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Determination of three metabolites of a new angiotensin-converting enzyme inhibitor, imidapril, in plasma and urine by gas chromatography–mass spectrometry using multiple ion detection

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ABSTRACT

A specific and sensitive gas chromatographic–mass spectrometric method for the determination of three metabolites of the angiotensin-converting enzyme inhibitor, imidapril, in plasma and urine was developed. The metabolites were isolated from plasma and urine using a Bond Elut C_{18} solid-phase extraction cartridge. The isolated metabolites were converted to sensitive derivatives by pentafluorobenzyl bromide and heptafluoro-*n*-butyric acid anhydride. Following derivatization, the sample solutions were analysed by wide-bore column gas chromatography–mass spectrometry with multiple ion detection. The detection limits of the three metabolites were each 1 ng/ml in plasma and 5 ng/ml in urine. Analysis of the spiked plasma and urine samples demonstrated the good accuracy and precision of the method. This method was very useful for use in pharmacokinetic and bioavailability studies of the three metabolites of imidapril in humans.

INTRODUCTION

(−)-(4*S*)-3-[(2*S*)-2-[[(1*S*)-1-Ethoxycarbonyl-3-phenylpropyl]amino]propionyl]-1-methyl-2-oxoimidazolidine-4-carboxylic acid hydrochloride (imidapril) is a new angiotensin-converting enzyme (ACE) inhibitor whose metabolic pathways were determined, as shown in Fig. 1, by animal studies using ^{14}C-labelled compounds [1]. It is a prodrug that is de-esterified *in vivo* by esterase to release an active metabolite (M1). Imidapril is now undergoing clinical trials for the treatment of hypertension and congestive heart failure.

Analytical methods in use at present for the measurement of an ACE inhibitor and its metabolites are inhibitor binding assay (IBA) [2], radioimmunoassay (RIA) [3], high-performance liquid chromatography (HPLC) [4] and gas chromatography (GC) [5,6]. The IBA and RIA methods are very sensitive, but it is difficult to determine several kinds of compounds simultaneously. The HPLC and GC methods are specific, but less sensitive than the others.

The determination of imidapril and its active metabolite has already been established using HPLC with 9-anthryldiazomethane (ADAM) as a fluorimetric derivatization reagent. The HPLC method was applied to the determination of imidapril and its active metabolite in human plasma and urine [7], and their pharmacokinetics was clarified. However, it is difficult to determine the three metabolites (M2, M3 and M4) of imidapril

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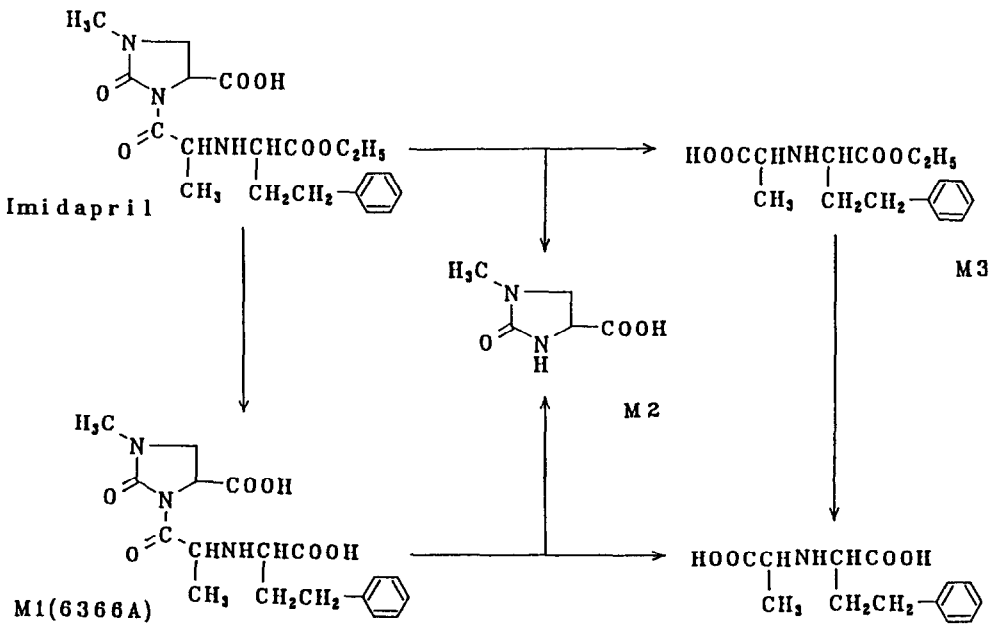

