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METHOD FOR DETERMINATION OF ANGIOTENSIN-CONVERTING ENZYME ACTIVITY IN BLOOD AND TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simplified method for an angiotensin-converting enzyme activity assay in biological samples was developed. Samples were incubated with hippurylhistidylleucine, an artificial substrate of angiotensin-converting enzyme. The reaction was terminated by the addition of metaphosphoric acid and liberated hippuric acid in the supernatant was quantitated directly by reversed-phase high-performance liquid chromatography. Tissues were homogenized in the presence of Nonidet-P40, a detergent, and the resulting supernatant was used for the assay of tissue angiotensin-converting enzyme activity by high-performance liquid chromatography. The present procedure made it possible to determine angiotensin-converting enzyme activity in whole blood and the total activity in tissues. A comparative study of angiotensin-converting enzyme activity in plasma, kidney and lung of five experimental animals showed a high degree of variation from species to species.

INTRODUCTION

Angiotensin-converting enzyme (E.C. 3.4.15.1, dipeptidyldipeptidase) (ACE) hydrolyzes angiotensin I to give the potent vasodepressor angiotensin II. The enzyme also degrades the vasodepressor peptide bradykinin [1]. ACE was found in various tissues including lung, kidney, serum, brain and testicles [2]. Elevated activity of ACE was demonstrated in serum of patients with sarcoidosis [3] and Gaucher's disease [4]. Recently, a competitive inhibitor of ACE has been shown to be a antihypertensive drug [5, 6] and plasma ACE activity might be a useful guide to plasma level of ACE inhibitor after its administration [7]. With these current findings, a convenient and exact method for the determination of ACE is desired. For the determination of ACE, numerous compounds have been used as substrate. With physiological

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substrate, the conversion of angiotensin I to angiotensin II has been followed by radioimmunoassay [8], contractile response [9], and the determination of released histidylleucine (His-Leu) with fluorimetric coupling agents [10—13]. However, these methods are still subject to errors due to the hydrolysis of both angiotensin I and His-Leu by the other peptidases when crude tissue preparation was used as the enzyme source of ACE.

Another approach is a method using artificial substrate, usually a tripeptide with a blocked amino-terminal. Hippurylhistidylleucine (Hip-His-Leu) may be the most widely used substrate among artificial substrates. The procedure is based on the estimation of His-Leu or hippuric acid liberated from the substrate. One of the products, His-Leu, can be measured fluorimetrically after reaction with *o*-phthaldialdehyde [11—13] or fluorescamine [10]. Although these methods have been applied to the assay of ACE activity in serum [13] and brain [14], they have the disadvantage that a part of His-Leu undergoes hydrolysis during incubation [11, 14, 15]. The other product, hippuric acid, can be determined spectrophotometrically at 228 nm after its extraction [16]. However, this method is somewhat tedious; for the crude tissue preparations, problems are encountered with high background absorbance caused by the substances which are extracted into ethyl acetate together with hippuric acid. Furthermore, lipemic sera result in a turbid sample solution [11, 17]. To avoid these problems, a modification procedure using a radioactive substrate of Hip-His-Leu was recently reported [17].

In this paper, a simple and widely applicable method for the determination of ACE in biological sample is presented by the high-performance liquid chromatographic (HPLC) determination of hippuric acid liberated from Hip-His-Leu.

EXPERIMENTAL

Animals and materials

Unless otherwise stated, male Wistar rats weighing about 200 g were used. Hip-His-Leu was purchased from the Institute for Protein Research (Osaka, Japan). Nonidet-P40 was obtained from Shell Chemicals (Manchester, Great Britain). All other chemicals were commercially available of reagent grade.

Enzyme preparations

Blood was collected from the abdominal aorta of rat anesthesized with diethyl ether and put into a heparinized test tube. Tissue samples were prepared as follows. Lung and kidney were removed immediately after sacrifice and rinsed gently with chilled saline. They were chopped into small pieces and suspended in 5 volumes of chilled Tris—HCl buffer (pH 7.8) containing 30 m*M* KCl, 5 m*M* magnesium acetate, 0.25 *M* sucrose and Nonidet-P40. The suspension was homogenized and centrifuged at 20,000 *g* for 20 min at 4°C. The resultant supernatant was used as the enzyme preparation.

Standard assay method of ACE activity

A 50-μl aliquot of blood or diluted enzyme preparation was incubated in a total volume of 250 μl of 100 m*M* phosphate buffer (pH 8.3) containing

300 m*M* NaCl and 5 m*M* Hip-His-Leu for 30 min at 37°C. The reaction was terminated by adding 0.75 ml of 3% metaphosphoric acid and the mixture was centrifuged for 5 min. A 20-μl aliquot of the resultant supernatant was injected into the chromatograph. Control incubation was also carried out in the absence of Hip-His-Leu or enzyme preparation. One unit of activity is defined as the amount of enzyme catalyzing the release of 1 μmol of hippuric acid from Hip-His-Leu per minute at 37°C [16].

