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An optimized fluoroenzymatic assay for the determination of angiotensin converting enzyme inhibitors in biological fluids

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Abstract: An easy, analytically sound fluoroenzymatic assay for angiotensin converting enzyme (ACE) inhibitors is described. Drug samples and standards are extracted with methanol and evaporated to dryness. Drug residues are then incubated with the substrate *N*-benzyloxycarbonyl-phenyllanyl-L-histidyl-leucine and human plasma ACE at 37°C, pH 7.65, for 1 h. Fluorescence of the *o*-phthaldialdehyde derivatized product is measured at wavelengths of 365 nm (excitation) and 490 nm (emission). A computer program converts fluorescence to percent of ACE activity inhibited and correlates this percent inhibition with drug concentration. The ester prodrug enalapril (MK-421) was measurable at levels of a 1 ng ml^{-1} in serum after base hydrolysis to enalaprilat. Lowest reliable detection limits for enalaprilat (MK-422) and lisinopril (MK-521) in serum were 0.7 ng ml^{-1}. This method is easily adapted to most other ACE inhibitors, is well suited to automation and avoids the use of radioactivity.

Keywords: *Angiotensin converting enzyme inhibitor; lisinopril; enalapril; enalaprilat; captopril; fluoroenzymatic assay; angiotensin converting enzyme.*

Introduction

Pharmacological investigations of the growing family of angiotensin converting enzyme (ACE) inhibitors (Fig. 1) are increasing as more of these antihypertensive drugs become available for testing and as they gain in clinical popularity. Several methods exist for the determination of ACE inhibitors in plasma and urine. Radioimmunoassay (RIA) has typically been the method of choice because both sample throughput and sensitivity are high [1, 2]. High-performance liquid chromatography (HPLC) and gas–liquid chromatographic (GLC) methods are either relatively insensitive or difficult and time consuming [3, 4]. An inhibitor binding assay (IBA) was recently introduced [5], but as with RIA, radiolabelling techniques are required. A newly published radioenzymatic assay (REA) uses an innovative and simple procedure for sample preparation [6]. Although a

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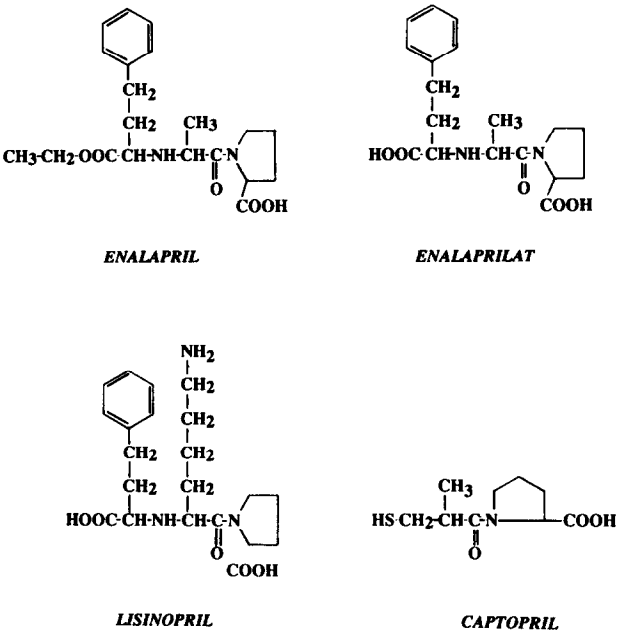

Figure 1
Chemical structures of the prodrug enalapril; its active metabolite, enalaprilat; the lysine analogue of enalapril, lisinopril; and the sulphydryl containing inhibitor, captopril.

fluoroenzymatic assay (FEA) is sensitive, it is labour intensive and costly [7]. The purpose of this report is to describe a new non-radioactive assay for ACE inhibitors which incorporates the REA method of sample preparation and other significant improvements into the original fluoroenzymatic technique.

