Biochemical Pharmacology, Vol. 41, No. 5, pp. 821–827, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

A MICROMETHOD FOR THE DETERMINATION OF THE ACTIVITIES OF KININASES IN RAT PLASMA

KINETICS AND INHIBITORY CHARACTERISTICS

JEAN BARABÉ*† and DENISE HUBERDEAU

Département de Pharmacologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, Québec, Canada J1H 5N4

(*Received* 17 *October* 1989; *accepted* 12 *September* 1990)

Abstract—Kininase II (EC 3.4.15.1) (KII) and kininase I (KI) (EC 3.4.12.7) activities of rat plasma were characterized by the hydrolysis of hippuryl-L-histidyl-L-leucine (HHL), hippuryl-L-arginine (HLA) [expressed as carboxypeptidase N1 (CN1) activity] and hippuryl-L-lysine (HLL) [expressed as carboxypeptidase N2 (CN2) activity]. Using a spectrophotometric assay, biochemical characteristics of the three enzymes were investigated. The Michaelis–Menten constants were as follows: KII: K_m 2.55 ± 0.22 mM, V_{max} 0.357 ± 0.017 μmol/min/mL; CN1: K_m 6.93 ± 0.32 mM, V_{max} 0.748 ± 0.019 μmol/min/mL; and CN2: K_m 35.8 ± 1.52 mM, V_{max} 13.11 ± 0.40 μmol/min/mL. EDTA and *O*-phenanthroline inhibited the three enzyme assays at the same K_i, whereas captopril and 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MERGETPA), allowed for the demonstration of the specificity of each assay. Furthermore, K_i values of MERGETPA against both CN1 (4.75 μM) and CN2 (2.36 μM) activities do not support the hypothesis that KI activity may be accounted for by the presence of isoenzymes in rat plasma.

Plasmatic degradation of bradykinin (BK‡) has been attributed to the presence of two important enzymes. Kininase II (KII) [or angiotensin-converting enzyme (EC 3.4.15.1)] acts as a carboxydipeptidase and splits the Pro^7-Phe^8 bond of BK; this enzyme is present in plasma in a low concentration [1]. The other enzyme is kininase I (KI) or arginine carboxypeptidase (EC 3.4.12.7); it is also called carboxypeptidase N (CN) to distinguish it from pancreatic carboxypeptidase A and B [2]. Based on results obtained by isoelectric focusing, Koheil and Forstner [3] have suggested that human blood contains two isoenzymes responsible for KI activity. Furthermore, Schweisfurth *et al.* [4] have characterized two carboxypeptidase N (CN) activities in human plasma by their substrate specificity and their inhibitory profile: CN1 splits the synthetic substrate hippuryl-L-arginine (HLA) and it is not inhibited by EDTA, whereas CN2 hydrolyzes hippuryl-L-lysine (HLL) and is inhibited by EDTA. Furthermore, *O*-phenanthroline and dimercaprol show different I_{50} values against both enzyme activities.

The present study was undertaken to characterize these three enzyme activities in rat plasma and to verify if both CN1 and CN2 activities are found in this species. We have described methods for determining KII, CN1 and CN2 activities using small volumes of plasma and HPLC procedures to measure hippuric acid (HA), one of the reaction products.

MATERIALS AND METHODS

HA, HLA, HLL, Hippuryl-L-histidyl-L-leucine (HHL), phosphoramidon and thiorphan were obtained from Sigma; MERGETPA (2-mercaptomethyl-3-guanidinoethylthiopropanoic acid) was obtained from Calbiochem and captopril was a gift from Squibb. All other chemicals were obtained from Fisher Scientific. All solutions were made with deionized, twice-distilled water.

Plasma samples. Heparinized (25 units/mL) blood was collected from the left carotid artery of male Wistar rats under pentobarbital anesthesia (60 mg/kg, intraperitoneal), in order to avoid hemolysis since hemoglobin may interfere with the enzymatic reaction [4]. Blood samples were centrifuged at 1400 *g* for 30 min at 4°, and plasmas were pooled and stored at −20° until used. In these conditions, enzymatic activities (KI and KII) of the plasma are not modified for at least 12 months [4, 5].

Kininase assays. All assays were performed in 1.5-mL polypropylene tubes. The assay buffer consisted of a potassium phosphate buffer (0.1 M, pH 8.3) containing 0.3 M NaCl and 0.2 mM $CoCl_2$ for the KII assay and 0.15 M NaCl and 0.1 mM $CoCl_2$ for the CN1 and CN2 assays. KII was evaluated by measuring the hydrolysis of HHL as described by Cushman and Cheung [6]. The two CN assays were carried out according to Schweisfurth *et al.* [4]: CN1 was evaluated by the hydrolysis of HLA, and CN2 was measured by the hydrolysis of HLL. Preliminary studies were made to determine the optimal

* Scholar of the Fonds de la Recherche en Santé du Québec.

