CHROMBIO. 3703

DETERMINATION OF A NEW ANGIOTENSIN CONVERTING ENZYME INHIBITOR AND ITS ACTIVE METABOLITE IN PLASMA AND URINE BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

GÜNTHER KAISER*, ROLAND ACKERMANN, WALTER DIETERLE and JEAN-PIERRE DUBOIS*

Research and Development Department, Pharmaceuticals Division, Ciba-Geigy Limited, Basle (Switzerland)

SUMMARY

A specific and sensitive gas chromatographic-mass spectrometric method for the simultaneous quantification of unchanged 3-{[1-ethoxycarbonyl-3-phenyl-(1S)-propyl]amino}-2,3,4,5-tetrahydro-2-oxo-1-(3S)-benzazepine-1-acetic acid (I) and its active metabolite, the dicarboxylic acid (II), in plasma and urine has been developed and validated. $^2H_5$-labelled analogues of I and II were used as internal standards. The compounds were isolated from plasma and urine under acidic conditions using XAD-2 resin or Extrelut 1 columns. Following derivatization with diazomethane, the samples were analysed by packed-column gas chromatography-electron-impact mass spectrometry with selected-ion monitoring. The analysis of spiked plasma and urine samples demonstrated the good accuracy and precision of the method, which is suitable for use in pharmacokinetic and bioavailability studies with the new angiotensin converting enzyme inhibitor prodrug I·HCl in humans.

INTRODUCTION

3-{[1-Ethoxycarbonyl-3-phenyl-(1S)-propyl]amino}-2,3,4,5-tetrahydro-2-oxo-1-(3S)-benzazepine-1-acetic acid hydrochloride (I·HCl, CGS 14824A, Fig. 1) is a new drug from the class of the angiotensin converting enzyme (ACE) inhibitors. I·HCl has been found to be a very potent agent in humans after oral administration [1]. The compound is a prodrug which, on absorption, is hydrolysed to a pharmacologically active metabolite, the dicarboxylic acid (II, CGS 14831, Fig. 1). A similar mechanism has been reported for the ACE inhibitors enalapril [2] and ramipril [3]. I·HCl is now undergoing clinical trials for the treatment of hypertension and congestive heart failure.

---

*Present address: Biopharmaceutical Research Center, Laboratories Ciba-Geigy SA, Rueil Malmaison, France.

Fig. 1. Structures of I and II.

This paper describes a gas chromatographic–mass spectrometric (GC–MS) method for the specific and sensitive determination of unchanged I and its metabolite II in plasma and urine. The application of the method is demonstrated by assaying plasma and urine samples of a healthy volunteer orally dosed with I·HCl.

EXPERIMENTAL

Materials and reagents

I·HCl and its active metabolite II were available from Ciba-Geigy (Basle, Switzerland). The internal standards, pentadeuterated I·HCl and II containing five deuterium atoms in the phenyl ring, were synthesized in the Isotope Laboratory of Ciba-Geigy. A solution of diazomethane in diethyl ether was prepared from N-nitroso-N-methyl-p-toluenesulphonamide (Fluka, Buchs, Switzerland) by analogy with the method of Fales et al. [4]. Amberlite XAD-2, particle size 0.2–0.25 mm, was obtained from Serva (Heidelberg, F.R.G.) and Extrelut 1 pre-packed glass columns from E. Merck (Darmstadt, F.R.G.). Diethyl ether, tetra-hydrofuran, 25% (w/v) ammonia solution, concentrated sulphuric acid, buffer Titrisol pH 3, sodium chloride, phosphorus pentoxide and sodium carbonate were reagent grade from E. Merck. Puriss. ethyl alcohol, hexane, methyl alcohol, methylene chloride, perfluorotributylamine, toluene and 32% (w/v) hydrochloric acid were from Fluka. Water used in the experiments was bidistilled.

Human plasma was obtained from Blutspendezentrum (Basle, Switzerland) and human urine from healthy volunteers. Plasma and urine were stored below −18°C until used for preparing spiked samples.