Fig. 1. Proposed metabolic pathways of imidapril.

at low concentrations in plasma and urine by HPLC. Thus, a specific and sensitive method is required for the determination of the three metabolites of imidapril in order to study its pharmacokinetics.

This paper describes the gas chromatographic–mass spectrometric (GC–MS) method with a wide-bore column and multiple ion detection (MID) for the specific and sensitive determination of the three metabolites of imidapril in plasma and urine. The application of this method is demonstrated by assaying plasma and urine samples of healthy volunteers orally dosed with imidapril.

EXPERIMENTAL

Materials and reagents

Authentic specimens of M2, M3 and M4 were synthesized by Hayashi *et al.* [8]. (2*S*)-2-[N-[(1*S*)-1-Methoxycatonyl-3-phenylpropyl]amino]-propionic acid (I.S.-1, Fig. 2) and (4*S*)-1-ethyl-2-oxoimidazolidine-4-carboxylic acid (I.S.-2, Fig. 2) were used as internal standards (I.S.) [8]. Hydrochloric acid, ammonium sulphate and triethylamine were reagent grade, and ethyl acetate, isopropyl alcohol, acetonitrile and dichloromethane were HPLC grade from Katayama Chemicals (Osaka, Japan). Pentafluorobenzyl (PFB) bromide and heptafluorobutyric (HFB) anhydride were purchased from Tokyo Kasei (Tokyo, Japan). The Bond Elut C_{18} solid-phase extraction cartridge (1 ml) was obtained from Varian (Harbor City, CA, USA).

Plasma and urine samples

Blood samples obtained from healthy volunteers were collected in heparinized containers and

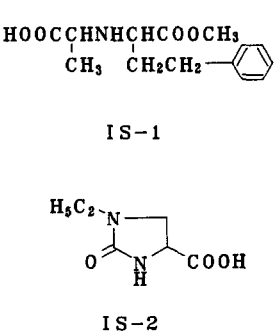

Fig. 2. Structures of the internal standards.

centrifuged to obtain the plasma. The plasma and urine samples were stored at −20°C until the time of analysis.

Sample preparation

Plasma samples (1 ml) were added to the internal standard (I.S.-1, 200 ng), acidified by 50 μl of 2 *M* hydrochloric acid. The plasma solutions described above were charged to the Bond Elut C_{18} solid-phase extraction cartridges, which were activated by first washing with 3 ml of methanol, 0.5 ml of 80% ethanol–hydrochloric acid (0.005 *M*), 3 ml of distilled water and 1 ml of 1 *M* hydrochloric acid. The cartridges were washed with 1 ml of 0.1 *M* hydrochloric acid, and M3, M4 and I.S.-1 retained in the cartridge were eluted with 0.5 ml of 80% ethanol–hydrochloric acid (0.005 *M*) into a reaction vial. The eluate was evaporated to dryness at 40°C under a stream of nitrogen gas for the derivatization.

For M3 and M4, the residual sample eluted from the C_{18} cartridge was taken up in 100 μl of dichloromethane; 5 μl of triethylamine were then added to the solution. The mixture was treated with 30 μl of PFB bromide, and heated at 60°C for 30 min to obtain the PFB esters of M3, M4 and I.S.-1. Subsequently the sample was evaporated to dryness under a stream of nitrogen gas. The residue was redissolved in 100 μl of acetonitrile and a 1 μl aliquot injected into the GC–MS system for the determination of M3 and M4.

