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Radioimmunoassay for imidapril, a new angiotensin-converting enzyme inhibitor, and imidaprilat, its active metabolite, in human plasma and urine

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Abstract

A radioimmunoassay (RIA) was investigated for the determination of imidapril and its active metabolite, imidaprilat, in human plasma and urine. Imidapril is a new angiotensin-converting enzyme inhibitor and an oral prodrug of imidaprilat. Imidapril was determined after conversion to imidaprilat with esterase. Antiserum was raised in rabbits against the *p*-amino derivative of imidaprilat conjugated to bovine serum albumin. Radioligand was prepared by iodination (^{125}I) of the *p*-hydroxybenzoylamino derivative of imidaprilat. Cross-reactivities of anti-imidaprilat antiserum for imidapril, its metabolites and several cardiovascular drugs were low. The calibration range was 0.1–100 ng ml^{-1} using a 100 μl of human plasma of urine. Intra- and inter-day variations of imidaprilat assay in plasma were 2.0–7.9 and 4.1–6.2%, respectively, and intra- and inter-day variations of imidapril assay in plasma were 5.4–10.7 and 7.9–18.1%, respectively. The variations of the assay in urine were a little smaller than those in plasma. The recovery of imidaprilat and imidapril spiked in plasma or urine samples was approximately 100%. A good correlation between RIA and high-performance liquid chromatograpy was observed for both plasma and urine samples. Furthermore, this method was applied to the determination of imidaprilat and imidapril in human plasma and urine samples, for the evaluation of the pharmacokinetics of imidapril in humans. From the results, it was demonstrated that the developed RIA was useful for the determination of imidaprilat and imidapril in human plasma and urine, and was applicable to pharmacokinetic studies in humans.

Keywords: Radioimmunoassay; Angiotensin-converting enzyme inhibitor; Imidapril; Imidaprilat; Pharmacokinetics; Humans

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1. Introduction

Imidapril hydrochloride, (−)-(4*S*)-3-[(2*S*)-2-[[(1*S*)-1-ethoxycarbonyl-3-phenylpropyl]amino]-propionyl]-1-methyl-2-oxoimidazolidine-4-carboxylic acid hydrochloride, is a new angiotensin-converting enzyme (ACE) inhibitor developed by Tanabe Seiyaku [1]. It is an oral prodrug and is converted into the de-esterified active metabolite, imidaprilat, (4*S*)-3-[(2*S*)-*N*-[(1*S*)-1-carboxy-3-phenylpropyl]alanyl]-1-methyl-2-oxo-4-imidazolidine carboxylic acid, in vivo. Imidapril has antihypertensive effects by oral administration once a day [2] and is reported to be less likely to produce a dry cough as a side-effect [3–5]. Therefore, it is considered to be a desirable antihypertensive drug.

A high-performance liquid chromatographic (HPLC) method [6] and a negative-ion desorption chemical ionization–tandem mass spectrometric (DCI/MS/MS) method [7] have been used for the determination of imidaprilat and imidapril in human plasma and urine. However, these methods are very complicated and time consuming. Therefore, they are not practical for the rapid analysis of large numbers of biological samples. The aim of the present study was the development of a sensitive radioimmunoassay (RIA) for imidaprilat and imidapril in human plasma and urine, and its application to pharmacokinetic studies in humans.

