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Determination of Metal Ions in the Plasma of Children with Tumour Diseases by Differential Pulse Voltammetry

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The aim of this study was to investigate the interaction between lead ions and ethylenediaminetetraacetic acid (EDTA) in plasma. EDTA is widely used in medicine as anticoagulant agent. Understanding of the influence of EDTA on the electrochemical lead ions determination is very suitable for the determination of lead ions at patients with increased metals levels in body fluids. Further, the changes of metal ions levels (Zn, Cd, Pb and Cu) were monitored in the blood plasma of child patients treated for various oncological diseases. Electrochemical method differential pulse voltammetry with fully automated system and atomic absorption spectrometry was used for determination of the metal ions. It was found an increased amount of metal ions in the blood plasma of patients suffering from cancer disease in comparison with physiological values in healthy people. In these patients there was also found that they have higher levels of metallothionein and the ratio of GSH/GSSG was reduced. These results suggest that tumour diseases cause important changes in the level of ions.

Keywords: Metals; Electrochemical Analysis; Plasma; EDTA; Interaction; Oncological Diseases

1. INTRODUCTION

Human exposure to metals is common due to wide presence in industry and long-term environmental persistence. Among the general population, exposure to a number of metals is

widespread but generally at substantially lower levels than have been found in industry. Accumulation of metal ions in fatty tissues and circulatory system, negative effects on central nervous system and functioning of internal organs as well as acting as triggers of several serious diseases including tumour ones can be listed as adverse effects of metal ions on humans [1,2]. Metals share certain physical and chemical features and it is reasonable to speculate that common mechanisms for carcinogenicity may take place. Specific carcinogenic pathways, however, are determined by numerous factors including metal type, speciation, solubility, possible metal-metal interactions, and others [3,4].

The carcinogenicity of arsenic(III), chromium(VI), and nickel(II) has been confirmed in humans [3]. Some experimental and epidemiologic studies suggest that lead may be a human carcinogen, but the evidence is inconclusive so far. Although epidemiologic data are less extensive for beryllium(II) and cadmium(II), the findings in humans of excess cancer risk are supported by the clear demonstration of carcinogenicity in experimental studies [5]. Other metals, including antimony and cobalt, may be carcinogens in human, but the experimental and epidemiologic data are limited yet [3]. Heavy metal adsorptions from the digestion system and haemoglobin sensitivity to these metals are much higher in children compared to adults [6-9]. The health risk is especially high for children, because of their tolerance to poisons is lower [10]. Further, chronic effects of metals that might not be immediately apparent represent an important issue that also needs to be taken into account. Thanks to the negative characteristics referred to metals, it is necessary to monitor their amount in the body [11-14]. In that case, it is often necessary to analyse complex biological matrices such as blood, serum, tissue and urine. A variety of methods has been reported in the literature, including inductively coupled plasma – mass spectrometry (ICP-MS) and other spectroscopic and electrochemical method [15-17]. It is clear that the monitoring of metals in biological samples is given great attention [12,18,19].

Blood as a biological material is collected most frequently for laboratory testing. The way of sampling is given by methodology of examination in the laboratory and its technical equipment. For haematological blood testing, the examined samples are whole blood, plasma or serum. During an analysis of plasma there is necessary to add the anticoagulant solution to the blood sample. The anticoagulant compositions take the form of liquid (mixed in a certain ratio with patient blood in collection tube) or a crystalline form (the crystalline evaporation residue on the walls of collection tube). The most common anticoagulant agents include heparin, sodium citrate 3.8% and K2 or K3 EDTA (di- or tri-potassium salt of ethylenediaminetetraacetic acid) [20-26]. In the sampling tubes (VACUETTE®) for haematology, the EDTA dry additive is applied to the inner wall. Alternatively**,** these tubes can contain an 8% liquid EDTA solution (0.3 μM). The EDTA binds calcium ions and thus blocked the coagulation cascade [26]. Besides EDTA anticoagulant effect, this substance has the ability to bind other metal ions. Therefore the influence of EDTA to the metal ions determination was studied. Electrochemical techniques were used for metal ions quantification because they belong to the best methods for metal ions detection. These methods have advantages associated with high sensitivity, low detection volumes, compact instrumentation and low cost [27-31]. Moreover, the optimized methods were applied to analyse blood samples obtained from patients with a tumour disease.

