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Ion-pair liquid chromatographic assay of angiotensin-converting enzyme activity

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Abstract: A rapid and specific high-performance liquid chromatographic assay for the quantitative determination of angiotensin-converting enzyme activity is described. Hippuryl-L-histidyl-L-leucine (Hip–His–Leu) is used as substrate and the released hippuric acid is measured. The procedure is accurate and precise and no extraction is required.

Keywords: *Angiotensin-converting enzyme; high-pressure liquid chromatographic enzyme assay; hippuric acid.*

Introduction

The angiotensin-converting enzyme (A.C.E., peptidyl-dipeptide hydrolase kininase II) is a specific component of the vascular endothelium in most mammalian organs [1], particularly the lungs. It plays an important role in the maintenance of systemic blood pressure by two mechanisms, by catalysing both the conversion of angiotensin I into the potent pressor octapeptide angiotensin II [2], and by the inactivation of the depressor peptide bradykinin [3]. These mechanisms involve the cleaving of the terminal dipeptides His–Leu or Phe–Arg, respectively. Both mechanisms contribute to increased blood pressure: specific inhibitors of angiotensin-converting enzyme have been developed as possible antihypertensive drugs.

Captopril (D-3-mercapto-2-methylpropanoyl-L-proline, SQ-225), one of the more potent orally active inhibitors of angiotensin-converting enzyme [4], blocks the effects of angiotensin II formation from angiotensin I and increases the action of bradykinin both *in vitro* and *in vivo*. The antihypertensive activity of captopril and the recently proposed model for the active site of antiotensin-converting enzyme [5] have opened new fields in research on antihypertensive drugs. Many analogues of captopril continue to be synthesized and tested for angiotensin-converting enzyme inhibitory activity. The development of simple methods for the quantitative assay of angiotensin-converting enzyme has thus been essential for studies of the analytical properties of the enzyme and for comparison of the activity of enzyme inhibitors. The activity of angiotensin-converting enzyme has been assayed by a large number of methods. Many have utilized

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small peptides such as hippuryl-L-histidyl-L-leucine (Hip–His–Leu) and benzyloxycarbonyl-L-phenylalanyl-L-histidyl-L-leucine as substrate. The hydrolysis product (His–Leu) obtained from the first substrate was measured fluorimetrically [6, 7]. The spectrophotometric determination of the hippuric acid [8] obtained from Hip–His–Leu is particularly useful for assay of angiotensin-converting enzyme activity under a variety of conditions and frequently adopted for determining the activity of the enzyme in blood [9]. A liquid chromatographic method [10] which required a preliminary extraction with ethylacetate was also described. In the present paper the assay of the hippuric acid from Hip–His–Leu by a simple reversed-phase, ion paired liquid chromatographic method is described. No preliminary extraction procedure is involved.

Experimental

Apparatus

Analyses were performed on a high-pressure liquid chromatograph (Perkin–Elmer) operated at room temperature and connected to a variable wavelength UV detector. A 25 cm, 4.6 mm i.d. C_{18} reversed-phase column with a 10 μm particle size was used. Samples were introduced into the column through a septumless injector with a 10 μl Hamilton syringe. Chromatograms were recorded on a strip-chart recorder.

Materials

Hippuric acid and Hip–His–Leu (Sigma), salicylic acid (Riedel-De Haën), monohydrogen potassium phosphate, phosphoric acid, silver oxide, sodium chloride and HPLC-grade method (Carlo Erba) were used without further purification.

Chromatographic conditions

The analyses were carried out at room temperature. The flow rate was adjusted to 1.8 ml min^{-1} (~2.000 psi). The detector sensitivity was 0.05 a.u.f.s. The chart speed was 0.5 mm min^{-1}. The solvent mixture was prepared daily and degassed before use. The detector wavelength was 230 nm.

