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Speciation of selenium (IV, VI) in urine and serum of thyroid patients by ultrasound-assisted dispersive liquid-liquid microextraction

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ABSTRACT

In-vitro speciation of inorganic selenium (Se^{IV} and Se^{VI}) in serum blood and urine of hyperthyroidism and hypothyroidism patients based on isopropyl 2-[(isopropoxycarbothiolyl) disulfanyl] ethane thioate (IICDET) as a complexing agent were studied by ultrasound-assisted dispersive liquid-liquid bio-microextraction procedure (USA-DLLMBE). In first stage, 100 µL (≈0.1 g) of hydrophobic ionic liquid of $[C_8MIM][PF_6]$ mixed with IICDET ligand and 100 µL of acetone. Then, the mixture injected to 10 mL of human samples at pH=4. After shacking, the Se (IV) ions were complexed by IICDET and extracted to IL at pH=4 (R-S: …Se). The IL phase was separated from sample by centrifuging and inorganic selenium (Se_{IV}) in remained samples was determined by electro thermal atomic absorption spectrometry (ET-AAS) after back extraction of Se (IV). As speciation, the Se (VI) reduced to Se (IV) in acidic pH (HCl, 130^{O}C) and the total Se(T-Se) was obtained at pH=4. Therefore, the Se (VI) was calculated by difference of T-Se and Se (IV). After optimized conditions, the enrichment factor (EF), Linear range and limit of detection (LOD) for inorganic Se (IV) were obtained 20.1, 0.75- 20 µg L^{-1} and 0.18 µg L^{-1} in serum and urine samples respectively. The results showed us, the concentration of selenium was decreased in thyroid patients as compared to healthy peoples. The validation of methodology was achieved by certified reference material (CRM) and ICP-MS.

1. Introduction

Selenium is an essential trace element in humans. The soluble selenium compounds can be easily absorbed through the lungs and the gastrointestinal tract. Selenium is mainly excreted in human urine [1]. When the exposure is very high it can also be excreted in exhaled air as dimethylselenide vapor (DMSe). Normal selenium concentrations in serum and urine are dependent on daily intake, which may vary considerably in different parts of the world but are usually below 15 µg per 100 mL^{-1} and 25 µg g^{-1} creatinine, respectively [2-5]. The concentration of selenium in urine is mainly a reflection of recent exposure. The relationship between the intensity of exposure and selenium concentration in urine has not been established yet. It seems that the concentration in plasma (or serum) and urine mainly reflects to short-term exposure, whereas the selenium content of erythrocytes reflects more long-term exposure

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[6]. Measuring selenium in blood or urine gives some information on selenium status. Currently it is more often used to detect a deficiency rather than an overexposure. Since the available data concerning the health risk of long-term exposure to selenium and the relationship between potential health risk and levels in biological media are too limited. So, the biological threshold value for Se wasn't reported [7]. The thyroid is the organ with the highest selenium content per gram of tissue because it expresses specific selenoproteins. The value of selenium supplementation in autoimmune thyroid disorders has been emphasized. Most authors attribute the effect of supplementation on the immune system to the regulation of the production of reactive oxygen species and their metabolites [8-10]. The mechanism and role of selenium in inflammation, immunity and hepatocytes was reported [11, 12] In patients with Hashimoto's disease and in pregnant women with anti-TPO antibodies, selenium supplementation decreases anti-thyroid antibody levels and improves the ultrasound structure of the thyroid gland [13]. Although clinical applications still need to be defined for Hashimoto's disease, they are very interesting for pregnant women given that supplementation significantly decreases the percentage of postpartum thyroiditis and definitive hypothyroidism. In Graves' disease, selenium supplementation results in euthyroidism being achieved more rapidly and appears to have a beneficial effect on mild inflammatory orbitopathy [14]. A risk of diabetes has been reported following long-term selenium supplementation, but few data are available on the side effects associated with such supplementation and further studies are required. One of the diseases that affect the thyroid gland is subclinical hypothyroidism, which is characterized by elevated serum levels of thyroid-stimulating hormone (TSH) at a concentration recommended for prohormone thyroxine (T4) and active hormone triiodothyronine (T3). The decompensated levels of thyroid hormones may contribute to atherosclerotic events and an increase in cardiovascular-related mortality [15]. Also, observational longitudinal studies have shown an inverse association between selenium exposure and risk of some cancer types but still to be confirmed [16]. It is estimated that subclinical hypothyroidism affects 3–8% of the general population and is more common in women than in men. In Brazil, an epidemiological study in elderly reported that prevalence of subclinical hypothyroidism was 6.5%. The thyroid gland contains high levels of selenium (Se) and expresses a variety of selenoproteins that are involved in protection of oxidative stress and metabolism of thyroid hormones (TH) [17]. Selenium deficiency impairs regular synthesis of selenoproteins and adequate TH metabolism. Therefore selenium species in serum and urine must be evaluated and determined by favorite techniques. The different methods such as, flame atomic absorption spectrometry [18], electrothermal atomic absorption spectrometry [19], liquid chromatography and liquid chromatography inductively coupled plasma mass spectrometry (LC and LC-ICP- MS) [20-22] and high-performance liquid chromatography coupled to hydride generation atomic fluorescence spectrometry [23,24] were used for Se determination in different human and water samples. A sample preparation is required to extract metals ions in different biological samples. The sample preparation such as microextraction techniques [25], suspended dispersive solid phase microextraction [26], ultrasonic assisted dispersive liquid-liquid microextraction method [27] and ultrasound assisted-ionic liquid-solid phase microextraction [28] were used for extraction metals in human samples. In this study, the mixture of hydrophobic ionic liquid of $[C_8MIM][PF_6]$, IICDET ligand and acetone was used for selenium speciation /extraction based on ultrasound-assisted dispersive liquid-liquid bio-microextraction procedure (USA-DLLMBE) and determined by ET-AAS. The Se (IV) ions were complexed by IICDET and extracted to IL at pH=4. Then speciation of Se was obtained by total determination of selenium. Validation methodology was confirmed by spiking of standard samples and ICP-MS.

