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Note

Determination of kininase I and kininase II activities in human urine by high-performance liquid chromatography

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Kininases are enzymes that deactivate kinins. The best known kininases, such as kininase I and II, are not (kinin) substrate specific, but they can cleave other peptides with susceptible bonds. Kininase I is arginine carboxypeptidase (EC 3.4.12.7) and also functions as an anaphylotoxin inactivator [1]. The dipeptidyl dipeptidase kininase II (EC 3.4.15.1) is identical with angiotensin-converting enzyme (ACE) [2–5]: it deactivates kinins, enkephalins [6,7] and insulin [8].

Human urinary kininase I and II increase in hypertension [9,10]. Recently, some competitive inhibitors of kininase II have been shown to be antihypertensive drugs [11], and urinary kininase II activity can be a useful guide to urine levels of ACE inhibitors after their administration [12].

In human urine, three kininases II of different molecular mass have been characterized [13]. A fraction of human urinary kininase partially purified by prolonged incubation hydrolyses bradykinin (Bk), producing Phe^8, Arg^9 and the heptapeptide Arg^1 — Pro^7 residue [14].

Bioassay techniques using the contraction of rat uterus [15–17], guinea pig ileum [18,19] or blood pressure change by Bk [20], have been used for the determination of kininase activity. Assays for kininases and other Bk-degrading enzymes have been based on dansylation and separation of the dansylated materials by thin-layer chromatography [21]. A fluorimetric assay of Bk-degrading enzymes, separating the residual intact Bk from its fragments and measuring the fluorescamine–Bk compounds, has also been described [22]. The activity of human urinary kininase was determined by radioimmunoassay [23]. Recently, a study was reported on the assay of human urinary kininase II with a hippuryl–phenylalanyl–arginine (Hip-Phe-Arg) substrate, showing that the condi-

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tions adopted for the Bk substrate [24] are identical with those used for the blocked tripeptide [25].

This paper describes a method for determination of human urinary kininase I and II based on the high-performance liquid chromatographic (HPLC) determination of hippuric acid (benzoylglycine, Bz-Gly) and hippuryl–phenylalanine (Hip-Phe) liberated from Hip-Phe-Arg.

EXPERIMENTAL

Materials

Hip-Phe-Arg was synthesized from Hip-Phe and Arg·HCl by the carbodiimide–hydroxysuccinimide procedure [25]. Hip-Phe was purchased from Serva (Heidelberg, F.R.G.), AG-50W-X8 from Bio-Rad Labs. (Richmond, CA, U.S.A.), and Bz-Gly and other chemical products from Fluka (Buchs, Switzerland).

Urinary sample assay

The urinary samples, which corresponded generally to 24-h excretion or some other specific interval before the assay, were covered with a layer of toluene. A 0.5-ml aliquot of urine diluted to 1.0 ml with 0.01 *M* Tris–HCl (pH 8.0) was dialysed overnight in 200 ml of the same buffer with magnetic stirring. A 0.5-ml aliquot of this sample, preincubated for 10 min at 37°C, was added to 50 μl of a solution containing 5 mg of Hip-Phe-Arg in 5 ml of 0.1 *M* Tris–HCl (pH 8.0) and incubated for 30 min at the same temperature. The reaction was stopped by adding 450 μl of acetonitrile–aqueous sulphuric acid (pH 3) (1:1, v/v) and left at 4°C. Before chromatographic analysis each sample was purified from macromolecules, peptides, amino acids and substrate excess using a small pasteur AG-50W-X8 column equilibrated with acetonitrile–aqueous sulphuric acid (1:1, v/v). A 0.200-ml aliquot of this sample, corresponding to 50 μl of urine, was injected into the chromatograph.

Control incubation was carried out in the absence of Hip-Phe-Arg or urine preparation.

The unit of activity (U) is defined as the amount of kininase I or II catalysing the release of 1 μmol of Hip-Phe or Bz-Gly, respectively, from Hip-Phe-Arg, per minute of incubation and per litre of urine.

High-performance liquid chromatographhy

The HPLC apparatus consisted of an HPLC pump, Model 1330 (Bio-Rad), a UV monitor, Model 1306 (Bio-Rad), a Leeds and Northrup Speedomax recorder furnished by C. Erba (Milan, Italy), an analytical column (25×0.4 cm I.D.) packed with 5-μm ODS2 prepared by Violet (Rome, Italy), and a Rheodyne 1725 injector valve fitted with a 200-μl loop.

The mobile phase consisted of acetonitrile–aqueous sulphuric acid (pH 3.0) (1:1, v/v) and the flow-rate was 1.5 ml/min.

Quantification of hippuric acid and Hip-Phe was done by using peak heights measured at 230 nm.

