U.K., Surrey, Great Britain), f.s.d. 10 mV, chart speed 2 mm/min.

2.3. *HPLC–MS interface system*

The outlet from the detector cell of the UV monitor was connected to the moving-belt interface (V.G. Analytical, Cheshire, Great Britain)[3] by a stainless-steel tube, 80 cm × 0.25 mm I.D. The belt speed was 15 mm/sec, the solvent evaporator setting was 2.50, the sample heater setting was 7.50 and the pressure in the interface housing was reduced to 50 Torr. The flow-rate of the mobile phase was 0.5 ml/min.

2.4. *Mass spectrometry*

A V.G. Micromass 7070H mass spectrometer (V.G. Analytical) was operated in the ammonia chemical ionisation (CI) mode at a source housing pressure of

$8 \cdot 10^{-5}$ Torr. The resolving power was 1000 (10% valley) and an electron beam current of 500 μA emission at 50 eV was used. The source was operated at 4 kV and 200°C. The mass spectrometer was interfaced to a V.G. 2035 data system (V.G. Analytical) for data acquisition and processing. The mass spectrometer was set to scan continuously at 3 sec per mass decade for qualitative HPLC–MS studies.

The SIM technique was used for the determination of ranitidine in urine. To check that the operating parameters of the mass spectrometer remained constant during each analysis, the background ion *m/z* 279, of low abundance, was monitored for 10-msec dwell periods throughout the analysis. For the determination of ranitidine the mass spectrometer was tuned to monitor for 500-msec dwell periods the ions *m/z* 281 from ranitidine and *m/z* 284 from [$^2H_3$]ranitidine.

2.5. Qualitative HPLC–MS analyses

2.5.1. Standard solution of ranitidine and metabolites. A test solution containing 100 μg each of ranitidine, desmethylranitidine, ranitidine-S-oxide and ranitidine-N-oxide per millilitre of methanol was prepared, and 10 μl of this solution were injected on to the column for HPLC and HPLC–MS analysis.

2.5.2. Identification of ranitidine and metabolites in urine. Two tablets containing a total of 250 mg of ranitidine were swallowed by a male volunteer. Urine was collected for 2 h after dosing and stored at −15°C prior to analysis.

The Prep I automated centrifugal sample processor (DuPont Instruments, Hitchin, Great Britain) was used to concentrate ranitidine and the metabolites. Urine (0.5 ml) was pipetted on the XAD-2 column, followed by 1.0 ml of 0.1 *M* sodium hydroxide. The column was placed in the centrifuge of the Prep I, and Program 15 selected. This caused the column to be washed with 2 ml of distilled water, followed by 2 ml of methanol; the latter was automatically collected in a cup and evaporated to dryness at 30°C by an air stream. The residue was dissolved in 50 μl of methanol, and 10 μl were analysed by HPLC–MS.

2.6. Quantitative HPLC–MS analyses

2.6.1. Preparation and analysis of calibration standards. The internal standard, 1.2 μg of [$^2H_3$]ranitidine per microlitre, was prepared by dissolving 6 mg of [$^2H_3$]ranitidine in 5 ml of distilled water.

Ranitidine hydrochloride (23.4 mg, equivalent to 21 mg of free base) was dissolved in 20 ml of distilled water giving a (base) concentration of 1.05 mg/ml. This was diluted ten-fold to give a concentration of 105 μg/ml.

Standards were prepared by adding the internal standard, 6 μg of [$^2H_3$]ranitidine, and 0–10.5 μg of ranitidine to samples of 0.5 ml control human urine. This gave a range of 0–21 μg of ranitidine per millilitre of urine, and the lowest standard corresponded to 1.05 μg/ml urine. Then 10 μl of each standard were analysed by HPLC–MS. The intensities of the ions *m/z* 281 and *m/z* 284 were recorded.

The ratio of the peak areas of the ions *m/z* 281 : *m/z* 284 for each standard was plotted against the ranitidine concentration in the sample, and a calibration line determined by the method of least-squares regression analysis.

