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Highly sensitive determination of imidapril, a new angiotensin I-converting enzyme inhibitor, and its active metabolite in human plasma and urine using high-performance liquid chromatography with fluorescent labelling reagent

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ABSTRACT

A method for the simultaneous determination of imidapril and its active metabolite in human plasma and urine has been developed using high-performance liquid chromatography with a fluorescent labelling reagent, 9-anthryldiazomethane. Imidapril and its active metabolite were extracted from human plasma and urine using a solid-phase extraction cartridge (Bond Elut C_{18}). Two compounds in the eluate were derivatized with 9-anthryldiazomethane and purified with a solid-phase extraction cartridge (Bond Elut SI). The derivatives were analysed using high-performance liquid chromatography with fluorometry. The detection limits of imidapril and its active metabolite were 0.2 ng/ml in plasma and 10 ng/ml in urine. This method could be applied to the pharmacokinetic study of imidapril.

INTRODUCTION

Imidapril hydrochloride (TA-6366, I), (4*S*)-1-methyl-3-{(2*S*)-2-[N-((1*S*)-1-ethoxycarbonyl-3-phenylpropyl)amino]propionyl}-2-oxo-imidazolidine-4-carboxylic acid hydrochloride, is an orally applicable active angiotensin I-converting enzyme (ACE) inhibitor, which has been newly developed by Tanabe Seiyaku Co. [1]. This compound is known to bioconvert easily into an active metabolite (6366A, II) in the body by the hydrolysis of its ethyl ester group to show strong ACE inhibitory action [2].

Various pharmacokinetic studies in animals have been conducted using exclusively ^{14}C-labelled I for convenience of assay [3,4]. However, to begin pharmacokinetic studies in humans, it is necessary to establish a highly sensitive assay

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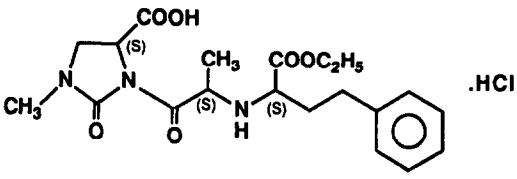
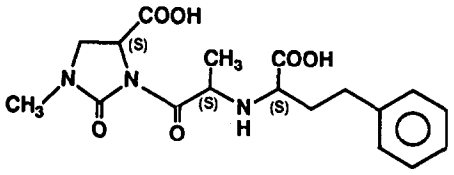


Imidapril (TA-6366)



Active metabolite (6366A)

Fig. 1. Structures of imidapril (TA-6366, I) and its active metabolite (6366A, II).

method for the determination of I and II in the plasma and urine samples, because the clinical dose of I is as low as 2.5–10 mg and hence the conventional high-performance liquid chromatographic (HPLC) technique with UV detection is thought to be not sensitive enough.

The structures of I and II are shown in Fig. 1. Because I and II have one and two carboxyl groups, respectively, we decided to apply the fluorescent-labelling technique using 9-anthryldiazomethane (ADAM), which has been used for the high-sensitive determination of fatty acids [5], prostaglandins [6] and biotin [7].

This paper describes the ADAM derivatization of I and II in biological fluids, and the simultaneous HPLC analysis of the derivatives.

EXPERIMENTAL

Materials

Compounds I and II, and the I analogue compound octyl-6366 (*i.e.* with an octyl group in place of the phenyl group of I) were synthesized in Tanabe Seiyaku. ADAM was synthesized according to Barker's method [8]. ADAM derivatives of I, II and myristic acid were synthesized according to Korte's method [9]. Solutions of ADAM and ADAM derivatives were prepared just before use. Analytical columns, Hypersil MOS-5 (250 mm × 4 mm I.D., particle size 5 μm) were purchased from Chemco (Osaka, Japan). Guard columns, LiChrosorb Si60 (4 mm × 4 mm I.D., particle size 5 μm) were purchased from E. Merck (Darmstadt, Germany). Disposable solid-phase extraction cartridges, Bond Elut C_{18} (100 mg) and Bond Elut SI (100 mg) were purchased from Varian (Harbor City, CA, USA). Bond Elut C_{18} cartridges were conditioned by washing with 3 ml of methanol, 3 ml of water and 1 ml of 0.1 *M* hydrochloric acid, and Bond Elut SI cartridges were conditioned by washing with 1 ml of acetone and 3 ml of *n*-hexane just before use. Other chemicals and solvents used were of reagent grade.

