Short Communication

HPLC Method for evaluation of urinary angiotensin-converting enzyme: some examples of normal subjects and patients with renal transplantation

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Introduction

Angiotensin-converting enzyme (ACE; EC 3.4.15.1; peptidyl-dipeptide hydrolase, kininase II) is localized in the vascular endothelium of several organs [1–6], mainly lungs [3] and kidneys [4]. It is also present in the epithelial cells of the renal proximal tubule [5–6].

Angiotensin-converting enzyme is present in the plasma [7] and urine of normal subjects [8]. ACE activity in urine probably derives not from plasma, but from renal tubules [9]; its value increases significantly in some renal diseases [10] and upper urinary tract infections [9].

Römmer *et al.* [11] have considered serum ACE activity in renal disorders, hemodialysis and transplantation. However, no data are available about ACE activity in urine after renal transplantation.

Other colorimetric [12] and HPLC [13] methods for ACE evaluation in serum and urine have been published in the past years; however, the proposed method offers many advantages. It is quick and simple: no extraction steps are required; it is versatile: it can be used for tissue extracts [14], serum [15] and urine; it is widely applicable: so far no interferences have been found.

In this preliminary work plasma and urinary ACE activity is examined in some patients with renal transplantation.

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Experimental

Materials

Hippuric acid and hippuryl–histidyl–leucine (Hip–His–Leu) were purchased from Sigma Chemical Co., St Louis, Missouri, USA; methanol for HPLC from Hoechst, Riedel-De Haën AG, Seelze-Hannover; tetrabutylammonium sulphate from Aldrich-Europe; salicylic acid, monohydrogen potassium phosphate, phosphoric acid, sodium chloride, boric acid and sodium carbonate, all analytical grade, from Carlo Erba, Milano, Italy. All reagents were used without further purification.

Urine samples

Twenty-four-hour urines were collected. Urine samples were treated as described by Kato *et al.* [10]. Aliquots (50 ml) were adjusted to pH 7 with NaOH 1 M and centrifuged at 3000 rpm for 20 min at 4°C. The supernatant was fractionated with 3.14 mol 1^{-1} ammonium sulphate and the precipitate was dissolved in 8 ml of phosphate buffer 10 mM, pH 7.8 and dialysed overnight against the same buffer through a Visking dialysis tubing, ¼ I, cut of 8000.

After dialysis the volume sometimes slightly increased. After centrifugation at 8000 rpm the supernatant was used for enzymatic determination (ACE urine preparation).

Assay of angiotensin-converting enzyme

A 50–100 µl sample of 'ACE urine preparation' or serum was incubated for 1 h in a total volume of 250 µl of 80 mM borate sodium carbonate buffer (adjusted to pH 7.8 with HCl 5 N) and 5 mM hippuryl–histidyl–leucine.

The 250 µl total incubation volume was obtained as follows: 100 µl ACE urine preparation (or serum) were added to 50 µl of water and 100 µl of 12.5 mM Hip–His–Leu in 200 mM borate buffer.

Smaller samples can be used, provided the total volume of 250 µl is obtained by adding water. After incubation the reaction was terminated by addition of 50 µl of 5 M HCl and 200 µl of internal standard solution (0.25 mg ml^{-1} in water). Salicylic acid was used as internal standard.

After centrifugation and neutralization of supernatant (with nearly 35 µl of 5 N NaOH) 20-µl aliquots of the samples and reference standard solution were chromatographed alternately using the chromatographic conditions already described.

The reference standard solution was prepared by pipetting 8 ml of the hippuric acid solution (0.5 mg ml^{-1}), 40 ml of the salicylic acid solution (0.25 mg ml^{-1}) in a 100 ml flask and bringing to volume with distilled water. The standard solution also contained borate sodium carbonate buffer, hydrochloric acid and sodium hydroxide in the same concentrations as the sample solutions.

Peak height ratios were used to evaluate the resulting chromatograms.

Two zero time samples were prepared: the first, by adding HCl before substrate; the second, by incubating the samples in absence of enzyme.

One unit of activity is defined as the amount of enzyme catalysing the release of 1 µmol hippuric acid from Hip–His–Leu per minute at 37°C under the described conditions. The specific activity is expressed in units day^{-1} for urine, and units ml^{-1} for serum.