2. Experimental

2.1. Instrument and Reagents

The electrothermal atomic absorption spectrophotometer (ET-AAS, GBC932plus, Australia)

equipped with a graphite furnace (Pal GF3000) were used for the validation and determination of selenium (Se) in samples (Wavelength 349.9 nm; slit 0.2 nm; current 10 mA). The working range as peak area and height was obtained 15- 400 µg L^{-1} and 15-210 µg L^{-1}, respectively. The linear range was achieved for peak area of 0.3 Abs for sample injection. Based on the manual book of ET-AAS, the Se determination was achieved by injecting 20 µL of sample to graphite tube with auto-sampler in three steps of drying, ashing, and atomization for Se. The ICP-MS (Perkin Elmer, USA) as ultra-trace analysis with high sensitivity was used for determining of Se(IV) and Se(VI) in human blood and water samples (1100 W; 15 L min^{-1}; 1.5 sec per mass; auxiliary gas 1.12 L min^{-1}). The Metrohm pH meter based on the glass electrode was used for measuring pH in serum, urine and blood samples (E-744, Switzerland). The vortex mixer were used for shaking of human samples based on 300 rpm speeds and centrifuged by Falcon accessory by 4000 rpm speeds (Thermo, USA). An ultrasonic bath was used for blood and urine samples with heat controller between 30- 120°C (Thomas, USA). The standard solution of Se (VI, VI) was purchased from Merck CO. (Germany) with a concentration of 1000 mg L^{-1} in 1 % HNO_3. The different concentration of Selenium was prepared by dilution of deionized water (DW) and ultrapure water was purchased from Millipore Company. The 1-Hexyl-3-methylimidazolium hexafluorophosphate as hydrophobic ionic liquid was purchased from Sigma Aldrich ([HMIM][PF_6], CAS N: 304680-35). Isopropyl 2-[(isopropoxycarbothioyl) disulfanyl] ethane thioate was synthesized and purified by Azad university laboratories (IICDET; $(CH3)_4$ $(CO)_2$ S_4). The acetate (CH_3COOH/ CH_3COONa) and phosphate buffer was used to adjust the pH between 2.8–6.2 and 6.2–8.2, respectively. The analytical grade of reagents such as polyoxyethylene octyl phenyl ether (TX-100) as the anti-sticking agent, HNO_3, HCl, acetone, and ethanol were purchased from Sigma Aldrich, Germany.

2.2. Preparation of human samples

All glass or PCV tubes were cleaned with a 1.0 mol L^{-1} HNO_3 solution for at least one day and then washed for ten times with ultrapure water. As low concentrations of Se(IV) and Se(VI) in human serum, blood and urine samples, the cations or anions contamination at any stage of sample preparation, saving and analytical processes can be affected on the results accuracy. Heparin was used as anticoagulants for human blood samples into Eppendorf (5 mL) tubes and kept at -20°C for two weeks. Each blood samples were prepared by 10 µL of pure heparin (free Se) to blood sample. The serum, blood and urine samples were collected from hypothyroidism patients (50) and healthy peoples (50) with aged between 25 - 60 years, Tehran (IRAN). In this study, the world medical association declaration of Helsinki (WMADH) based on guiding physicians in human body research was considered by project of Azad university (AZAD UN. SPN: 960250673). The human samples were prepared based on WMADH law and absolutely protected the life and health of the human subject.

2.3. Synthesis of IICDET ligand

The 2.0×10^{-3} mol of potassium O-isopropyl (ditiocarbomate) was dissolved in 20 mL of DW and cooled in an ice bath for 10 minutes. The 2.0×10^{-3} mol of iodine solution and potassium iodide as drop wise was added to 20 ml of DW. After stirring of mixture for 1h, the aqueous phase was extracted with CH_2Cl_2 and washed with 30 mL of aqueous $Na_2S_2O_3$(10%) and DW. The organic phase was dried and evaporated with powder anhydrous calcium chloride Ca Cl_2 (99.99%; CAS Number: 10043-52-4). The purification was obtained by recrystallization in hexane. A pale yellowish crystal of isopropyl 2-[(isopropoxycarbothiolyl) disulfanyl] ethane thioate with a yield of 95% was achieved. The structure and isopropyl 2-[(isopropoxycarbothiolyl) disulfanyl] ethane thioate were confirmed by NMR spectroscopic methods (Fig. 1). ^{1}H NMR ($CDCl_3$). δ (ppm): 1.43 (d, 12H, CH_3), 5.63 (m, 2H, CH). ^{13}C NMR ($CDCl_3$). δ (ppm): 22.2, 80.6, 207.1. IR (KBr). νmax (cm−1): 2979.8 (s), 2869.9 (w), 1463.9 (s), 1442.7 (s), 1373.0 (s), 1271.1 (s, b), 1145.6 (s), 1082.2 (s), 1048.0 (s, b) 898.8 (s), 796. 5 (s), 690. 5 (m).