Apparatus

HPLC was carried out using a Shimadzu Model LC-6A liquid chromatograph pump equipped with a Toso Model AS-48 automated sample injector, a Shimadzu Model CTO-2A column oven, a Shimadzu Model RF-535 fluorometer and a Shimadzu Model C-R3A data processor. The measurements of fluorescent spectra were carried out using a Shimadzu Model RF-510 fluorescence spectrometer. A Savant Model SVC-200H Speed Vac concentrator was used to evaporate solvents in the extraction procedures (displacement, 200 l/min; ultimate vacuum, $1.0 \cdot 10^{-2}$ Pa; controlled temperature, 45°C).

Collection of blank plasma and urine

Blood samples were collected with heparinized syringes from healthy male volunteers who were not taking any drugs. Plasma samples were immediately obtained by centrifugation of the blood at 1800 *g* for 10 min. Urine samples were also collected from the volunteers. All samples were stored at −20°C until assay.

Extraction procedure from plasma

To 1 ml of plasma sample, 0.2 ml of 2 *M* hydrochloric acid, 0.2 ml of water, and 10 ml of acetone were added. After shaking for 10 min, the mixture was centrifuged at 1500 *g* for 10 min.

Supernatant (10 ml) was taken and evaporated to dryness. The residue was dissolved in 1 ml of 0.1 *M* hydrochloric acid, and then 10 ml of diethyl ether were added. The mixture was shaken for 5 min and centrifuged at 1500 *g* for 5 min, and the organic layer was discarded. The aqueous layer was loaded on a Bond Elut C_{18} cartridge. The cartridge was washed with 2 ml of 0.1 *M* hydrochloric acid and then with 1 ml of methanol–0.1 *M* hydrochloric acid (20:80). Compounds I and II were eluted from the cartridge with 1 ml of methanol–0.1 *M* hydrochloric acid (60:40). The eluate was evaporated to dryness.

Extraction procedure from urine

A 1-ml volume of urine was diluted to 25 ml with water after the addition of 0.2 ml of water, 0.2 ml of the internal standard (octyl-I), 15 ml of 0.5 *M* disodium hydrogenphosphate. Next, 10 ml of diethyl ether were added to 2 ml of the diluted sample, and the mixture was shaken for 5 min and centrifuged (1500 *g*, 5 min). After the organic layer had been discarded, 10 ml of chloroform were added to the aqueous layer. The mixture was shaken for 5 min and centrifuged (1500 *g*, 5 min). Then 1 ml of the aqueous layer was loaded on a Bond Elut C_{18} cartridge. The cartridge was washed with 2 ml of 0.1 *M* hydrochloric acid and 1 ml of methanol–0.1 *M* hydrochloric acid (20:80). Compounds I and II were eluted with 1 ml of methanol–0.1 *M* hydrochloric aicd (60:40). The eluate was evaporated to dryness.

ADAM derivatization

The residue of the eluate was dissolved in 0.2 ml of the same solvent used for the elution. The solution was transferred to a Reacti Vial (32 mm × 12 mm I.D., Pierce, Rockford, IL, USA), and evaporated to dryness. This dissolving procedure was duplicated to recover I and II completely. The residue was dissolved in 25 μl of water and 25 μl of acetone. After ultrasonication for 10 min, 50 μl of 0.2% (w/v) ADAM acetone solution was added to the vial. The mixture was incubated at 40°C for 60 min to esterify I and II with ADAM. The derivatization and subsequent procedures were performed in light-tight conditions to prevent degradation. The reaction mixture was transferred to another test-tube and evaporated to dryness. The residue was dissolved in 50 μl of acetone and 1 ml of *n*-hexane. Then the solution was loaded on a Bond Elut SI cartridge. Excess ADAM and any by-products were washed out with 1 ml of *n*-hexane–ethyl acetate (70:30), and then the ADAM derivatives of I and II were eluted with 1 ml of *n*-hexane–ethyl acetate (50:50). The eluate was evaporated to dryness.