On the other hand, the solution containing metabolite M2 passed through the C_{18} cartridge was collected in a centrifuge tube containing 2 g of ammonium sulphate. After 6 ml of ethyl acetate–isopropyl alcohol (4:1, v/v) were added, the mixture was shaken vigorously for 10 min and centrifuged at 1500 *g* for 5 min. The organic layer was taken and evaporated to dryness at 40°C under a stream of nitrogen gas. The residue was transferred to a reaction vial with 0.5 ml of ethyl acetate–isopropyl alcohol (4:1, v/v); this step was repeated three times. The mixture was evaporated to dryness again at 40°C under a stream of nitrogen gas for the derivatization.

For M2, the residual sample extracted with organic solvent was taken up in 100 μl of I.S.-2 solution (5 μg/ml in dichloromethane); 5 μl of triethylamine were then added to the solution. After PFB derivatization and evaporation in accordance with the procedure for M3 and M4, the residue was taken up in 50 μl of acetonitrile. The mixture was treated with 100 μl of HFB anhydride at room temperature for 15 min to obtain the PFB–HFB derivative of M2 and I.S.-2. After all derivatizations 1 μl was injected into the GC–MS system for the determination of M2.

Chromatographic conditions

The gas chromatograph-mass spectrometer was a Hitachi M-80A equipped with an M-003 computer system. The wide-bore fused-silica column was DB-1 (15 m × 0.53 mm I.D., 1.5 μm film thickness; J&W, Rancho Cordova, CA, USA). The carrier gas was helium at a flow-rate of 15 ml/min. The temperature of the injection port and the separator was 270°C. The column temperature was initially set at 200°C for 1.0 min, and the temperature was then raised at 5°C/min to 245°C for analysis of metabolites M3 and M4 in plasma samples, and was maintained at 230°C for urine samples. The column temperature for metabolite M2 was 170°C. This temperature was maintained during the chromatography. The mass spectrometer was operated in the electron-impact (EI) mode at 70 eV; the temperature of the ion source was 150°C. The stable fragment ions selected for MID were as follows: *m/z* 386 for the PFB derivatives of M3, M4 and I.S.-1, *m/z* 295 for the PFB–HFB derivative of M2 and *m/z* 309 for the PFB–HFB derivative of I.S.-2.

Calibration curves, accuracy and precision

The calibration curves for determination of the three metabolites were prepared by the addition of M2, M3 and M4 (5, 10, 25, 50 and 100 ng/ml in plasma; 10, 50, 100, 250, 500, 1000 and 2000 ng/ml in urine). These spiked samples were analysed by the procedure describe above.

The accuracy and precision of the proposed method were determined by adding known amounts of M2, M3 and M4 to control plasma and urine.

RESULTS AND DISCUSSION

Sample preparation

To develop a highly sensitive and reproducible analytical method for biological samples, it is necessary to extract and separate the compounds of interest from a mixture. The GC–MS method is selective and specific and was considered to make the preparation procedure as simple as possible.

First, we investigated assaying the metabolites M2, M3 and M4 converting them to their ADAM derivatives as in the HPLC method for the determination of imidapril and its active metabolite. It was difficult to separate the ADAM-derivatized metabolites from excess ADAM reagent because the metabolites had high polarity. The next analytical method investigated was the GC–MS method for determination of the three metabolites. Using the GC–MS method the compounds could be identified by their retention times and characteristic fragment ions. This method has high selectivity and sensitivity.

The C_{18} solid-phase extraction cartridge that was used for the isolation of imidapril and its active metabolite was chosen to isolate M3 and M4 from plasma and urine. The plasma and urine samples with added hydrochloric acid were charged into the C_{18} cartridge. M3, M4 and I.S.-1 were retained in the cartridges and M2 passed through it by the procedure described above. Thus M3 and M4 were isolated by elution with acidic ethanol, but further extraction from the waste with ethyl acetate–isopropyl alcohol (4:1, v/v) was required to isolate M2. The same size of sample, only 1 ml of plasma and 0.5 ml of urine, was used to analyse M2, M3 and M4; the recoveries of this isolation procedure were quantitative.

