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Short Communication

Direct injection assay of angiotensin-converting enzyme by high-performance liquid chromatography using a shielded hydrophobic phase column

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ABSTRACT

A rapid and sensitive method for the determination of angiotensin-converting enzyme activity in serum and tissue extracts is described. The procedure is based on the high-performance liquid chromatographic separation of the synthetic substrate hippuryl-L-histidyl-L-leucine from the hydrolysis product hippuric acid. The separation is accomplished by direct injection of biological assay mixtures onto a shielded hydrophobic phase column with isocratic elution.

INTRODUCTION

Angiotensin-converting enzyme (ACE) is a dipeptidyl carboxypeptidase (EC 3.4.15.11) which cleaves the decapeptide angiotensin I to the octapeptide angiotensin II, a potent vasoconstrictor. ACE is a key enzyme in the renin–angiotensin system, an important regulator of blood pressure and fluid balance in mammals. It is membrane-bound in lung arterial endothelia where conversion of angiotensin I to angiotensin II occurs [1]. ACE has also been detected as a transmembrane peptidase in a variety of other epithelial and neuroepithelial tissues including the brush borders of placenta, kidney, intestine and choroid plexus [2,3] as well as in various areas of the brain [4] and the male genital tract [5]. Soluble forms of the enzyme occur in various body fluids including blood, amniotic fluid, semen and cerebrospinal fluid [6,7]. Although levels of ACE in human plasma are normally very low (0.4 μg/ml) [8], changes in ACE activity in a variety of conditions have been noted and these changes may be of clinical significance. For example, plasma ACE levels are elevated in several lung inflammatory conditions such as sarcoidosis, silicosis, asbestosis and exposure to underground coal mine dusts [9].

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Using either the physiological substrate, angiotensin I, or various synthetic N-blocked tripeptide analogues, assays of ACE activity have been developed which are based on spectrophotometric [10–12], fluorimetric [13–17], radiochemical [18,19] and high-performance liquid chromatographic (HPLC) [20–23] methodology. These procedures suffer from one or more of the following limitations: (1) expensive, radioactive or not readily available reagents are required; (2) coupling of peptide hydrolysis with chromogenic or fluorogenic reagents is necessary; (3) extensive purification by extraction or chromatography is needed to remove macromolecules and other interferences or to concentrate the product prior to analysis. An enzyme-linked immunosorbent assay (ELISA) has been reported for bovine endothelial cells [24] but it is lengthy and requires the preparation of specific antibodies.

We report here an HPLC method for the assay of ACE in tissues and body fluids which utilizes the readily available tripeptide hippuryl-L-histidyl-L-leucine (HHL) as substrate and monitors the appearance of the hippuric acid product on a shielded hydrophobic phase (SHP) column [25]. The assay method allows for the direct injection of reaction products onto the chromatographic column without prior sample clean-up or modification.

EXPERIMENTAL

Materials

Bicinchoninic acid reagents for protein quantification were obtained in kit form from Pierce (Rockford, IL, USA). Hippuric acid and HHL were obtained from Sigma (St. Louis, MO, USA). All solvents were HPLC grade and other chemicals were of reagent grade. Human serum was obtained fresh in-house. Extracts of ACE were prepared from lungs of rats obtained from CAMM Research Institute (Wayne, NJ, USA).

Lung tissue was homogenized with a Teflon tissue grinder in twenty volumes of ice-cold 50 m*M* phosphate buffer, pH 8.3, and the homogenate was centrifuged at 1000 *g* for 10 min. The supernatant was decanted and re-centrifuged at 30 000 *g* at 4°C for 60 min. The resulting pellet was re-suspended in 3.0 ml of phosphate buffer and stored frozen at −40°C prior to use in enzyme assays. Total protein in the membrane suspension was determined using the bicinchoninic acid method [26].

Enzyme assay

ACE activity was determined using a modification of the spectrophotometric method of Cushman and Cheung [10] in which the hippuric acid produced upon hydrolysis of the substrate HHL is measured. For our assays a final volume of 650 μl containing HHL (5 m*M*), phosphate buffer (pH 8.3, 100 m*M*) and NaCl (276 m*M*) was incubated with either rat lung membrane suspension or serum at 37°C in 1.5-ml polyethylene centrifuge tubes. For rat lung extracts, after a suitable incubation period (15–60 min), the incubation mixture was centrifuged for 2.0 min at 735 *g* to sediment the membranes prior to removing a 20-μl aliquot for hippurate determination by HPLC. For serum ACE assays, a 20-μl aliquot was removed from the incubate and injected directly onto the chromatographic column for hippurate determination.