To evaluate the accuracy and precision of the assay, 6 μg of [$^2H_3$]ranitidine and either 0.525 μg or 5.25 μg of ranitidine were added to each of six samples of 0.5 ml control urine. The peak area ratio of *m/z* 281 : *m/z* 284 was obtained, and the concen-

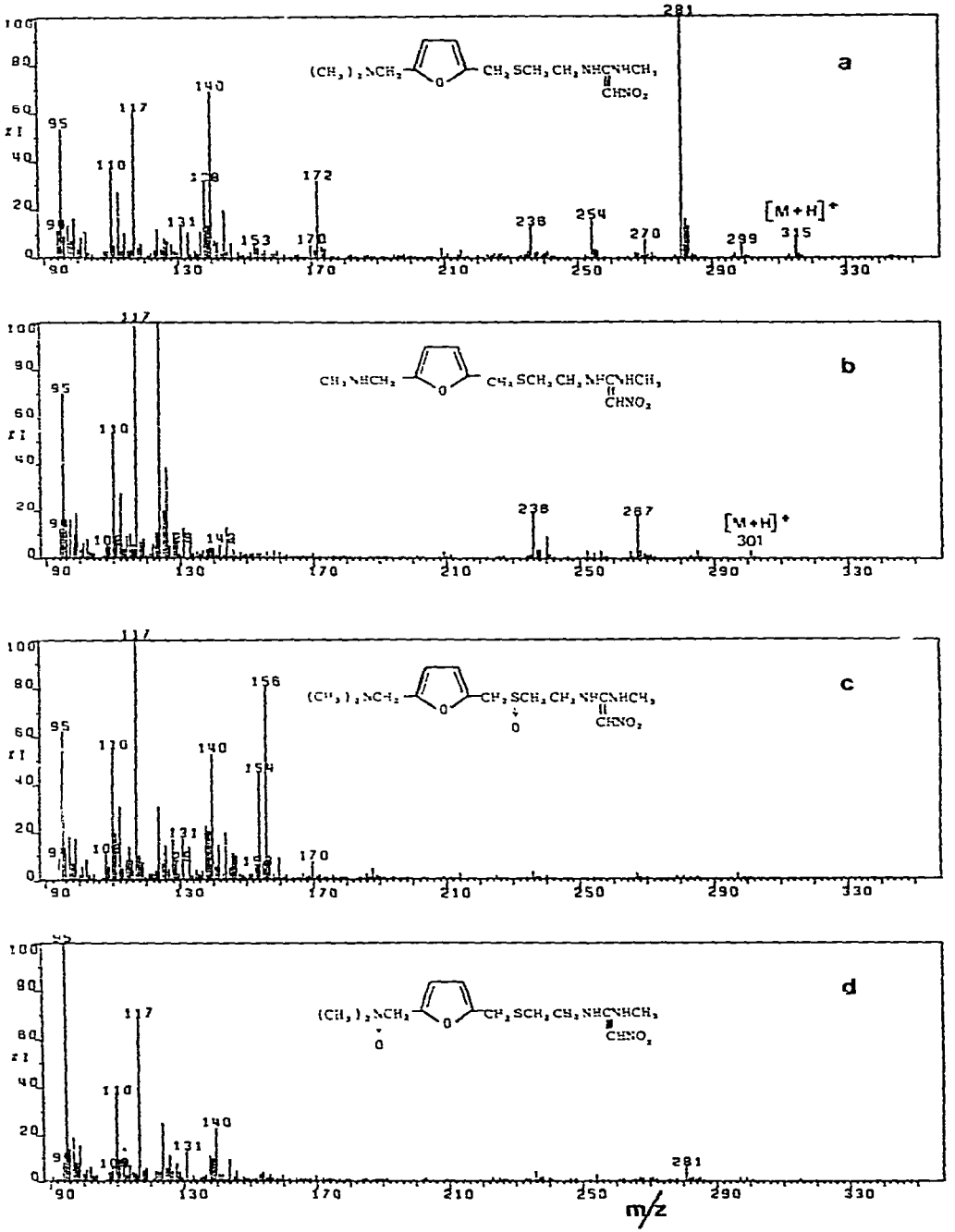

Fig. 1. Ammonia CI mass spectra, obtained by HPLC–MS analysis, of (a) ranitidine, (b) desmethylranitidine, (c) ranitidine-S-oxide, (d) ranitidine-N-oxide. Chromatographic conditions are described under Experimental.

tration of ranitidine was determined from the calibration line.