For plasma samples, the residue was dissolved in 0.3 ml of acetonitrile–water (80:20) containing the ADAM derivative of myristic acid as an internal standard (20 ng/ml), and 200 μl of the final solution were injected into the chromatographic system.

For urine samples, the residue was dissolved in 0.25 ml of acetonitrile–water (80:20), and 25 μl of the final solution were injected into the chromatographic system.

About fifty samples can be analysed by one person in two days (day 1, extraction from plasma or urine; day 2, ADAM derivatization).

HPLC operating conditions

A Hypersil MOS-5 (250 mm × 4 mm I.D.; 5 μm particle size) and a LiChrosorb Si60 (4 mm × 4 mm I.D.; 5 μm particle size) were used as the analytical column and the guard column, respectively. The mobile phase was acetonitrile–0.02% (v/v) triethylamine aqueous solution (80:20), and the pH was adjusted to 3.0 with 10% phosphoric acid. The flow-rate of the mobile phase was 1.0 ml/min. The chromatography was carried out at 40°C. The fluorescence was measured at 412 nm, with excitation at 254 nm.

Pharmacokinetic studies in humans

Six healthy male volunteers gave their own written informed consent and participated in the study. After overnight fasting, each subject received a 5-mg tablet of I with 150 ml of water. Blood samples were collected at 0 (predose), 1, 2, 3, 4, 6, 8, 12, 24 and 48 h postdose. Each blood sample was taken into a heparinized tube and centrifuged to obtain the plasma. Urine samples were collected at intervals of 0–4, 4–8 and 8–24 h

postdose. The plasma and urine samples were stored frozen at −20°C until assay. This study was performed under the supervision of M.D. Y. Hirota, Osaka Medical College.

RESULTS AND DISCUSSION

Fluorescence spectra

ADAM is a compound with a highly reactive diazomethyl group in its molecule, which can quantitatively form anthryl derivatives with an intense fluorescence by coupling with a carboxyl group of organic compounds (Fig. 2). In preliminary studies, we established that the carboxyl groups of I and II would react with ADAM and form the corresponding fluorescent ADAM derivatives (I-ADAM and II-ADAM) even under mild conditions. Fig. 3 shows the fluorescence spectra of the authentic samples of both ADAM derivatives in acetonitrile–water (80:20). The observed fluorescence intensity of II-ADAM was almost double with that of I-ADAM, corresponding the number of carboxyl groups in the original compounds. No difference was found in the spectra between the two compounds, and the spectra obtained were similar to those of the ADAM derivatives of fatty acids reported by Nimura and Kinoshita [5]. Of the various combinations of excitation and fluorescence emission wavelength tested the highest fluorescence intensity was obtained at excitation 412 nm and emission 254 nm, as used for the highly sensitive detection in HPLC analysis by Kanazawa *et al.* [7]. The fluorescence properties were not dependent on the composition of the mobile phase.

Effect of trimethylamine concentration in mobile phase

Because anthryl derivatives generally have high lipophilicity, a Hypersil MOS column, a reversed-phase analytical column prepacked with dimethyloctyl-bonded silica gel, was chosen for the separation of weakly polar compounds in the HPLC analysis of I-ADAM and II-ADAM. A mobile phase to provide suitable retention times was then sought. Fig. 4 shows the effect of the triethylamine concentration and the pH in the mobile phase (acetonitrile–triethylamine aqueous solution) on retention time. The retention times of I-ADAM and II-ADAM became shorter when either the triethylamine concentration or the pH of the mobile phase was increased, whereas the retention time of the internal standard was unchanged. This may be due to the physical interaction between the silanol groups in the column and the primary amine group of I and II. This result suggested that the retention times of I-ADAM and II-ADAM could be finely adjusted and their peaks could be separated from interfering peaks by altering the concentration of triethylamine and the pH of the mobile phase. The most suitable composition of the mobile phase was determined by varying the pH, the triethylamine concentration and fraction of acetonitrile–water for HPLC analysis of both ADAM derivatives, namely eight parts of acetonitrile and two parts of 0.02% triethylamine aqueous solution, adjusted to pH 3 with phosphoric acid.

