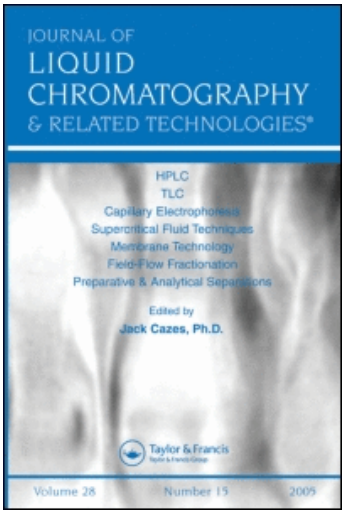


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# A Direct Injection Assay of Angiotensin Converting Enzyme in Tissue Extracts

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# A DIRECT INJECTION ASSAY OF ANGIOTENSIN CONVERTING ENZYME IN TISSUE EXTRACTS

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## ABSTRACT

A rapid, sensitive and selective method for the determination of angiotensin converting enzyme (ACE) activity in tissue extract and serum is described. The procedure is based on the high performance liquid chromatographic separation of the synthetic substrate furylacryloylphenylalanyl-glycyl-glycine (FAPGG) from the hydrolysis product furylacryloylphenylalanine (FAP). Baseline separation is accomplished in ten minutes by direct injection of biological assay mixtures onto a 15 cm shielded hydrophobic phase column with isocratic elution using 180 mM ammonium acetate/ 15% acetonitrile as the mobile phase. Both substrate and product are detected by absorbance at 305 nm.

## INTRODUCTION

Angiotensin-converting enzyme (ACE) is a key component of the renin-angiotensin system, an important regulator of blood pressure and fluid balance in mammals.[1] Physiologically, ACE is a dipeptidyl carboxypeptidase ( EC 3.4.15.11) which cleaves the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II, an octapeptide. ACE is known to participate in the degradation of bradykinin and other neuropeptides. [2] Specific inhibitors of ACE have been used for the control of hypertension in man.[3,4]

ACE is a zinc metallopeptidase which occurs in both soluble and membrane-bound forms. The enzyme has been detected in a wide variety of tissues and organisms.[5-9] The determination of ACE activity is clinically significant in human serum, as well as physiologically important in many other tissues.[10] Currently, assays of ACE are carried out using synthetic substrates, most often hippuryl-histidyl-leucine (HHL).[11,12] Here, we describe an HPLC based assay using an alternative substrate FAPGG which has several advantages over HHL.[13] Chromatography is performed on a shielded hydrophobic phase (SHP) column which allows for direct injection of assay mixtures without prior protein precipitation or product extraction. The procedure is simple, rapid, and more sensitive than the spectrokinetic assay using this substrate.[14]



## EXPERIMENTAL

### Materials

FAP, FAPGG, captopril, and purified rabbit lung ACE were obtained from Sigma. All solvents were HPLC grade and other chemicals were of reagent grade. Human serum was obtained fresh in-house. The marine worm, *Nereis diversicolor* was obtained from Marine Biological Laboratory, Woods Hole and crude extracts were prepared by homogenization of tissue in 20 volumes of ice-cold 50 mM phosphate buffer, pH 8.3.

### Methods

### Chromatographic analysis

Shielded hydrophobic phase high performance liquid chromatography (SHP-HPLC) was carried out using a Varian Model 2510 liquid chromatograph

equipped with a 250 mm x 4.6 mm I.D. HISEP SHP column with a 20 mm x 4.6 mm I.D. HISEP guard column (Supelco) and a Rheodyne Model 7125 injection valve with a 20 μL injection loop. The mobile phase used for isocratic elution at a flow rate of 1.5 mL/min was 180 mM ammonium acetate-acetonitrile (85:15, V/V). Absorbance at 305 nm was measured using a Varian Model 2550 spectrophotometer with an 8 μL flow cell and peak areas were determined using a Spectra-Physics Model 4270 integrator.

**Measurement of ACE activity**

Angiotensin-converting enzyme (ACE) activity was determined using a modification of the spectrophotometric method of Baudin and Giboudeau[13] in which the hydrolysis of the substrate FAPGG to FAP is measured. Each assay had a total volume of 650 μL containing FAPGG (1 mM), phosphate buffer (100 mM, pH 8.3), NaCl (276 mM), and either human serum, crude *Nereis* extract, or purified rabbit lung ACE solution, and was incubated at 37°C in a 1.5 mL polyethylene centrifuge tube in a shaker bath. After a suitable incubation period (15-60 min), the incubation mixture for crude extacts was centrifuged for 2.0 min at 735 g prior to removing a 20 μL aliquot for HPLC analysis. For serum assays, a 20 μL aliquot was removed from the incubate and injected directly onto the chromatographic column.