High-performance liquid chromatography

The following instruments were obtained from Shimadzu Seisakusyo (Kyoto, Japan): an LC-3A pump, a spectrophotometric detector SPD-2A, and a Chromatopac C-R1A recorder. Injection was achieved using a Rheodyne Model 1725 injector valve fitted with a 20-μl loop. The analytical column was 25 × 0.4 cm I.D. packed with 7.5 μm Nucleosil 7 C_{18}, Macherey-Nagel & Co. (Düren, G.F.R.). The mobile phase consisted of methanol—10 m*M* KH_2PO_4 (1:1) and adjusted to pH 3.0 with phosphoric acid (flow-rate 1.0 ml/min). Quantitation was done by using peak heights at 228 nm.

RESULTS

Determination of hippuric acid

Fig. 1A shows the chromatographic separation of 20 μl of standard mixture of 135 μ*M* hippuric acid and 5 m*M* Hip-His-Leu. Retention times were 3.5 min for the former and 5.2 min for the latter. Under the same conditions, His-Leu and benzoic acid were eluted at 2.0 min and 7.3 min, respectively. These indicated that hippuric acid could be clearly separated from the other hydrolyzed products of Hip-His-Leu.

Recovery of hippuric acid was examined by adding several protein precipitants such as metaphosphoric acid, perchloric acid, ethanol, trichloroacetic acid, and sulfosalicyclic acid. Among them, metaphosphoric acid gave a sharp peak (Fig. 1A) and good recovery (Table I) of hippuric acid. Hippuric acid was stable during the incubation for 30 min at 37°C.

Typical chromatograms obtained in the present study were shown in Fig. 1B—E where the upper diagrams showed the formation of hippuric acid after incubation of biological samples with Hip-His-Leu. No endogenous interfering substance with the determination of hippuric acid was detected even in the sample containing Nonidet-P40, a detergent used for solubilizing tissue ACE (lower diagrams).

Measurement of ACE activity in blood and tissues

As shown in Fig. 2, hippuric acid formation by serum and whole blood increased linearly with incubation time up to 60 min and with a sample volume of up to 100 μl.

ACE activity in whole blood, serum and plasma of rats measured by the present method is shown in Table II. No hemolysis was noticed during the course of incubation of whole blood with Hip-His-Leu. No difference in activity was observed between serum and plasma. The activity in whole blood was nearly equal to the predicted value both from the activity in plasma and the

