Ionized Calcium Measurement in Serum and Plasma by Ion Selective Electrodes: Comparison of Measured and Calculated Parameters

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Abstract To determine the concentration of ionized calcium (iCa) collected in lithium heparin and gel tubes and to correlate the measured iCa with calculated iCa. Anaerobic fasting blood samples were simultaneously collected from healthy laboratory workers in lithium-heparin and gel tubes. iCa, pH, total calcium (CaT), total protein and albumin were measured. Ionized Ca was calculated with albumin and globulin values using an appropriate formula. Mean iCa in gel tubes showed a positive constant bias of 0.08 mmol/L ($p < 0.001$) when compared to lithium heparin results. The mean pH of blood taken in lithium heparin and gel tubes was non-significant ($p = 0.3$). Measured iCa poorly correlated with CaT ($r = 0.2$, $p = 0.1$) and calculated iCa ($r = 0.2$, $p = 0.2$). To evaluate the calcium status direct measurement of iCa must be done rather than using the formulae for iCa determination. In addition serum is recommended as the choice of sample for iCa determination in comparison to plasma samples.

Keywords Calcium · Ionized calcium · Serum · Albumin

Abbreviations

iCa Ionized calcium
CaT Total calcium
ISE Ion selective electrode
PTH Parathyroid hormone

CLSI Clinical Laboratory Standards Institute
IFCC International Federation of Clinical Chemistry
SPSS Statistical Package of Social Sciences

Introduction

Ionized calcium (iCa) testing which was previously available for research and at referral facilities only is now available for routine clinical use with the advent of calcium (Ca) ion selective electrodes (ISE) [1]. Advances in technology for its direct measurement have not only reduced the cost but have also improved availability in the clinical care. Since the 1990s, it has become an important component in emergency and intensive care units as a feature of nearly all blood gas analyzers as a point of care testing analyte. Its strict sample handling requirements and difficult to manage pre-analytical conditions such as diet, the choice of anticoagulant, pH dependence and anaerobic conditions limits its use in a clinical laboratory [2].

Forty five to fifty percent of the total calcium (CaT) in plasma is iCa, which is the free and biologically active form [3, 4], 40 % is protein bound and the remaining 10–15 % is Ca complexed to anions like phosphates, sulphates, lactate and citrate [5, 6]. Redistribution of Ca among the three plasma pools can occur acutely or chronically, by changes in the concentration of protein, small anions or alteration in pH [7–9]. iCa is kept in the narrow reference range of 1.15–1.33 mmol/L by an integrated homeostatic mechanism involving parathyroid hormone and 1, 25 dihydroxy vitamin D axis. The concentration of CaT is protein dependent and decreases as serum albumin levels fall and vice versa [10]. Equations to adjust CaT for albumin, such as the regularly cited corrected Ca [$\text{CaT mmol/L} + 0.02 (40 - \text{albumin g/L})$] are

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routinely used in clinical practice in patients with hypoalbuminemia [11].

Consistent pre-collection variables like relaxing the patient with normal breathing for 10 min, ensuring that the individual has not eaten for at least 4 h, anaerobic handling of the specimen, timing of sampling, patient posture, pH of the specimen and the effect of anticoagulants should be controlled to minimize variation in iCa that is not related to the disease being investigated. A high degree of accuracy is needed for iCa measurement to diagnose a Ca disorder and issues of specimen collection and choice of collection container are important for accurate iCa determination [12, 13].

The International Federation of Clinical Chemistry (IFCC) recommends heparin as the anticoagulant of choice for iCa determination [14]. Both sodium/lithium heparin and calcium titrated heparin can be used. However, the concentration of sodium/lithium heparin should not exceed 15 IU/ml blood while calcium titrated heparin should not be more than 50 IU/ml blood. Heparin binds small but significant amount of calcium depending upon on the quantity and type of heparin used [13, 15]. As for the Clinical Laboratory Standards Institute (CLSI) guidelines plasma derived from evacuated tube collection is unacceptable for iCa measurement as it is influenced by both heparin binding and completeness of tube filling. According to CLSI whole blood specimens (specimen in syringes) with modified heparin preparations can be used for iCa analysis. The CLSI guidelines state that although not recommended if evacuated tubes must be used they should be completely filled and kept sealed until analysis [16].

