Direct Determination of Angiotensin-Converting Enzyme Inhibitors in Plasma by Radioenzymatic Assay

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We describe a simple, rapid, specific radioenzymatic assay for "CGS 16617," a new, potent inhibitor of angiotensin-converting enzyme (ACE; EC 3.4.15.1) in human plasma. This assay is based on the principle that the inhibitor (i.e., the drug) binds to the ACE in plasma and hence the amount of free ACE in plasma is inversely related to the amount of active inhibitor present. Free enzyme is reacted with a radiolabeled substrate, and the radioactive product is selectively extracted into the scintillation cocktail for quantification. Fivefold-diluted plasma samples are incubated with [3H]hippuryl-glycyl-glycine enzyme substrate at 37 °C for 30 min and the liberated [3H]hippuric acid is selectively extracted into scintillation cocktail. The radioactivity is counted in a liquid scintillation counter. Both within-run and between-run, the variability (CV) of the assay is <10%. As little as 200 ng of the drug per liter can be quantified in 50-μl plasma samples. The method can also be used to assay two other ACE inhibitors, pentopril and CGS 14831, demonstrating that the method can be readily adapted to any ACE inhibitor having a single active component in plasma. The ester prodrug pentopril can also be assayed after ester hydrolysis. This method is suitable for analysis of large numbers of samples in clinical laboratories for routine monitoring of the concentrations of active ACE inhibitors in blood.

Additional Keyphrases: CGS 16617  CGS 14831  pentopril  monitoring therapy  hypertension  heart disease

In recent years, inhibitors of angiotensin-converting enzyme (ACE; dipeptidyl carboxypeptidase I, EC 3.4.15.1) offer a new approach to the treatment of hypertension and congestive heart failure (1, 2). Two prototype compounds, captopril and enalapril, are being marketed, and several others are undergoing clinical investigation (3-7). For improved oral absorption, some of these ACE inhibitors are administered as more lipophilic prodrug esters (3-5), which are hydrolyzed in vivo by esterase to release an active ACE inhibitor. Because of their high potency, most of these ACE inhibitors are therapeutically active at unusually low (nanogram) concentrations in plasma. Their paucity of intrinsic ultraviolet absorbance or fluorescence properties has added to the challenge of monitoring these drugs' concentrations in plasma. The analytical methods currently available for ACE inhibitors include radioimmunoassay (8, 9), gas chromatography/mass spectrometry (10), and liquid or gas-liquid chromatography (11-13) for some relatively less potent inhibitors. Generation of antibodies for use in radioimmunoassay requires several months; moreover, many clinical laboratories lack facilities for antibody production or a mass spectrometer to measure concentrations of individual drugs.

The present radioenzymatic assay is based on the principle that the inhibitor (drug) binds to the ACE in plasma and hence the amount of free ACE in plasma is inversely related to the amount of active inhibitor present. Free enzyme is reacted with a radiolabeled substrate and the radioactive product is selectively extracted into the scintillation cocktail for quantification. The concentration of active ACE inhibitor in plasma is, therefore, measured in the patient's plasma by comparison with a standard curve made from results for additions of analyte to the same patient's blank plasma. Plasma containing orally administered prodrugs is hydrolyzed to release the ACE inhibitor, which is then extracted from the sample; the concentrations of the total inhibitor (ester plus acid) are measured after an aliquot of blank plasma is added as the source of enzyme. We hydrolyze the samples with esterases from rat plasma rather than by chemical hydrolysis.

Here we describe the development of a radioenzymatic assay for three ACE inhibitors currently under clinical investigation—pentopril, CGS 14831, and CGS 16617 (Figure 1). Pentopril, like enalapril, is an ester prodrug that, on hydrolysis, releases an active ACE inhibitor (CGS 13934). Detailed data for CGS 16617 are presented to demonstrate the precision, accuracy, and usefulness of this radioenzyme assay.

Materials and Methods

Reagents and Chemicals

The pH 8.0 assay buffer ("A.C.E. Microvial Buffer"; Ventrex Laboratories, Inc., Portland, ME) contained, per liter, 50 mmol of NaAc (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1 mol of NaCl, 0.6 mol of Na2SO4, and 1.0 g of sodium azide. The enzyme substrate ("A.C.E. Microvial Substrate"); [3H]hippuryl-glycyl-glycine in pH 8.0 buffer and the assay scintillation cocktail ("Scintillation Cocktail no. 1"); a mixture of toluene, ethyl acetate, and scintillators) were also purchased from Ventrex Labs. We used "Scintisol"...
(Isolab Inc., Akron, OH), a scintillation cocktail for $^3$H and $^{14}$C, for determining total-counts radioactivity.

All CGS drug compounds were obtained from the Pharmaceuticals Division, Ciba-Geigy Corp., Suffern, NY.