2. EXPERIMENTAL PART

2.1. Chemicals

All chemicals used in this study were purchased from Sigma Aldrich (St. Louis, MA, USA) in ACS purity unless noted otherwise. HPLC-grade methanol (>99.9%; *v*/*v*) was obtained from Merck KGaA (Darmstadt, Germany). Pipetting was performed by pipettes from Eppendorf (Hamburg, Germany). Working standard solutions were prepared daily by diluting the stock solutions. Stock solutions of metals (1 mg/ml) was prepared by dissolving appropriate amount of zinc(II) nitrate, cadmium(II) nitrate, lead(II) nitrate and copper(II) nitrate in ACS water and diluted to 50 ml volumetric flask. Acetate buffer of pH 5 was prepared with 0.2 M acetic acid and 0.2 M sodium acetate and diluted with water and used as a supporting electrolyte. High purity deionised water (Milli-Q Millipore 18.2 MΩ/cm, Bedford, MA, USA) was used throughout the study. There was also used human blood plasma for sample preparation.

2.2. Preparation of deionised water and pH measurement

The deionised water was prepared using reverse osmosis equipment Aqual 25 (Brno, Czech Republic). The deionised water was further purified by using apparatus MiliQ Direct QUV equipped with the UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany). Deionized water was used for rinsing, washing, and buffer preparation.

2.3. Preparation of samples

Blood plasma samples were obtained from 10 child in patients at Department of Paediatric Haematology and Oncology of Faculty Hospital Motol with newly diagnosed solid tumours (non-Hodgkin lymphoma (n=2), Ewing sarcoma, hepatoblastoma, testicular germ cell tumour, embryonal rhabdomyosarcoma, neuroblastoma (n = 2), medulloblastoma, nephroblastoma; average age of 12.2 years). The samples were stored in -80 °C until assayed.

2.3.1. Microwave digestion for electrochemical and spectrometric determination of metal ions

10 µl of blood plasma was pipetted into digestion vials. Nitric acid (65 %, *v*/*v*) and hydrogen peroxide (30 %, *v*/*v*) were used as the digestion mixture. There was used 500 µl volume of this mixture, while the volume ratio between nitric acid and hydrogen peroxide was always 7:3 (350 µl HNO_3 and 150 µl H_2O_2). Samples were digested by Microwave 3000 (Anton Paar GmbH, Austria) using rotor MG-65. The program begins and ends with the same ten-minute-long-step, beginning with the power of 50 W and ending with the power 0 W (cooling). Microwave power was set to 100 W in the main part of the programs (30 minutes) at temperature of 140 °C. After the digestion, the samples with digestion mixture were pipetted into Eppendorf vials and electrochemical determination of zinc, lead, cadmium and copper followed [27,32,33].

2.3.2. Samples preparation for metallothionein electrochemical determination

Samples were prepared by heat treatment by the help of the automated pipetting system epMotion 5075 (Eppendorf, Germany), because the heat treatment effectively denatures and removes the high molecular weight proteins from real samples [34]. Briefly, the samples were kept at 4°C then samples were transferred to the 96 well plates (Eppendorf) containing 0.2 M phosphate buffer pH 7. This mixture was kept at 99 °C for 15 min. The last step was cooling down of samples to 4 °C. The cooled samples were then centrifuged for 30 minutes at 15000 × g at 4 °C. The supernatant was collected and measured.

