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Optimisation and validation of a method for determination of selenium in human plasma and blood by ETAAS and its clinical application

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Selenium is a part of the active center of glutathione peroxidase, thioredoxin reductase, and iodothyronine deiodinase which have antioxidant activity and play an important role in the metabolism of thyroid hormones. Therefore, blood selenium concentration is strongly correlated with immune function, thyroid disease, and tumourigenesis. In this work, a method for the determination of selenium in blood and plasma by electrothermal atomic absorption spectrometry (ETAAS) was optimized and validated. Samples were prepared by dilution in 0.2% detergent (Triton X-100, Tween 80, or SDS) in 0.1% HNO_3. The pyrolysis was performed at 1100ºC for 30 s for plasma and 35 s for blood, while atomization at 2500˚C for 3 s with a Pd matrix modifier. LOD and LOQ for plasma samples were 0.56-0.62 μg/L and 1.87-2.07 μg/L depending on the detergent used, while LOD and LOQ for blood samples were 0.64-0.72 μg/L and 2.12-2.41 μg/L, respectively. The validation results show a recovery of 98.25-102.65%. The precision of the method ranges from 1.55% to 2.63%. The method was applied for the determination of selenium in plasma and whole blood in healthy patients. The obtained selenium concentrations in plasma are in the range of 31.40–47.01 μg/L, while the concentrations in whole blood samples are higher by 23-25%.

Keywords: selenium; blood; plasma; electrothermal atomic absorption spectrometry

INTRODUCTION

Selenium in the form of selenocysteine is an essential component of the glutathione peroxidase enzymes (GPx) and thioredoxin reductase (TR). These enzymes protect tissues from oxidative damage by removing oxygen free radicals [1]. This micronutrient has been studied over the last two decades, and scientific reports have revealed its crucial role in biological processes, such as free radical catabolism, immune response, endocrine function and tumourigenesis [2].

Oxidative stress due to decreased glutathione peroxidase activity, as a result of low Se concentration or increased free radical damage has been proposed to be an important event in the pathogenesis of different diseases such as Alzheimer's disease, coronary heart disease, lung and prostate cancers [3]. Based on observational studies, several meta-analyses have summarized inverse associations between levels of selenium biomarkers and lung [4] and prostate [5] cancers. Also, there are several studies available in the literature which reported the associations between brain selenium levels and Alzheimer's disease [6]. A recent meta-analysis of 14 prospective studies found a modest but statistically significant inverse association between selenium levels and coronary heart disease [7].

On the other hand, being incorporated into iodothyronine deiodinases D1 and D2, selenium also plays an essential role in the metabolism of thyroid hormones [8]. Regarding thyroid pathology, selenium intake has been particularly associated with autoimmune disorders. The literature suggests that selenium supplementation of patients with autoimmune thyroiditis is associated with a reduction in antithyroperoxidase antibody levels, improved thyroid ultrasound features, and improved quality of life. Selenium supplementation in Graves' disease is associated with an improvement in quality of life and eye involvement, as well as a delayed progression of ocular disorders [9].

In plasma Se is incorporated in selenoprotein P which may serve as a transport protein for Se and facilitate whole body Se distribution [10]. Selenium levels in the body are dependent on the population's characteristics and its diet and geographical area [11]. Among all human tissues, the thyroid gland contains the largest concentrations of selenium. The main sources of selenium in the form of selenocysteine are mammalian meat, chicken, and fish. On the other hand, selenomethionine is found in vegetable sources such as pasta or rice, bread or cereals, garlic (*Allium sativum*), Indian mustard (*Brassica juncea*), canola (*Brassica napus*), and some mushrooms [12].

Understanding of selenium biochemistry and its importance resulted in rapid growth in the number