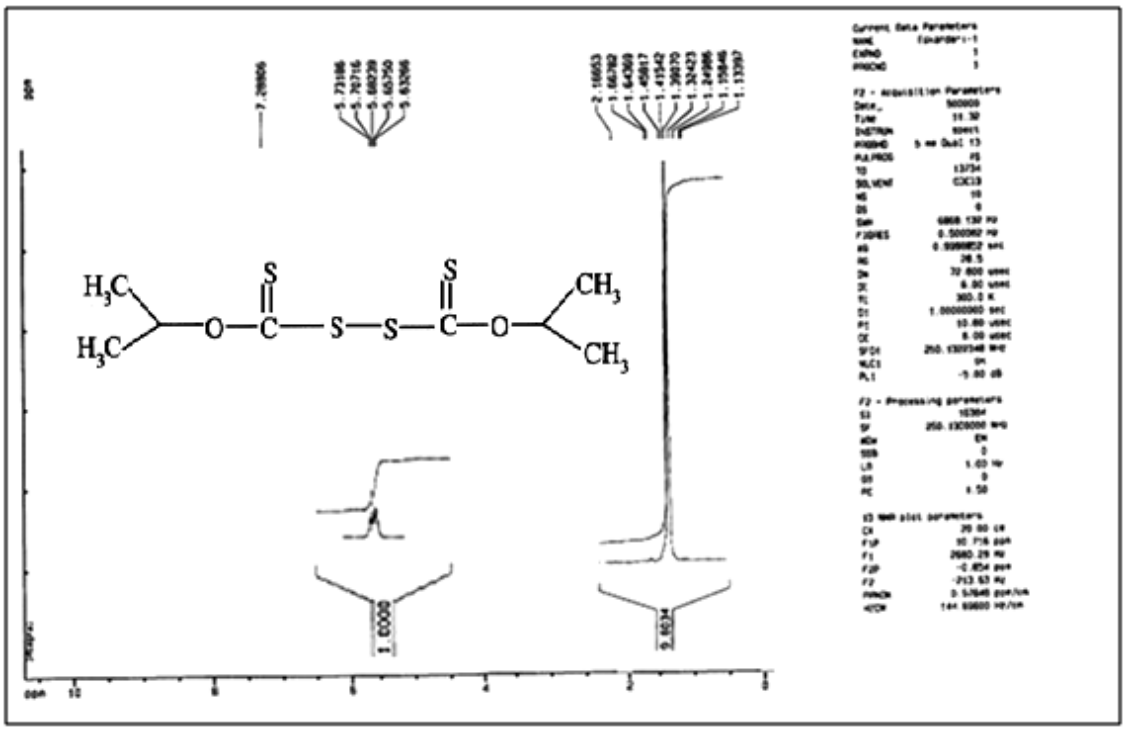

Fig.1. NMR spectroscopic for isopropyl 2-[(isopropoxycarbothiolyl) disulfanyl] ethane thioate

2.4. Extraction Procedure

The Se (IV) based on IICDET ligand was extracted by ultrasound-assisted dispersive liquid-liquid bio-microextraction procedure (USA-DLLMBE). By procedure, 100 µL (≈0.1 g) of hydrophobic ionic liquid of $[C_8MIM][PF_6]$ mixed with 0.35×10^{-6} mol L^{-1} of IICDET solution and 100 µL of acetone at pH of 4. The mixture based on Triton X-100, an emulsifier and anti-sticking agent was injected to 5 mL of blood, serum and urine samples which was diluted with 5 mL of DW. For optimizing, 1.0 µg L^{-1} and 20 µg L^{-1} of standard solution of Se (IV) as LLOQ and ULOQ was used instead of the blood and serum samples. After shacking, the Se (IV) ions were complexed by IICDET and extracted to IL at pH=4 (R-S: …Se). By centrifuging (3 min), the IL phase was separated from sample and inorganic selenium (Se_{IV}) was back- extracted from IL phase in basic pH (0.25 mL of 1.0 mol L^{-1} NaOH). Finally, the remained samples was determined by electro thermal atomic absorption spectrometry (ET-AAS). the Se (VI) reduced to Se (IV) in acidic pH (HCl, 130^{o}C) and the total Se(T-Se) was obtained at pH=4. Therefore, the Se (VI) was calculated by difference of T-Se and Se (IV) amount (Fig.2).

3. Results and Discussion

The human blood, serum and urine samples based on IICDET was used for selenium speciation with high accuracy by USA-DLLMBE procedure. The results showed us, the mean concentrations of Se (IV and VI) in human biological samples in hypothyroidism patients (50) were significantly higher than the healthy peoples (50). As linear range of selenium between 0.75-20 µg L^{-1}, the human samples can be diluted before using by proposed procedure. Based on results, the mean concentration of total selenium in urine and serum of hypothyroidism patients was obtained 11.8 µg g^{-1} creatinine and 52.6 µg L^{-1}, respectively which was less than 25 µg g^{-1} creatinine for urine samples and 150 µg L^{-1} for serum samples as TLVs in standard references.

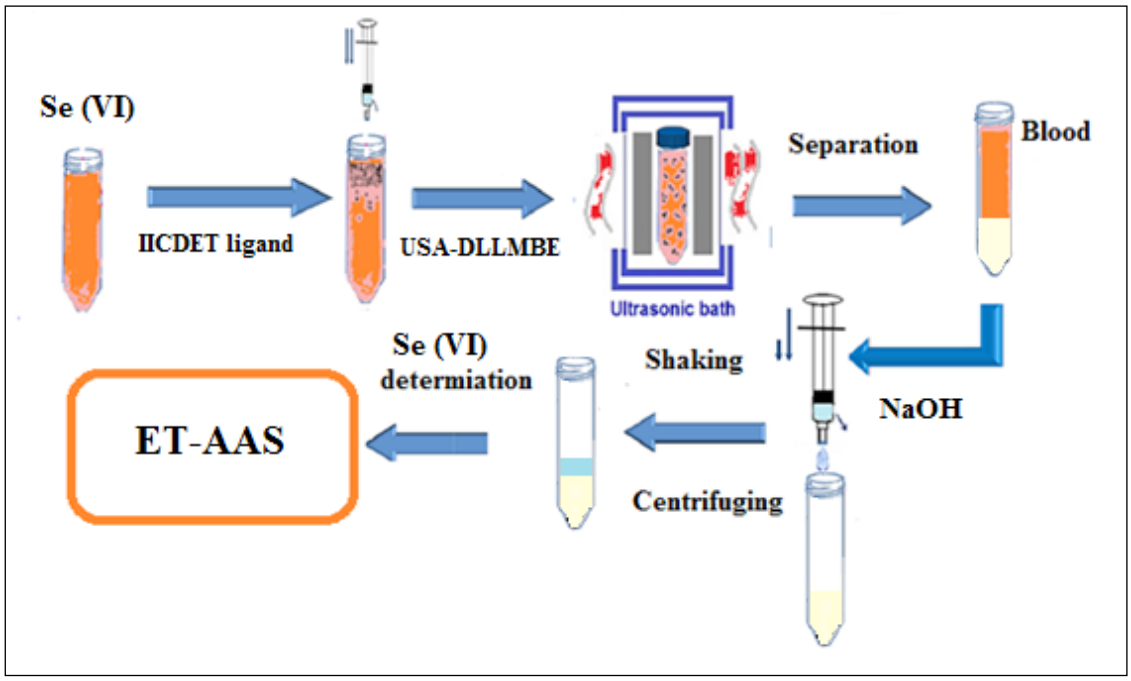

Fig. 2. Separation of selenium (IV) by ultrasound-assisted dispersive liquid-liquid bio-microextraction procedure (USA-DLLMBE)