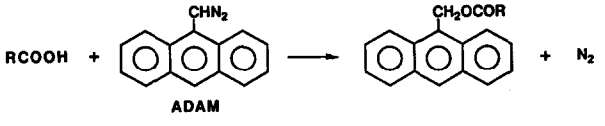


Fig. 2. Esterification of carboxyl group with 9-anthryldiazomethane (ADAM).

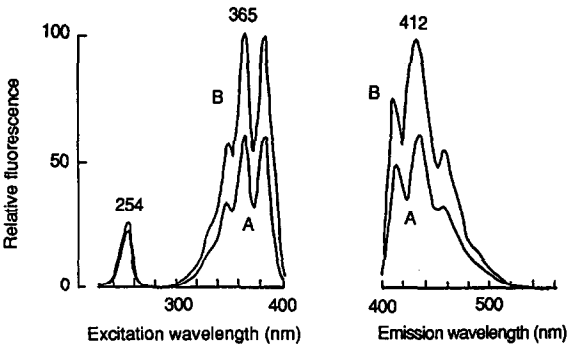


Fig. 3. Fluorescence spectra of I-ADAM (A) and II-ADAM (B), at a concentration of 10 μg/ml.

Stability of I-ADAM

Short-term stability tests were performed for I-ADAM to determine whether chemical degra-

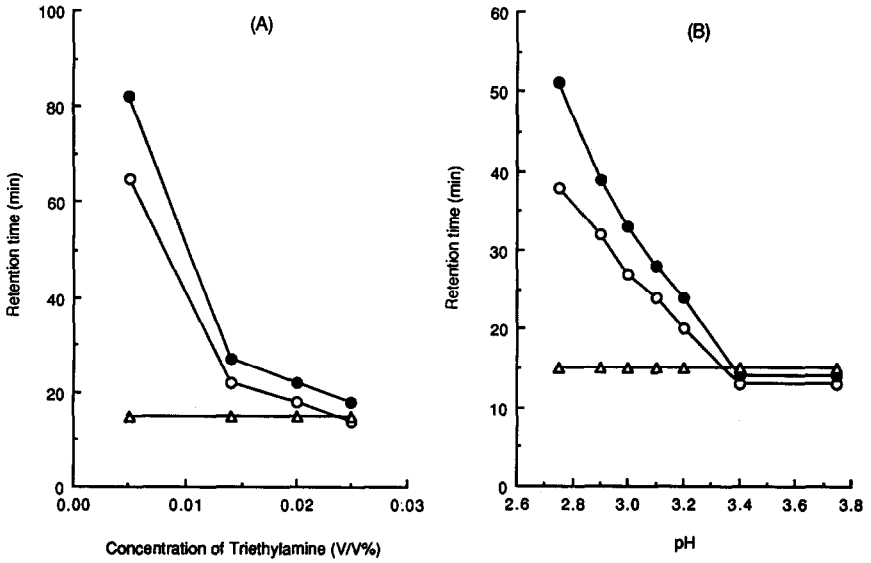


Fig. 4. Effect of concentration of triethylamine and mobile phase pH on the retention times of I-ADAM (○), II-ADAM (●) and myristate-ADAM (△). The mobile phase was acetonitrile–triethylamine aqueous solution (80:20). (A) Triethylamine concentration in the range 0.005–0.025% (v/v) at pH 3.00 adjusted with 10% phosphoric acid. (B) pH in the range 2.70–3.75 at the triethylamine concentration of 0.020% (v/v).

dation would occur during ADAM derivatization or the analytical processes. When I-ADAM in methanol was exposed to light of 1000 lux at 25°C, considerable degradation occurred. The degradation rate seemed to obey first-order kinetics, and the apparent degradation half-life was *ca.* 16 h. I-ADAM was stable in methanol–0.01 *M* phosphate buffer from pH 3 to pH 7 at 25°C under light-tight conditions. Therefore, all the procedures after ADAM derivatization should be carried out in the absence of light.

