Characterization of a Spectrophotometric Assay for Angiotensin Converting Enzyme

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Although serum angiotensin converting enzyme (EC 3.4.15.1) activity has generally been shown to be increased in patients with sarcoidosis, considerable variation in the diagnostic usefulness of this test has been reported. We investigated the possibility that this variation may be the result of inhibition of the widely used spectrophotometric assay by various substances. We also prospectively examined the predictive value of measurements of this enzyme in serum from 100 patients being evaluated for sarcoidosis. The following did not significantly affect the results: storage at 4 or 25 °C for one week, hemoglobin, lipoproteins, or corticosteroid medications. Bilirubin, at concentrations of 20 mg/L of serum or more, significantly inhibited the assay. Sera with increased activity showed nonlinear reaction rates during the usual 60-min reaction interval. This was corrected by shortening the reaction time to 30 min. We found a predictive value of 88% when the serum angiotensin converting enzyme test was applied to the diagnosis of active sarcoidosis.

Additional Keyphrases: dipeptidyl carboxypeptidase - sarcoidosis - interference by bilirubin

Angiotensin converting enzyme (dipeptidyl carboxypeptidase; EC 3.4.15.1), or ACE, converts angiotensin I to the pressor compound, angiotensin II, by cleavage of the C-terminal dipeptide, histidylleucine (1, 2). ACE also inactivates circulating bradykinin by cleavage of a dipeptide moiety (3). Although ACE is present in various tissues (4), its activity is highest in the capillary endothelial cells of the lung (5-8).

Increased ACE activity has been found in serum (9-17) and granulomatous tissue (18) from patients with active sarcoidosis. Increased serum ACE activity has also been reported in patients with less-common diseases such as leprosy (19), asbestosis (20), silicosis (20), and Gaucher's disease (21). Electron microscopic and histochemical data suggest that increased serum and tissue ACE activity in sarcoidosis results from its enhanced production by epithelioid cells (22).

Various methods for ACE assay have been described, including spectrophotometric (23), fluorometric (24-27), radioisotopic (28), and chromatographic (29) procedures. In general, patients with active sarcoidosis have been reported to have increased serum ACE activity, but reports vary greatly as to the usefulness of the test (9-17). Possible sources of variability are (a) differences in patient populations, (b) differences in diagnostic criteria for sarcoidosis, (c) differences between methodologies, or (d) the influence of interfering substances on these assays, or some combination of these.

In this study the sensitivity, specificity, and predictive value of increased serum ACE activity were calculated for a large group of patients being evaluated for sarcoidosis. Sarcoidosis was diagnosed according to strict, well-accepted criteria, by persons unaware of the ACE results. We also examined the effect of the following on a widely used spectrophotometric assay for ACE activity: substrate concentration, reaction time, hemoglobin, lipoproteins, bilirubin, storage temperature, and corticosteroid medications.

Materials and Methods

Materials

Serum samples: Blood was obtained by venipuncture from either patients or normal volunteers. Serum was separated and frozen at -20 °C until analysis. In the predictive value study, blood was obtained from 100 consecutive patients referred to a pulmonary medicine clinic for evaluation of possible sarcoidosis. Criteria for diagnosis of sarcoidosis were (a) typical radiographic and clinical findings and (b) biopsy demonstration of noncaseating granulomas in the absence of tuberculous, fungal, or neoplastic diseases (30).

Pools of sera with increased ACE activity (abnormal pools) were obtained by combining sera from eight to 12 patients with active sarcoidosis and ACE activity >35 kU/L (9). Normal pools were prepared by mixing sera from six to eight normal subjects.

Reagents and drugs. Hippuryl-L-histidyl-L-leucine and hippuric acid were obtained from Sigma Chemical Co., St. Louis, MO 63178. Previous reports (9, 23, 24) do not agree on the relative molecular mass of the former preparation, perhaps because of variations in water content. We assumed the theoretical Mr for the anhydrous compound of 429.5 g/mol and took into account the manufacturer’s claim of 95.5% purity for lot no. 57C-6396. Therefore, 56 mg was required to make 10 mL of substrate (12.5 mmol/L).