Derivatizations such as silylation, esterification and acylation of the carboxylic acid and amino groups were investigated. After trimethylsilylation of the metabolites, the reaction proceeded quantitatively, but this derivatization could not be used for determination owing to interference by substances in plasma and urine. To avoid interference from biological samples, M3 and M4 were converted to the PFB and di-PFB esters, respectively, which were much heavier than the trimethylsilyl derivatives. However, M2, whose molecular weight is much less than M3 and M4, was converted to the PFB derivative for the same reason. This derivatization was used for the determination of M2 in spiked plasma samples; however, M2 determination in urine could not be achieved because of many interfering substances, especially variant fatty acids in the urine. Thus esterification and acylation of M2 were investigated. Metabolite M2 in plasma and urine was determined without interference by HFB acylation of the amino group after PFB esterification of the carboxylic acid group.

The optimum conditions for PFB derivatization of the metabolites were investigated in terms of reaction solvent (dichloromethane, acetone and acetonitrile), amounts of PFB bromide (5, 10, 25, 50 and 100 μl), amounts of triethylamine (0, 1, 5, 10 and 30 μl) and reaction time (0, 15, 20, 60 and 90 min at 60°C). It was found that the yield was maximum in dichloromethane and the optimum amounts of PFB bromide and triethylamine were 30 and 5 μl, respectively. Fig. 3 shows the reaction time for derivatization, requiring 30 min at 60°C for completion.

For derivatization of metabolite M2, esterification and acylation were investigated with PFB and HFB anhydride (HFBA). The optimum con-

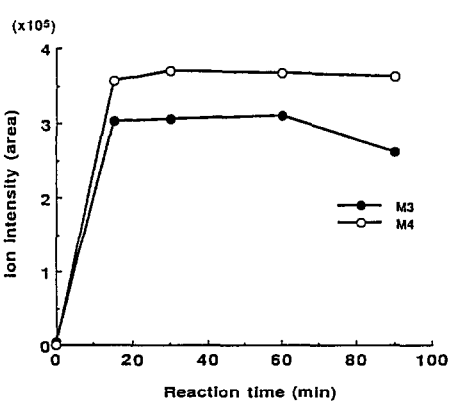

Fig. 3. Effect of reaction time for pentafluorobenzyl derivatization of metabolites M3 and M4.

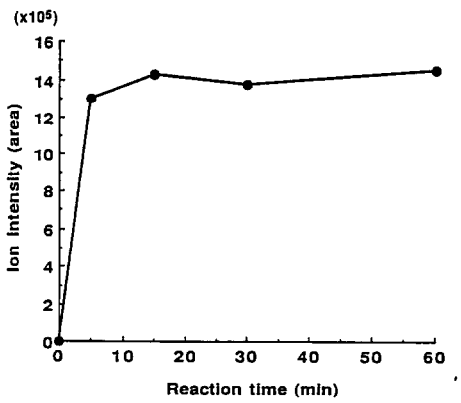

Fig. 4. Effect of reaction time for heptafluorobutyryl derivatization of metabolite PFB-M2.

centration and reaction time (Fig. 4) were observed with HFBA.

From the results, the proposed derivatization procedure with PFB and HFBA was established. It was found that esterification with PFB bromide proceeds quantitatively at 60°C for 30 min, and HFB acylation at room temperature for 15 min with HFBA in a screw-capped reaction vial. After derivatization, the samples were subjected to GC–MS without further purification.

