Liquid-Chromatographic Assay of Angiotensin-Converting Enzyme in Serum

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This assay of angiotensin-converting enzyme is based on release of hippuric acid from a synthetic tripeptide, hippuryl-L-histidyl-L-leucine. Hippuric acid is separated and quantitated on a reversed-phase column under conditions similar to those used for assay of theophylline and acetaminophen. The method is rapid and free from interferences.

Additional Keyphrases: sarcoidosis - hippuric acid

Angiotensin-converting enzyme (ACE; peptidyl dipeptidase; EC 3.4.15.1) is a dipeptidase that catalyzes the conversion of angiotensin I to form the pressor octapeptide, angiotensin II. The enzyme also catalyzes hydrolysis of many synthetic substrates and inactivates the peptide bradykinin.

Clinically, ACE is measured in serum for the diagnosis and follow-up of patients with sarcoidosis (1-3).

Because the catalytic activity of this enzyme in serum is low, sensitive methods such as radioisotopic (4) and fluorometric (5) are quite desirable, but these methods require special instrumentation and extra steps for labeling. The ultraviolet-spectrophotometric methods for ACE (2, 6) are not very sensitive, necessitate a long incubation, a high ratio of serum to substrate, and solvent evaporation, and they are subject to interferences from lipids (5).

"High-performance" liquid chromatography (HPLC) includes the capability of measuring relatively small changes in absorbance. Here we describe an HPLC method that has the advantage that it can be performed on the same instrument and under the same conditions as used for the assay of theophylline. The method is sensitive, involves a short incubation, requires no solvent evaporation, and is not subject to interferences.

Materials and Methods

Equipment

We used a Model 110 A pump (Altex Scientific Inc., Berkeley, CA 94710) to deliver the solvent through a C_{18} 100-mm cartridge compressed in a Model RCM-60 Radial Compression Module (Waters Associates, Milford, MA 01757) at a flow rate of 2.5 mL/min. The samples were introduced through a 20-μL loop injector (Model 7120; Rheodyne Inc., Berkeley, CA 94710). The effluent was monitored at 254 nm at 0.020 absorbance full scale with a Model 440 ultraviolet detector (Waters Associates).

Reagents

Phospho-saline buffer: Dissolve 21.77 g of K_{2}HPO_{4} and 21.91 g of NaCl in 900 mL of water. Adjust the pH to 8.3 and dilute to 1 L with water.

Substrate: Dissolve 8.0 mg of hippuryl-L-histidyl-L-leucine (Sigma Chemical Co., St. Louis, MO 63178) in 1 mL of 20 mmol/L NaOH. Add 4 mL of phospho-saline buffer and adjust the pH to 8.3 if necessary. Leave overnight in the refrigerator before use (stable for one week). Check each batch of substrate for the presence of free hippuric acid by running a blank assay.

Standard (555.6 μmol/L): Dissolve 100 mg of N-benzoyl-glycine in 1 L of water. Store refrigerated.

Sulfamic acid solution: Dissolve 100 g of sulfamic acid in 1 L of water (stable for two months).

Extraction solvent: Dissolve 30 mg of β-hydroxyethyl-theophylline (the internal standard) in 1 L of a mixture of dichloromethane/n-amyl alcohol (2/1 by vol).

Pump solvent: Dilute 110 mL of acetonitrile to 1 L with acetate buffer (26 mmol/L, pH 4.0).

Procedure

Incubation: The substrate, 100 μL in 1.5-mL centrifuge tubes, was equilibrated at 37 °C, 25 μL of serum was added, and the mixture incubated at 37 °C for 10 min. The reaction was stopped by adding 25 μL of sulfamic acid solution.

Extraction and chromatography: The extraction method for ACE is similar to that used for theophylline (7). Extraction solvent, 100 μL, was added to the incubation tubes and vortex-mixed for 10 s. The tubes were centrifuged at 9000 × g for 120 s (Microfuge; Beckman Instruments, Inc., Fullerton, CA, 92643). A 25-μL aliquot from the organic (bottom) layer was injected onto the column.

Blanks: Blanks are not really needed if the column can separate acetaminophen, hippuric acid, and theophylline, and if the substrate does not contain free hippuric acid. Otherwise, a sample at zero time must be run for each patient.

Standard: Dilute 25 μL of standard with 100 μL of water, add acid and solvent as described for patient’s sample.

Calculation: Enzyme activity (U/L; μmol min/L) = [(peak height of sample/peak height internal std) × 555.6 μmol/L]/[(peak height of std/peak height of internal std) × 10 min].

Results and Discussion

In this assay, ACE liberates hippuric acids from the synthetic tripeptide hippuryl-L-histidyl-L-leucine (5). Hippuric acid, acetaminophen, and theophylline are all eluted on a reversed-phase column with acetonitrile solvent, near one another (Figure 1). The three compounds are reasonably soluble in amyl alcohol, absorb well at 254 nm, and can all be detected electrochemically. The distribution ratio of hippuric acid between organic and aqueous phase is 84:16. In essence, the three compounds can be extracted, eluted, and quantitated by the same method and with the same internal standard. This capability is quite desirable in the clinical laboratory. In about 6000 theophylline assays (7) we saw no other compounds on the chromatograms. If the column can separate these three compounds, there is no need for blanks if the patient is not taking drugs that generate hippuric acid as a metabolic product. However, the column must also separate the amyl alcohol and the dichloromethane to avoid solvent evaporation, otherwise the peak will be nonsymmetrical. In addition to the Radial Compression Module, we found two other columns to be suited for this analysis: the C_{18} μBondapak (Waters Associates) and the C_{18} Alltech (Alltech Associates, Deerfield, IL 60015). The Radial Compression Module gave the best separation most quickly, 5 min (Figure 1). The incubation is
brief in this method, 10 min as compared to 60 min for the spectrophotometric methods (2, 6).

The extraction method in this procedure yields clean chromatograms. Caffeine and the excess of the substrate hippuryl-L-histidyl-L-leucine do not appear on the chromatogram. Figure 2 depicts representative chromatograms of a standard and serum from a normal individual and a patient with sarcoidosis. Hippuric acid production vs time is illustrated in Figure 3. Evidently the reaction is linear for a long period.

In addition to sarcoidosis, ACE is increased in neonatal idiopathic distress syndrome (8) and in Gaucher's disease (9). Unlike some enzymes measured routinely in clinical chemistry, the increase in ACE in sarcoidosis rarely exceeds three times the upper value for the normal range (1, 2). This necessitates good precision. The CV for 36 duplicate assays done during 40 days was 8.3%, with a mean of 45 U/L. Our inclusion of an internal standard improves the reproducibility.

We compared results by our method with those by the method of Frieland and Silverstein (6) (Figure 4). The correlation coefficient is 0.98 ($y = 1.8 + 1.03x; r = 0.98; n = 30$).

The reference interval for 63 adults (mean ± 2 SD) was 9–53 U/L, in agreement with Frieland and Silverstein (5). Ten patients with known sarcoidosis showed a mean of 86 (range, 56 to 145) U/L.

Another procedure for ACE assay by HPLC that has been described (10) requires a special eluent and solvent evaporation. The present method is quite suited for the routine laboratory, because it can be performed on the same column as that used for theophylline assay, without changing solvents and without an evaporation step. The procedure is sensitive, rapid, and free from interferences.

References


