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Abstract: The aim of this study was to compare zinc (Zn) level in EDTA, heparin, citrate plasma and serum. The blood samples from the same healthy volunteers (n = 10) were sampled by use of different anticoagulants (EDTA, heparin and citrate) or with no anticoagulant. Zn level was determined by flame atomic-absorption spectrometer (AAnalyst 800, PerkinElmer, USA). Zn level in EDTA, heparin, citrate plasma and serum was in reference range (0.7 to 1.5 μg mL^{-1}), and the difference between sample groups was not observed (P = 0.079). However, correlation was observed only between Zn level in heparin plasma and citrate plasma (r_s = 0.72; P = 0.016) and citrate plasma and serum (r_s = 0.76; P = 0.012). In conclusion, in Zn blood analysis, sample collection step is important and should be observed when planning study and when comparing the results of Zn level from various studies.

Keywords: anticoagulants, atomic-absorption spectrometer, serum, plasma, zinc.

INTRODUCTION

TRACE element zinc (Zn) is essential to all organisms. In human body Zn, as a component of more than 300 enzymes, participates in the synthesis and degradation of carbohydrates, lipids, proteins and nucleic acids, and thus is required for normal cell growth, division and integrity.[1,2] Zn requirements are the highest for infants (120–140 μg kg^{-1} b.w. / day) and adolescents (60–80 μg kg^{-1} b.w. / day) while adult requirement is lower (30–40 μg kg^{-1} b.w. / day).[1] The clinical manifestations of Zn deficiency include: growth retardation, delayed sexual and bone maturation, skin lesions, diarrhoea, alopecia, impaired appetite, defect of immune system and behavioural changes.[1] Development of some chronic diseases such as atherosclerosis, neurological disorders, autoimmune diseases, ageing and cancers are associated with Zn deficiency.[2] However, high concentration of Zn is toxic (signs of Zn toxicity: nausea, vomiting, diarrhoea and fever), so upper intake level of Zn for adults is set at 45 mg / day.[1] Therefore, monitoring Zn levels in human biological samples is important.

In order to associate the level of Zn with the development of specific human disease, numerous studies have monitored its level in biological fluids such as plasma, serum or urine.[3–5] However, in some studies, sample collection and sample preparation step is not clearly described.[6]

Due to their physiological functions, the levels of trace elements inside and outside the cell are different. Therefore, collection of plasma/serum sample is the most critical step for trace element analysis.[7] For example iron (Fe) and Zn levels in erythrocyte are 10-times higher than in plasma and haemolysis or micro-haemolysis can give false higher results of Fe and Zn in plasma. Also, anticoagulants can affect the level of trace elements in plasma since anticoagulants cause fluid shift (inside / outside cell). Additionally, the use of anticoagulants that chelate metals ions (such as EDTA and citrate) is limited. Thus for trace element analysis it is important to standardise the method of pre-analysis, i.e. sample collection step as well. However, due to multi-parameter analysis and study limitations, such as sample size, in some studies sample collection is neglected. Therefore, the aim of this study was to test various anticoagulants normally used in clinical laboratories (EDTA, heparin and citrate) and compare the level of Zn detected in obtained plasma and serum.

EXPERIMENTAL

Samples

Blood samples were collected from 10 healthy volunteers (Caucasians, aged 30–48 years, of both genders), from Zagreb (Croatia), all of which had an annual medical check-up at Clinical Hospital Centre "Sestre Milosrdnice", Zagreb, Croatia. The blood samples were collected in the morning (between 8.00 and 9.00 a.m.) from fasting subjects. The exclusion criteria were the use of vitamin and/or mineral supplements in the past month. From each volunteer venous blood was collected in sterile conditions in plastic (PET) blood collection tubes containing K_3EDTA, lithium heparin and trisodium citrate solution as anticoagulant (Greiner Bio-One, Kremsmünster, Austria) or in serum tubes (Greiner Bio-One, Kremsmünster, Austria) to obtain serum. To obtain plasma, tubes were immediately centrifuged (4000 rpm / 10 min), and to obtain serum, tubes were left to sit for 30 min, and then centrifuged (4000 rpm / 10 min). Plasma or serum was transferred to polyethylene Eppendorf tubes and stored at –20 °C until analysis. During sample collection procedure it was taken care that no Zn contamination occurs.