Experimental

Reagents and standard solutions

All chemicals were of reagent grade quality or better. The assay buffer consisted of 0.1 M Tris-HCl in 0.3 M sodium chloride, pH 7.65 at 25°C. The HPLC-grade methanol was obtained from Fisher Scientific Co. (King of Prussia, PA, USA). Substrate solution was prepared by dissolving *N*-benzyloxycarbonyl-phenylalanyl-L-histidyl-leucine (Bachem, Torrence, CA, USA) in methanol to form a 4 mg/ml stock solution. This was diluted 1 + 9 with assay buffer to form the working substrate reagent. The working ACE solution was prepared by diluting human serum possessing high ACE activity (9–10% met min^{-1} ml^{-1} as assayed by the method of Swanson *et al.*) [8] with assay buffer to give a concentration which produces 20% substrate hydrolysis under the prescribed incubation conditions. Alternatively, a solution of purified serum ACE [9] in Tris-HCl buffer with equivalent ACE activity may be employed. Using purified ACE allows the omission of the final centrifugation step. Histidyl-leucine (HIS-LEU; Sigma Chemical, St. Louis, MO, USA) standards (0, 3.3, 10, and 33.3 μM) were prepared daily from refrigerated stock solutions, which were prepared fresh every two weeks. *O*-Phthaldialdehyde reagent (OPD) (Sigma Chemical, St. Louis, MO, USA) was prepared daily by dissolving

2.6 g in 14 ml of methanol and then adding 190 ml of 0.28 M sodium hydroxide to yield the working solution. Lisinopril (MK 521), enalapril (MK 421), and enalaprilat (MK 422) were supplied by Merck, Sharp and Dohme Research Laboratories (West Point, PA, USA). Captopril (SQ 14,225) and captopril disulphide (SQ 551) were supplied by E.R. Squibb and Sons (Princeton, NJ, USA). Plasma drug standards containing lisinopril, enalapril or enalaprilat were prepared by diluting aqueous stock solutions to yield final concentrations of 0.0, 0.4, 0.8, 1.5, 3.0, 6.0, 8.0, 10.0, 20.0, and 35.0 ng ml^{-1}. All final dilutions in the preparation of the standard solutions were 1 + 9 with plasma. Captopril plasma standards were prepared at a 10-fold higher concentration. Urine drug standards were prepared by diluting aqueous stock solutions to yield final concentrations of 0.0, 40, 80, 150, 300, 600, 800, 1000, 2000, and 3500 ng ml^{-1}. As with plasma, all final dilutions were 1 + 9 with urine. In cases where dilution of plasma samples prior to analysis was necessary, dilutions were made with blank plasma to maintain a constant background fluorescence. A 1 + 99 dilution of urine standards and samples with water was required to avoid interference from background fluorescence. Quinine sulphate (1 μg ml^{-1}) in 0.05 M sulphuric acid was used as a daily check of spectrofluorometer performance.

Assay procedure

A schematic diagram illustrating the various steps involved in the assay procedure is presented in Fig. 2. All pipetting steps were automated through the use of a Micromedic® automatic pipetting station and a Hamilton® diluter/dispenser. Micromedic® racks were fitted with spacers to accommodate the tubes used in the assay