Purification of samples after ADAM derivatization

Because the ADAM reagent is highly reactive and usually contains some impurities, excess ADAM and any interfering substances generated by the ADAM reaction must be removed prior to HPLC analysis. When the acetonitrile solution containing I-ADAM, II-ADAM and ADAM reagent was directly injected, the accurate quantitative determination of each compound is impossible owing to severe interference by large impurity peaks (Fig. 5A). Thus, the solid-phase extraction technique using a Bond Elut SI cartridge was applied for the purification, and the operating conditions required to remove the interfering substances from the mixture were examined. As shown in Fig. 5B and C, most impurities were eluted out by passage through a Bond Elut SI cartridge and washing with a small amount of *n*-hexane–ethyl acetate (70:30). After removal of the impurities, I-ADAM and II-ADAM were quantitatively recovered from the cartridge by elution with *n*-hexane–ethyl acetate (50:50), as shown in Fig. 5D.

Optimization of ADAM derivatization

The solvent used for the ADAM reaction is likely to influence the yield of the ADAM derivatives. To select the most suitable solvent system, the ADAM reaction was carried out in seven organic solvents: *n*-hexane, chloroform, diethyl ether, ethyl acetate, acetone, methanol and acetonitrile; the yields obtained are compared in Table I. It was found that the reactivity varied greatly, and that the yield of II-ADAM was considerably lower than that of I-ADAM in every solvent. The

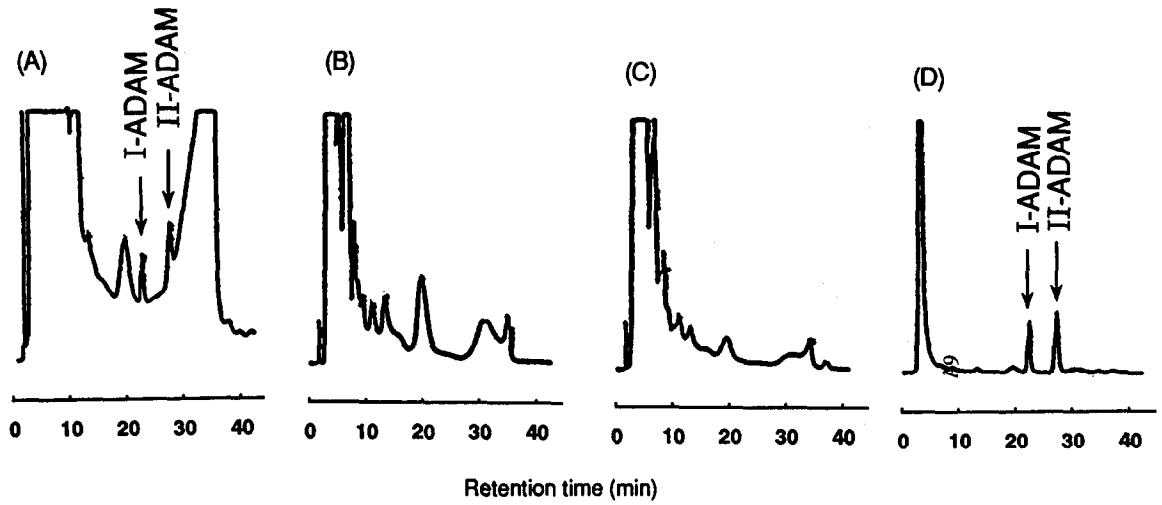


Fig. 5. Chromatograms after treatment by Bond Elut SI for the mixture of I-ADAM (5 ng/ml), II-ADAM (5 ng/ml) and ADAM reagent (0.2%, w/v, 50 μl). (A) No treatment by Bond Elut SI; (B) eluate passed through Bond Elut SI in *n*-hexane; (C) eluate with 1 ml of *n*-hexane–ethyl acetate (70:30) after (B); (D) eluate with 1 ml of *n*-hexane–ethyl acetate (50:50) after (C).

correlation between the dielectric permittivities of the solvents and the yields of I-ADAM suggested that the solvent with high permittivity tended to provide high yields (Fig. 6). The reaction was carried out in acetone–water with various compositions in order to raise the polarity, and good yields of both I-ADAM (91%) and II-ADAM (97%) were finally obtained when acetone–water (3:1) was used as the solvent.