Stock solutions and working solutions

Stock solutions of I·HCl and pentadeuterated I·HCl in water and of II and pentadeuterated II in ammonia solution (0.5%, v/v) were individually prepared at concentrations between 0.1 and 1.0 nM. The solutions were stored at 4°C and were stable for at least two months. For each assay, four working solutions were prepared by dilution with 0.01 M hydrochloric acid: S containing I·HCl and II, S_1 containing I·HCl, S_2 containing II, and R containing 3 μM pentadeuterated I·HCl and 5 μM pentadeuterated II. The concentrations of I·HCl and II were 1 μM each for plasma assays and 1 μM and 4 μM, respectively, for urine assays. These values are only indicative and were adapted to each particular assay. Solution S was used for preparing the calibration samples, S_1 and S_2 were used for the control (given/found) samples.

Preparation of spiked samples

With each assay, six calibration samples and five control samples were prepared by spiking plasma and urine in 5-ml glass ampoules and 20-ml plastic vials, respectively.

For plasma calibration samples, 0.5 ml of blank plasma was mixed with 10–500 μl of solution S, 400–890 μl of 0.01 M hydrochloric acid, 100 μl of solution R and 0.5 ml of buffer (Titrisol pH 3, undiluted). The total volume of each sample was 2.0 ml. For urine calibration samples, to 1 ml of blank urine were added: 15–800 μl of solution S, 0–785 μl of 0.01 M hydrochloric acid, 200 μl of solution R and 2 ml of a mixture of 2.5 M hydrochloric acid and saturated sodium chloride solution (4:1, v/v) to a total volume of 4.0 ml.

Using the solutions S_1 and S_2 instead of S, quality-control samples were prepared containing both I·HCl and II with comparable or different concentrations.

The spiked plasma mixtures were shaken on a vibration shaker, type Vortex evaporator (Haake-Buchler, Saddle Brook, NJ, U.S.A.) at speed 8 for 5 min, the urine mixtures on a shaker, type Vortex Genie (Scientific Industries, Bohemia, NY, U.S.A.) at speed 9 for at least 15 s.

Preparation of clinical samples

Clinical plasma samples were homogenized by shaking on a vibration shaker (Vortex Genie) for a few seconds. Then 0.5 (or 1.0) ml of each sample was weighed on a Type AC 100 micro-balance (Mettler, Greifensee, Switzerland) into 5-ml ampoules. The plasma was diluted with 0.9 (or 1.9) ml of 0.01 M hydrochloric acid and 0.1 ml of the standard working solution R. After addition of 0.5 (or 1.0) ml of buffer (Titrisol pH 3, undiluted), the ampoules were shaken in the same way as the spiked samples.

Clinical urine samples were homogenized likewise, and a defined volume (0.05–1.0 ml) was weighed into 20-ml vials. Blank urine was added to a final volume of 1 ml. The urine was diluted with 0.8 ml of 0.01 M hydrochloric acid and 0.2 ml of the internal standard working solution R. After addition of 2 ml of a mixture of 2.5 M hydrochloric acid and saturated sodium chloride solution (4:1, v/v), the vials were shaken in the same way as the spiked samples.

Isolation procedure for plasma

Glass columns with a sintered-glass filter and a volume of 25 ml were filled with ca. 200 mg of XAD-2. After washing with 3 ml of methanol, the XAD-2 was degassed by pressing in 5 ml of methanol by a pasteur pipette, and then washed three times with 5 ml of water. The chromatographic system was acidified with 3 ml of 0.1 M hydrochloric acid. The centrifuged plasma sample (5 min at 1250 g; type Multex centrifuge, MSE Scientific Instruments, Crawley, U.K.) was applied to the XAD-2 column. The column was washed three times with 5 ml of water, and the water was displaced by adding 0.1 ml of methanol. The adsorbed compounds were eluted with 4 ml of methanol into a 5-ml ampoule. The eluate was evaporated to dryness under a stream of nitrogen at 40°C, and the residue was dried in a vacuum desiccator over phosphorus pentoxide for at least 30 min.

