Sensitivity of colorimetric assay for angiotensin converting enzyme in serum

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A sensitive colorimetric procedure has been developed for the assay of angiotensin converting enzyme (EC 3.4.15.1) in serum. Serum (10 μL) is incubated for 30 min with hippuryl-glycyl-glycine as described earlier (Clin Chem 28: 1352-1355, 1982). After a Folin–Wu deproteinization, the liberated glycyl-glycine is derivatized with a borate-buffered (pH 9.3) trinitrobenzenesulfonate solution (60 mmol/L) to form trinitrophenyl-glycylglycine, the absorbance of which is read at 420 nm vs a serum blank. The linear range extends to an activity of more than 900 U/L and the detection limit is <4 U/L. The mean activity for serum from 50 blood donor samples and 25 patients with active sarcoidosis was 281 (SD 77) and 693 (SD 81) U/L, respectively. The method demonstrates good precision (CVs < 2.8%) and correlates well (r = .99) with results from a “high-pressure” liquid chromatographic procedure for determining hippuric acid. In addition, the proposed method is widely applicable, involving only commonly available apparatus.

Additional Keyphrases: sarcoidosis • diseases affecting ACE values

At present, measurement of angiotensin converting enzyme (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1) in serum is indispensable for detecting clinically active sarcoidosis; it is also valuable in monitoring the effectiveness of steroid therapy in this disease (1). Recently, several procedures for the determination of ACE have been published, but have one or more of the following disadvantages: extensive extraction and redissolution steps (2), the need for radioactive substrates (3, 4), the necessity of delicate or expensive auxiliary enzymatic reactions (5, 6), the use of noxious reagents (7), or the need for special instrumentation such as a liquid scintillation counter (3, 4), “high-pressure” liquid chromatography equipment (8), or a fluorometer (9, 1O). These disadvantages preclude widespread use and limit the clinical applications of ACE determinations.

Here we describe a new assay system for ACE, involving only stable and inexpensive reagents and a photometer; it can thus be successfully applied in every clinical laboratory. The sensitivity of the assay is such that only 10 μL of serum will provide reliable and accurate results.

Materials and Methods

Materials

Serum samples: Blood was sampled from blood-bank donors, ages 18–65 years. The blood was allowed to clot, centrifuged within 2 h, and the serum was stored at −25 °C.

Samples were assayed for enzyme activity within a month after collection.

Reagents: Hippuryl-glycyl-glycine (Hep-Gly-Gly) was purchased from Bachem Feinchemikalien, Bubendorf, Switzerland. Sodium tungstate and 2,4,6-trinitrobenzene sulfonic acid (TNBS, grade II) were obtained from Sigma Chemical Co., St. Louis, MO 63178. Glycyl-glycine (Gly-Gly) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Calbiochem, La Jolla, CA 92037. All other reagents were analytical grade from E. Merck, Darmstadt, F.R.G.

Buffered substrate solution. Add to a 20.0-mL flask the following (final concentrations in parentheses): 0.238 g of HEPES (50 mmol/L), 0.351 g of NaCl (300 mmol/L), 1.136 g of Na₂SO₄ (400 mmol/L), and 0.179 g of Hep-Gly-Gly (30 mol/L). Add distilled water and 50 μL of saturated NaOH; after complete dissolution, adjust the pH to 8.15 at 25 °C (8.0 at 37 °C) with NaOH. Dispense 100-μL portions of this solution into test tubes (75 x 8 mm) and store stoppered at −25 °C until used.

Borate buffer (100 mmol/L). Dissolve 9.53 g of Na₂B₄O₇·10H₂O in 250 mL of distilled water and bring to pH 9.6 with NaOH.

TNBS solution (60 mmol/L). Dissolve 406 mg of TNBS in 20 mL of distilled water, and store at −25 °C.

Instruments: Spectrophotometric readings were performed with a Model 300-N micro-sample spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH 44074), or with a Model 8450A spectrophotometer (Hewlett-Packard, Palo Alto, CA 94304). A water bath (37 °C ± 0.1 °C) was used for incubations, and centrifugations were done in a table-top model Labofuge III (Heraeus-Christ, Osterode am Harz, F.R.G.). The pipetting was performed either with a Dilutrend dispenser (Clinicon, Mannheim, F.R.G.) or manually.