Evacuated blood collection tubes containing lithium heparin and gel separator material are widely used in clinical laboratories. For acutely ill patients or during a course of surgery the need for fast and dependable iCa results can be best met by using plasma samples. Advantages of using lithium heparin is that it is time saving in urgent tests as gel separator tubes take 20–30 min for the blood sample to completely clot prior to being spun-down and another advantage of using lithium heparin tube is that the entire volume of the specimen is usable for analysis. The use of serum as the sample avoids biases from erythrocytes and heparin, but larger sample volume and more time before analysis is required.

As the Ca titrated heparin containing vacutainers are not commonly available and their use would add to the complexity of the logistics of everyday practice we performed a validation study to optimize the testing of iCa in our laboratory with the objective to compare the specimen integrity collected in lithium heparin tubes (plasma) and gel tubes (serum). Another objective was to compare calculated iCa (calculated from the results of serum albumin, globulin and CaT) with the measured iCa.

Materials and Methods

Subjects and Blood Collection and Handling

For the purpose of optimization of methodology for iCa determination at the Section of Chemical Pathology, Department of Pathology and Microbiology, Aga Khan University this study was undertaken from 1st June to 1st July 2010. It was given exemption from the ethical review board of our institution as it was part of optimizing this test methodology in our laboratory for patient service. Samples were collected after consent from forty healthy laboratory workers with no known co-morbid conditions and with no obvious disturbances of Ca homeostasis. Blood sampling was performed after overnight fast.

At the time of specimen collection all subjects were made to sit, relax and breathe normally for 10 min. Tourniquets were applied for less than 1 min and subjects were not allowed to exercise the forearm or make a fist while sampling. Blood was taken simultaneously in both lithium heparin (spray dried form 17 IU/ml) and gel tubes (manufactured by Becton, Dickinson and Company, USA). Gel tubes were filled first followed by collection in lithium heparin vacutainers to avoid heparin contamination. The specimens were transported to the clinical chemistry laboratory within 10 min after sample drawing and kept under ambient temperature (22–25 °C) until analysis. All tubes were filled to their nominal volume, kept sealed under analysis and handled anaerobically. Lithium heparin specimens were thoroughly mixed to distribute the heparin and minimize the formation of micro clots.

Laboratory Analysis

Lithium heparin specimens were centrifuged at 3000 rpm for 5 min and analyzed within 20–30 min after collection. Gel tubes specimens were analyzed for iCa within 1 h of collection after clot formation and centrifugation at 3000 rpm for 5 min. Serum was then used for estimation of CaT, protein and albumin.

Ionized calcium and pH were estimated using ISE on Cobas 121 analyzer (Roche Diagnostics, USA). Sampling was repeated if pH was not in the range of 7.2–7.6. Total calcium was analyzed by means of a bichromatic end-point methodology using Arsenazo III reagent on Synchron CX 9 (Beckman USA). The system was closely monitored by routine practice of running both high and low controls with every batch analyzed. The within run and total CV were 2 and 3 % respectively at a concentration of 2.5 mmol/L. Total protein was measured by means of a rate biuret method on an auto analyzer Synchron CX 9 (Beckman USA). Both normal and abnormal controls were run with each batch. The within run and total CV were 2 and 3 %

respectively at a concentration of 100 g/L. Serum albumin was analyzed using the bromocresol purple method on Synchron CX9 (Beckman USA). Two levels of controls were analyzed daily. The three analytes CaT, protein and albumin were closely monitored by successful biannual participation in external proficiency program of CAP.

Selection of formula for estimating iCa

Values for total protein, albumin and globulin were utilized for derivation of calculated iCa by using these formulae

1. % protein bound Calcium = $0.8 \times \text{albumin(g/L)} + 0.2 \times \text{globulin(g/L)} + 3$
2. Calculated iCa (mg/dl) = CaT – protein bound Calcium
3. Calculated iCa (mmol/L) = Calculated iCa (mg/dl) $\times 0.25$

Literature has published several equations to estimate iCa from other blood biochemistry results. Sava et al. few years back used the above mentioned equation in Indian population and hence we chose the same equation for our study [17].

