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Clinica Chimica Acta 371 (2006) 124–129
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A rapid and sensitive assay for the quantitation of carboxypeptidase N, an important regulator of inflammation

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Received 19 January 2006; received in revised form 24 February 2006; accepted 24 February 2006
Available online 17 April 2006

Abstract

Background: Carboxypeptidase N is a plasma zinc metallocarboxypeptidase which is constitutively expressed in the liver and was identified as the enzyme responsible for inactivating bradykinin and kallidin by removing the C-terminal arginine. Because CPN can cleave the C-terminal arginine of C3a, C4a and C5a it is often referred to as anaphylatoxin inactivator. Markedly reduced levels of circulating CPN are associated with recurrent angioedema and abnormal cutaneous polymorphonuclear cell infiltration.
Methods: In this paper we describe a fast kinetic coupled enzymatic assay for the sensitive measurement of carboxypeptidase N activities in serum samples. The assay makes use of the excellent CPN substrate Benzoyl-*L*-Alanyl-*L*-Arginine.
Results: This novel assay is very fast, easy to perform and combines good reliability and reproducibility with excellent correlation with the HPLC-assisted assay ($r=0.927$; $n=140$).
Conclusion: The presented assay can be used for high throughput screening of this important regulator of inflammation in clinical plasma or serum samples.

Keywords: Anaphylatoxin inactivator; Carboxypeptidase N; Inflammation; Kinetic assay

1. Introduction

Human carboxypeptidase N (CPN; arginine carboxypeptidase, kininase I, anaphylatoxin inactivator; EC 3.4.17.3) is a 280-kDa tetrameric enzyme consisting of two 83-kDa regulatory subunits and two catalytic 50-kDa subunits. Erdos and Sloane discovered CPN as an enzyme that inactivates bradykinin by cleaving its carboxy-terminal arginine [1]. When cleaved by CPN, bradykinin changes its receptor specificity, from B2 to B1 [2]. CPN also cleaves the C-terminal arginine from the anaphylatoxins C3a, C4a and C5a – released during complement activation – thereby reducing their biological activities by 10–100 fold [2,3]. In a patient, genetically determined low levels of plasma CPN were associated with recurrent bouts of angioedema, probably due to enhanced levels of intact kinins. During attacks, CPN serum levels remained unchanged but there was an increase in C3a levels as well as an increase in histamine and polymorphonuclear cell infiltration [4,5]. The severe phenotype of a partial deficiency indicates that CPN has an important role in vivo [4,5]. Recently, a frameshift mutation was identified in this patient's DNA encoding exon 1 of the catalytic subunit, a possible cause of the low CPN level [6]. A recent paper of Davis and coworkers identifies CPN as the enzyme responsible for the C-terminal cleavage of stromal cell-derived factor-1α, a chemokine that controls hematopoiesis, lymphocyte homing, B-lineage cell growth and angiogenesis, in the circulation. This cleavage reduces the activity of the chemokine by approximately 50% as a chemoattractant and pre-B cell growth factor [7].

All data above confirm that CPN is an enzyme which is on the crossroad of several important physiological systems and strengthen the need for an assay to measure CPN activities in a fast, easy and accurate way. Our aim now is to introduce a fast kinetic assay which is easy to use. This novel kinetic assay uses

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[1] J. Willemse is a research assistant of the Fund for Scientific Research Flanders (FWO-Vlaanderen).

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doi:10.1016/j.cca.2006.02.035

the auxiliary enzymes arginine kinase, pyruvate kinase and lactate dehydrogenase and the substrate Bz–Ala–Arg.

2. Materials and methods

2.1. Serum and plasma 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 kept at 37 °C for 2 h before storage at − 80 °C and assayed within a month after collection.

Plasma was collected from healthy volunteers in accordance with NCCLS guidelines. Blood was collected into buffered 32 g/L sodium citrate anticoagulant (109 mmol/L) using an evacuated tube system with a final ratio of nine parts blood to one part buffered citrate, by volume. Plasma was prepared by centrifugation at 3000 *g* for 15 min at room temperature and was stored at − 80 °C until analysis.