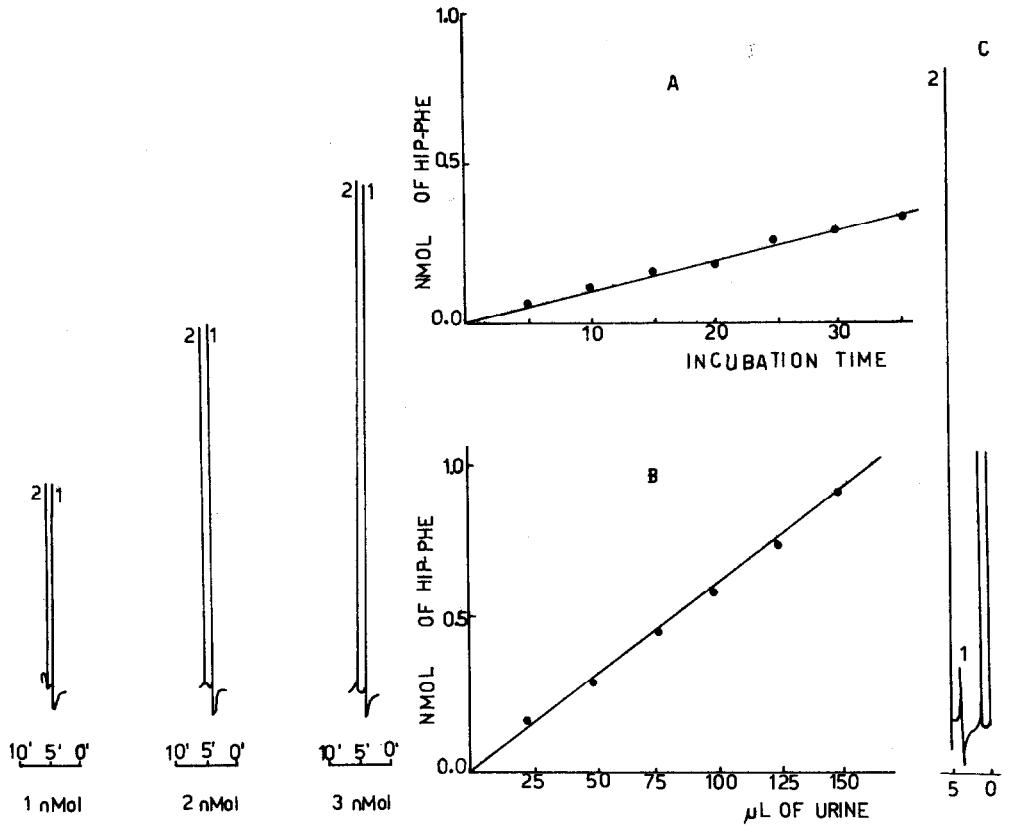

Fig. 1. Partial chromatograms from three samples containing, respectively, standard mixtures of 5, 10 and 15 nmol of both Bz-Gly and Hip-Phe in 1 ml of a solution of 0.550 ml of 0.01 *M* Tris–HCl (pH 8.0) and 0.450 ml of acetonitrile–aqueous sulphuric acid (pH 3.0) (1:1). A 0.2-ml aliquot of samples, previously purified on a AG-50W-X8 column, was injected into the chromatograph. Peaks: 1 = Bz-Gly; 2 = Hip-Phe.

Fig. 2. Dependence of (A) incubation and (B) sample volume of Hip-Phe formation from Hip-Phe-Arg catalysed by kininase I contained in a urinary sample from a newborn human. Each value of the incubation time and of the sample volume diagrams represents the mean of three determinations. Analytical conditions described in Experimental. In diagram C, peak 1 is Bz-Gly and peak 2 is Hip-Phe.

RESULTS

Standard mixtures containing the same amounts of Bz-Gly and Hip-Phe show the same peak heights measured at 230 nm (Fig. 1).

A series of three mixtures containing 5, 10 and 15 nmol of both Bz-Gly and Hip-Phe in 300 μl of the buffer were added to 250 μl of dialysed urine, previously incubated at 37°C for 10 min. Each sample, with or without a further incubation for 30 min at 37°C, was collected in a tube containing 450 μl of acetonitrile–aqueous sulphuric acid (1:1). After purification on an AG-50W-X8 small column, a good recovery of Bz-Gly and Hip-Phe was obtained (Table I).

TABLE I

RECOVERY OF Bz-Gly AND Hip-Phe WITH AND WITHOUT INCUBATION

Determination by chromatography of Bz-Gly and Hip-Phe in samples prepared under the conditions given in Results section.

Amount of Bz-Gly and Hip-Phe added (nmol)	Recovery (mean, $n=3$) (%)			
	Before incubation		After incubation	
	Bz-Gly	Gly-Phe	Bz-Gly	Hip-Phe
1	102	98	101	101
2	103	99	98	101
3	97	99	102	103

Table II shows the increased formation of Bz-Gly that occurred when the same urinary samples were incubated with Hip-Phe-Arg in comparison with the amount of Bz-Gly formed from the Hip-His-Leu substrate.