To determine suitable conditions for ADAM derivatization, the effects of the concentration of ADAM reagent, reaction temperature and time were also investigated (Fig. 7). Based on all the results obtained in these studies, the ADAM derivatization was finally performed under the following conditions: 0.1% (w/v) as the ADAM concentration, 40°C as the reaction temperature, and 60 min as the reaction time.

TABLE I

EFFECT OF REACTION SOLVENT ON THE FORMATION OF ADAM DERIVATIVES OF I AND II

Solvent	Yield (mean ± S.D., n = 3) (%)	
	I	II
n-Hexane	25 ± 2	2 ± 2
Chloroform	46 ± 5	7 ± 3
Diethyl ether	9 ± 2	2 ± 1
Ethyl acetate	52 ± 8	8 ± 3
Acetone	77 ± 8	7 ± 3
Methanol	61 ± 11	34 ± 5
Acetonitrile	61 ± 12	12 ± 3

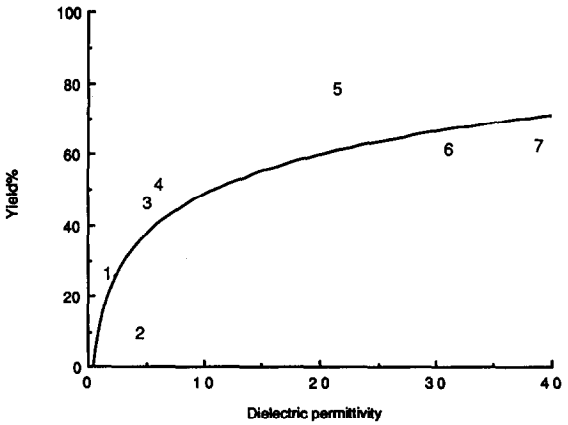


Fig. 6. Correlation between dielectric permittivity of solvent and formation of I-ADAM. 1, *n*-hexane; 2, diethyl ether; 3, chloroform; 4, ethyl acetate; 5, acetone; 6, methanol; 7, acetonitrile.

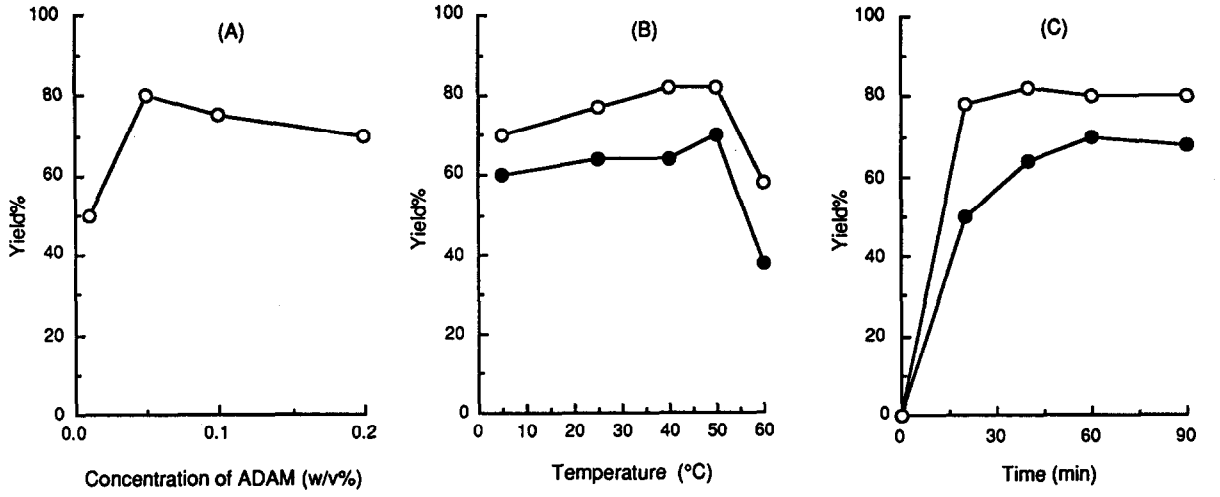


Fig. 7. Effect of ADAM concentration (A), reaction temperature (B) and reaction time (C) on I-ADAM (○), II-ADAM (●). Conditions: (A) for 60 min at 40°C; (B) 0.2% (w/v) of ADAM for 60 min; (C) 0.2% (w/v) of ADAM at 40°C.