2.2. Chemicals and peptides

The substrates Bz–Gly–Arg and Bz–Ala–Arg were purchased from Bachem (Bubendorf, Switzerland). Bz–Gly (hippuric acid) was obtained from Fluka (Buchs, Switzerland). *o*-methylhippuric acid was synthesized from *o*-methylbenzoylchloride by a procedure analogous to that used for the synthesis of hippuric acid. Phosphoenolpyruvate (PEP), reduced nicotinamide-adenine dinucleotide (NADH), adenosine 5′-triphosphate (ATP), lactate dehydrogenase and pyruvate kinase mixture were purchased from Roche Diagnostics (Basel, Switzerland). All other reagents were of high purity grade and were obtained from Merck (Darmstadt, Germany).

2.3. Instruments

The HPLC system consisted of a model 302 pump, a model 303 solvent delivery system, a model 802C manometric module, a model 401 dilutor, a model 117 UV–VIS detector and a model 231 autosampling injector all from Gilson (Paris, France) and a C-18 Chromolith performance 100–4.6 mm column from Merck (Darmstadt, Germany). The microtiter plate reader (SpectraMAX 340) was from Molecular Devices (Sunnyvale, CA).

2.4. Purification of the enzymes

CPN was purified according to the protocol described by Wang et al. [8]. Isolation of arginine kinase from *Peneaus* sp tail muscle was accomplished as described previously [9].

2.5. Determination of K_m and k_{cat} of CPN for Bz–Gly–Arg and Bz–Ala–Arg

The K_m of CPN for Bz–Gly–Arg and Bz–Ala–Arg was derived by nonlinear curve fitting carried out with the computer program GraFit using our previously described protocol [9]. The k_{cat} for both substrates was determined in one single run using a substrate concentration 10-fold the K_m and protein concentration determination was performed using Coomassie brilliant blue G-250 according to the method of Bradford [10].

2.6. Assay

The assay is based on the coupled enzymatic determination of the cleaved arginine by carboxypeptidase N. The method uses the coupling enzymes arginine kinase, pyruvate kinase and lactate dehydrogenase as auxiliary enzymes. Arginine kinase phosphorylates the generated arginine using ATP as a cofactor. The ATP used in this reaction is regenerated by pyruvate kinase, which catalyzes the transfer of the reactive phosphorus in PEP to adenosine diphosphate (ADP). The final step in this coupled enzymatic assay is the reduction of pyruvate to lactate using lactate dehydrogenase with a concomitant oxidation of NADH that can be followed continuously at 340 nm (Fig. 1).

For the determination of carboxypeptidase N in serum/plasma a reaction mixture was prepared consisting of 2.9 mM $MgSO_4$, 11.1 mM KCl, 2.5 mM PEP, 0.6 mM NADH, 2.9 mM ATP, 5500 U/L of both pyruvate kinase and lactate dehydrogenase, 11000 U/L arginine kinase and Bz–Ala–Arg (1.5 mM) in 100 mM Hepes adjusted to pH 8.0. Prior to adding to the reaction mixture, the pyruvate kinase/lactate dehydrogenase suspension was centrifuged for 5 min at 14,000 *g*, and the pellet was redissolved ex tempore in an amount equal to 100 mM Hepes buffer (pH 8.0).