2.6.2. Determination of ranitidine in human urine. The urine passed by a patient with renal disease during 24 h after an intravenous dose of 50 mg of ranitidine was collected, the volume measured and a sample stored at −15°C until analysis. [$^2H_3$]ranitidine (6 μg) was added to 0.5 ml of urine, and 10 μl of the urine were analysed by HPLC–MS. The ratio of the areas of the ion intensities *m/z* 281 : *m/z* 284 was measured, and the concentration of ranitidine determined from the calibration graph.

3. RESULTS

3.1. Qualitative HPLC–MS analyses

The standard solution containing ranitidine and its metabolites was analysed by HPLC, and the capacity factors (k') obtained were respectively 1.4, 2.1, 2.5 and 4.0 for ranitidine, desmethylranitidine, ranitidine-S-oxide and ranitidine-N-oxide. A 10-μl volume of the standard solution containing 1.0 μg of each of the components was injected on column, and the sample analysed by HPLC–MS using ammonia as the reagent gas. There were background ions present in the mass spectrum of each component. These ions originated from impurities in the solvents, and the Kapton belt. They remained constant during an analysis, and the computer was programmed to subtract the background ions from the mass spectra of the analyte.

The CI mass spectra obtained, after subtraction of background ions, are shown for each component in the mixture (Fig. 1).

3.1.1. Identification of ranitidine and metabolites in urine. The total ion current (TIC) chromatogram obtained after HPLC–MS analysis of 10 μl of the XAD-2 methanol extract of the 0–2 h urine from the subject who took an oral dose of 250 mg of ranitidine is shown in Fig. 2. Five peaks are present in the chromatogram; the first, peak A, was due to endogenous material. Peaks B, C, D and E were assigned to ranitidine, desmethylranitidine, ranitidine-S-oxide and ranitidine-N-oxide, respectively, by comparison of both their ammonia CI spectra (Fig. 2b) with reference spectra (Fig. 1) and the k' values for each component which were, respectively, 1.3, 2.0, 2.3 and 3.8.

3.2. Quantitative HPLC–MS analyses

3.2.1. Determination of ranitidine in urine. The base peak in the ammonia CI mass spectrum of ranitidine (Fig. 1a) was at *m/z* 281 and was formed by loss of an oxygen atom and a molecule of water from the protonated molecular ion. Extensive fragmentation of the ranitidine molecule occurred even under the mild ionisation conditions used, and the abundance of the *m/z* 281 ion was only 11% of the total ion current (recorded from *m/z* 90 – 350). The base peak in the mass spectrum of [$^2H_3$]ranitidine was at *m/z* 284 (Fig. 3).

The SIM chromatograms of the ions *m/z* 281 and *m/z* 284 obtained by analysis of a standard of 1.05 μg of ranitidine per millilitre of standard and of a control urine, both containing [$^2H_3$]ranitidine internal standard, are shown in Fig. 4. Tris-deuterated ranitidine contains 1.5% unlabelled analogue which is the major component of the peak with a retention time of 6 min 6 sec in the *m/z* 281 chromatogram of the control urine.

