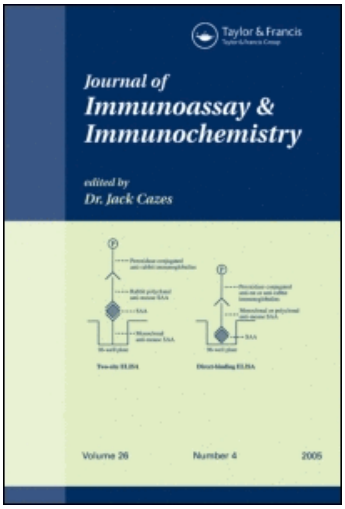


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# ENZYME IMMUNOASSAY OF THE ACTIVE METABOLITE (RS-5139) OF ANGIOTENSIN CONVERTING ENZYME INHIBITOR (CS-622)

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## ABSTRACT

A simple and sensitive enzyme immunoassay (EIA) for determination of the active metabolite (RS-5139) of a new angiotensin converting enzyme inhibitor (CS-622) was developed. The *N*-succinimmidyl ester of RS-5139 was coupled with bovine serum albumin (BSA) and its conjugate was used as an immunogen. Horse radish peroxidase (HRP; EC 1, 11, 1, 7) was used as a labeled enzyme and 3, 3', 5, 5'-tetramethylbenzidine was used as a substrate. The antiserum was used at a final dilution of 1:10000 and sensitivity was 10 pg in plasma and 20pg in urine.

CS-622 exhibited cross-reactivity (22.3%), but other ACE inhibitors didn't exhibit cross-reactivity.

The plasma levels determined by EIA and GC/MS was good agreement (y=6.58e-2+1.07x, R^2=0.985, n=52).

(KEY WORDS: Enzyme immunoassay, Angiotensin converting enzyme).

## Introduction

CS-622 (I, (+)-{(2S,6R)-6-[(1S)-1-Ethoxycarbonyl-3-phenyl-propyl]amino-5-oxo-2-(2-thienyl)perhydro-1, 4-thiazepin-4-yl} acetic

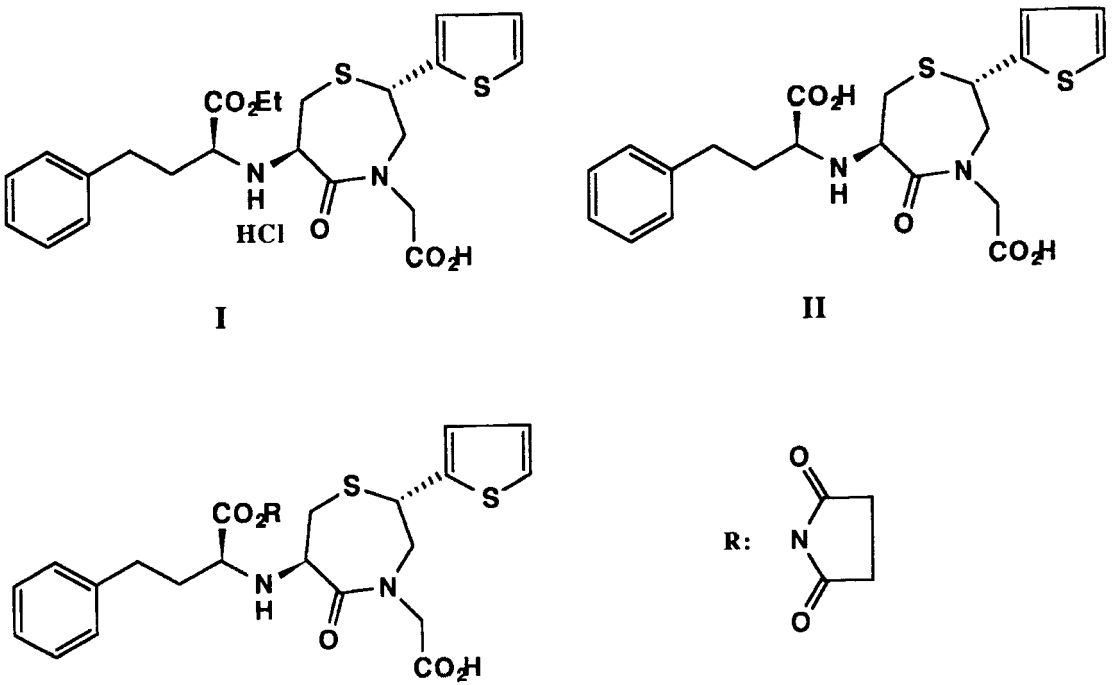


Fig. 1 Structures of I, II and *N*-succinimidyl ester of II.

acid hydrochloride, Fig. 1) is a new ACE inhibitor and it is a prodrug which is deesterified in vivo by esterase to release an active metabolite RS-5139 (II, Fig. 1). II has a very high potency so that it is therapeutically active at low concentration in plasma. So it is very important to determine the concentration of II in plasma and urine with respect to pharmacokinetics and pharmacodynamics. The analytical methods for their measurement were inhibitor binding assay (IBA) (1), radioimmunoassay (2) and liquid or gas chromatography (3, 4).