2. Experimental

2.1. Materials and reagents

Imidapril hydrochloride, its active metabolite, imidaprilat, its main metabolites M2((4*S*)-1-methyl-2-oxo-4-imidazolidine carboxylic acid), M3-((2*S*) - *N* - [(1*S*) - 1 - carboxy - 3 - phenylproply]alanine), 2-ethyl ester) and M4((2*S*)-*N*-[(1*S*)-1-carboxy-3-phenylpropyl]alanine) (Fig. 1), and several cardiovascular drugs, such as captopril, enalapril, enalaprilat, diltiazem and glibenclamide, were synthesized at Tanabe Seiyaku. Hydrochlorothiazide, furosemide, nifedipine, atenolol, digoxin, warfarin, cimetidine, rifampicin and diclofenac were purchased from Sigma (St. Lous, MO, USA), acenocoumarol from Ciba-Geigy (Basle, Switzerland), 1,5-difluoro-2,4-dinitrobenzene (DFDNB) from Fluka (Buchs, Switzerland), bovine serum albumin (BSA) and esterase (from porcine liver) from Sigma, Freund Complete Adjuvant from Difco Laboratories (Detroit, MI, USA), chloramine-T from Wako Pure Chemical (Osaka, Japan) and sodium [^{125}I]iodide from Amersham (Buckinghamshire, UK). Anti-rabbit IgG goat IgG (secondary antibody) was obtained from Eiken Chemical (Tokyo, Japan). All solvents and reagents were of special grade.

2.2. Preparation of immunogen

Imidaprilat is a pharmacologically active com-

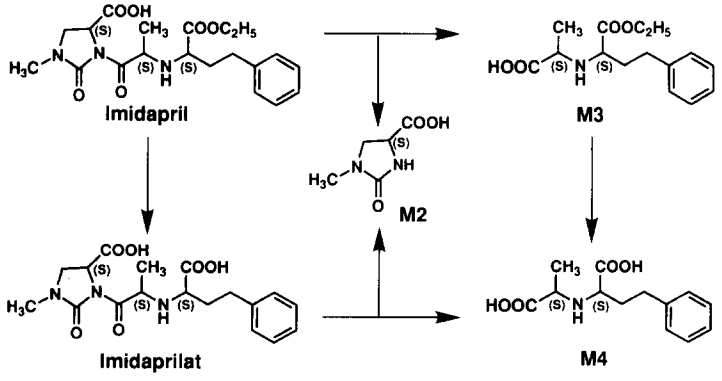

Fig. 1. Main metabolic pathway of imidapril in humans.

pound and imidapril is determined after conversion to imidaprilat with esterase. Therefore, anti-imidaprilat antiserum was prepared for determination of imidaprilat. Imidaprilat is not immunogenic and has no function site; thus the *p*-amino derivative of imidaprilat was used as a hapten.

As shown in Fig. 2, immunogen was prepared by coupling hapten to BSA using DFDNB. For this, 150 mg of DFDNB in 5 ml of methanol was added to solution of 15.6 mg of hapten in 1 ml of 0.1 M phosphate buffer (pH 7.2). After reaction for 20 min, 2.5 ml of 0.1 M phosphate buffer (pH 7.2) was added to the mixture. It was washed three times with 20 ml of diethyl ether and the residual diethyl ether in the water layer was evaporated. A 250 mg amount of BSA in 4 ml of 0.1 M borate buffer (pH 10) was added to the water layer. After stirring for 24 h at room temperature in the dark, the reaction mixture was dialysed against 5 l of water for 12 h. The dialysis was repeated four times. The dialysed solution was stored in a deep freezer (−80°C). From the ultraviolet absorbance at 350 nm, the molar ratio of hapten molecules to BSA was calculated to be 12.9.

2.3. Immunization

The immunogen solution was emulsified with twice its volume of Freund Complete Adjuvant. Three rabbits (Japanese White Rabbit) were injected subcutaneously at six different sites on the back with 80 μl of emulsion. Booster injections were given at 2 week intervals for 44 weeks. At 1 week intervals after each injection, blood samples were taken from the marginal ear vein for determination of the antibody titre. After the final injection, the rabbits were bled by heart puncture under anaesthesia. The serum was stored in small aliquots at −80°C. Each serum sample was diluted (1:800) and its B/T value was measured. The serum taken 44 weeks after immunization was used as an antiserum, since the B/T value of the serum was about 50%.

