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# A Reliable Radiometric Assay for the Determination of Angiotensin I-Converting Enzyme Activity in Urine

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**Summary:** We present a radiometric assay for the determination of urinary angiotensin-converting enzyme activity, using benzoyl-[1-$^{14}$C]glycyl-*L*-histidyl-*L*-leucine as the substrate. An optimal pH of 8.3, an optimal chloride concentration of 0.375 mol/l and complete inhibition by EDTA-$Na_2$, captopril and enalaprilat confirm the specificity of the assay. Comparison of dialysis and ultrafiltration for concentration of urine showed the existence of angiotensin-converting enzyme inhibitors in human urine. Dialysis against water was the more effective method for avoiding enzyme inhibition. After dialysis of urine, the assay was linear with time and with enzyme concentration; it was highly sensitive (60 mU/l) and showed good reproducibility. Under our technical conditions, we found angiotensin-converting enzyme activity in urine samples with quantitatively abnormal protein contents, but not in normal urine. Urinary angiotensin-converting enzyme did not correlate with proteinuria nor with water-salt parameters or creatinine. We confirm the kidney tubular epithelial origin of the enzyme, and propose the use of our assay to study urinary angiotensin-converting enzyme as a marker of renal tubular damage.

## Introduction

Angiotensin I-converting enzyme is the peptidyldipeptide hydrolase (dipeptidylcarboxypeptidase EC 3.4.15.1) which cleaves angiotensin I to the potent vasopressor angiotensin II and inactivates the vasodilator bradykinin. Angiotensin-converting enzyme activity is measurable in plasma and in most organs; the enzyme is located on the luminal surface of the endothelial cells as an ecto-glycoprotein (1).

The brush border of kidney tubules is one of the extravascular sites richest in angiotensin-converting activity (2). Because of this location some authors have suggested the potential use of urinary angiotensin-converting activity as an index of renal tubular damage (3, 4). Studies on urinary angiotensin-converting enzyme are, however, rare, possibly because its activity is low in urine and the accuracy of the present assays not proved; in particular, no criteria of reliability have been specified (3–5). In addition, mammalian urine may contain angiotensin-converting enzyme inhibitors (5).

In this study, we describe a sensitive and specific radiometric assay for determination of urinary angiotensin-converting activity. We draw attention to the presence of inhibitors of the enzyme in human urine, and propose a prior dialysis of urine for an accurate determination of the enzyme. Concentration of urine before the measurement must be avoided.

## Materials and Methods

### Reagents

All reagents were of analytical grade. Potassium phosphates, sodium chloride and ethyl acetate were from Merck (Darmstadt, FRG). EDTA-$Na_2$ and 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) were provided by Sigma Chemical Co. (Saint Louis, Mo., USA). Benzoylglycyl-*L*-histidyl-*L*-

[1]) Deceased on September 1988

leucine was from Calbiochem (San Diego, Cal., USA) and benzoyl-[1-$^{14}$C]glycyl-*L*-histidyl-*L*-leucine was purchased from New England Nuclear (Boston, Ma., USA). Instafluor was from Packard Instruments (Warrenville, Ill., USA).

Captopril and enalaprilat were gifts from Squibb & Sons (Princeton, NJ., USA) and Merck Sharp & Dohme (West Point, Pa., USA), respectively. PM-10 (cut-off $M_r$ 10 000) and XM-50 (cut-off $M_r$ 50 000) ultrafiltration membranes were from Amicon Ltd. (Lexington, Ma., USA), dialysis membranes from Union Carbide (Chicago, Ill., USA) (cut-off $M_r$ 1000).

### Angiotensin-converting enzymatic assay

All urinary analyses were performed on 24-hour, fresh urine containing no preservatives, which was centrifuged 10 min at 2000 *g*, +4 °C. Extensive dialysis and concentration by ultrafiltration were performed at +4 °C with constant agitation on 10 ml aliquots of centrifuged urine.