Mass chromatography and mass fragmentography with MID

The mass spectra of the PFB derivatives of M3, M4 and I.S.-1 are shown in Fig. 5. Molecular ions of appreciable abundance were observed for all compounds: *m/z* 445 for the PFB ester derivative of I.S.-1, *m/z* 459 for the PFB ester derivative of M3 and *m/z* 611 for the di-PFB ester derivative of M4. For MID analysis of M3, M4 and I.S.-1, the more abundant ions at *m/z* 386 were used. These fragment ions were obtained after cleavage of the carboxy ester (I.S.-1, M − 59; M3, M − 73; M4, M − 225) in the side-chain. The mass spectra of the PFB–HFB derivatives of M2 and I.S.-2 are shown in Fig. 6. Molecular ions of abundance were observed for the PFB–HFB derivatives of M2 and I.S.-2 at *m/z* 520 and 534, respectively. For MID analysis of M2 and I.S.-2, the most abundant ions of base peaks *m/z* 295 and 309, respectively, were used.

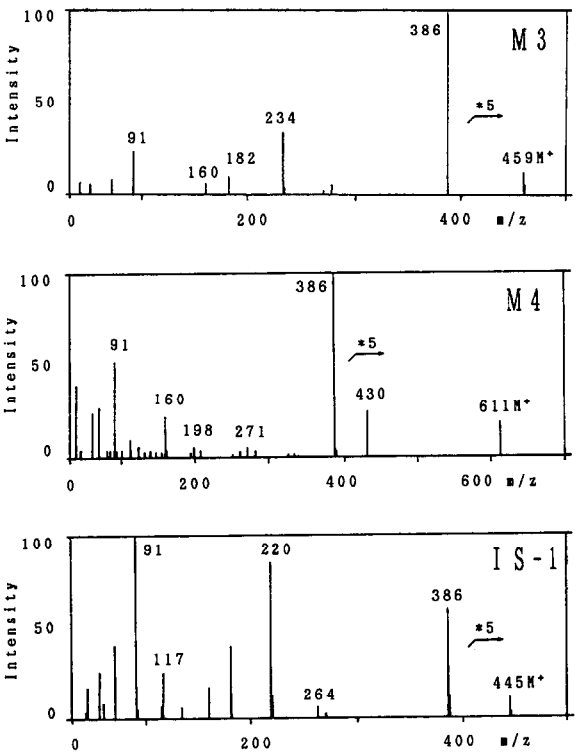

Fig. 5. Mass spectra of pentafluorobenzyl derivatives of M3, M4 and I.S.-1.

These fragment ions were obtained after the cleavage of the carboxy ester (M − 225).

Typical mass fragmentograms of extracts from control plasma and urine spiked with the three metabolites are shown in Fig. 7. No interference peak due to endogenous components was observed in either plasma or urine.

Generally, PFB derivatives of the compounds could be analysed with high sensitivity and selectivity by the negative-ion chemical ionization (NICI) method. Goto *et al.* [9] reported the determination of 2 fg of bile acids using the PFB derivative and NICI. The mass spectra of a carboxylic acid PFB ester obtained by the NICI method showed that the fragment ions were obtained after cleavage of PFB ester from the molecular ion. However, the NICI method has the drawback of bringing about a change in ion intensity as a result of the change in chemical ionization gas pressure. So, it was not suitable for using for many