† Correspondence should be sent to: Jean Barabé, Ph.D., Institut de recherches cliniques de Montréal, 110 Avenue des Pins Ouest, Montréal, Québec, Canada H2W 1R7.

‡ Abbreviations: BK: bradykinin; KII: kininase II; KI: kininase I; CN: carboxypeptidase N; HA: hippuric acid; HLA: hippuryl-L-arginine; HLL: hippuryl-L-lysine; HHL: hippuryl-L-histidyl-L-leucine; and MERGETPA: 2-mercaptomethyl-3-guanidinoethylthiopropanoic acid.

conditions (substrate concentrations, incubation time, volume of plasma) for each assay. All tests were done in a final volume of 0.1 mL. The plasma diluted in the buffer was preincubated at 37° for 5 min before testing to allow the warming of the solution, since the samples were kept on ice before the experiment, and to allow the activation of the enzymes by cobalt ions, as suggested by Erdos and Yang [2]. Then the substrate solution, maintained at 37°, was added to the tubes and the reaction was allowed to proceed. The enzymatic reaction was stopped by the addition of 0.1 mL of 1 N HCl and the tubes were kept at room temperature until the extraction procedure.

The hydrolysis product measured for the three reactions was HA. The HA formed was extracted by the addition of 0.6 mL of ethyl acetate to the tubes; the tubes were vortexed for 15 sec and centrifuged for 20 sec in an Eppendorff centrifuge. Then 0.4 mL of the organic phase was taken in a clean tube and evaporated to dryness under air flow at room temperature overnight; 0.05 mL of NaCl (0.15 M) was added to the tubes which were thoroughly agitated for 15 sec. The HA formed was evaluated by HPLC using HA solutions as standard. The solution of HA (5 mM) was sequentially diluted to make standard solutions which were processed in parallel with test samples.

HPLC procedure. The method of Hendriks *et al.* [7] was used with slight modifications. A reversed phase HPLC system consisting of a pump (Waters, model M-45), an injector (Waters, model U6K), a spectrophotometer set at 230 nm, and a Vydack C-18 column was used. HA was eluted using isocratic conditions; the mobile phase consists of 40% methanol in sodium phosphate buffer (1 mM, pH 3.0) at a flow rate of 1.5 mL/min. Under these conditions, HA has an elution volume of 1.7 mL, HLA and HLL are eluted at the injection peak, and HHL has an elution volume of 4.5 mL; thus, the various substrates do not interfere with the measurement of HA. The minimal detectable amount of HA was 0.37 nmol at a sensitivity of 0.2 AUFS (absorbance units full scale). Injections of standard solutions were followed by the unknown samples, and the amount of HA was evaluated by the height of the peak. A typical tracing is illustrated in Fig. 1.

Characterization of enzyme activities. Each enzyme activity was analyzed using the Michaelis–Menten equation and was characterized by the K_m value of the substrate and the V_{max} of the hydrolysis using the Lineweaver–Burk transformation.

In another series of experiments, K_i values of various inhibitors were determined in each assay in order to demonstrate its specificity. The inhibitors, at the desired concentration, were added to the reaction tubes just before the preincubation period and kept in contact with the plasma 5 min prior to the addition of the substrate. EDTA and *O*-phenanthroline were used to differentiate CN1 and CN2 activities as described by Schweisfurth *et al.* [4]; MERGETPA, a specific and competitive inhibitor of CN activity [8], and captopril, a specific and competitive inhibitor of KII activity [9], served to show the specificity of each reaction. Furthermore,

