Assay of Carboxypeptidase N Activity in Serum by Liquid-Chromatographic Determination of Hippuric Acid

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We describe conditions for determining carboxypeptidase N (EC 3.4.17.3) activity by liquid chromatography. Serum (10 μL) is mixed with the artificial substrates hippuryl-L-arginine (30 mmol/L) and hippuryl-L-lysine (100 mmol/L) in 50 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solution at pH 8.2 and 7.8, respectively. The hippuric acid product is separated from the substrate in less than 2 min by reversed-phase "high-performance" liquid chromatography and measured spectrophotometrically. α-Methyl hippuric acid is used as internal standard. By this method, optimized for activity and sensitivity of detection, carboxypeptidase N activities are 60-fold greater than those by another procedure (J Chromatogr 266:173–177, 1983). The mean value for 80 normal control subjects was 74.8 (SD 10.3) nmol of hippuric acid released per milliliter of serum per minute for hippuryl-L-arginine substrate, 378 (SD 55) for hippuryl-L-lysine substrate. The sensitivity and precision of the method make it suitable both for routine clinical determinations and as a reference procedure.

Additional Keyphrases: enzyme activity · hippuryl-L-arginine and hippuryl-L-lysine synthetic substrates · chromatography, reversed-phase · kininase I · inflammation · kinins · propeptides · fibrinopeptides · reference interval

Carboxypeptidase N (kininase I, arginine carboxypeptidase, EC 3.4.17.3) cleaves carboxy-terminal basic amino acids from various peptides in human plasma. The enzyme shows high affinity for kinins (bradykinin and kallidin) and anaphylatoxins (C5a and C5a), removing their C-terminal arginine (1, 2), and for the fibrinopeptides FDP-6A and -6D, cleaving the C-terminal lysine (3). New interest in the effect of carboxypeptidase N on the metabolism of inflammatory propeptides was engendered by the discovery that kinin metabolites (produced by the action of carboxypeptidase N) have significant pharmacological actions. Indeed, as has been shown recently (4), carboxypeptidase N does not abolish the activities of bradykinin and anaphylatoxin C5a; rather, it promotes a qualitative change in their biological behavior. Observations that carboxypeptidase N activity is altered in various pathological conditions (5) have generated the need for an accurate, clinically applicable assay.

Carboxypeptidase N exists in two forms: carboxypeptidase N₁ (CN₁) and carboxypeptidase N₂ (CN₂) (6), which can be differentiated by their activities towards hippuryl-L-arginine (Hip-Arg) and hippuryl-L-lysine (Hip-Lys), respectively, as stated by Erdős et al. (7). Here we describe a method for determining CN₁ and CN₂ activities in human serum by using a chromatographic procedure. Detection of the enzyme-produced product is simple, fast, and specific, and is partly automated. The sensitivity of the assay is such that as little as 10 μL of serum provides reliable and accurate results.

Materials and Methods

Materials

Serum samples: Blood obtained from blood-bank donors, ages 18 to 65 years, was allowed to clot at room temperature and centrifuged within 2 h (2000 × g, 15 min). The serum was stored at −25 °C and assayed within a month after collection.

Reagents: Hip-Arg and Hip-Lys were from Bachem Feinchemikalien, Bubendorf, Switzerland; HEPES and HEPES were from Calbiochem, La Jolla, CA. All other reagents were of high purity and were from Merck, Darmstadt, F.R.G. α-Methylhippuric acid was synthesized from glycine and α-methylbenzoylchloride (UCB, Drogenbos, Belgium) by a procedure analogous to that used for the synthesis of hippuric acid (8). In all assays we used distilled, de-ionized water.