## RESULTS

A series of chromatograms of standard assay mixtures containing increasing amounts of human serum are shown in Fig. 1. Baseline separation of the substrate FAPGG and the product FAP of the ACE catalyzed reaction is accomplished in 10 minutes. ACE activity is detectable in as little as 1.0 μL of human serum if an incubation time of 60 min is utilized. Of course, much shorter incubation times may be used if larger volumes (up to 100 μL) are available. A typical assay of ACE activity in 100 μL of human serum is accomplished in 15 min.

Note that although the peak at 10.40 min is not obvious in Frame A of Fig. 1 at 1.0 aufs, the signal was well above background for the electronic integrator.

It can be seen in Fig. 2 that the production of FAP was linear with respect to time for at least 60 min over a ten-fold range of enzyme activity units and that 0.5 mU ACE was reliably detected.

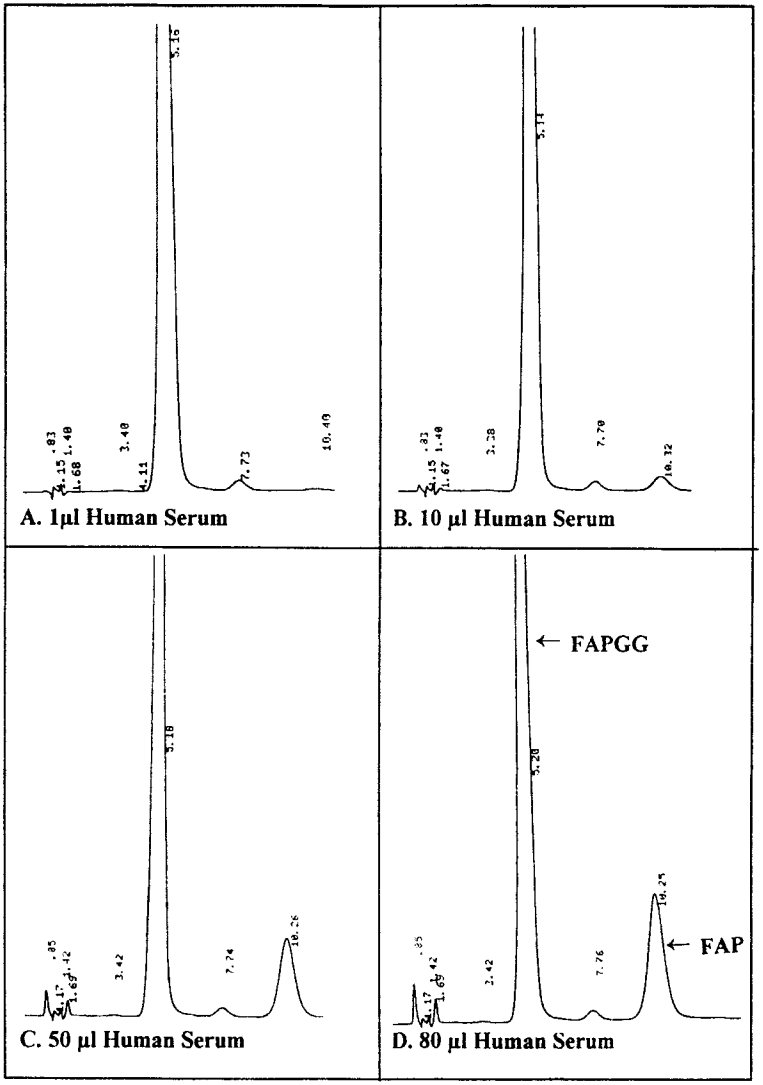


**Figure 1**. Chromatograms of ACE assay mixtures containing 1, 10, 50, and 80 µL human serum (panels A-D, respectively) showing development of the FAP peak with a retention time of approximately 10 minutes. Assays mixtures were incubated for 60 min at 37°C prior to chromatography. Chromatographic conditions: column, HISEP (4.6 x 20 mm); mobile phase, 180 mM ammonium acetate:acetonitrile (85:15); flow rate , 1.5 mL/min; temperature, ambient; detection, UV at 305 nm; injection volume, 20 µL; aufs = 1.0.