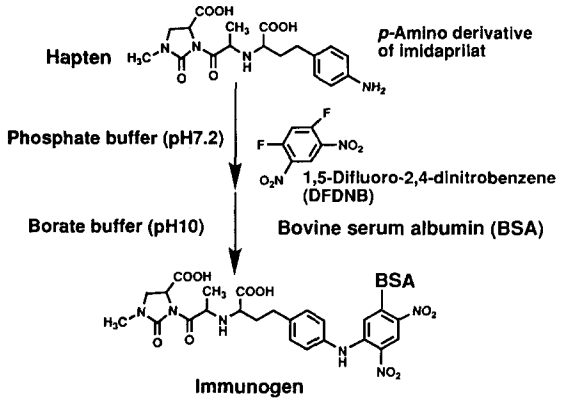

Fig. 2. Synthesis of immunogen.

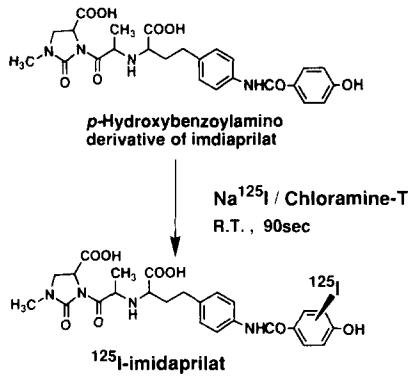

Fig. 3. Synthesis of radioligand.

2.4. Preparation of radioligand

Radioligand was prepared from the *p*-hydroxybenzoylamino derivative of imidaprilat by radioiodination via the chloramine-T reaction [8] as shown in Fig. 3. A 40 μl volume of a 4 MBq μl^{-1} solution of sodium [^{125}I]iodide dissolved in 1 mM sodium hydroxide was added to a solution of 10 μl of a 0.1 mg ml^{-1} solution of *p*-hydroxybenzoylamino derivative dissolved in 0.1 M phosphate buffer (pH 7.2) and was followed by 20 μl of a 1.0 mg ml^{-1} solution of chloramine-T dissolved in 0.5 M phosphate buffer (pH 7.5). After 90 s at room temperature, the reaction was stopped by addition of 50 μl of a 2.5 mg ml^{-1} solution of sodium thiosulphate dissolved in 0.5 M phosphate buffer (pH 7.5). A 500 μl volume of 0.1 M Tris–HCl buffer was added to the solution. The reaction mixture was purified by reversed-phase HPLC using a column (4.6 mm

i.d. × 250 mm) packed with TSK gel ODS-120T (5 μm) (Tosoh, Tokyo, Japan). The mobile phase consisted of mixture of 0.05 M Tris–HCl buffer (pH 7.2) and methanol. First, the mobile phase contained 0% methanol for 10 min, with a subsequent gradient to 100% methanol after 60 min, maintaining 100% methanol for 10 min. The flow rate was 0.7 ml min^{-1} and the column was operated at room temperature. The eluate was collected every 1 min and the radioactivity of each fraction was counted by a gamma scintillation counter (Cobra; Packard, Meriden, CT, USA).

2.5. Assay procedure

In the determination of imidaprilat in plasma, 100 μl of acetate buffer (pH 5.0), 200 μl of antiserum and 200 μl of radioligand solution (total counts ca. 30 000 cpm per 200 μl) were added to 100 μl of sample solution in a tube. After mixing on a vortex mixer, the tubes were incubated for 16–20 h at 4°C. A 200 μl volume of secondary antibody was added to the tubes. After mixing on a vortex mixer, the tubes were incubated for 60 min at room temperature. After centrifugation (2000*g* for 30 min at 4°C), the supernatant was removed by aspiration. The radioactivity of the pellets was counted for 3 min using a gamma scintillation counter. Concentrations of samples were calculated using the standard curve, which was fitted to a four-parameter logistic function using an automated data processing program [9]. Imidapril was determined after conversion to imidaprilat with esterase. That is, 100 μl of esterase solution (64 U per 100 μl) was added to 100 μl of sample solution in a tube and the mixture was incubated for 3 h at 37°C. The imidapril concentration was calculated by subtracting the amount of imidaprilat after and before enzymatic hydrolysis. Imidaprilat and imidapril in urine were determined according to the same assay procedure as for plasma.

