Enzymatic Method for Assaying Calcium in Serum and Urine with Porcine Pancreatic α-Amylase

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We developed a kinetic assay for calcium in serum and urine, based on the activation of porcine pancreatic α-amylase (EC 3.2.1.1) with 2-chloro-4-nitrophenyl-α-maltotrioside as substrate. The kinetic generation of 2-chloro-4-nitrophenol, monitored at 405 nm, was proportional to the concentration of calcium in serum and urine. The assay was developed and evaluated with the Cobas Bio centrifugal analyzer. The average within-run and between-day imprecision (CV) was 0.96/1.26% and 1.07/1.63%, respectively, for serum calcium and 1.50/2.54% and 1.70/2.64%, respectively, for urine calcium. Results of the proposed method (y) correlated well with those determined by atomic absorption spectrophotometry (x): y(serum) = 1.005x + 0.028 mmol/L (S_yx = 0.058, r = 0.974, n = 50), and y(urine) = 1.017x - 0.115 mmol/L (S_yx = 0.30, r = 0.981, n = 25). We also present data showing that the method is highly sensitive, rapid, relatively free of interference, and amenable to automation.

Indexing Terms: kinetic enzymatic assay/synthetic substrates/2-chloro-4-nitrophenyl-α-maltotrioside/atomic absorption spectrophotometry compared

Calcium (Ca) is required in the homeostatic regulation of inorganic compounds. Analyses for Ca in serum and urine are especially important in the diagnosis of hypocalcemia (i.e., hypoparathyroidism, renal failure) and hypercalcemia (i.e., hyperparathyroidism, sarcoidosis) (1, 2). Major conventional methods for assaying Ca are atomic absorption spectrophotometry (3) and colorimetry of Ca complexes with o- cresolphthalein (4). The former method is reliable and specific for Ca, but requires special and expensive equipment and is unsuitable for routine assay in clinical chemistry. The latter method, with o-cresolphthalein complexone (CPC), is widely available and has been improved by Kessler and Wolfman (5) so as to be more specific for Ca by the addition of 8-hydroxyquinoline to mask any magnesium present.3 This modified method has been made more rapid and precise by adaptation to the AutoAnalyzer and other discrete analyzers. Sunderland et al. (6) and Barnett et al. (7) demonstrated that several commercially available kits based on the same reaction systems still showed differences in reproducibility and precision.

3 Nonstandard abbreviations: CNP, 2-chloro-4-nitrophenol; CNP-G3, 2-chloro-4-nitrophenyl-α-maltotrioside; CNP-G5, 2-chloro-4-nitrophenyl-α-maltopentaoside; MES, 2-(N-morpholino)ethanesulfonic acid; BAPTA, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid; CPC, o-cresolphthalein complexone; and SRM, Standard Reference Material.

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Tabata et al. (8) reported an enzymatic assay for calcium ion (Ca2+) based on the activation of phospholipase D (EC 3.1.4.4). This method is specific for Ca and is performed under mild conditions, but is subject to slight interference from magnesium. Here we report an original, sensitive, and specific enzymatic assay for total Ca in serum and urine, based on the activation of porcine pancreatic α-amylase (EC 3.2.1.1) (9, 10).

Materials and Methods

Principle of the Reaction

The reaction sequence in our method is as follows:

\[ \text{Ca}^{2+} + \text{CNP-G3} \rightarrow \text{G3} + \text{Cl}^- + \text{NO}_2^- \]

Calcium activates α-amylase in the initial reaction, converting 2-chloro-4-nitrophenyl-α-maltotrioside (CNP-G3) into 2-chloro-4-nitrophenol (CNP) and α-maltotriose (11, 12). The generation of CNP is monitored at 405 nm. The increase in the concentration of CNP is proportional to the concentration of Ca.

Instrumentation and Reagents

Instruments. We used the Cobas Bio analyzer (Roche Diagnostics Systems, Nutley, NJ) for the enzymatic Ca assay. For comparison studies, we used a TBA-80S biochemical discrete analyzer (Toshiba, Tokyo, Japan) for the CPC method and an atomic absorption spectrometer (Model AA-680; Shimadzu, Kyoto, Japan).

Reagents. Calcium carbonate [Standard Reference Material (SRM) 915] and SRM 909a (consisting of two different analytic concentrations) were from the National Institute of Standards and Technology, Gaithersburg, MD. Tris; 2-(N-morpholino)ethanesulfonic acid, monohydrate (MES); α-amylase (from porcine pancreas); reduced and oxidized glutathione; bovine serum albumin; and maltose were from Boehringer Mannheim Yamanouchi, Tokyo, Japan. The chelating agent, 1,2-bis(2-aminophenoxy)ethane-N,N',N'-tetraacetic acid tetrapotassium salt, hydrate (BAPTA), was from Dojindo Labs., Kumamoto, Japan. CNP-G3 and the reagent kit for the α-amylase assay [Dia-Color-AMY rate, with 2-chloro-4-nitrophenyl-α-maltopentaoside (CNP-G5) as substrate] were obtained from Ono Pharmaceutical, Osaka, Japan.