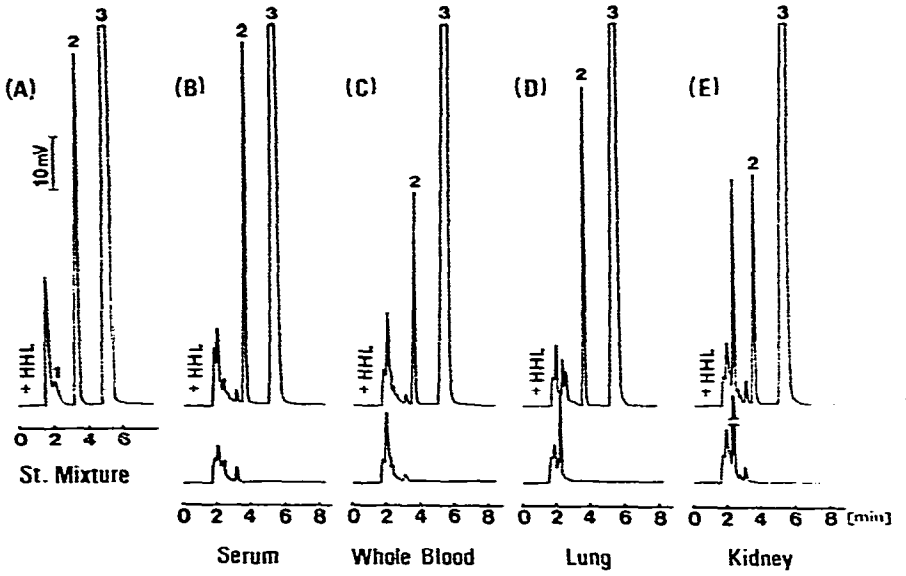

Fig. 1. Chromatograms obtained from various samples incubated with (upper diagram) or without (lower diagram) Hip-His-Leu (HHL). (A) Standard mixture of 2.7 nmol His-Leu, 2.7 nmol hippuric acid and 100 nmol Hip-His-Leu. (B) A 50-μl aliquot of serum or (C) whole blood was incubated with (upper diagram) or without (lower diagram) 5 m*M* Hip-His-Leu according to the standard ACE assay method as described in Experimental. After 30 min, 0.75 ml of 3% metaphosphoric acid was added and centrifuged. (D) Lung or (E) kidney was homogenized in 5 volumes of chilled Tris—HCl buffer containing 0.5% Nonidet-P40, and centrifuged. The supernatant was incubated with (upper diagram) or without (lower diagram) 5 m*M* Hip-His-Leu. In the case of lung, the supernatant was diluted 20 times with the buffer prior to incubation with Hip-His-Leu. Analytical conditions as described in Experimental. Peaks: 1 = His-Leu, 2 = hippuric acid, 3 = Hip-His-Leu.

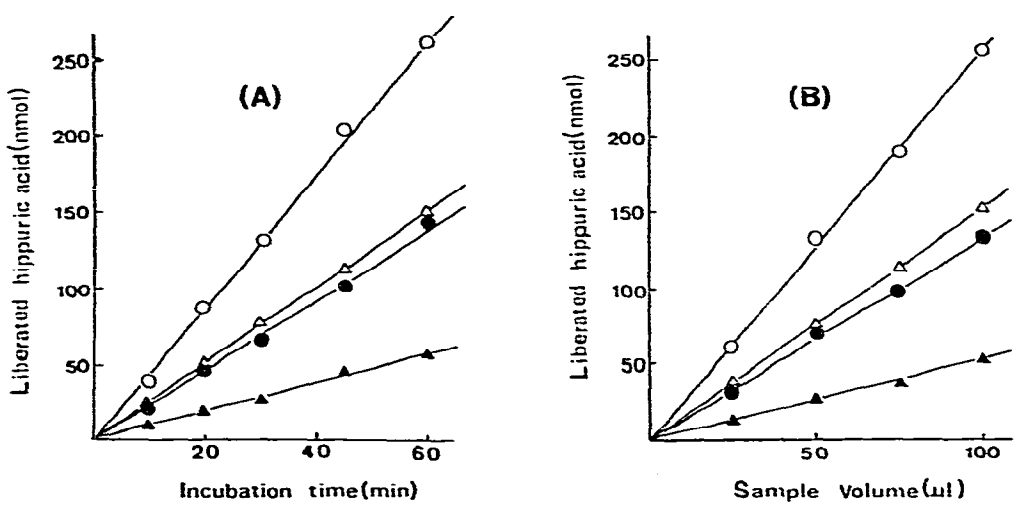

Fig. 2. Dependence of incubation time (A) and sample volume (B) of hippuric acid formation from Hip-His-Leu catalyzed by ACE in serum (○), whole blood (●), lung (△) and kidney (▲). (A) A 50-μl aliquot of sample was used; (B) incubation time was 30 min. Each point represents the mean of three determinations.

TABLE I

PERCENT RECOVERY OF HIPPURIC ACID WITH OR WITHOUT INCUBATION

To a 200-μl aliquot of 125 m*M* phosphate buffer (pH 8.3) containing hippuric acid were added 50 μl of blood or tissue sample, and then 0.75 ml of 3% metaphosphoric acid was added before or after incubation for 30 min. Hippuric acid in the supernatant was determined by chromatography under the conditions given in Fig. 1.

	Hippuric acid added (nmol)	Recovery (%)*	
		Before incubation	After incubation
Serum	75	99	102
	150	99	99
Whole blood	75	100	101
	150	97	100
Lung**	75	99	103
	150	97	98
Kidney**	75	102	103
	150	103	101

*Each value represents the mean of three determinations.
**Tissue samples of lung and kidney were prepared as described in the legend to Fig. 1.

TABLE II

ACE ACTIVITY OF SERUM, PLASMA AND WHOLE BLOOD IN RATS

Each value represents the mean of three determinations.

Rat No.	Hematocrit (%)	Liberated hippuric acid (nmol/min/ml)			
		Serum	Plasma	Whole blood*	Whole blood**
1	42	76.4	76.9	42.4	46.2
2	42	97.4	86.8	45.1	49.5
3	43	100.6	96.3	52.7	54.9
4	44	90.3	88.8	42.1	49.7
5	42	92.1	94.2	48.6	54.6

*Observed value.
**Calculated value from the activity in plasma and the estimated hematocrit.

estimated hematocrit, indicating that ACE in whole blood originates from plasma ACE.

As shown in Table III, addition of Nonidet-P40 to the homogenization medium of lung and kidney of rats resulted in a solubilization of most of the ACE activity in these tissues. Hippuric acid formation by the resulting supernatants increased linearly with both incubation time and volume of the sample (Fig. 2).

