February 2003

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EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 35, No. 1, 17-22, February 2003

Enzymatic method for assaying calcium in serum with Ca^{++}-ATPase

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Accepted 8 January 2003

Abbreviations: AAS, atomic absorption spectrophotometry; AKUH, The Aga Khan University Hospital; CPC, o-cresolphthalein complexone; CV, Coefficient of variation; EC_{50}, 50% activation; Ca_f^{++}, free Ca^{++}; MOPS, 3-[N-morpholino] propanesulfonic acid; PEP, phosphoenolpyruvate; PK, pyruvate kinase; RM, reaction mixture; HEPES, N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid]; Ca^{++}-ATPase, Ca^{++}-dependent adenosine triphosphatase

Abstract

A kinetic assay for total calcium in serum was developed which is based on the activation of Ca^{++}-ATPase by free Ca^{++} $[Ca^{++}]_f$ maintained by EGTA in the reaction mixture. The concentration of Ca_f^{++} was dependent on total reference calcium added or serum calcium. Ca^{++}-ATPase activity was coupled to the reduction of NADH by pyruvate kinase (PK) and lactate dehydrogenase (LDH) and monitored by change in absorbance at 340 nm. The calcium in normal serum was 10.08±0.24 mg/dl (n = 35) by our method while with o-cresolphthalein complexone (CPC) method, the total calcium in the same 35 serum samples was 10.14± 0.54 mg/dl. The range of within-run coefficient of variations (CVs) by this method was 0.9-2.87% at 8-12 mg/dl and day-to-day CVs were 0.72-3.17%. The presence of other ions and standard clinical interfering agents did not affect this assay system. The correlation between values obtained with our method (y) and CPC method (x) for normal serum was: y = 1.064x-0.580 mg/dl (r = 0.912, n = 59).

Keywords: Ca^{++}-ATPase; calcium, serum; enzymatic assay; kinetics

Introduction

Analysis of calcium in serum and urine is important in the diagnosis of hypocalcaemia (i.e. hypoparathyroidism, renal failure) and hypercalcaemia (hyperparathyroidism) (Kanis, 1996). The concentration of calcium in body fluids has been widely determined by atomic absorption spectrophotometry (AAS) (Madsen *et al.*, 1996) and colourimetry of Ca^{++} complexes with o-cresolphthalein complexone (CPC) and Arsinazo III dye (Endres and Rude, 1994). The AAS method is reliable and specific for calcium but requires expensive equipment and is thus unsuitable for routine assay in clinical laboratories, especially in developing countries. The CPC method with 8-hydroxyquinoline is widely available but does not eliminate the interference of Mg^{++} ions sufficiently while IgM has been shown to interfere with assay of Arsinazo III dye method (John *et al.*, 1997).

Several enzymatic methods have been developed for the determination of calcium using phospholipase-D (EC 3.1.4.4) (Tabata *et al.*, 1986., Morishita, *et al.*, 1999), α-amylase (EC 3.2.1.1) (Kayamori and Katayama, 1994) and urea amidolyase (EC 3.5.1.45) (Kimura, *et al.*, 1996). These methods are based on activation or inhibition of the enzymes by calcium present in the sample. The choice of enzymes for use as analytical tools requires that they have high substrate specificity and catalyze sufficiently rapid reaction to make it possible to perform the assay without any separation procedures (Valero and Garcia-Carmona, 1996; Reg, 1998). Ca^{++}-dependent adenosine triphosphatase (Ca^{++}-ATPase; EC 3.1.6.38) is activated directly by Ca_f^{++} ions (Michelangeli and Munkonge, 1991). This enzyme can be readily obtained by partial purification from rabbit skeletal muscle sarcoplasmic reticulum as described (Michelangeli and Munkonge, 1991), which makes it a cheap and accessible enzyme for routine studies. Therefore, we have utilized this enzyme for the determination of calcium in serum.

Materials and Methods

Subjects

For normal serum Ca^{++} level determinations, blood

was drawn from the antecubital vein of healthy adult volunteers, 18 males (aged 20-55 years) and 17 females (aged 18-40 years). Serum was prepared by centrifugation at 3,000 rpm (approximately 1,500 *g*) in tabletop centrifuge at room temperature for about 10 min. This study followed the protocol of the Human Subject Protection Committee of AKUH and all volunteers were informed prior to participation.