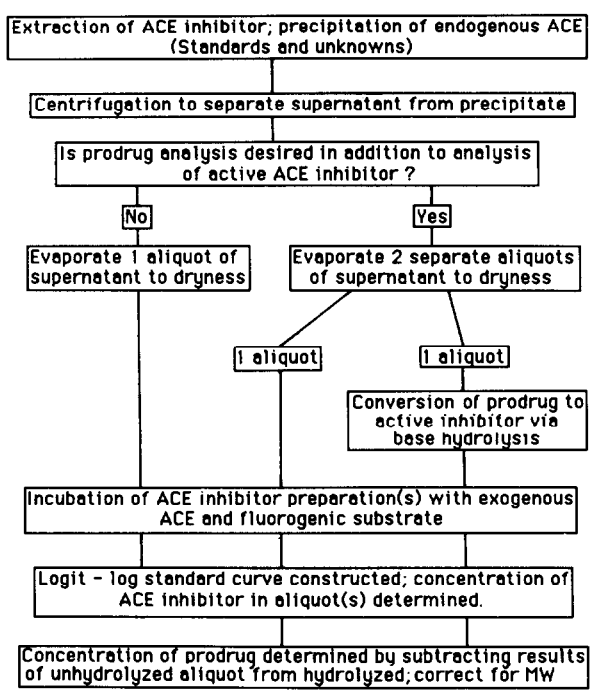

Figure 2
Algorithm for the ACE inhibitor assay procedure.

(Sarstedt 72.708 tubes; Princeton, NJ, USA). Blank tubes were placed between samples to eliminate possible carryover. To 300 μl of undiluted plasma or urine (diluted 1 + 99 with deionized water) in borosilicate glass tubes (12 × 75 mm) 1.2 ml of methanol was added. This precipitates endogenous ACE and simultaneously extracts the ACE inhibitor. The tubes were capped, vortexed and centrifuged at 10,000 **g** for 2 min. The supernatant solution (0.6 ml) was transferred to another test tube and evaporated to dryness in a vortex evaporator at 45°C. When ester prodrugs (such as enalapril) were assayed, an additional 0.6 ml of supernatant solution was dried, base hydrolyzed by reconstituting with 0.10 ml of a 0.5 M sodium hydroxide solution, and incubated at 37°C for 1 h. Hydrolyzed samples were then neutralized by adding 0.10 ml of 0.5 M hydrochloric acid. The 0.5 M sodium hydroxide and 0.05 M hydrochloric acid stock solutions were made equivalent in strength by titrating one against the other using a phenolphthalein indicator and then appropriately diluting the stronger of the two reagents. Hydrolyzed and non-hydrolyzed samples were then placed on ice. The working ACE and substrate solutions (0.25 ml of each) were then added and the tubes were vortexed immediately to reconstitute the drug residue. All tubes were kept on ice until the pipetting was complete and then they were placed in a 30°C water bath for a 1 h incubation period. The enzyme reaction was stopped by placing the incubation mixtures back on ice and 1.7 ml of the OPD solution was added to incubation mixtures and to HIS-LEU standards and the tubes were incubated at room temperature. The derivatization reaction was stopped after 20 min by the addition of 0.2 ml of 3 M hydrochloric acid. Samples were then centrifuged at 2500 **g** for 10 min at 25°C to remove the protein that precipitates under acidic conditions. The fluoroescence of the final supernatant solution was determined in a fluorometer (Aminco-Bowman Model J4-8960A equipped with an automatic sample sipper) at an excitation wavelength of 365 nm and an emission wavelength of 490 nm. The instrument zero was set using the 0 μM HIS-LEU standard solution. A digital reading was taken for all other samples. A logit–log relationship between inhibitor concentration and percent enzyme inhibition for the standards was constructed by computer fit using a Hewlett–Packard Model 97 calculator program (courtesy Dr. Ulm; Merck, Sharp and Dohme Research Laboratories, West Point, PA, USA). The unknown ACE inhibitor concentrations were calculated from this logit–log relationship by interpolation. To calculate ester prodrug concentrations, the results for hydrolyzed and unhydrolyzed samples were first determined from their respective standard curves. The result from the unhydrolyzed sample was then subtracted from that of the corresponding hydrolyzed sample. The difference was multiplied by a molecular weight conversion factor to reflect the initial parent drug concentration that was converted to active inhibitor by base hydrolysis.