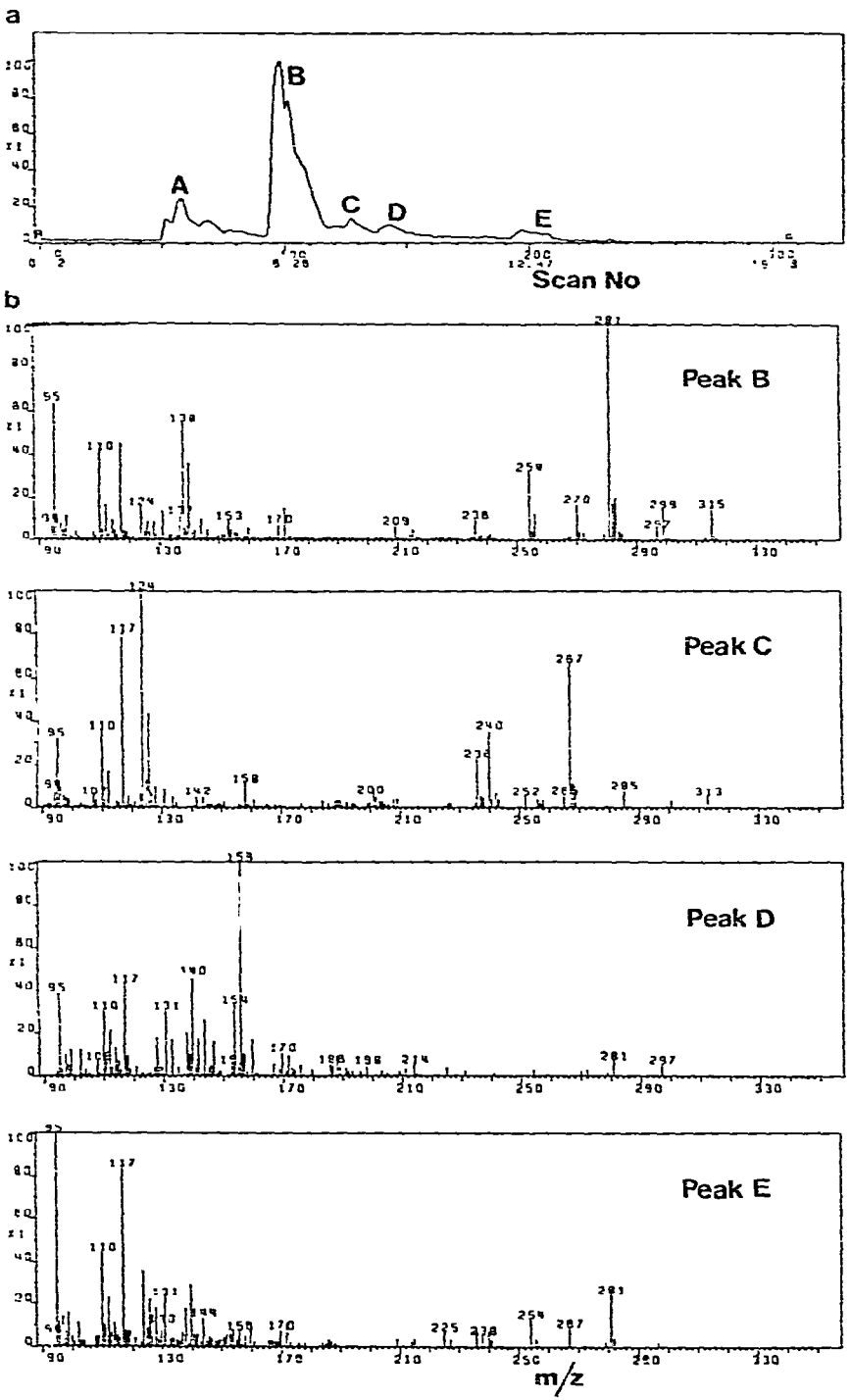

Fig. 2. (a) Reconstructed total ion current chromatogram obtained by HPLC–ammonia CI-MS of an XAD-2 extract of human urine that was collected 0–2 h after oral administration of 250 mg of ranitidine. Instrumental conditions are described under Experimental. (b) Ammonia CI mass spectra from TIC peaks: B = ranitidine; C = desmethylranitidine; D = ranitidine-S-oxide; E = ranitidine-N-oxide.