High-performance liquid chromatography

SHP HPLC was carried out using a Varian Model 2510 liquid chromatograph (Varian, Walnut Creek, CA, USA) equipped with a 250 mm × 4.6 mm I.D. HISEP SHP column with a 20 mm × 4.6 mm I.D. HISEP guard column (Supelco, Bellefonte, PA, USA) and a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, USA) with a 20-μl injection loop. The mobile phase used for isocratic elution at a flow-rate of 1.0 ml/min was 180 m*M* ammonium acetate–acetonitrile (95:5, v/v). Absorbance at 254 nm was measured using a Varian Model 2550 spectrophotometer with an 8.0-μl flow-cell, and peak areas were determined using an SP4270 integrator (Spectra-Physics, San Jose, CA, USA).

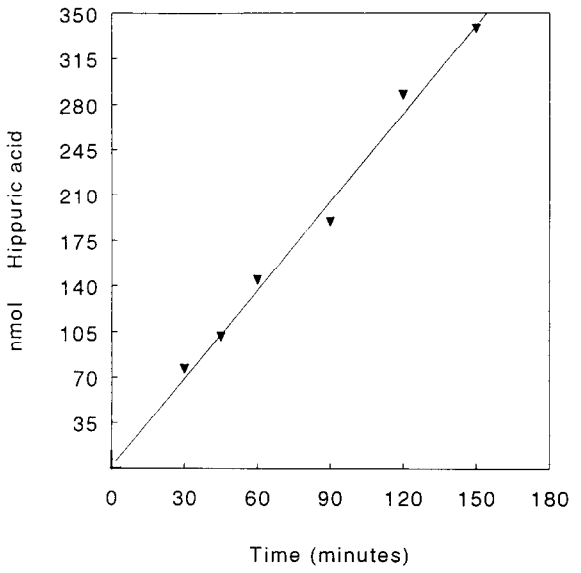

Fig. 1. Time dependence of hippuric acid formation from hippuryl-L-histidyl-L-leucine under standard assay conditions containing 100 μl of human serum.

RESULTS

Baseline separation of hippuric acid from HHL was achieved in less than 7 min on the HISEP column. Activity was quantified by measuring the production of hippuric acid. Detector response was linear over the range $0.2 \cdot 10^{-9}$–

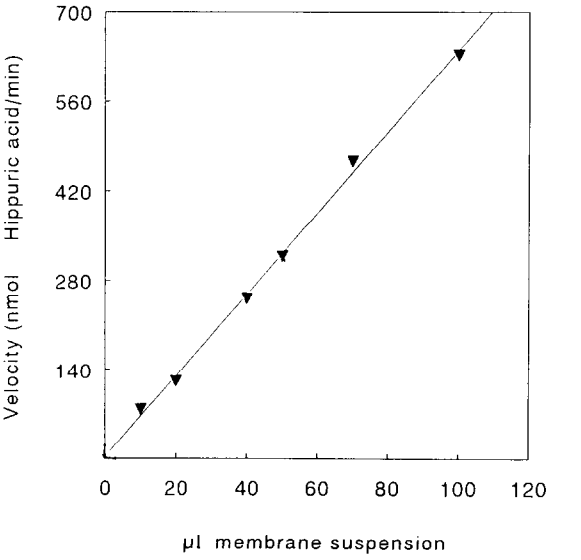

Fig. 2. Hydrolysis of hippuryl-L-histidyl-L-leucine as a function of rat lung membrane suspension volume under standard assay conditions with an incubation time of 30 min.

