

Calcium Determination in EDTA Treated Plasma by Colorimetric Method and Microplate Reading Format

S. Adeleh Razavi,¹ Laleh Hoghoohirad,² Hoda Golab-Ghadaksaz,² Mehdi Hedayati*²

1. Department of Biology, Faculty of Basic Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran
2. Cellular and Molecular Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Article information	Abstract
<p>Article history: Received: 16 June 2013 Accepted: 1 Oct 2013 Available online: 24 Dec 2013 ZJRMS 2015 Feb; 17(2): 7-10</p> <p>Keywords: Colorimetry Calcium Plasma EDTA</p> <p>*Corresponding author at: Cellular and Molecular Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran. E-mail: Hedayati@endocrine.ac.ir</p>	<p>Background: Calcium has different functions in metabolic processes and increase or decrease of calcium level in blood may lead to different disorders. Therefore, measurement of calcium level in blood is an important clinical issue. It is not possible to measure calcium in plasma samples which contain calcium chelators and we need serum samples to do so. This research aims to investigate the simple and direct colorimetric method for calcium measuring in EDTA treated plasma.</p> <p>Materials and Methods: In this experimental study, through adding a patented solution to EDTA treated plasma, calcium ions are released from calcium chelators and then reacted with ortho cresolphthalein complexone (o-CPC) as a coloring reagent and finally product of calcium reaction is measured using microplate reading format. The details of methodology are patented on 29.04.2012 in State Organization for Registration of Deeds and Properties with registration number of 74804, and are the exclusive property for the owners. Sensitivity, precision, accuracy and the correlation of plasma calcium with serum calcium level were investigated.</p> <p>Results: Sensitivity of this method was 0.4 mg/dL. Coefficient of variation for intra assays and inter assay were less than 5% and 6% respectively. According to recovery and parallelism tests, ratio and recovery percent ranged from 90 to 110% and correlation coefficient in compare with determination of calcium in serum by the same reagent was 0.92.</p> <p>Conclusion: This method is capable of measuring calcium in EDTA treated plasma and in addition, it has the required sensitivity, precision and accuracy for common clinical tests.</p> <p>Copyright © 2015 Zahedan University of Medical Sciences. All rights reserved.</p>

Introduction

Calcium is the most important mineral in human's body. As well as a structural role in teeth and bones, it has important physiological functions such as muscle contraction, neural excitation, blood clotting, secretion, fertilization, enzyme activation, proliferation, gene expression and apoptosis [1, 2]. There are three forms of calcium in plasma: free calcium ion, binding to proteins such as albumin and binding to small anion molecules such as phosphate, bicarbonate, and citrate [3]. According to the importance of various physiological roles of calcium in human's body, measuring serum level of calcium is a daily demand of physicians and referring of medical laboratories [4]. Usually some tests such as complete blood count (CBC) and measurement of plasma glucose requires plasma sample. The most common anticoagulant to obtain plasma is ethylene diamine tetra acetic acid (EDTA). Due to the lack of calcium assay on plasma samples containing EDTA, laboratories should provide serum samples for measurement of calcium. Conventional methods for measuring blood calcium include: Clark-Colip method which is the oldest one and involves complicated procedures such as the precipitation of calcium as the oxalate, separation of precipitated oxalate by filtration,

release of oxalate ions in hot H₂SO₄ solution, and titration of the oxalate with potassium permanganate. This standard method is usually used to check the accuracy of the other methods [5], complexometry or the titration method of calcium with EDTA is a rapid and accurate one but recognition of endpoint of titration is difficult [6]. The various enzymatic assays have been reported but they have limitations due to their instability of the reactants [7]. Methods such as atomic absorption spectrometry (AAS) [8], ion selective electrodes (ISE) [9], ion chromatography [10], nuclear magnetic resonance (NMR) [11], high performance liquid chromatography (HPLC) [12], capillary electrophoresis [13] are analytical methods which are sensitive but they are sophisticated and their procedures include tedious and cumbersome extraction or preparation steps. In addition, the costs of these instruments are high [14]. Currently the most common method for measuring calcium levels in clinical laboratory is a spectrophotometric method using Ortho cresolphthalein complexone (o-CPC) [15, 16]. The most important limitation of o-CPC is its inability in measuring calcium in EDTA plasma because EDTA with calcium ion forms a stable complex. Thus, for measuring calcium by this reagent, it is strongly emphasized not to use

EDTA plasma. Since it has not been reported any routine laboratory method for measuring of calcium levels in EDTA plasma samples, the need to conduct such a study was found. In this study a new patented method has been presented, according which we are able to measure calcium in EDTA treated plasma by o-CPC.