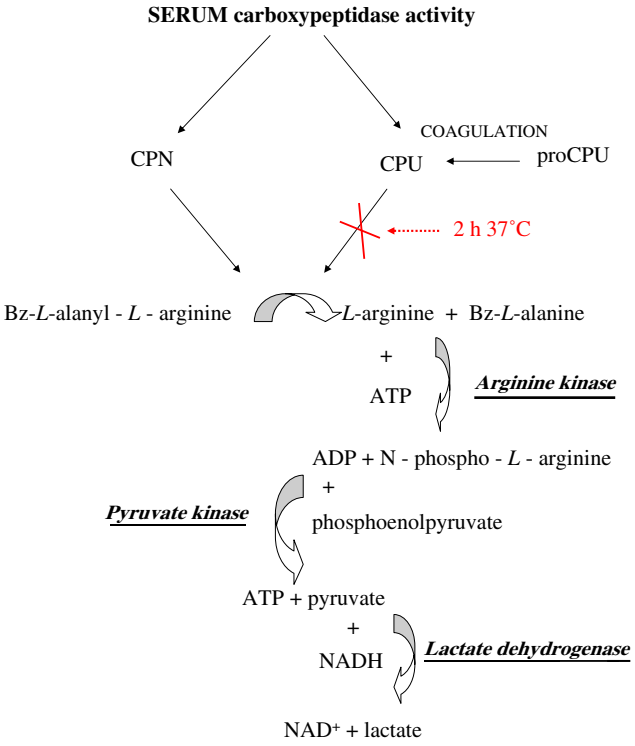

Fig. 1. Reaction mechanism of the assay. In fresh serum samples 2 basic carboxypeptidases are present: CPN, which is constitutively active and CPU, activated upon coagulation from proCPU by thrombin. This CPU activity is highly unstable at 37 °C. Incubating serum at 37 °C for 2 h completely abolishes this labile carboxypeptidase activity. CPN cleaves off the C-terminal arginine of the synthetic substrate Bz–Ala–Arg. The generated arginine is phosphorylated by arginine kinase using ATP as a cofactor. The ATP used in this reaction is regenerated by pyrute kinase which catalyzes the transfer of the reactive phosphorus in phosphoenolpyruvate to ADP. The final step in this coupled enzymatic assay is the reduction of pyruvate to lactate with a concomitant oxidation of NADH that can be followed continuously at 340 nm.

The assay procedure is as follows: after preparation of the reaction mixture it is preincubated for 5 min at 37 °C. 10 µl plasma or serum is added in different wells of a microtiterplate. Afterwards 190 µL of preincubated reaction mixture was added to the wells and the decline in absorption is measured continuously at 340 nm for 5 min.

2.7. Activity calculation

One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze 1 µmol of substrate per minute at 37 °C under the conditions described. Carboxypeptidase N activity can be calculated using the following equation:

$$\frac{U}{L} = \frac{\Delta v}{6220} \times \frac{1}{L} \times \frac{200}{10} \times 10^6$$

where Δv (OD/min) is the difference in velocity between sample and blank; 6220 M^{-1} cm^{-1} is the molar extinction coefficient of NADH (340 nm); L is the optical path length (cm), 10 µL is the volume of the plasma/serum in each well of the microtiterplate, 200 µL is the total volume in the wells and 10^6 is the factor for converting mol to µmol.

2.8. Blank determination

Theoretically two blank measurements should be performed. One for correcting for interfering plasma/serum arginine and one for correcting decline in absorption at 340 nm due to free arginine in substrate and spontaneous oxidation of NADH. Because the signal due to plasma/serum arginines is less than 1% we do not perform individual serum blank measurements but perform only a blank measurement to check for the substrate and NADH quality. To 10 µL 20 mM Hepes buffer pH 7.4, 190 µL reaction mixture is added and measured at 340 nm.

2.9. Linearity as a function of the concentration of auxiliary enzymes and as a function of dilution

As described in our previous publication the performance of the coupled enzymatic assay was tested by using fivefold and twofold lower and higher concentrations of the coupling enzymes and it was checked that there was no effect of varying the amount of coupling enzymes on the measured CPN activity [9].

To determine linearity in function of dilution, a serum sample with a high CPN activity (1200 U/L) was diluted several