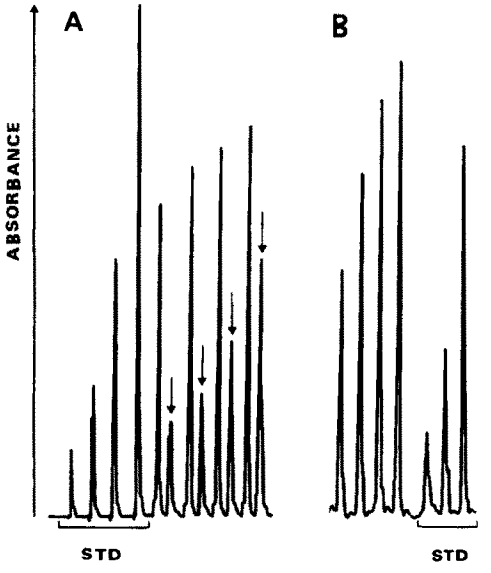


Fig. 1. Typical chromatogram of kininase activities. (A) Kininase II assay: standard solutions (STD) of HA (0.375 to 3 nmol) followed by unknown samples. HHL (1.5 to 6 mM) was incubated in the presence of 5 μL of rat plasma for 20 min. Note the presence of HHL in the extract (arrows). (B) Kininase I assay: unknown samples followed by standard solutions of HA (0.93 to 3.75 nmol). HLL (30 to 120 mM) was incubated in the presence of 2.5 μL of rat plasma for 10 min. Ordinate: absorbance at 230 nm.

phosphoramidon and thiorphan, two known inhibitors of endopeptidase (EC 3.4.24.11) [10, 11], were used to exclude the possibility that the hydrolysis of HHL was due to this enzyme.

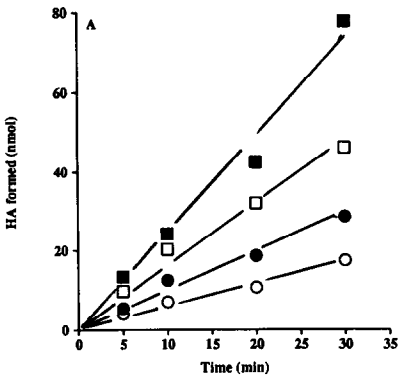
Analysis of the results. Results are expressed in nanomoles of HA formed, and analysis was performed by linear regression using a microcomputer and the statistical program of Tallarida and Murray [12]. All values were corrected by a factor of 66.7% to take into account the fractional volume of ethyl acetate evaporated (0.4 mL of 0.6 mL), and the recovery of the extraction procedure was not considered since standard solutions were treated in parallel with the unknown samples.

The intraassay variation (within-day variation) was evaluated, in a limited number of experiments, by measuring the enzyme activities of the same plasma. The interassay variation (between days) was also evaluated by comparing the activities of the same plasma measured in different experiments.

RESULTS

In all assays, the recovery of HA after extraction was evaluated to be between 80 and 85%. At 0.1 mM or less (KII and CN1 assays) the recovery averaged 85%, and at 1 mM (CN2 assay) the mean of the recovery was 80%. The regression lines obtained from the standard solutions of HA gave a correlation coefficient of 0.99 in all tests performed.

KII assay. KII activity was measured by the



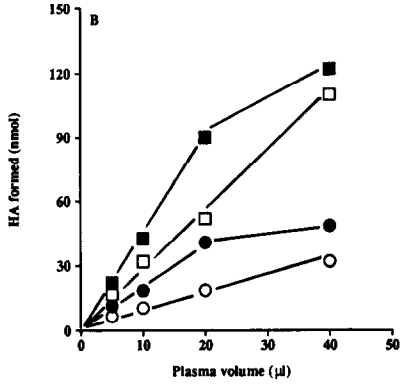
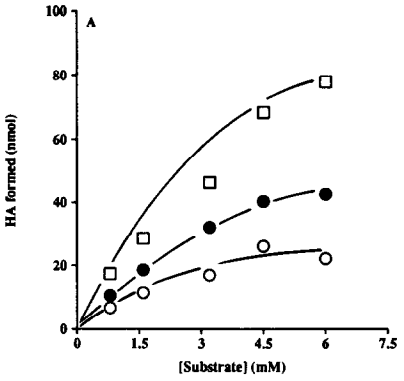


Fig. 2. Hydrolysis of HHL as a function of time (A) and plasma volume (B). In panel A, substrate concentrations of 0.8 mM (○), 1.6 mM (●), 3.2 mM (□) and 6 mM (■) were incubated in the presence of 10 µL of plasma. In panel B, the same concentrations were incubated in the presence of an increasing volume of plasma for 20 min.