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Consequently, there is a need for methods that are rapid, accurate, and require only a small amount of sample. Methods based on direct analysis by electrothermal atomic absorption spectrometry (ETAAS) [13], hydride generation atomic absorption spectroscopy (HG-AAS) [14], and inductively coupled plasma - mass spectrometry (ICP-MS) [15], are widely used. ETAAS is the most useful spectrometric method for mono-elemental analysis due to its high selectivity, low quantification limit, and acceptable costs [16]. Most of the matrix is destroyed during the ashing step and thus sample pre-treatment can be simplified (simple sample dilution), thereby minimizing contamination risk or analyte loss. Also, low injection volume (10-20 μL) is used, which is important for the analysis of biological samples, such as blood or blood plasma [17]. The analytical problems associated with the determination of selenium in the blood can be divided into three major classes: spectral interferences, chemical interferences, and thermal pre-atomisation losses. Spectral interferences are generally minimized if higher ashing temperatures can be achieved. Optimization of this step is crucial for the method's accuracy, because of the organic matrix complexity. Many modifiers have been tested to improve the thermal stabilization of selenium. The most commonly used modifiers are: copper [18], nickel [19], palladium [20], platinum [21], silver [22], iridium and mixtures of some of them [23]. Palladium is one of the most suitable and commonly used modifiers in the determination of selenium in the blood. It provides better sensitivity compared to other modifiers, does not cause contamination of the graphite tube, and palladium is not determined for clinical purposes, unlike copper and nickel.

Spectral interference in the determination of selenium can occur as a result of high concentrations of chromium, cobalt, iron, nickel, and phosphorus because of spectral lines around 196.0 nm, where the selenium signal is measured. However, the concentrations of chromium, cobalt, and nickel in the blood are very low and errors from them can be eliminated when Zeeman background correction is used [16].

Usually, the biological sample preparation involves treatment of the blood or serum with nitric acid and Triton X-100 as a detergent, which provides lysis of cell membranes. However, there are no reports in the scientific literature on sample preparation with different types of detergents and the influence of their concentration. The purpose of this study was to define optimal sample preparation procedures, instrument parameters, and calibration procedures for direct ETAAS determination of selenium in plasma and whole blood. The proposed method was applied to the determination of selenium in the plasma and whole blood of healthy individuals to obtain blood test results that include complete blood count (CBC), biochemical parameters, and thyroid status.

METHODS

Instrumentation

A Varian SpectrAA 640Z Zeeman electrothermal atomic absorption spectrometer equipped with a GTA-100 graphite furnace (Varian, USA) and PSD-100 autosampler (Varian, USA) was used. Pyrolytically coated tubes were used as atomizers. A Varian selenium hollow cathode lamp was used and the measurements were performed at 196.0 nm. Argon was applied as a protective gas and 10 μL samples were injected into the graphite furnace (GF). The graphite furnace operating parameters are presented in Table 1.

Table 1. Optimal parameters for Se determination by Zeeman ETAAS.

Parameter	Se
Wavelength	196.0 nm
Lamp current	10.0 mA
Calibration mode	Absorbance, peak height
Background correction	Zeeman
Drying	
Temperature	85; 95; 120°C
Time	5; 40; 10 s
Pyrolysis	
Plasma	
Temperature	1100°C
Ramp time	5 s
Hold time	30 s
Blood	
Temperature	1100°C
Ramp time	5 s
Hold time	35 s
Atomization	
Temperature	2500°C
Ramp time	0 s
Hold time	3 s
Cleaning	
Temperature	2500°C
Time	2 s
Gas	Argon

Only integrated absorbance values (peak height) were used for quantification.

Reagents

All reagents and standards were of analytical grade. Stock standard solutions for selenium were 1000 μg/mL Solution Plus Inc. (USA). The working

standard solutions were prepared weekly by appropriate dilution and kept refrigerated at 4°C. The palladium matrix modifier solution (500 ppm) was prepared by the dilution of 10 g/L $Pd(NO_3)_2$ (Merck, Darmstadt, Germany) in 20% HCl (*V*/*V*) (Merck, Darmstadt, Germany). Sample diluting agents (nitric acid with a concentration in the range of 0.005-0.2% (*V*/*V*) and corresponding detergent with concentrations from 0.05 to 0.3% (*V*/*V*) were prepared by dilution of pure concentrated nitric acid (65% *V*/*V*) (Merck, Darmstadt, Germany) and Triton X-100, Tween 80, sodium dodecyl sulphate, digitonin and tauroglycocholic acid (Merck, Darmstadt, Germany) were used. Doubly distilled water with a conductivity of 0.3 μS/cm was used in all operations. All disposable devices were rigorously cleaned before use by brief immersion in hot concentrated nitric acid, cleaned with tap water and detergent, and rinsed twice with doubly distilled water.

Study design

The study group consisted of 57 apparently healthy volunteers, 31 women, and 26 men, with an average age of 44 years. All study participants signed an agreement for selenium testing of their blood. Clinical experiments were approved and performed according to the Ethics Committee provisions of the Department of Nuclear Medicine at Clinical Hospital „Dr. Trifun Panovski“ in Bitola (North Macedonia).