Procedures

Preparation of plasma. Whole blood was collected into heparinized Vacutainer Tubes (Becton-Dickinson, Inc., Rutherford, NJ) from normal, healthy volunteers. After centrifugation, we removed the plasma by aspiration and pooled it for storage at −20 °C for several months until analysis.

Preparation of standards and other drug-supplemented samples. We prepared aqueous stock 1.0 g/L solutions of CGS 16817 and stored these in 0.5-mL aliquots for as long as two months at −20 °C. A fresh 10.0–μL aliquot of the 1.0 g/L stock, thawed for preparing each standard curve, was diluted with 990 μL of distilled water to yield 10.0 mg of CGS 16817 per liter. Further dilution of the 10.0 mg/L stock with distilled water resulted in stock solutions of 20, 30, 50, 100, 200, and 250 μg/L. We added 10 μL of each of these dilute stock solutions to separate 990-μL aliquots of plasma to yield standards having respective final concentrations of 0.2, 0.3, 0.5, 1.0, 2.0, and 2.5 μg of CGS 16817 per liter. Inhibitor-supplemented samples were prepared similarly by adding 10 μL of aqueous stock solutions (concentration 20 μg/L to 10 mg/L) to 990 μL of plasma to yield samples with ACE inhibitor concentrations of 0.2 to 100 μg/L.

Determination of ACE activity. Standards and samples were analyzed with the A.C.E. Microvial Radioassay System, as detailed by the manufacturer. Plasma samples with a drug concentration of more than 2.5 μg/L were diluted with blank plasma to be within the standard curve range of 0.2 to 2.5 μg/L; all plasma standards and samples were diluted fivefold with the HPEFs buffer. Triplicate 50-μL aliquots of the diluted standard or sample were added to separate 2.0-mL Wheaton microvials and pre-incubated for 5 min at 37 °C. Substrate (50 μL) was added to each microvial and the incubation continued for 30 min at 37 °C. We stopped the enzyme reaction by adding 50 μL of 0.5 mol/L HCl and vortex-mixing. After the incubation with ACE substrate was stopped, the samples were allowed to come to room temperature (>10 min), at which time any product formed (Hippuric acid) was separated from unreacted substrate (Hippuric-glycyl-glycine) by addition of 1.5 mL of scintillation cocktail to each microvial and vigorous shaking for 30 s. Under these conditions, approximately 69% of the hippuric acid and less than 1% of the unreacted substrate enter the organic phase. Because radioactivity in the aqueous phase does not interfere with measurement of radioactivity in the organic phase, phase separation is not necessary and the radioactivity in the microvials can be counted immediately after shaking. The assay blank was determined by substituting buffer for the diluted plasma during the 30-min incubation. Total counts of radioactivity per microvial were determined by adding 50 μL of substrate and 1.5 mL of Scintisol to triplicate microvials and vortex-mixing for 30 s. We used a Tricarb 4530 liquid scintillation counter (Packard Instrument Co., Downers Grove, IL) equipped for quench correction by external standardization. We assayed all samples and standards in triplicate and used the average disintegrations per minute (dpm) in our calculations.

Hydrolysis of prodrugs. After diluting 50 μL of plasma sample with 200 μL of HPEFs buffer, we added 100 μL of rat plasma. After incubating the samples at 37 °C for 30 min, we acidified them to pH 1–2 with 50 μL of phosphoric acid (pH 0.6), then extracted the hydrolyzed metabolite into 4 mL of methylene chloride, vortex-mixed for 30 s, then centrifuged for 10 min at 500 × g. We transferred 3 mL of the organic (lower) layer to a 12 × 75 mm test tube and evaporated the solvent under nitrogen. After redisolving the residue in 250 μL of HPEFs buffer and vortex-mixing for 30 s, we transferred 200 μL of this to a microvial and mixed it with 50 μL of fivefold-diluted blank plasma. This sample, which now contained hydrolyzed metabolite, was then analyzed for active ACE inhibitor as described above.

Calculation of results. We converted dpm to ACE "Activity Units" (AU) by means of equation 1 (14).

\[
AU = \frac{V}{T} \left\{ \ln \left( \frac{1}{S - Bl} \right) \left( \frac{1}{K_p(\TC) - (Bl)} \right) \right\} \times 100
\]

where:
- \( V \) = reaction volume/sample volume
- \( T \) = time of reaction (min)
- \( S \) = dpm of sample
- \( Bl \) = dpm of blank
- \( TC \) = total dpm
- \( K_p \) = extraction efficiency of scintillation cocktail

To convert AU to percentage inhibition, we used equation 2:

\[
\% \text{ inhibition} = 100 \left[ 1 - \left( \frac{\text{sample AU}}{\text{blank plasma AU}} \right) \right]
\]