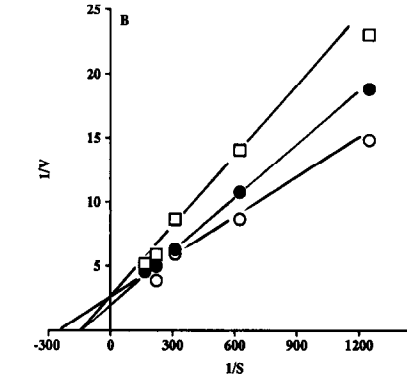
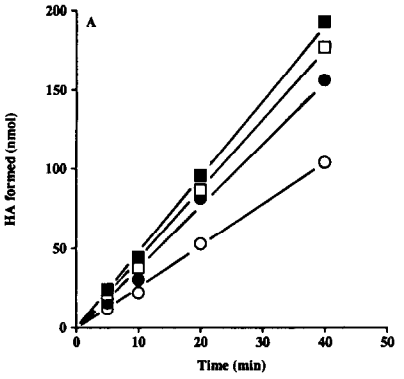


Fig. 3. (A) Saturation curves of KII. HHL concentrations of 0.8 to 6 mM were incubated for 20 min with 5 µL (○), 10 µL (●) and 20 µL (□) of plasma. (B) Lineweaver–Burk transformation of the curves presented in panel A. On the ordinate V is expressed in µmol/min/mL, and on the abscissa S is expressed in mM.

hydrolysis of HHL. Figure 2A illustrates the formation of HA as a function of incubation time when 0.8 to 6 mM HHL was incubated in the presence of 10 µL of plasma for 5–30 min. The figure shows that hydrolysis was linear and proportional to the substrate concentration for an incubation period of up to 30 min. Incubation periods of 20 min were used in all subsequent experiments. Figure 2B shows the formation of HA as a function of plasma volume ranging from 5 to 40 µL; the results indicate that the hydrolysis was proportional to the plasma volume up to 20 µL; then the curves became flat when the plasma volume was increased to 40 µL. Following this analysis, saturation curves were made using 0.8 to 6 mM HHL and various volumes of plasma for an incubation period of 20 min; the results are illustrated in Fig. 3A. For a plasma volume equal to or lower than 20 µL, maximal hydrolysis was reached at a 4.5 mM concentration of the substrate. A Lineweaver–Burk analysis of these curves is presented in Fig. 3B. These curves show a mean K_m value of 5.2 ± 0.58 mM and a V_{max} of 0.411 ± 0.017 µmol/min/mL. In light of these results and in order to avoid substrate consumption higher than 10%, in routine assays we used a plasma volume of 5 µL, 1.5 to 6 mM HHL, and an incubation period of 20 min to determine K_m and V_{max} values of HHL for plasma KII.

KI assays: CN1 and CN2. The same protocols were applied to study the hydrolysis of HLA, the substrate for CN1, and HLL, the substrate for CN2. Figure 4A illustrates the hydrolysis of HLA in concentrations ranging from 5 to 40 mM when incubated in the presence of 10 µL of plasma for a period of 5 to 40 min and that of HLL, in concentrations from 7.5 to 120 mM for a period of 2.5 to 20 min in the presence of 2.5 µL of plasma (Fig. 4B).

Hydrolysis of HLA proceeded linearly at each concentration of substrate used up to 40 min; the hydrolysis of HLL was linear from 2.5 to 20 min. For both substrates, the plasma volume influenced the rate of hydrolysis, as illustrated in Fig. 5; the hydrolysis of both substrates (HLA and HLL) was linear between 2.5 and 20 µL of plasma but the curves flattened at 40 µL. Saturation curves were made and are illustrated in Figs. 6 and 7 with their linear transformations. As shown in Fig. 6, CN1



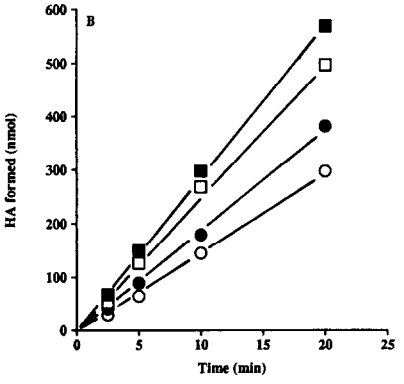
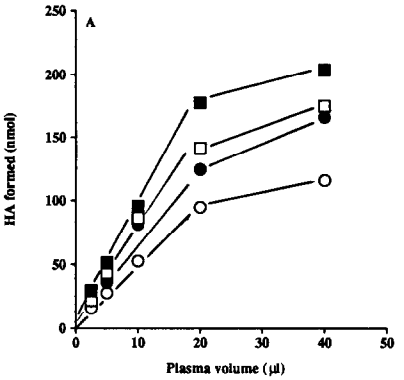


Fig. 4. (A) Hydrolysis of HLA (CN1 activity) as a function of time. Substrate concentrations of 5 mM (○), 10 mM (●), 20 mM (□) and 40 mM (■) were incubated in the presence of 10 μL of plasma. (B) Hydrolysis of HLL (CN2 activity) as a function of time. Substrate concentrations of 7.5 mM (○), 15 mM (●), 30 mM (□) and 120 mM (■) were incubated in the presence of 2.5 μL of plasma.



