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Research Article

The Effect of Anticoagulants on Zinc Measurement: A Comparative Study in Serum and Plasma Samples Using a Colorimetric Method

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ABSTRACT

Objectives: Zinc (Zn) is a vital trace element, and its accurate measurement in biological samples is important for diagnosing Zn deficiency. However, the difference in Zn concentration between serum and plasma samples and the effect of anticoagulants on results are major methodological challenges. The aim of this study was to compare the effect of common anticoagulants (EDTA, citrate and heparin) on Zn concentration in blood samples using a routine colorimetric method..

Methods: This research was conducted on 20 healthy volunteers (10 women and 10 men). Samples of serum, EDTA, citrate and heparin plasma were collected and Zn levels were measured using a routine colorimetric method based on 5-Br-PAPS.

Results: The results of this study showed that Zn concentrations in EDTA- and citrate-anticoagulated plasma were significantly lower than those measured in serum ($P<0.05$). In contrast, no statistically significant difference was observed between serum and heparin-anticoagulated plasma ($P>0.05$). Spearman's correlation analysis showed a strong positive correlation between serum Zn concentrations and those measured in heparin ($r=0.831$, $P<0.001$) and citrate ($r=0.704$, $P<0.01$) plasma samples, whereas the correlation between serum and EDTA plasma was positive but weak and not statistically significant ($r=0.099$, $P>0.05$).

Conclusion: The choice of anticoagulant is a critical pre-analytical factor in Zn measurement using colorimetric methods. These findings show that chelating anticoagulants like EDTA and citrate spuriously lower Zn concentration, whereas heparin-anticoagulated plasma yields Zn measurements that are comparable to those obtained in serum. These results are of great importance for the accurate interpretation of results in clinical laboratories.

Keywords: Zinc, Anticoagulant, Colorimetric method, Serum, Plasma

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Introduction

Zinc (Zn) is a vital trace element essential for human health and proper biological function. It acts as a cofactor in more than 300 enzymes in the body and plays a central role in key metabolic processes such as protein synthesis, nucleic acid metabolism, cell growth, immune function, and inflammatory responses (1). Given zinc's widespread roles in the body, its deficiency can lead to a variety of health problems, including growth impairment, skin issues, and immune system dysfunction. Therefore, the accurate measurement of Zn concentration in biological samples, particularly blood, is of high importance for the timely diagnosis of Zn deficiency and its clinical management (2).

Zinc measurement in biological samples is accompanied by numerous methodological complexities and challenges. One of the most important of these challenges is the influence of pre-analytical conditions on the final Zn concentration (3). There has been a long-standing debate in the scientific community regarding the difference in Zn concentration between serum and plasma samples. Some researchers have observed that Zn concentration in serum is significantly higher than in plasma and have attributed this difference to the release of Zn from platelets during the clotting process (4). This phenomenon can affect final results due to the high Zn content in platelets. In contrast, other studies have found no statistically significant difference between Zn concentrations in serum and plasma, considering both sample types suitable for Zn measurement (5, 6). These contradictions in results highlight the importance of precise standardization of sample collection and preparation procedures.

The use of different types of anticoagulants in plasma samples has also been identified as a significant factor in causing measurement errors. Anticoagulants such as EDTA and citrate are known as potent chelating agents for metal ions, and they can bind to Zn, thus affecting its concentration in the sample (7). Studies have shown that these substances themselves or the blood collection tubes can be a source of Zn contamination. For instance, research has demonstrated that tubes containing EDTA, due to their chelating ability, can cause Zn to leach from rubber stoppers, artificially increasing plasma Zn concentration (8, 9). Likewise, in some commercial heparin preparations, Zn has been identified as an inherent contaminant (8). Consequently, the effect of these factors on the results underscores the importance of choosing the correct anticoagulant and controlling for contamination.

Another important point is the influence of measurement method on the final results. In most previous research, Zn measurement was performed using spectroscopic methods such as Flame Atomic Absorption Spectrometry (AAS) or Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (10) (11, 12). These techniques, by atomizing the sample at high temperatures, break the chemical bonds between Zn and chelating agents. For this reason, the effect of Zn chelation by anticoagulants might not be fully revealed by these methods, and they might provide a more accurate result for the total Zn concentration in the sample. For example, a study showed that the presence of chelating agents like EDTA does not affect the results in the AAS method (10).

