Short Communication

Electrochemical Investigation of Strontium – Metallothionein Interactions – Analysis of Serum and Urine of Patients with Osteoporosis

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Abstract
The main aim of this paper is to study interaction between strontium and metallothionein (MT), and to determine changes in strontium and thiols (MT, reduced glutathione, cysteine, and homocysteine) level in plasma, serum, and urine samples of patients treated with strontium ranelate (SrR). To investigate the interactions between MT and strontium(II) ions, adsorptive transfer stripping technique coupled with differential pulse voltammetry (DPV) the Brdicka reaction was employed. Besides standard Brdicka signals (Co, RS_2Co, Cat1, Cat2, Cat3), we observed new signal related to Sr-MT interaction. Further we investigated the effect of various time of interaction, concentration of strontium(II) ions and temperature of supporting electrolyte on Brdicka signals. Optimal time of interaction was 240 s. Under temperature of supporting electrolyte 20 °C, we measured linear dependence of Cat3 signal height on strontium(II) ions concentration. After that we have investigated the possibility of strontium-MT interactions, we were interested in strontium, MT and low molecular mass thiols levels in serum and urine of patients treated with strontium(II) ions to cure osteoporosis. Strontium concentration determined by atomic absorption spectrometry was 55 ± 5 μg/L before and $10{,}500 \pm 1{,}400$ μg/L at the 30th day of SrR administration. Levels of metallothionein in serum ranged from 0.1 to 6.4 μM. Correlation between serum strontium concentration and MT level was determined and correlation coefficient was $R = 0.93$.

Keywords: Strontium, Metallothionein, Differential pulse voltammetry, Brdicka reaction, Heavy metal-protein interaction, Osteoporosis, Human blood serum and urine

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Dedicated to Professor Joseph Wang, on the Occasion of His 60th Birthday

Strontium ranelate (SrR, 5-(bis(carboxymethyl)amino)-3-(carboxymethyl)-4-cyano-2-thiophenecarboxylic acid) is a drug consisted of two strontium(II) atoms and ranelic acid. Strontium ranelate has been shown to prevent bone loss by decreasing osteoclast resorption and inducing osteoblast formation in vivo [1] and in vitro [2]. Hence, this drug is used to treat post-menopausal osteoporosis and increase bone mineral density. Safety and tolerance of drugs are particularly key issues in treatments of post-menopausal osteoporosis, because pharmaceutics are mainly administered over a long period of time to elderly people. Similar to other members of group 2 in periodic table such as calcium and magnesium, strontium forms a divalent cation in biological fluid, and is a trace element in the body [3]. Due to the fact that strontium is located above barium in the group 2, potential for similar complications, such as hypokalemia, is considered during administration [4]. Acute toxicity of strontium has not been described in humans [3, 5]. In animals, high-dose strontium causes hypocalcaemia, rickets, dental caries, endocrine abnormalities, in-coordination and phosphorus deficiency [3, 6, 7]. Chronic exposure of humans is associated with elevated levels of this element, but documented toxicity is lacking. Neither delayed toxicity nor chronic toxicity can be excluded in patients treated with SrR.

Metallothioneins (MT) were discovered in 1957 by Margoshes and Vallee as cadmium binding cysteine rich proteins [8]. They are a group of low molecular mass (about

6.5 kDa) single-chain proteins. MT are a family of proteins with a large degree of sequence homology, which have been described in bacteria, fungi, plants, and eukaryotic species. They are found in cytoplasm, subcellular organelles like lysosomes or mitochondria and nuclei of cells, particularly of liver, kidney, and intestine [9 – 11]. There is no conclusive data on the functional significance of their distribution in different cellular compartments. The highest cytoplasmic concentration of MT was found in the late G1 and G1/S cell cycle phase [12]. Depending on the cell cycle phase, cell differentiation or in case of toxicity, MT-1 + MT-2 are transported to the nucleus, as it was shown under oxidative stress or during early S-phase [13 – 16]. Cells have been also demonstrated to actively secrete MT-1 and MT-2 in vitro, although there is not known signaling pathway for this cellular export [17, 18].

One may suggest that MT as heavy metal binding protein can play considerable role in treatment of patients with SrR. However the relationship between SrR administration and MT level has not been investigated yet. Hence, the main aim of this paper is to study interaction between strontium and MT, and to determine changes in strontium and thiols (metallothionein, reduced glutathione, cysteine, and homocysteine) level in plasma, serum, and urine samples of patients treated with SrR. For determination of MT several groups of techniques are used and have been reviewed [19 – 23]. Electrochemical ones belong to the most sensitive, low cost and easy to use [24 – 35]. Therefore, we employ Brdicka reaction [27] and/or liquid chromatography with electrochemical detection [36, 37] to determine MT and/or reduced glutathione, cysteine, and homocysteine.