Hemolysates used to study hemoglobin interference were prepared by hypotonic disruption and centrifugation of washed human erythrocytes.

We prepared lipoprotein precipitates used in the turbidity study from normal human serum, using the dextran sulfate method of Proksch and Bonderman (31).

The effect of bilirubin on the assay was evaluated by use of both a bilirubin control serum (Dade Division, American Hospital Supply Co., Miami, FL 33152) and aqueous bilirubin standards (Sigma Chemical Co.) prepared without dimethyl sulfoxide according to the method of Doumas et al. (32).

Hydrocortisone (A-hydroCort®) was purchased from Abbott Laboratories, North Chicago, IL 60064. Methylprednisolone (Solus-Medrol®) and prednisone (Deltasone®) were purchased from The Upjohn Co., Kalamazoo, MI 49001.

Instrument. All absorbance measurements were made with a Beckman Model DB-G Spectrophotometer. Its wavelength accuracy was verified by use of the emission lines of mercury. Photometric accuracy was verified using Standard Reference Material No. 903c from the National Bureau of Standards, Washington, DC 20234. The photomultiplier tube was a
1P28A and the slit program gave a spectral bandwidth of 1.6 nm at 228 nm. The panel meter was connected to a digital voltmeter capable of indicating the nearest 0.1% transmittance.

Procedure

The spectrophotometric assay described by Cushman and Cheung (23) as modified by Lieberman (9) was used in this study. In this assay, hippurate, removed from the artificial substrate, hippuryl-L-histidyl-L-leucine, by the action of ACE, is extracted into ethyl acetate. This solvent is evaporated and the hippurate is dissolved in 1.5 mol/L sodium chloride. The absorbance is measured at 228 nm vs an appropriate blank (ΔA228). The blank for each sample was prepared and treated identically to the test specimen, except that acid (used to stop the reaction at the end of the incubation interval) was added to the blank just before incubation (9). A unit of serum ACE activity is defined as the release of one nanomole of hippurate per minute per milliliter of serum under the reaction conditions described (9).

Statistics. Statistical comparisons were performed with Student's t-test for nonpaired data. Probabilities ≤0.05 were considered significant.

Results

Some Analytical Variables

Storage temperature. Aliquots of an abnormal pool were stored at either refrigerator temperature (4 to 6 °C) or room temperature (23 to 26 °C) for up to seven days before analysis. As shown in Figure 1, there was no significant loss of activity when sera were stored at either temperature for this length of time.

Precision. Results of within-day and between-day precision studies are shown in Table 1. All analyses were performed with strict adherence to the procedure described above. Between-day precision was evaluated by weekly measurements of ACE activity in normal and abnormal pools during a six-month interval. The results did not show a downward trend during the six-month period, so this study confirms an earlier report (19) that ACE is stable for at least six months at <20 °C.

Incubation time and substrate concentration. The effect of varying the duration of incubation and substrate concentration are shown in Figures 2 and 3, respectively. In each case the assay was performed as described in Methods, except for alterations in the variable examined. As shown in Figure 2, the reaction velocity was linear throughout the usual 1-h incubation when sera with normal (30 kU/L) or decreased (15 kU/L) ACE activity were used. A significant deviation from linearity was observed when the pool with elevated activity (60 kU/L) was incubated for longer than 30 min.