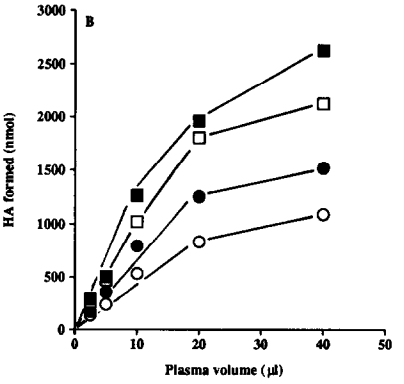


Fig. 5. (A) Hydrolysis of HLA (CN1 activity) as a function of plasma volume. HLA concentrations of 5 mM (○), 10 mM (●), 20 mM (□) and 40 mM (■) were incubated in the presence of plasma for 20 min. (B) Hydrolysis of HLL (CN2 activity) as a function of plasma volume. HLL concentrations of 7.5 mM (○), 15 mM (●), 30 mM (□) and 120 mM (■) were incubated in the presence of plasma for 10 min.

activity showed saturation at approximately 30 mM HLA and Lineweaver–Burk transformation of two such experiments gave a K_m value of 4.75 ± 0.53 mM and a V_{max} value of 0.549 ± 0.033 μmol/min/mL. The same analysis for the HLL (CN2 activity) is illustrated in Fig. 7 which shows that higher concentrations of HLL were needed to saturate the enzyme (120–240 mM); linear transformation (Fig. 7B) gives a K_m value of 30.19 ± 2.97 mM and a V_{max} value of 13.79 ± 0.37 μmol/min/mL.

In light of these results, in routine assays we used the following parameters: for the CN1 assay: 10 μL of plasma with substrate concentrations ranging from 5 to 40 mM and an incubation of 20 min; for the CN2 assay: 2.5 μL of plasma, HLL concentrations ranging from 15 to 120 mM, and an incubation period of 10 min.

Under these conditions, the intraassay variations for the three assays were 4.32 ± 0.65% (N = 7) for the KII assay, 6.27 ± 0.76% (N = 7) for the CN1 assay, and 5.22 ± 0.23% (N = 4) for the CN2 assay. The interassay variations were from 5.27 to 7.5%. These methods were used in a limited number of plasma samples from Wistar rats; the results are listed in Table 1 which gives the means of K_m and V_{max} values of each substrate together with the correlation coefficient (R) of the Lineweaver–Burk lines. The latter value being near 1.0 demonstrates again the accuracy of the methods.

Characterization of enzyme activities. To characterize the hydrolysis of each substrate as representative of the activity of each enzyme, various enzyme inhibitors were tested and their K_i values determined in each assay. The results, shown in Table 2, indicate that EDTA and *O*-phenanthroline, two chelating agents and nonspecific inhibitors, have the same potency to inhibit the three enzyme activities. Captopril, a specific and competitive inhibitor of KII, had a K_i value of 1.6 μM for the hydrolysis of HHL but higher concentrations (*ca.* 1.0 mM) were needed to inhibit the hydrolysis of HLA and HLL. MERGETPA, a potent and specific inhibitor of KI, inhibited both CN1 and CN2 activities but was totally inactive in the KII assay in concentrations up to 0.1 mM. This compound was twice as potent an inhibitor of CN2 activity (K_i = 2.36 μM) than CN1 (K_i = 4.75 μM) activity. Two