Reagents and standards

$CaCl_2$, MOPS (free acid), NADH, PEP, PK, ATP, LDH, EGTA, CPC kit (procedure number 587), and most of the interfering reagents were from Sigma (St Louis, MO). Sarcoplasmic reticulum membranes containing Ca^{++}-ATPase from rabbit skeletal muscle were prepared as described previously (Michelangeli and Munkonge, 1991), which had protein concentrations of 12.6 mg/ml. Due to non-availability of lipaemic material, we used serum from a patient having 6,000 mg/dl triacylglycerol. Calcium reference standard (10 mg/dl), calcium controls level-I (mean 8.9 mg/dl, range 8.1-9.7 mg/dl), level-II (mean 10.8 mg/dl, range 9.8-11.8 mg/dl) and level-III (mean 13.1 mg/dl, range 12-14.4 mg/dl) were from Beckman as used in Synchron Clinical System. All solutions were made in deionized water. Most of the solutions for interfering substances were made as 100 fold stock in water and 2 μl was added in 0.2 ml serum. Bilirubin was made in 0.02 N KOH while NaCl and albumin for desired amounts were added as such in serum.

Principle

The reaction sequence in this method is following:

$$ATP \xrightarrow[Ca_f^{++}]{Ca^{++}\text{-ATPase}} ADP+Pi$$

$$ADP+PEP \xrightarrow{PK} ATP+Pyruvate$$

$$Pyruvate+NADH+H^+ \xrightarrow{LDH} NAD^++lactate$$

Ca_f^{++} activates the Ca^{++}-ATPase in the initial reaction, converting ATP into ADP, which was coupled to PEP and NADH using PK and LDH, respectively. The decrease in NADH absorbance was monitored at 340 nm. We added EGTA in the reaction mixture which not only converted all calcium of serum (both ionized and albumin bound) into Ca^{++}-EGTA complex (Simonides and van Hardeveld, 1990) but also maintained Ca_f^{++} at a constant level (Michelangeli and Munkonge, 1991). The Ca_f^{++} was dependent on the externally added total calcium in the form of standard $CaCl_2$ or serum/urine/other biological fluids. Since Ca^{++}-ATPase showed a sigmoidal behaviour with Ca^{++} as a substrate (Simonides and van Hardeveld, 1990), we calculated calcium in samples by comparing 50% activation (EC_{50}) of Ca^{++}-ATPase obtained by serum with that of reference $CaCl_2$.

Procedure

We used the following conditions in Beckman DU 70 spectrophotometer: 470 μl of the reaction mixture (RM) was added in 1 ml plastic cuvette. The composition of the RM was almost the same as used by Simonides and van Hardeveld (1990) with some modifications. We prepared 18.6 ml of RM, which contained about 20 mM MOPS, 200 mM KCl, 15 mM $MgCl_2$, 10 mM NaN_3, 10 mM PEP and 0.5 mM ATP. The pH of the RM was adjusted to 7.4 with KOH. This mixture was stored at -20°C in aliquots and was stable for several months. Just before measuring Ca^{++}-ATPase activation, we added 18 u/ml LDH/PK (where one unit (u) was defined as the amount of LDH or PK that utilized one μmole of NADH/min), EGTA (final concentration in RM was 0.05 mM), and Ca^{++}-ATPase (0.3 u/ml, where one unit of enzyme was defined as the amount of ATPase that utilized one mole of NADH per min by using 40 μM $CaCl_2$ in the reaction mixture under our assay conditions). The temperature of the RM was maintained at 30± 0.1°C using Multitemp II Thermostatic Circulator (LKB-Produkter, AB, Bromma, Sweden). Adding NADH to the RM started background reaction and the reaction rate was monitored at 1 min intervals for about 5 min at 340 nm. Ca^{++}-ATPase activity (i.e., activation) was then measured either by successive addition of reference standard or sample (3 μl each time) until the enzyme was maximally activated. The reaction rate was measured for 1 min. The EC_{50} (50% activation) of unknown (serum or urine) was compared with reference $CaCl_2$ to calculate the calcium in serum or urine by the following equation;

$$\text{Calcium (mg/dl)} = \frac{\mu\text{l of reference at } EC_{50}}{\mu\text{l of unknown at } EC_{50}} \times \text{Concentration of reference (mg/dl)}$$

The results are expressed in mg/dl±standard deviation (SD).

The activation of Ca^{++}-ATPase was linearly dependent on the amounts of Ca^{++}-ATPase up to 0.4 u/ml in the RM. The molar absorption coefficient for NADH of 6.22×10^3 M^{-1} cm^{-1} at 340 nm was used for calculations (Simonides and van Hardeveld, 1990). NADH

(0.2 mM) was added to start the reaction of non-specific ATPase. After about 5 min (when $\triangle A$ was approximately 0.002/min), the activation of Ca^{++}-ATPase was measured by adding various concentrations of either reference standard, serum, urine or control serum (or any other biological fluids), 3 μl at a time. The total volume of the sample added was always 18 μl. To differentiate between serum and serum supplemented with 0.2 mg/dl (0.05 mM); we had decreased the EGTA concentration to 25 μM in some experiments.

