Journal of Chromatography, 568 (1991) 309–314
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5921

Determination in plasma of angiotensin-converting enzyme inhibitor by inhibitor-binding assay

HIROAKI SHIOYA*, MASAKO SHIMOJO and YUKINORI KAWAHARA

Product Development Laboratories, Sankyo Co., Ltd., 2-58, Hiromachi 1-chome, Shinagawa-ku, Tokyo 140 (Japan)

(First received July 31st, 1990; revised manuscript received March 29th, 1991)

ABSTRACT

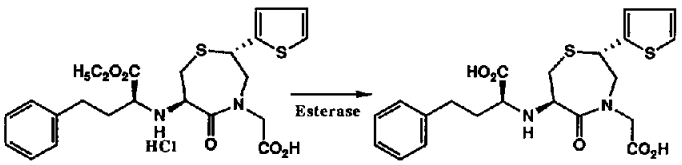
A method of determining a new angiotensin-converting enzyme inhibitor (CS-622) and its active metabolite (RS-5139) in plasma by inhibitor-binding assay has been developed using high-performance liquid chromatography. The assay is based on the principle that the amount of inhibitor bound to the enzyme is inversely related to the amount of hippuric acid liberated on hydrolysis from the artificial substrate (hippuryl-L-hystidyl-L-leucine). Plasma was heated at 60°C for 15 min, to inactivate endogenous enzyme, and preincubated with rabbit-lung angiotensin-converting enzyme at 37°C for 3 min. The artificial substrate (5.75 mg/ml in pH 8.3 phosphate buffer containing sodium chloride) was added to the resulting solution, and the mixture was incubated for 30 min. The reaction was terminated by the addition of 2 *M* hydrochloric acid. The hippuric acid liberated on hydrolysis was extracted with ethyl acetate and determined by reversed-phase chromatography using methylparaben as an internal standard. The total concentration of the inhibitor and its metabolite were determined by this method after de-esterification by rat-plasma esterase. The standard curve was obtained by the regression analysis of log concentration against logit response. The within-day and day-to-day precision were satisfactory. The proposed method is simple, rapid and sensitive enough to determine angiotensin-converting enzyme inhibitor in plasma.

INTRODUCTION

Angiotensin-converting enzyme (ACE, EC 3.4.15.1), also known as kininase II, catalyses the cleavage of the C-terminal dipeptide L-His-L-Leu of the decapeptide angiotensin I, generating the biologically highly active vasopressor angiotensin II.

In recent years, ACE inhibitors have offered a new approach to the treatment of hypertension. CS-622 (I, (+)-{(2*S*,6*R*)-6-[(1*S*)-1-ethoxycarbonyl-3-phenylpropyl]amino-5-oxo-2-(2-thienyl)perhydro-1,4-thiazepin-4-yl}acetic acid hydrochloride) is a new ACE inhibitor, which is a prodrug to enhance oral absorption, and it is deesterified *in vivo* by esterase to release an active metabolite, RS-5139 (II, Fig. 1). *In vitro*, the metabolite is a strong inhibor of ACE, whereas I has virtually no inhibitory effect on ACE at concentrations present in plasma. It is very important to determine the concentration of I and II in plasma, in order to establish their pharmacokinetics and pharmacodynamics. Analytical methods

0378-4347/91/$03.50



I II

Fig. 1. Structures of CS-622 (I) and its active metabolite RS-5139 (II).

used for their measurement include inhibitor-binding assay (IBA) [1], radioimmunoassay [2], and liquid or gas chromatography (GC) [3,4]. In previous paper [5], we reported a new method using gas chromatography–mass spectrometry (GC–MS), but this is not suitable for routine analysis in clinical investigations. Thus we attempted to develop an IBA involving high-performance liquid chromatography (HPLC). In this paper, a simple, rapid and sensitive method for the determination of an ACE inhibitor in plasma is presented.

EXPERIMENTAL

Chemicals

Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) was purchased from Protein Research Foundation (Osaka, Japan). Rabbit-lung angiotensin-converting enzyme was purchased from Sigma (St. Louis, MO, USA). All reagents were of analytical grade and used without further purification.

High-performance liquid chromatography

All enzymic reaction products were separated and quantified by an HPLC system consisting of a Model SIL-6A autosampler (Shimadzu, Kyoto, Japan) and a Model LC-6A liquid chromatograph (Shimadzu) connected to a Model SPD-6A UV spectrophotometric detector (Shimadzu). Chromatograms were recorded on a Model C-R3A integrator (Shimadzu). The column effluent was monitored at 228 nm with a sensitivity of 0.08 a.u.f.s. The column used for all separations was a TSKgel ODS-80^{TM} (150 mm × 4.6 mm I.D.; 5 μm particle size; Tosoh, Tokyo, Japan). The mobile phase was acetonitrile–water–acetic acid (30:70:1, v/v). The flow-rate was 1.0 ml/min at ambient temperature.

Plasma samples

All blood samples were drawn from peripheral veins into heparinized blood-collecting tubes. The plasma was collected following centrifugation of the samples, and stored at −20°C until analysis.