Procedures

Spectrophotometric assay. Incubate 10 μL of serum and 100 μL of substrate solution for 30 min at 37 °C. Stop the reaction by adding consecutively 100 μL of sodium tungstate (100 g/L) and 100 μL of dilute (0.33 mol/L) H₂SO₄. Vortex-mix for 10 s and add 1000 μL of water. After mixing, centrifuge for 10 min at 2000 × g. To 750 μL of the supernate, add 1000 μL of borate buffer followed by 50 μL of TNBS solution. Mix, allow to stand for 15 min at 37 °C, and read the absorbance against a serum blank, prepared by adding the deproteinization agents (sodium tungstate and H₂SO₄) to the substrate solution before the serum.

The procedure can be interrupted between deproteinization and arylation for longer intervals (up to at least three days). After addition of the TNBS solution, however, the absorbance must be read within 3 h.

Calculation or calibration procedure: Measure absorbance at 420 nm with an optical pathlength of 1 cm. ACE, U/L = (A serum sample − A serum blank) × 670

\[ \Delta A \times 10^3 \times 1310 \times 1.8 \times 10^{-3} \]

\[ = \frac{15 650 \times 1 \times 30 \times 10^{-5} \times 750} {15 600 \times 1 \times 30 \times 10^{-5} \times 750} \]

The conversion factor (670) was derived from the following:

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1 Nonstandard abbreviations: ACE, angiotensin converting enzyme; Hep-Gly-Gly, hippuryl-glycyl-glycine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TNBS, 2,4,6-trinitrobenzenesulfonic acid; and TNP-Gly-Gly, trinitrophenyl-glycyl-glycine.

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TNP-Gly-Gly = 15 650 L · mol⁻¹ · cm⁻¹; optical path-length = 1 cm; incubation time = 30 min; volume of serum assayed = 10⁻⁵ L; conversion factor from the mol/L dimension of Beer’s law to the ACE units = 10⁵, the ratio of formed split-product transferred for arylation = 750/1310, and the final dilution volume = 1.8 × 10⁻³ L.

One unit (U) of ACE activity is defined as the amount of enzyme required to release 1 μmol of hippuric acid per minute per liter of serum.

If a wavelength other than 420 nm is used (for wavelength interval, see Figure 1), a calibration curve can be prepared with a Gly-Gly standard solution as follows. Dissolve 52.9 mg of Gly-Gly in 10 mL of water (40 mmol/L). Dilute this solution, which contains 0.4 μmol of Gly-Gly per 10 μL, to the following concentrations: 0.2, 0.1, 0.06, 0.04, and 0.02 μmol/10 μL. These quantities correspond to the Gly-Gly produced after 30 min by 10 μL of serum containing 1333, 667, 333, 267, 200, 133, and 67 U of ACE per liter. Use these solutions in place of serum in the previously described procedure, read the absorbance against distilled water, and calculate a calibration line as a regression between the absorbance and the activity.

Comparison method: For correlative studies we used the liquid-chromatographic method of Neels et al. (8).

Results and Discussion

Development and Evaluation of the Method

Using a previously developed incubation procedure (8), we have obtained an enzymatic activity 10-fold greater than that with the currently popular method of Lieberman (2). This rate of hydrolysis was sufficient to allow the quantification of the split dipeptide by a simple derivatization procedure. Although 1-fluoro-2,4-dinitrobenzene is frequently used to derivatize amino acids and peptides (11), we preferred to use TNBS, which reacts in milder conditions and, unlike the dinitro derivative (12), does not react with alcohols, sugars, and lipids.

Deproteinization: Because TNBS reacts with proteins as well as amino acids (13), we could not use a one-step approach but had to deproteinize the incubation mixture. Sulfosalicylic acid, trichloroacetic acid, and perchloric acid were evaluated as deproteinizing reagents, but their supernates were not completely clear after centrifugation. By modifying the Folin–Wu procedure (14), we obtained clear supernates and a reasonable blank value (range 0.15 to 0.3 A).