Given these considerations, the current study aims to directly compare Zn concentrations in different samples (serum, plasma with EDTA, heparin, and citrate) using a routine colorimetric kit, which is commonly applied in clinical laboratories. In contrast to AAS methods, which break chelate bonds, our main hypothesis is that colorimetric kits are sensitive to the chemical form of Zn in the solution and will clearly demonstrate the interference caused by chelation from anticoagulants. The objective of this study is to compare the results obtained with this method to the results of previous studies, thereby clarifying the role of pre-analytical factors, particularly the type of anticoagulant and the analytical method, in the accuracy of Zn measurement under routine laboratory conditions and providing a new insight in this field.

Materials and Methods

Blood Sample Collection

This study was conducted in compliance with ethical principles (Ethical code: IR.TUMS.MEDICINE.REC.1402.008) and based on the ethical principles of the Declaration of Helsinki, commencing after obtaining informed consent from all participants. 20 volunteers (10 women and 10 men) with an age range of 35 to 55 years participated in this study from clinical laboratory of Yas Hospital complex, Tehran University of Medical Sciences, Tehran, Iran. Blood sampling was performed in a fasted state between 7 and 9 a.m. To prevent potential contamination, stainless steel needles and sterile equipment were used. Blood samples were immediately transferred to four types of blood collection tubes, which were: a serum tube (without anticoagulant), a plasma tube containing EDTA, a plasma tube containing heparin, and a plasma tube containing citrate. Exclusion criteria for the study included the presence of any chronic disease such as liver disease, kidney disease, or diabetes that affects Zn metabolism, the use of Zn, vitamin, or mineral supplements within one month prior to sampling, a history of any blood disorder or anemia, and blood samples that showed hemolysis.

Sample Preparation

All tubes containing anticoagulants (EDTA, heparin, and citrate) were centrifuged immediately after blood collection at 3000 rpm for 10 minutes to separate plasma

from the cells. This was done to prevent the effects of pre-analytical factors such as fluid shifts and the release of Zn from cells. To prepare serum samples, anticoagulant-free tubes were left at room temperature for 30 minutes to allow clotting process to complete. Subsequently, the tubes were centrifuged at 3000 rpm for 10 minutes. The plasma and serum samples were carefully separated and stored in polypropylene tubes until analysis. Samples were stored at -20°C until analysis. Any hemolyzed samples were carefully discarded to prevent a false increase in Zn levels.

Zinc Measurement Method

Zn concentration in all samples was measured using a colorimetric method kit from Delta Darman Part Company, Tehran,Iran. The basis of this method is the formation of a colored complex between Zn ions and chromogen 5-Bromo-2-pyridylazo-5′-(N-propyl-N-sulfopropylamino)-phenol (5-Br-PAPS) in an alkaline medium. The intensity of this colored complex is directly proportional to Zn concentration in the sample. The measurement was performed at a wavelength of 570 nm, and the results were read using an ELISA reader (BioTek, USA). The lowest and highest detection limits of this kit were 5.25 µg/dL and 500 µg/dL respectively. All samples were measured in duplicate to ensure the accuracy and repeatability of the results.

Statistical Analysis

Before analysis, the data were evaluated for normality of distribution using Shapiro-Wilk test. Since the data did not follow a normal distribution, non-parametric tests were used for statistical analysis. To compare Zn concentrations among four sample groups (serum, EDTA, heparin, citrate plasma), Kruskal-Wallis test, followed by Dunn's post-hoc test was used. Furthermore, to evaluate the correlation between Zn concentration in each of the plasma samples and serum sample, Spearman's rank correlation coefficient was used. A p-value of less than 0.05 ($P<0.05$) was considered statistically significant for all analyses.

Results

Measurement Method Validation

To confirm the accuracy and reliability of the measurement method, the performance of the colorimetric kit was evaluated by preparing a standard curve (Figure 1). The results showed that the kit has excellent linearity over a wide concentration range, from 0 to 300 mg/dL. All measured Zn concentrations in the present study fell within this linear range. This linearity across a broad range confirms the kit's ability to accurately measure various Zn concentrations. Furthermore, the correlation coefficient (R^2) of this standard curve was 0.99, indicating a strong and positive correlation between Zn concentration and the optical absorption signal. This high coefficient indicates acceptable linearity and reliable performance of the method for quantitative measurements in biological samples.

