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Automated Kinetic Determination of Angiotensin-Converting Enzyme in Serum

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In this automated kinetic modification of a previous method (*Anal Biochem* **95**: 540–548, 1979) for determining angiotensin-converting enzyme (EC 3.4.15.1), 3-(2-furylacryloyl)-L-phenylalanylglycylglycine is used as the substrate. The change in absorbance at 340 nm is used to monitor hydrolysis of the substrate. The rate of hydrolysis is roughly threefold greater than with previously reported substrates, so assay time and sensitivity are improved.

Additional Keyphrases: *enzyme activity · reference interval*

Requests for determination of angiotensin-converting enzyme (ACE, EC 3.4.15.1) in serum, for differential diagnosis of lung sarcoidosis and its treatment with corticosteroids, have been increasing in recent years.[1] Of the several published methods for assay of this enzyme, most either require special equipment or are very laborious. Moreover, none is adaptable to automated analysis.

I describe here an automated kinetic modification of the ACE method of Holmquist et al. (*1*), later adapted to serum by Ronca-Testoni (*2*), in which a furanacryloyl-blocked tripeptide, 3-(2-furylacryloyl)-L-phenylalanylglycylglycine (FAPGG) is used as substrate. FAPGG absorbs strongly in the low-wavelength visible and high-wavelength ultraviolet region, with a maximum at 305 nm. Measurement of ACE activity is based on the change in the absorption spectrum when the substrate undergoes hydrolysis to furylacryloyl-L-phenylalanine (FAP) and glycylglycine. The absorbance change after this reaction is greatest at 328 nm but the difference is well measurable up to 352 nm. This method can be applied to automated enzyme analyzers that allow measurements to be made at 340 nm. ACE activity can be measured kinetically in either equilibrium mode or two-point mode.

Materials and Methods

Reagent and buffer solution. FAPGG was purchased from Sigma Chemical Co., St. Louis, MO. I prepared 80 mmol/L borate buffer containing 0.3 mol of NaCl per liter from sodium tetraborate decahydrate and NaCl and adjusted the pH to 8.2 (at 37 °C) with 1 mol/L HCl. The solution was filtered and stored refrigerated. The 0.8 mmol/L substrate solution, prepared by dissolving 32 mg of FAPGG in 100 mL of the buffer, was stored in a brown glass bottle in the refrigerator; it can be used for several weeks. Just before use in an assay, the substrate solution was allowed to come to room temperature.

Samples. Serum was separated from patients' blood without delay and stored at −20 °C, a temperature at which the analyte seems to be stable for several months. Even if samples are thawed and then stored in the refrigerator, the ACE activity is essentially unchanged if the measurements are done within one or two weeks.

Apparatus. I used an Olli C Compact Clinical Analyzer (Kone Oy, Espoo, Finland) with program revision 8.1, which allows a kinetic measurement of enzymes in the two-point mode. ACE assays were made at 340 nm and 37 °C, with a measuring time of 10 or 15 min, depending on the mode used. The two-point mode is preferred, owing to the easy,

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[1] Nonstandard abbreviations: ACE, angiotensin-converting enzyme; FAPGG, 3-(2-furylacryloyl)-L-phenylalanylglycylglycine; FAP, furylacryloyl-L-phenylalanine.

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rapid handling. The value for the zero standard (distilled water) was entered into the program and the mean value for duplicate determinations of samples was automatically calculated.

Enzyme assay. Pipet 50 µL of distilled water, control sera, or serum samples into two 4-mL plastic Olli-tubes of 10-mm diameter. Place the tubes in a prewarmed heating block (the Olli equipment holds 24 samples). Add 0.5 mL of substrate solution and mix the tubes' contents by shaking in a mechanical shaker for 15 s. Incubate the samples at 37 °C for 5 min. Transfer the set of samples into the Olli C measuring head and start measuring the reaction. After 10 min, the analyzer prints the results in U/L at 37 °C. The means of the double assays are calculated according to the program, and the individual absorbance differences (ΔA) and units are also printed.

One unit (U) of ACE is that amount of the enzyme that will hydrolyze 1 µmol of the substrate FAPGG into FAP and glycylglycine in 1 min at 37 °C.