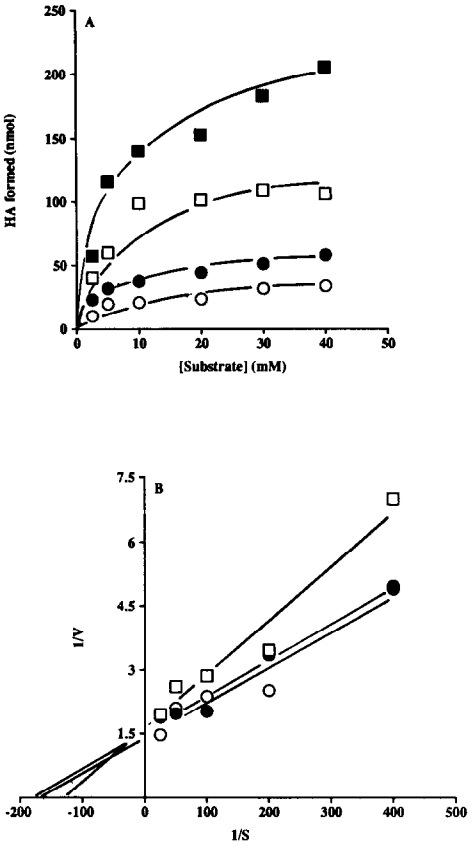


Fig. 6. Saturation curves of HLA (CN1 activity). Increasing concentrations of HLA were incubated with 2.5 (○), 5 (●), 10 (□) and 20 (■) μL of plasma for 20 min. (B) Lineweaver–Burk transformation of the curves presented in panel A. On the abscissa S is expressed in mM, and on the ordinate V is expressed in μmol/min/mL.

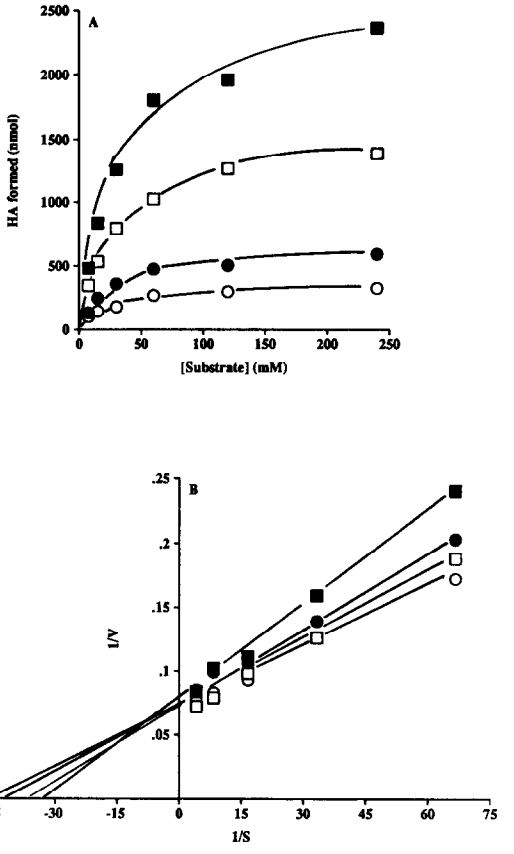


Fig. 7. (A) Saturation curves of HLL (CN2 activity). Increasing concentrations of HLL were incubated with 2.5 (○), 5 (●), 10 (□) and 20 (■) μL of plasma for 10 min. (B) Lineweaver–Burk transformation of the curves presented in panel A. Units for S and V on the abscissa and ordinate respectively, are as given in the legend of Fig. 6.

inhibitors of the neutral-endopeptidase (NEP, enkephalinase, EC 3.4.24.11) showed interesting results; while thiorphan (0.1 mM) was totally inactive in the three assays, phosphoramidon (0.29 mM) exerted inhibitory activity against the hydrolysis of HHL.

DISCUSSION

The methods applied in the present study to estimate kininase (KII and KI) activities have allowed for precise and accurate measurements of such activities, using very small volumes of plasma, as shown by the good intraassay and interassay coefficients. The availability of small synthetic substrates leading to the same reaction product (HA) and the utilization of HPLC procedures to purify and measure the HA formed led to the setting up of a sensitive and very rapid test. In fact, under the conditions used in the present study, as low as 0.37 nmol of HA could be detected and many samples could be analyzed in a few hours since the retention time of HA is very short.

The utilization of HPLC to measure the formation of HA confers higher specificity to the KII assay. As shown in Fig. 1, the presence of the substrate (HHL) was always observed in the ethyl acetate extract and the proportion of the substrate increased at the higher concentrations and may be equivalent to 50% of the total absorbance of the sample. Without this purification step or without a blank extraction of the substrate for each concentration used, one can overestimate the rate of hydrolysis at higher substrate concentrations, resulting in higher K_m and V_{max}.

Kinetic analysis of the hydrolysis of HHL (KII activity) shows a linear relationship with time (up to 30 min) and plasma volume (up to 20 μL). The saturation experiment shows that the enzyme saturated between 4.5 and 6.0 mM and, at substrate concentrations of 6 mM or more, a decrease of the rate of hydrolysis was observed regularly; this effect has also been observed by many workers, who used HHL or other synthetic substrates [6, 13].