The buffered substrate contained 0.165 mmol/l radiolabelled substrate and 2.175 mmol/l cold substrate in 0.5 mol/l potassium phosphate – 0.75 mol/l sodium chloride – pH = 8.3 buffer. Incubation was for one hour at 37 °C in 100 × 8 mm glass tubes, started by the addition of 25 µl of urine to 25 µl of buffered substrate. The hydrolysis was stopped by the addition of 50 µl HCl 1 mol/l. Benzoyl-[1-$^{14}$C]glycine was then extracted with 375 µl of ethyl acetate. After vigorous mixing and centrifugation (10 min at 1000 *g*, +4 °C), 250 µl of the upper ethyl acetate layer were directly dropped into 5 ml of Instafluor and counted (Mini-Maxi-Tricarb Packard – Packard Instruments). Angiotensin-converting enzyme activity was determined in duplicate and a blank was run with 0.15 mol/l sodium chloride replacing the urine.

Results are expressed as angiotensin-converting enzyme units per litre of urine, one unit (U) corresponding to the release of one µmol of benzoylglycine in one minute at 37 °C.

The formula used is derived from that described in l. c. (6) after calculation of the specific activity of the isotopic dilution (SA):

$$U/l = \frac{[Bq\ (dosage) - Bq\ (blank)]}{SA \times 0.91 \times 0.67 \times 60 \times 25 \times 10^{-6}} = \frac{\Delta Bq}{SA \times 915 \times 10^{-6}},$$

where 0.91 is the benzoylglycine fraction extracted by ethyl acetate; 0.67 is the counted organic fraction; 60 is time in minutes and 25 × $10^{-6}$ the volume of the sample in litres.

For example, with radiolabelled substrate of activity 88000 Bq/µmol, SA becomes 7000 Bq/µmol, thus

$$U/l = \frac{\Delta Bq}{6.4}.$$

### Other determinations

Proteinuria, creatininuria, urinary sodium (Na) and potassium (K) were measured on the multiparametric discrete analysers Greiner G300 (Langenthal, CH) and Astra 8 (Beckman Instruments Inc. – Fullerton, Ca., USA).

Albumin/globulins ratio was determined with a Cliniscan densitometer (Helena Laboratories – Beaumont, Te., USA) after electrophoretic separation on agarose gel (Paragon SPE-II Kit, Beckman).

### Urines

Normal urines were from 15 apparently healthy people from the laboratory staff (8 women, mean age 27.0 ± 10.8 years, and 7 men, mean age 30.1 ± 10.7 years) without any renal disorder as judged by a normal diuresis and normal urinary biological criteria: proteins < 0.15 g/l, Na = 131.1 ± 49.0 mmol/l, K = 48.5 ± 15.8 mmol/l, creatinine = 12.2 ± 4.7 mmol/l.

Urines with an abnormally high protein content were selected from 44 urinary samples sent to the laboratory for electrophoretic analysis of quantitatively abnormal proteinuria (i.e. > 0.15 g/day).

### Data analysis

Results are expressed as mean ± standard deviation. Coefficients of variation (CVs) for reproducibility studies were obtained from 20 determinations. Statistical comparisons were performed with the non-parametric *Mann-Whitney* U-test.

## Results

As we did not find any angiotensin-converting activity in urines from normal subjects, we performed further experiments on urines with high protein contents. A direct determination showed the presence of angiotensin-converting activity in 16 of the 44 urine samples tested. Concentration of these urines by ultrafiltration on Amicon PM-10 did not increase angiotensin-converting activity, but 24-hour dialysis against pure water increased the enzymatic activity by 85 ± 42%. Dialysis against water was more effective than dialysis against the phosphate buffer of the enzymatic assay, or against 0.375 mol/l sodium chloride, or a 25 mmol/l HEPES – 0.375 mol/l NaCl – pH = 8.3 buffer. Concentration of the dialysates on Amicon PM-10 never increased the enzymic activity, and more often led to a substantial loss of activity. Dialysis against water did not reveal any angiotensin-converting activity in the other 28 urines with a high protein content or in the normal urines. For the experiments reported below, all urines were dialysed for 24 hours against pure water. Under these conditions we determined the main analytical parameters of the radiochemical assay.