Enzyme activity is calculated as follows:

$$\frac{V_t\,1000}{\Delta e V_s d} \times \Delta A/\text{min} = \text{activity, in U/L at 37 °C}$$

where V_t is the final assay volume (550 µL), V_s the sample volume (50 µL), d the cuvette diameter in cm (1), and Δe is the maximum change in absorbance at 340 nm produced by hydrolysis of 1 mmol of FAPGG. The absorbance decreases during the reaction, so the ΔA/min will have a negative value. At 340 nm, Δe is about −0.58; thus the factor is −1897 for the 10-min measuring time.

Results

I compared values for ACE for 66 ostensibly normal persons, as measured by the method of Cushman and Cheung (*3, 4*), and by the present method, with the following results: The respective mean values (and SD and range) were 26.2 (8.5, 12–43) µmol/min per liter and 94.1 (30, 34–156) U/L. For 2 SD limits, the normal interval for the present method would thus be 35–155 U/L (16–45 µmol/min per liter for the Cushman method).

On comparing values for a total of 100 patients with both normal and elevated ACE activity, I obtained the following linear-regression equation: $y = 3.574x - 6.06$, $\bar{x}$ (Cushman) = 39.58 (SD 16.9), $\bar{y}$ (present method) = 135.40 (SD 64.3), r = 0.946. The factor for multiplication of the Cushman values to kinetic values will thus be about 3.6.

Control sera with normal and above-normal activities were assayed for within-run and between-run precision studies; the results are reported in Table 1.

Table 1. Precision Data

	ACE, U/L			
	Mean	SD	CV, %	n
Within-run	126	2.9	2.3	17
	168	3.1	1.9	10
Between-run	62	2.8	4.4	20
	165	4.1	2.5	20

Discussion

The K_m value of the substrate is 0.31 mmol/L (*2*); the substrate concentration used in this assay was 0.80 mmol/L, or 2.5-fold the K_m value. Results by this reaction varied linearly with activity concentration up to 300 U/L, i.e., to a ΔA of −0.160 (about 40% hydrolysis of FAPGG). Reaction time was kept at 10 min because at longer times (>30 min) the reaction velocity of sera having high ACE activity tends to decrease. On the other hand, because the total activity of ACE is relatively small in comparison with many other serum enzymes measured kinetically, an assay time of 1 to 2 min is too brief, the absorbance difference in serum with normal ACE activity being only about −0.005 per minute.

In my first studies, I obtained about the same results as those reported here by using a substrate concentration of 0.40 mmol/L, slightly more than the K_m value of 0.31 mmol/L. Later, however, a new batch of substrate yielded lesser results. Because the substrate is at present available at a reasonable price, it is better to keep the substrate concentration clearly in excess over the K_m value; one can thus be sure that the substrate hydrolysis is linear with time and also proportional to enzyme concentration.

The pH of the substrate solution tends to decrease slowly and one must check that the optimal narrow pH range of 8.0 to 8.2 is maintained during the reaction (*2*).

The measurements I report were done in a batch-type analyzer, in which 24 tubes were measured simultaneously. With this system and measuring ΔA for 10 min, one can easily analyze 30 to 40 samples in duplicate per hour, which is much faster than the older methods (*3, 4*).

I chose to use 340-nm wavelength and the 10-mm cuvette diameter because these are commonly used in clinical laboratories. Under these conditions the initial absorbance of the substrate solution is about 1.5, which most analyzers can measure accurately.

The millimolar absorbance difference (Δe) at a certain wavelength can be determined with partly purified rabbit lung enzyme. Its value can be approximated from the difference absorption spectrum between FAPGG and FAP (*2*). At 328 nm Δe is $-2.300\ \text{cm}^{-1}$; at 345 nm the complete hydrolysis of 1 mmol of FAPGG gives Δe of −0.50 (*1, 2*).

FAPGG has a great affinity for the enzyme and is hydrolyzed faster than the ACE substrates used in previous assays, such as hippuryl-L-histidyl-L-leucine (*3*). Consequently, the normal reference interval will also be higher; I found a normal range of 35 to 155 U/L at 37 °C.

This method application is readily adapted to any clinical analyzers capable of measuring ΔA at 340 nm during 10 to 15 min at 37 °C; the ACE activity can be calculated either manually or with a built-in microprocessor. With this new rapid method more clinical laboratories can measure ACE activity easily, reliably, and inexpensively.

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