Statistical Analysis

Statistical Package of Social Sciences (SPSS) version 19 and Analyze-it for Microsoft Excel was used for statistical analysis of the data. Mean and standard deviation (SD) for quantitative variables was computed. Comparison was done by applying paired t test between iCa in the two vacutainers and also between iCa measured and calculated. Using Bland–Altman technique comparison between iCa in lithium heparin and gel tubes was done. Regression analysis taking measurement errors for both methods into account was done using Deming procedure. Deming regression was used to obtain slopes adjusted for measurement error and regression to the mean. Two-tailed $p < 0.05$ were considered significant and <0.01 as highly significant.

Results

Forty individuals participated in the study, majority (75 %) were females. Mean age of the group was 27 ± 5 years. The values for measured and calculated biochemical parameters of the study group are illustrated in Table 1. All of the participants were normocalcemic.

Comparison of pH and iCa concentration in lithium heparin versus gel tubes

The mean pH of blood taken in lithium heparin and gel tubes was not significant ($p = 0.3$). Comparison of mean

Table 1 Measured and calculated biochemical parameters

Biochemical parameters	Mean ($\pm$ SD)
pH in lithium heparin	7.39 (± 0.02)
pH in gel tube	7.40 (± 0.02)
Measured iCa (mmol/L) in lithium heparin	1.15 (± 0.03)
Measured iCa (mmol/L) in gel tube (serum)	1.22 (± 0.03)
Serum total calcium (mmol/L)	2.23 (± 0.13)
Serum total protein (g/L)	73.5 (± 38.7)
Serum albumin (g/L)	41.9 (± 34.9)
Serum globulin (g/L)	31.9 (± 2.8)
Calculated iCa (mmol/L)	1.16 (± 0.1)

iCa ionized calcium

iCa in both the evacuated tubes showed a statistically significant mean difference bias of 0.08 mmol/L when paired t test was applied. ($p < 0.001$). Deming fit and Bland–Altman plot showed bias of 0.07 (95 % CI 0.59–0.57) and 0.08 mmol/L (95 % CI 0.06–0.08) respectively between the two evacuated tubes (Fig. 1a, b).

Relation of measured iCa with calculated iCa

Statistically significant mean difference existed between calculated iCa (1.16 ± 0.10 mmol/L) and measured iCa (1.22 ± 0.03 mmol/L) ($p < 0.01$) in gel tubes and correlation of calculated iCa with measured iCa in gel tubes was $r = 0.2$. Deming fit analysis between iCa estimation and iCa calculated depicted a slope of 10.17 (95 % CI 0.68–19.66) and an intercept of -11.3 (95 % CI 22.9–0.33) (Fig. 2a)

Mountain plot (Fig. 2b) provides information about the distribution of the differences between measured iCa results from gel tubes compared to measured iCa from lithium heparin tubes and calculated iCa. Long tails of calculated iCa in the plot reflect large differences between the measured serum iCa and calculated iCa. The median of the differences is close but not coinciding with zero for both iCa measured in lithium heparin tubes and also for calculated iCa. The differences of iCa measured in gel tubes and in lithium heparin tend to be smaller than the differences of iCa measured and calculated Ca from formula.

Relation of iCa and CaT with proteins

The association of measured iCa was non-significant with CaT ($r = 0.2$, $p = 0.1$). Serum protein and globulin showed inverse but non-significant correlation with iCa ($r = -0.3$, $p = 0.8$ and $r = -0.1$, $p = 0.4$ respectively). The association of CaT was significant with total protein

Fig. 1 Comparison of iCa values in lithium heparin and in gel tubes. **a** The Deming regression analysis demonstrates close agreement between the two methods. The *solid line* indicates the slope of observed measurements (slope = 0.95) plotted against the *perfect-fit line* for which slope = 1 and **b** Bland–Altman between the two methods reveals majority of values lying around 0 and within confidence interval

($r = 0.5$, $p < 0.001$) and albumin ($r = 0.6$, $p < 0.001$) whereas non-significant with globulin ($r = -0.3$, $p = 0.8$).