All the participants signed the informed consent form and approved their participation in the study. The study was approved by the Clinical Hospital Centre "Sestre Milosrdnice" Ethics Committee and conducted in accordance with the ethical principles of the Declaration of Helsinki.

Standard Preparation

Standard stock solution of Zn (1000 µg mL^{-1} in 2 % HNO_3) was procured from PerkinElmer (Waltham, MA, USA). Working standards in concentration range (0.05–1.0 µg mL^{-1}) were prepared fresh daily by dissolving stock solution in MilliQ water.

All glassware used for standard preparation was soaked in 15 % HNO_3 (*p.a.* Kemika, Zagreb, Croatia) overnight, and thoroughly rinsed with MilliQ water before use.

Equipment

Analysis of Zn level was performed on flame atomic-absorption spectrometer, FAAS (AAnalyst 800 PerkinElmer,

Table 1. Optimal conditions set of flame atomic-absorption spectrometer (FAAS) for Zn analysis in plasma and serum.

Type of flame / fuel	Acetylene / air
Wavelength	213.9 nm
Slit width	0.7 nm
Lamp current	15 mA

Waltham, MA, USA) with deuterium background corrector (DBC). The results were collected and processed by AAWinlab 32 Software (PerkinElmer, Waltham, MA, USA).

For Zn analysis FAAS was set as follows: hallow cathode lamp at 15 mA and λ = 213.9 nm; as oxidants acetylene / air was used (pressure / flow: 0.9×10^5 / 5; 5.5×10^5 / 17 for acetylene and air, respectively) (Table 1).

Sample Procedure

Sample procedure step was performed according to the standard method described by PerkinElmer[8] with minor modification. Plasma or serum samples were thawed and centrifuged (9000 rpm / 3 min) to remove debris that could clog FAAS. Afterwards, samples were diluted with MilliQ water (5×), homogenised and aspirated to FAAS. Concentration of Zn was calculated from calibration curve. Each sample was analysed in triplicate and results have been reported as a mean value. Plastic (polyethylene) tubes were used throughout sample preparation procedure.

Statistical Analysis

Data are presented as a median and interquartile range and, to be able to compare the results with other studies, as a mean ± standard deviation. The difference in Zn concentration in EDTA, heparin, citrate plasma and serum was tested with Kruskal-Wallis One-Way Analysis of Variance Ranks. To test for correlation, Spearman correlation was used. Probability values of $P < 0.05$ were considered statistically significant. For statistical analysis, Sigma Stat 3.0 was used.

RESULTS AND DISCUSSION

Selection of Analytical Technique

Nowadays, AAS (flame or graphite), inductively coupled plasma atomic emission spectroscopy (ICP-AES), inductively coupled plasma mass spectrometry (ICP-MS) or X-ray fluorescence spectrometry is used for the assessment of trace elements in biological samples. Analytical techniques, such as ICP-AES, ICP-MS or total reflection X-ray fluorescence (TXRF), allow multi-element analysis and have lower limit of detection (as low as ppq, 10^{15}), thus in comparison with AAS they need smaller sample volume, and have better sensitivity. Nevertheless, AAS is still used for trace elements analysis in the majority of clinical laboratories. AAS is robust, sample preparation is not time consuming, and handling of instrument is simple, making the analysis cost-effective.

Validation of the Method

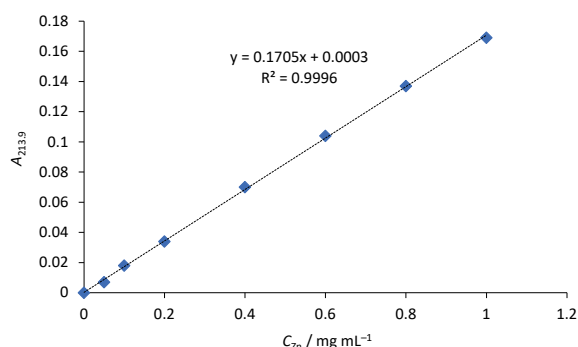The method validation parameters are presented in Table 2. The linearity of the method was tested by preparing