Assay validation

The optimized FEA assay was assessed for application to plasma and urine, on three consecutive days by using lisinopril as a model ACE inhibitor. Standard curves (prepared in duplicate) and plasma samples containing low (0.7 ng ml^{-1}), medium (7.5 ng ml^{-1}) and high (30 ng ml^{-1}) concentrations of lisinopril and urine samples containing low (70 ng ml^{-1}), medium (750 ng ml^{-1}) and high (3000 ng ml^{-1}) concentrations of lisinopril were analyzed in replicates of six on each of the three consecutive days. The mean, standard deviation and relative standard deviation (RSD) of each set of determinations were evaluated to assess assay variation. In addition, plasma samples were obtained from normal volunteers [10] and patients with renal failure who received lisinopril and were

analyzed for lisinopril content using this optimized fluoroenzymatic assay and an RIA assay [11].

Results

A plot of ACE activity (percent inhibition) versus the logarithm of lisinopril concentration (ng ml^{-1}) obtained on each of three consecutive days is shown in Fig. 3 for plasma and Fig. 4 for urine. For both plasma and urine, the standard curves were sigmoidal in shape with no appreciable systematic fluctuations in curve characteristics on different days. Selected assay statistics for the lisinopril plasma and urine standard curves as well as the results obtained for the plasma urine quality control samples analyzed on one of the days are presented in Tables 1 and 2. The logit–log curve-fitting algorithm used in the construction of the standard curve produced a good line of fit based on the agreement between the nominal and readback values for each of the standards. Intra-assay variability was low in plasma and urine indicated by the low RSD values of the quality control samples (Tables 1 and 2). The inter-assay RSD computed by pooling all of the quality control data generated for each specimen over the three day period are shown in Table 3. Variability on different days was acceptable for all plasma and urine control samples evaluated.

In order to show the applicability of this method in the analysis of other ACE inhibitors, standard curves and control samples for enalaprilat and its prodrug form, enalapril, were prepared and analyzed. A representative plot of ACE activity (percent inhibition) versus the logarithm of drug concentration (ng ml^{-1}) for enalaprilat and enalapril in plasma is shown in Fig. 5. As with lisinopril, a well-defined sigmoidal

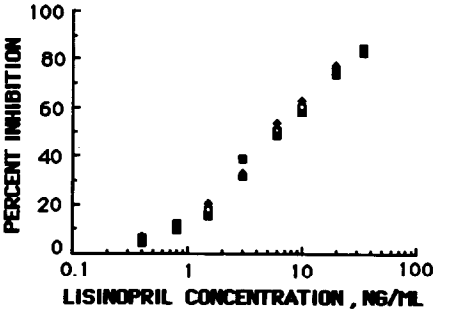

Figure 3
Standard curves obtained for lisinopril in serum on three consecutive occasions (run 1: □; run 2: ◆; run 3: ■).

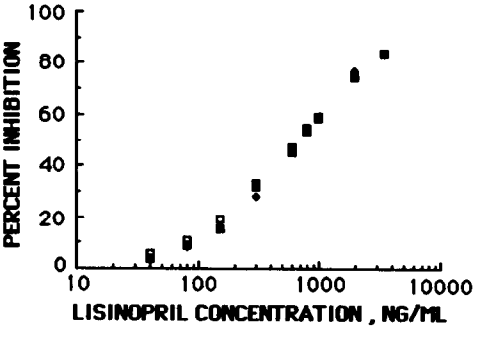

Figure 4
Standard curves obtained for lisinopril in urine on three consecutive occasions (run 1: □; run 2: ◆; run 3: ■).