The specificity was assessed by

(i) an optimal pH of 8.3 (fig. 1 b) and activation by an increase in the chloride concentration (fig. 1 a), both of which are characteristic of angiotensin-converting enzyme activity determined with benzoylglycine-histidyl-leucine as the substrate;

(ii) a complete inhibition of urinary angiotensin-converting activity by 60 mmol/l EDTA-$Na_2$ (angiotensin-converting enzyme is a zinc metallopeptidase) and 100 µmol/l captopril or 40 µmol/l enalaprilat (two specific synthetic inhibitors of the enzyme). As phosphates have been implicated as inhibitors of angiotensin-converting activity, we verified that the concentration used (250 mmol/l) was not inhibitory. In

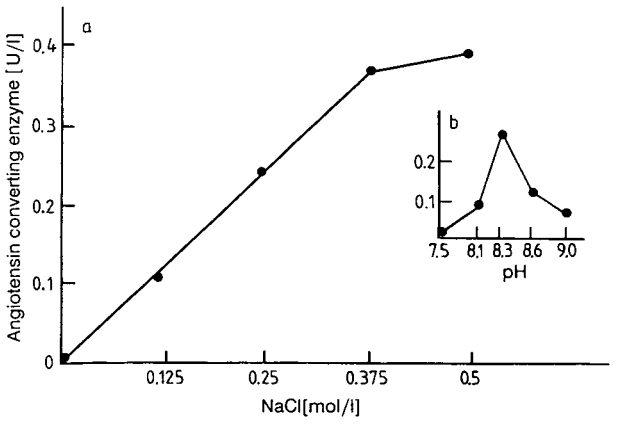


Fig. 1. Urinary angiotensin-converting enzyme activity; pH and chloride activation curves. Each point is the mean of three determinations of a pool of urinary samples dialysed against water.

particular, substitution of the phosphate buffer by a 50 mmol/l HEPES – 0.75 mol/l NaCl – pH = 8.3 buffer did not increase the urinary angiotensin-converting activity.

The assay was linear as a function of enzyme concentration up to 4.0 U/l and as a function of time up to 120 minutes (fig. 2). When the urines were not dialysed, the activity curves fell rapidly, suggesting an inhibitory agent in the urines which can be eliminated by dialysis (not shown). When, after dialysis, the urines were concentrated on PM-10, the activity curve swiftly decreased but not when a XM-50 membrane was used instead of the PM-10 membrane (fig. 2a). These data suggest the existence of a second inhibitory substance in human urines eliminated neither by dialysis nor by ultrafiltration on PM-10 membrane, but excluded by ultrafiltration on XM-50.

The sensitivity of the assay was determined by measuring the radioactivity of 30 blanks in the same set of experiments; the precision of the counts corresponded to an assay sensitivity of 44 mU/l. Linear dilution of urines, previously dialysed against water, showed the limit of detection to be 66 mU/l (fig. 2b). Within-day reproducibility CVs were less than 7% for two pools of urines with activities of 1.44 U/l and 3.21 U/l. Their between-days CVs were 10.3 and 4.4% respectively. CVs were between 10 and 20% for a urine pool containing 124 mU/l angiotensin-converting activity.