In the previous papers (5, 6), we reported GC/MS and IBA for determination of II. In this paper, we reported enzyme immunoassay for determination of II in plasma and urine.



## Materials and Methods

### Reagents

HRP (EC 1, 11, 1, 7) (Type XII, 335 units/mg) was obtained from Sigma (St. Louis, MO, U.S.A.). 3, 3', 5, 5'-Tetramethylbenzidine was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Goat anti-rabbit immunoglobulin G (IgG) antiserum and normal rabbit serum were purchased from Daiichi Radioisotope Labs., Ltd. (Tokyo, Japan). These sera were diluted with 0.05M phosphate buffer (pH 7.3) (PB) containing 0.1% gelatin and 0.9% NaCl (assay buffer).

All reagents were of analytical-reagent grade and used without further purification.

Preparation of II-BSA conjugate

The *N*-succinimidyl ester of II was prepared in these laboratories (7). In short, tert-butyl ester of I was deesterfied by LiOH to yield tert-butyl ester of II. Condensation of tert-butyl ester of II with *N*-hydroxysuccinimide in the presence of water-soluble carbodiimide provided the *N*-succinimidyl ester. It was deesterified by HCl in dioxane to yield the *N*-succinimidyl ester of II.

A solution of *N*-succinimidyl ester of II (50 mg) and BSA (100 mg) in pyridine (1 ml) was added to 0.05 mol phosphate buffer (pH 7.3) and stirred at 4°C overnight (7). After addition of acetone, the mixture was centrifuged at 3000 rpm for 20min. This procedure was repeated until the contaminated II was completely removed. The precipitate was dissolved in aqueous pyridine and dialyzed against distilled water at 4°C overnight and lyophilized to give a hapten-BSA conjugate (90 mg) as fluffy powder. The number of II molecules linked to a BSA molecule was found to be 16 by UV spectrometric analysis at 230nm (7).

Immunization of rabbits

Three male albino rabbits were used for immunization. The antigen (1mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into rabbits subcutaneously at multiple sites over the scapulae and in the thighs. This procedure was repeated at intervals of 2 weeks for 5 months. The rabbits were bled 10 days after the booster injection. The sera were separated by centrifugation at 3000 rpm for 10 min and stored at -20°C.



Preparation of II-enzyme conjugate

Dioxane solution (0.2 ml) containing *N*-succinimidyl ester of II corresponding to *N*-succinimidyl ester of II/HRP molar ratios of 30 (molecular weight of HRP, 40000) was added to the solution of HRP (5mg) in PB (1ml) at 0°C, and the mixture was incubated at 4°C overnight (8). The resulting solution was dialyzed against cold PB (1 l) for 2 days, and stored at a concentration of 2.5 mg/ml adjusted with assay buffer.

Preparation of assay samples

All blood samples were drawn from a peripheral vein into heparinized blood collecting tubes. The plasma was collected on

centrifugation of the samples and stored at -20°C until analysis. Urine samples were stored at -20°C until analysis.

Plasma samples (0.2ml) were deproteinized by methanol (1ml) and centrifuged. The supernatant (0.6ml) was transferred to another vessel and evaporated to dryness. Assay buffer (0.1ml) was added to the vessel and an aliquot of this was subjected to the assay.

Urine samples were diluted with assay buffer (x10~x100) and 0.1ml of which were subjected to the assay.



