A Scheme for Determining the Correct Activity of the Kinetic Angiotensin-Converting Enzyme

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The absorbance difference measured when angiotensin-converting enzyme (EC 3.4.15.1) hydrolyzes the substrate N-[3-(2-furyl)acryloyl]-l-phenylalanlyglycylglycine is the basis for measuring its activity. We show this difference to be instrument dependent, and describe a method for deriving it that is applicable to manual or automated procedures.

Additional Keyphrases: enzyme activity  analytical error

Kinetic spectrophotometry of angiotensin-converting enzyme (ACE; EC 3.4.15.1),1 first proposed by Holmquist et al. (1) and later used for determining ACE activity in human serum (2), appears simple and suitable for the clinical laboratory. Recently, this method has been adapted for the Multistat III centrifugal analyzer (3) and the Olli C Compact Clinical Analyzer (4).

The method involves hydrolysis of the substrate, N-[3-(2-furyl)acryloyl]-l-phenylalanlyglycylglycine (FAPGG), to N-[3-(2-furyl)acryloyl]-l-phenylalanine (FAP) and glycylglycine and measurement of the consequent change in absorbance (ΔA) at 340 nm or 345 nm. Ronca-Testoni (2) found ΔA at 345 nm to be 0.50 for the complete hydrolysis of 1.0 mmol of FAPGG per liter. Neels et al. (3) and Harjanne (4), found ΔA to be 0.80 and 0.58, respectively, for 340 nm and 1-cm lightpath. The different ΔA values reported (3, 4) have not been elaborated upon, nor was it quite clear how ΔA was determined, especially in automated instruments with fixed wavelength settings. Because ΔA is the basis for quantifying the enzyme activity, its accurate determination is necessary to measure ACE activity by methods such as that of Ronca-Testoni (2). When we determined ΔA, based on the absorbance of FAPGG and FAP solutions and of mixtures of them, we obtained a different set of results, as was also the case when measurements were repeated in other spectrophotometers. Evidently, the ΔA value is not a constant but is instrument dependent. In this paper, we propose a method for deriving ΔA that is applicable both to manual and automated procedures.

Materials and Methods

Reagents

FAPGG was purchased from Sigma Chemical Co., St. Louis, MO 63178. FAP was a generous gift from S. Ronca-Testoni (Institute of Biological Chemistry, Faculty of Medicine, University of Pisa, Italy); it can also be purchased from Laboratorio Baldacci, Via S. Michele delgi Scalzi, 56100 Pisa, Italy.

**Borate buffer**, 0.16 mol/L, pH 8.2, containing 0.6 mol of NaCl per liter.

**FAPGG**, 2.0 mmol/L in borate buffer, is stored in a dark bottle at 4 °C and is stable for several weeks.

**FAP**, 2.0 mmol/L in borate buffer.

**Potassium dichromate solution**, 0.2 g/L.

Measurement of ΔA

On enzymic hydrolysis, FAPGG forms FAP and glycylglycine. FAPGG and FAP absorb at 345 nm; glycylglycine does not. A decrease of 1.0 mmol of FAPGG per liter causes an increase of 1.0 mmol/L in FAP, and the change in absorbance measures the net absorbance change due to this decrease in FAPGG and increase in FAP. Because FAPGG absorbs more strongly than FAP, the net absorbance is a negative change.

To simulate an enzyme reaction to deplete 0.1 mmol of FAPGG per liter and form 0.1 mmol of FAP per liter, prepare the following solutions:

(a) Dilute solutions of FAPGG and FAP with the borate buffer to give three concentrations of each: 1.0, 0.9, and 0.8 mmol/L. Measure the absorbance of these six solutions at 345 nm, using matched glass cuvettes of 1-cm lightpath. Use the borate buffer as a blank for the FAP solutions, but use the dichromate blank for the FAPGG solutions because of the high absorbances involved. Plot the measured absorbances of the FAPGG and FAP solutions vs concentration and determine the line of best fit of the slope for each to get the ΔA per 0.1 mmol/L. The difference gives the theoretical net ΔA.

(b) Alternatively, using 1.0 mmol/L solutions of FAPGG and FAP, prepare mixtures of both solutions in the following proportions: 10 + 0, 9 + 1, and 8 + 2, respectively. Thus, the three mixtures contain, per liter, 1.0 mmol of FAPGG, 0.9 mmol of FAPGG and 0.1 mmol of FAP, and 0.8 mmol of FAPGG and 0.2 mmol of FAP. Measure the absorbances of these three mixtures as before vs the dichromate blank and determine ΔA per 0.1 mmol/L as before, which in this case is the net ΔA.