All volunteers were asymptomatic, did not have a history of chronic disease, and had normal or only trivial clinical findings. Clinical findings were considered to be trivial if there were no associated symptoms, if they were not caused by a potentially important medical illness, and if they did not induce any diagnostic or therapeutic activity (an old surgical scar or mild obesity would qualify as such a finding).

Health status was also checked by a blood test which included a complete blood count (CBC), different biochemical parameters, and thyroid status. The CBC parameters included white blood cell count, leukocyte differential counts of neutrophil, lymphocyte, monocyte, eosinophil, basophil, red blood cell count, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, platelets, and red blood cell distribution width. The biochemical parameters determined were glucose, aspartate aminotransferase, alanine aminotransferase, serum creatinine, blood urea nitrogen, low-density lipoproteins, high-density lipoproteins, and triglycerides. The thyroid parameters included free triiodothyronine, free thyroxine, thyroid-stimulating hormone, antithyroglobulin antibody, and thyroperoxidase antibody. The inclusion criteria were values in the reference ranges for all examined parameters in the blood test.

The exclusion criteria were individuals with a personal history of chronic diseases or any disease, low or high values for some examined parameters in a blood test, and individuals treated with selenium as a supplement.

Procedures

The blood samples were collected with plastic i.v. cannula with an injection valve and sodium citrate as an anticoagulant. Whole blood samples (500 μL) were diluted 1+2 with a sample diluting agent in a centrifuge tube. The diluted sample was vortexed vigorously for 60 s to produce a lysis of the blood cells. The samples were then centrifuged at 3000 rpm for 5 min. For selenium determination, 10 μL of clear red supernatant were introduced into the graphite furnace with a 5 μL solution of palladium modifier.

Plasma samples were obtained by centrifugation of blood for at least 15 minutes at 2500 rpm. After centrifugation, 500 μL of the clear supernatant were diluted 1+2 with a sample diluting agent and 10 μL were introduced into the graphite furnace with a 5 μL solution of palladium modifier.

The selenium concentration was determined by triple injections from each cup (57 volunteers, n=171) into the graphite furnace, operated under the conditions given in Table 1.

RESULTS AND DISCUSSION

Sample pre-treatment

The ability to tune a particular detergent for complete lysis of cell membranes and isolation of selenium-containing proteins is a major goal in the determination of total selenium in the blood. In order to investigate the influence of the different types of detergent (anionic, cationic, and nonionic) and their concentration on the determination of the total selenium concentration in blood and plasma, a series of experiments (with 3 replicates from each of 57 blood samples) were performed applying the instrumental parameters given in Table 1. In this study Triton X-100, Tween 80, sodium dodecyl sulfate (SDS), digitonin, tauroglycocholic acid, and urea, were used (RSD from 2.6% to 9.1%). As can be seen (Figure 1) the best results are obtained by using Triton X-100, Tween 80, and sodium dodecyl sulfate (SDS). Although the highest values of absorbance are obtained by using SDS, the difference in the absorbance obtained with these three detergents is not significant. Therefore, it can be concluded that these three detergents give satisfactory results in determining of total selenium concentration in blood and plasma.

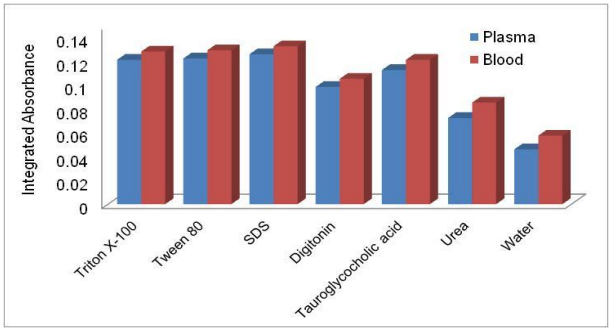

Figure 1. The influence of the different types of detergent on the determination of total selenium concentration in blood and plasma.

The efficiency of detergents is probably due to their structure. Digitonin and tauroglycocholic acid have hydrophobic tails with a steroidal structure, while Triton X-100, Tween 80, and SDS have a long alkyl chain. On the other hand, Triton X-100, Tween 80 and SDS have less hydrophilic heads compared to digitonin and tauroglycocholic acid. Specifically, the larger the detergent head, the lower is the detergent's propensity to break up lipid–lipid and protein-lipid interactions in biological membranes [24]. Also, it can be concluded that long alkyl chain detergents are more effective than detergents with steroid cores as hydrophobic tails [25].