Previously, we investigated interactions of MT with heavy metals (cadmium, zinc, platinum, palladium) by using hanging mercury drop electrode (HMDE) and differential pulse voltammetry [25, 36, 38]. We used these results to suggest new heavy metal biosensors based on metal-protein interactions. In this work, we studied the influence of strontium(II) ions on catalytic signals evolving due to presence of free –SH moieties of MT and cobalt(III) complex as supporting electrolyte. MT (100 nM) was accumulated onto HMDE under open circuit. Optimal time of MT accumulation onto HMDE was verified in several papers. The most suitable time for accumulation of MT onto HMDE within the interval from 120 s to 240 s was chosen (not shown). DP voltammogram of MT accumulating onto the HMDE surface for 120 s is shown in Figure 1A. We observed following signals: i) Co peak at – 0.95 V could result from reduction of $[Co(H_2O)_6]^{2+}$; ii) RS_2Co signal is associated with formation of protein free –SH moieties and cobalt ions (– 1.1 V); iii) catalytic signal Cat1 at 1.2 V; iv) catalytic signal Cat2 at – 1.3 V; v) catalytic signal Cat3 at negative potential about – 1.7 V.

To investigate the interactions of MT with strontium(II) ions we used the following scheme. MT modified HMDE was rinsed in phosphate buffer (0.2 M, pH 6.9) and transferred to aqueous solution of strontium(II) ions. The electrode was further rinsed in the phosphate buffer and transferred to an electrochemical cell containing Brdicka supporting electrolyte. The changes in DP voltammogram of MT modified HMDE after interaction with strontium(II) ions are shown in Figure 2B. We observed a new signal called Sr due to strontium(II). Sr signal appeared at – 0.8 V