Chromatography

Typical chromatograms obtained by the proposed analytical method for the human plasma and urine samples are shown in Fig. 8. The retention times of I-ADAM, II-ADAM and the internal standard for plasma samples were 23, 26 and 17 min, respectively, and those for urine samples were 20, 22 and 42 min, respectively. Each ADAM derivative and internal standard was detected as a single symmetric peak. The clean-up

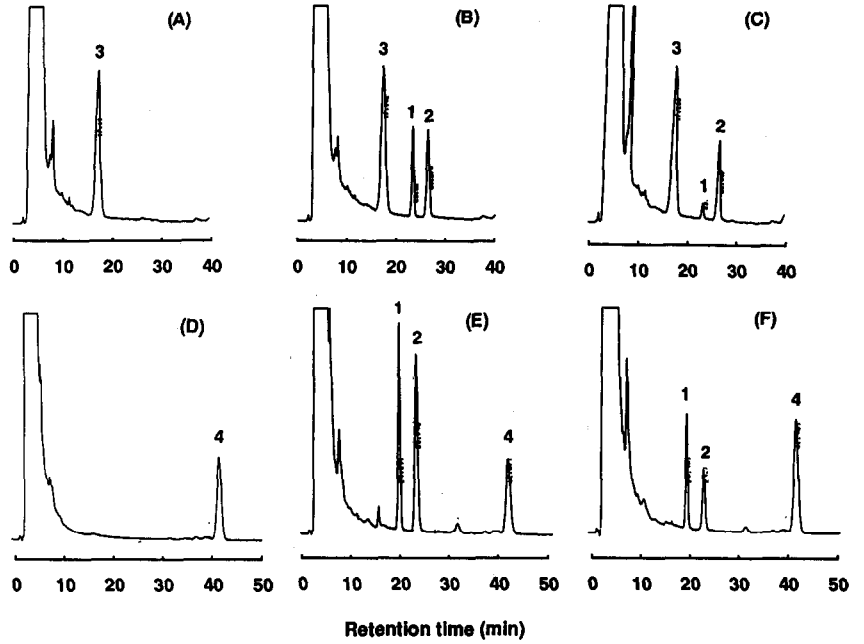


Fig. 8. Typical chromatograms of plasma and urine samples. (A) Blank plasma; (B) plasma spiked with I and II (10 ng/ml); (C) plasma after oral administration of I; (D) blank urine; (E) urine spiked with I and II (2.5 μg/ml); (F) urine after oral administration of I. Peaks: 1 = I-ADAM; 2 = II-ADAM; 3 = internal standard for plasma; 4 = internal standard for urine.

TABLE II

LINEARITY OF STANDARD CURVES FOR I AND II IN HUMAN PLASMA AND URINE

Compound	Concentration added (ng/ml)	Regression line (mean ± S.D., n = 3)		
		Slope	Intercept	Correlation
Plasma				
I	0.5–50	0.0275 ± 0.0021	0.0100 ± 0.0094	0.999 ± 0.000
II	0.5–50	0.0422 ± 0.0002	0.0136 ± 0.0099	0.999 ± 0.000
Urine				
I	10–20 000	0.618 ± 0.036	0.052 ± 0.005	0.999 ± 0.000
II	10–20 000	0.942 ± 0.021	0.071 ± 0.004	0.999 ± 0.000

procedure and the selective fluorescence monitoring effectively eliminated interfering peaks.

Linearity and detection limit

Compounds I and II were spiked to 1 ml of blank plasma at concentrations ranging from 0.5 to 50 ng/ml, and to 1 ml of blank urine in the range 10–20 000 ng/ml, and then each was assayed according to the procedure described. The calibration graphs obtained by plotting the peak-area ratio *versus* concentration were linear for plasma and urine in the respective concentration ranges. Table II shows the regression lines and the coefficients of correlation. The detection limits of I and II (at a signal-to-noise ratio of 3) were 0.2 ng/ml in plasma and 10 ng/ml in urine.