Mobile phase

The mobile phase was a 60:40 v/v mixture of water and methanol, containing 0.005 M tetrabutylammonium cation and 0.005 M monohydrogen potassium phosphate and buffered to pH 7.4 with phosphoric acid. It was prepared in the following manner. An appropriate quantity of tetrabutylammonium iodide (Fluka AG), calculated to give the desired molar concentration in the final solvent mixture was dissolved in methanol and stirred at room temperature for 1 h with an equivalent quantity of finely divided silver oxide. After filtration the methanolic solution was diluted with the same solvent and was mixed with an adequate volume of water to give the desired solvent ratio. The aqueous solution contained an amount of monohydrogen potassium phosphate calculated to obtain a final 0.005 M concentration. The prepared solution was adjusted to pH 7.4 with an 85% aqueous phosphoric acid solution.

Stock solution

A stock solution of salicylic acid (internal standard) was prepared by dissolving 100 mg of the pure compound in 100 ml distilled water. A stock solution of hippuric acid (50 mg 100 ml^{-1}) was also prepared using distilled water as solvent.

Calibration curve

Accurately pipetted volumes of 1.0, 2.0, 4.0, 8.0. and 16.0 ml of hippuric acid stock solution and the internal standard stock solution, 10 ml, were placed into 100 ml volumetric flasks and water was added to volume. Aliquots of 7 μl of each solution were injected into the chromatograph. Calibration curves were prepared by plotting the height ratios of the hippuric acid and internal standard peaks against the concentrations of hippuric acid in mg 100 ml^{-1}. The five concentrations of hippuric acid were subjected to regression analysis and the slope and intercept were calculated (Table 1).

Table 1
Calibration data for hippuric acid

Final concentration (mg 100 ml^{-1})	Hippuric acid/int. st. ratio	Slope	Intercept	r
0.5	0.09004 + 0.0011*	0.1704	0.00036	0.9999
1	0.1686 + 0.0008			
2	0.3390 + 0.0011			
4	0.6810 + 0.0013			
8	1.365 + 0.0009			

*Confidence limits at $p = 0.5$ (n=5).

Preparation of angiotensin-converting enzyme

Converting enzyme was extracted from rabbit lungs according to a modified method of Dorer *et al.* [11]. Fresh rabbit lungs were frozen and thawed, cut into small pieces and homogenized with cold 0.05 M sodium phosphate buffer (pH 7, 2 ml/g tissue) in a Waring blendor three times for 30 s. The homogenate was strained through gauze, frozen and thawed, and centrifuged at 2000*g* for 30 min. The supernatant solution was fractionated with ammonium sulphate in the range of 1.0–1.6 M. The resulting sediment was suspended in 0.05 M sodium phosphate buffer (pH = 7.0) and reprecipitated with 1.6 M ammonium sulphate. The protein content was determined by the method of Lowry *et al.* [12] using bovine serum albumin as standard.

Incubation conditions

Incubation was carried out for 30 min at 37°C. The final volume of the incubation medium was 250 μl. The final concentrations of the components were: 100 mM potassium phosphate buffer (pH 8.3), 300 mM NaCl, 5 mM Hip–His–Leu. The enzymatic reaction was initiated by addition of the enzyme suspension (40–50 μg) and terminated by addition of 50 μl of 3 M HCl. The internal standard stock solution was accurately diluted (1:4) and 200 μl of this solution was added to the test tube. After centrifugation the supernatant was used for the chromatographic assay.

Reference standard preparation

The reference standard solution was prepared by pipetting 8 ml of the hippuric acid stock solution and 10 ml of salicylic acid (internal standard) stock solution into a 100 ml flask and bringing to volume with distilled water. The standard solution also contained phosphate buffer, sodium chloride and hydrochloric acid in the same concentrations as the sample solutions.

Sample preparation and recovery

Solutions of the samples were prepared as follows. To six centrifuge tubes were added quantities of potassium phosphate buffer (pH 8.3), NaCl and Hip–His–Leu calculated to give final 50, 150 and 2.5 mM concentrations respectively. Accurately measured volumes (0, 5, 10, 20, 40 and 80 μl) of the hippuric acid stock solution were added to each tube, followed by 50 μl of 3 M HCl and 40–50 μg of the suspension of the enzyme. The enzyme suspension was added after the addition of hydrochloric acid in order to avoid hippuric acid formation. The volume was adjusted to 300 μl with distilled water. The solutions were incubated for 30 min at 37°C. The internal standard stock solution was accurately diluted (1:4) and 200 μl of the resulting solution was added to each sample. The final volume was 500 μl. The concentrations of hippuric acid in the sample solutions were 0, 0.5, 1.0, 2.0, 4.0 and 8.0 μg 100 μl^{-1} respectively. The internal standard concentration was 10 μg 100 μl^{-1}. The mobile phase was pumped through the column until a stable baseline was obtained. Aliquots (7 μl) of the standard solution obtained after centrifugation were injected into the chromatograph. When the peak height ratios of hippuric acid to the internal standard were reproducible within ± 1% alternate sample and standard injections (7 μl) were made.