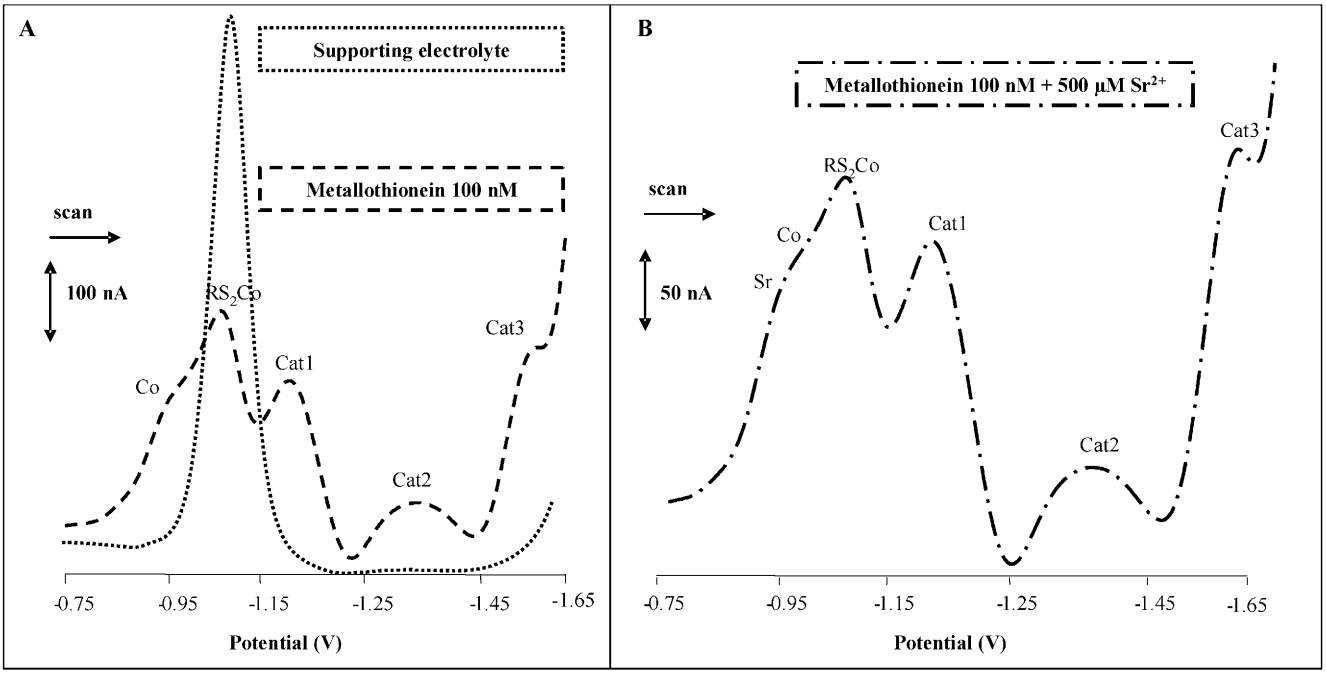

Fig. 1. A) DP voltammogram of supporting electrolyte (dotted line) and MT (100 nM) adsorbed onto HMDE (continuous line), time of accumulation 120 s. B) DP voltammogram of MT modified HMDE after 240 s exposition to 500 µM strontium(II) ions.

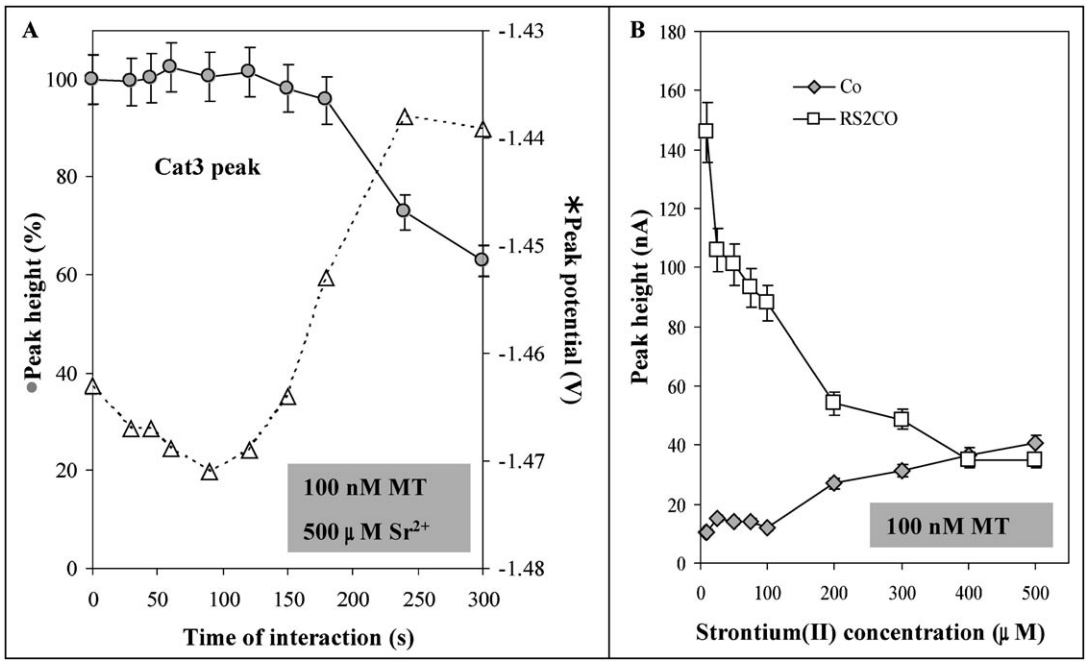

Fig. 2. A) Dependence of Cat3 signal height and potential on time of interaction of strontium(II) ions with MT modified HMDE. B) The changes of Co and RS_2Co heights with increasing strontium(II) ions concentration.

(100 mV in front of Co signal). Based on the results obtained we can state that Sr signal relates to the presence of strontium(II) ions in MT accumulated onto HMDE. This finding is supported by papers published previously, where we found that cadmium(II), zinc(II), palladium(II) and cisplatin could not be accumulated at open circuit onto HMDE and transferred [25, 26, 36, 38 – 40]. The Co, RS_2CO and Cat signals were changed too. Enhancement of Cat3 signal and decline of Cat1 and Cat2 was observed (Fig. 2B).