Assay procedure

This was carried out in duplicate in glass tubes (10 ml). A solution of standard II in assay buffer (0.1ml) or assay sample (0.1ml) and II-enzyme conjugate (HRP label, 5ng/tube) in buffer (0.1ml) were added to diluted antiserum (0.1ml), and the mixture was incubated at 4°C for 6 h. Goat anti-rabbit IgG antiserum (0.1ml) diluted 1:20 with assay buffer containing 0.3% EDTA was added to the incubation mixture, and the solution was mixed by voltex mixer, then allowed to stand at 4°C for 16 h. After addition of assay buffer (2ml), the resulting solution was centifuged at 3000rpm for 10 min and the supernatant was removed. The immune precipitate was diluted with 0.05 M acetate-citric acid buffer, pH 5.5 (1.8ml), containing 0.42mM 3, 3', 5, 5'-tetramethylbenzidine and 3% dimethyl sulfoxide, mixed by voltex mixer, and preincubated at 37°C for 3 min. Hydrogen peroxide

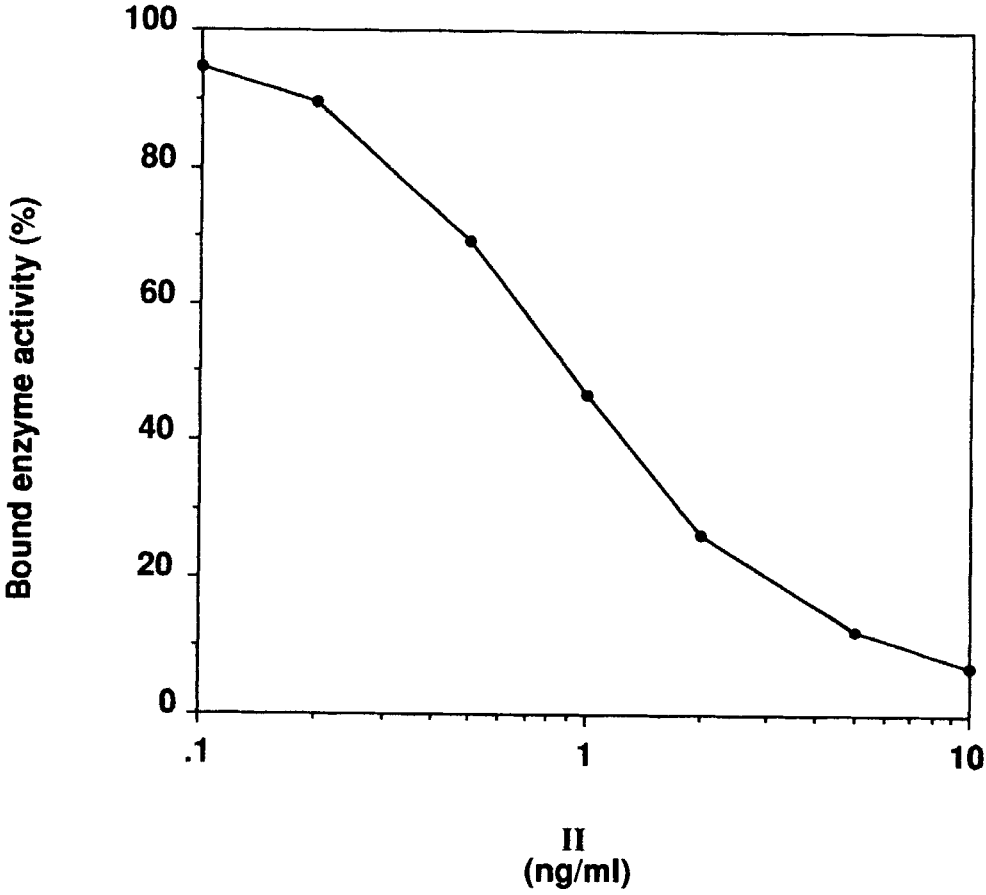


Fig. 2 Dose-response curve for enzyme immunoassay of II.

Table 1 Per cent cross-reactions of anti-II antisera with selected compounds.

| Compounds | %Cross-reactivity (50%) |
|---|---|
| II | 100.0 |
| I | 22.3 |
| Enalapril | <0.1 |
| Enalaprilat | <0.1 |
| Ramipril | <0.1 |
| Ramiprilat | <0.1 |



(0.02%, 0.2ml) was added to the resulting solution, and the mixture was incubated for 30 min. The reaction was terminated by addition of 0.5M $H_2SO_4$ (2ml) and the absorbance was measured at 450 nm.

