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RAPID ASSAY OF HUMAN PLASMA CARBOXYPEPTIDASE N BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF HIPPURYL-LYSINE AND ITS PRODUCT

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SUMMARY

A rapid and sensitive method for measuring carboxypeptidase N (CPN) activity in human plasma is described. The procedure is based on the hydrolysis of a high-specificity/low-affinity substrate, hippuryl-L-lysine, to its products hippuric acid and lysine. The substrate and product are separated quantitatively by high-performance liquid chromatography in less than 10 min following a minimal sample preparation time. The advantages of this method over previous ones are discussed and data are presented demonstrating the reliability of this method for the routine clinical determination of CPN activity.

INTRODUCTION

Plasma carboxypeptidase N (CPN) (E.C. 3.4.12.7), also known as kininase I, was discovered as a bradykinin (BK)-destroying enzyme of human plasma by Erdos and Sloane[1]. This same enzyme was found independently by Bokish and Muller-Eberhard[2] in the course of their search for the plasmatic "anaphylatoxin inactivator". Previous work by our group involving the HPLC separation of BK and its fragments following incubation in plasma has identified CPN as the main kininase in human plasma[3]. Recent findings in the pharmacology of kinins and anaphylatoxins reinforce the suggestion that CPN plays a role in the regulation of inflammatory peptides. It has been shown recently that CPN does not abolish the activities of BK and anaphylatoxin C5a, but rather promotes a qualitative change in their biological actions[4,5].

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Because of the suspected involvement of inflammatory peptides in various pathological conditions such as edema, certain allergies and arthritis[6], there is an evident need for a rapid and sensitive method for measuring CPN on a routine basis. The assays of CPN activity at present available are laborious, requiring multiple reaction and/or extraction steps[7–10]. In general, the enzyme activity has been determined by measuring substrate degradation by either biological assays or UV extinction or by examining product formation with spectrophotometry following reaction with ninhydrin. Also, most of the reported methods rely on preincubation of the enzyme with $CoCl_2$, a procedure which artificially elevates CPN activity 3-6-fold[8].

The aim of this study was to establish and validate a rapid CPN assay applicable to crude human plasma for routine clinical measurement. Similar to the method proposed by Erdos *et al.*[8], this procedure is based on the hydrolysis of hippuryl-L-lysine to hippuric acid and lysine. Because this assay is designed to facilitate the detection of plasmatic competitive inhibitors of the enzyme, a compound demonstrating high specificity but low affinity such as Hip-Lys was chosen as the substrate, although substrates of higher affinity are available[11].

The method described here takes advantage of the unique resolving power of high-performance liquid chromatography (HPLC) using a reversed-phase column to achieve a rapid and selective separation of substrate and product, thus permitting the simultaneous, quantitative measurement of both.

EXPERIMENTAL

CPN assay

Hippuryl-L-lysine and hippuric acid were purchased from Sigma (St. Louis, MO, U.S.A.).

Human blood (4.5 ml) was collected by venipuncture in evacuated tubes containing 0.5 ml of 0.129 *M* sodium citrate buffer (Vacutainer 6468). The plasma ob-

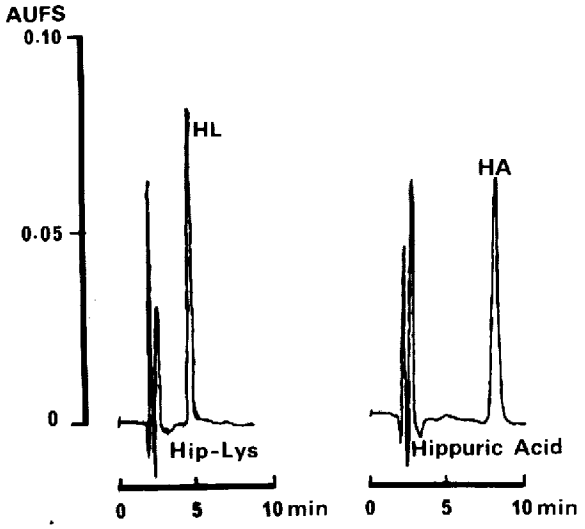

Fig. 1. HPLC traces of standard solutions (32 μg/ml) of Hip-Lys (HL) and hippuric acid (HA).

tained by centrifugation was frozen (−70°C) until utilization. The reaction mixture was composed of human plasma and 0.05 *M* HEPES buffer (3:1, v/v) containing 0.25 *M* NaCl, pH 7.75. The final concentration of the substrate Hip-Lys was $4.07 \cdot 10^{-4}$ *M*, and the volume of the reaction mixture was 0.5 ml. After 15–60 min of incubation at 37°C with constant agitation, the reaction was stopped by the addition of 0.5 ml of absolute ethanol. The mixture was then centrifuged and the clear supernatant applied to the HPLC column. Control experiments have shown a complete recovery of Hip-Lys and hippuric acid following this deproteinization procedure.