Table 1
Lisinopril plasma standard curve data and quality control statistics for a validation run

Lisinopril plasma concentration (ng ml^{-1})	ACE activity (%Inhibition)	Concentration of lisinopril in plasma, found (ng ml^{-1})
0.40	6.2	0.40
0.80	12.0	0.84
1.50	18.0	1.4
3.0	31.7	2.9
6.0	50.2	6.4
8.0	55.5	7.9
10.0	60.8	9.8
20.0	76.4	20.6
35.0	84.4	34.5

Logit versus log concentration plot

$r^2 = 0.999$
I_{50}* = 6.30 ng ml^{-1} (15.6 × 10^{-9}M)
Slope = 1.00

Within-assay variation of quality control samples

Lisinopril nominal concentration (ng ml^{-1})	Measured concentration (ng ml^{-1}) Mean ± SD, $N = 6$	%RSD
0.70	0.67 ± 0.06	9.0
7.5	7.5 ± 0.2	2.7
30.0	30.3 ± 1.2	4.0

*Lisinopril concentration at which 50% ACE inhibition occurs.

Table 2
Lisinopril urine standard curve data and quality control data for a validation run

Lisinopril urine concentration (ng ml^{-1})	ACE activity (%Inhibition)	Concentration of lisinopril in urine, found (ng ml^{-1})
40	5.6	39
80	10.9	81
150	19.0	154
300	31.8	307
600	47.3	591
1000	59.5	967
2000	75.4	2018
3500	84.3	3534

Logit versus log concentration plot (for 100-fold diluted standard curve)

$r^2 = 1.000$
I_{50}* = 6.58 ng ml^{-1} (16 × 10^{-9}M)
Slope = 1.00

Within-assay variation of quality control samples

Lisinopril nominal concentration (ng ml^{-1})	Measured concentration (ng ml^{-1}) Mean ± SD, $N = 6$	%RSD
70	69.2 ± 6.2	9.0
750	714 ± 10.2	1.4
3000	3020 ± 10	3.3

*Lisinopril concentration at which 50% ACE inhibition occurs.

Table 3
Selected variability statistics for plasma and urine lisinopril quality control samples analyzed over a three day period ($N = 18$)

Specimen Nominal concentrations, ng ml^{-1}	Mean ± SD ng ml^{-1}	%RSD
Plasma		
0.7	0.69 ± 0.05	7.2
7.5	7.6 ± 0.4	5.3
30.0	29.6 ± 1.3	4.4
Urine		
70	69.3 ± 6.3	9.1
750	739 ± 7	3.6
3000	2948 ± 85	2.9

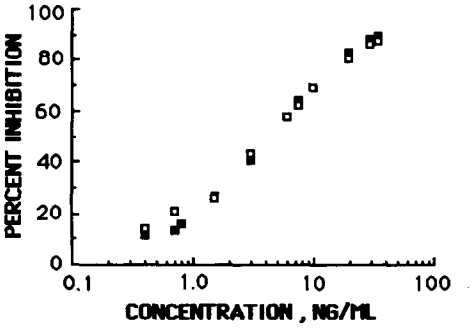

Figure 5
Standard curves obtained for plasma standards containing (1) enalaprilat (■) and, (2) enalapril following base hydrolysis to enalaprilat (□).

relationship between percent ACE inhibition and enalaprilat (or enalapril) concentration exists.

To compare the results derived from the assay with those obtained by RIA [1], plasma specimens from a normal volunteer following two single doses of lisinopril and from a renal failure patient following acute and chronic doses of lisinopril were analyzed by each method. The relationships between lisinopril concentrations determined by the optimized FEA and RIA were evaluated using perpendicular least squares regression which assumes that experimental error is present in each assay [12]. The results of this analysis are presented for each dose in Fig. 6. Excellent agreement between assays was obtained following single doses of lisinopril in the normal volunteer [Fig. 6: Panels (A) and (B)] as well as in the renal failure patient [Fig. 6: Panel (C)]. While a highly significant relationship existed between the results obtained by each assay following multiple doses of lisinopril in the patient with renal failure, the present assay produced results which were generally higher than those measured by the RIA method. This difference in results may be related to the manner in which each assay detects the ACE inhibitor (see Discussion).

