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A simple high-performance liquid chromatographic method for estimating human serum angiotensin-converting enzyme activity

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Abstract: A simple method for measuring angiotensin-converting enzyme activity in human serum was developed. Samples were incubated with hippurylhistidylleucine and the liberated hippuric acid was determined directly by reversed phase ion-pair high-performance liquid chromatography with UV spectrometric detection.

Keywords: *Ion-pair high-performance liquid chromatography; hippuric acid determination; angiotensin-converting enzyme assay.*

Introduction

Angiotensin-converting enzyme (ACE) is an important component of the renin–angiotensin system implicated in the pathogenesis of hypertension. It hydrolyses angiotensin I to very active vasopressor angiotensin II by cleavage of the C-terminal dipeptide, histidyl-leucine. It also inactivates the depressor peptide bradykinin by the same mechanism [1]. Various authors have studied the correlation between ACE plasma levels and blood pressure in different physiological and pathological situations. Elevated ACE activity was demonstrated in the sera of patients with sarcoidosis [2] and Gaucher's disease [3].

Such studies may provide a better interpretation of the mechanism of hypertension. But a convenient and accurate method for the determination of ACE is clearly desirable. Many compounds have been used as ACE substrates. With physiological substrate, the conversion of angiotensin I to angiotensin II has been followed by radioimmunoassay, and by the determination of released histidyl-leucine (His-Leu) with fluorimetric coupling agents [4, 5]. The more frequently used methods utilize small peptides such as hippuryl-L-histidyl-L-leucine as substrates, the procedure being based on the estimation of the liberated His-Leu or hippuric acid. His-Leu can be measured fluorimetrically after reaction with *o*-phthaldialdehyde or fluorescamine [6, 7]. These methods have the disadvantage that His-Leu undergoes partial hydrolysis during incubation [8].

The other product, hippuric acid, can be determined spectrophotometrically, after extraction with ethylacetate [2, 9], but this method is somewhat tedious. Two HPLC

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methods have been reported. One was based on the ethyl acetate extraction of hippuric acid, evaporation of the organic layer, redissolution in water and subsequent determination of hippuric acid [10]. In the second method, no internal standard was used, and the incubation medium was injected directly into the chromatograph [11].

In this paper a simple method for the determination of ACE activity in human blood is presented, using ion-pair liquid chromatographic determination of hippuric acid liberated from hippuryl-histidyl-leucine (Hip-His-Leu). The incubation medium is injected, directly or after neutralization, into the chromatograph, and salicylic acid is used as the internal standard. In this way preliminary extractive procedures are avoided.

Experimental

Apparatus

Analyses were performed on a high pressure liquid chromatograph operated at room temperature and connected to a variable wavelength UV detector. A 25 cm, 4.6 mm i.d. reversed phase column with a 10 mm particle size was used. Samples were introduced into the column through a Rheodyne 7105 injector with a 10 μl syringe. Chromatograms were recorded on a Perkin–Elmer strip-chart recorder.

Materials

Hippuric acid and Hip-His-Leu were purchased from the Sigma Chemical Co., St. Louis, Missouri, USA, salicylic acid from Riedel-De Haën AG, Seelze–Hannover, and methanol for HPLC, monohydrogen potassium phosphate, phosphoric acid, silver oxide and sodium chloride, all analytical grade, from Carlo Erba, Milano, Italy. All reagents were used without further purification.

Chromatographic conditions

Analyses were carried out at room temperature. The flow rate was adjusted to 1.5 ml/min. The detector sensitivity was 0.02 AUFS. The chart speed was 0.5 mm/min. The detector wavelength was 230 nm.

Mobile phase

The mobile phase was a 60:40 v/v mixture of water–methanol, containing 0.005 M tetrabutylammonium cation, 0.005 M monohydrogen potassium phosphate and buffered to pH 7.4. It was prepared as previously described [12].

Stock solution

The stock solution of salicylic acid (internal standard) was prepared by dissolving 250 mg of the pure compound in 1000 ml of distilled water. The same solution may be prepared in ethanol if desired (100 mg/100 ml). The stock solution of hippuric acid (50 mg/100 ml) was also prepared using distilled water as solvent.