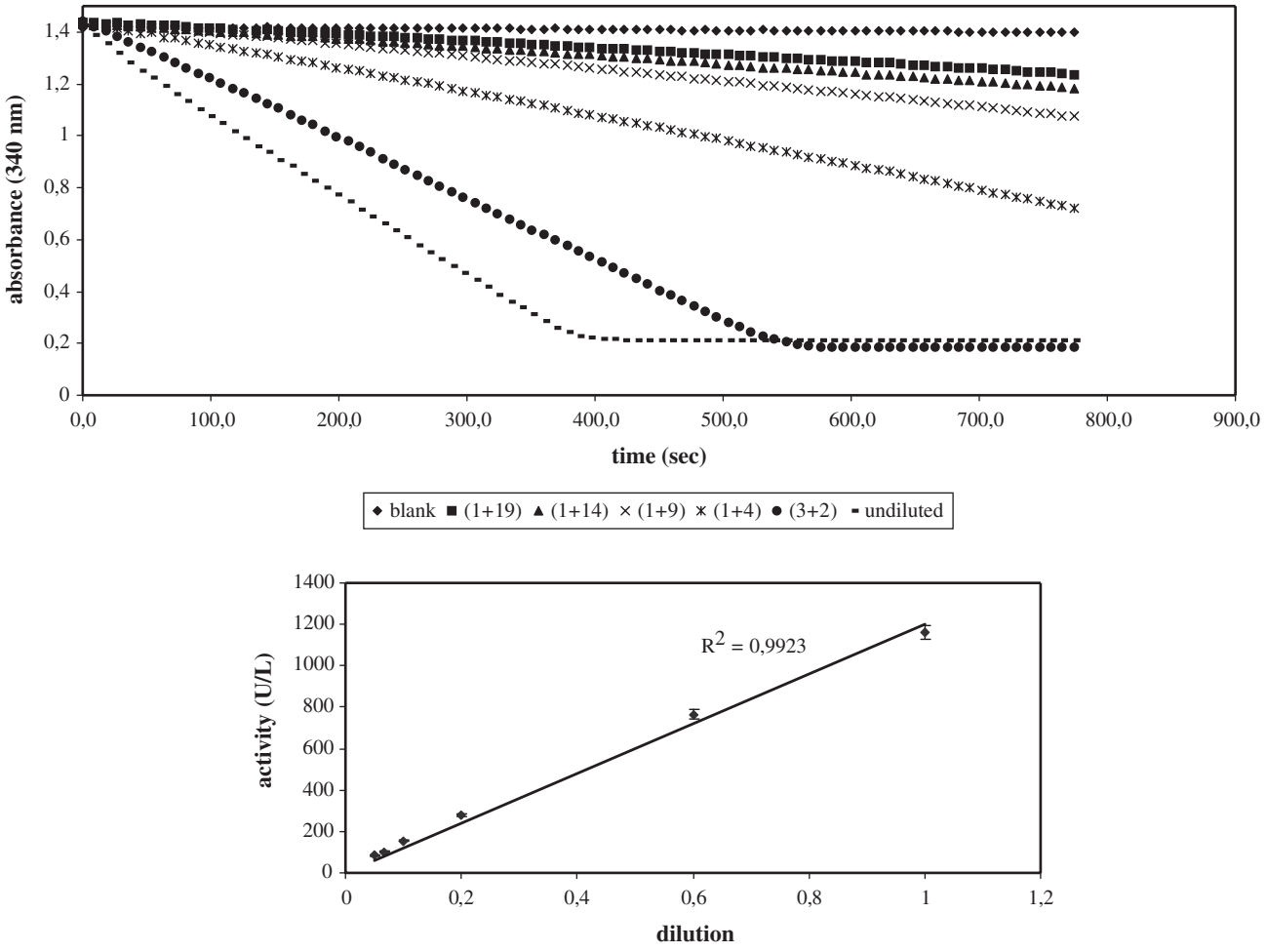

Fig. 2. Linearity in function of dilution. A serum sample with a CPN activity of 1200 U/L was diluted in HEPES 20 mM pH 7.4 and subjected to the kinetic assay. The upper panel shows the progression curves at 340 nm of the blank and the different serum dilutions. In the lower panel the measured CPN activity is plotted against the dilution factor. Good linearity was seen in function of dilution.

Table 1
Within and between-day variation

	Activity (U/L)
Mean value measured on the same day ($n=20$)	1302±27
	401±8
Mean value measured on different days ($n=15$)	1298±50
	399±15

Mean (± SD) CPN activity of two serum samples measured 20 times on the same day and once on 15 different days.

fold (3+2), (1+4), (1+9) and (1+14) and (1+19) in 20 mM HEPES buffer pH 7.4 and afterwards subjected to the kinetic assay.

2.10. Variation of the assay and correlation with the HPLC-assisted assay

To show the reliability and repeatability of this assay, within-run ($n=20$) and between-run ($n=15$) variation was determined using a serum sample with a high CPN activity (1300 U/L) and a low CPN activity (400 U/L). The kinetic assay was correlated with the HPLC-assisted assay using serum samples from 140 healthy volunteers [11].