We used the Unicam SP8000 spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.) with the slit width set at 1.0 mm to minimize noise. We also used the Techtron 635 spectrophotometer with 1.0-mm slit width (Varian Techtron Pty. Ltd., Melbourne, Australia) and the Ultrospec 4050 spectrophotometer (LKB-Produkter AB, Bromma, Sweden), which has no slit width adjustments.

Kinetic Assay for ACE

The kinetic ACE assay is performed as follows: Place 1.2 mL of FAPGG solution and 1.0 mL of water in a test tube and warm to 37 °C in a water bath. Add 0.2 mL of serum, mix, and transfer the mixture into a glass cuvette kept at 37 °C in the thermostated spectrophotometer. Adjust the initial absorbance, with respect to the dichromate blank, to about 0.8. In the Unicam SP8000, one can assay four tests.
simultaneously; the absorbances are printed at 5-min intervals for 20 min.

Results and Discussion

The net absorbance change (ΔA) resulting from a decrease of FAPGG by 0.1 mmol/L and an increase of FAP by 0.1 mmol/L as derived by methods a and b is shown in Table 1. When this was determined by the theoretical difference between FAPGG and FAP solutions (method a), the mean value was 0.073 per 0.1 mol/L with the Unicam. However, ΔA as determined from the actual mixture (method b) is 0.083. Because the mixture more realistically reflects the change arising from the enzymic hydrolysis of FAPGG, we used the value 0.083 for calculating enzyme activity.

Thus with the Unicam SP8000, 0.83 is the millimolar ΔA of FAPGG "hydrolyzed". The ACE activity, in U/L, according to Ronca-Testoni (2), is (ΔA/min) × 2.4 × (1000/0.83) × (1/0.2) or (ΔA/min) × 14 458.

Interestingly, ΔA as measured with the Varian and the LKB spectrophotometers gave results that were different not only for the two methods but also for different spectrophotometers (Table 1). As before, ΔA was greater for the mixture than the value derived by difference. The differences in ΔA values as determined by use of the three spectrophotometers confirm that ΔA is instrument dependent.

We compared results by the kinetic method (y) with those obtained by colorimetry (x) (5) for 87 samples. The resulting least-squares linear-regression equation, calculated according to Deming, was y = 1.67x + 13, and the correlation coefficient (r) was 0.86. The kinetic ACE assay gave a mean activity of 68 U/L with a 2 SD range of 32–105 U/L for samples from 54 blood donors. This reference interval for normal subjects is lower than those previously reported (2–4) because of the higher ΔA used in calculating the enzyme activity. Like Rømer (6), we have not observed any significant sex-related differences in reference interval, although this has been reported by others.

The within-day precision (CV) of the kinetic method was 3.9 and 4.0% for normal and above-normal ACE activity in serum for respective activities (mean ± SD) of 72 ± 2.8 and 116 ± 4.7 U/L (n = 12 each). For between-day precision, the CVs were 3.0 and 3.5%, respectively, for ACE activities of 85 ± 2.6 and 141 ± 4.9 U/L (n = 12 each).

The kinetic assay we outline is essentially a modification of Ronca-Testoni’s method (2), to use standard cuvettes with a 1-cm lightpath. We consider this kinetic ACE method to be the easiest and most convenient method available for the clinical laboratory. The method is also ideal for measuring the low ACE activity in bronchoalveolar lavage fluid, where a larger sample volume, e.g., 1.2 mL, may be used in the assay method and the water omitted.

The scheme we propose allows ΔA to be easily and accurately measured on manual and automated systems. The finding that ΔA is instrument dependent is consistent with the different ΔA values previously reported (2–4), although the importance of this observation has not been previously appreciated. Unless ΔA is accurately established, the kinetic measurement of ACE activity may not be reliable.

We thank Dr. C. Beng for his interest and helpful discussions.

References


Table 1. Measurement of ΔA from the Substrate/Product Difference and from the Mixture, by Different Spectrophotometers

<table>
<thead>
<tr>
<th>Instrument</th>
<th>FAPGG</th>
<th>FAP</th>
<th>FAPGG – FAP (method a)</th>
<th>Mixture (method b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pye Unicam</td>
<td>0.162</td>
<td>0.089</td>
<td>0.073</td>
<td>0.083</td>
</tr>
<tr>
<td>Varian</td>
<td>0.135</td>
<td>0.069</td>
<td>0.066</td>
<td>0.079</td>
</tr>
<tr>
<td>LKB</td>
<td>0.106</td>
<td>0.061</td>
<td>0.045</td>
<td>0.056</td>
</tr>
</tbody>
</table>

Results are the mean of quadruple analyses.