Isolation procedure for urine

Prepacked glass columns (Extrelut 1) were washed twice with 4 ml of methylene chloride–ethanol (95:5, v/v) and dried at 50°C in vacuum for at least 1 h prior to use. A 1.0-ml aliquot of each urine sample was transferred to the top of the column and allowed to soak for 10 min. Then the column was eluted twice into a 10-ml glass ampoule with 4 ml of methylene chloride–ethanol (95:5, v/v) each. The eluate was evaporated to dryness, and the residue was dried as described for plasma samples.

Derivatization

The plasma or urine extract was dissolved in 0.1 ml of tetrahydrofuran. After vortexing for 10 s, 0.3 ml of a solution of diazomethane in diethyl ether were added, the mixture was shaken for 30 min at 300 rpm in a horizontal shaker, type TR 1 (Infors, Hofstetten, Switzerland) and evaporated to dryness (nitrogen, 40°C).

Work-up procedure for plasma

To the derivatized sample, 0.5 ml of 0.5 M sulphuric acid and 1 ml of hexane were added. The mixture was vortexed for 5 min and centrifuged for 1 min at 1250 g, and the two phases were separated by freezing the aqueous phase in a dry ice–ethanol mixture and discarding the organic phase. The aqueous phase was made alkaline by addition of 0.5 ml of 2 M sodium carbonate and extracted with 1.6 ml of diethyl ether–methylene chloride (2:1, v/v). The organic phase was transferred into a conical vial and evaporated to dryness (nitrogen, 40°C).

Work-up procedure for urine

The residue of the derivatized sample was dissolved in 1.5 ml of diethyl ether. The solution was vortexed for 5 min, transferred into a conical vial and evaporated to dryness (nitrogen, 40°C).

Chromatographic conditions

GC–MS was performed on an HP 5985 B or HP 5987 A system (Hewlett-Packard, Palo Alto, CA, U.S.A.). A 1.5 m × 2 mm I.D. Pyrex glass column packed with 3% OV-101 on Gaschrom Q, 80–100 mesh (Ciba-Geigy) was used. The carrier gas was helium with a flow-rate of 30 ml/min. The GC injector port and the column oven were operated isothermally at 275°C. Under these conditions, the retention times for the methyl ester derivatives of I and II were 2.55 and 2.3 min, respectively.

The separator and the GC–MS interface heaters were set to 260°C and the mass spectrometer ion source temperature to 200°C. The mass spectrometer was operated under electron-impact (EI) ionization conditions at 70 eV. Perfluorotributylamine (PFTBA) was used for the ion-source tuning. For selected-ion monitoring (SIM), the ion-source parameters were adjusted to obtain the highest signal on the fragment ion m/z 414 of PFTBA. To detect and quantitate I and II, SIM was performed at the following positive fragment ions: m/z 365 for the methyl esters of I and II, m/z 370 for the methyl esters of pentadeuterated I and II. The
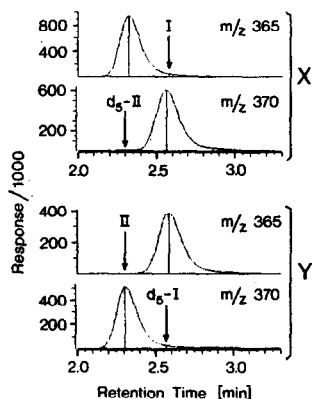
Fig. 2. SIM chromatograms of plasma extracts showing the mutual contributions of the derivatives of I and II (or their deuterated analogues) to their peak heights. (X) Sample (0.5 ml) spiked with 4.4 nmol of II and 1.9 nmol of pentadeuterated I·HCl. (Y) Sample (0.5 ml) spiked with 1.4 nmol of I·HCl and 3.9 nmol of pentadeuterated II. Conditions as described in Experimental.

analyte residues were redissolved in 5–10 μl of toluene, and 1 μl was injected for GC–MS–SIM analysis.