2.6. HPLC method

Imidaprilat and imidapril in plasma and urine were also determined according to the HPLC method [6].

2.7. Human study

A tablet of imidapril (10 mg) was administered orally to six healthy male volunteers. Blood samples were taken at 0, 1, 2, 4, 6, 8, 10, 14 and 24 h. Urine samples were collected for 0–4, 4–8 and 8–24 h. Plasma and urine samples were frozen at −20°C until used.

2.8. Pharmacokinetic analysis

The maximum plasma concentration (C_{max}) and the time to reach C_{max}(T_{max}) were determined from the observed plasma concentrations of imidapril and imdaprilat. The area under the plasma concentration–time curve (AUC) was calculated by the trapezoidal rule up to 24 h (AUC_{24}). The elimination half-life ($t_{1/2}$) was calculated by log–linear regression for the declining plasma concentrations against time after administration.

3. Results and discussion

3.1. Purification of [^{125}I]imidaprilat

By the chloramine-T method [8], the *p*-hydroxybenzoylamino derivative of imidaprilat was labelled with ^{125}I and the radioligand was purified by the HPLC method. As shown in Fig. 4, three radioactive peaks (F5, F42 and F47) were observed, and two of the peaks, F42 and F47,

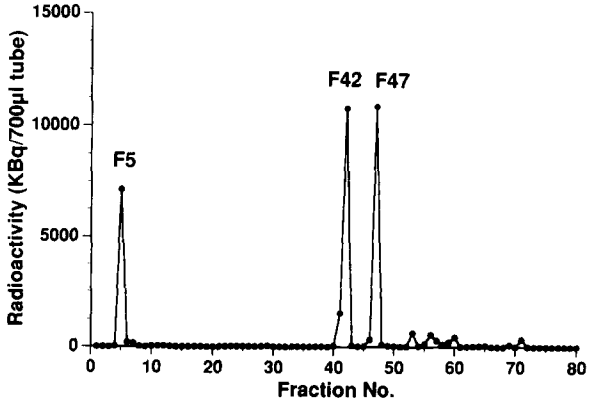

Fig. 4. Chromatographic separation of radioligand.

Table 1
Cross-reactivity of anti-imidaprilat antiserum

Type	Compound	Cross-reactivity (%)
Imidapril and its metabolites	Imidaprilat	100
	Imidapril	0.45
	M2	<0.001
	M3	<0.001
	M4	<0.001
ACE inhibitors	Captopril	<0.001
	Enalapril	<0.001
	Enalaprilat	<0.001
Cardiovascular drugs	Hydrochlorothiazide	<0.001
	Furosemide	<0.001
	Diltiazem	<0.001
	Nifedipine	<0.001
	Atenolol	<0.001
	Digoxin	<0.001
	Warfarin	<0.001
	Acenocoumarol	<0.001
Joint-use drugs	Cimetidine	<0.001
	Rifampicin	<0.001
	Glibenclamide	<0.001
	Diclofenac	<0.001

reacted with antiserum. F47 was adopted as a radioligand, since the B_0/T value of F47 was higher than that of F42.

3.2. Specificity of the antiserum

Cross-reactivity of the antiserum was examined for imidapril, its metabolites (M2, M3 and M4), ACE inhibitors, antihypertensive drugs, cardiovascular drugs and joint-use drugs. It was defined as the ratio (%) of the concentration of imidaprilat to that of the related compounds which is necessary to cause 50% displacement of the radioligand. As shown in Table 1, the cross-reactivity for imidapril was only 0.45%. The cross-reactivity was below 0.001% for other compounds. Also, the cross-reactivity was low for isomers of imidaprilat and imidapril (data not shown). Therefore, it was concluded that the anti-imidaprilat antiserum was specific for imidaprilat.