The K_m value for HHL measured in our assay is in close agreement with that reported by other workers for rat serum [14, 15]. The V_{max} value (0.357 μmol/min/mL) is close to the value reported

Table 1. Characteristics of kininases activities in rat plasma

	Substrate (mM)	K_m (mM)	V_{max} (μmol/min/mL)	R^*
Kininase II	HHL (1.5–6)	2.55 ± 0.22†	0.357 ± 0.017	0.953 ± 0.007
Kininase I				
CN1	HLA (5–40)	6.93 ± 0.32	0.748 ± 0.019	0.988 ± 0.004
CN2	HLL (30–120)	35.8 ± 1.52	13.11 ± 0.40	0.976 ± 0.006

* R: correlation coefficient.
† Values are means ± SEM of 27 animals.

Table 2. K_i values obtained with enzyme inhibitors in three kininase assays with rat plasma

	K_i		
	KII assay	KI assays	
Compound	HHL	CN1:HLA	CN2:HLL
EDTA	0.48 mM	0.34 mM	0.22 mM
O-Phenanthroline	0.44 mM	0.29 mM	0.36 mM
Captopril	1.6 μM	0.93 mM	1.52 mM
MERGETPA	None*	4.75 μM	2.36 μM
Thiorphan	None	None	None
Phosphoramidon	0.29 mM	None	None

* No inhibition was observed at a concentration up to 0.1 mM.

by Ibarra-Rubio *et al.* [13] but higher than that reported by Santos *et al.* [14]. The discrepancy may be explained by the fact that these authors did not activate the enzyme by the use of $CoCl_2$ as suggested by Cushman and Cheung [6].

The measurement of KI activities, both CN1 and CN2, does not need the purification of the extract by HPLC for a precise evaluation since the substrates (HLA and HLL) are not extracted with ethyl acetate, as illustrated in Fig. 1. The rate of hydrolysis of HLA and HLL was linearly correlated to the time of incubation and with the plasma volume (up to 20 μL).

The K_m values for HLA (CN1 activity) (6.93 ± 0.32 mM) and HLL (CN2 activity) (35.8 ± 1.52 mM) are close to those reported by Schweisfurth *et al.* [4] for human plasma but higher than the values reported by Hendricks *et al.* [7]; we have no explanation for this discrepancy. The V_{max} values calculated in this study are higher than values reported by others in rat plasma [2]; the difference may be due to the pH of the buffer since those authors used a pH of 7.4 in their assay and the enzyme has an optimal pH at 8.0 to 8.4 [4, 7]. This value is also higher than that found for human plasma [4, 7]; however, this result confirms the observation of Erdos *et al.* [16] that rat plasma contains higher concentrations of KI activity than human plasma.

In light of the results obtained by Schweisfurth *et al.* [4] on the inhibitory pattern of CN1 and CN2 activities in human plasma, the same inhibitors were tested in rat plasma. Furthermore, the analysis was extended to other enzyme inhibitors and to the hydrolysis of HHL (KII activity).

It is well known that EDTA and *O*-phenanthroline are good inhibitors of both KI and KII activities [2]; the results of the present study confirm this observation: EDTA and *O*-phenanthroline inhibited the hydrolysis of the three substrates with approximately the same K_i values. This result does not confirm the observation of Schweisfurth *et al.* [4], who used these inhibitors to characteize CN1 and CN2 activities and showed that EDTA does not inhibit the hydrolysis of HLA by human plasma. However, our results confirm the observation of Erdos *et al.* [17] that EDTA inhibits the hydrolysis of HLA. Furthermore, in two experiments, EDTA effectively inhibited the hydrolysis of HLA by human plasma (data not shown).

The results obtained with captopril and MERGETPA demonstrate the specificity of each assay. While captopril was active against the hydrolysis of HHL at micromolar concentrations, millimolar concentrations were needed to inhibit the KI activities. On the contrary, MERGETPA, at micromolar concentrations, inhibited the hydrolysis of HLA and HLL and was inactive in the KII assay in concentrations up to 0.1 mM. The inhibitory potencies of captopril (in KII assay) and MERGETPA (in KI assays) are lower than those reported by others [8, 13]; the difference may be accounted for by the presence of $CoCl_2$ as enzyme activator, in our assay, or by the short preincubation time used in the present experiment (5 min).

Phosphoramidon and thiorphan were inactive

against the hydrolysis of HLL and HLA and only phosphoramidon exerted some inhibitory effect in the KII assay. This result confirms the observation that phosphoramidon is less specific than thiorphan as an inhibitor of the neutral endopeptidase 24.11 [18].