Derivatization. The TNBS method, as described by Snyder and Sobocinski (15), was not directly applicable to the concentrations of Gly-Gly produced in this assay. We adjusted the pH of the arylation reagent to 9.6 rather than 9.3, to compensate for the sulfuric acid used in the deproteinization procedure. The final pH of the reaction mixture (9.3) is critical, because lower values slow the reaction and higher values decrease the stability of TNP-Gly-Gly.

Because TNBS hydrolizes to picric acid (13, 16) in alkaline conditions, we evaluated this reaction by using parallel-access spectrophotometry. First-order kinetics (k = 2.1 × 10⁻³ min⁻¹; 37 °C) was observed, resulting in a loss of TNBS at a rate of about 10%/h (initial concentration 1.67 mmol/L). This reaction is responsible for the slowly increasing sample and blank readings. Because we used relatively low TNBS concentrations, the net absorbance was not affected, as shown in Figure 2. Thus it is not necessary to quench the formation of picric acid, as some previous investigators have done (13, 16).

Calibration. The calibration curve (see Procedure, and Figure 3) had a correlation coefficient of 0.99, while the CV for the readings varied between 1.4 and 2.0%. The molar absorptivity for TNP-Gly-Gly (15 650 L · mol⁻¹ · cm⁻¹) did not alter when the Gly-Gly was added either to a HEPES salt buffer without substrate or to a deproteinized serum-containing blank.

<table>
<thead>
<tr>
<th>Table 1. Procedure</th>
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<tr>
<td><strong>Sequence</strong></td>
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<tr>
<td><strong>Mix</strong></td>
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<tr>
<td>Buffer substrate solution</td>
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<tr>
<td>Tungstate solution, 100 g/L</td>
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<tr>
<td>H₂SO₄, 0.33 mol/L</td>
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<tr>
<td>Preincubate at 37.0 °C for 5 min, then add:</td>
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<tr>
<td>Serum</td>
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<tr>
<td>Gly-Gly solution, 20 mmol/L</td>
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<tr>
<td>Incubate, stoppered, for exactly 30 min at 37.0 °C, then add:</td>
</tr>
<tr>
<td>Tungstate solution, 100 g/L</td>
</tr>
<tr>
<td>H₂SO₄, 0.33 mol/L</td>
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<tr>
<td>Vortex-mix immediately, then add:</td>
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<tr>
<td>H₂O</td>
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<tr>
<td>Mix, centrifuge (approximately 2000 × g), then mix the following:</td>
</tr>
<tr>
<td>Supernate</td>
</tr>
<tr>
<td>Borate buffer</td>
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<tr>
<td>TNBS solution</td>
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Mix, allow to stand for 15 min at 37 °C or least 30 min at room temperature. Read absorbance of serum sample at 420 nm in a 1-cm cuvette against serum blank.

Fig. 1. Absorbance spectra of TNBS and TNP-Gly-Gly

TNBS, 1.67 mmol/L; TNP-Gly-Gly, concentration corresponding to serum sample with ACE activity of 667 U/L, read against serum blank.

Fig. 2. Color yield vs time

The arylation of the enzymatically liberated Gly-Gly was continuously monitored against the respective serum blank by use of sera with normal and high ACE activity.

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Linearities. That the linear range extends to an ACE activity of more than 900 U/L of serum was demonstrated with an unusually high pathological serum sample (more than the mean serum activity + 2 SD of 25 patients with active sarcoidosis), which showed a straight-line dilution curve (Figure 4) and an activity of 945 U/L. This sample consumed less than 9% of the substrate during the 30-min incubation time, an indication that substrate depletion will not occur with this method. Figure 2 shows a plot of color yield vs. time for both a normal serum and a pathological serum. The TNBS consumption during incubation with a serum having 500 U of ACE activity per liter, including an approximation for the serum blank consumption, is <4%.

Sensitivity. With the procedure described we are able to detect ACE as low as 4 U/L of serum. Using a twofold concentrated derivatization procedure leads to detection of less than 2 U/L of serum.

Precision. Table 2 shows results of within-day and between-day precision studies for both a normal and an abovenormal serum; pipetting was done with a dispenser. A within-day precision study, with manual pipetting of the same sera, had respective CVs of 2.4 and 2.1% (n = 2 x 10).

