Automation of a Kinetic Method for Determining Angiotensin-Converting Enzyme in Serum, James L. Groff,1 Roman B. Rutkowski,2,3 and Nancy B. Brantley3

Serum angiotensin-converting enzyme (ACE) has for many years been particularly valuable in the diagnosis of pathological changes in lung tissue, such as occurs in sarcoidosis (1) and asbestosis (2). Additionally, the enzyme plays a very important role in circulatory homeostasis, and recent studies on polymorphism of the gene controlling the synthesis of ACE have shown that certain genotypes predispose to higher concentrations of serum ACE and have been identified as a risk factor for myocardial infarction and cardiomyopathy (3–5). This interest in ACE as a possible participant in cardiovascular disease has stimulated the search for improved methods for monitoring serum concentrations of the enzyme.

We report an automated method for assay of serum ACE, based on modification of a manual method that we published previously (6). The reagents used in the automated method are the same as those in the manual method. However, in adapting the method to the automated system, some reagents were able to be combined to reduce the number of programmed reagent additions.

All assays were conducted on a Hitachi Analyzer, Model 911 (Boehringer Mannheim, Indianapolis, IN). The optional two-point rate mode was selected for the assay. Serum samples were collected from inpatients at a local hospital. Samples were stored under refrigeration (5°C), and were assayed within 2 days after collection.

ACE, N-hippuryl-glycylglycine (Hip-Gly-Gly), HEPES, l-glutamyl-3-carboxy-4-nitroanilide (GGCN), glycyglycine (Gly-Gly), and l-glutamyltransferase (GGT) were all purchased from Sigma Chemical Co. (St. Louis, MO). Reagent solutions, including buffered ACE substrate, HEPES/saline buffer, GGT solution, and GGCN solution, were prepared as described (6). All solutions were stored frozen in suitable aliquots. However, because of its high concentration of salts, the buffered ACE substrate must be mixed thoroughly after thawing just before use and, once thawed, it cannot be subjected to another freeze/thaw cycle. Analytical reagents were prepared in the following proportions:

Analytical reagent 1. Add 260 μL of the GGT solution to 8.0 mL of buffered ACE substrate solution. This reagent must be prepared just before use.

Analytical reagent 2. Add 400 μL of the GGCN solution to 7.6 mL of HEPES/saline buffer. We prepare this reagent just before use, although it is stable refrigerated (5°C) for at least 48 h.

Before beginning an analysis, reagents 1 and 2 should be allowed to equilibrate to the temperature of the reagent storage compartment of the instrument. This takes ~10 min for our instrument, which has a storage temperature of 10°C.

The following protocol was used in the assay: To 20 μL of serum was added 200 μL of reagent 1. After a programmed incubation period of 10 min at 37°C, 250 μL of reagent 2 was added. Assay points (absorbance readings) were at 1 min and 6 min after addition of reagent 2. The incremental change in absorbance at 415 nm during this 5-min reaction time was recorded. For calibration, we selected the two-point calibrator mode. Pertinent instrument settings for the Hitachi 911 Analyzer include: assay code/reaction time, 2-point rate/15 min; assay point, [33–48]; sample volume, 20 μL; absorbance limit, 32 000; reagents, T1 (reagent 1), 200 μL, T4 (reagent 2), 250 μL; calibration type, linear; number of calibration points, 2; wavelength (primary), 415 nm.

We used a serum pool and a fourfold dilution of the pool as working calibrators. The ACE activity of the pool was determined from comparative assay values of calibrator solutions of Gly-Gly, 5 and 10 mmol/L. One unit of ACE activity is defined as the amount of enzyme required to release 1.0 μmol of Gly-Gly from Hip-Gly-Gly per minute per liter of serum. Therefore, corrected for sample volume and incubation time, sera with activities equivalent to these calibrators would contain 500 and 1000 U/L, respectively. Our working calibrator sera, which were assayed to contain 580 U/L and 145 U/L, were stored frozen in 500-μL portions and thawed when needed.

We determined the range of linearity of the method by measuring the activity of commercially obtained ACE, which we serially diluted in serum. Activity values were linear to at least 3000 U/L, which is nearly 6 times the mean value of 512 U/L obtained for 48 inpatients’ sera. These sera were also used for a correlation study between the present method (y) and another automated method for ACE (x) based on the ACE-catalyzed spectral shift of N-(3-[2-furyl]acryloyl)-l-phenylalanylglycylglycine (7). Results of the correlation study yielded the following linear regression equation: y = 9.13x + 178 (r = 0.914, S_y|x = 84.3).

A between-run statistical evaluation of the method conducted with a serum having normal ACE activity yielded the following results: mean = 444 U/L, SD = 18.5 U/L, CV = 4.17%, n = 10.