Several other optimized FEA related parameters are worthy of mention. HIS-LEU standard curves are linear up to concentrations yielding about 20 arbitrary fluorescence units. HIS-LEU product formation is linear over the full 60 min incubation period. Fluorescence may be measured up to 1.5 h post-derivatization. Standard curves of percent ACE inhibition versus the logarithm of drug concentration are usable over the

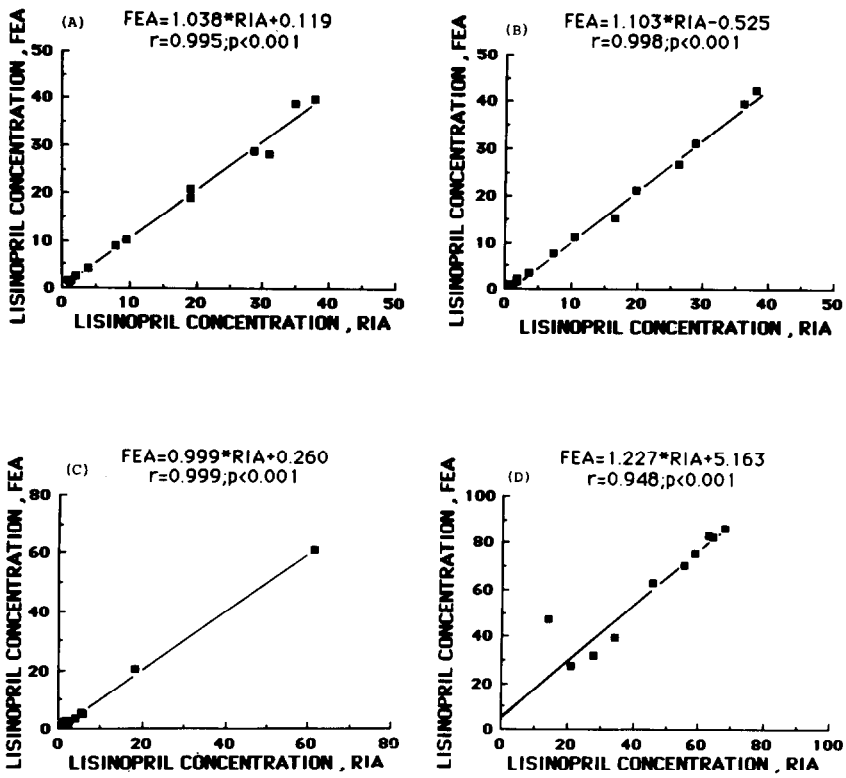

Figure 6
Comparison of lisinopril concentrations (ng ml^{-1}) measured by FEA versus RIA in: (1) serum samples obtained from a normal volunteer following the oral administration of a single dose of lisinopril on two separate occasions — Panels (A) and (B); and (2) plasma samples obtained from a patient with renal failure following single (Panel (C)) and multiple (Panel (D)) oral doses of lisinopril.

range of about 6–90% inhibition. The detection limit is 0.4 ng ml^{-1} for plasma and 40 ng ml^{-1} for urine.

Attempts to measure captopril were complicated by the instability of captopril in plasma. Methods of preserving captopril activity using *N*-ethylmaleimide (5 mg ml^{-1}), dithiothreitol (100 μM), sodium metabisulphite (5 mg ml^{-1}) and vitamin E (100 μM) were found to be unsatisfactory. Dithiothreitol acted to preserve captopril but also converted captopril dimer into active captopril. A procedure which involves performing the assay immediately after the evaporation stage and holding the residues frozen until analysis is currently under investigation. An HPLC technique [13] which employs an alternative sample preparation procedure that derivatizes the drug may help to resolve this problem.

Discussion

The optimized FEA described in this report combines the advantages of two existing assays to produce a relatively easy, sensitive, and specific method for the measurement of ACE inhibitory activity in plasma and urine. A comparison of the characteristics of assays capable of measuring ACE inhibitors in biological fluids is presented in Table 4.