Calculations

Quantification was based on the peak-height ratio of the substance and its pentadeuterated standard. The chromatographic resolution of the derivatives of I and II (or their pentadeuterated analogues) was not complete. To determine the possible mutual contributions to the peak heights, two spiked control samples X and Y were prepared with each assay. X contained II and pentadeuterated I, Y contained I and pentadeuterated II. Fig. 2 shows typical SIM chromatograms of these samples. The determination of II (first peak) was not influenced by the presence of I (second peak). In contrast, the tailing of the first peaks (II and pentadeuterated II) affected the determination of the heights of the second peaks (I and pentadeuterated I). Using X and Y, the percentage contribution of the first peaks to the peak heights of the second peaks was calculated. For each analytical sample, the peak heights for I and its labelled analogue were corrected by the contributions calculated from the peak heights for II and its labelled analogue.

Least-squares linear regression analysis ($y = a + bx$) of the peak-height ratio y versus concentration x was used to quantify unknowns. In most cases, a regression line through a fixed positive intercept a was chosen. The value of a was determined as an average from twenty samples spiked with internal standards only ($x = 0$) and analysed on different days.

Method validation

The method was validated by analysis of spiked plasma and urine samples. The sample volume was 0.5 ml for plasma and 1.0 ml for urine. All concentrations of method validation experiments are related to "sample used for analysis" and are given in pmol per sample.

RESULTS AND DISCUSSION

Isolation procedures

Conventional liquid–liquid extraction of I and II from aqueous solution resulted in low extraction yields, especially for the dicarboxylic acid II. Therefore, the glass columns prepacked with Extrelut 1 and columns filled in our laboratory with XAD-2 resin were chosen to isolate drug and metabolite from urine and plasma, respectively. The recoveries of both methods were almost quantitative. For isolation on XAD-2, 2 or 4 ml of the diluted plasma mixtures could be applied to the same amount of resin without affecting the isolation efficiency. This enabled us to use 0.5 or 1.0 ml of clinical plasma samples for analysis, dependent on the expected concentrations of I and II.

Derivatization

The convenient derivatization with diazomethane at room temperature yielded the methyl esters of I and pentadeuterated I and the dimethyl esters of II and pentadeuterated II. Methanol had to be excluded from this reaction, since it caused partial re-esterification of the ethyl ester group of I and its labelled analogue. Tetrahydrofuran had no such influence and was used as co-solvent.

After derivatization, the urine samples were subjected to GC–MS without further purification. The plasma samples were purified to remove co-extracted endogenous compounds, which may affect the chromatographic resolution. Purification was achieved by back-extraction into acidified aqueous solution and re-extraction into the organic phase.

Gas chromatography and selected-ion monitoring

Packed columns and widebore fused-silica capillary columns (HP Series 530 μ, methyl silicone, 10 m, Hewlett-Packard) were tested for GC of the methyl ester derivatives of I and II. In none of these columns were the two compounds completely resolved. Wide-bore capillary columns, however, made higher demands on the purity of the analyte solution injected than packed columns. Therefore, a packed column (3% OV-101, 1.5 m) was preferred for the present method.

The fragmentation patterns of the methyl ester derivatives of I, II, and their labelled analogues under EI conditions are shown in Figs. 3 and 4. Molecular ions of appreciable abundance were observed for all compounds. For SIM analysis the more abundant fragment ions at m/z 365 (I and II) and 370 (internal standards) were used. These fragments were obtained by the cleavage of the carboxyethyl $(M-73)$ or carboxymethyl group $(M-59)$ in the side-chain.

Typical SIM chromatograms of extracts from blank plasma (A), spiked plasma (B) and plasma of a volunteer orally dosed with I·HCl (C) are shown in Fig. 5. No interference peak derived from endogenous components was observed. Likewise, no interference was noted in urine.