High-performance liquid chromatography

Analyses were performed on a high-performance liquid chromatograph (Knauer FR-30) operating at room temperature and connected to a variable wavelength UV detector (Kontron LCD 725); chromatograms were recorded on a strip-chart recorder.

The analytical column was 250×4.6 mm i.d. packed with Lichrosorb RP 18, particle size 7 μm (Knauer, AG, Berlin, FRG).

Samples were introduced into the column through a Reodyne 7125 injector equipped with a 20-μl loop.

The mobile phase was water–methanol (60:40 v/v) containing 0.005 M tetrabutyl-ammonium cation, 0.005 M K_2HPO_4 and buffered to pH 7.4 as previously described [14].

The detector wavelength was 230 nm. The detector sensitivity was 0.05 a.u.f.s. The flow rate was adjusted to 1.5 ml min^{-1}.

Results and Discussion

Calibration curves were reported in a previous paper [14] and were prepared by plotting the peak height ratios of the hippuric acid peak and internal standard peak against the concentration of hippuric acid (mg 100 ml^{-1}). The slope was 0.1704, the intercept 0.0003 and the correlation coefficient 0.9999.

In Fig. 1 a chromatogram of a denatured sample (zero time) is shown. No interfering substances were eluted at the same retention time as hippuric acid, either for sera or for urine samples. The samples for determining the recovery in urine were evaluated under two different conditions, as reported in Table 1. A typical chromatogram is reported in Fig. 2. It is therefore demonstrated that no hippuric acid is present in the substrate, nor is any produced by HCl or incubation alone. Moreover, hippuric acid produced from enzymatic reaction is stable during incubation.
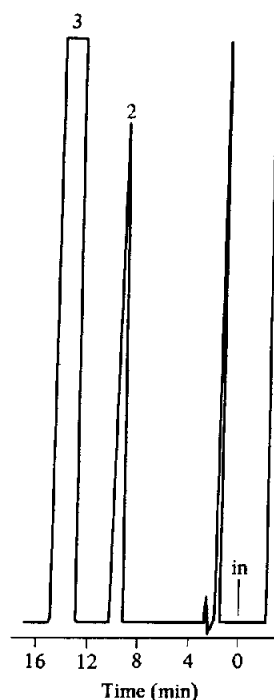
Figure 1
Chromatogram of a denatured 'zero time sample' containing salicylic acid (2), Hip–His–Leu (3).

recovery of hippuric acid in urine with and without incubation*

	Recovery (%)			
	No incubation		Incubation	
tration (µg ml^{-1})		SD(%)		SD(%)
	103	(1,24)	104	(1,03)
	101	(1,21)	99	(1,13)
	99	(1,46)	100	(1,14)
	98	(1,17)	99	(1,37)
	97	(1,24)	100	(1,28)
	98	(1,35)	102	(1,27)

nber of estimations = 4.
uric acid was added to a final volume of 250 µl containing 80 mM borate
carbonate buffer adjusted at pH 7.8 with HCl 5N.
e first case hippuric acid was added in the presence of ACE preparation.
istrate was added after denaturation of the enzyme with HCl (Fig. 2).
second case no enzyme was added and HCl was added at the end of 60 min
ion at 37°C.

chromatogram of a urine sample containing
alicylic acid (2), Hip–His–Leu (3) and
acid (1).

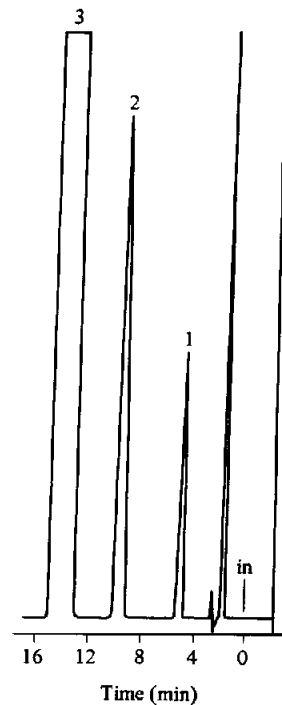

re 3 shows the hippuric acid formed during incubation of a biological sample
with Hip–His–Leu. ACE activity in urine and serum for normal (average of eight
ts) and transplanted subjects are summarized in Table 2.
values of serum activity are significantly higher than those reported in a previous
15]. This is attributable to the use of borate instead of phosphate buffer, as
ed by Pre and Bladier [16].