HMDE modified by MT was immersed into 10 µL drop of aqueous solution of strontium(II) ions (500 µM) under various times of interaction within the interval from 10 to 300 s. We determined that height of Cat3 signal changed slightly under shorter times of interaction (between 10 and 100 s). Whether the interaction was longer than 100 s, marked decrease in Cat3 signal height (for more than 40%) was observed. Potential of this signal shifted to more positive values with increasing time of interaction (Fig. 2A). It follows from Figure 2A, the time of interaction

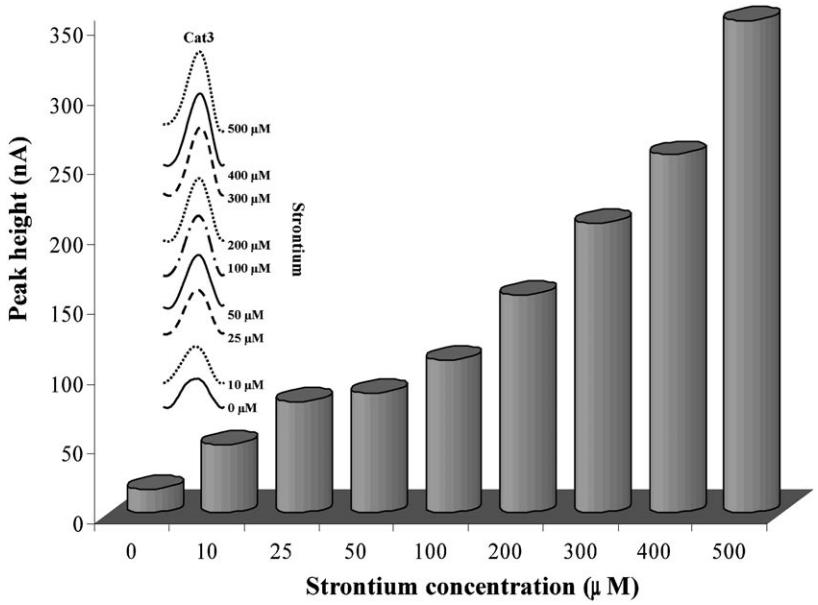

Fig. 3. Changes of Cat3 signal height after interaction with 0, 10, 25, 50, 100, 200, 300, 400, and 500 µM strontium(II) ions. Inset: DP voltammograms of Cat3 after baseline correction. Time of interaction: 240 s, temperature of Brdicka supporting electrolyte: 20 °C.

of strontium(II) ions with MT modified electrode lasting for more than 240 s resulted in Cat3 signal 30% decline ($n = 6$, *RSD* 5%). The change in Cat3 signal unambiguously shows on interaction of strontium(II) ions with MT accumulated on HMDE. Moreover we aimed our attention on study of effects of various strontium(II) concentration on MT signals. MT modified HMDE was immersed to solution containing strontium(II) ions for 240 s, and then the changes were observed. We found that Co signal moderately increased with the increasing strontium(II) ions concentration (from 100 μM). RS_2Co signal declined markedly with increasing strontium(II) ions concentration up to 200 μM, and then the decline was slowed down (Fig. 2B). These changes can be related to cobalt ions exchange by strontium(II) ions in MT molecule adsorbed onto HMDE. The changes in Cat1 and Cat2 signals were negligible with increasing concentration of strontium(II) ions. Dependence of Cat3 signal height on strontium(II) ions concentration is shown in Figure 3. The obtained dependence was linear with the following equation: $y = 0.5797x + 44.154$, $R^2 = 0.9762$. Determined Cat3 signals were well repeatable with relative standard deviation 6.6% ($n = 6$).

Temperature is very important condition at measurements using Brdicka reaction. It clearly follows from the results obtained that lower temperature of supporting electrolyte enhanced Cat1 and Cat2 signals. Temperature of 4 °C was estimated as the most suitable for the highest Cat1 and Cat2 signal response measurement. However Cat2 and Cat1 changed slightly due to presence of strontium(II) ions, as we mentioned above. Co and RS_2Co signals did not change much under various temperatures. Due to this fact we aimed further our attention on Cat3 signal. Compared to Cat1 and Cat2 signals, the height of signal decreased and relative standard deviation increased with decreasing temperature. The repeatability of Cat3 signals markedly increases with increasing temperature. At 20 °C of supporting electrolyte, relative standard deviation of Cat3 signal decreased under 7% ($n = 6$).

After that we have investigated the possibility of strontium-MT interactions, we were interested in strontium, MT and low molecular mass thiols levels in serum and urine of patients treated with strontium ions to cure osteoporosis. For the own treatment drugs such as SrR with high strontium content resulting in high plasmatic concentration of these ions is used. We determined that plasmatic strontium ions concentration was extremely higher (2000 times) in patients treated by SrR compared to that at the beginning of the treatment. Particularly, strontium concentration in plasma was determined as 55 ± 5 μg/L before and 10,500 ± 1,400 μg/L at the 30th day of SrR administration. Moreover, we found significant correlation between strontium levels in plasma and serum ($R = 0.98$). Concentration of strontium in urine was 22 ± 3 mg/L, which resulted in excretion of 47 ± 7 mg of strontium per 24 hours.