In the 16 urines containing angiotensin-converting activity the mean activity was 2.6 ± 4.7 U/l with a range of 0.1 to 14.5 U/l and a median value of 440 mU/l, but the distribution of the values was not unimodal. There was no apparent difference between men and women. For these 16 urines, proteinuria was

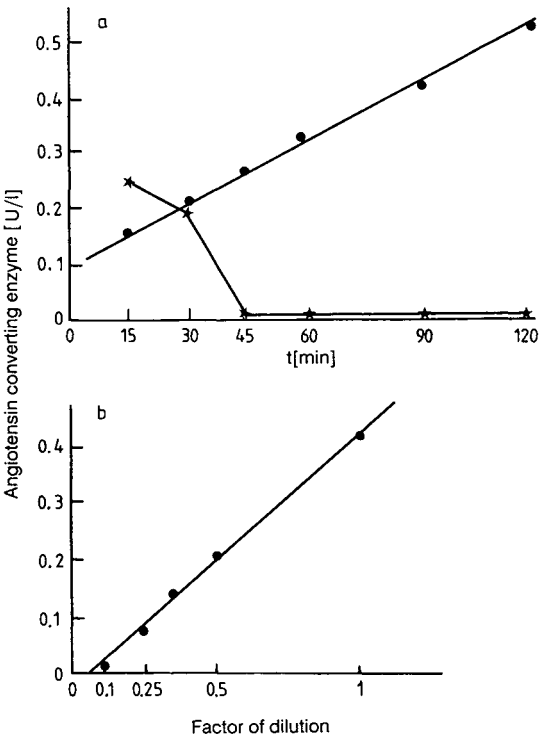


Fig. 2. Radioassay of angiotensin-converting enzyme in urines, showing activity with time (a), and linearity (b). Each point is the mean of three determinations of a pool of urinary samples, dialysed only (●) or concentrated on Amicon PM-10 after dialysis (★).

6.7 ± 15.1 g/l (range: 0.35–62.4 g/l). Urinary angiotensin-converting activity did not correlate with proteinuria (fig. 3), or with the albumin/globulins ratio (1.79 ± 1.06), or with any kind of abnormality on the agarose electrophoresis pattern, such as a peak in the γ-globulins or patterns of selective or non-selective proteinuria (not shown).

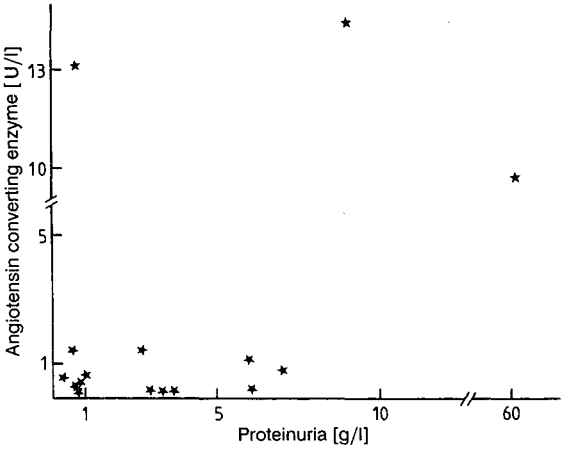


Fig. 3. Lack of correlation (r = 0.29) between angiotensin-converting activity and proteinuria measured in 16 urine samples with detectable enzyme activity.

Angiotensin-converting activity in urines was not related to water-salt excretion; thus, no statistical link appeared between the enzyme activity and urinary Na (39.7 ± 35.1 mmol/l), K (34.0 ± 13.9 mmol/l), or Na/K (1.45 ± 1.49). Moreover, angiotensin-converting activity was not linked to creatininuria (8.16 ± 7.05 mmol/l). These data were the same when enzyme activity was expressed as U/g of protein or as U/mmol of creatinine.

## Discussion

We modified the radiometric method of *Rohrbach* (6) for the determination of urinary angiotensin-converting enzyme activity. Dilution of the radiolabelled substrate, benzoyl-[1-$^{14}$C]glycyl-*L*-histidyl-*L*-leucine, with cold substrate gives a sensitivity suitable for a direct determination on urine samples, i.e. without prior concentration.