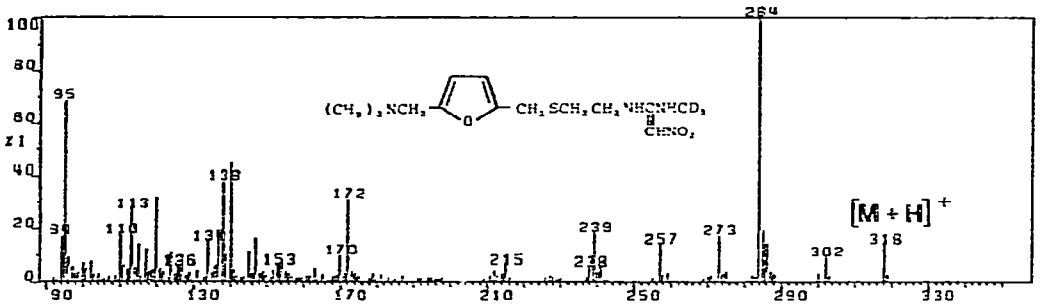

Fig. 3 Ammonia CI mass spectrum of [$^2H_3$]ranitidine obtained by HPLC–MS.

The calibration graph (Fig. 5) obtained from analysis of a series of standards of ranitidine in urine was linear over the range 0–21 μg/ml (0–210 ng of ranitidine injected on column). Six separate HPLC–MS analyses were carried out on urine samples containing either (a) 1.05 μg or (b) 10.5 μg of ranitidine per millilitre. The mean value found for (a) was 1.05 μg/ml, the standard deviation was 0.15 μg/ml, and the coefficient of variation was 13.3%; the mean value found for (b) was 10.6 μg/ml, the standard deviation was 0.45 μg/ml, and the coefficient of variation was 4.2%.

The SIM chromatogram obtained during the analysis of 10 μl of the 0–24-h urine from the patient given an intravenous dose of 50 mg of ranitidine is shown in Fig. 6. The concentration of ranitidine in the sample, calculated from the calibration line, is 7.8 μg/ml. Thus only 20% of the dose was excreted as unchanged drug in the 0–24-h urine (1285 ml); this low level of excretion was attributed to the patient's renal disease.

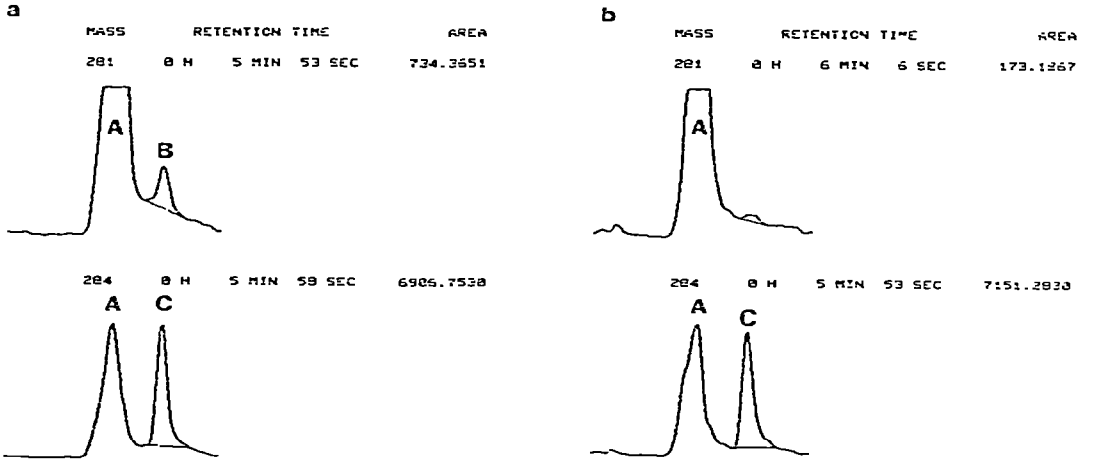

Fig. 4. SIM chromatograms of (a) 10 μl urine standard containing 1.05 μg/ml ranitidine and 12 μg/ml [$^2H_3$]ranitidine, and (b) 10 μl control urine containing 12 μg/ml [$^2H_3$]ranitidine. Peak A, endogenous material; peak B (m/z 281) ranitidine, peak C (m/z 284) [$^2H_3$]ranitidine. HPLC–MS instrumental conditions are described under Experimental. The chromatograms were smoothed during data processing.