For comparison purpose we determined calcium concentration in normal serum by CPC method according to the method described by manufacturer (Sigma Diagnostics, St. Louis, MO). The statistical evaluation for comparison was made by linear regression analysis.

Results

The activation of Ca^{++}-ATPase by reference standard, as well as by serum or other biological samples showed a sigmoidal curve (Figure 1). The EC_{50} for samples with higher value of Ca^{++} (such as urine and hypercalcaemic samples) requires less volume and samples with lower concentrations of Ca^{++} (such as hypocalcaemic serum) require more volume (Figure 1). In 35 normal serum samples, the Ca^{++} was found to be 10.08±0.24 mg/dl (ranged 9.5-10.7 mg/dl) by our enzymatic method. In the same 35 samples, the calcium level was found to be 10.2±0.54 mg/dl (ranged 9.2-11.1 mg/dl) by CPC method. These results show that SD was less with our enzymatic method compared to CPC method. Under our assay conditions (when EGTA concentration was 50 μM), the EC_{50} values were essentially linear from about 8 mg/dl to 21 mg/dl (Figure 2). However, by decreasing EGTA concentration to 25 μM we observed a linear relationship between Ca^{++} concentration and EC_{50} values up to 2 mg/dl (Figure 2). In urine, this method clearly differentiated the normal samples and samples supplemented with 0.26 mg/dl (0.060 mM) $CaCl_2$ (Figure 3A) ($P < 0.002$). In serum this method was unable to measure the difference between normal serum and serum supplemented with 0.5 mg/dl (0.125 mM) $CaCl_2$. However, by decreasing EGTA concentration to 25 μM in RM, our method was able to differentiate between normal serum and serum supplemented with 1 mg/dl (0.25 mM) $CaCl_2$ (Figure 3B) ($P < 0.005$ above 6 μl serum). The analytical recovery of calcium was approximately 100% above the detection limit.

Comparison study

The comparison data of normal serum between our method (y) and CPC (x) correlated well (Figure 4) with the regression line $y = 1.064x - 0.580$ mg/dl ($r = 0.912$, $n = 59$).

Precision

The statistical analysis of the precision study for three different sera is shown in Table 1. This method exhibits an excellent precision as assessed by within-assay (CVs ranged from 0.9 to 2.87%) and day-to-day CVs (ranged from 0.72 to 3.17%). The calcium level

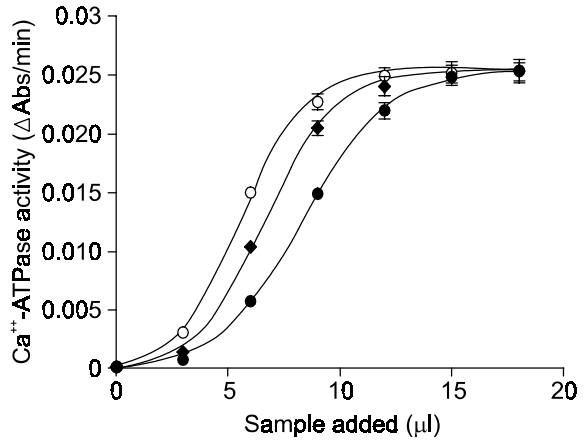

Figure 1. Stimulation of Ca^{++}-ATPase activity by normal (10 mg/dl, ◆-◆), hypocalcaemic (8 mg/dl, ●-●) and hypercalcaemic sera (12 mg/dl, ○-○). The serum was added in 480 μl of the reaction mixture in small increment (3 μl) with recording of ATPase activity after each addition. The data points are the mean of between 3-5 experiments where the standard deviations were less than 5% of the mean value.