Instruments: We used an FK₂ water bath (Haake, Berlin, F.R.G.) for incubations (37.0 ± 0.1 °C). To add serum samples and reagents, we used a "Dilutrend" pipettordilutor (Boehringer, Mannheim, F.R.G.). The hippuric acid and internal standard were quantified with a "high-pressure" liquid-chromatography system (Waters Associates, Brussels, Belgium), consisting of an M45 solvent-delivery system, a Wisp 710B automatic sampler, and a Data Module automatic integrator. We separated enzyme products on a 100 × 8 mm (i.d.) C₁₈ reversed-phase micro-Bondapak column fitted in a radial compression module, at a flow rate of the mobile phase of 5.5 mL/min [8.0 MPa (1200 psi) at ambient temperature]. The absorbance of the column effluent was monitored at 228 nm, sensitivity 0.02 A full scale, with a Model 450 variable-wavelength detector, all from Waters Associates.

Mobile phase: We used a 17/3 (by vol) mixture of potassium phosphate buffer (10 mmol/L, pH 3.5) and acetonitrile for the mobile phase.

Standard solution: Dissolve 40.3 mg of hippuric acid in 100 mL of ethanol. Dilute 1.0 mL of this stock solution to

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1 Nonstandard abbreviations: CN₁ and CN₂, carboxypeptidase N₁ and N₂; Hip-Arg, hippuryl-L-arginine; Hip-Lys, hippuryl-L-lysine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; and HEPES, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid.

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Received July 1, 1985; accepted September 5, 1985.

1936 CLINICAL CHEMISTRY, Vol. 31, No. 12, 1985
100 mL with ethyl acetate to a concentration of 22.5 \mu mol/L; store at 4 °C. Prepare the internal standard, \( o \)-methylhippuric acid, by dissolving 291 mg in 25 mL of ethanol and diluting to 100 mL with distilled water. Store at -20 °C.

**Buffered substrate solutions:** For CN\(_1\) dissolve 30 mmol of Hip-Arg per liter of 50 mmol/L HEPES buffer (pH 8.2 at room temperature); for CN\(_2\) dissolve 100 mmol of Hip-Lys per liter of 50 mmol/L HEPES buffer at pH 7.8. Store frozen in aliquots at -20 °C. Check each batch of substrate for the presence of free hippuric acid by assaying a sample blank (substituting 10 \mu L of distilled water for the serum sample). Stored under the conditions described, the standard solution, internal standard, and buffered substrate solution are stable for at least six months.

**Procedures**

**Chromatographic assay:** To determine CN\(_1\), incubate 10 \mu L of serum and 40 \mu L of substrate solution for 45 min at 37.0 ± 0.1 °C. Stop the reaction by adding 50 \mu L of 1 mol/L HCl. After adding 10 \mu L of internal standard, extract the hippuric acid and internal standard from the acidified solution into 300 \mu L of ethyl acetate, vortex-mixing for 30 s. Centrifuge (5 min, 1000 \times g), then evaporate 100 \mu L of the supernatant ethyl acetate layer at 120 °C (this takes about 10 min). Dissolve the dry residue in 200 \mu L of the chromatographic mobile phase, and inject 10 \mu L into the chromatograph.

For CN\(_2\), the procedure is similar: use 10 \mu L of serum, 80 \mu L of substrate solution, 20 min of incubation time, 100 \mu L of HCl, 10 \mu L of internal standard, and 600 \mu L of ethyl acetate, and evaporate 200 \mu L of the supernate.

**Calibration procedure:** Evaporate 1000- and 2000-\mu L aliquots of the standard hippuric acid solution (22.5 \mu mol/L) to dryness (120 °C, 60 min) in 10 × 76 mm glass tubes. To each tube add 10 \mu L of distilled water and perform the assay procedure described above, beginning with the addition of the substrate. Calculate the calibration line as a regression between the ratio of the peak-areas or peak-heights of hippuric acid and the internal standard vs the concentrations of hippuric acid. One unit (U) of carboxypeptidase N activity is defined as the amount of enzyme required to release 1 \mu mol of hippuric acid per minute at 37 °C under the assay conditions described. Results calculated by automated integration correlated very well with those obtained by measuring the peak heights (\( r = 0.992, n = 20 \)).