Cross-reaction study

The specificity of antisera raised against II-BSA conjugate was assessed by cross-reaction studies with related compounds. The

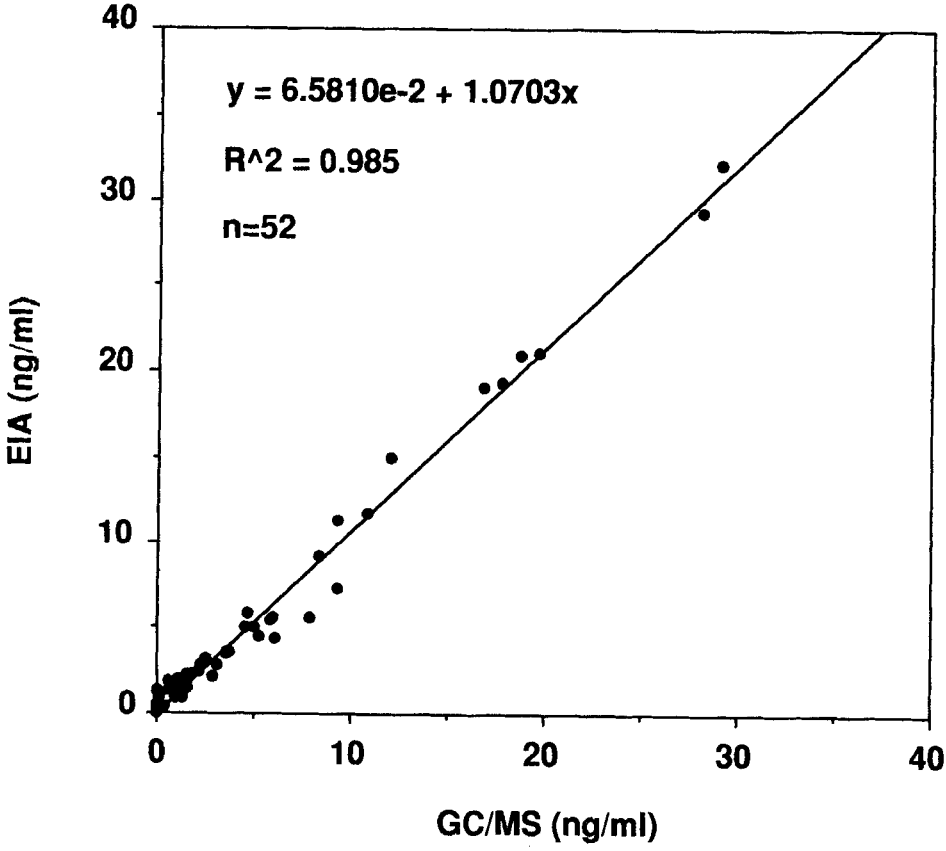


Fig. 3 Relationship between plasma concentrations of II determined by EIA and GC/MS.

Table 2 Assay variations of the proposed method for II in plasma.

| Sample | Intra-assay (Mean+/-S.E., N=6) (ng/ml) | CV(%) | Sample | Inter-assay (Mean+/-S.E., N=6) (ng/ml) | CV(%) |
|---|---|---|---|---|---|
| A | 1.3 +/- 0.1 | 4.1 | F | 1.0 +/- 0.1 | 12.6 |
| B | 5.8 +/- 0.4 | 7.2 | G | 4.2 +/- 0.3 | 8.2 |
| C | 8.3 +/- 0.2 | 2.6 | H | 6.8 +/- 0.7 | 10.1 |
| D | 32.9 +/- 0.5 | 1.7 | I | 47.4 +/- 4.5 | 9.5 |
| E | 51.1 +/- 1.8 | 3.5 | J | 57.5 +/- 4.7 | 8.1 |



relative amounts required to reduce the initial binding of enzyme-labeled II by half, where the mass of unlabeled II was arbitrarily taken as 100%, were calculated from a standard curve (9).

## Results and Discussion

II-BSA conjugate and II-HRP conjugate were prepared by the activated ester method. The anti-II antiserum used in the EIA was raised against the II-BSA conjugate. The bound and free II-HRP conjugates were separated by the double antibody technique, and the enzymic activity of immune precipitate was determined

Table 3 Assay variations of the proposed method for II in urine.

| Sample | Intra-assay (Mean+/-S.E., N=6) (ng/ml) | CV(%) | Sample | Inter-assay (Mean+/-S.E., N=6) (ng/ml) | CV(%) |
|---|---|---|---|---|---|
| A | 19.3 +/- 0.3 | 1.8 | F | 23.9 +/- 1.4 | 5.8 |
| B | 64.8 +/- 1.8 | 2.8 | G | 73.5 +/- 4.5 | 6.1 |
| C | 90.6 +/- 2.3 | 2.5 | H | 101.6 +/- 7.2 | 7.1 |
| D | 282.0 +/- 5.4 | 1.9 | I | 264.7 +/- 12.3 | 4.7 |
| E | 640.9 +/- 22.8 | 3.6 | J | 542.7 +/- 28.3 | 5.2 |



colorimetrically with 3, 3', 5, 5'-tetramethylbenzidine as a substrate. The dose-response curve with 5ng of II-HRP conjugate was 10-1000pg at a 1:10000 dilution of the antiserum (Fig. 2).