Correlation. Results obtained by the derivatization technique (y) and those obtained with an assay based on "high-pressure" liquid chromatography of hippuric acid (x) on the same serum specimens, but different incubation mixtures, were correlated by least-squares linear-regression analysis: y = 0.99x + 3.6, r = .99 (n = 50). A least-squares linear-regression analysis of data obtained when the two methods were used to assay the same incubation mixtures gave y = 0.95x + 4.8, r = .99 (n = 39).

Sample preservation. Decreases in ACE activity under various conditions of serum preservation are moderate (Figure 5). Storage at −25 °C until assay seems to be preferred. A control serum stored at −25 °C showed a CV of 4.5% (mean 202 U/L, n = 48) when assayed monthly for a year.

Reference interval. For samples from 50 blood bank donors (25 men, 25 women, ages 18–65 years), the mean ACE activity was 281 (SD 77) U/L.

Clinical Applications of Serum ACE

Sera from 25 untreated patients with active sarcoidosis had increased ACE activity, ranging from 575 to 945 U/L (mean 693, SD 81 U/L). Other studies have confirmed that increased clinical activity of sarcoidosis (2, 17–19) or the need for therapy of sarcoidosis with steroids (1), may be reflected by high ACE activity. The highest ACE activity was found in patients with severe parenchymal lung infiltration due to sarcoidosis, activities being lowest in patients with inactive disease or after successful treatment with steroids (1). Recent reports (20) have also focused attention on the correlation between serum ACE and the 67Ga scan in detecting and monitoring sarcoidosis. Interest in these non-invasive procedures is growing, given the other procedures for investigation of sarcoidosis, such as biopsy, the Kveim–Siltzbach skin test, and serum lysozyme activity. A biopsy

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**Table 2. Precision of Colorimetric Assay for ACE**

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
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<tbody>
<tr>
<td>Within-day</td>
<td>200</td>
<td>4</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>945</td>
<td>15</td>
<td>1.6</td>
</tr>
<tr>
<td>Between-day</td>
<td>197</td>
<td>5</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>944</td>
<td>26</td>
<td>2.7</td>
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n = 10 each.

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**Fig. 5. Stability of pooled sera under various storage conditions**

Each point is the mean of six determinations. Activity was measured within 4 h after sampling (*); after storage at 4 °C (●); after storage at about 20 °C (□); after storage for 24 h at −25 °C; followed by storage at 4 °C (●); and after one, two, and three freeze-thaw cycles (○).
sample may fail to be from an involved site, and granulomas are not necessarily diagnostic of sarcoidosis (20). The Kveim–Sillbach skin test is not generally available, and the serum lysozyme activity is less specific for sarcoidosis than ACE (21).

Some other pathological conditions can also be responsible for increased activities of ACE. In hyperthyroidism, ACE can be significantly increased, probably because excessive thyroid hormone increases the release of this enzyme from vascular endothelial cells (22, 23). Increased ACE activities are also found in some patients with viral hepatitis and liver cirrhosis, probably originating from an altered pulmonary circulation (24–26). Patients suffering from diabetes mellitus, especially when associated with microangiopathy and retinopathy, may also show increased ACE activity (27), as do patients with silicosis and asbestosis (28, 29), leprosy (30), or Gaucher's disease (18, 31, 32). In Gaucher's disease, ACE activity is also increased in the spleen (32). Another condition with increased ACE activity is hypoxia, which can occur in idiopathic respiratory distress syndrome of the newborn (33).

ACE activities are not significantly increased and may even be decreased in four other granulomatous conditions: Crohn's disease, primary biliary cirrhosis, Hodgkin's disease, and active tuberculosis (1, 34–37). ACE activities reportedly are decreased in chronic asthmatics (38, 39), chronic obstructive pulmonary disease (2), and adult respiratory distress syndrome (40). Low activity seems to be associated with a poor prognosis in lung cancer (41), and preoperative ACE values may be a useful prognostic indicator. The normal values for ACE in patients with farmer's lung (42) and pig-elevator's disease (43) differentiate these diseases from sarcoidosis. Normal pregnancy or use of an oral contraceptive (44, 45) does not significantly modify ACE activity.

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References