Standard assay procedure

A 100-μl aliquot of sample plasma was mixed with 100 μl of 1/15 *M* phosphate buffer (pH 7.4) and heated for 15 min at 60°C, to inactivate the endogenous ACE, and then it was preincubated with rabbit-lung ACE at 37°C for 3 min. A 100-μl volume of substrate (Hip-His-Leu, 5.75 mg/ml in pH 8.3 phosphate buffer containing 500 m*M* sodium chloride) was added to the resulting solution, and the mixture was incubated for 30 min. The reaction was terminated by the addition of 100 μl of 2 *M* HCl, and 100 μl of methylparaben in ethyl acetate were added as an internal standard. For the extraction step, 1.5 ml of ethyl acetate was added, followed by shaking for 5 min and centrifugation at 5000 *g* for 5 min. Then 1 ml of the ethyl acetate layer was transferred to another vessel and evaporated to dryness. The residue was redissolved in 200 μl of mobile phase, and 10 μl of this solution were injected into the HPLC system.

For the assay of I in plasma, a 100-μl aliquot of sample plasma was incubated with rat plasma (100 μl) for 30 min at 37°C to de-esterify I to II. The above method was repeated, and the concentration of I was estimated by difference from the result obtained without rat plasma.

Standard curve

Plasma standards spiked with II to concentrations of 0.78, 1.56, 3.125, 6.25, 12.5, 25, 50 and 100 ng/ml were analysed by the above method, and standard curves were drawn using a logit plot.

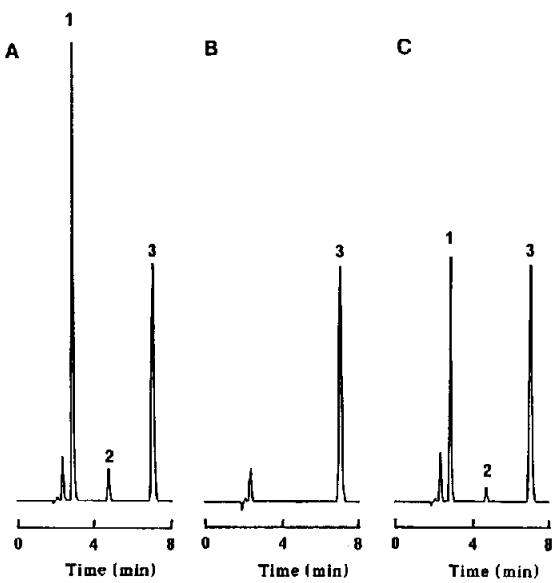
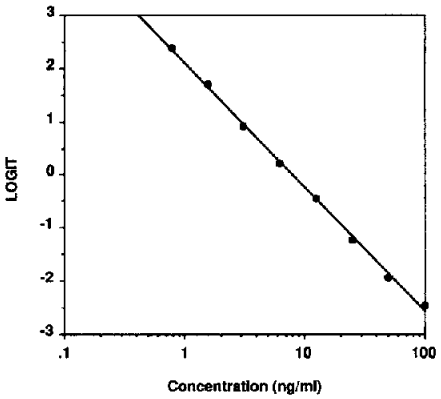


Fig. 2. Chromatograms obtained from (A) plasma without inhibitor, (B) plasma without addition of artificial substrate and (C) standard plasma sample (II, 12.5 ng/ml). Peaks: 1 = hippuric acid; 2 = Hip-His-Leu; 3 = internal standard.



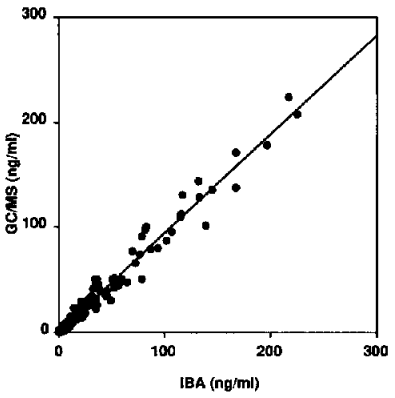


Fig. 3. Standard curve of II. The fitted line is the regression line, for which the equation is $y = 2.100 - 2.337\log(x)$, $r^2 = 0.999$.

Fig. 4. Correlation of concentrations of II in plasma measured by GC–MS and by the IBA. The fitted line is the regression line, for which the equation is $y = 0.942x + 0.245$, $r^2 = 0.970$ ($n = 123$).

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 2: (A) a plasma sample without inhibitor; (B) a plasma sample without addition of artificial substrate; and (C) a

TABLE I

RECOVERY TEST OF I IN HUMAN PLASMA ($n = 3$)

Sample	Concentration of I (ng/ml)			Recovery (%)
	Added	Expected	Found	
A	0[a]	–	6.25[a]	–
	12.5	18.75	18.15	95.2
	25.0	31.25	31.05	99.2
	50.0	56.25	56.55	100.6
B	0[a]	–	12.5[a]	–
	12.5	25.0	25.9	107.2
	25.0	37.5	38.7	104.8
	50.0	62.5	62.9	100.8
C	0[a]	–	25.0[a]	–
	12.5	37.5	38.5	108.0
	25.0	50.0	51.4	105.6
	50.0	75.0	74.9	99.8
Mean				102.4
S.E.				1.4

[a] Added 0 is added II in each concentration to estimate hydrolysis rate from I to II as recovery.