2.11. Determination of the measurable plasma CPN activity range using standard assay conditions

The measurable CPN activity range was obtained by spiking serum with purified CPN. Activity and recovery were measured using standard assay conditions and the interval allowing linear CPN activity measurement was determined.

3. Results

3.1. K_m and k_{cat} of CPN for Bz–Gly–Arg and Bz–Ala–Arg

The K_m values for Bz–Gly–Arg and Bz–Ala–Arg were 1.50±0.20 mM ($n=2$) and 0.25±0.03 mM respectively. The k_{cat} values were estimated as 10.0±1.1 and 150.0±9.8 s^{-1}. These results clearly show that Bz–Ala–Arg is a much superior substrate for CPN compared to Bz–Gly–Arg especially for the development of spectrophotometric assays. Indeed Bz–Ala–Arg allows the spectrophotometric determination of CPN activity using only microquantities of serum (10 μL).

3.2. Effect of dilution

A serial serum dilution was made and serum carboxypeptidase N activity was measured using the protocol described above. Results are shown in Fig. 2. Excellent linearity in function of dilution was seen.

3.3. Within and between run variation

The mean CPN concentration of two serum samples was determined within a single run ($n=20$) and on several days ($n=15$). The within-day variation and between-day variation of the method are 2.0% and 3.9% respectively, proving that this kinetic assay is very reproducible (Table 1).

3.4. Correlation with HPLC-assisted assay

The novel kinetic assay was compared with the HPLC assay using serum samples from 140 healthy volunteers [11]. Excellent

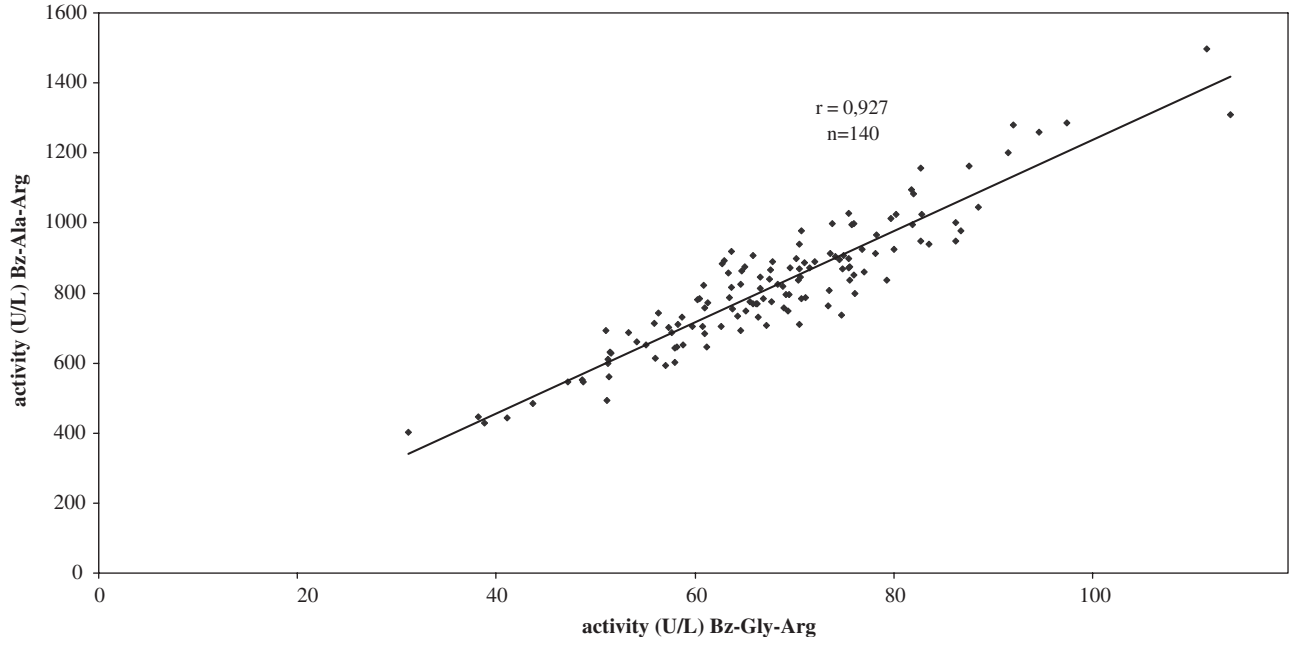

Fig. 3. Correlation with HPLC-assisted assay. The kinetic assay was correlated with the HPLC-assisted assay using 140 serum samples of healthy blood-bank donors. Good correlation was seen ($r=0.927$, $p<0.0001$).