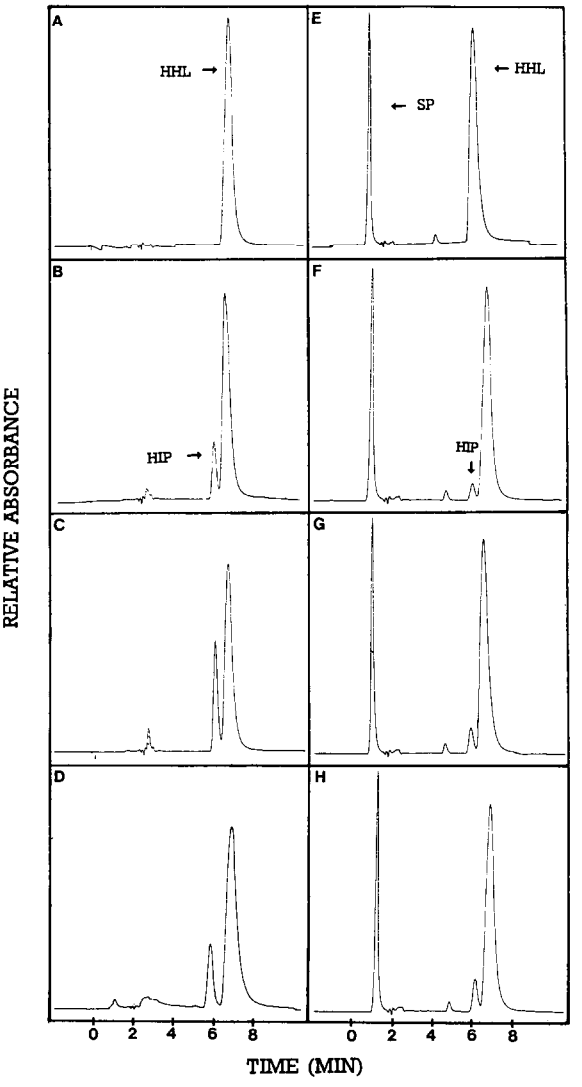

Fig. 3. Chromatograms of ACE assay mixtures prepared from rat lung suspensions (A–D) and human serum (E–H). Chromatograms A, B and C show development of the hippuric acid peak (HIP) at 0, 30 and 60 min incubation at 37°C. Chromatogram D shows an assay of ACE activity after 60 min incubation of the crude rat lung extract (1000 *g* supernatant) prior to sedimentation of the membrane fraction. Chromatograms E, F, G and H show development of the hippuric acid peak at 0, 60, 90 and 120 min incubation. SP = serum protein; HHL = hippuryl-L-histidyl-L-leucine. Chromatographic conditions: column, HISEP (250 mm × 4.6 mm I.D.); mobile phase, 180 m*M* ammonium acetate–acetonitrile (95:5, v/v); flow-rate, 1.0 ml/min; temperature, ambient; detection, UV at 254 nm; injection volume, 20 μl; 0.500 a.u.f.s.

$4 \cdot 10^{-9}$ mol of hippurate. This limit of detection corresponds to 0.02 ACE units for a 150-min incubation period. Under the conditions of our assay, the production of hippuric acid from HHL by human blood serum ACE was linear with respect to incubation time for at least 150 min (Fig. 1) with the mean ± S.D. (n = 6) for ACE activity being 23.3 ± 3.4 units (1.0 unit = 1.0 nmol hippuric acid per min per ml plasma). Rat lung ACE activity was determined to be 249 ± 2.9 units per mg protein (n = 6). Fig. 2 shows a linear response in our assay over a wide range (15–100 μl) of rat lung membrane suspension volumes. A typical series of chromatograms showing baseline separation of hippuric acid from HHL is seen in Fig. 3. Serum proteins are not retained on the HISEP column and appear as a large peak in the void volume. No similar large protein peak is evident in the chromatograms from the rat lung membrane preparation or crude extract where the protein concentration (1.65 mg/ml) is greater than three orders of magnitude less than that found in serum. Captopril at a concentration of $4 \cdot 10^{-4}$ M completely inhibited the production of hippuric acid by ACE.

DISCUSSION

The present study was prompted by a need for a simple, rapid assay for ACE activity that could be applied to crude extracts of biological tissues from a variety of species. A number of new HPLC columns and packing materials are available which eliminate the need for extensive sample preparation by protein precipitation and/or extraction into an organic solvent [27,28]. In SHP columns, macromolecules are excluded from the internal hydrophobic phase by a hydrophilic outer surface and are eluted at the void volume while analytes are selectively retained and chromatographically resolved [27]. To date, these columns have been used primarily for monitoring drugs in serum and have not been utilized for enzyme assays. The assay we have described is simplified by the direct injection of crude assay mixtures. Baseline separation of the substrate and product is accomplished in less than 7 min with no interference from macromolecules at the void volume. A serious disadvantage of common reversed-phase columns is protein adsorption leading to column clogging and concomitant deterioration of column performance. We have noted no such deterioration after several hundred injections and Gisch *et al.* [29] reported no reduction in performance with over 1000 direct injections of drug-spiked serum. It should be noted that our serum samples (100 μl) are diluted into assay mixture (650 μl) prior to HPLC analysis.

The versatility of the assay was demonstrated by application to human serum and to rat lung tissue. The ACE activity in these tissues has been extensively studied previously and the results reported here, 23 units/ml in human serum and 2.9 units/mg tissue (249 units/mg protein) in rat lung are consistent with those reported by others using the same substrate. In human serum, reported normal values of ACE activity using HHL as a substrate have ranged from 7.8 to 29.3 units/ml with a mean of around 20 ± 2 units/ml [22,30,31]. An additional advantage of the procedure reported here is that the presence of hemoglobin in serum from slightly hemolysed blood samples would not interfere as it does in other absorbance procedures because any hemoglobin present would elute at the void volume of the column. In rat lung membrane suspensions, Lanzillo and Fanburg [32] have reported 200 units/mg protein in their P3 fraction.

We believe that the advantages afforded by the new HPLC columns which allow direct injection of crude biological extracts should be widely applicable in enzymology in addition to their current use in therapeutic drug monitoring.

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