Zn concentration in serum and EDTA, citrate, and heparin plasma samples

Statistical analysis showed that Zn concentrations in EDTA- and citrate-treated plasma samples were significantly different from those measured in serum samples. The results of the multiple comparison tests indicated that Zn concentration in serum (89.58±9.17)

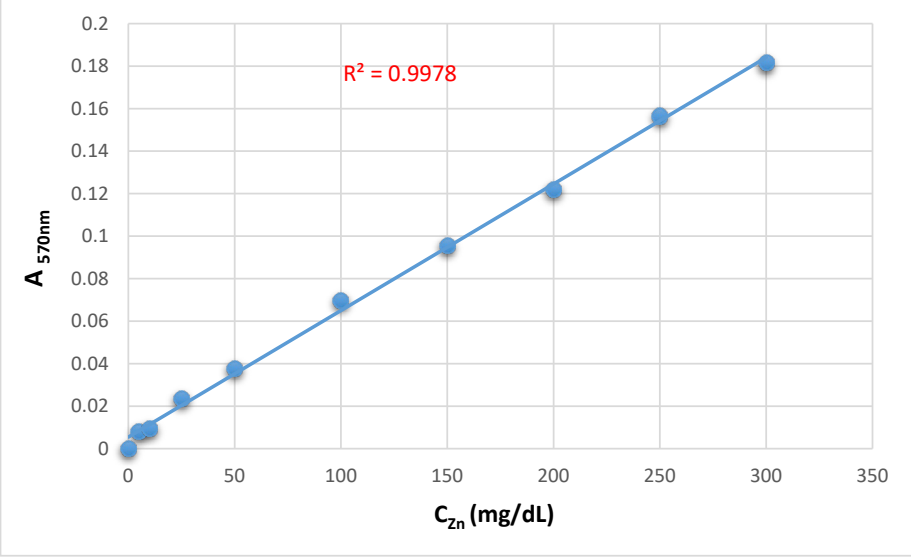

Figure 1. Standard curve for the colorimetric Zn assay. The plot demonstrates the excellent linearity of the method over a concentration range of 0 to 300 mg/dL, with a correlation coefficient of 0.99, confirming the reliability and accuracy of the kit for quantitative Zn measurements at 570 nm.

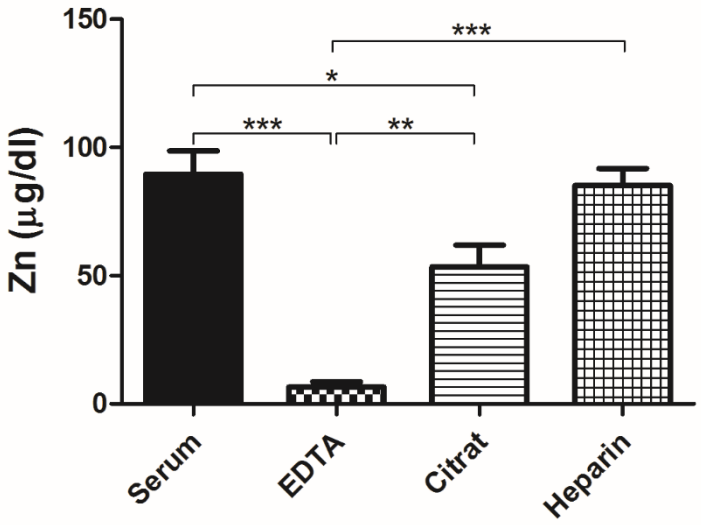

Figure 2. Results of Zn concentration in serum and EDTA, citrate, and heparin plasma samples. EDTA: Ethylenediaminetetraacetic acid. The Kruskal-Wallis test, followed by Dunn's post-hoc test, was used to analyze the data. A p-value of < 0.05 was considered statistically significant. P<0.05 (*), P<0.01 (**), and P<0.001 (***).

Table 1. Correlation Analysis between Zn levels in serum and EDTA, citrate and heparin plasma samples.

	EDTA	Citrate	Heparin
Serum	r =0.099	r=0.704	r=0.831
	P>0.05	P<0.01	P<0.001

Correlation Analysis. EDTA: Ethylenediaminetetraacetic acid. r: Spearman's rank correlation coefficient. A p-value of < 0.05 was considered statistically significant.

samples was significantly higher than in EDTA plasma (6.5±5.63, *P*< 0.001) and citrate plasma (55.88±11.01, *P*<0.001). In contrast, no statistically significant difference was observed between Zn concentration in serum and heparin plasma (85.20±6.61, P> 0.05). Based on these findings, the order of Zn concentration in the samples from highest to lowest was: serum > heparin > citrate > EDTA. These results are displayed in Figure 2.

Correlation Analysis

The correlation of Zn concentration between the serum and plasma samples was evaluated using Spearman's rank correlation coefficient. The correlation between Zn concentrations in serum and EDTA plasma was positive but weak, whereas strong positive correlations were observed with both heparin and citrate plasma, with the strongest association detected between serum and heparin samples (Table 1).