Table 2
Comparison of the kinetic assay with the HPLC-assisted assay

	Bz–Gly–Arg (HPLC)	Bz–Ala–Arg (kinetic assay)
Range	31.2–113.9 U/L	401–1497 U/L
Arithmetic mean	66.1 U/L	821 U/L
Standard deviation	13.1 U/L	179 U/L

The table represents range, mean and standard deviation of 140 serum samples of healthy volunteers measured with HPLC and the novel kinetic assay.

correlation was seen ($r=0.927$, $p<0.0001$) (Fig. 3). Activity range, mean and standard deviation are shown in Table 2.

3.5. Determination of the CPN detection range using standard assay conditions and recovery

Low CPN activity serum (400 U/L) was spiked with purified CPN thereby creating serum samples with different CPN activities between 400–1720 U/L against Bz–Ala–Arg. Good linearity was seen in this CPN activity range ($r^2=0.997$, $n=6$). Recovery of the added CPN was in the range between 97–102%. The detection limit for CPN activity measurement using standard assay conditions is 125 U/L. Using 20 μl plasma/serum – instead of 10 μl – 65 U/L can be adequately detected.

4. Discussion

Carboxypeptidase N (E.C. 3.4.17.3) is a constitutively active carboxypeptidase in human plasma and is recognized to be an important enzyme, mainly because of its modulating effect on bradykinin and anaphylatoxin activity [1–3]. Partial deficiency of CPN (21% of normal CPN activity) is associated with recurrent bouts of angioedema and the genetic basis of this deficiency has been recently described [4–6]. Many papers deal with the determination of carboxypeptidase N activities in human serum of patients with various pathologies. Most of the assays to measure basic carboxypeptidase activity make use of the synthetic substrate hippuryl-*L*-arginine [11–14]. We introduce here a very fast and sensitive assay for the high throughput screening of CPN activity in clinical serum samples. The assay is based on the quantification of the cleaved arginine from Bz–Ala–Arg by CPN. In our experiments Bz–Ala–Arg is a superior substrate for CPN compared to hippuryl-*L*-arginine with a 75-fold higher k_{cat}/K_m value. The assay described here combines several advantages. First of all the assay is easy to use: after preparing a reaction mixture, 10 μL serum is added and measurement can be started at 340 nm; also it allows a very fast measurement of CPN activity (results are obtained within 5 min). The kinetic assay shows a similar sensitivity compared to the more time-consuming and cumbersome HPLC-assisted assay [11]. Within and between-day variations are as low as 2% and 3.9% respectively. The assay shows a high precision and broad linear detection range using standard assay conditions. The detection limit using standard assay conditions is 125 U/L. The lowest CPN serum activities reported in literature are 20% of the average normal CPN serum level [4,5]. The average CPN activity in serum was 821 U/L in our experiments corresponding with a 20% value of 164 U/L. Good linearity was seen until a CPN activity of 1720 U/L, whereas the highest CPN activity seen in our studied population was 1497 U/L. This means that standard assay conditions can be used for the screening of clinical samples. However, if necessary, a higher sensitivity can be reached when higher serum volumes are used to perform the kinetic assay. We showed that serum samples with high CPN activity (1200 U/L) can be linearly diluted until a dilution (1+19) (Fig. 2).

Because the substrate used in this assay is not specific for CPN, interfering CPU activity should be accounted for. Indeed on coagulation of blood proCPU is activated to CPU by thrombin and this CPU activity, present in fresh serum samples, can interfere with the CPN determination as described by Hendriks et al. [15,16]. CPU activity has a reported half life of 8–15 min at 37 °C [17,18]. Therefore serum samples should be kept at 37 °C for 2 h to remove this labile carboxypeptidase activity in serum before the CPN analysis is performed. No CPU activity is present in plasma samples, so that plasma samples can be measured straightforward.

Acknowledgements

The authors acknowledge Yani Sim for technical assistance.

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