The automated method follows the same chemical principle as the manual method from which it evolved (6). However, in planning for the method’s adaptability to analyzers that may be limited to two programmed reagent additions, we consolidated some of the reagents. For example, the sample is incubated with reagent 1, which contains both the GGT reagent and the ACE substrate. The presence of the GGT in the incubation mixture causes the rate of the ACE-catalyzed reaction to be increased by nearly 30%—probably because of binding of Gly-Gly to its catalytic site on GGT and the consequent enhancement effect on the ACE-catalyzed reaction. This speeding of the reaction rate increases the sensitivity of the method, which is particularly important in automated systems because of the limited incubation times available. The positive GGT effect on the reaction, and our use of sub-
strate having a composition optimal for ACE activity (8), account for the relatively high ACE unit values we report for this method. The presence of GGT does, however, accelerate an autolytic release of Gly-Gly from Hip-Gly-Gly in the reagent 1 solution, falsely increasing apparent ACE activity. Nonetheless, because calibrator activity and sample activity are affected equally, recalibration before each run fully corrects for this effect, even over a period of several days. This is shown by our between-run CV of 4.17% for replicate sampling over a 3-day period with the same calibrator sera.

In the absence of Gly-Gly, GGT catalyzes the transfer of a γ-glutamyl moiety of a GGCN (donor) molecule to a second (recipient) GGCN molecule. The reaction releases the 3-carboxy-4-nitroaniline chromophore from the GGCN donor molecule, just as it does when Gly-Gly is the acceptor substrate. Referred to as the autotransfer reaction (6), this therefore causes a significant ΔA/t even at zero ACE activity (no released Gly-Gly). However, the contribution to ΔA/t by the autotransfer reaction is constant, irrespective of the presence of or concentration of Gly-Gly, the preferred acceptor substrate in the reaction. Reaction rates are therefore strictly linear with respect to Gly-Gly concentrations, and consequently to ACE activity (6).

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References

Free Amino Acids in Amniotic Fluid and the Prenatal Diagnosis of Homocystinuria with Methylnalonic Aciduria, Philippe Parvy,1 Jacqueline Bardet, Bernadette Chadeaux-Vekemans, Daniel Rabier, Martine Gasquet, Joelle Aupetit, and Pierre Kamoun (Lab. de Biochim. Méd. B, Hôpital Necker–Enfants Malades, 149, rue de Sèvres, 75743 Paris Cedex 15, France; 1 author for correspondence: fax 33-1-44-49-51-60)

Measurement of free amino acids in amniotic fluid obtained between 11 to 13 and 17 to 22 weeks of gestation is an important step for the prenatal diagnosis of only three amino acidopathies: citrullinemia, argininosuccinic aciduria, and sulfite oxidase deficiency (1–3). Recently, we studied free amino acids in amniotic fluid from an at-risk family for homocystinuria with methylnalonic aciduria (CblC or CblD mutant). Diagnosis of the index case (dead at age 6 months) was performed by enzymatic study of N5-methylenetetrahydrofolate homocysteine methyltransferase activity.

Amniotic fluid from a subsequent pregnancy was obtained by transabdominal amniocentesis at 13 weeks of gestation. Centrifugation at 4 °C and deproteinization with sulfosalicylic acid (40 g/L) were immediately performed to prevent formation of disulfide bonds between homocysteine and proteins. Ion-exchange analysis for free amino acids was performed with a Beckman 6300 Amino Acid Analyzer, with use of ninhydrin detection, a 10-cm high-performance column for lithium methodologies, and four lithium buffers (A, D, E, and F) all purchased from Beckman (Gagny, France). The manufacturer's methodology was slightly modified. The modified operating settings for buffers were: A, 132 min; D, 32.8 min; E, 74.0 min; F, 80.0 min; and regeneration reagent, 130.0 min; for temperature, the settings were: T1, 132.5, 33.8 °C; T2, 14.0 min, 65.5 °C; and T3, 75.0 min, 70.0 °C. These modifications allow the detection of homocysteine and cysteine–homocysteine mixed disulfide. The profile clearly showed the presence of homocysteine, cysteine–homocysteine mixed disulfide, and a small peak of homocysteine (Fig. 1).

None of these three compounds was detectable in amniotic fluid controls obtained from amniotic fluid withdrawn for cytogenetic analysis (n = 74; 10 to 32 weeks of gestation) according to ethical standards of the institution's Committee for Ethics. Measured concentrations of sulfur-containing amino acids (μmol/L) were: half-cystine, 67 (for nine controls, at 10 to 13 weeks of gestation; range 53–106, mean 84); methionine, 26 (controls: range 28–41,

Fig. 1. Amino acid chromatography of the amniotic fluid: 1, cystine; 2, homocysteine; 3, methionine; 4, cysteine–homocysteine disulfide; 5, homocysteine.
bc, buffer change.