Variation of ACE activity in animals

Table IV shows ACE activity of serum, lung and kidney obtained from various species including rat, rabbit, mouse, guinea pig, and dog. The value obtained from rat lung was approximately similar to that reported by Lazo and Quinn [18] using 1-O-*n*-octyl-β-glucopyranoside as detergent. For serum ACE, the highest value was observed in the guinea pig. The decreasing order of variation was guinea pig > mouse > rat = rabbit > human > dog. On the other hand, in the lungs of animals studied, the lowest and highest ACE activities were found in the guinea pig and mouse, respectively. In kidney, high activity was found in the rabbit and mouse.

TABLE III

EFFECT OF 0.5% NONIDET-P40 ON THE EXTRACTION OF ACE FROM TISSUES

Rat lung and kidney were homogenized in 5 volumes of chilled Tris—HCl buffer (pH 8.3) with or without 0.5% Nonidet-P40 and centrifuged.

Tissue	ACE activity* (%)			
	Without Nonidet-P40		With Nonidet-P40	
	Supernatant	Pellet	Supernatant	Pellet
Lung	26.8	73.2	98.7	1.3
Kidney	16.9	83.1	90.0	10.0

*ACE activity in the supernatant and pellet fraction was expressed as percent against the sum of the values in the supernatant and pellet fractions. Each value represents the mean of three determinations.

TABLE IV

ACE ACTIVITY IN SERUM, LUNG AND KIDNEY OBTAINED FROM VARIOUS SPECIES

Values are expressed as mean ± S.D. obtained from five men and five animals.

Animal	Age or body weight	ACE activity		
		Serum (U/ml)	Lung (U/g wet wt.)	Kidney (U/g wet wt.)
Human	25—40 years	0.028 ± 0.006	—	—
Dog (Beagle ♂)	7.5 months	0.005 ± 0.001	—	—
Rabbit (albino ♂)	3.5 months	0.077 ± 0.019	7.19 ± 1.76	4.76 ± 1.82
Rat (Wistar ♂)	7.5 weeks	0.090 ± 0.021	6.02 ± 0.57	0.13 ± 0.03
Mouse (ICR ♂)	7.0 weeks	0.368 ± 0.043	12.04 ± 0.94	5.29 ± 0.29
Guinea pig (Hartler ♂)	270—310 g	1.176 ± 0.178	0.84 ± 0.44	0.14 ± 0.06

DISCUSSION

The use of HPLC for the assay of serum ACE has been reported by Chiknas [19] and Nagamatsu et al. [20], both using Hip-His-Leu as substrate. The former assay is based on the ethyl acetate extraction of hippuric acid, evaporation of the organic layer, redissolution into water and subsequent HPLC quantification of hippuric acid. The latter employed an ion-exchange column for the determination of hippuric acid in the filtrate after stopping the enzymatic reaction by boiling.

The procedure described here can be performed in a single tube; the analysis time of hippuric acid is 6 min per sample. No pretreatment other than deproteinization of the sample is required. Additionally, supernatant samples were found to be stable for at least five days at 4°C. These features are advantageous when handling a large number of samples as in the clinical screening of patients with elevated serum ACE [21], and for monitoring the effect of administrated ACE inhibitor [14]. In the latter case, there are many reports that action of a sulfhydryl group-containing ACE inhibitor, such as Captopril, changes depending on the time between blood sampling and assay of ACE activity [22—24]. This is a severe problem when an extremely low level of a highly potent inhibitor was present in the blood. There is no report using whole blood for the assay of blood ACE activity. Our observation that whole blood ACE activity directly reflects plasma ACE activity (Table II) is advantageous to evaluate in vivo action of ACE inhibitor since assay of ACE activity can start rapidly when using whole blood without having to separate serum or plasma. Furthermore, the present method can be applied to the assay of tissue ACE activity by combination with detergent. In quantitating tissue ACE, it is necessary to disrupt the cell membrane in order to solubilize ACE. Detergents such as Triton X-100 [25] and Nonidet-P40 [26] have been used widely to solubilize and isolate the membrane-bound ACE. However, these detergents have a high absorbance at 228 nm and cause uncertainty in the spectrophotometric assay of hippuric acid. The use of HPLC made it possible to determine hippuric acid even in the presence of detergent as shown in the present study.

Cushman and Cheung reported that ACE activity in 5000 *g* supernatant of tissues varied with species [27] and organs [2]. On the other hand, Soffer [1] pointed out a marked discrepancy among the reported values of the enzyme activity in tissues and the presence of methodological problems. We applied the present method to the assay of the enzyme in lung and kidney of some experimental animals, and also confirmed a high degree of species difference of the total activity in these tissues.

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