Figure 3
Chromatogram of a urine sample incubated as
described in experimental:hippuric acid liberated (1),
salicylic acid (2) and Hip–His–Leu (3).
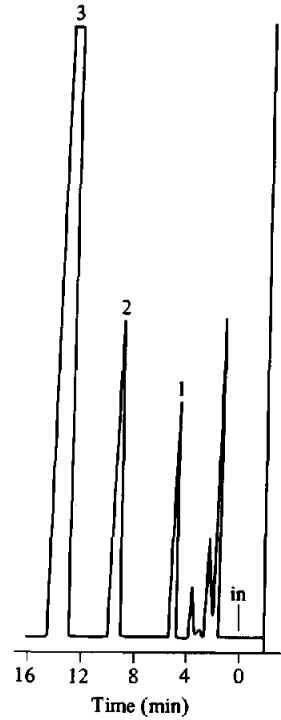
Figure 4
Angiotensin-converting enzyme activity in urine
samples from normal and transplanted subjects.
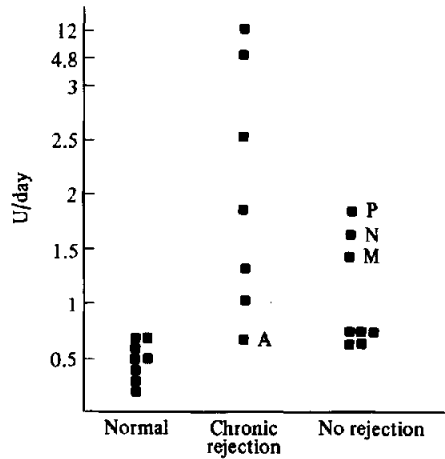
Prior to the present work, an HPLC method had not been applied to measurements of ACE activity in urine. The proposed method, previously applied to serum, has proved useful and valid for urine.

Three groups of subjects were examined (Table 2, Fig. 4). For the first group (normal subjects) values were found with a mean ($\pm$SD) of 0.46 ± 0.15 U day^{-1} for urinary ACE. In the second group (patients with chronic rejection) only patient A shows a low

Table 2
Angiotensin-converting enzyme activity in urine and serum from normal and transplanted subjects

	Normal		Transplanted			
			Chronic rejection		No rejection	
	Urine (U day⁻¹)	Serum (mU ml⁻¹)	Urine (U day⁻¹)	Serum (mU ml⁻¹)	Urine (U day⁻¹)	Serum (mU ml⁻¹)
(1)	0.4	20.2	(A) 0.7	41.1	(H) 0.6	35.1
(2)	0.2	25.2	(B) 1.01		(I) 0.7	18.3
(3)	0.6	38.1	(C) 2.5		(L) 0.7	
(4)	0.5	18.0	(D) 1.3		(M) 1.4	31.1
(5)	0.3	38.1	(E) 1.83	20.2	(N) 1.6	
(6)	0.65	32.2	(F) 12.1	24.1	(O) 0.7	
(7)	0.5	25.4	(G) 4.5	58.0	(P) 1.8	27.2
(8)	0.4	28.2			(Q) 0.6	25.4
Mean	0.46	28.17				
SD	0.15	7.59				

value of urinary ACE (0.7 U day^{-1}), having been treated with captopryl 75 mg day^{-1} because of hypertension. The drug, partially excreted in urine, could have lowered the value of ACE.

Of the nine patients in the third group, who had undergone transplantation and had a well-functioning graft, five had normal values of urinary ACE activity. Three showed high ACE activity values: patient M had had an acute rejection a few months before testing (probably his tubular damage had not yet been resolved). Patient N, just after testing, developed hydronephrosis of the transplanted kidney (tubular damage is always associated with this disease). Patient P had serious polyuria (5000 ml day^{-1}), which evidently expressed an altered tubular function. Serum ACE activities were also examined for some normal subjects and transplanted patients, but no consistent changes were observed, in agreement with the data given by Römmer [11].

The sample of subjects examined in this preliminary work is not sufficiently representative to draw more definite conclusions. More extensive screening is being carried out in order to elucidate the problems proposed.

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