In conclusion, the present results show that both KII and KI activities can be evaluated precisely and accurately in small volumes of rat plasma using HPLC techniques to measure the rate of hydrolysis of small synthetic substrates. The rate of hydrolysis of HHL and HLL or HLA is specific for the activity of KII and KI, respectively, as demonstrated by the K_i values of captopril and MERGETPA. The identical K_i values obtained with EDTA and *O*-phenanthroline against the hydrolysis of HLA and HLL and the inhibitory potency of MERGETPA in CN1 and CN2 assays do not support the hypothesis [4] that KI activity can be accounted for by two isoenzymes in rat plasma.

These methods can be applied to the plasma of other species and allow for a rapid and precise measurement of both KI and KII activities. Since the rat is used frequently to induce experimental pathological states, our methods allow a rapid test to study the modulation of both kininase activities under pathophysiological conditions.

Acknowledgements—This work was supported by a grant from the Medical Research Council of Canada (MA-6730) and from the Quebec Heart Foundation.

REFERENCES

1. Erdos EG, Kininases. *Handbook of Experimental Pharmacology (Suppl)* (Ed. Erdos EG), Vol. 25, pp. 427–487. Springer, New York, 1979.
2. Erdos EG and Yang HYT, Kininases. *Handbook of Experimental Pharmacology* (Ed. Erdos EG), Vol. 25, pp. 289–323. Springer, New York, 1970.
3. Koheil A and Forstner G, Isoelectric focusing of carboxypeptidase N. *Biochim Biophys Acta* **524**: 156–161, 1978.
4. Schweisfurth H, Reinhart E, Heinrich J and Brugger E, A simple spectrophotometric assay of carboxypeptidase N (kininase I) in human serum. *J Clin Chem Clin Biochem* **21**: 605–609, 1983.
5. Lijnen P, Staessen J and Amery A, Assay of plasma angiotensin-converting enzyme activity in captopril-treated subjects. *Methods Find Exp Clin Pharmacol* **4**: 413–415, 1982.
6. Cushman DW and Cheung HS, Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem Pharmacol* **20**: 1637–1648,1971.
7. Hendriks D, Scharpe S and van Sande M, Assay of carboxypeptidase N activity in serum by liquid-chromatographic determination of hippuric acid. *Clin Chem* **31**: 1936–1939, 1985.
8. Plummer TH and Ryan TJ, A potent mercapto bi-product analogue inhibitor for human carboxypeptidase N. *Biochem Biophys Res Commun* **98**: 448–454, 1981.
9. Ondetti M, Rubin M and Cushman DW, Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. *Science* **196**: 441–444, 1977.
10. Fulcher IS, Matsas R, Turner AJ and Kenny AJ, Kidney neutral endopeptidase and the hydrolysis of enkephalin by synaptic membranes show similar sensitivity to inhibitors. *Biochem J* **203**: 519–522, 1982.
11. Matsas R, Fulcher IS, Kenny AJ and Turner AJ, Substance P and [Leu]enkephalin are hydrolyzed by an enzyme in pig caudate synaptic membranes that is identical with the endopeptidase of kidney microvilli. *Proc Natl Acad Sci USA* **80**: 3111–3115, 1983.
12. Tallarida RJ and Murray RB, *Manual of Pharmacologic Calculation with Computer Programs*. Springer, Berlin, 1984.
13. Ibarra-Rubio ME, Pena JC and Pedraza-Chaverri J, Kinetic and inhibitory characterisics of serum angiotensin-converting enzyme from nine mammalian species. *Comp Biochem Physiol [B]* **92**: 399–403, 1989.
14. Santos RAS, Krieger EM and Greene LJ, An improved fluorometric assay of rat serum and plasma converting enzyme. *Hypertension* **7**: 244–252, 1985.
15. Yamaguchi T, Hiratsuka N, Ikekita M, Kizuki K and Moriya H, Rat testicular angiotensin I converting enzyme: Purification and comparison with rat pulmonary enzyme. *Adv Exp Med Biol* **198A**: 461–467, 1986.
16. Erdos EG, Sloane EM and Wohler IM, Carboxypeptidase in blood and other fluids. I. Properties, distribution and partial purification of the enzyme. *Biochem Pharmacol* **13**: 893–905, 1964.
17. Erdos EG, Renfrew AG, Sloane EM and Wohler JR, Enzymatic studies on bradykinin and similar peptides. *Ann NY Acad Sci* **104**: 222–235, 1963.
18. Matsas R, Kenny AJ and Turner AJ, The metabolism of neuropeptides. The hydrolysis of peptides, including enkephalins, tachykinins and their analogues by endopeptidase-24.11. *Biochem J* **223**: 433–440, 1984.