Peak responses

The peak responses were measured as peak height. The amount of hippuric acid was calculated using:

$$\mu g \text{ of hippuric acid} = \frac{h_1 h_2' v}{h_2 h_1'} c$$

where h_1 was the peak height of hippuric acid in the sample solutions, h_2 was the peak height of the internal standard in the sample solutions, h_1' was the peak height of hippuric acid in the standard solution. h_2' was the peak height of the internal standard in the standard solution, c was the concentration (μg/μl) of the hippuric acid in the standard solution, and v was the volume of the sample solutions (μl). The results are listed in Table 2.

Results and Discussion

The present high-pressure liquid chromatographic method requires no elaborate sample preparation procedures, and is accurate and reproducible. The average hippuric

Table 2
Recovery of hippuric acid from sample solutions

Amount added (μg)	Amount found (μg)	Recovery (%)
2.5	2.55	102.0
5.0	5.08	101.6
10.0	10.08	100.8
20.0	20.20	101.0
40.0	39.59	98.97
Mean recovery (%)		100.87
RSD (%)		1.16

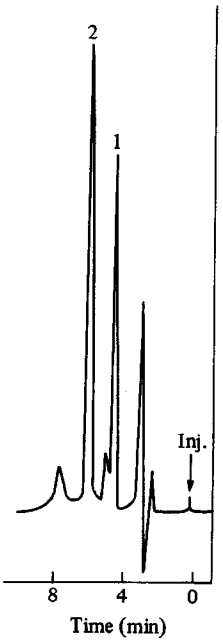

Figure 1
Chromatogram of the reference standard solution containing hippuric acid (peak 1) and salicylic acid (peak 2) as internal standard.

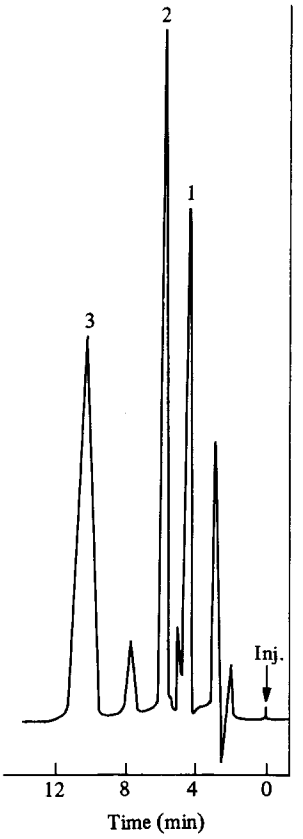

Figure 2
Chromatogram of a sample solution containing hippuric acid (peak 1), salicylic acid (internal standard — peak 2) and Hip–His–Leu (peak 3).

acid recovery is 100.87% ± 1.16 (Table 2). Typical standard and sample chromatograms are shown in Figs 1 and 2. From the reference standard solution chromatogram (Fig. 1) it is seen that the retention times of hippuric acid and salicylic acid (internal standard) are 4.4 and 6 min, respectively. Other internal standards and mobile phases were used in a study carried out to evaluate the possibility of determining hippuric acid in the presence of interfering angiotensin-converting enzyme inhibitors, which can be eluted in a manner similar to hippuric or salicylic acid. Methyl-*p*-oxybenzoate, which elutes before hippuric acid, is a suitable internal standard (t_r = 3 min). The mobile phase composition was varied using the following considerations. The ion-pair technique utilizes the equations