3.1. Effect of ETAAS

The effect of pyrolysis temperature on the absorbance of Se was studied up to 1000 ˚C. The maximum absorbance was achieved within a range of 600–800 ˚C. Therefore, 700 ˚C was selected as the working pyrolysis temperature for selenium by nickel nitrate as modifier at concentration of 0.05 to 0.1% Ni. In addition, a drying as a 25 s was chosen for water evaporation, and a long ramp time of 40 s was chosen as it allowed gradual elimination of trace ionic liquid solution in liquid phase (350 ˚C) and avoided Se loss in pyrolysis temperature. The effect of atomization temperature on chromium signal was studied within the range of 2000–2500 ˚C, and the maximum signal was obtained at approx. 2400 ˚C. Cleaning time and temperature were ordered at 2 s and 2500 ˚C, respectively, and argon flow rate was 350 mL min^{-1}

3.2. Optimization of pH

The sample pH for extraction Se(IV) ions based on IICDET ligand was studied and optimized in different pH ranges between 2-11 for 0.75µg L^{-1} as a lower limit of quantification (LLOQ) and 20 µg L^{-1} selenium as upper limit of quantification (ULOQ). The complexation Se with sulfur of IICDET ligand was strongly depended on the pH of serum, blood and urine samples and caused to increase the recovery of extraction by ligand. Based on experimental results, the extraction efficiency of Se(IV) ions was perfectly achieved at pH =3.5-4.5. Therefore, the USA-DLLMBE procedure was used to speciation of selenium at pH=4 by IICDET ligand. The mechanism of Se extraction was obtained based on the complex formation of IICDET ligand between Se(IV) ions and sulfur covalence bonding of IICDET at optimized pH. The sulfur groups can be deprotonated (SH^-) at pH range of 3.5-8 and pH=4 was used as favorite pH for extraction of Se(IV) from human biological samples which was shown in Figure 3. As hydroxyl form of $Se(OH)_4$ in above pH (more than 6), the extraction capacity of Se(IV) in basic pH may be attributed to the affinities of OH groups.

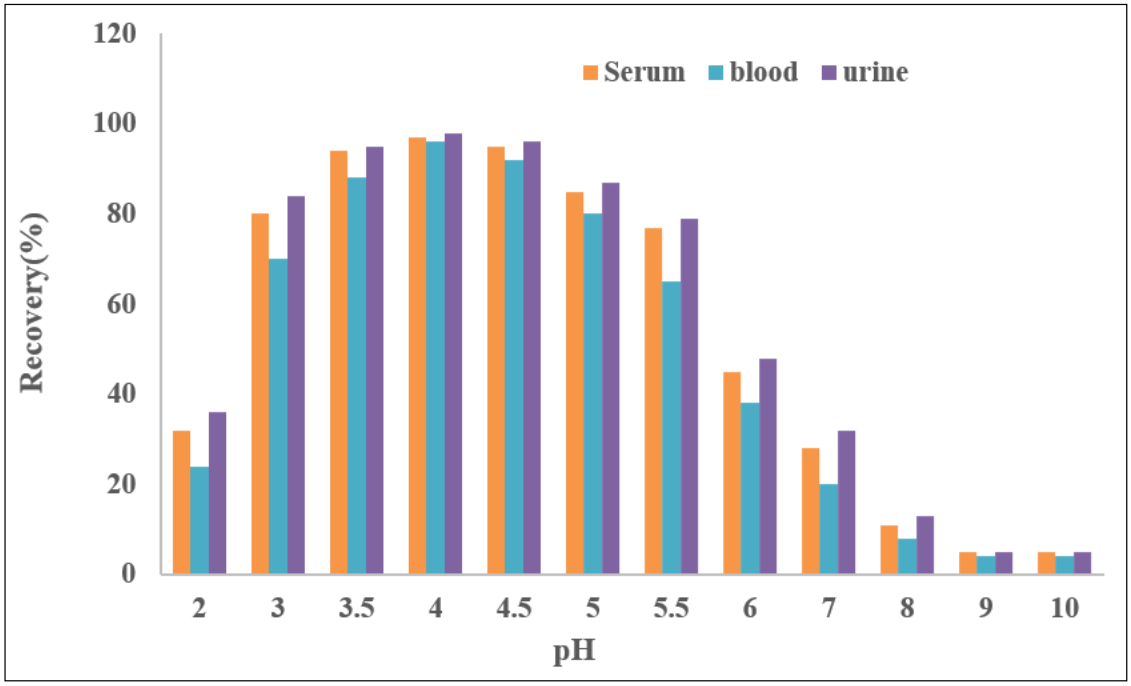

Fig.3. The effect of pH on Extraction Se(IV) based on IICDET ligand by USA-DLLMBE procedure

3.3. Optimization of IICDET ligand

The concentration of IICDET ligand as important parameters must be studied and optimized by USA-DLLMBE procedure. For optimizing, the concentration of 0.1×10^{-6}-1.0×10^{-6} mol L^{-1} of IICDET ligand was used for evaluation. Due to results, the more concentration of 0.33×10^{-6} mol L^{-1} of IICDET, has no effect on recoveries. So, the concentration of 0.35×10^{-6} mol L^{-1} of IICDET was selected as optimum ligand concentration for high extraction efficiency. The signal remained constant from 0.35×10^{-6} mol L^{-1} up to at least 1.0×10^{-6} mol L^{-1} IICDET for 0.75µg L^{-1} Se as a LLOQ range. Therefore 0.35×10^{-6} mol L^{-1} of IICDET concentration was used for further works. As 20 µg L^{-1} of Se as a ULOQ range, the signal remained constant from 0.4×10^{-6} mol L^{-1} up to at least 1.0×10^{-6} mol L^{-1} and 0.4×10^{-6} mol L^{-1} was selected as an optimized IICDET concentration. By adjusting pH, the best performance of the Se extraction was achieved between 0.3-0.4 µmol L^{-1} (Fig. 4).