Determination of drug concentration. We plotted standard curve data as AU vs concentration (μg/L), using a computer curve-fitting program (Fit Function, RS/1; BBN Research Systems, Cambridge, MA) to fit the following exponential function for determining the standard curve.

\[
AU = A \times e^{B \cdot \text{conc}}
\]

where A and B are parameters of the exponential standard curve, and "conc" is the concentration of the ACE inhibitor in plasma. Inhibitor concentrations in unknowns were calculated by interpolation from the standard curve. We also used a Michaelis–Menten function (equation 4) to determine the inhibitor concentration in plasma that gave 50% of maximum inhibition (IC50). This function allows the comparison of relative potencies among the ACE inhibitors.

\[
\% \text{ inhibition} = \frac{I_{\text{MAX}} \times \text{conc}}{IC_{50} + \text{conc}}
\]

where \( I_{\text{MAX}} \) is the maximum inhibition.

Results

Comparison of three ACE inhibitors. Figure 2 shows a comparison of the inhibition curves for the three ACE inhibitors in drug-supplemented plasma. The concentration in plasma for 50% of maximum inhibition is 19.8, 1.98, and 0.75 μg/L for CGS 13934, COS 14831, and CGS 16817, respectively.

Measurement of ester prodrug. Figure 3 shows the percent inhibition of pentopril (prodrug of CGS 13934), hydrolyzed pentopril, and CGS 13934 in drug-supplemented human plasma. Between 10 and 100 μg/L, the inhibition by both hydrolyzed pentopril and CSG 13934 were similar, while the inhibition of unhydrolyzed pentopril, even at a 10-fold higher concentration (100–1000 μg/L) was less than 10%. This suggests almost no cross reactivity of unchanged
Fig. 2. Plasma inhibition curves with fitted Michaelis-Menten curves for three active ACE inhibitors: CGS 13934 (O), CGS 14831 (△) and CGS 16617 (□).

Concentrations for CGS 13934 are in μg/L; those for CGS 14831 and CGS 16617 are in 10⁻² μg/L. Fitted curves: CGS 13934 [---, 14.6x(1.98 + x); CGS 16617 [-----, 125x(0.75 + 0.01x)].

Fig. 3. Comparison of inhibition of plasma ACE by pentopril (●), hydrolyzed pentopril (○), and its metabolite CGS 13934 (△) added to plasma.

Concentrations of pentopril are as shown × 10. Fitted curves: pentopril [-----, 0.0056x + 5.4]; hydrolyzed pentopril (-----, 0.51x + 17); and CGS 13934 (----, 0.46x + 22).

The inhibition from the lowest concentration in the standard curve, 200 ng/L (20.5% inhibition).

Stability of CGS 16617 and ACE activity on storage. Plasma samples (n = 6, each in duplicate) supplemented with CGS 16617 and stored at −70 °C for three months showed a mean (± SD) recovery of 102.8% (± 5.6%) at concentrations ranging from 1.0 to 80.0 μg/L. In addition, plasma samples collected from a normal volunteer after a 20-mg oral dose of CGS 16617 were analyzed once a month for three months. The mean CV was 10.7%, and it did not indicate any instability in either drug concentration or enzyme activity.

Specificity of the radioenzymatic inhibition assay. Concentrations in 64 samples of plasma from four volunteers were also measured by a gas chromatographic–mass spectrometric procedure (unpublished data, Ciba-Geigy Corp.) and the results compared in order to determine the specificity of the radioenzymatic determination (Figure 4). The linear relationship between concentrations determined by the two methods, with a slope of 0.93, intercept of almost zero (0.32), and coefficient of determination of 0.97, indicates that the radioenzymatic method is specific for analysis of human plasma samples after oral doses of CGS 16617. A paired t-test analysis also showed no significant difference (p >0.05) in concentrations in plasma as determined by these two methods.

Fig. 4. Comparison of concentrations in plasma as determined by GC-MS and by radioenzymatic inhibitor methods.

The fitted line is the mean regression line, for which the equation is 0.93x + 0.32

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Table 1. Between-Run Variability of Measurements of CGS 16617 in 12 Drug-Supplemented Samples of Human Plasma

<table>
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<tr>
<th>Mean*</th>
<th>SD</th>
<th>CV, %</th>
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</table>

* n = 4 each

The inhibition from the lowest concentration in the standard curve, 200 ng/L (20.5% inhibition).