Reproducibility and accuracy

The within-day variation was evaluated by replicate analyses for plasma samples at concentrations of 2 and 10 ng/ml and for urine samples at 500 ng/ml. The coefficient of variation (C.V.) was less than 10% in every analysis for both plasma and urine (Table III). These results prove that

TABLE III

WITHIN-DAY REPRODUCIBILITY AND ACCURACY OF MEASUREMENTS OF I AND II IN HUMAN PLASMA AND URINE

Compound	Concentration added (ng/ml)	Mean concentration found (n = 5) (ng/ml)	Coefficient of variation (%)
Plasma			
I	2	2.2	7.9
	10	11.5	7.7
II	2	2.1	4.7
	10	10.1	7.3
Urine			
I	500	453	1.6
II	500	459	10.3

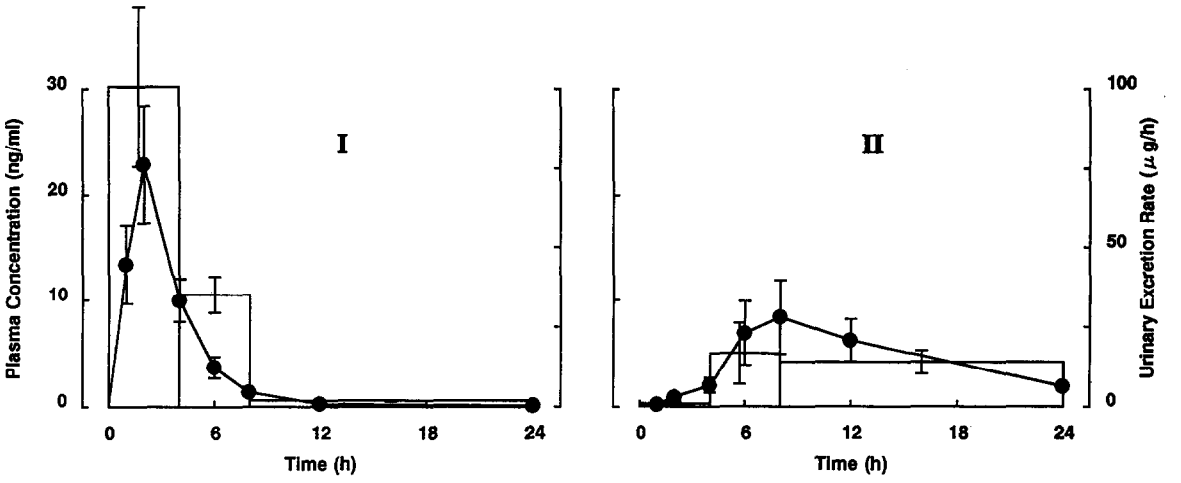


Fig. 9. Concentration–time curves for plasma (●) and urine (□) excretion of I and II after oral administration of 5 mg of I to six healthy volunteers. Each point represents the mean ± standard error.

this assay method is sensitive, reproducible and accurate enough for the determinations of the plasma concentration and the urinary excretion of I and II in humans.

Application to biological samples

The proposed method was applied to the determination of I and II in plasma and urine samples for the evaluation of the pharmacokinetics of I in humans. Plasma and urine samples were periodically collected up to 24 h after oral administration of one tablet containing 5 mg of I to six healthy male volunteers after an overnight fast. Fig. 9 shows the mean plasma concentration and the urinary excretion rate *versus* time profiles of I and II. The plasma level of I reached a maximum 2 h after the administration, and thereafter the plasma level declined with an elimination half-life of *ca.* 2 h. On the other hand, II gradually appeared after the administration of I and was long lasting in plasma. The urinary excretion rates of I and II showed similar profiles to the corresponding plasma concentration curves.

CONCLUSIONS

Through the present study, we have established a highly sensitive assay method for imidapril (I) and its active metabolite (II) in human plasma and urine by HPLC with fluorescent labelling. The linearity, reproducibility and accuracy have been proved. The proposed method is useful for the evaluation of the pharmacokinetics of imidapril in humans.

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