3.3. Standard curves

Plasma concentrations of imidaprilat and imidapril were at the ng ml^{-1} level following oral administration of imidapril (dose 5 and 10 mg) to humans, and therefore a range of 0.1–100 ng ml^{-1} for the standard curve was considered to be suitable for the determination of imidaprilat and imidapril in the samples. Fig. 5 shows a typical standard curve for [^{125}I]imidaprilat binding to anti-imidaprilat antiserum by adding imidaprilat (0.1–100 ng ml^{-1}) in the presence of 100 μl of human plasma.

The inter-assay variations of the standard curves for imidaprilat in plasma were 0.7–5.0% as relative standard deviation (RSD) and the accuracy of the standard curve was very high (data not shown). Also, inter-assay variations and accuracy for urine were as same as those for plasma.

3.4. Precision and recovery

Table 2 shows the intra- and inter-assay variations of RIA for imidaprilat and imidapril using plasma spiked with different levels of imidaprilat and imidapril. The intra-assay variation was determined from the data obtained for six assays performed in a single day, whereas the inter-assay variation was calculated form data for six assays obtained on six different days. The RSD values of the intra- and inter-assays for imidaprilat in plasma were 2.0–7.9 and 4.1–6.2%, respectively, and those for imidapril were 5.4–10.7 and 7.9–18.1%, respectively. The RSD values in urine samples were slightly smaller than those in plasma samples (data not shown). The recoveries of imidaprilat and imidapril were 101.1–121.7 and

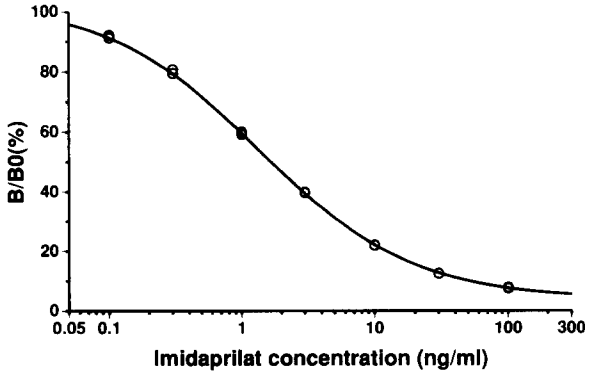

Fig. 5. Typical standard cuve for imidaprilat in plasma obtained by RIA.

Table 2
Precision and recovery of imidapril and imidaprilat in plasma

Parameter	Concentration (ng ml^{-1})					
	Imidaprilat			Imidapril		
	0.1	1	10	0.1	1	10
Intra-assay ($n = 6$)						
Mean (ng ml^{-1})	0.11	1.11	10.11	0.08	1.10	10.38
RSD (%)	7.85	2.38	2.00	10.73	5.35	7.55
Recovery (%)	114.0	110.8	101.1	78.0	109.5	103.8
Inter-assay ($n = 6$)						
Mean (ng ml^{-1})	0.12	1.04	10.19	0.12	0.96	9.21
RSD (%)	6.19	4.14	6.06	18.10	10.01	7.94
Recovery (%)	121.7	104.2	101.9	122.5	96.2	92.1

78.0–122.5%, respectively, for plasma, and 97.5–119.3 and 86.8–107.1%, respectively, for urine.

3.5. Comparison of RIA and HPLC

After oral administration of 10 mg of imidapril to healthy male volunteers, 48 plasma and 11 urine samples were analysed by RIA and HPLC. As shown in Fig. 6, the correlation coefficients between the RIA and HPLC methods were 0.975 and 0.991 for imidaprilat and imidapril, respectively, in plasma. The correlation coefficients were 0.996 and 0.979 for imidaprilat and imidapril, respectively, in urine.