HPLC separation and measurement of Hip-Lys and hippuric acid

The chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisted of a Model 6000 A pump, coupled with a U6K injector and a variable-wavelength detector (Model 450) fixed at 230 nm. The isocratic mobile phase was a 1:4 mixture of methanol (Omnisolv; BDH, Poole, Great Britain) and 0.001 *M* K_2HPO_4 + H_3PO_4 (pH 3.0). It was pumped through a C_{18} reversed-phase column (μBondapak, 30 × 0.4 cm) at a rate of 1.5 ml/min. The analytical column was protected by a short (4-cm) pre-column. Because of the high percentage of plasma used in this assay, it was necessary to re-pack the pre-column with Bondapak C_{18} Corasil (27–50 μm; Waters Assoc.) after approximately 50 sample injections. Also, if the pressure exceeded 2500 p.s.i., the analytical column was washed with 25 ml of water, followed by 50 ml of methanol. During the methanol washing, four 2-ml injections of dimethyl sulfoxide were made. The column was then washed with 50 ml of methanol. Under these conditions the column efficiency had not declined after more than 200 injections. The injection volume was 15 μl.

Calibration graphs, with which plasma samples were compared, were prepared by injecting known concentrations of Hip-Lys and hippuric acid ranging from 16 to 313 μg/ml and measuring peak heights.

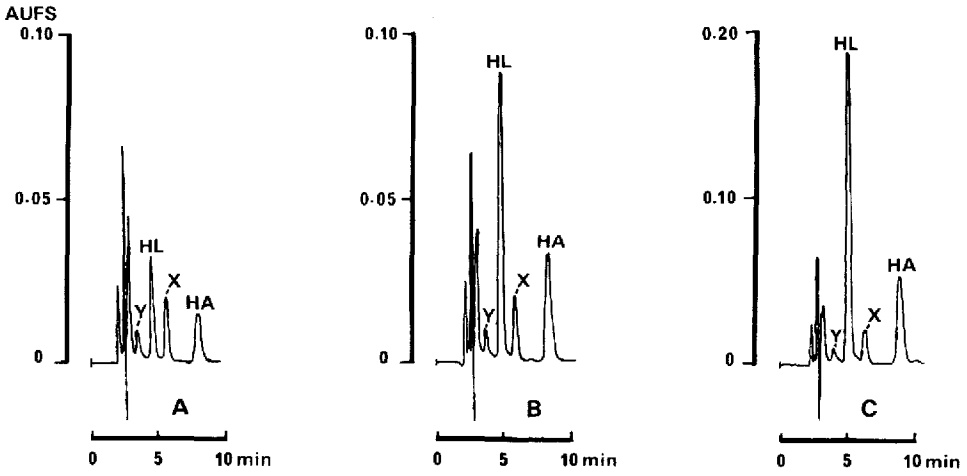

Fig. 2. HPLC traces of 15-min incubated solutions of Hip-Lys containing 75% human plasma. The substrate concentration was (A) $4.07 \cdot 10^{-4}$, (B) $1.02 \cdot 10^{-3}$ and (C) $2.03 \cdot 10^{-3}$ *M*. X and Y represent unknown substances from plasma.

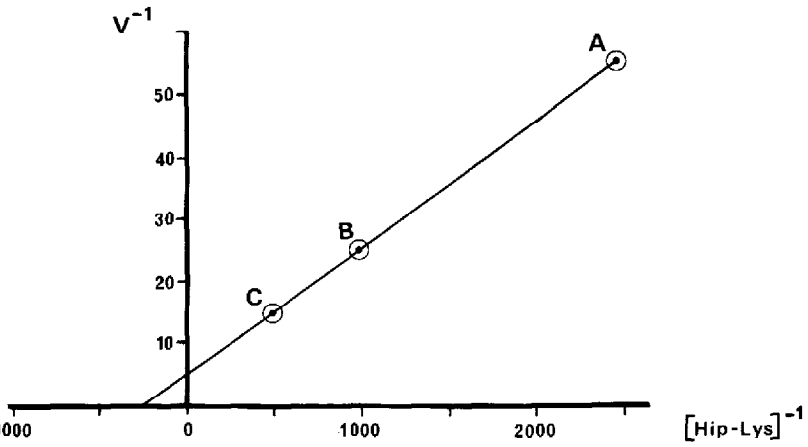

Fig. 3. Lineweaver-Burk analysis of the chromatographic data in Fig. 2. Abcissa, reciprocal of the initial molar concentration of substrate; ordinate, reciprocal of the formation rate (μmol/min/ml plasma) of hippuric acid.