$$K = E_{QX}\,[Q^+] \text{ and } E_{QX} = [QX]_{org}/[Q^+]_{aq}.\,[X^-]_{aq},$$

where K is the distribution ratio, E_{QX} is the extraction constant, Q^+ is the counter-ion and X^- is the organic anion extracted by the counter-ion from the aqueous phase as an ion-pair. Increasing the pH thus produces an increased k (capacity factor) because of the augmented complex concentration. The same result is obtained by utilizing a greater quantity of the aqueous component in the mobile phase, owing to the reduced solubility of the ion pair. The capacity factor k should increase with increasing concentrations of the counter-ion: however, no increase in retention time was observed at 0.01 M tetrabutylammonium concentration, probably because ion-pair formation is quantitative even at 0.005 M concentration.

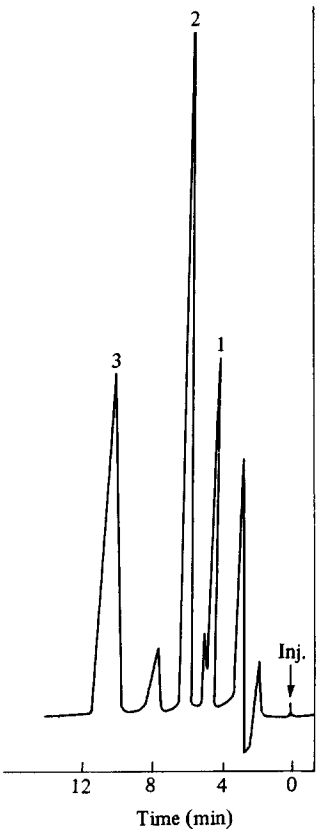

Figure 3
Chromatogram of a sample containing Hip–His–Leu (2.5 mM), salicylic acid (10 μg 100 μl^{-1}) and 50 μg of A.C.E. (1) Hippuric acid formed by enzyme action; (2) salicylic acid (internal standard); (3) Hip–His–Leu (unhydrolysed).

A calibration curve was constructed to verify the linearity of the detector response. The linearity was confirmed over the range 35–560 ng injected (0.005–0.08 mg/ml). Regression analysis gave a slope of 0.1704 and an intercept of 0.00036. The correlation coefficient was 0.9999. Quantitation was carried out with this regression equation using data obtained after alternate injections of standard and sample solutions. This procedure gave good results in terms of recovery and its standard deviation (Table 2). Time-consuming quantitation methods using a daily recalculated calibration curve, or through the mean slope and intercept as determined over a longer period of time, are not strictly required.

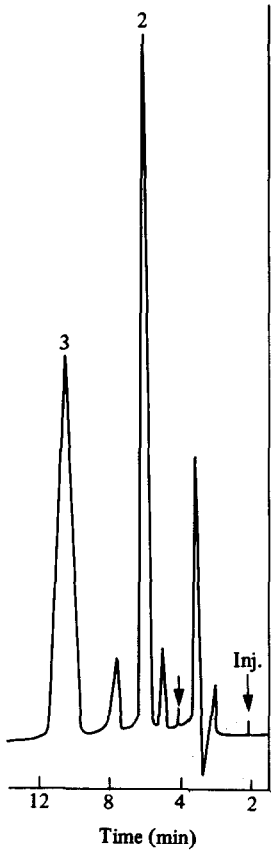

Figure 4
Chromatogram of a sample containing Hip–His–Leu (2.5 mM), salicylic acid (10 μg 100 μl^{-1}), 50 μg of A.C.E., and captopril at a 8×10^{-5} M concentration. Key: (2) salicylic acid (internal standard); (3) Hip–His–Leu.

Figure 2 illustrates a chromatogram of a sample solution and shows that the substrate peak (Hip–His–Leu) emerges with the greatest retention time. Hippuric acid formed by action of the angiotensin-converting enzyme on Hip–His–Leu was determined using the same conditions with the addition of hydrochloric acid after 30 min incubation (Fig. 3). The activity of the angiotensin-converting enzyme was found to be 50 nmol/mg protein/min. The effect of incubation time and substrate concentration on the specific activity was in good agreement with that previously described [8]. Captopril was shown to produce 100% inhibition of the enzyme at a 8×10^{-5} M concentration [13] (Fig. 4).

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