3.4. Optimization of volume and ionic liquid amount

The sample volume as main parameters for Se extraction based on IICDET ligand and must be optimized at pH=4. So, the different volume of sample urine, blood and serum from 1-20 mL was used for extraction of Se ions by USA-DLLMBE procedure as 0.75 µg L^{-1} and 20 µg L^{-1} of selenium (IV). Perfect extraction more than 95% was achieved by sample volume of 1 - 10 mL. By increasing of sample volumes, the extraction efficiency was reduced. On the other hand, in high sample volumes, the partially solubilized the ionic liquid phase was increased and decreased accuracy and precision of results. So, a sample volume of 5 mL was selected as optimum volume for Se(IV) extraction based on ICDET ligand by USA-DLLMBE procedure (Fig. 5). Furthermore, the amount of ionic liquid effected on extraction recovery of Se in serum, blood, urine samples. Therefore, the different amount of ($[C_8MIM][PF_6]$ as hydrophobic ionic liquid was studied from the range of 0.05–0.35 g. Quantitative extraction was observed at higher than 0.08 g. So, 0.1 g of

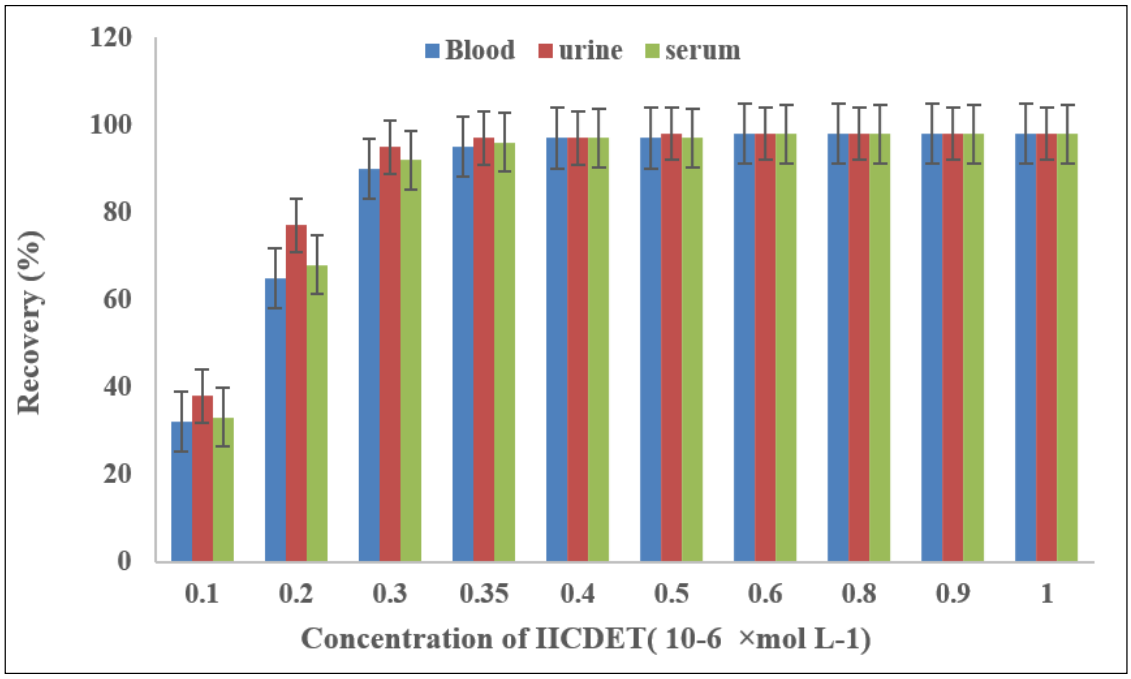

Fig. 4. The effect of IICDET ligand on Extraction Se(IV) by USA-DLLMBE procedure

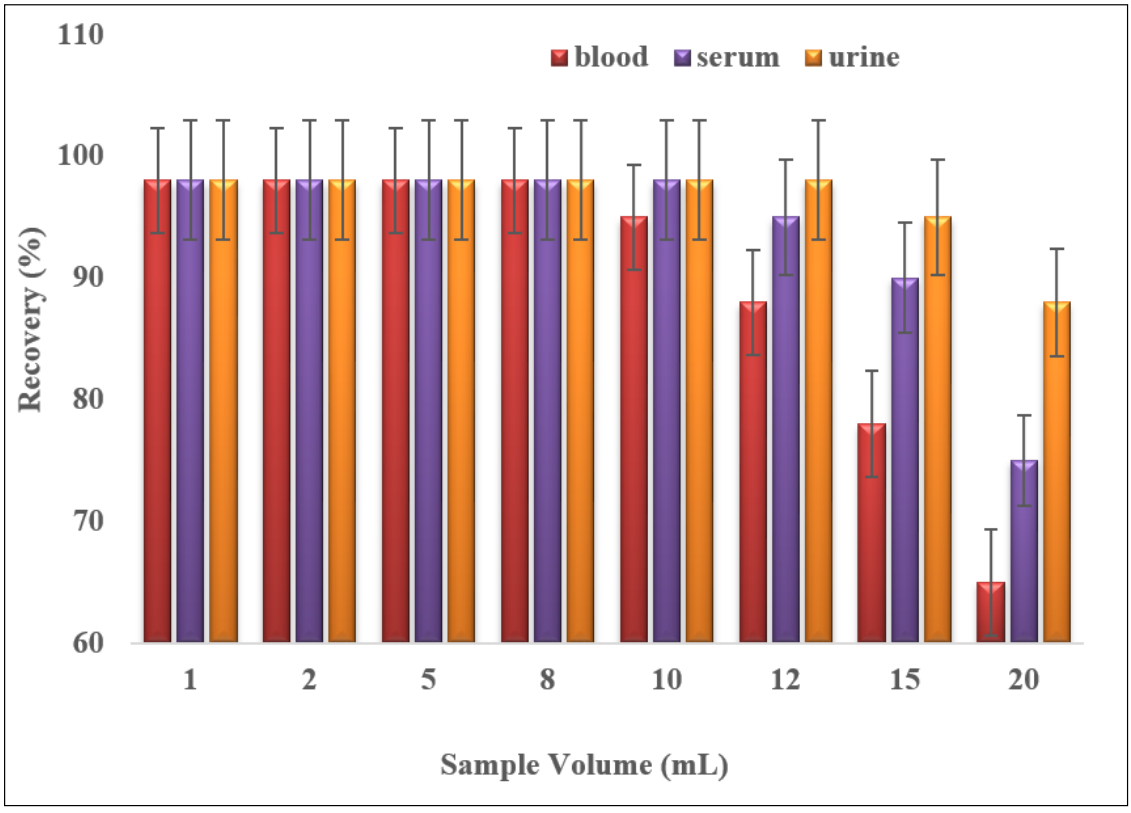

Fig. 5. The effect of sample volume on Extraction Se(IV) by USA-DLLMBE procedure