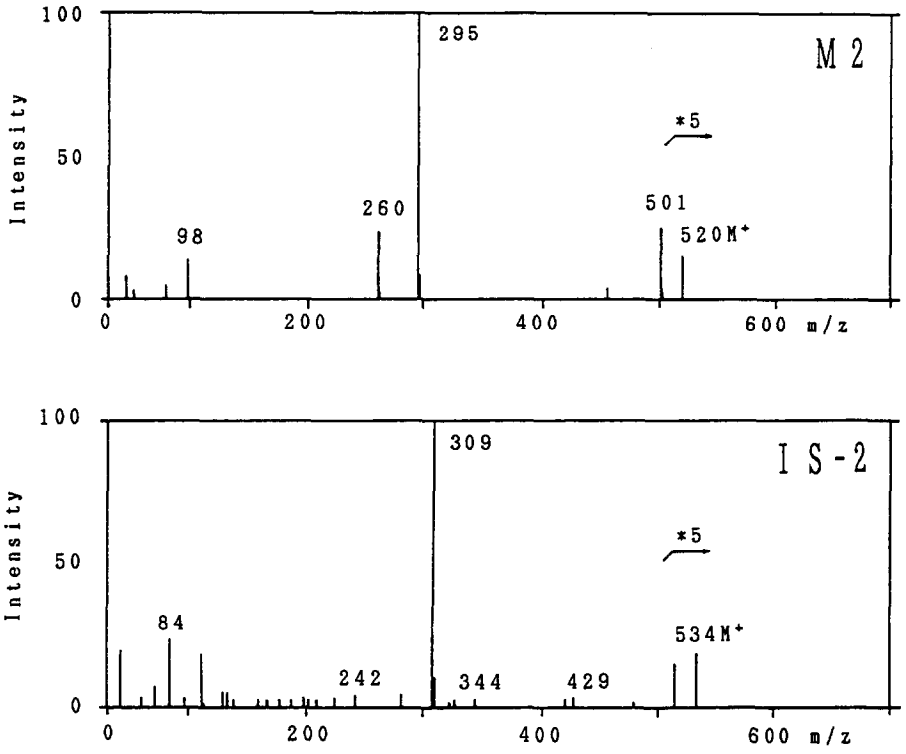

Fig. 6. Mass spectra of pentafluorobenzyl–heptafluorobutyryl derivatives of M2 and I.S.-2.

hours and analysing many samples, as is necessary to study pharmacokinetics. Thus, we determined the metabolites PFB esters using the characteristic fragment ions in the positive-ion mode.

In addition, it may be possible to analyse a mixed solution of the derivatives of M2, M3 and M4 as a single sample. However, it was difficult to chromatograph a solution containing M2, M3 and M4 as a single sample because of large differences of the polarity and the volatility between the derivatized M2 and M3, M4. Therefore, the derivatized M2, M3 and M4 were analysed under two different chromatographic conditions.

Assay linearity, accuracy, precision and sensitivity

Calibration curves were obtained by plotting the ratio of the peak area of the derivatives of M2, M3 and M4 spiked in plasma and urine to that of the derivative of the internal standards against concentration. In plasma samples, the linear calibration range was 5–100 ng/ml for M2, M3 and M4, which corresponds to the regression equations $y = 0.0075x - 0.0031$ ($r = 0.9999$), $y = 0.011x + 0.0083$ ($r = 0.9999$) and $y = 0.0029x + 0.019$ ($r = 0.9933$), respectively.

In urine samples, the calibration curves in the range 10–2000 ng/ml for M2, M3 and M4 similarly showed good linearity.

The accuracy and precision of the method were determined for plasma and urine samples spiked with three metabolites (Table I). The found concentrations of M4 in plasma and M2 in urine were low compared with the added concentrations, however, the coefficient of variation (C.V.) indicated good precision. It is evident from the data in Table I that the proposed method is almost satisfactory in terms of both accuracy and precision. The concentrations in working samples were calculated from the concentrations of standard solutions in control plasma and urine spiked with known amounts of three metabolites (M2, M3 and M4) using the proposed method.