The assay enables both substances I and II to be determined simultaneously in one run, although the chromatographic resolution was not complete. Using the peak height for quantification, the determination of the metabolite II was not
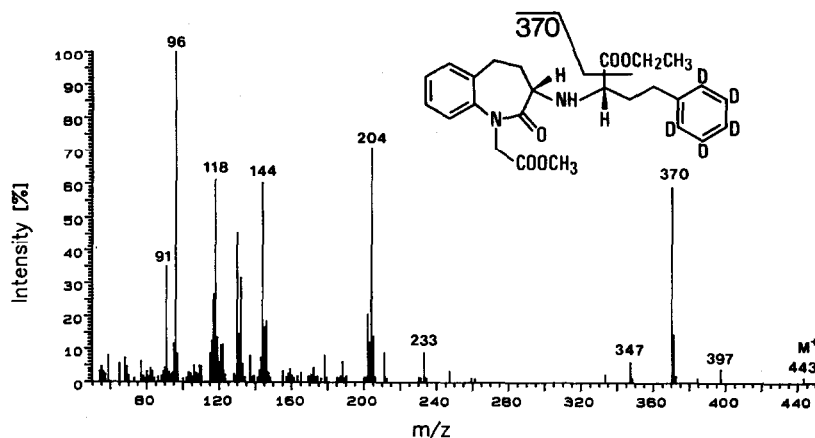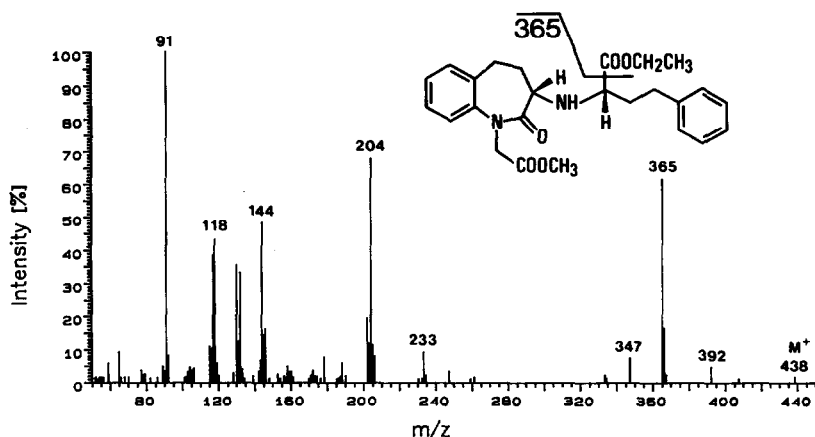
Fig. 3. Electron-impact mass spectra of the methyl ester derivatives of I (top) and pentadeuterated I (bottom). Conditions as described in Experimental.

affected by unchanged I. Contributions of II to the peak height of I were taken into account by the correction procedure described in Experimental.

Assay linearity, accuracy, precision and sensitivity

Calibration curves for I and II in plasma showed a linear response in the range 10–700 pmol per sample. In urine, the linear calibration range was 12–800 pmol per sample for I and 60–3500 pmol per sample for II. Typical parameters for calibration curves in plasma were $y = 0.004 + 0.00388x$, $Sy = 0.0119$, $r = 0.99996$ for I and $y = 0.006 + 0.00209x$, $Sy = 0.0062$, $r = 0.99998$ for II, where y denotes the peak-height ratio, x the concentration, Sy the estimated standard deviation and r the coefficient of correlation.