As shown in Fig. 2, the extent of formation of Hip-Phe in a urinary sample from a newborn human increased linearly with incubation time and with sample volume. The same result was obtained for Bz-Gly formation detected in urine from a pregnant human (Fig. 3).

When urine samples were subjected twice to dialysis, a 50% loss of the enzymic activity was apparent.

DISCUSSION

The simultaneous assay of kininase I and II in human urine by HPLC shows whether there are physiological (see Figs. 2 and 3) or pathological differences between the two enzymes. For ACE assay, only substrates structurally related to

TABLE II

URINARY KININASE II ACTIVITY DETERMINED USING Hip-His-Leu OR Hip-Phe-Arg AS SUBSTRATE

Between the amount of Bz-Gly formed from Hip-His-Leu and Hip-Phe-Arg substrates, a significant correlation ($r=0.71, p<0.05$) was found.

Sample	Volume of 24-h excretion (ml)	Bz-Gly liberated (mean, $n=3$) (μmol per 24 h)	
		Hip-His-Leu	Hip-Phe-Arg
1	1200	0.200	0.816
2	1400	0.149	0.560
3	1200	0.120	0.400
4	1400	0.093	0.242
5	1850	0.271	1.060
6	1150	0.115	0.483
7	1900	0.221	2.077
8	1800	0.182	1.317

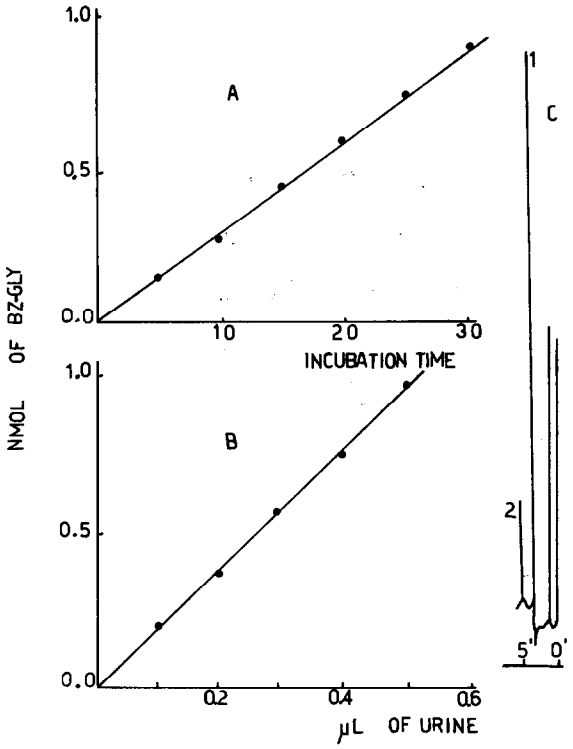

Fig. 3. Formation of Bz-Gly as a function of (A) incubation time and (B) sample volume, from Hip-Phe-Arg catalysed by kininase II contained in a urine sample from a pregnant human. Each value represents the mean of three determinations. Analytical conditions described in Experimental. In diagram C, peak 1 is Bz-Gly and peak 2 is Hip-Phe.

the C-terminal end of angiotensin I, such as Hip-His-Leu or Z-Pro-Phe-His-Leu (benzyloxycarbonyl–prolyl–phenyl–histidyl–leucine), were used. When kininase II was shown to be identical with ACE and was found to cleave the peptide bonds of a wide variety of amino acids, other substrates were synthesized [26].

According to Kokubu et al. [13], who used angiotensin I and the related substrate Hip-His-Leu, the Michaelis constant (K_M) of human urinary ACE was 2.9 mM, whereas using Bk [26] or Hip-Phe-Arg [25] the K_M values were $0.93 \cdot 10^{-7}$ M and $3.22 \cdot 10^{-7}$ M, respectively. In addition, the low affinity of kininase II for angiotensin I or Hip-His-Leu and the increased sensitivity for the deactivation of Bk or the degradation of Hip-Phe-Arg (see Table II) suggest a greater enzymic specificity for Bk and substrates structurally related to its C-terminal end.

The assay procedure can be performed with an ODS2 column equilibrated in acidified water (sulphuric acid, pH 3.0), eluted by acetonitrile–aqueous sulphuric acid (1:1) and washed with acetonitrile–aqueous sulphuric acid (8:2). The total analysis time for Bz-Gly and Hip-Phe determination is ca. 20 min.

Before purification on the AG-50W-X8 column, the samples were found to be stable at 4 °C for one week at the most. These features are advantageous when handling a large number of samples in clinical screening. Before the assay, the

dialysis or the membrane filtration of the urinary samples must be performed to remove the high concentrations of the pre-existent Bz-Gly.

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