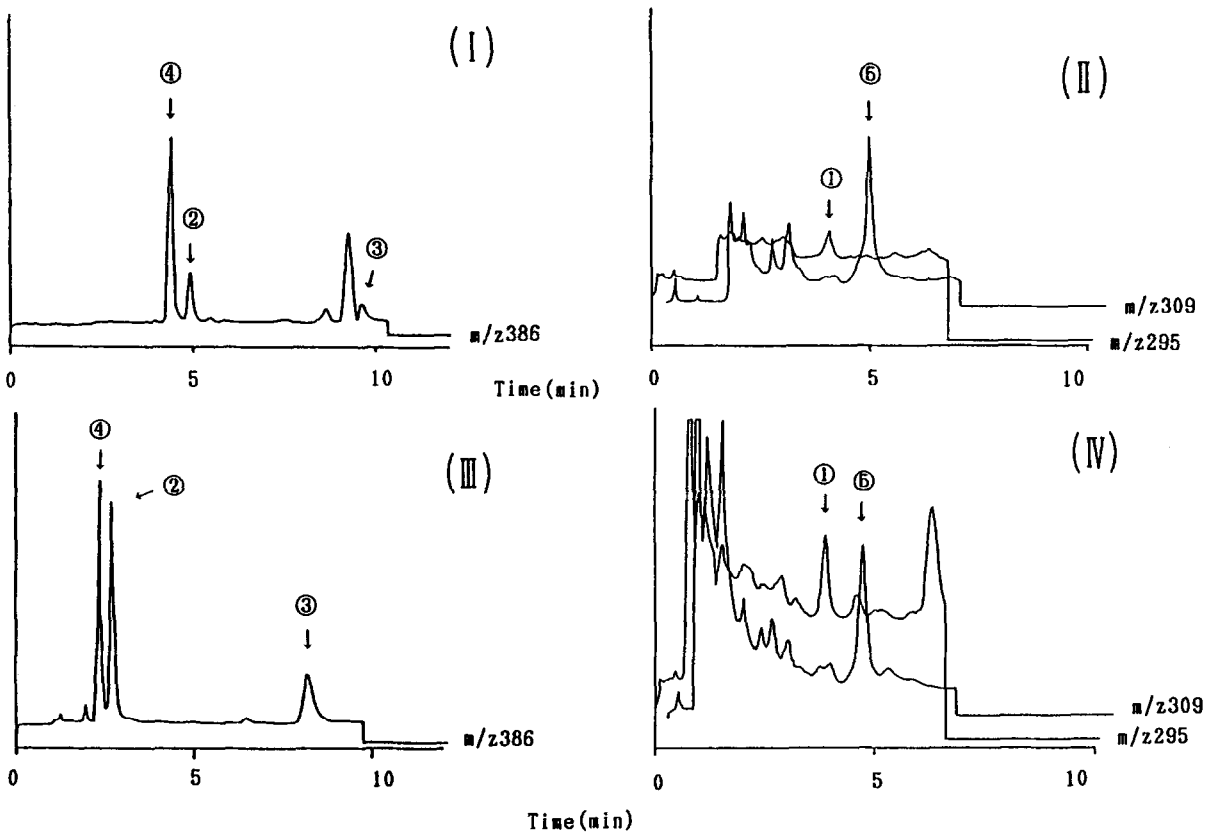

Fig. 7. Mass fragmentgrams of control plasma and urine spiked with metabolites M2, M3 and M4. I, Plasma spiked with 25 ng of M3 and M4; II, plasma spiked with 25 ng of M2; III, urine spiked with 100 ng of M3 and M4; IV, urine spiked with 100 ng of M2. Peaks: 1 = M2; 2 = M3; 3 = M4; 4 = I.S.-1; 5 = I.S.-2.

TABLE I

ACCURACY AND PRECISION OF THE PROPOSED METHOD FOR THE DETERMINATION OF THE THREE METABOLITES (M2, M3 AND M4) IN PLASMA AND URINE

Data are expressed as the values calculated using direct standards with no extraction procedure.

Compound	Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)
Plasma			
M2	5	4.0 ± 0.5	11.9
	25	24.8 ± 1.1	4.6
M3	5	5.7 ± 0.2	3.7
	25	25.6 ± 0.8	3.0
M4	5	2.8 ± 0.2	7.5
	25	14.6 ± 1.0	6.7
Urine			
M2	50	26.5 ± 1.5	5.8
	200	94.4 ± 3.7	3.9
M3	30	30.4 ± 1.7	5.7
	100	97.7 ± 0.6	0.6
M4	30	26.6 ± 2.3	8.7
	100	92.6 ± 5.8	6.3