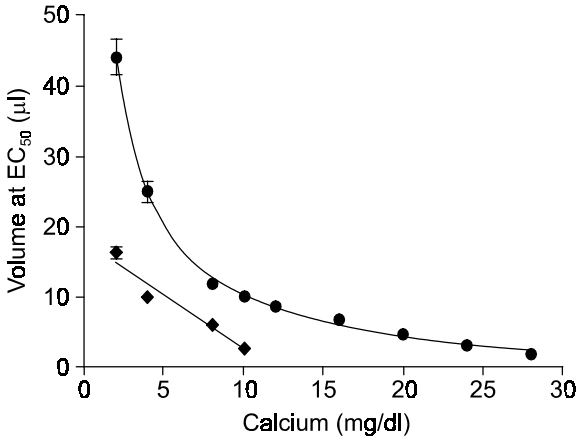

Figure 2. Standard curves of $CaCl_2$ with enzymatic method. The activation of ATPase was carried out as described in the Materials and Methods. The circles are for 50 μM EGTA while squares are for 25 μM EGTA.

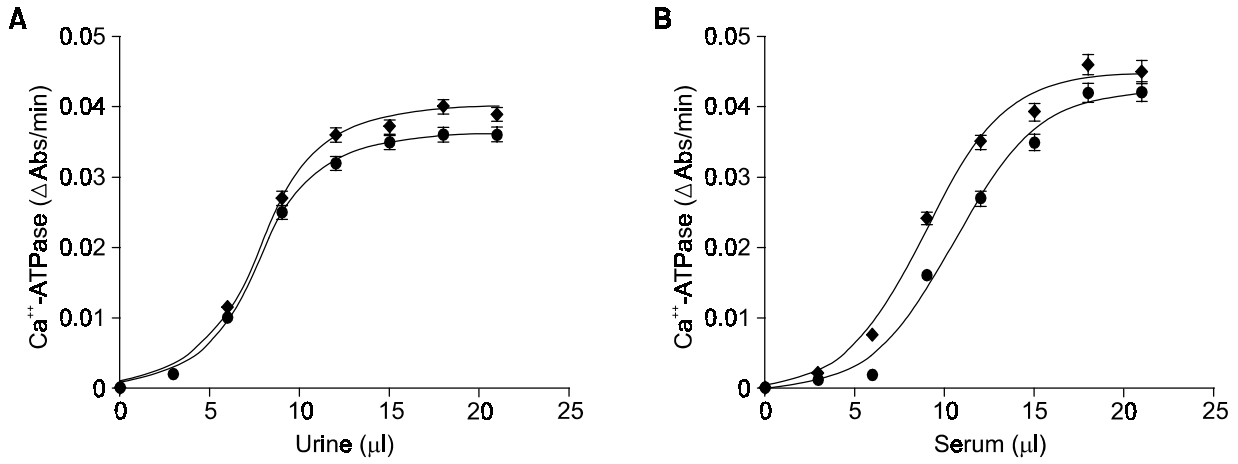

Figure 3. Differentiation between normal samples and samples supplemented with $CaCl_2$ by enzymatic method. A) urine and B), serum. The data points are the mean of between 6-10 experiments where the standard deviations were less than 3% of the mean value. The circles are for normal samples while squares are for samples supplemented with calcium.

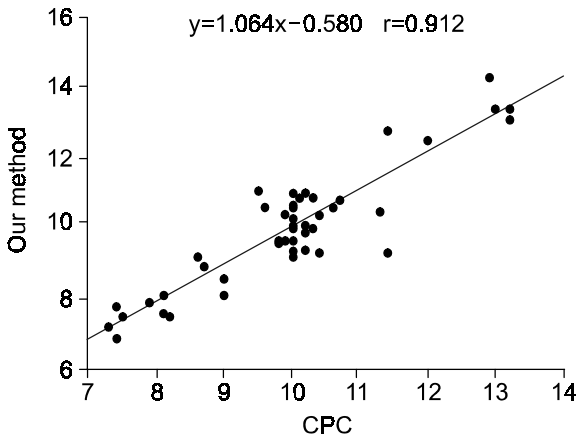

Figure 4. Correlation between the Ca^{++}-ATPase and CPC methods for calcium in normal serum. Some of the sera were diluted with saline and in some external calcium was added to get hypocalcaemic and hypercalcaemic sera, respectively. To minimize the bias effect, our colleagues modified some of the sera.

Table 1. Precision of the proposed method.

	Serum 1	Serum 2	Serum 3
Within run			
Mean	8.27 (n = 6)	10.0 (n = 10)	12.2 (n = 10)
SD	0.075	0.287	0.207
CV, %	0.9	2.87	1.7
Between-day			
Mean	8.22 (n = 8)	10.05 (n = 8)	12.17 (n = 7)
SD	0.26	0.12	0.09
CV, %	3.17	1.22	0.72

in controls I, II and III was always within the manufacturer's limits.