TABLE II

ASSAY VARIATIONS OF THE PROPOSED METHOD FOR II IN HUMAN PLASMA

Sample	Concentration (mean ± S.E.) (ng/ml)	C.V. (%)
Intra-assay (n = 5)		
A	5.3 ± 0.1	4.3
B	20.9 ± 0.4	4.7
C	72.2 ± 1.9	6.0
Inter-assay (n = 5)		
D	4.7 ± 0.3	12.3
E	27.6 ± 0.6	4.6
F	98.6 ± 1.7	3.9

standard plasma sample containing 12.5 ng/ml II. The retention times of hippuric acid, Hip-His-Leu and the internal standard were 2.3, 4.6 and 7.0 min, respectively. This indicated that the hippuric acid and the internal standard could be clearly separated.

The standard curve (Fig. 3) was calculated as the logit of the peak-height ratio of the hippuric acid to internal standard *versus* the concentration of II. The equation of the fitted line is $y = 2.100 - 2.337 \log(x)$, $r^2 = 0.999$.

In order to determine the specificity of the IBA, we compared the concentrations of II in plasma from volunteers orally given I, as determined by the IBA and by GC–MS [5]. The regression of concentrations of II in plasma by IBA against by GC–MS was $y = 0.942x + 0.245$. The correlation coefficient was $r = 0.970$ ($n = 123$) (Fig. 4).

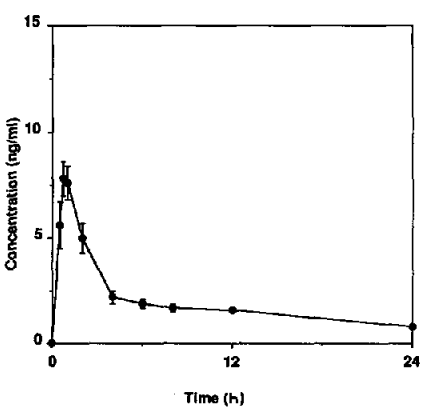


Fig. 5. Plasma concentration of II in healthy volunteers after a single dose of I (0.5 mg) ($n = 6$, mean ± S.E.).

The assay procedure was tested for accuracy and precision. The accuracy was tested by a recovery test, by adding I to plasma (Table I, $n = 3$). Each recovery rate was satisfactory (102.4 ± 1.4%, mean ± S.E.). The precision was tested by within-day and day-to-day analyses (Table II). For within-day precision, aliquots of a normal human plasma sample produced a coefficient of variation (C.V.) of 4.3–6.0% ($n = 3 \times 5$). For day-to-day precision, aliquots of a normal human plasma sample produced a C.V. of 3.9–12.3% ($n = 3 \times 5$).

Pharmacokinetics

The method described was used to follow the level of II in plasma of healthy volunteers orally given 0.5 mg of I. The plasma concentration of II is shown in Fig. 5.

The proposed method for the determination of II in human plasma is satisfactory with respect to sensitivity and specificity. It is thus suitable for the determination of other active ACE inhibitors (enalaprilat [6], lisinopril [6], ramiprilat [7], CGS 13934 [8], CGS 16617 [9], etc.) and during routine analysis in clinical investigations.

REFERENCES

1 D. J. Tocco, F. A. deLuna, A. E. W. Duncan, T. C. Vassil and E. H. Ulm, *Drug Metab. Dispos.*, 10 (1982) 15.
2 M. Hikens, E. L. Hand and W. S. Mulcahy, *Ligand Q.*, 4 (1981) 43.
3 A. Rakhit and V. Tipnis, *Clin. Chem.* 30 (1984) 1237.
4 V. Tipnis and A. Rakhit, *J. Chromatogr.*, 345 (1985) 396.
5 H. Shioya, M. Shimojo and Y. Kawahara, *J. Chromatogr.*,, 496 (1989) 129.
6 J. Biollaz, J. L. Schelling, B. Jacot des Combes, D. B. Brunner, G. Desponds, H. R. Brunner, E. H. Ulm, M. Hichens and H. J. Gomez, *Br. J. Clin. Pharmacol.*, 14 (1982) 363.
7 P. U. Witte, R. Irmisch, P. Hajdú and H. Metzger, *Eur. J. Clin. Pharmacol.*, 27 (1984) 577.
8 A. Rakhit, M. E. Hurley, V. Tipnis, J. Coleman, A. Rommel and H. R. Brunner, *J. Clin. Pharmacol.*, 26 (1986) 156.
9 P. Reydel-Bax, E. Redalieu and A. Rakhit, *Clin. Chem.*, 33 (1987) 549.