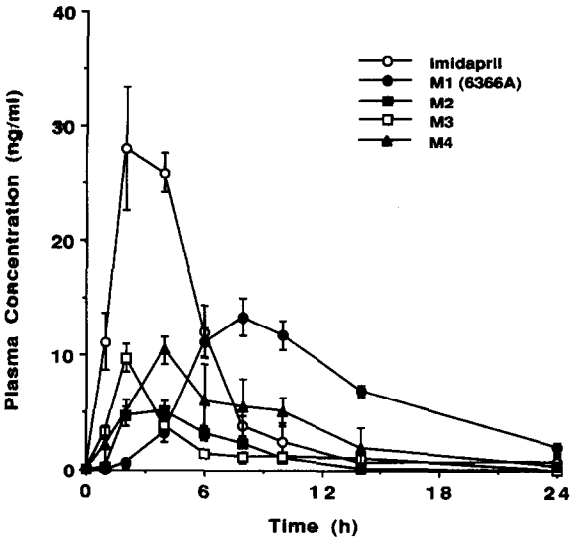

Fig. 8. Plasma concentration of imidapril, its active metabolite (M1) and three other metabolites (M2, M3 and M4) after oral administration of an imidapril tablet (10 mg) in four volunteers. Data are expressed as the mean values ± S.E.

Therefore, it was considered that the proposed method is suitable for use in pharmacokinetic and bioavailability studies in humans.

The detection limits of the PFB derivatives of M2, M3 and M4 were 1 ng/ml in plasma and 5 ng/ml in urine.

Applications

The described method was applied to the determination of M2, M3 and M4 in the plasma and urine of four healthy volunteers orally dosed with a 10-mg tablet of imidapril. The plasma concentrations of M2, M3 and M4 are shown in Fig. 8, in which the plasma concentrations of imidapril and its active metabolite M1 were measured by the HPLC method with ADAM reagent [7]. It was difficult to determine sensitively the PFB derivatives of imidapril and its metabolite M1 by the GC–MS method. The quantitative determination of imidapril and M1 has already been established using the HPLC method with ADAM, therefore we investigated the quantitative GC–MS method for three metabolites (M2, M3 and M4) which could not be determined by the HPLC method.

The urinary excretion rates of M2, M3 and M4 are shown in Table II. It is apparent that the metabolites are rapidly excreted into urine, since their level is low in plasma and comparatively high in urine.

It has been reported from animal studies using ^{14}C-labelled compounds after oral administration that the amount of urinary excretion is equal to the amount of faeces excretion [1]. Thus, it was considered that excretion of the dose other than urinary excretion was in faeces.

CONCLUSIONS

The GC–MS method described here permits the determination of the three metabolites in plasma and urine with a high specificity and sen-

TABLE II

CUMULATIVE URINARY EXCRETION OF IMIDAPRIL, ITS ACTIVE METABOLITE (M1) AND THREE METABOLITES (M2, M3 AND M4) AFTER ORAL ADMINISTRATION OF AN IMIDAPRIL TABLET (10 mg) IN FOUR VOLUNTEERS

Data are expressed as the mean values ± S.E.

Time (h)	Percentage of dose				
	Imidapril	M1 (6366A)	M2	M3	M4
0–4	4.8 ± 0.3	0.1 ± 0.0	5.3 ± 0.8	2.6 ± 0.6	2.4 ± 0.5
4–8	3.1 ± 0.2	1.7 ± 0.3	3.7 ± 0.5	0.8 ± 0.2	1.9 ± 0.6
8–24	0.6 ± 0.1	4.1 ± 0.4	2.3 ± 0.5	0.8 ± 0.3	2.4 ± 0.8
Total	8.5 ± 0.5	5.8 ± 0.5	11.2 ± 1.6	4.1 ± 0.8	6.7 ± 1.7

sitivity owing to direct purification with a solid-phase extraction cartridge and high-mass derivatization. The method is suitable for use in pharmacokinetic and bioavailability studies with the new ACE inhibitor imidapril.

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