The accuracy and day-to-day precision of the method were determined by assaying sets of five plasma and urine samples (A–E) spiked with I and II (Tables
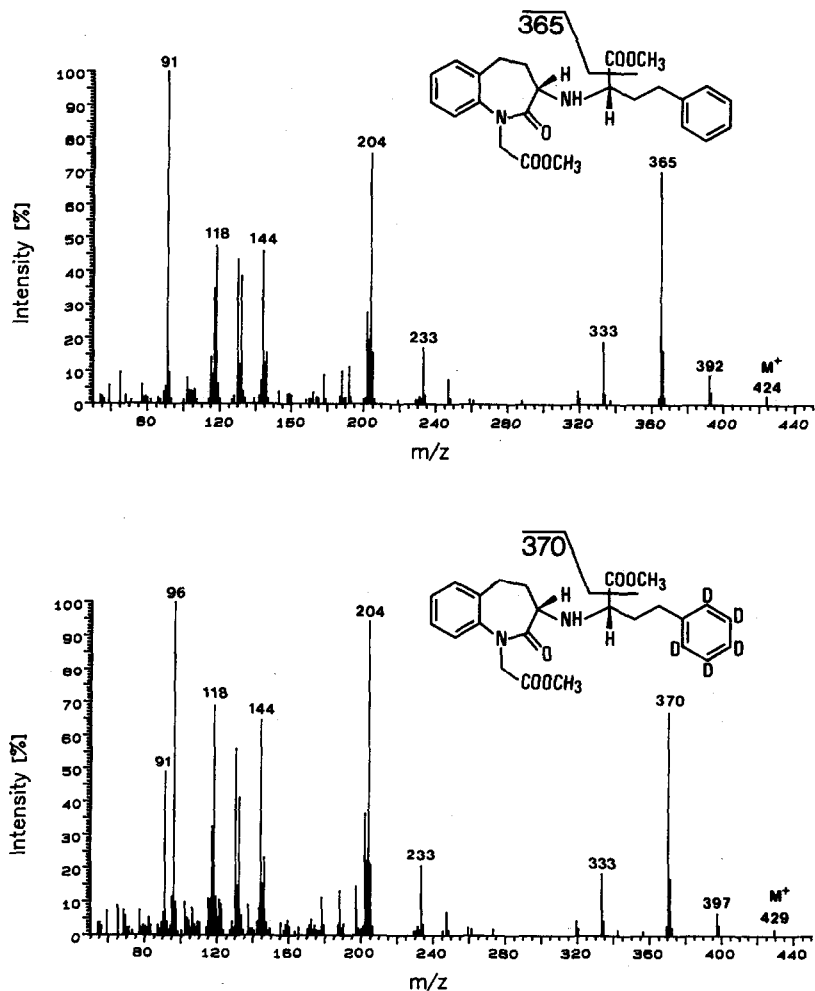
Fig. 4. Electron-impact mass spectra of the dimethyl ester derivatives of II (top) and pentadeuterated II (bottom). Conditions as described in Experimental.

I and II). The samples contained I in amounts increasing from sample A to sample E and decreasing amounts of II.

With the exception of the lowest concentration each, the mean recoveries for both compounds were in the range 97.4–102.4% of the given plasma or urine concentration and demonstrated the good accuracy of the method. The coefficients of variation (C.V.) also indicated a good precision of the method for plasma concentrations of I and II above 70 pmol per sample and urine concentrations above 140 and 360 pmol per sample for I and II, respectively.

The limits of quantification (LOQ, C.V. $\leq$ 10%) and the limits of detection (LOD, C.V. $\leq$ 100%) were estimated from the above spiked samples. For I, the LOQ was 30 pmol per sample in plasma and 80 pmol per sample in urine. The corresponding values for the LOD were 4 and 15 pmol per sample. For II, the LOQ
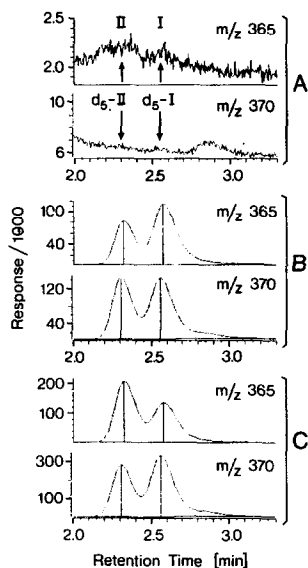
Fig. 5. SIM chromatograms of plasma extracts. (A) Blank human plasma showing no interferences at the retention times of the derivatives of I, II and their deuterated analogues. (B) Sample (0.5 ml) spiked with 212 pmol of I·HCl, 270 pmol of II, 258 pmol of pentadeuterated I·HCl and 484 pmol of pentadeuterated II. (C) 1-h plasma sample (0.5 ml) of a subject orally dosed with 10 mg of I·HCl, added standards as listed for B. Conditions as described in Experimental.

was 30 and 170 pmol per sample and the LOD 3 and 20 pmol per sample, for plasma and urine, respectively.

Stability of spiked samples

Plasma samples spiked with I and II were stored below $-18°C$ and analysed after twelve months. The mean recoveries were 98.3% for I and 101.6% for II ($n=4$). These results demonstrate that both compounds are stable under the usual storage conditions of biological samples for at least one year.