Intralipid was from KabiVitrum AB, Stockholm, Sweden. Bilirubin, sodium chloride, heparin, sodium azide, and ascorbic acid were from Wako Pure Chemicals, Osaka, Japan. Human pancreatic amylase, for
study of interfering substances, was from Scripps Labs., San Diego, CA. The CPC reagent kit for the calcium assay, Calcium Assay Reagent A, used for the comparison studies, was from International Reagent, Kobe, Japan.

Specimens. Blood (anticoagulated with NaF, EDTA, or sodium citrate), and urine specimens (fresh 24-h urine), were collected from hospitalized patients.

The study was approved by the committee for ethical standards of the National Cardiovascular Center Hospital.

Standards. Both the enzymatic and the CPC method were calibrated with calcium carbonate (SRM 915) prepared in a concentration of 2.5 mmol/L.

Procedure

Analytical conditions. We used the following Cobas Bio analyzer settings: specimen volume 5 μL (diluent 10 μL), reagent 1 (R-1) 150 μL, start reagent (R-2) 75 μL (diluent 20 μL), type of analysis 3 (enzyme kinetic mode), temperature 37°C, wavelength 405 nm. After mixing the specimen and R-1, the reaction was started by adding 75 μL of R-2; after 60 s, the absorbance at 405 nm was monitored at 10-s intervals for 3 min.

Reagent composition. We considered the optimal conditions to be those that afforded zero-order reaction kinetics, broad linearity, the greatest sensitivity, the least drift of the reagent blank value, and stable working solutions. In the assay we used a two-reagent system. Reagent 1 consisted of 7 mmol/L Tris-HCl (pH 7.5), 3.2 kU/L α-amylase (activity determined with CNP-G5 substrate), 200 mmol/L NaCl, and 0.4 mmol/L BAPTA. Reagent 2 was 100 mmol/L MES, 200 mmol/L NaCl, 150 mmol/L maltose, and 0.25 mmol/L CNP-G3. For reagent 1, pH 7.5 was selected to avoid losses of α-amylase and BAPTA in storage. The final pH in the reaction mixture was pH 5.9, which was optimal for color intensity of developing CNP, rather than for the activity of α-amylase (optimal pH 7.5). Because porcine pancreatic α-amylase is also activated by the chloride ion, we included enough sodium chloride (200 mmol/L) to avoid interference from endogenous chloride ion in the specimen. The presence of chloride ion increased dramatically the affinity of α-amylase for the Ca²⁺ (9, 10) and the protein-bound calcium also bound to α-amylase. By increasing the CNP-G3 concentration and α-amylase activity, the overall reaction rate proportionally increased, but the range of assay linearity decreased. To improve the reaction rate, we added to reagent 1 BAPTA, a calcium chelator. At the given concentrations of BAPTA and CNP-G3, and at the given activity of α-amylase, the overall reaction was proportional to the concentration of Ca²⁺ in the specimen, with adequate sensitivity and extended linearity. Maltose, another substrate of α-amylase, was added to mildly reduce CNP-G3 hydrolysis to minimize any drift of the reagent blank. Sodium azide, as an activator and a preservative, was included at 7.7 mmol/L. Reagents 1 and 2 were stable for at least 2 weeks at 2–8°C.

Results

Time courses. Fig. 1 shows typical time courses of the readings with three different specimens (SRM 915 at 2.0, 2.5, and 3.0 mmol/L) after addition of reagent 2. A lag phase was observed up to 60 s, but after that the reaction was linear. Serum and urine specimens showed similar time courses. Therefore, we used the change in absorbance between 60 and 300 s to assay Ca with the Cobas Bio analyzer.

Linearity studies. The standard curve with aqueous standards (SRM 915) showed linearity from 0 to 7.8 mmol/L (Fig. 2). The standard curve with SRM 915 aqueous standards in (1:9 by vol) human serum matrix showed similar linearity.

Recovery. Three pooled sera and urines of known Ca concentration (1.90, 2.13, and 2.28 mmol/L and 0.61, 1.17, and 3.26 mmol/L, respectively) were supplemented with Ca (0.37, 0.75, and 1.25 mmol/L), and assayed on
Within-run imprecision. Table 1 shows the within-run and between-day imprecision data for serum and urine specimens. The within-run and between-day CVs were 0.96–1.26% and 1.07–1.63% for serum specimens and 1.50–2.54% and 1.70–2.64% for urines, respectively. Each of these values was better than the corresponding imprecisions obtained with the CPC method.