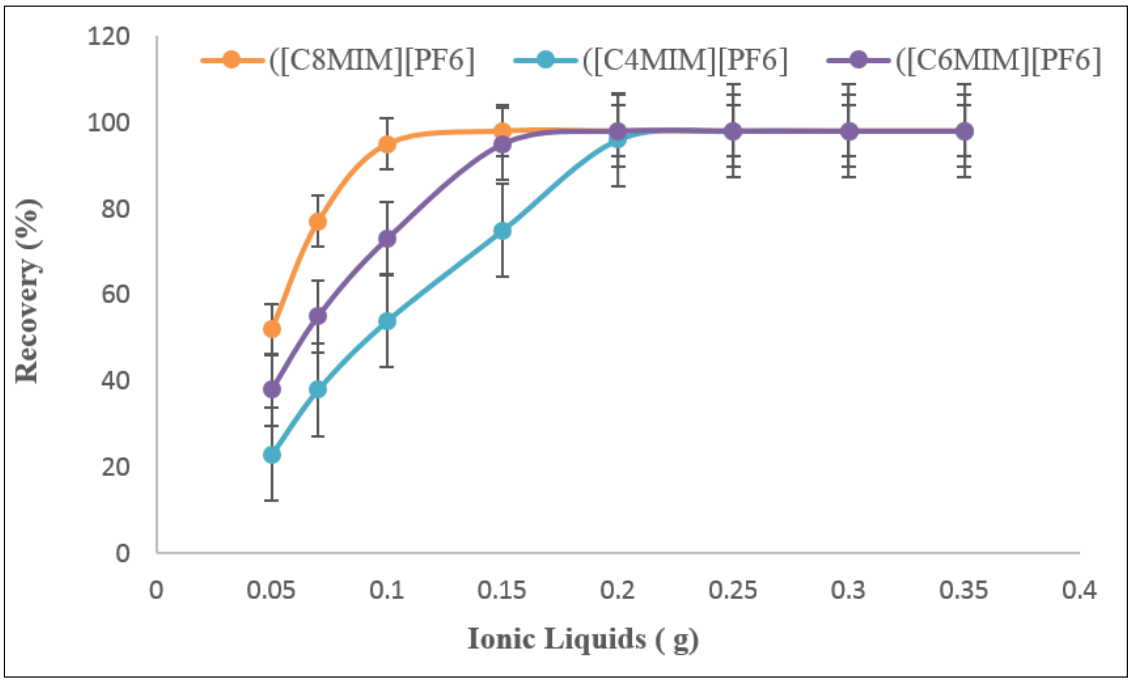

Fig. 6. The effect of amount of ionic liquid on Extraction Se(IV) based on IICDET ligand by USA-DLLMBE procedure

$[C_8MIM][PF_6]$ was chosen as optimum mass for Se extraction in 10 mL of samples at pH=4 (Fig. 6). The results showed, the amounts of IL have changes a little mass in different samples. The amounts of IL for serum, blood and urine samples were obtained 0.09 g, 0.1 g and 0.07 g, respectively.

3.5. Optimization of Eluent

The ionic liquids cannot apply directly by ETAAS as a viscose solution with high ash point temperature. So, the se ions were back-extracted from $[C_8MIM][PF_6]$ by different eluents such as a mineral acidic/basic solution. By changing pH, the complexation of Se-ligand leads to dissociation and Se ions release into the aqueous phase. Therefore, the varying concentration of mineral reagents such as HCl, HNO_3, H_2SO_4, KOH and NaOH from 0.5─3 mol L^{-1} were used for Se back-extraction from IL by elution processes (Fig. 7). Based on results, 1.0 mol L^{-1} of NaOH at 25^{O}C can be back-extracted Se (IV) from the IL phase). By procedure, 0.25 mL of 1.0 mol L^{-1} of NaOH was added and shacked for I minute at 25 ^{O}C. Finally, Se(IV) the remain solution determining by ET-AAS after dilution with DW up to 0.5 mL.