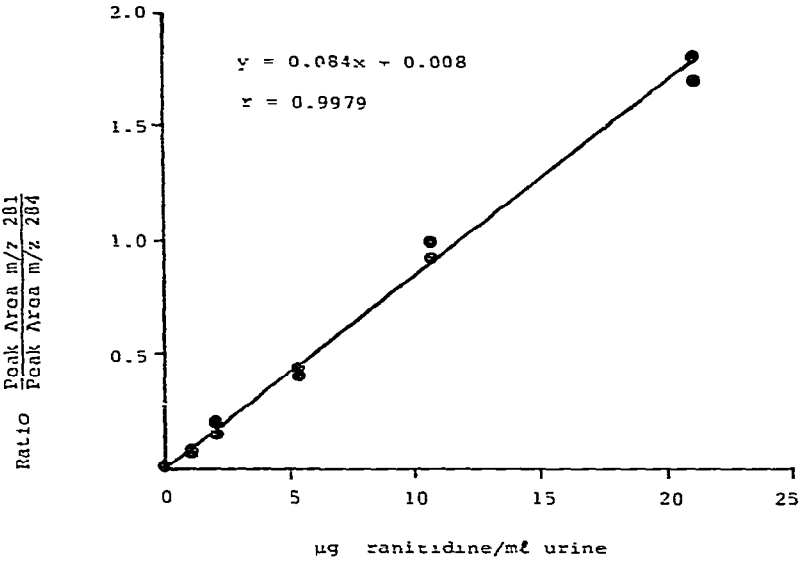

Fig. 5. Calibration graph for the quantification of ranitidine in human urine by HPLC–MS and SIM of the ions m/z 281 and m/z 284; 10 μl of each standard were analysed.

4. DISCUSSION

Studies in animals using [^{14}C]ranitidine have shown that ranitidine is oxidatively metabolised with the formation of ranitidine-N-oxide, ranitidine-S-oxide, and desmethylranitidine[4].

Ion-pair HPLC analyses of urine, from four men given separate 100-mg intravenous and oral doses of ranitidine, showed that the mean percentage of the dose

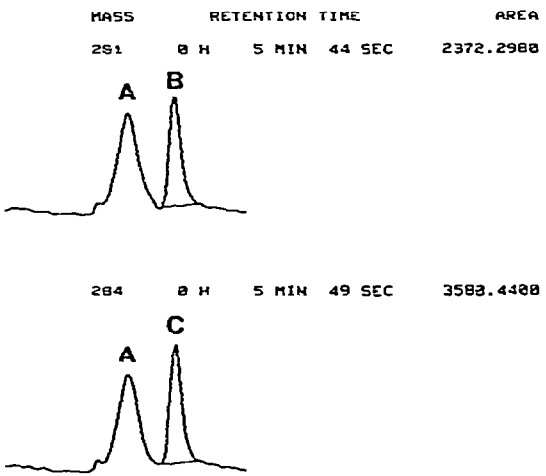

Fig. 6. SIM chromatograms of 10 μl of a sample of human urine collected 0–24 h after an intravenous injection of 50 mg of ranitidine. Peak A, endogenous material; Peak B, (m/z 281) ranitidine; Peak C, (m/z 284) [$^2H_3$]ranitidine. HPLC–MS instrumental conditions are described under Experimental. The chromatograms were smoothed during data processing.

excreted in the 0–24-h urine were, respectively, 68% and 27% as ranitidine, 5% and 4% as ranitidine-N-oxide, 2% and 1% as ranitidine-S-oxide and 2% and 2% as desmethylranitidine.

The ion-pair chromatography method was unsuitable for HPLC–MS because of the involatile counter-ions and buffers present in the mobile phase. Methanol–water–ammonium acetate reversed-phase systems for separating ranitidine and its metabolites were investigated as mobile phases for the HPLC–MS analyses of ranitidine and its metabolites. It was found that the drug and metabolites were thermally degraded at the temperature required to evaporate the mobile phase from the moving belt in HPLC–MS interface. To overcome this problem the more volatile non-aqueous mobile phase used in this study was developed[1]. The sensitivity of qualitative and quantitative on-line HPLC–MS analyses of ranitidine and its metabolites was limited for two reasons. The first was the use of normal-phase chromatography, which restricted the volume of urine which could be injected to 10 μl because larger volumes caused deterioration of the chromatography. The second was the extensive fragmentation of molecules of ranitidine and its metabolites, even under the mild conditions of ammonia chemical ionisation.