Figure 1. The calibration curve obtained from Zn standards in concentration range 0.05–1.0 µg mL⁻¹.

calibration curve from working Zn standards in MilliQ water in concentration range from 0.05 to 1.0 µg mL⁻¹. Due to the dilution of plasma / serum samples 0.05 µg mL⁻¹ standard was included in the calibration curve. The calibration curve was linear (correlation coefficient, R^2 = 0.9996; Figure 1). The sensitivity of the method was given as limit of detection (LOD) and limit of quantification (LOQ). LOD was calculated as 3.3 times standard deviation of the intercept divided with slope of calibration curve and it was 0.01 µg mL⁻¹. LOQ was calculated as 10 times standard deviation of the intercept divided by the slope of calibration curve and it was 0.04 µg mL⁻¹.

For precision (repeatability and reproducibility) experiments pooled serum sample was obtained. The pooled serum sample was prepared as described in Sample Procedure and aspired to the FAAS in ten replicates within the day. The calculated mean coefficient of variation (CV) for repeatability (intraday precision) was below 1.0 %. For reproducibility (inter day precision) the sample of pooled serum was aspired to the FAAS trough six consecutive days and calculated mean CV was 1.0 %. These data indicate high precision of the method.

For accuracy experiment a certificate reference material (CRF) Human Multi-Sera Level 3 (Randox, Crumlin, UK) was used. CRF was prepared as described in Sample Procedure, aspired to FAAS and analysed in triplicate. The calculated accuracy of the method was 101.06 %.

Table 2. Validation parameters of the method for determination of Zn level in plasma and serum by flame atomic-absorption spectrometer (FAAS).

Limit of detection, LOD / µg mL⁻¹	0.01
Limit of quantification, LOQ / µg mL⁻¹	0.04
Repeatability / %	0.72
Reproducibility / %	1.0
Accuracy / %	101.06

Influence of Anticoagulants on Zn Level Detected in Plasma

Several factors affect blood Zn level. Nutrition is one factor. After the meal immediate increase of serum Zn level is observed.[9] Also, diurnal variations in serum Zn level among fasted individuals is recorded.[9] Both these factors should be observed when collecting blood for Zn analysis. However, in trace element analysis the most critical step is sample collection.[7] This is especially important for Zn, since Zn level is higher in the cell than in extracellular fluid. Milne[7] stated that Zn level is 5–15 % higher in serum than in plasma because of the release of Zn from erythrocytes and platelets during clotting. Additionally, anticoagulants can cause osmotic fluid shifts from blood cells, thus leading to plasma dilution. Therefore, for Zn analysis, as biological sample, serum is preferred over plasma.

In this study several anticoagulants, commonly used in clinical laboratory, were selected and Zn level in obtained plasma was compared to Zn level in serum. Previous studies compared Zn level detected in plasma to Zn level detected in serum. However, in those studies either only one type of plasma sample (heparin plasma)[10] was used, or the type of plasma sample (type of anticoagulant used) was not stated.[11] More detailed study was done by Frank *et. al.*[12] however, citrate plasma was not included in that study. Therefore, in this study, the Zn level detected in EDTA, heparin and citrate plasma samples, was compared to the Zn level detected in serum samples. The obtained results are presented in Table 3. In this study, the Zn level detected in EDTA, heparin, citrate plasma or serum was within the accepted reference interval 0.7 to 1.5 µg mL⁻¹ (10.7 to 22.9 µmol L⁻¹)[7] and was similar to the one reported in previous studies for healthy (control) subjects. In the study by Wei-Jie *et. al.*[9] the Zn level in serum and heparin plasma of healthy individuals determined by FAAS was 0.87 ± 0.22 µg mL⁻¹ and 0.90 ± 0.24 µg mL⁻¹, respectively. Similarly, Prasad *et. al.*[4] reported serum Zn level of 12.71 ± 3.24 µmol L⁻¹ (equivalent to 0.83 ± 0.21 µg mL⁻¹; assessed by FAAS) in control subjects and Feng *et. al.*[3] reported serum Zn level of 1.06 µg mL⁻¹ (assessed by FAAS) in control subjects.