Materials and Methods

In this experimental study about 4 mL blood was taken from 40 apparently healthy volunteers. Each sample was divided into two aliquots (2 mL for preparation of serum and 2 mL for preparation of plasma). For plasma preparation, tubes containing EDTA was used. Samples were collected in tubes and stored in microtubes without any contamination of calcium. For preventing surface evaporation, microtubes caps were completely sealed. Until testing, the samples were placed at 2-8°C and for longer periods they were kept at -20°C. All chemicals were purchased from Merck, Germany.

For calcium determination at first 10 µL of plasma samples, standard as a calibrator and distilled water as a blank, were added to the wells of a 96 wells microplate. Calcium standard solution (10.5 mg/dL) was purchased from Pars Azmoun Co., Tehran, Iran. Solution of X was prepared. (X is a patented solution that can dissociate and release calcium from EDTA. It is patented on 29.04.2012 in State Organization for Registration of Deeds and Properties with registration number of 74, 804, and is the exclusive property for the owners). Then 10 µL of X solution was added to wells containing plasma and blank. For having the same volume, 10 µL double distilled water was added to the standard solution. For 15 min, microplate was placed on a microplate shaker (Stat Fax-2200, Awareness Technology, Inc., USA). Four parts of ethanolamine solution, 0.8 M/L, pH=10.7, and one part of solutions containing o-CPC, 0.06 mM, 8-hydroxyquinoline, 7 mM and hydrochloric acid, 20 mM, pH=1.1 for making a homogenous solution were mixed. Then 300 µL of the homogenous solution were added to the all wells containing plasma, standard and blank. Finally, after 5 min the absorbance was measured at 578 nm with a microplate reader (Sun Rise, TECAN A-5082, TECAN Groups Ltd., Salzburg, Austria).

The validity of the method has been evaluated as follows: Precision of the method was assessed by intra and inter-assay coefficient of variations percent for three samples with low, medium, and high calcium content. Replication number was eight in all steps. Sensitivity of the method was calculated based on average optical density of zero standards plus twice the standard deviation. To evaluate the accuracy based on parallelism test, a sample was serially diluted with double distilled water. Using the expected and measured data, the ratio percentage of parallelism tests was determined. Also, the recovery test was determined by adding 10 µL of standard solution into three samples with different concentrations. Then using the expected and measured data, the recovery percent was calculated. For comparing two methods, the obtained results of this new method in plasma samples

were compared with o-CPC colorimetric method in the same serum samples and the correlation coefficient was calculated.

Results

After theoretical studies and pilot tests, X was chosen as a displacer of calcium ion from EDTA as a chelators. Proper concentration and volume of X and the required time for releasing of calcium from the EDTA-Ca complex were optimized. Table 1 contains the data of inter/intra assay. As the findings show, the precision of the method is acceptable.

Table 1. Results of precision assessment, intra and interassay coefficients

Run	Concentration	Replications No.	Mean±SD	CV%
Intra-assay	Low	8	7.98±0.20	2.57
	Medium	8	10.03±0.44	4.34
	High	8	11.64±0.48	4.10
Inter-assay	Low	8	8.92±0.52	5.83
	Medium	8	10.62±0.62	5.85
	High	8	12.16±0.65	5.36

Based on the average of zero standard absorption plus twice the standard deviation, the sensitivity was calculated 0.4 mg/dL

The results of parallelism and recovery tests were presented in tables 2 and 3. These tables showed that the method has an acceptable accuracy. Figure 1 contains the results of method comparison.