Table 1. Precision of the Spectrophotometric Assay for ACE

<table>
<thead>
<tr>
<th></th>
<th>No. measurements</th>
<th>ACE activity, kU/L</th>
<th>Mean</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
</tr>
<tr>
<td>Within-day</td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
</tr>
<tr>
<td>Normal pool</td>
<td>10</td>
<td>19.2</td>
<td>2.0</td>
<td>10.5</td>
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<tr>
<td>Abnormal pool</td>
<td>10</td>
<td>46.1</td>
<td>2.9</td>
<td>6.4</td>
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<tr>
<td>Between-day</td>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>CV, %</td>
</tr>
<tr>
<td>Normal pool</td>
<td>20</td>
<td>18.3</td>
<td>1.7</td>
<td>9.3</td>
<td></td>
</tr>
<tr>
<td>Abnormal pool</td>
<td>20</td>
<td>40.8</td>
<td>4.0</td>
<td>9.8</td>
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</tbody>
</table>

**Fig. 1.** Effect of storing serum at refrigerator (4–6 °C) (●—●) and room (23–26 °C) (○—○) temperatures before ACE measurements. Each point represents mean and SD of three measurements.

**Fig. 2.** Effect of duration of incubation on ACE activity in pools with above-normal (-O-O-), normal (●—●), and decreased (-Δ-Δ-) ACE activity. Broken lines represent extrapolation of the initial linear portion of the reactions.

**Fig. 3.** Effect of substrate concentration on ACE activity. The Michaelis constant (Km) was graphically determined to be 1.8 mmol/L.

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Figure 3 shows the effect of substrate concentration on reaction velocity. The substrate concentration ordinarily used (5 mmol/L, final reaction mixture) is threefold the measured Michaelis constant ($K_m$) and results in a reaction velocity that approaches the $V_{max}$ for these conditions.

**Interference Studies**

Hemoglobin. Hemolysate was added to an abnormal pool to a final hemoglobin concentration of 10 g/L. There was no significant inhibition of the assay by hemoglobin at any concentration used (Figure 4).

Lipemia/turbidity. Lipoprotein precipitates, lyophilized and reconstituted to enhance turbidity, were added to an abnormal pool. Figure 5 demonstrates that turbidity from these denatured lipoproteins does not affect the spectrophotometric assay. The qualitative descriptions (Figure 5) of "clear," "slightly turbid," and "very turbid" represent a consensus of persons not involved in the study.

Bilirubin. The effect of bilirubin was examined by adding either icteric control serum or aqueous bilirubin standards (described in Methods) to aliquots of an abnormal pool before measuring ACE activity. As seen in Figure 6, there was a significant decrease ($p < 0.05$) in ACE activity when the bilirubin concentration in the serum sample was adjusted to >20 mg/L with either the icteric control serum or the aqueous standards. When the bilirubin concentration was increased to 45 mg/L there was a 60% decrease in activity measured with the spectrophotometric assay.

Corticosteroid preparations. There have been anecdotal reports (9, 19) that the increased ACE activity associated with active sarcoidosis returns to normal during corticosteroid therapy. It has generally been assumed that this decrease in serum ACE activity reflects decreased production by epithelioid cells, but the possibility that this change represents inhibition of ACE by corticosteroids has not been investigated. Figure 7 depicts the effect in vitro of two corticosteroid agents,
methylprednisolone and hydrocortisone, on ACE activity. Methylprednisolone did not significantly affect the assay. Hydrocortisone was a significant inhibitor only when present in concentrations much higher than likely to be found in patients' serum specimens. The in vivo effect of prednisone, the corticosteroid preparation most widely used in the treatment of sarcoidosis, was assessed by measuring ACE activity in normal volunteers immediately before and 2 h after ingestion of 40 mg of prednisone. ACE measurements were performed in triplicate on each sample, and no significant difference between pre- and post-dose ACE activity was found.

ACE in Patient Groups

Group comparison. ACE activity in normal persons, patients with non-sarcoid lung diseases, and patients with active sarcoidosis is shown in Table 2. There was a significant difference (p < 0.05) in ACE activity when the active sarcoidosis group was compared with either the control group or the non-sarcoid lung disease group. The difference in ACE activity between the normal control and non-sarcoid lung disease groups was not significant.