**Results**

**Enzymic assay:** CN\(_1\) and CN\(_2\) liberate hippuric acid from the synthetic dipeptides Hip-Arg and Hip-Lys, respectively. Typical chromatograms are shown in Figure 1. Hippuric acid and the internal standard are separated by virtue of the low acetomitrile content of the mobile phase. Little substrate is extracted with the procedure described, and the low pH of the mobile phase prevents it from co-eluting with the hippuric acid and the internal standard. Elution of the components takes less than 2 min. The column efficiency had not declined after more than 1000 injections.

We investigated the pH dependence of CN\(_1\) and CN\(_2\) activity, using HEPES, 50 mmol/L, as buffer solution. CN\(_1\) showed a pH optimum between 8.0 and 8.2, whereas CN\(_2\) yielded highest enzyme activities at pH 7.8 (Figure 2). In subsequent enzyme assays, we therefore used pH 8.2 for CN\(_1\) and pH 7.8 for CN\(_2\) (pH was determined at room temperature).

We studied the effect of the buffer composition on the enzyme activities of both CN\(_1\) and CN\(_2\), using several buffer solutions (HEPES, HEPES, Tris, borate, and phosphate) at different concentrations. HEPES, HEPES, and Tris (all at a concentration of 50 mmol/L) yielded similar results; with the borate buffer solution we obtained enzyme activities 10% lower, whereas the phosphate buffer showed a marked decrease in enzyme activity (both for CN\(_1\) and CN\(_2\)) parallel with increasing potassium phosphate concentration. HEPES, 50 mmol/L, was chosen for routine use in the enzyme assays.

Because some authors use sodium chloride in their determinations of carboxypeptidase N activities (6, 9, 10), we studied the effect of this halide ion, in different concentrations, on CN\(_1\) and CN\(_2\) activities in a HEPES 50 mmol/L buffer solution at pH 8.2 and 7.8. Both for CN\(_1\) and CN\(_2\) activity decreased with increasing sodium chloride concentration (Figure 3). Therefore, no NaCl was used in subsequent assays.

**Linearity:** The relative low substrate concentration assures linearity. For a serum with normal CN\(_1\) activity, only 2 to 3% of the substrate (Hip-Arg) is hydrolyzed in 2 h; for CN\(_2\), the higher enzymatic activity is compensated by using higher substrate concentrations. Results for CN activities vs time were: CN\(_1\), \( r = 0.999 \) (time 0–120 min); CN\(_2\), \( r = 0.999 \) (time 0–50 min). For CN\(_2\), linearity diminished after more than 50 min of incubation time (for a serum with normal enzyme activity). Therefore, we chose an incubation period of 20 min for CN\(_2\).

Linearity of the assay could also be demonstrated by
assaying serially diluted (with isotonic saline) sera with above-normal carboxypeptidase N activities (CN₁ = 184 U/L; CN₂ = 620 U/L). Linear regression analysis gave \( r = 0.999 \) (CN₁) and \( r = 0.999 \) (CN₂) for sera diluted 1:8, 1:4, 1:2, 3:4, and undiluted sera.

Michaelis–Menten constants (\( K_m \)) were determined by a direct linear plot (II). CN₁ activity was determined at substrate concentrations ranging from 1.0 to 50.0 mmol/L; CN₂ at concentrations of 1.25 to 80.0 mmol/L (Figures 4 and 5). The result yielded a \( K_m \) for CN₁ of 1.8 mmol/L, for CN₂ 10.5 mmol/L. Thus the affinity of CN₁ for Hip-Arg exceeds the affinity of CN₂ for Hip-Lys.

**Sensitivity:** By exploiting the ultraviolet absorbance maximum of hippuric acid at 228 nm, we have been able to detect as little as 0.5 nmol of hippuric acid, corresponding to a 45-min enzymatic activity of 1.1 U/L (CN₁) and a 20-min enzymatic activity of 2.5 U/L (CN₂) in 10 \( \mu \)L of serum, when measuring peak heights. By using the automatic integrator, fivefold lower activities can be detected.