Standard assay method for ACE

The incubation was carried out for 60 min at 37°C in a final volume of 250 μl, containing 100 mM potassium phosphate buffer (pH 8.3), 300 mM NaCl and 5 mM Hip-His-Leu. The enzymatic reaction was initiated by addition of 100 μl of a solution containing potassium phosphate buffer, NaCl and Hip-His-Leu, prepared as described by Lieberman [2], to a mixture of 100 μl serum and 50 μl water (final volume 250 μl).

After incubation the reaction was terminated by addition of 50 μl of 5 M HCl and 200 μl of internal standard stock solution was added to each sample (final volume 500 μl). After centrifugation, 5 μl supernatant was used for the chromatographic assay. One unit of activity is defined as the hippuric acid formed (nanomoles) per minute at 37°C, under standard assay conditions. The specific activity is expressed as the number of units per ml of serum. The incubation medium was sometimes neutralized before injection.

Reference standard solution

The reference standard solution was prepared by pipetting 8 ml hippuric acid stock solution, 40 ml salicylic acid stock solution or 10 ml ethanolic stock solution, 20 ml of the solution used to dissolve the substrate for incubation [2], and 10 ml of 5 M HCl into a 100 ml flask subsequently made up to volume with distilled water.

Analysis of standard solutions

Solutions of the samples were prepared as follows: To seven centrifuge tubes were added potassium phosphate buffer (pH 8.3), NaCl and Hip-His-Leu in the same quantities as in the ACE assay method. Accurately measured volumes (0, 5, 10, 25, 40, 60 and 80 μl) of the hippuric acid stock solution were added to each tube followed by 50 μl 5 M HCl, 100 μl serum and water (final volume 300 μl). The serum was added after the hydrochloric acid to avoid hippuric acid formation. The solutions were incubated for 60 min at 37°C. Then 200 μl internal standard solution was added to each sample. The solutions were also analysed before incubation. With the ethanolic stock solution of internal standard 50 μl was used, with 150 μl of water. Samples were sometimes neutralized before injection. The samples and reference standard solution were chromatographed alternately using the chromatographic conditions already described. Peak height ratios were used to evaluate the resulting chromatograms. The results are listed in Table 1 for standard solutions and in Table 2 for sample solutions.

Results and Discussion

Calibration curves were reported in a previous paper [12] and were prepared by plotting the peak height ratios of the hippuric acid peak and the internal standard peak against the concentration of hippuric acid (mg/100 ml). The slope was 0.1704, the

Table 1
Recovery of hippuric acid in ACE assay

Amount added (μg)	Amount found after incubation (μg)	Recovery* (%)	Amount found before incubation (μg)	Recovery* (%)
2.5	2.51	100.40	2.53	101.20
5	5.04	100.86	4.88	97.60
12.5	12.65	101.01	9.88	98.80
20	19.4	98.00	20.53	102.65
30	30.2	100.70	30.71	102.36
40	41.1	102.70	39.83	99.57
Mean		100.61		100.36
RSD (%)		1.5		2.05

* Each value is the mean of three determinations.

Table 2
Serum activity in 20 normal subjects

U/ml				
4.12	4.58	5.34	5.67	5.86
5.95	6.60	6.88	7.07	7.25
7.44	7.81	7.81	8.18	8.37
8.56	8.93	9.20	11.25	13.02
Mean	7.48			
SD	±2.17			

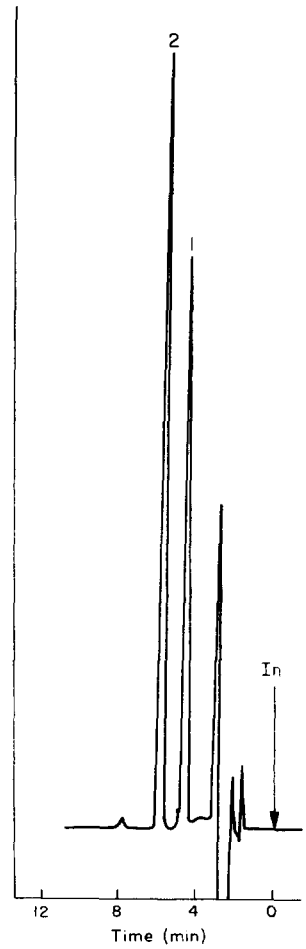

Figure 1
Chromatogram of the reference solution containing hippuric acid (peak 1) and salicylic acid (peak 2) as internal standard.

intercept 0.0003 and the correlation coefficient 0.9999. Figure 1 shows the chromatographic separation of 5 μl standard solution containing 4 mg/100 ml hippuric acid and 10 mg/100 ml salicylic acid (internal standard).