TABLE I

ACCURACY AND DAY-TO-DAY PRECISION FOR PLASMA

I and II were determined simultaneously in samples spiked with both compounds ($n=8$).

Sample	Concentration added (range, pmol per sample)		Mean recovery (%)		C.V. (%)	
	I	II	I	II	I	II
A	15– 17	624–671	105.2	100.2	30.1	2.8
B	75– 89	375–404	97.4	101.3	3.2	2.1
C	151–177	189–201	99.6	100.8	4.0	3.7
D	294–358	94–100	99.9	102.4	3.4	2.8
E	494–594	18– 20	97.6	90.9	5.7	12.9

TABLE II

ACCURACY AND DAY-TO-DAY PRECISION FOR URINE

I and II were determined simultaneously in samples spiked with both compounds ($n=11$).

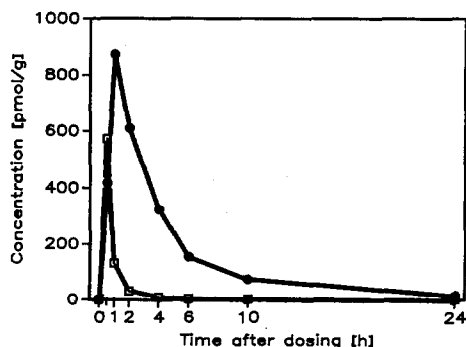| Sample	Concentration added (range, pmol per sample)		Mean recovery (%)		C.V. (%)	
	I	II	I	II	I	II
A	9– 14	2701–2936	61.1	102.0	116.5	2.9
B	71– 72	1350–1468	101.6	101.3	10.2	3.2
C	144–144	666– 734	101.7	101.1	3.5	3.6
D	287–288	361– 367	100.5	101.5	4.3	5.1
E	575–577	51– 73	99.4	87.1	4.7	28.1

Fig. 6. Plasma concentrations of unchanged I (□) and metabolite II (●) in a healthy male volunteer after a single oral dose of 20 mg of I·HCl.
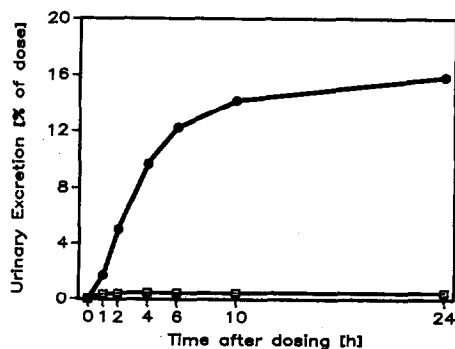
Fig. 7. Cumulative excretion of unchanged I (□) and metabolite II (●) in urine of a healthy male volunteer after a single oral dose of 20 mg of I·HCl.

Application

The described method was applied to the determination of unchanged I and metabolite II in plasma and urine of healthy volunteers orally dosed with I·HCl in the dose range 5–20 mg. For illustration, typical plasma concentration–time profiles of I and II are shown in Fig. 6. The cumulative urinary excretion of the two compounds is depicted in Fig. 7. The graphs demonstrate that the prodrug I·HCl was rapidly absorbed and extensively metabolized to the pharmacologically active metabolite II. Only trace amounts were excreted unchanged in urine.

CONCLUSIONS

The GC–MS method described here permits the simultaneous determination of unchanged drug I and its active metabolite II in plasma or urine with a high specificity and sensitivity. The method is suitable for use in pharmacokinetic and bioavailability studies with the new ACE inhibitor prodrug I·HCl.

REFERENCES

1   M.D. Schaller, J. Nussberger, B. Waeber, J.P. Bussien, G.A. Turini, H. Brunner and H.R. Brunner, Eur. J. Clin. Pharmacol., 28 (1985) 267.
2   E.H. Ulm, Drug Metab. Rev., 14 (1983) 99.
3   P.U. Witte, R. Irmisch, P. Hajdu and H. Metzger, Eur. J. Clin. Pharmacol., 27 (1984) 577.
4   H.M. Fales, T.M. Jaouni and J.F. Babashak, Anal. Chem., 45 (1973) 2302.