Table 2. Results of parallelism test for accuracy assessment

Sample	Dilution	Expected	Measured	Ratio%
1	1	7.38	7.38	100
2	2	3.69	3.75	101.69
3	4	1.84	1.58	85.42
4	8	0.92	0.88	94.92

Table 3. Results of recovery test for accuracy assessment

Sample	Standard	Expected	Measured	Recovery%
1	11.10	10.35	10.76	103.92
2	11.10	11.28	11.29	100.07
3	11.10	11.81	12.99	110.00

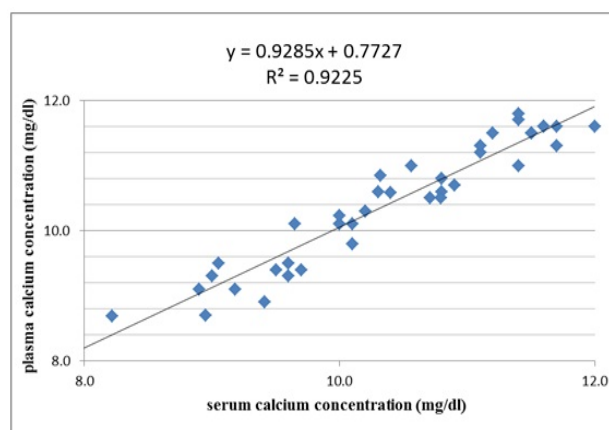


Figure 1. Comparison results of the new assay for plasma calcium with serum calcium levels (N=40)

Discussion

According to data collected in present study, patented solution X, can dissociate and release calcium ions from EDTA chelator and provides the possibility of measuring calcium concentration in EDTA plasma. Since it has not been reported any routine assay for calcium determination in EDTA treated plasma, comparative evaluating of patented method is not possible. Therefore, the scientific basis of patented solution, efficiency and its advantages compared with other methods are discussed. In EDTA treated plasma, calcium is highly chelated by EDTA and is not exposed to o-CPC, because EDTA has a high tendency to form complex with calcium ion. The scientific basis of the novel method is formed using a displacer. Accordingly, if a displacer which has a greater tendency to EDTA is added to EDTA plasma, Ca is released by replacement. After the release of calcium ions, they can be determined by o-CPC reagent.

Despite the importance of calcium in various metabolic processes, there is not a method for calcium determination in plasma. Additionally many methods for calcium determination require analytical instruments which are not proper for clinical laboratories because of their high costs, high experienced personnel and low rate of measurement [17]. However, these methods have the high accuracy and are considered as the reference method [5-14]. So a simple and inexpensive method is needed.

Colorimetric method for calcium determination by o-CPC reagent due to its simplicity, sensitivity and efficiency, is common. But this method is limited to use of urine, serum and heparin plasma samples [15, 16]. Heparin is more expensive than the other anticoagulant reagents (e.g. EDTA) and its effect is temporary. Heparin does not stop clotting of blood and it just delays the process for 8 to 12 h [18]. Thus, the most commonly anticoagulant is EDTA. So according to o-CPC method, laboratory has to take serum and plasma, 2 kinds of samples from patients who need complete blood count (EDTA treated blood) and serum calcium. Due to the mentioned reasons, efforts was started for removing the limitation of o-CPC method and eventually leads to develop a new method for measuring calcium in EDTA treated sample which is patented on 29.04.2012 in State Organization for Registration of Deeds and Properties with registration number of 74804. Generally, solving the

limitations of common method of calcium determination improves methodological parameters and expands the range of applied science in this field. Another advantage of this method is that calcium can be measured in plasma and serum simultaneously. Thus, the laboratory costs are reduced. Thirdly, this method is a simple method that can directly measure the calcium. Also microplate reading format increased speed of the assay time and shorten the total assay time.

The obtained results of this new method in plasma samples should have been compared with another method that determines the calcium levels in EDTA plasma sample, but as mentioned before the reported methods for calcium determination have been used serum sample not plasma. Since the difference between plasma and serum calcium is low and it is not possible to determine EDTA plasma calcium level, so they obtained results of the new method were compared with identical serum samples. Final conclusion of this study is the proposed method for measuring calcium levels in EDTA plasma has an acceptable sensitivity, precision and accuracy. According to this method, limitation of colorimetric method for EDTA plasma calcium determination by o-CPC reagent was solved. Since the most clinical laboratories use auto analyzer for biochemical analysis and measurements, it is suggests to study the possibility of adjust this new method on common automated instruments.

Acknowledgements

This paper has been conducted based on the MSc thesis entitled: "Design of a simple direct colorimetric method for calcium determination in EDTA treated plasma" in which a novel method has been presented, patented on 29.04.2012 in State Organization for Registration of Deeds and Properties with registration number of 74804. The authors of this paper appreciate kind of cooperation of Dr. Shahriar Norouzpour, Mrs. Elaheh Ostad and Ms. Aghileh Ostad.