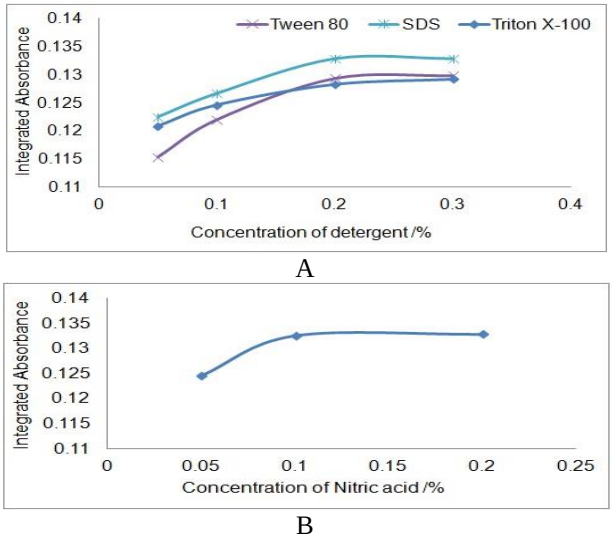

Figure 2. The influence of concentration of detergent (A) and nitric acid (B) on the integrated absorbance of selenium in the blood.

Furthermore, the influence of concentration of detergent and nitric acid on the measurement of selenium in blood was investigated (n=3). As can be seen from the results presented in Figure 2 the highest results were obtained by using 0.2% detergent solution and 0.1% nitric acid solution (RSD in the range of 1.9-6.8%).

Optimisation of instrumental parameters for ETAAS measurements

Optimization of the temperature program for Se determination by electrothermal atomic absorption spectrometry in human plasma and blood samples was performed. According to our previous results, parameters of the drying step were selected to allow the sample drop to dry slowly without sputtering [26]. Optimization of ashing temperatures was done by construction of pyrolysis–atomization curves from blood and plasma samples in the presence of palladium (500 ppm) modifier for thermal stabilization in ETAAS. The modifier was applied through the autosampler directly into the graphite furnace with a volume of 5 µL for 10 µL plasma or blood sample.

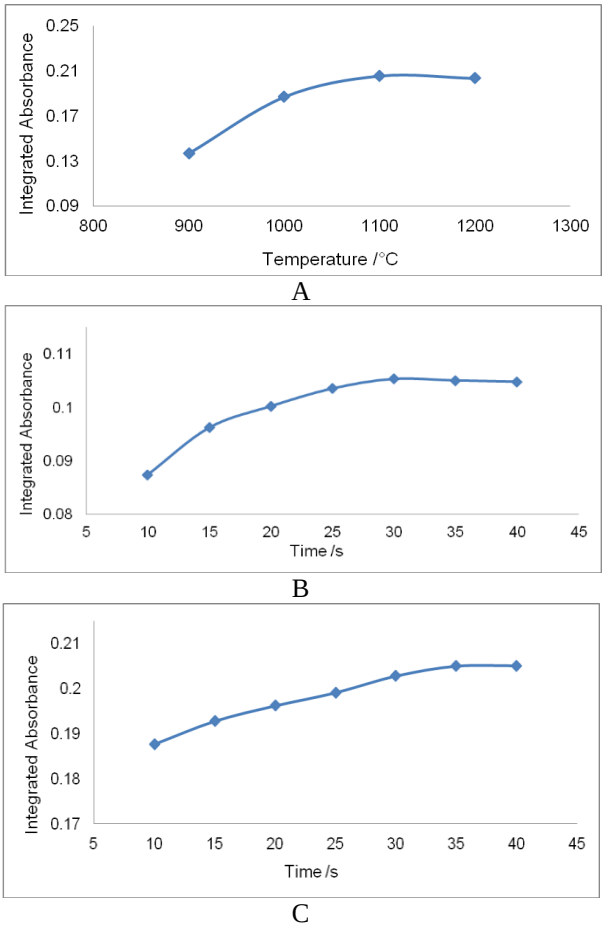

Figure 3. Effects of pyrolysis temperatures (A) and times on integrated absorbance for plasma (B) and blood (C) samples with Pd modifier.