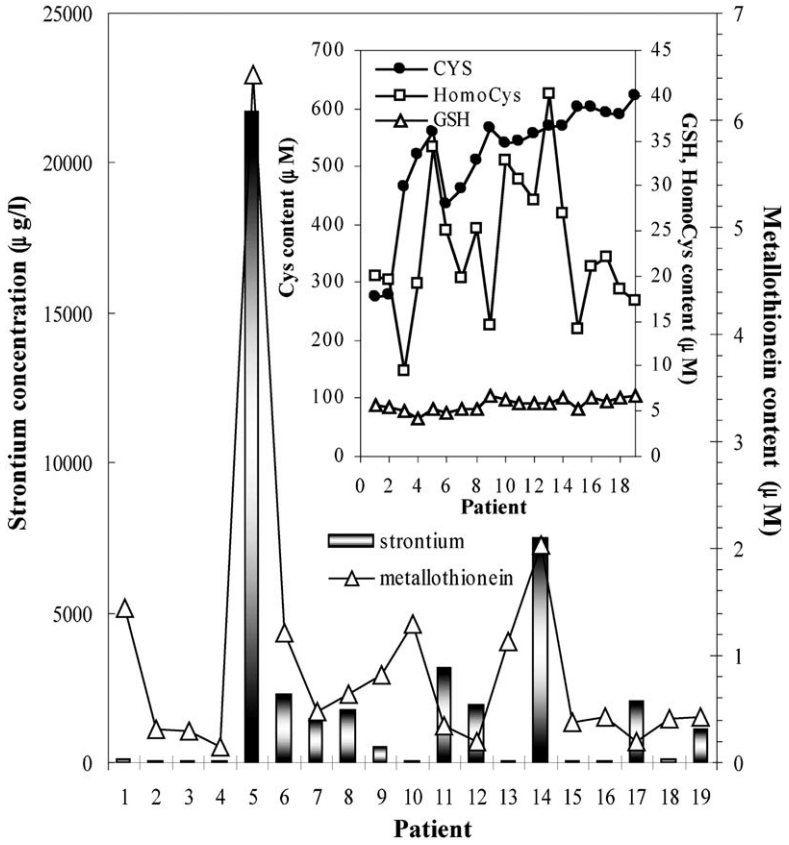

Fig. 4. Concentration of strontium ions and metallothionein in plasma of patients treated with SrR. Inset: concentration of cysteine (Cys), reduced glutathione (GSH) and homocysteine (HomoCys).

Metallothionein determination was carried out by automated electrochemical analyzer by method of differential pulse voltammetry Brdicka reaction [41]. Metallothionein were highly elevated as protein involved in detoxification of heavy metals. Levels of metallothionein in serum ranged from 0.1 to 6.4 μM, whereas mean value in serum of healthy volunteers was below 1 μM (Fig. 4). Correlation between serum strontium concentration and MT level was determined and correlation coefficient was $R = 0.93$. Moreover we quantified level of low molecular mass thiols (cysteine-Cys, reduced glutathione-GSH and homocysteine-HomoCys) in serum of patients treated with SrR. Mean serum concentration of cysteine was 2 times higher (540 ± 80 μM) than physiological one. Serum level of HomoCys was elevated too as 23 ± 7 μM, but normal values were ranged between 5 and 12 μM. We found GSH concentration 5.7 ± 0.8 μM in patients after treatment with SrR.

Investigation of heavy metal ions interactions with biologically important molecules including peptides, proteins and nucleic acids still belongs to the main aims of biochemistry. We show that differential pulse voltammetry Brdicka reaction can be used to reveal heavy metal based drug strontium ranelate interaction with heavy metal binding protein metallothionein. It can be concluded that interactions proceeds between these target molecules. This presumption is evaluated by analysis of blood serum and urine of patients treated with strontium ranelate. Level of metallothionein and strontium very well correlates, which can be associated with the interaction between the protein and metal taking place inside a cell.

Experimental

Rabbit liver MT (MW 7143 g/mol), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma Aldrich (St. Louis, USA). Acetic acid was purchased from Fluka (USA). All other reagents used were ACS purity and purchased from Sigma Aldrich unless stated otherwise. Stock standard solutions were prepared with ACS water (Sigma-Aldrich, USA) and stored in the dark at $-20\,^{\circ}$C. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through a 0.45 μm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to high performance liquid chromatographic (HPLC) analysis. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany).