2.3.3. Samples preparation for reduced and oxidized glutathione determination

Serum was separated from whole blood by centrifugation at 4000 × g for 10 min (Model 5402, Eppendorf, Germany), and the samples were stored at -80˚C until assayed. When required, the denatured samples were centrifuged at 15000 × g at 4˚C for 30 min (Model 5402; Eppendorf AG) and directly analysed using an optimised high performance liquid chromatography with electrochemical detection. Prior to chromatographic analysis, precipitation of proteins with trifluoroacetic acid (TFA) to avoid excessive clogging of filters and precolumns, which protect the separation column from contaminations, was required. The denatured sample (100 µl of plasma and 100 µl of 10% (*v*/*v*) TFA) was than centrifuged and the resulting supernatant was directly injected to the chromatographic column.

2.4. Atomic absorption spectrometry (AAS)

Measurements were carried out on 240 FS AA Agilent Technologies flame atomic absorption spectrometer with deuterium lamp background correction or 280Z Agilent Technologies atomic absorption spectrometer (Agilent, USA) with electrothermal atomization and Zeeman background correction. Zinc, cadmium, lead and cooper were measured on primary wavelengths: Zn 213.9 nm (spectral bandwidth 1.0 nm, lamp current 5 mA); Cd 228.8 nm (spectral bandwidth 0.5 nm, lamp current 4 mA); Pb 217.0 nm (spectral bandwidth 1.0 nm, lamp current 10 mA) and Cu 324.8 nm (spectral bandwidth 0.5 nm, lamp current 4 mA). Elements measured by electrothermal AAS were determined in the presence of palladium chemical modifier. The samples were modified in accordance with chapter 2.3.1.

2.5. Determination of Zn, Cd, Pb and Cu in plasma of children with malignant tumours

Determination of zinc, cadmium, lead and cooper by differential pulse voltammetry were performed with 797 VA Computrace instrument connected to 813 Compact Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. The three electrode system consisted of a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 as the working electrode, an Ag/AgCl/3 M KCl reference electrode and a platinum as the auxiliary electrode. 797 VA Computrace

software by Metrohm CH was employed for data processing. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999%). Acetate buffer (0.2 M CH_3COONa + 0.2 M CH_3COOH, pH 5) was used as a supporting electrolyte. The supporting electrolyte was replaced after an analysis. The parameters of the measurement were as follows: purging time 90 s, deposition potential -1.15 V, accumulation time 240 s, equilibration time 5 s, modulation time 0,057 s, interval time 0.04 s, initial potential of -1.3 V, end potential 0.2 V, step potential 0.005 V, modulation amplitude 0.025 V, volume of injected sample: 15 µl, volume of measurement cell 2 ml (15 µl of sample and 1985 µl acetate buffer).

Moreover, lead was determined in the presence of EDTA by differential pulse voltammetry (DPV) using the same instrument and conditions mentioned in the previous paragraph with the following exceptions: initial potential of –0.6 V, end potential 0.2 V, deposition potential -0.5 V, accumulation time 300 s, deoxygenating with argon 90 s, volume of injected sample: 500 µl, volume of measurement cell 2 ml (500 µl of sample and 1500 µl acetate buffer pH = 5.0).

2.6. Determination of metallothionein in plasma of children with malignant tumours

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 693 VA Processor and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes and cooled sample holder and measurement cell to 4 °C (Julabo F25, JulaboDE). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was used as the working electrode. An Ag/AgCl/3M KCl electrode was the reference and platinum electrode was auxiliary. For data processing VA Database 2.2 by Metrohm CH was employed. The analysed samples were deoxygenated prior to measurements by purging with argon (99.999 %) saturated with water for 120 s. Brdicka supporting electrolyte containing 1mM $Co(NH_3)6Cl_3$ and 1M ammonia buffer (NH_3(aq) + NH_4Cl, pH = 9.6) was used. The supporting electrolyte was exchanged after an analysis. The parameters of the measurement 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} = 0 V, volume of injected sample: 10 µl, volume of measurement cell 2 ml (10 µl of sample and 1990 ml of Brdicka solution).