Ashing temperatures (from 900ºC to 1200ºC) and ashing time (from 10 s to 40 s) were carefully optimized to ensure complete matrix decomposition and removal during this step by using wall atomization (pyrolytically graphite coated graphite tubes). Effects of pyrolysis temperatures and times on integrated absorbance for plasma and blood

samples with a Pd modifier are given in Figure 3 (n=3; RSD from 1.7% to 5.3%).

The optimal pyrolysis temperature was found to be 1100ºC, while the optimal pyrolysis ramp time was established to be 5 s, and the hold time was 30 s for plasma and 35 s for blood samples. The complexity of the blood sample matrix affects the ashing time and the determination of total selenium. The atomization was performed at 2500ºC by using a Pd modifier. The atomization ramp time was 1 s and hold time 3 s.

Calibration

Matrix interferences were evaluated according to the slopes of calibration curves obtained in the presence of the matrix (serum or blood), and for aqueous standard solutions. Results obtained are shown in Table 2 and clearly illustrate how strongly serum and blood matrices affected the degree of atomization of Se.

Table 2. Ratio of the slopes (b_m/b_o) for calibration curves in the presence of matrix (b_m) and aqueous standards (b_o), n=3.

Sample	b_m/b_o (mean±s)
Plasma	0.76 ± 0.11
Plasma + Triton X-100	0.96±0.02
Plasma + Tween 80	0.97±0.01
Plasma + SDS	0.95±0.02
Blood	0.63 ± 0.13
Blood + Triton X-100	0.95±0.02
Blood + Tween 80	0.94±0.03
Blood + SDS	0.94±0.02

The ratios of the slopes of the calibration graphs obtained in the presence of detergent (Triton X-100, Tween 80, or SDS) and Pd modifier are in the range of 0.94–0.97. These results confirm the capability of this detergent and modifier to reduce the spectral interferences and high background absorption values encountered with serum or blood matrices and at the same time to improve the degree of atomization of Se. Therefore, with detergent (Triton X-100, Tween 80, or SDS) and Pd modifier, calibration could be performed against a calibration curve prepared with an aqueous standard solution of Se.

Method validation

To test the accuracy of the proposed procedure for direct ETAAS determination of selenium in human plasma and blood, recovery experiments were performed. Plasma and blood samples were spiked with Se in the concentration range of 5–60 µg/L. Recovery experiments performed with human plasma and blood samples in 0.1% nitric acid and 0.2% detergent (Triton X-100, Tween 80, or SDS) mixtures spiked with a known amount of selenium standard solution have shown 98.25–102.65 % yield, which is within the acceptable limits for the accuracy of the analytical methods (95-105%) [26]. The obtained results of recovery are presented in Table 3 (*n*=3, RSD 2.3-9.7%).

The limit of detection (LOD) and limit of quantification (LOQ) were evaluated based on three repeated analyses of blanks. LOD and LOQ were calculated as the average Se level in the blank plus 3 times and 10 times the standard deviation of the blank, respectively. For plasma samples the LOD was in the range of 0.56-0.62 µg/L and LOQ was in the range of 1.87-2.07 µg/L depending on the different detergents used. LOD and LOQ for blood samples were in the range of 0.64-0.72 µg/L and 2.12-2.41 µg/L, respectively. The obtained results are shown in Table 4. The linearity range was 0.56–80 µg/L Se. The precision of the methods was evaluated and the percent relative standard deviation (% RSD) was found to be from 1.55 % to 2.63 %. The obtained % RSD did not exceed 15% for all studied concentrations. These values for precision fell well within the criteria normally accepted in bioanalytical method validation.

Analytical application

The method was applied for the determination of Se in plasma and whole blood samples of 57 healthy volunteers (31 females and 26 male) with an average age of 44 years. The results obtained revealed that the selenium content varied in the range of 31.40–47.01 µg/L. No significant difference was revealed (below 11 %) between women 38.84 ± 4.23 (31.40-47.01) µg/L and men 40.43 ± 3.62 (34.02-46.70) µg/L. The concentration of selenium in whole blood was higher by 23-25% in all cases (49.73–58.28 µg/L) compared to the determined concentration of selenium in plasma as a result of the distribution of selenoproteins in plasma and blood cells. In healthy humans, plasma Se is incorporated in two selenoproteins: selenoprotein P (SePP1) 40–70%, glutathione peroxidase (GPx3) 20–40%, while 6–10% of Se is bound to albumin in the form of selenomethionine, through the replacement of methionine. Free Se accounts for less than 1% of total plasma Se [27]. On the other hand, selenium in blood cells is incorporated in erythrocyte glutathione peroxidase. Plasma Se, very often used in various Se investigations, reflects rather short-term Se status, while platelet, leukocyte, and erythrocyte Se reflect its longer-term status. Our data reveal that the plasma selenium levels of healthy people in Macedonia are