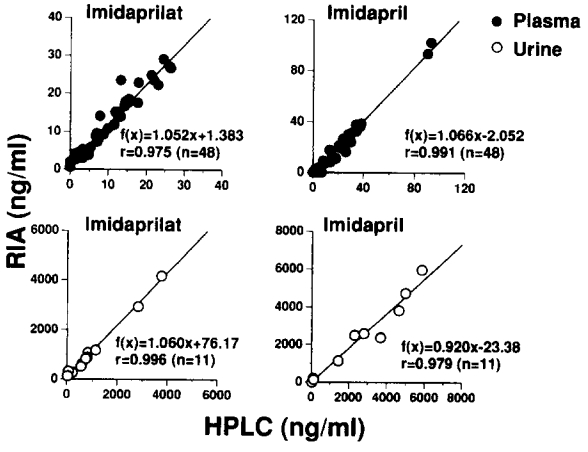

Fig. 6. Correlaton between RIA and HPLC.

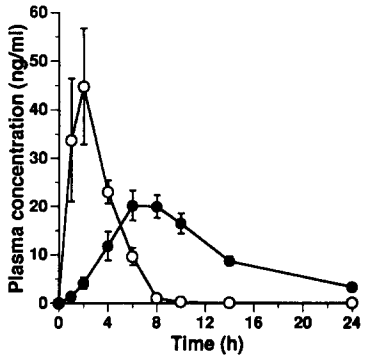

Fig. 7. Mean plasma concentrations of (○) imidapril and (●) imidaprilat in six volunteers after a single oral administration of imidapril at dose of 10 mg (mean ± SE).

3.6. Application to pharmacokinetics of imidapril in humans

The RIA method was applied to the determination of imidapril and imidaprilat in human plasma and urine samples for the evaluation of the pharmacokinetics in humans. Plasma and urine concentrations of imidapril and imidaprilat were determined up to 24 h after oral administration of one tablet containing 10 mg of imidapril to six healthy male volunteers.

Fig. 7 shows the mean plasma concentrations of imidapril and imidaprilat. Table 3 gives pharmacokinetic parameters for imidapril and imidaprilat. The plasma concentration of imidapril reached a maximum 2.2 h after administration, and thereafter decreased with an elimination half-life of 1.4 h. On the other hand, imidaprilat appeared gradually in plasma, and the plasma concentration reached a maximum 7.0 h after the administration. Also, the plasma concentration of imidaprilat showed a prolonged duration. The ratio of C_{max} for imidaprilat to imidapril was 0.47 and the ratio of AUC_{24} was 1.41.

Fig. 8 shows the urinary excretion of imidapril and imidaprilat. The urinary excretion rates of imidapril and imidaprilat showed similar profiles to the plasma concentration curves, and the urinary excretion levels of imidapril and imidaprilat were 7.8 and 8.8%, respectively.

In conclusion, it was found that RIA is useful for the determination of imidaprilat and imidapril in human plasma and urine, and that it is applicable to pharmacokinetic studies in humans.

Table 3
Pharmacokinetic parameters in humans for imidapril and imidaprilat

Compound	C_{max}(ng ml^{-1})	T_{max}(h)	AUC_{24}(ng h ml^{-1})	$t_{1/2}$(h)
Imidapril	45.2 ± 11.7	2.2 ± 0.4	169.9 ± 36.9	1.4 ± 0.2
Imidaprilat	21.1 ± 2.8	7.0 ± 0.4	239.1 ± 30.9	6.4 ± 0.3

Each value represents the mean ± SE for six subjects.

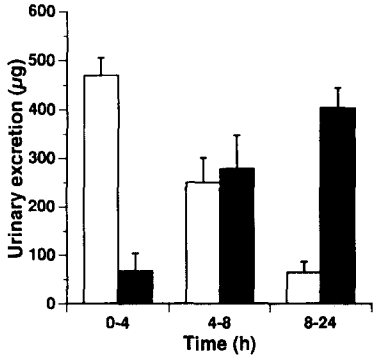

Fig. 8. Urinary excretion of (□) imidapril and (■) imidaprilat in four healthy volunteers after a single oral administration of imidapril at dose of 10 mg (mean ± SE).

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