After an oral dose of 250 mg of ranitidine the concentration of metabolites in the urine was too low to obtain mass spectra suitable for their characterisation by on-line HPLC–MS of the urine[1]. When the metabolites were concentrated ten-fold by XAD-2 chromatography a satisfactory total ion current chromatogram showing the presence of each metabolite was obtained, and each metabolite was identified from its mass spectrum.

The profiles of the SIM chromatograms were not as smooth as those obtained under GC–MS conditions. One of the reasons for this could be that during evaporation of the mobile phase the solid formed is not uniformly deposited on the belt, and this could result in a small difference in area and thickness of the sample which could affect the transfer yield into the source. Also during volatilisation and ionisation of the samples there may be small changes in the operating conditions in the source. The reproducibility of evaporation, volatilisation and ionisation of a sample during HPLC–MS was assessed by measuring the coefficient of variation of the peak area of the ion m/z 284 obtained from six replicate injections of 120 ng of [$^2H_3$]ranitidine in 10 μl of urine. The coefficient of variation found was 19%; this value is much greater than could be attributable to errors in dilution or injection of the sample. Regression analysis of the data from which the calibration graph (Fig. 5) was obtained showed the correlation coefficient was 0.9979. This confirmed that any variation in evaporation profile of the samples had been corrected by using [$^2H_3$]ranitidine as an internal standard.

The limit of detection of ranitidine was 10.5 ng injected on column, *i.e.* 1.05 μg/ml urine. To measure lower concentrations of ranitidine in urine a pre-concentration step would be necessary. The area in the peak of the total ion current chromatogram due to endogenous material in the urine is considerably reduced when ranitidine and its metabolites are concentrated by chromatography of urine on XAD-2 resin. Therefore, using this procedure probably up to 2 ml of urine could be taken for concentration of ranitidine and its metabolites. For drugs and metabolites which are thermostable and can be analysed by on-line reversed-phase HPLC–MS, up to 1 ml of urine can be injected on column. Therefore the limit of detection of such compounds

by direct HPLC–MS analysis would be much increased over that of ranitidine and its metabolites.

5. CONCLUSION

The technique of on-line HPLC–MS has been evaluated in human drug metabolism studies. Ranitidine and three of its metabolites were characterised in a urine concentrate and the drug was quantified by direct analysis of urine using a deuterium-labelled internal standard.

6. SUMMARY

High-performance liquid chromatography has been used for the qualitative analysis of metabolites and the quantitative analysis of ranitidine in the urine from subjects given oral and intravenous doses of ranitidine.

Ranitidine, ranitidine-N-oxide, ranitidine-S-oxide and desmethylranitidine were identified in extracts of the urine obtained by the XAD–methanol procedure.

A selected-ion monitoring technique, using [$^2H_3$]ranitidine as the internal standard, was used to determine ranitidine.

A normal-phase system consisting of methanol–propan-2-ol–5 *M* ammonium acetate (50:50:1) was used, and because of this the volume of urine which could be injected on-column without deterioration of the chromatography was limited to 10 μl. This limited the sensitivity of the method to 1.0 μg of ranitidine per millilitre of urine.

REFERENCES

1 L. E. Martin, J. Oxford and R. J. N. Tanner, *Xenobiotica*, in press.
2 B. J. Millard, *Quantitative Mass Spectrometry*, Heyden and Son, London, 1978.
3 D. S. Millington, D. A. Yorke and P. Burns, in A. Quayle (Editor), *Advances in Mass Spectrometry*, Vol. 8, Heyden and Son, London, 1980, p. 1819.
4 J. A. Bell, F. A. A. Dallas, W. N. Jenner and L. E. Martin, *Biochem. Soc. Trans.*, 8 (1980) 93.
5 P. F. Carey, L. E. Martin and P. E. Owen, *J. Chromatogr.*, 225 (1981) 161.