**Precision:** Table 1 shows results of within-day and between-day precision studies for sera with low, normal, and

### Table 1. Precision of the Assay for Carboxypeptidase N

<table>
<thead>
<tr>
<th></th>
<th>Activity, U/L</th>
<th>CV, %</th>
</tr>
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<tbody>
<tr>
<td><strong>CN₁</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-day</td>
<td>44.3</td>
<td>2.05</td>
</tr>
<tr>
<td></td>
<td>72.5</td>
<td>2.62</td>
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<tr>
<td></td>
<td>263.8</td>
<td>12.76</td>
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<tr>
<td>Between-day</td>
<td>43.2</td>
<td>2.51</td>
</tr>
<tr>
<td></td>
<td>72.2</td>
<td>3.52</td>
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<tr>
<td></td>
<td>281.8</td>
<td>14.04</td>
</tr>
<tr>
<td><strong>CN₂</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Within-day</td>
<td>213.1</td>
<td>4.83</td>
</tr>
<tr>
<td></td>
<td>409.3</td>
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<td>4.28</td>
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<tr>
<td></td>
<td>410.9</td>
<td>8.15</td>
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<tr>
<td></td>
<td>576.9</td>
<td>24.12</td>
</tr>
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\( n = 15 \) each.
above-normal activity. We evaluated the precision of the liquid-chromatography system by injecting aliquots of the same extract 10 times. The results were: mean 72.9 (SD 0.46) U/L, and CV 0.6%.

Reference interval: For samples from 80 blood-bank donors (40 men, 40 women, ages 18–65 years) the mean values were: CN₁, 74.8 (SD 10.3) U/L; CN₂, 378 (SD 55) U/L. No statistically significant sex-related difference was observed (CN₁; 0.60 < p < 0.70; CN₂ 0.05 < p < 0.10).

Accuracy: Accuracy could not be determined because a standard reference method is not available. However, we compared the results by our technique, using Hip-Lys (x), and those obtained with the method of Plummer and Kimmel (y) (10) for 80 sera. Correlation by least-squares linear regression analysis was very good (y = 2.53x + 160; r = 0.93).

Discussion

Few methods are available for determination of carboxypeptidase N activities. In most of them the synthetic substrates Hip-Arg or Hip-Lys are used, either by following the released hippuric acid with an ultraviolet-photometric spectrophotometer (12) or by measuring the liberated hippuric acid spectrophotometrically after an extraction procedure (6). CN₃ activities have been determined by measuring the decrease in absorbance at 336 nm, using 3-(2-pyridylcarboxy)-L-alanyl-L-lysine as the substrate (10). Recently, an assay of human plasma carboxypeptidase N by "high-performance" liquid chromatographic separation of the hippuric acid and its substrate was described (9). These authors used Hip-Lys (only) as a substrate, the buffer solution contained sodium chloride, and no internal standard procedure was used. Activities of CN₁ found by these authors were in the range of 6 μmol/min per liter of plasma (6 U/L), whereas in our assay we obtained 60-fold greater activities. The better performance of our method is probably due to the carefully chosen conditions of substrate concentration and buffer solution and the omission of NaCl. The short separation time for the hippuric acid and its substrate (less than 2 min) also favors our proposed procedure. The spectrophotometric assay introduced by Schweifurth et al. (6), who measured CN₁ and CN₂ activities in serum with the same substrates we used in our study, detected enzyme activities in normal individuals that were only half as great as the values we obtained. This is very probably ascribable to the buffer composition; a potassium phosphate buffer, 0.5 mol/L, was used by these authors. We observed a marked decrease in activity, both for CN₁ and CN₂, directly related to the concentration of the potassium phosphate in the buffer solution.

Because carboxypeptidase N is a major enzyme in the inactivation of bradykinin and other blood-borne peptides, changes in its activity in the blood may be important in various pathological conditions (5, 12, 13). Low activities of carboxypeptidase N in serum were found in cystinosis of the liver (14), in cystic fibrosis (5), in hereditary angioedema (15), and in hyperbradykininism or familial orthostatic hypotension (16). Increased carboxypeptidase N activities were reported in pregnancy (12), in patients with sarcoidosis (17), and in malignancies such as lung cancer (6) and Hodgkin's disease (13).

References