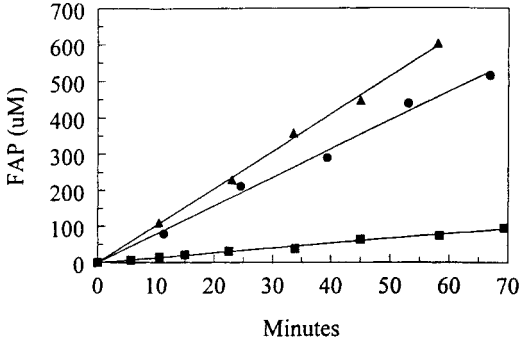


**Figure 2**. Assay linearity over time with three different concentrations of purified rabbit lung ACE . Each value is the mean of replicate assays. In no instance is the variance greater than 10%. ■ = 5 x $10^{-4}$ U ACE, ● = 1 x $10^{-3}$ U ACE, ▲ = 5 x $10^{-3}$ U ACE.



Production of FAP was also linear with respect to the amount of enzyme (Fig. 3) for all three sources of enzyme. Captopril ( data not shown) at a concentration of 4 x $10^{-4}$ M completely inhibited the production of FAP by each of the ACEs.

## DISCUSSION

The present study was prompted by a need for a sensitive assay for ACE that could be applied to crude extracts of biological tissues from diverse sources. These extracts are complex mixtures that often contain high concentrations of light-absorbing species and enzymes, other than ACE, which have proteolytic activity. The combination of the substrate FAPGG with SHP-HPLC analysis obviates many of the problems associated with the determination of ACE activity in such extracts.

A major advantage of the substrate FAPGG over the most frequently used synthetic substrate HHL is that both FAPGG and the hydolysis product FAP absorb maximally above 300 nm. Therefore, the assay using FAPGG and monitoring absorbance at 305 nm is less subject to interferences encountered with HHL and the hydrolysis product hippurate which have absorbance maxima at 238 nm and are usually monitored at 254 nm.

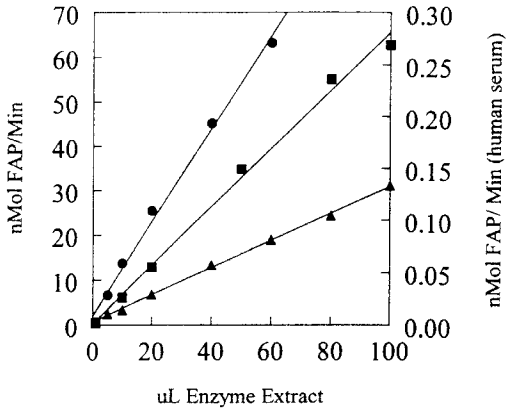


**Figure 3**. FAP production as a function of enzyme concentration from three different tissue sources. Each value is the mean of replicate assays. In no instance was the variance greater than 10%. ● = rabbit lung ACE, ▲ = *Nereis diversicolor* crude extract, ■ = human serum. The left Y axis is scaled for FAP production by rabbit lung and *Nereis diversicolor* ACE and the right Y axis is scaled for FAP production by human serum ACE



Additional advantages of FAPGG are that stock solutions of the substrate are stable at 4°C for more than a year, the rate of hydrolysis is considerably faster, and, most importantly, FAPGG is a more specific substrate for ACE in that it is not cleaved by carboxypeptidase A as is HHL[13]. This is particularly important in studies involving tissues from the digestive tract.

Although FAPGG is the preferred substrate for ACE activity measurement because of its specificity and lack of background interference problems, the poor sensitivity of the spectrophotometric assay has limited its use. This assay is based on a blue shift in the absorbance spectrum upon substrate hydrolysis,[14] but the difference absorption spectrum is relatively small at its maximum at 328 nm. In the modified procedure reported here, separation of the substrate and product by HPLC allow for a 23-fold increase in sensitivity by utilizing the absorbance maximum at 305 nm and 60 minute incubation times. An additional advantage of the HPLC-based assay is that both the disappearance of substrate and the appearance of product are simultaneously monitored. Moreover, the use of SHP columns allows for direct injection of assay mixtures without need for prior protein precipitation or product extraction.[15] Thus, the assay is simple, rapid, sensitive, and applicable to a wide range of tissues without interference.

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