Interference by cations and other reagents

We found no interference by adding to human sera the 0.1 mM of Mn^{++}, Ni^{++}, Cd^{++}, Ba^{++}, Cu^{++}, Fe^{+++} (chloride) and Zn^{++} (sulfate), 350 μM bilirubin, 10 g/l human serum albumin, 100 USP unit/ml heparin, 300 μM glucose, 3.25 mM reduced glutathione, 1.63 mM oxidized glutathione, 5.7 mM ascorbic acid, 600 mM NaCl, 10 mM of NaI, KBr, KNO_3, $NaNO_2$, Na_2CO_3, CH_3COONa, Na_2SO_4, 20 mM of $NaHCO_3$, Na thiosulfate, 1 mM citrate, 3 mM lithium lactate, 7.8 mM haemoglobin, 16.7 mM urea, 0.5 mM creatinine, and 1,000 mg/dl triacylglycerol. However, the triacylglycerol above 1,000 mg/dl gave a negative interference due to turbidity effect.

Discussion

In recent years, many enzymatic assays have been developed for the determination of some ions in biological samples (Tabata *et al.*, 1986; Kimura *et al.*, 1992; Kayamori and Katayama, 1994; Kimura *et al.*, 1996; Kimura *et al.*, 1997; Reg, 1998; Morishita *et al.*, 1999). This is due to the fact that enzymes have extremely high catalytic power and specificity, which may quite often be detectable with great ease without any separation procedures (Valero and Garcia-Carmona, 1996; Favero *et al.*, 1998). Here, we have developed a new method for serum calcium using Ca^{++}-ATPase, which is directly activated by Ca^{++} (Simonide and van Hardeveld, 1990; Michelangeli and Mun-

konge, 1991; Favero *et al.*, 1998; Xu *et al.*, 2002). The "r" value (0.912) is reasonably good (Kim *et al.*, 2002) when compared with CPC on normal sera, and the values obtained by control sera were always within the limits of prescribed values. This shows that our method is reproducible, reliable and accurate. Tabata *et al.* (1986) and Morishita *et al.* (1999) have used phospholipase-D, while Kayamori and Katayama (1994) have observed the activation of porcine pancreatic alpha-amylase for the measurement of calcium. The major drawback with phospholipase-D was the interference with Mg^{++}, bilirubin, hemoglobin, phosphates, IgM and other reduced substances (Tabata *et al.*, 1986; Morishita *et al.*, 1999), while endogenous alpha-amylase caused positive interference with amylase method in patients with acute pancreatitis and hyperamylasaemia (Kayamori and Katayama, 1994). The Mg^{++} interference could not even be eliminated by CPC method, which has been extensively used in clinical laboratories (Endres and Rude, 1994; Kimura *et al.*, 1996). The RM of our method already contained saturated concentration of Mg^{++} (15 mM), which is far exceeded from the normal concentration of Mg^{++}in plasma (1 mM) and urine (5 mM or 3-5 mM/day) (Milne, 1994). Also saturated amount of ammonium ions were already present in solution of LDH/PK. Thus, there would be no problem of interference of these chemicals (Mg^{++} and ammonium ions) with our method. Furthermore, pyruvate is formed during reaction and immediately utilized by saturated level of LDH, we believe it will also not create any problem. Similarly, Ca^{++}-ATPase is a membrane-bound enzyme and there is no known clinical condition where this enzyme has been shown to increase in serum. Kimura *et al.* (1996) has reported a kinetic assay for serum calcium based on inhibition of urea amidolyase by Ca^{++} in serum. They pointed out that serum proteins interfere with the assay and the reagents were stable only for 3 days at 4°C.

The values of calcium in normal serum (10.08± 0.24 mg/dl) with our method and CPC method (10.14 ±0.54 mg/dl) were almost equal to reported values by other methods (Tabata *et al.*, 1986; Endres and Rude, 1994; Kayamori and Katayama, 1994; Morishita *et al.*, 1999). Our method had good precision, with reasonable analytical recovery without any interference with clinical analytes and gave good correlation with CPC method.

In enzymatic assays, cost of the enzyme is an important issue. If the enzyme is costly there is a natural tendency that lower concentration of the enzymes will be used, which may lead to incomplete reaction, the non-attainment of steady state, and non-linearity with time (Valero and Garcia-Carmona, 1996). In our method we used same reaction mixture for different concentrations of standard calcium and samples to get the standard curve and sample curve, respectively. We have also measured the reaction rates in separate reaction mixtures for each calcium concentration, but there was no significant difference in reaction rate in general and particularly EC_{50}. Therefore, we analyzed the calcium in serum or urine using successive addition of the samples in same reaction mixture, and thus minimized the cost.

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