Table 4
Comparison of assays capable of analyzing ACE inhibitors in biological fluids*

	Original FEA Assay	Optimized FEA Assay	REA Assay	RIA	IBA
Prodrug analysis	Yes	Yes	Yes	Yes	N.A.†
Plasma	Yes	Yes	Yes	Yes	Yes
Urine	Yes	Yes	Yes	Yes	N.A.
Minimum sample volume	0.7 ml	0.3 ml	0.1 ml	0.01 ml	0.1 ml
Lowest reliable detection limit (plasma)	0.7 ng ml^{-1}	0.7 ng ml^{-1}	<4 ng ml^{-1}	≤0.4 ng ml^{-1}	N.A.
Specificity‡	3	3	3	2	2
Relative standard deviation	3–10%	3–10%	11–14%	6–8%	N.A.
Range of standard curve	0.7–14 ng ml^{-1} (20-fold)	0.4–35 ng ml^{-1} (88-fold)	16–168 ng ml^{-1} (11-fold)	1–400 ng ml^{-1} (400-fold)	N.A.
Radiochemicals	No	No	Yes	Yes	Yes
Radiolabelling techniques	No	No	No	Yes	Yes
Automation	Partial	Yes	Partial	Yes	Yes
Throughput‡	5	1	2	1	1
Assay development time‡	2	1	1	5	3
Estimated reagent cost‡	3	2	2	2	2
Technician cost per assay‡	5	2	2	2	2
Equipment	Fluorometer	Fluorometer	Scintillation counter	Gamma counter	Gamma counter

*Drug data is based on measurement of enalaprilat in plasma; see text for assay abbreviations; HPLC and GLC data are not available.
†Data not available.
‡Rated from 1 (optimal) to 5 (poor).

The optimized FEA compares well analytically to the RIA and REA techniques. Each method is generally similar with regard to the lowest reliable detection limit, %RSD, and concentration range of the standard curve. Compared to RIA, the optimized FEA method offers the advantages of relatively short assay development time and also avoids the use of radioactivity. Like the RIA, it can be completely automated, allowing high sample throughput and reduced technical cost per assay.

Cross analysis of plasma samples containing lisinopril demonstrated excellent agreement between optimized FEA and RIA methods for samples obtained from a normal volunteer and a uremic patient following single doses of lisinopril. Following the administration of chronic doses of lisinopril to a uremic patient, however, the lisinopril plasma concentrations determined by the FEA method tended to be higher than those determined by RIA. While the cause for this discrepancy is currently unknown, its predominance following chronic doses of lisinopril suggests that a minor metabolite of lisinopril may exist which possesses ACE inhibitory activity. In uremic patients this metabolite would be formed in increasing amounts and could accumulate to relatively high plasma concentrations following chronic doses of the drug. Since both the optimized and original FEA non-specifically measure ACE inhibitory activity, this metabolite would be detected in the present assay system, whereas it might not interfere with the RIA. It is worthwhile to note, however, that the present data suggests that lisinopril does not undergo significant metabolism.

Since this assay measures ACE inhibitory activity non-specifically, it may prove useful in the further evaluation of endogenous ACE-inhibitors which have been isolated from the sera of humans, rabbits, guinea pigs, and rats [14–16]. While the presence of these substances has been recognized for some time [14], their characterization and physiologic roles remain to be determined. The present assay, however, might require modifications to enhance its sensitivity, as these natural substances are not currently detected in serum obtained from normal volunteers.

By expanding the working range of a sensitive, reliable fluoroenzymatic technique and combining this with a rapid method of sample extraction, an analytically sound, more cost effective assay has been developed that is relatively easy to perform. The optimized FEA may be the method of choice for clinical pharmacology laboratories where expense, convenience, and analytical performance are all important criteria used in selecting assay methodology. The assay is easily adapted to most other ACE inhibitors, lends itself well to automation, and avoids the use of radioactivity.

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