The specificity of the antiserum was assessed by the cross-reaction study using related compounds. I which was the prodrug of II exhibited the cross-reactivity (22.3%), but the other compounds (enalapril, enalaprilat, etc) exhibited no cross-reactivity (Table 1). The high cross-reactivity was due to the position of conjugation between II and BSA being the carboxyl function of the ethyl ester of I. So, to avoid high cross-reactivity, it is necessary to conjugate by using the free carboxyl function of II.

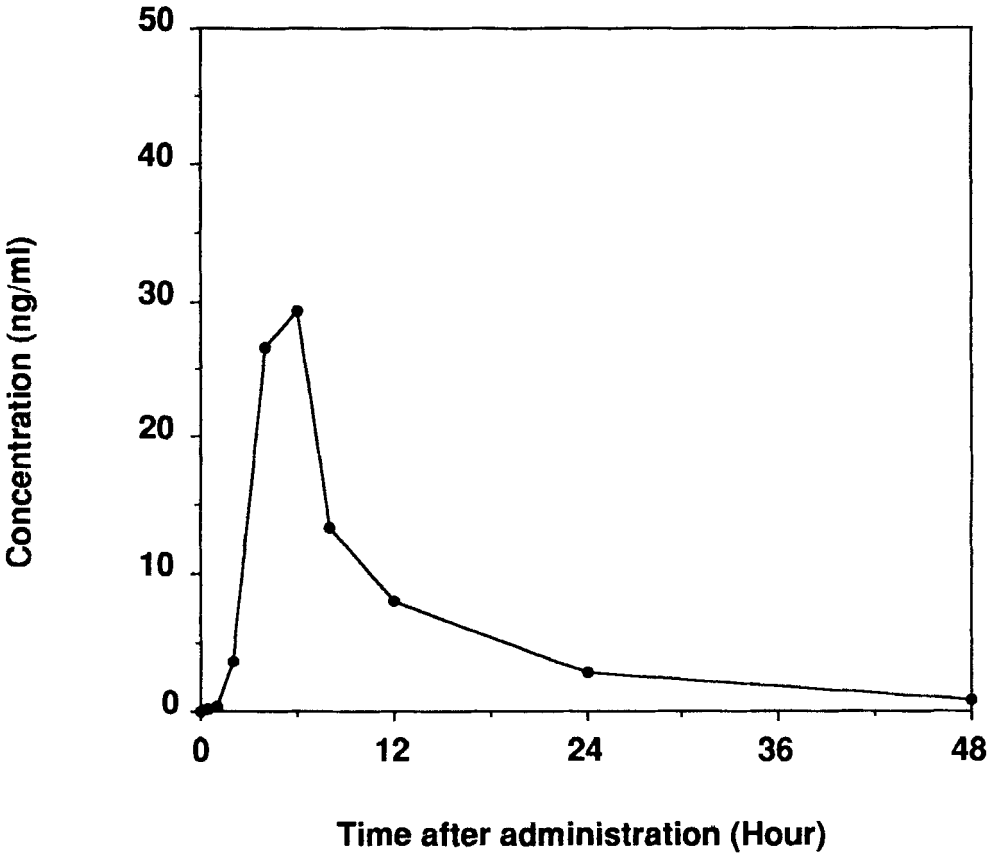


Fig. 4 Plasma concentration of II after an oral administration of I (2 mg) to volunteers (N=2).

The plasma levels of II determined by EIA and GC/MS showed good agreement (y=6.58e-2+1.07x, R^2=0.985, n=52, Fig. 3).

The reproducibility of the present method was assessed by intra-assay and inter-assay with five samples each of different levels in plasma and urine. In plasma, intra-assay and inter-assay coefficients

of variation were found to be 1.7-7.2 % and 8.1-12.6 %, respectively (Table 2). In urine, intra-assay and inter-assay coefficients of variation were found to be 1.8-3.6 % and 4.7-7.1 %, respectively (Table 3).

The plasma concentration of II after an oral administration of I (2mg) to volunteers determined by EIA is shown in Fig. 4.

The newly developed EIA is capable of determining II in plasma and urine. It is hoped that availability of a reliable method may serve for the studies of pharmacokinetics and pharmacodynamics of II.



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