RESULTS AND DISCUSSION

Separation of Hip-Lys and hippuric acid by HPLC

The separation of Hip-Lys from hippuric acid is demonstrated in the chromatograms in Fig. 1. The retention times were 4.8 and 8.0 min, respectively. Under the experimental conditions used the calibration graphs were linear up to 10^{-3} *M*. Solutions containing less than $5 \cdot 10^{-5}$ *M* of these compounds were not reliably detected at this sensitivity.

Biochemical validation of the assay

Fig. 2 shows the results of a 15-min incubation of normal plasma containing three different concentrations of Hip-Lys. The Lineweaver-Burk plot (Fig. 3) based on the chromatographic data of Fig. 2 shows excellent linearity. From extrapolation of the three-point linear regression[12], the maximal rate of hydrolysis (V_{max}) is shown to be 0.197 μmol/min/ml plasma and the Michaelis-Menten constant (K_M) is equal to $4.0 \cdot 10^{-3}$ *M*. The latter value is higher than that reported by Erdos *et al.*[8], who found a K_M value of $1.4 \cdot 10^{-3}$ *M* using the same substrate. However, direct comparisons are not possible because in their procedure the enzyme was activated by cobalt. Considering that the latter treatment might be expected to produce a lower apparent K_M, the two values might not be dissimilar. Taken together the data suggest that the high proportion of plasma (75%) in the incubation media does not interfere with the cleavage of Hip-Lys by CPN. The advantage of minimal sample dilution resides in the fact that drug-induced inhibition of CPN in certain plasma samples can be more readily detected. We intend to study the effect of slow-acting anti-rheumatic drugs on CPN *in vivo* using the present assay[13].

CPN levels in a normal population

To verify the feasibility of this assay for the routine determination of the

enzyme, CPN activity was measured in the plasma of 27 normal persons (16 females, 11 males). With the addition of $4.07 \cdot 10^{-4}$ *M* of Hip-Lys and an incubation period of 60 min at 37°C, the mean consumption of substrate was found to be 64.7%. This corresponds to a rate of 5.84 ± 0.606 nmol/min/ml of plasma (mean ± standard deviation). Although Erdos *et al.*[8] reported a substantially higher rate of Hip-Lys hydrolysis, divergent experimental procedures may again account for the discrepancy. Additional analyses of CPN activity in pathological conditions have been performed and will be published elsewhere.

CONCLUSION

The simplicity of the method described eliminates several sources of error inherent in previous methods used to measure CPN activity. It does not require extraction of plasma components; the enzyme does not need to be artificially activated by the addition of cobalt; samples are subjected to only a minimal dilution; and post-column derivatization with ninhydrin is not necessary. Finally, as the sample preparation time is minimal and each analysis requires only 10 min, the procedure lends itself readily to complete automation.

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REFERENCES

1 E. G. Erdos and E. M. Sloane, *Biochem. Pharmacol.*, 11 (1962) 585.
2 V. A. Bokish and H. J. Muller-Eberhard, *J. Clin. Invest.*, 49 (1970) 2427.
3 F. Marceau, M. Gendreau, J. Barabé, S. St.-Pierre and D. Regoli, *Can. J. Physiol. Pharmacol.*, 59 (1981) 131.
4 D. Regoli, F. Marceau and J. Lavigne, *Eur. J. Pharmacol.*, 71 (1981) 105.
5 H. D. Perez, I. M. Goldstein, R. O. Webster and P. M. Henson, *J. Immunol.*, 126 (1981) 800.
6 F. Marceau, A. Lussier, D. Regoli and J.-P. Giroud, *Gen. Pharmacol.*, (1982) in press.
7 M. C. Corbin, T. E. Hugli and H. J. Muller-Eberhard, *Anal. Biochem.*, 73 (1976) 41.
8 E. G. Erdos, E. M. Sloane and I. M. Wohler, *Biochem. Pharmacol.*, 13 (1964) 893.
9 A. Koheil and G. Forstner, *Biochim. Biophys. Acta*, 524 (1978) 156.
10 T. H. Plummer and M. T. Kimmel, *Anal. Biochem.*, 108 (1980) 348.
11 T. H. Plummer and E. G. Erdos, *Methods Enzymol.*, 80 (1981) 442.
12 H. Lineweaver and D. Burk, *J. Amer. Chem. Soc.*, 56 (1934) 658.
13 F. Marceau, A. Drumheller, S. St.-Pierre and A. Lussier, *Eur. J. Pharmacol.*, in press.