Interference by cations and other agents. Aqueous 0.1 mmol/L solutions of Mn


2+, Ni


2+, Cd


2+, and Ba


2+ (chloride), and Zn


2+ and Cu


2+ (acetate) and 25 mmol/L Mg


2+ (acetate) were individually added to a pooled serum to examine whether these cations changed the expected Ca concentration (2.13 mmol/L) on the Cobas Bio analyzer. The Ca measured was 104.6%, 102.8%, and 102.3% of that expected when Ba


2+, Fe


3+, and Cu


2+, respectively, were present. All other measurements ranged between 100.0% and 101.8%. We conclude that all these cations tested did not significantly affect the assay results. Less than 0.1 mmol/L interference resulted from the addition of the following substances to serum or urine: α-amylase (from human pancreas, activity determined with CNP-G5 substrate), 5000 U/L; bilirubin, 342 μmol/L; albumin, 100 g/L; heparin, 100 USP units/mL; glucose, 278 μmol/L; reduced glutathione, 3.25 mmol/L; oxidized glutathione, 1.63 mmol/L; ascorbate, 5.68 mmol/L; pyruvate, 5.68 mmol/L; NaCl, 300 mmol/L; KI, KBr, NaNO


3, NaNO


2, Na


2CO


3, CH


3COONa, Na


2SO


4, and NaH


2PO


4, 100 mmol/L; NaF, 50 mmol/L; NaHCO


3 and NaSCN, 20 mmol/L; hemoglobin, 7.8 μmol/L; and Intralipid, 1.6 mL/L.

SRM 909a. This serum was assayed on five separate occasions. The mean results of SRM 909a 1 and 2 by the present method were 2.25 and 3.29 mmol/L; the labeled values were 2.322 ± 0.04 and 3.338 ± 0.047 mmol/L, respectively.

Intermethod comparison data. We assayed samples independently by atomic absorption spectrophotometry, by the TBA-80S biochemical analyzer CPC method, and by our method with the Cobas Bio analyzer. The comparison data of serum and urine specimens between our method (\(y\)) and atomic absorption spectrophotometry (\(x\)) correlated very well (Fig. 3). Conversely, results of the TBA-80S biochemical analyzer CPC method (\(x'\)) showed a proportional error of 5% less Ca in serum and urine specimens: 

\[
y = 0.953x + 0.07 \text{ mmol/L} (n = 132, r = 0.986, S_{x'x} = 0.074) \text{ for serum, and } \]

\[
y = 0.941x' + 0.19 \text{ mmol/L} (n = 100, r = 0.985, S_{x'x} = 0.160) \text{ for urine.}
\]

Comparing standard deviation of the regression line for the serum and urine specimens showed that by the CPC method the random error was higher for urine specimens \((S_{x'x} = 0.074 \text{ vs } 0.160)\) than for serum specimens, in comparison with our method.

**Discussion**

A widely used method in the clinical laboratory for determining Ca in serum and urine involves a chelating agent specific for calcium. This method is based on the following principle: In the first step Ca is liberated from protein and other ligands and then is reacted with

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<th>Table 1. Imprecision of the proposed method performed with the Cobas Bio.</th>
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<td><strong>Aqueous solution</strong></td>
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<td><strong>Within-run (n = 10)</strong></td>
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<td>Mean, mmol/L</td>
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<td>CV, %</td>
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<td><strong>Between-day (n = 7)</strong></td>
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<td>Mean, mmol/L</td>
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<td><strong>Intralipid</strong></td>
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<td>Urine 1</td>
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<td>Urine 3</td>
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<td><strong>Within-run (n = 25)</strong></td>
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o-cresolphthalein. In the color development step, 8-hydroxyquinoline is added as a magnesium masking agent. However, the CPC method with 8-hydroxyquinoline did not eliminate the interference of magnesium sufficiently. The linear range of this method was also lower. For determination of urine Ca, the CPC method required the dilution of urine specimens. Urine specimens with high magnesium concentrations may produce higher Ca values. Analyses of serum and urine specimens revealed that the regression slope for our method vs the CPC method was less than that obtained by our method vs atomic absorption spectrophotometry. This lower regression slope, especially with urine specimens, might be caused partly by the interference of magnesium. The difference in standard deviation of the regression line $S_{reg}$ between the serum and urine specimens by the CPC method might be due, in part, to a matrix effect.

Moreover, in the enzymatic method reported by Tabata et al. (5), magnesium gave a positive interference (9.2% at 4.10 mmol/L); the peroxidase–hydrogen peroxidase–chromogen method was subject to negative interference by bilirubin and other reduced substances. In contrast, the method we describe was unaffected by various cations, especially magnesium, the reduced substances, and albumin. However, endogenous $\alpha$-amylase caused positive interference at concentrations $>5000$ U/L, the interference being 4% at 10 000 U/L. Therefore, our method might be inaccurate for patients with acute pancreatitis and hyperamylasemia.

Because the linearity extends to 7.49 mmol/L, urine specimens can be assayed without dilution. The assay system uses a one-step reaction and, compared with the method reported by Tabata et al., is simpler and less expensive. Our method should increase the flexibility of selecting reaction conditions for kinetic measurements of substrates consisting of enzymes.

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References