Sensitivity, specificity, predictive value (34). The results of ACE activity measurements in 100 consecutive patients being evaluated in a pulmonary medicine clinic for possible sarcoidosis are shown in Table 3. Forty of these patients met the diagnostic criteria for sarcoidosis; the remainder had other pulmonary diseases. We have defined a "positive" test as one in which the ACE activity was ≥35 kU/L.

Discussion

The spectrophotometric assay for ACE has previously been criticized for two reasons: (a) interference by "significantly high levels of lipids" (25) and (b) "non-linearity with serums having higher (than normal) activity" (9). We investigated the unsubstantiated claim of lipid interference and found no significant inhibition of the spectrophotometric assay for ACE when lipoproteins were mixed with serum before analysis. We have, however, confirmed the observation that the reaction was linear for the usual 1-h incubation interval only when sera with normal ACE activity (up to 30 kU/L) were used (Figure 2). Lieberman has suggested that sera with above-normal activities be diluted and re-assayed (9). We have found that even for sera with elevated activity, the reaction remains linear throughout a 30-min incubation and prefer to use this shorter incubation period. The 30-min reaction time offers four advantages over the usual 1-h incubation interval: (a) errors associated with sample dilutions are avoided; (b) the overall time for analysis is decreased; (c) the expense of re-assay is avoided except for those specimens with ACE activity exceeding 60 kU/L; and (d) 30 min is the incubation interval initially recommended for the spectrophotometric assay (25).

We routinely exclude from analysis by this method all specimens with above-normal bilirubin concentrations, because we have found that even a modest increase in serum bilirubin concentration produces marked interference. This inhibition of the spectrophotometric assay for ACE by bilirubin has not previously been reported, and it is possible that some of the variability in diagnostic usefulness of this test reported in other studies may be the result of it. Mayock et al. (35) report that 21% of a large series of patients with sarcoidosis had liver involvement as part of the disease process. In addition, some of the patients in other series may have had increased serum bilirubin concentrations for other reasons.

The excellent predictive value of a positive test (88%) reflects, in part, our patient selection. We did not attempt to use ACE measurements as a screening test for sarcoidosis in the general population, and if we had done so our predictive value would undoubtedly have been much lower. Instead, we examined 100 consecutive patients referred to a pulmonary medicine clinic for whom a diagnosis of sarcoidosis was actually considered. This allowed for uniform application of diagnostic criteria by experienced physicians and most realistically approximated the specific patient population that would be considered for this test.

In summary, we think the spectrophotometric assay for serum ACE activity is useful in the evaluation of patients for possible sarcoidosis. We recommend a 30-min reaction time and exclusion from analysis by this method of specimens with above-normal bilirubin concentrations.

Table 2. ACE Activity in Normal and Patient Groups

<table>
<thead>
<tr>
<th>No. subjects</th>
<th>Normal</th>
<th>Non-sarcoid lung disease</th>
<th>Active sarcoidosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. subjects</td>
<td>80</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>ACE activity, kU/L, mean</td>
<td>19 (6)</td>
<td>26 (6) *</td>
<td>44 (8) b</td>
</tr>
<tr>
<td>(and SD)</td>
<td></td>
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</tbody>
</table>

* Not significantly different from control group. b Significantly different from normal and non-sarcoid lung disease group.

Table 3. Sensitivity, Specificity, and Predictive Value (34) of Serum ACE Measurements in Active Sarcoidosis

<table>
<thead>
<tr>
<th></th>
<th>No. negative results</th>
<th>No. positive results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients with sarcoidosis</td>
<td>4</td>
<td>36</td>
</tr>
<tr>
<td>Patients without sarcoidosis</td>
<td>55</td>
<td>5</td>
</tr>
</tbody>
</table>

Diagnostic sensitivity: [36/(36 + 4)] × 100 = 90%.
Diagnostic specificity: [55/(55 + 5)] × 100 = 92%.
Predictive value of a positive test: [36/(36 + 5)] × 100 = 88%.

References