Authors' Contributions

All authors had equal role in design, work, statistical analysis and manuscript writing.

Conflict of Interest

The authors declare no conflict of interest.

Funding/Support

This paper had been done by personal expenses.

References

1. Tandogan B, Ulusu N. Importance of calcium. *Perspect Medl Sci.* 2005; 35(1): 197-201.
2. Naghii MR, Ebrahimpour Y, Darvishi P, et al. Effect of consumption of fatty acids, calcium, vitamin D and boron with regular physical activity on bone mechanical properties and corresponding metabolic hormones in rat. *Indian J Exp Biol.* 2012; 50(3): 223-231.
3. Lanham-New SA. Importance of calcium, vitamin D and vitamin K for osteoporosis prevention and treatment. *Proc Nutr Soc.* 2008; 67(2): 163-176.
4. Lihong Shi, Xiaoqing Liu, Haijuan Li and Guobao Xua. Determination of total calcium in plasma by flow injection analysis with tris (2,2'-bipyridyl) ruthenium(II) electrochemiluminescent detection. *Electroanalysis.* 2006; 18(16): 1584-1589.
5. Clark EP, Collip JB. A study of TISDAL method for the determination of blood serum calcium with a suggested modification. *J Biol Chem.* 1925; 63(1): 461-464.
6. Schwarzenbach G, Gysling H, Metallindikatoren I. Murexid las Indikator auf Calcium- und andere Metall-

- Ionen. Komplexbildung und Lichtabsorption. *Helv Chim Acta*. 1949; 32(4): 1314-1325.
7. Sugano M, Yamauchi K, Sugano K, et al. New enzymatic assay using phospholipase D to measure total calcium in serum. *Clin Chem*. 2005; 51(6): 1021-1024.
 8. Thin CG, Thomson PA. Estimation of calcium and magnesium in serum and urine by atomic absorption spectrophotometry. *J Clin Pathol*. 1967; 20(3): 280-282.
 9. Urban P, Buchmann B, Scheidegger D. Facilitated determination of ionized calcium. *Clin Chem*. 1985; 31(2): 264-266.
 10. Yu BS, Yuan QG, Nie LH and Yao SZ. Ion chromatographic determination of calcium and magnesium cations in human saliva and urine with a piezoelectric detector. *J Pharm Biomed Anal*. 2001; 25(5-6): 1027-1032.
 11. Song SK, Hotchkiss RS, Neil J, et al. Determination of intracellular calcium in vivo via fluorine-19 nuclear magnetic resonance spectroscopy. *Am J Physiol*. 1995; 269(2 Pt 1): C318-22.
 12. Qian M, Gallo JM. High-performance liquid chromatographic determination of the calcium channel blocker nimodipine in monkey plasma. *J Chromatogr*. 1992; 578(2): 316-20.
 13. Zhang R, Shi H, Ma Y. Quantitative determination of ionized calcium and total calcium in human serum by capillary zone electrophoresis with indirect photometric detection. *J Microcolumn Sep*. 1994; 6(3): 217-221.
 14. Xian-Li W, Hong-Bi Z, Shu-Jie W and Bao-Xian Y. Determination of magnesium and calcium in biological samples by potentiometric stripping analysis. *J Chin Chem Soc*. 2010; 57(4A): 647-652.
 15. Baron DN, Bell JL. A simple specific titration method for serum calcium. *Clin Chim Acta*. 1957; 2(4): 327-331.
 16. Connerty HV, Briggs AR. Determination of serum calcium by means of orthocresolphthalein complexone. *Am J Clin Pathol*. 1966; 45(3): 290-296.
 17. Hedayati M, Ordoorkhani A, Daneshpour MS and Azizi F. Rapid acid digestion and simple microplate method for milk iodine determination. *J Clin Lab Anal*. 2007; 21(5): 286-292.
 18. McNeely TB, Griffith MJ. The anticoagulant mechanism of action of heparin in contact-activated plasma: Inhibition of factor X activation. *Blood*. 1985; 65(5): 1226-1231.

Please cite this article as: Razavi SA, Hoghooghird L, Golab-Ghadaksaz H, Hedayati M. Calcium determination in EDTA treated plasma by colorimetric method and microplate reading format. *Zahedan J Res Med Sci*. 2015; 17(2): 7-10.