Figures 2 and 3 show the chromatographic separation of 5 μl of a sample incubated according to the standard ACE assay. In Fig. 3 the sample contained added hippuric acid (12.5 μg), and HCl was added before the serum sample.

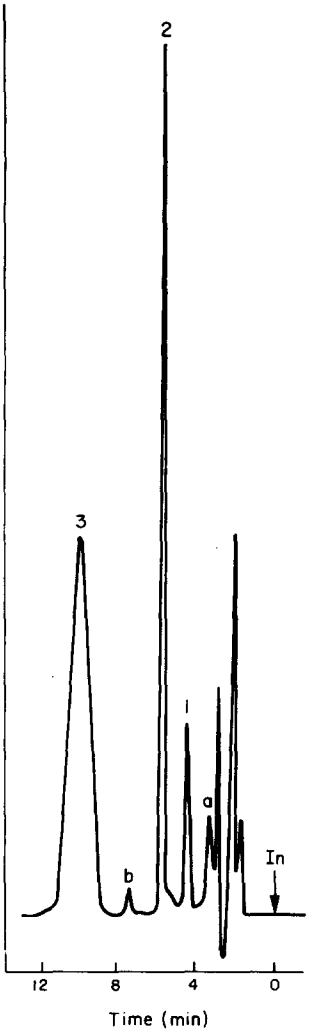

Figure 2
Chromatogram of a sample incubated as described in Experimental containing hippuric acid liberated (peak 1), salicylic acid as internal standard (peak 2) and Hip-His-Leu (peak 3). (a) Histidylleucine; (b) an unidentified peak.

In the experimental conditions His-Leu, another hydrolysis product of Hip-His-Leu, eluted after 3.2 min. Sometimes a peak partially overlapping that of hippuric acid was observed. This effect could be avoided if the incubation medium was neutralized with NaOH before injection into the chromatograph.

The method gives good results in terms of recovery and standard deviation, and has advantages over the methods previously described. The use of an internal standard allows direct injection of the incubation medium into the chromatograph without

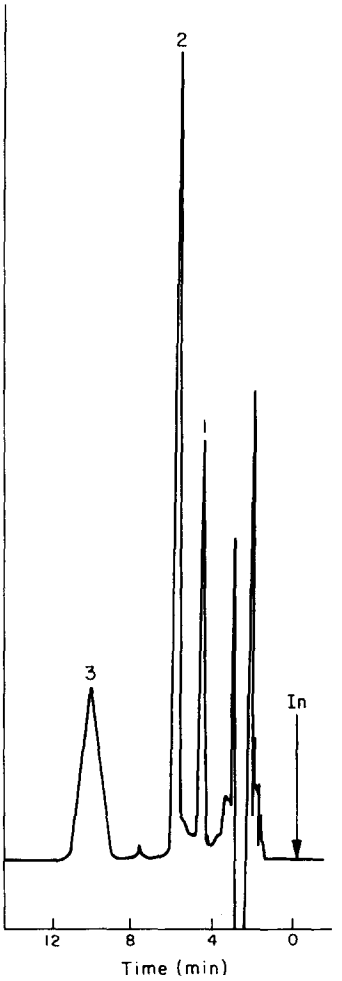

Figure 3
Chromatogram obtained form a sample incubated as described in Experimental containing hippuric acid added (peak 1), salicylic acid (peak 2) and Hip-His-Leu (peak 3). HCl was added before incubation.

preliminary extraction procedures. Hippuric acid was shown to be stable during the incubation for 60 min at 37°C. ACE levels in the blood of 20 normal subjects were determined. The age of the subjects was between twenty and forty years without distinction with regard to sex. The mean value of the enzyme activity was 7.48 ± 2.17 units/ml serum.

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