Statistical analysis revealed that Zn level in EDTA, heparin and citrate plasma and Zn level in serum did not differ (Table 3; P = 0.079). This is in agreement with previous observation of Wei-Jie *et. al.*[9] who demonstrated that there is no difference in Zn level detected in heparin plasma and Zn level detected in serum. Also, Makino and Takahara[10] observed no difference in Zn level between serum and plasma (in this study the type of plasma / anticoagulant used was not specified). On the other hand, in the study of Frank *et. al.*,[12] in which the Zn level in serum and several types of plasma samples were compared, significantly higher Zn level was detected in EDTA plasma.

Table 3. Zn level in EDTA, heparin, citrate plasma and serum.

	EDTA	heparin	citrate	serum	$P^{(a)}$
Median (interquartile range) / µg mL^{-1}	0.905 (0.811–0.949)	0.863 (0.780–1.040)	0.740 (0.690–0.805)	0.900 (0.794–0.911)	0.079
Mean ± SD$^{(b)}$ / µg mL^{-1}	0.88 ± 0.11	0. 89 ± 0.16	0.76 ± 0.09	0.88 ± 0.14	

[a] *P*-level of significance (Kruskal-Wallis One-Way Analysis of Variance Ranks).

[b] SD-standard deviation.

The higher level of Zn in EDTA plasma was related to the contamination of collection container / anticoagulant with Zn. The authors analysed EDTA in the collection containers and found that the Zn level in EDTA containers was 0.7 ± 0.4 µg mL^{-1}. Such high Zn concentration contributed to the higher level of Zn detected in EDTA plasma. In this study, all the used types of collection tubes were tested for the presence of Zn, and in collection tubes (with EDTA, heparin and citrate as anticoagulants and for serum) detected Zn level was below LOQ. In the study by Frank *et. al.*[12] glass collection tubes (producer was not specified) were used, whereas in this study collection tubes were made of PET plastic (as specified by the producer). It can thus be concluded that the blood collection tube production technique improved over time (2001–2018), and that no contamination occurs as a consequence. Although in this study no statistical difference in Zn level between EDTA, heparin, citrate plasma and serum was observed, the lowest Zn level was detected in citrate plasma. Compared to the other types of samples, Zn level detected in citrate plasma was up to 18 % lower. This could be due to reversible binding of calcium ions by citrate,[13] thus citrate except calcium ions is able to bind Zn leading to lower Zn level in citrate plasma.

Furthermore, results of Zn level in EDTA, heparin, citrate plasma or serum were tested for correlation. Correlation coefficients (r_s) are presented in Table 4. Correlation was observed only between Zn level in heparin plasma and citrate plasma (r_s = 0.72; *P* = 0.016) and citrate plasma and serum (r_s = 0.76; *P* = 0.012). Lack of correlation between the sample groups points to the importance of sample collection step in Zn blood analysis. When planning the study, it is important to set protocol for blood collection and that only one type of collection tube is used. Use of

collection tubes for trace element is recommended, but in the case of multi-parameter analysis, or lack of the sample, only one type of sample should be used. Also, the type of sample (serum/plasma) and anticoagulant should be considered when comparing results from different studies.

CONCLUSION

Since trace element Zn has numerous functions in the body and is associated with the development of human chronic diseases, numerous studies monitor its level in biological samples. This study confirms that FAAS is a simple, precise and robust analytical technique that gives reliable results for Zn level in plasma or in serum. Although this study found that there is no difference in the Zn level detected in plasma samples obtained by use of various anticoagulants and in serum, the lowest Zn level was detected in citrate plasma. Additionally, correlation was observed only between Zn level in citrate and heparin plasma samples and citrate plasma samples and serum samples. Therefore, collection of plasma/serum samples is an important step in Zn blood analysis and should not be neglected when planning the study and comparing results from different studies.

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Table 4. Spearman's correlations coefficients (r_s) and level of significance (*P*) between Zn levels in EDTA, heparin, citrate plasma and serum samples.

	Serum r_s; *P*	Citrate r_s; *P*	Heparin r_s; *P*
EDTA	0.60; 0.102	0.58; 0.087	0.65; 0.050
Serum	–	**0.76; 0.012**	0.54; 0.124
Citrate		–	**0.72; 0.016**

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