Blood and urine samples were collected during routine biochemical examination from 28 patients with osteoporosis before SrR administration (2 g/day) and in 30th day of the therapy. All patients subscribed informed consent with the utilization of their biological samples for the experimental purposes. *Metallothionein.* Blood sample was kept at 99 °C in a thermomixer (Eppendorf 5430, USA) for 15 min. with occasional stirring, and then cooled to 4 °C. The denatured homogenates were centrifuged at 4 °C, 15000 g for 30 min. (Eppendorf 5402, USA). Heat treatment effectively denatures and removes high molecular weight proteins out from samples [32]. The supernatant was used for analysis. *Low molecular mass thiols.* The supernatant obtained was filtered through a membrane filter (0.45 μm Nylon filter disk, Millipore). The prepared samples were stored at -20° and prior to analysis the sample was 1000 fold diluted with ACS water [27, 36, 42].

Electrochemical measurements by adsorptive transfer stripping technique (AdTS) coupled with differential pulse voltammetry (DPV) Brdicka reaction were performed with AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. HMDE with a drop area of 0.4 mm^2 was employed as the working electrode. An Ag/AgCl/3 M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. The Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq) + NH_4Cl$, pH 9.6) was used and changed after five measurements; surface-active agent was not added. The samples of the MT were reduced before each measurement by 1 mM tris(2-carboxyethyl)phosphine addition according to [43]. AdTS DPV Brdicka reaction parameters were as follows: an initial potential of -0.35 V, an end potential -1.8 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV, a modulation amplitude of 250 mV, E_{ads} = open circuit, injected sample volume 5 μL. All experiments were carried out at 4 °C (Julabo F12, Germany) [27].

Electrochemical measurements by differential pulse voltammetry Brdicka reaction were performed with AUTOLAB Analyzer (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. HMDE with a drop area of 0.4 mm^2 was employed as the working electrode. An Ag/AgCl/3 M KCl electrode served as the reference electrode. Glassy carbon electrode was used as the auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. The Brdicka supporting electrolyte was changed per one analysis. The DPV parameters were as follows: initial potential of -0.7 V, end potential of -1.75 V, modulation time 0.057 s, time interval 0.2 s, step potential 2 mV, modulation amplitude -250 mV, E_{ads} = open circuit, injected sample volume 1 μL, accumulation time 120 s. All experiments were carried out at temperature 4 °C (Julabo F12, Germany).

Strontium concentration was determined by atomic absorption spectrometry at 460.7 nm on instrument Varian 220 FS (Australia). Acetylene-air flame in 10-fold-diluted plasma and in solution consisted from 20 g/L $LaCl_3$, 4 g/l KCl, and 80 mL/L 1-butanol was used. Samples with strontium concentrations below 500 μg/L were measured by graphite-furnace AAS with Zeeman background correction (Varian 220Z, Australia). Plasma (100 μL) was diluted 1:19 with solution containing Triton X-100 (0.2%, v/v), antifoam A (0.2%, v/v), and deionized water [44].

High performance liquid chromatography coupled with electrochemical detector system consisted of two solvent delivery pumps operating in the range of 0.001 – 9.999 mL/

min (Model 582 ESA Inc., Chelmsford, MA), Metachem Polaris C18A reverse-phase column (150.0 × 4.6 mm, 3 μm particle size; Varian Inc., CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes three flow cells (Model 6210, ESA, USA). Each cell consists of four analytical cells containing working carbon porous electrode, two auxiliary and two reference electrodes. Both the detector and the column were thermostated at 30 °C. The sample (50 μL) was injected using autosampler (Model 540 Microtiter HPLC, ESA, USA) at 4 °C. Mobile phase: 80 mM trifluoroacetic acid (solvent A) and methanol (solvent B); gradient: 3% methanol constant for 8 minutes, then increase of methanol to 15% during 1 min, after 8 minutes of constant methanol content (15%) decrease to 3% during 1 min; mobile phase flow rate 0.8 mL min^{-1}, the potential of working electrodes was 900 mV. For other experimental see the following papers [36, 37].

Data were processed using MICROSOFT EXCEL (USA). Results are expressed as mean ± standard deviation (*SD*) unless noted otherwise. Differences with $p < 0.05$ were considered significant (t-test was applied for means comparison). Relations between individual parameters were assessed using Spearman's correlation coefficient (*R*).

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