Discussion

There is extensive literature on the clinical usefulness of iCa measurement in clinical practice. It is known that CaT concentration changes in parallel to the albumin concentration. Measurement of CaT is misleading in cases with hypoalbuminemia like in those suffering from hepatic or renal disease, burns, cardiac failure or malnutrition [18]. Even in the presence of a normal serum albumin, changes in blood pH can alter the equilibrium constant of the albumin-Ca complex, with acidosis reducing the binding and alkalosis enhancing it. Thus, in critically ill or post-surgical patients, correcting CaT for albumin is not necessarily accurate because of changes in pH and affinity of Ca binding. Dickerson et al. proposed in his study ($n = 100$) done on trauma patients to directly measure iCa

Fig. 2 A comparison of iCa values measured and calculated. **a** The Deming regression analysis demonstrates close agreement between the two methods. The *solid line* indicates the slope of observed measurements (slope = 10.17) plotted against the *perfect-fit line* for which slope = 1 and **b** Mountain plot between the iCa measured in gel tubes and iCa calculated and measured in lithium heparin tubes reflect the biases between the measured and calculated results of iCa as both mountains are not centered around 0. Taking iCa in gel tubes as reference method long tails of calculated iCa, reflect large differences from iCa measured in gel tubes

instead of calculated Ca for assessing Ca status in this population. In this study seven equations that estimated iCa based upon total calcium, albumin, total protein or anion gap were applied to each patient to evaluate the reliability of these predictive formulae to estimate Ca status. The mean sensitivity and specificity for predicting hypocalcemia by these equations was 32.8 and 88.2 % respectively. The mean sensitivity and specificity for predicting hypercalcemia by these equations was 33.2 and 60.4 % respectively [19].

Information regarding preference of iCa over CaT during acid base disturbances and in critical care units has markedly expanded during recent years [3]. A Japanese study reported sudden drops in iCa during cardiopulmonary bypass of pediatric patients ($n = 30$) and hypothesized

citrate added to the stored blood as the cause of these changes [20, 21]. Another study ($n = 115$) reported iCa determination as an optimal method of Ca homeostasis control during cardiopulmonary bypass [21]. In 691 consecutive chronic kidney disease patients who had simultaneous measurement of CaT, iCa and serum albumin, CaT failed to identify 40 % patients who had low iCa and 21 % patients who had a high iCa [22]. The utility of iCa has been reported in cardiopulmonary bypass and during hemodialysis where maintenance of good cardiac function is essential [23]. Several prospective and retrospective studies show that iCa rather than CaT is more useful in malignancies, hypo/hyperparathyroidism and pancreatitis [23–25].

The scope of our study was to determine the optimal sample type for its estimation. Findings from this study show minimal pH changes in gel separator and in lithium heparin vacutainers. Though the use of heparin-containing tubes allows rapid analysis, it should not be ignored that plasma iCa was 0.08 mmol/L (0.32 mg/dl) lower in these tubes than in gel separator tubes. The reference interval for iCa quoted in the product inserts, literature and guidelines is the same for serum and plasma samples (1.15–1.33 mmol/L). In an ideal situation laboratory using lithium heparin tubes should have separate reference interval as a constant negative bias of 0.08 mmol/L in lithium heparin tubes was noted compared to gel tubes.

As per IFCC guidelines the binding of iCa with liquid heparin is 2 % as long as the heparin concentration is <15 IU/ml of blood and it is 5 % if the concentration of dry heparin is <50 IU/ml of blood. It is recommended to use Ca titrated heparin as the best measure to minimize Ca binding but we did not have the facility of such vacutainers and used dry heparin in this study.

The sample of choice for iCa analysis is serum from this study. The advantages of using serum is that the specimen contains no anti-coagulant to bind and alter the iCa concentration, the specimen is suitable for a variety of tests regularly performed in clinical chemistry laboratories and hemolysis is readily detected. Another advantage is the incorporation of barrier gels that minimize changes in pH and other variables that affect iCa [12]. However the disadvantages are that the analysis is delayed as serum requires 20–30 additional minutes for the blood to clot which can be accelerated by centrifugation and the volume of serum obtained is only about one half of the amount of blood actually drawn.

This study shows that CaT and calculated iCa correlate poorly with iCa analyzed using ISE. The results from this study conclude that serum is the sample choice for iCa determination and discourage the use of equations and formulae for determination of iCa.

Conflict of interest None.

Ethical standards The study was given exemption by Ethical review committee (ERC) as per AKU ERC guidelines section B. <http://www.aku.edu/res-office/res-erc.shtml>.

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