among the lowest in Europe, but comparable with those in the Balkan region as shown in Table 5. These results are in agreement with previous reports for this biogeochemical region by Maksimović [28]: 35±7 µg/L and Čuparigova and Stafilov [26], 44.47±5.13 µg/L for selenium in blood plasma.

In this study, the obtained selenium plasma level was in a relatively narrow range of values compared to the results of previous reports. These differences are probably due to including criteria for chosen healthy individuals based on blood test results which include complete blood count (CBC), biochemical parameters and thyroid status

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Table 3. Recovery results of plasma and blood samples.

Triton X-100					
Plasma			Blood		
Added (µg/L)	Found (µg/L)	Recovery (%)	Added (µg/L)	Found (µg/L)	Recovery (%)
0	31.51	-	0	39.09	-
10	41.48	99.70	10	49.16	100.7
20	51.67	100.80	20	59.62	102.65
30	60.92	98.03	30	68.85	99.20
Tween 80					
Plasma			Blood		
Added (µg/L)	Found (µg/L)	Recovery (%)	Added (µg/L)	Found (µg/L)	Recovery (%)
0	32.4	-	0	40.29	-
10	42.25	98.50	10	50.54	102.5
20	52.12	98.60	20	59.94	98.25
30	62.51	100.37	30	69.87	98.6
SDS					
Plasma			Blood		
Added (µg/L)	Found (µg/L)	Recovery (%)	Added (µg/L)	Found (µg/L)	Recovery (%)
0	33.38	-	0	41.46	-
10	43.64	102.6	10	51.39	99.3
20	53.44	100.3	20	61.51	100.25
30	63.23	99.5	30	71.85	101.30

Table 4. Statistical parameters of the calibration curve.

Detergent	Triton X-100		Tween 80		SDS	
Sample	Plasma	Blood	Plasma	Blood	Plasma	Blood
LOD µg/L	0.58	0.72	0.62	0.69	0.56	0.64
LOQ µg/L	1.92	2.41	2.07	2.30	1.87	2.12
Number of replicates	5	5	5	5	5	5
Calibration range µg/L	5–60	5–60	5–60	5–60	5–60	5–60

Table 5. Plasma selenium levels of healthy people in some countries in Europe.

Country	Plasma selenium level, µg/L	Reference
Croatia	69±17	Becker et al. 1992 [29]
Bulgaria	66.5±15.5	Tsalev et al. 2001 [30]
Bosnia-Herzegovina	64±19	Maksimović et al. 1991 [28]
Greece	63±14	Thorling et al.1986 [31]
Montenegro	51±26	Maksimović et al. 1991 [28]
Hungary	50±11	Cser et al. 1996 [32]
Czech Republic	46±14	Kvičala et al. 1995 [33]
Serbia	41±20	Maksimović et al. 1991 [28]

CONCLUSION

The method for total selenium determination in human whole blood and plasma by ETAAS was optimized. The proposed method is simple, rapid, accurate and suitable for routine clinical analysis. The samples of blood and plasma were prepared by their dilution at a ratio of 1+2 with 0.2% detergent solution (Triton X-100, Tween 80, or SDS) and 0.1% nitric acid solution. It was found that the Pd modifier should be applied with the optimal pyrolysis temperature of 1100ºC for 30 s for plasma and 35 s for blood and an optimal atomizing temperature of 2500ºC. The method was applied for the determination of selenium in plasma and whole blood in healthy individuals. Health status was estimated by a blood test which includes complete blood count (CBC), biochemical parameters, and thyroid status. The obtained data for the selenium concentrations in plasma were in the range of 31.40–47.01 μg/L, which is a relatively narrow range of values compared to the results of previous reports. The concentrations of selenium in whole blood were higher by 23-25% in all samples due to the distribution of selenoproteins in plasma and blood cells.

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