3.6. Effect of ultrasound and matrix

By procedure, the different ultrasound times was studied for selenium extraction in urine, blood and serum samples from 30 to 300 seconds. The results showed us, the extraction efficiency of Se improved by increasing the ultra-sonication time and then the relative response increased. Based on results, the maximum extraction was shown at 132 seconds and then remained constant. So, 2.2 minutes was selected as optimum time as ligand complexation (IICDET). Many techniques such as ETAAS have low sensitivity to metal interference ions. Therefore, the most interference ions can be occurred during the pre-concentration or extraction processes which was effected on accuracy of results. So, the important metals based on potential interfering ions for selenium determination were studied and optimized by procedure. 10 mL sample containing 20 µg L^{-1} of Se and 1–4 mg L^{-1} different concentration of matrix ions was used. The tolerate amounts of each ion were tested and results showed the absorbance alteration of interfering metals

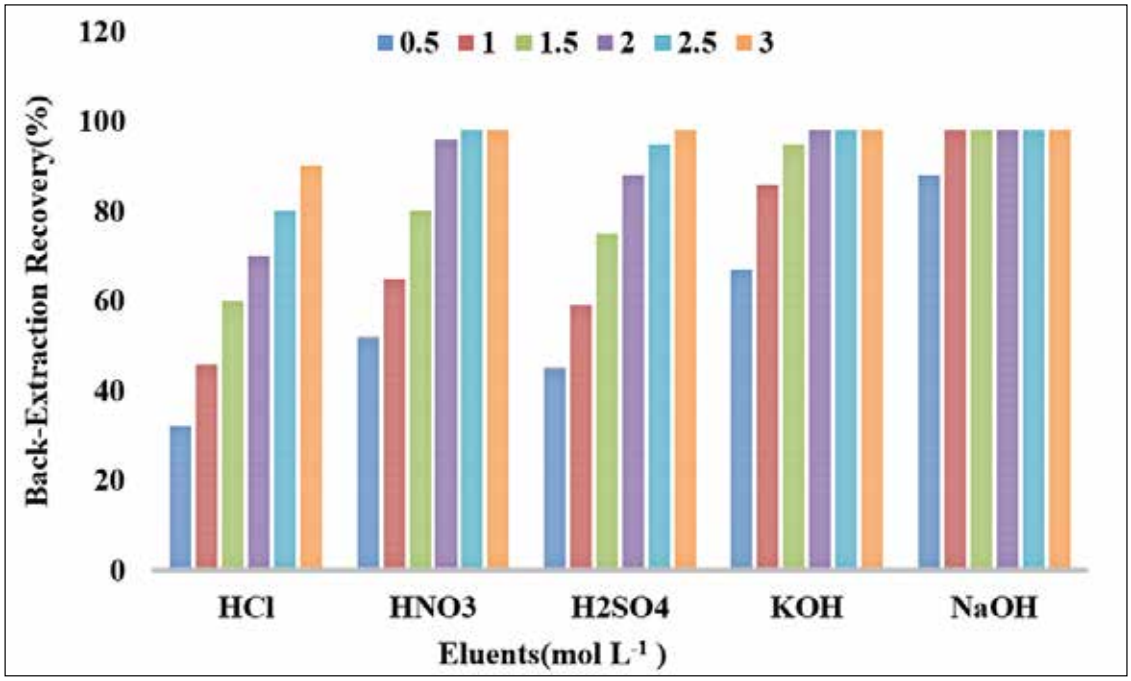

Fig. 7. The effect of different eluents on Extraction Se(VI) based on IICDET ligand by USA-DLLMBE procedure

were less than 5%. So, the interfering metals don't effected on extraction Se in optimized conditions (Table 1).

3.7. Analytical features

Analytical figures of merit were evaluated by USA-DLLMBE procedure for 10 mL of standard aqueous solutions, serum, urine and blood samples at pH=4 (Table 2). After preconcentration steps, the calibration curve was linear from 0.75 – 20 µg L^{-1} as a lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ). Detection limits (LOD) and precision (RSD %) was evaluated for selenium extraction by proposed ligand. The LOD were calculated as the concentration providing an analytical signal three times higher than the background noise. The LOD was obtained 186 ng L^{-1} and 174 ng L^{-1} for 10 mL of human and standard samples, respectively (MLOD=180 ng L^{-1}). As precision (RSD %), it was calculated from ten individual standards. The RSD (%) of Se (IV) in different concentrations of 0.75, 1.0 5.0, 10, and 20 µg L^{-1} were obtained 3.8, 3.2, 2.7, 2.6 and 2.45, respectively (MRSD% =2.95). The enrichment factor (EF), calculated as the ratio of the concentration of Se after preconcentration to that prior preconcentration based on curve fitting calibration rule. The EF of 21.2 and 18.9 for human and standard samples, respectively (M PF=20.1).

3.8. Validation of Results

The selenium was extracted and determined in human samples based on IICDET ligand with USA-DLLMBE procedure for 10 mL of hypothyroidism patients (50) and healthy peoples (50) with aged between 25 - 60 years (Table 3). The mean concentration of Se(IV) more than Se(VI) in human samples and the mean concentration of Se(IV) and Se(VI) in hypothyroidism patients lower than healthy peoples. The coloration analysis (r) of total Se(IV and VI) in hypothyroidism patients and healthy peoples were less than 0.19 in blood samples. The spiked urine, serum and blood were used to demonstrate the reliability of the method for determination of Se(IV) and Se(VI) in hypothyroidism patients by USA-DLLMBE procedure (Table 4). The recovery of spiked samples showed a satisfactorily results with the

Table 1. The effect of interferences ions on extraction of Se(IV) in human samples by USA-DLLMBE procedure

Blood, Serum (I)	Mean ratio (C_I /$C_{Se(IV)}$)	Recovery (%)
	Se(IV)	Se(IV)
Al^{3+} , Cr^{3+}	550	96.8
Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, Pb^{2+}	750 - 850	97.6
I^- , Br^-, F^-, Cl^-	1250	98.9
Na^+, K^+, Ca^{2+}, Mg^{2+}	1100	97.7
CO_3^{2-}, PO_4^{3-}, NH_4^+	950	99.3
Mn^{2+}, As^{3+}	150 - 250	98.1 - 97.5
Cd^{2+}	200	98.4
Hg^{2+}	45	97.3
Urine (I)	Mean ratio (C_I /$C_{Se(IV)}$)	Recovery (%)
	Se(IV)	Se(IV)
Cl^-, NO_3^-	1200	98.2
Na^+, K^+	1200	98.6
Ca^{2+}, Mg^{2+}	1000	98.0
Zn^{2+}, Cu^{2+}	700	97.5
CO_3^{2-}, PO_4^{3-}, NH_4^+	900	96.9
Hg^{2+}	50	97.4
Pb^{2+}	800	98.3
Ni^{2+}, Co^{2+}	700	97.2
Cd^{2+}	150	98.5
Mn^{2+}	100	96.6