Day-to-day variability in ACE concentration. Blood sampled from one subject in the placebo portion of a clinical study with CGS 16617 was analyzed to determine the changes in the ACE concentration in plasma during a usual study period. Plasma ACE was measured at 0, 8, and 24 h on days 1, 15, and 29. The mean AU was 5.46. The within-subject CV for enzyme activity was only 5.2%. Changes in activity ranged up to a maximum of 15.3%, which is below
Discussion

Inhibition curves for the three ACE inhibitors we studied show the generality in application of the radioenzymatic method for active ACE inhibitors after appropriate dilution of plasma samples. Although the theoretical maximum inhibition is 100%, the asymptotic estimation of $I_{\text{MAX}}$ was as high as 146% for CGS 14831. This is because the maximum inhibition measured was only about 80%. Above 80% inhibition, the error in estimation of free enzyme activity is relatively high because of the asymptotic nature of the inhibition curve. The coefficient of determination of the regression line determined within the range 0 to 80% inhibition, however, is quite high (multi $r^2 > 0.999$).

The estimated IC$_{50}$, the concentration for 50% of maximum inhibition, indicated that the potencies of the three ACE inhibitors were in the order CGS 16617 > CGS 14831 > CGS 13934. More than 75% of plasma ACE activity is inhibited at plasma CGS 16617 concentrations of 1–2 $\mu$g/L.

As seen in Figure 3, the percent inhibition of ACE activity in plasma after hydrolysis of pentopril was comparable with that for supplemented metabolite (CGS 13934), suggesting that conversion of pentopril on hydrolysis is complete, while cross reactivity of the intact prodrug, pentopril, was minimal. Even at a pentopril concentration of 1000 $\mu$g/L, the enzyme activity was not appreciably inhibited (<1%). Because of the incomplete recovery of the hydrolyzed inhibitor during extraction, the sensitivity of this method is slightly less than in situ determination of the active ACE inhibitor. Use of the esterase in rat plasma to hydrolyze the prodrug gave more consistent results than did chemical hydrolysis with sodium hydroxide.

Swanson et al. (15) recently reported a similar method based on radioenzymatic assay. In contrast to our method of direct monitoring of the active ACE inhibitor concentration in untreated plasma, their method requires precipitation of endogenous ACE followed by the addition of exogenous ACE and separation of the aqueous phase from water-immiscible scintillation cocktail. In contrast to their study, in our method the standard curve for CGS 16617 is plotted as enzyme activity, rather than % inhibition, versus concentration. This has the advantage that each data point is directly measured and is not influenced by any error in the measurement of activity in blank plasma (equation 2). This method also allows logarithmic transformation to linearize the standard curve instead of the required nonlinear regression of a Michaelis–Menten function.

The in situ determination of the active inhibitor concentration in plasma is simple, rapid, and accurate. The method requires no expensive instrument such as a mass spectrometer or liquid- or gas-chromatograph, or expertise in any unusual analytical technique. The method involves several dilution steps and incubation at 37 °C followed by measurement of the radioactivity of the product in a scintillation counter.

An average of 30 plasma samples, each in triplicate, can be analyzed each day by an analyst using standard hand pipettors. Although the method has been developed with triplicate runs for each sample, duplicate analysis would suffice for the necessary accuracy of determination and would considerably improve the efficiency of analysis. Analysis time can also be significantly shortened by using automated pipettors.

Although this method would not be expected to have absolute specificity, we find the cross reactivity to be minimal. The contribution of activity by the prodrug is negligible (<5%). Except for captopril, most of the active ACE inhibitors are almost entirely excreted unchanged via the kidney (3, 16). CGS 16617 is excreted >90% unchanged in urine after its intravenous administration to rats (unpublished data, Ciba-Geigy Corp.). The method can also be extended to determine the inhibitor concentration in a patient's plasma for whom the corresponding blank plasma is not available. Two methods can be used, depending on the patient's drug concentration in plasma. If the lowest expected drug concentration is at least fivefold the lowest standard concentration, the patient's plasma is simply diluted fivefold with any blank plasma, and the standard curve is derived by use of the same blank plasma. This minimizes any inter-subject enzyme variability and allows one standard curve from blank plasma to be used with many patients' plasma samples. If the patient's drug concentration is too low for it to be diluted in blank plasma, the drug must be assayed directly. This would require extraction of the active inhibitor as discussed for prodrugs, followed by reconstitution in any blank plasma. The inhibitor concentration in the sample can then be determined from the standard curve derived from use of the same blank plasma.

In conclusion: the specificity of this method was demonstrated by comparison with a gas chromatographic–mass spectrometric selective-ion-monitoring procedure, the results of which showed no significant difference (paired t-test, $p > 0.05$) in measured concentrations in plasma after oral administration of CGS 16617 to humans. Except for captopril, inhibition of plasma ACE activity is in general contributed entirely by the single active ACE inhibitor, whether given directly (CGS 16617 or lisinopril (17)) or as prodrug (enalapril (17), pentopril (3), ramipril (18)). Hence, this method would be suitable for use in analysis of large numbers of samples in clinical laboratories for routine monitoring of active ACE inhibitors in blood.

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References


14. ACE Microvial Radioassay System, manufacturer's brochure.