Dialysis of the urines is necessary to eliminate at least one inhibitor, which, as previously suspected (5), is of low molecular weight. According to the results obtained with PM-10 and XM-50 ultrafiltration membranes, another inhibitor may also be present; this inhibitor, relative molecular mass 10 000 – 50 000, is less inhibitory than the dialysable inhibitor since we could not reveal its inhibitory effect on non-concentrated urines. Some ions and metals have been implicated as physiological angiotensin-converting enzyme inhibitors (5, 7); *Lieberman* described inhibition by human plasma but did not identify the agent responsible (8). *Hazato & Kase* found an inhibitor in pig plasma and identified it as an oligopeptide (9). Such low molecular weight inhibitors have been detected also in urine. Potential physiological inhibitors of higher molecular weight have also been reported, i.e. albumin and some of its fragments (10), or fibrinogen fragments (11). In our study, it was difficult to correlate the inhibitory power of the urines with their protein concentration because we had only a few samples with both low angiotensin-converting activity and high protein content. Further investigations are necessary to characterize these urinary inhibitors of angiotensin-converting enzyme activity.

The reliability of our radiochemical enzyme assay after dialysis of urines is assessed by its specificity, linearity and reproducibility. The sensitivity is higher than that of most the methods described for the determination of angiotensin-converting activity in plasma. Only fluorimetric assays attain this sensitivity (12), but radioassays avoid the interferences frequently encountered with fluorimetric as well as photometric methods (13). *Kokubu* et al. (14) adapted *Cushman*'s spectrophotometric assay (13) to urines but their method involves partial purification of the enzyme from the urine. *Pitotti* et al. (15) described a HPLC method but, once again, with difficult pretreatment of the urinary samples, and with no indication of the reliability or precision. A colorimetric assay was also proposed but without evidence of specificity for angiotensin-converting enzyme (3). *Ryan* et al. (5), like the present authors (personal data), were unable to adapt *Cushman*'s assay for the determination of angiotensin-converting activity in mammalian urines, since the latter contain high concentrations of pigments. On the other hand, the radiochemical assay of *Rohrbach* (6) has poor sensitivity, detecting 500 mU/l, which is about 10-fold the limit of detection of our assay; thus *Rohrbach*'s assay cannot be directly adapted to urine determination.

Under our technical conditions we could not detect angiotensin-converting activity in the urines of normal subjects, but we were able to measure the enzyme activity in urines with a quantitatively abnormal protein content. Some authors (14, 15) found angiotensin-converting activity in normal, 24-hour urines; possibly they were measuring the physiological replacement of the renal tubular brush-border whose epithelial cells are sedimentable. In view of the high specific angiotensin-converting activities in the male genital tract (16, 17), a gonadic or prostatic origin of urinary angiotensin-converting enzyme might have been expected, but we found no difference between males and females; in particular, normal men did not excrete angiotensin-converting activity in the urine. The absence of a correlation between this urinary enzymatic activity and proteinuria, and even more the lack of dependence of this activity on the indices of glomerular function, such as creatininuria or albuminuria, eliminate the possibility of a passage of the plasmatic enzyme throughout the glomerular filter. Damage of renal glomeruli cannot explain a significant enzymuria, since glomerular epithelial and endothelial cells contain only a little angiotensin-converting enzyme (18, 19). Thus, in agreement with other authors (3, 4), we conclude that urinary angiotensin-converting enzyme is of tubular origin, as in the case of most enzymurias (20). We also showed that urinary angiotensin-converting enzyme excretion is independent of sodium and potassium excretion, so that physiological or pathological variations of this enzyme in response to haemodynamic or dietary factors would not be expected.

Our specific, sensitive and reproducible radioassay might enable the comparison of angiotensin-converting enzyme with other materials of tubular origin for their suitability as specific, early and precise markers

of tubular damage during renal disorders. The assay will also be of use in investigating putative inhibitors of angiotensin-converting enzyme in mammalian biological fluids.

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