Table 2. The analytical features for selenium determination by USA-DLLMBE procedure

Features	value
Working pH	4.0
Concentration of IICDET	0.35×10^{-6} mol L^{-1}
Sample volume of Blood, Serum, Urine (mL)	10 .0
Volume of sample injection	20 µL
Linear range (Peak Area)	0.75-20 µg L^{-1}
Linear range (Peak Height)	0.75-10.4 µg L^{-1}
Mean RSD %, n=10	2.95
LOD for human sample	0.187 µg L^{-1}
LOD for standard sample	0.174 µg L^{-1}
Enrichment factor for human blood or serum	21.2
Enrichment factor for standard	18.9
Volume and concentration of NaOH	0.25 mL,1M
Shaking/Centrifuging time	2.2 min, 3.0 min
Correlation coefficient	$R^2 = 0.9997$

ability of procedure for determination of Se(IV) and Se(VI) in hypothyroidism patients. Furthermore, the real blood, serum and urine samples were analyzed with ICP-MS and used as a CRM by USA-DLLMBE procedure. The results showed, the favorite efficiency and reliability of proposed method for determination and speciation of Se(IV) and Se(VI) in hypothyroidism patients (Table 5).

4. Conclusions

A simple and efficient method based on IICDET ligand was used for the speciation and determination of trace amount of Se(IV) and Se(VI) in hypothyroidism patients by USA-DLLMBE procedure coupled to ET-AAS. The main parameters such as sample volume, pH and ligand amount were optimized. This procedure introduced a sensitive, efficient and low cost method for speciation and separation of the Se(IV) and Se(VI) in human biological samples. The performance of USA-DLLMBE procedure for quantification extraction of Se(IV) and Se(VI) in blood, urine and serum samples was satisfactory. The favorite LOD, LOQ and RSD% achieved 0.18, 0.75 and 2.95, respectively and are comparable to previous reported methods. Based on results, the selenium concentration in thyroid patients was decreased as compared to healthy peoples. The method was validated by certified reference material (CRM) and ICP-MS analysis in real samples.

Table 3. Speciation and determination of Se(IV) and Se(VI) in serum, blood and urine samples based on IICDET ligand by USA-DLLMBE procedure **(**Serum and blood: $\mu g L^{-1}$, Urine: $\mu g\ g^{-1}$)

Sample	Patients (n=50)		Healthy peoples (n=50)		Patients /healthy	
	Se(IV)	Se(VI)	Se(IV)	Se(VI)	r	P value
Serum	77.9 ± 11.9	16.8 ± 4.8	130.7 ± 21.7	21.8 ± 4.3	0.202	<0.001
Urine	14.5 ± 3.7	2.7 ± 0.9	22.7 ± 7.8	3.6 ± 0.8	0.187	<0.001
Whole Blood	81.8 ± 13.8	17.4 ± 5.6	122.4 ± 18.6	34.5 ± 6.6	0.194	*<0.001*

*Correlations are based on Pearson coefficients (r). Statistical significance will be observed if $P < 0.05$
Mean of three determinations of samples ± standard deviation (P = 0.95, n =10)

Table 4. Analytical results of Se(IV), Se(VI) and T-Se determination in serum, blood and urine samples with USA-DLLMBE procedure and ICP-MS ($\mu g\ L^{-1}$)

Sample	Added		*Found ($\mu g\ L^{-1}$)			* ICP-Ms	Recovery (%)		
	Se(IV)	Se(VI)	Se(IV)	Se(VI)	T-Se	T-Se	Se(IV)	Se(VI)	T-Se
	-----	-----	67.5 ± 3.3	12.8± 0.6	80.3 ± 4.2	79.2 ± 2.7	----	----	----
Blood	50	-----	115.3 ± 5.6	12.6 ± 0.5	127.6 ± 6.1	129.3 ± 3.5	95.6	-----	94.6
	-----	10	67.3 ± 3.4	22.7 ± 1.1	90.0 ± 4.7	88.8 ± 2.9	-----	99.0	97.0
	-----	-----	81.6 ± 3.8	17.5 ± 0.8	99.1 ± 5.1	100.3 ± 3.4	-----	-----	----
Serum	100	-----	180.2 ± 8.5	17.2 ± 0.7	197.4 ± 9.3	195.6 ± 5.8	98.6	-----	98.3
	-----	20	82.2 ± 4.2	37.2 ± 1.8	119.4 ± 5.5	120.5 ± 3.6	-----	98.5	101.5
	-----	-----	12.6 ± 0.6	3.8 ± 0.2	16.4 ± 0.8	15.8 ± 0.3	-----	-----	-----
Urine	10	-----	22.4 ± 1.2	3.7 ± 0.2	26.1±1.3	26.5 ± 0.5	98.0	-----	97.0
	-----	5	12.7 ± 0.6	8.6 ± 0.4	21. 3 ± 1.1	20. 8 ± 0.6	-----	96.0	98.0

* Mean of three determinations ± standard deviation (P= 0.95, n=5)
All blood and serum samples diluted with DW (1: 10)

Table 5. Validation of methodology for determination selenium based on certified reference material (CRM) by USA-DLLMBE procedure

Sample	Certified (µg L^{-1})	Added (µg L^{-1})	*Found (µg L^{-1})	Recovery (%)
CRM1598a	13.44 ± 0.58	10	23.22 ± 1.14	97.8
S-ICP-MS	15.56 ± 0.46	10	25.13 ± 1.23	95.7

* Mean of three determinations ± standard deviation (P= 0.95, n=5)

All blood and serum samples diluted with DW (1: 10)

CRM1598a selenium in animal serum

S-ICP-MS: Human serum analyses with ICP-MS

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