Discussion

Zn status is increasingly recognized as a clinically relevant indicator in a wide range of conditions, including immune dysregulation, metabolic disorders, inflammatory states, and nutritional deficiencies (13). As a result, serum or plasma Zn measurement is frequently requested in routine laboratory practice as well as in epidemiological and interventional studies. Unlike many biochemical analytes, circulating Zn exists in different chemical forms, and only a fraction is readily measurable depending on the analytical approach (14, 15). This makes Zn assessment particularly vulnerable to methodological and pre-analytical variations, including sample type, anticoagulant choice, and assay principle. Consequently, inaccurate Zn measurement may lead to misinterpretation of an individual's Zn status, underscoring the necessity of understanding how routine laboratory methods respond to common pre-analytical conditions.

This study was conducted with the aim of evaluating the effect of various common anticoagulants on Zn concentration in blood samples using a colorimetric method. The main findings of this research showed that Zn concentration in plasma samples containing chelating anticoagulants such as EDTA and citrate was significantly lower than in serum and heparin plasma samples. These results confirm the central hypothesis of this study regarding the interference of chelating anticoagulants with colorimetric measurement methods. Moreover, Spearman's correlation analysis confirmed that although absolute concentrations differ, there is a positive and strong correlation between Zn concentration in serum and heparin and citrate plasma, which indicates that the trend of Zn concentration changes among individuals follows a similar pattern regardless of the

sample type. The findings of this study are based on measurements obtained using a colorimetric method, indicating that this approach demonstrates high validity and accuracy. The prepared standard curve exhibited excellent linearity over a broad concentration range of 0 to 300 mg/dL, with a correlation coefficient of 0.99, confirming the suitability and precision of this method for quantitative measurements.

The significant reduction in Zn concentration observed in EDTA- and citrate-treated samples can be attributed to the strong chelating properties of these compounds. EDTA and citrate bind to free Zn ions, thereby reducing the amount of Zn available to react with the 5-Br-PAPS chromogen. Since the colorimetric method used in this study primarily measures free Zn ions, the formation of stable Zn–EDTA or Zn–citrate complexes results in decreased absorbance and, consequently, an apparent reduction in Zn concentration in these samples (7). In contrast, the absence of a statistically significant difference between Zn concentrations in serum and heparinized plasma indicates that heparin, unlike EDTA and citrate, lacks strong chelating activity toward divalent metal ions such as Zn and does not interfere with the availability of free Zn ions for the colorimetric reaction. As a result, Zn measurements in heparinized plasma are closer to those obtained in serum and can be considered analytically comparable (5).

In some previous studies in which zinc was measured using AAS, only minor or non-significant differences between Zn concentrations in serum and EDTA-anticoagulated plasma have been reported. For example, Jablan et al (10), observed only a small difference between these two sample types. This discrepancy in findings is not necessarily due to differences in Zn distribution between samples, but rather may be attributed to differences in the analytical principles of the measurement methods. In AAS, the high-temperature atomization process dissociates metal–ligand complexes, allowing the measurement of total Zn regardless of whether it is present in a free or chelated form(16). In contrast, the colorimetric method employed in the present study primarily reflects the concentration of free Zn ions; therefore, the presence of chelating anticoagulants such as EDTA and citrate can exert a more pronounced effect on the results obtained with this method.

Additionally, in the present study, correlation analysis demonstrated statistically significant and positive correlations between serum Zn concentrations and those measured in citrate- and heparin-anticoagulated plasma, whereas no significant correlation was observed between serum and EDTA plasma. In contrast, Jablan et al. (10), who assessed Zn concentrations using AAS, reported a significant correlation only between serum and citrate plasma, while correlations between serum and both heparin- and EDTA-anticoagulated plasma samples were not statistically significant. Despite these methodological differences, both studies consistently indicate that EDTA-anticoagulated plasma shows poor agreement with serum Zn concentrations, underscoring the critical impact of anticoagulant choice on Zn assessment.

One of the main limitations of this study is its small sample size. Although the results were statistically significant, to confirm the findings and ensure their generalizability to larger populations, it is essential to replicate this research with a wider sample size and consider demographic variables such as age and gender. Additionally, it is suggested that future studies investigate the effect of other pre-analytical factors, such as sample storage time and centrifugation temperature, on Zn measurement results using colorimetric kit.

In conclusion, the results of this study indicate that the choice of anticoagulant is a critical pre-analytical factor in Zn measurement using colorimetric methods. The use of anticoagulants like EDTA and citrate can spuriously lower Zn concentration, whereas heparin plasma provides results that are close to serum. These findings are of significant importance for clinical laboratories that use colorimetric kits for Zn measurement.

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Conflict of Interest

The authors declare that they have no conflicts of Interest.

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