2.7. Determination of GSH and GSSG in plasma of children with malignant tumours

The HPLC-ED system consists of two chromatographic pumps (Model 582; ESA, Inc., Chelmsford, MA, USA; working range 0.001-9.999 ml/min), a chromatographic column with reverse phase Zorbax eclipse AAA C18 (Agilent Technologies, Inc., Santa Clara, CA, USA; 150×4.6 mm; 3.5-µm particles) and a twelve-channel CoulArray electrochemical detector (Model 5600A; ESA, Inc.). The detector consists of three flow analytical chambers (Model 6210; ESA, Inc.). A chamber contains four analytical cells and one analytical cell contains two referent (hydrogen-palladium), as well as two counters and porous graphite working electrodes. The ED is situated in the thermostated control module. A 20 µl sample was injected using an autosampler (Model 542; ESA, Inc.), which has

thermostated space for the column. The column was termostated at 35˚C. Flow rate of mobile phase was 1 ml/min. The mobile phase consisted of A: trifluoroacetic acid (80 mM), and B: 100% methanol (*v*/*v*). Substances were eluted with the following linear increasing gradient: 0-1 min (3% B) 1-2 min (10% B), 2-5 min (30% B), 5-6 min (98% B). Detection of the separated compounds was carried out at the applied potential of 900 mV. Analysis time was 20 minutes.

2.8. Mathematical treatment of data and estimation of detection limits

Data were processed using MICROSOFT EXCEL (Microsoft, Redmond, WA, USA) and STATISTICA.CZ Version 8.0 (Stat-Soft CR, Prague, Czech Republic). The results are expressed as mean ± standard deviation (SD) unless otherwise noted. The detection limits (3 signal/noise, S/N) were calculated to Long and Winefordner [35], whereas N was expressed as a standard deviation of noise determined in the signal domain unless otherwise stated.

3. RESULTS AND DISCUSSION

3.1. Optimization of lead determination

Among the different electrochemical techniques voltammetric and potentiometric techniques are the most reported for heavy metals detection [27,30,32,33,36-44]. In addition to conventional hanging mercury drop electrode the most commonly used electrode material for the detection of metals is carbon. Determination of metals on the electrodes made of different modifications of carbon (tips, rods, glassy carbon, carbon paste with different content of carbon particles with various shapes and sizes) can be found elsewhere [45-47]. Based on the facts mentioned in Introduction section, lead(II) ions are somehow connected with carcinogenetic processes, however, their specific roles remains unclear. To investigate their roles, some analytical methods able to study these ions under various environments are needed. Therefore, we primarily optimized electrochemical determination of these ions using differential pulse voltammetry. This method was optimized for the standard solution of $Pb(NO_3)_2$ within the concentration range of metal ions from 0.2 to 25 ng/ml. Various times of accumulation (120, 240, 300 and 360 s), pH of supporting electrolyte (4.4, 4.6, 4.8, 5.0, 5.2 and 5.4) and deposition potentials (-1.0, -0.8,-0.6, -0.55 and -0.5 V) were tested. The obtained results are shown in Figs. 1A, B and C, respectively. The relative peak heights of lead are related to the highest value. It clearly follows from the results obtained that the accumulation time of 300 s was the optimal value to measure sufficient signal during reasonable time. As the optimum pH of the acetate buffer 5.0 was chosen. The highest signal in the optimization of the deposition potential was achieved at -0.5 V. Therefore the accumulation time of 300 s, pH 5.0 and deposition potential -0.5 V were selected for the following experiments. Further, the calibration dependence was measured. The obtained concentration dependence for lead(II) ions was linear within the range from 0.2 to 25 ng/ml with equation of dependence as follows: $y = 0.4173x$; $R^2 = 0.9909$, n = 3, RSD = 3.9 % (Fig. 1D). Limit of detection (3 S/N) was estimated as 0.07 ng/ml of lead(II) ions. More analytical parameters are shown in Table 1.

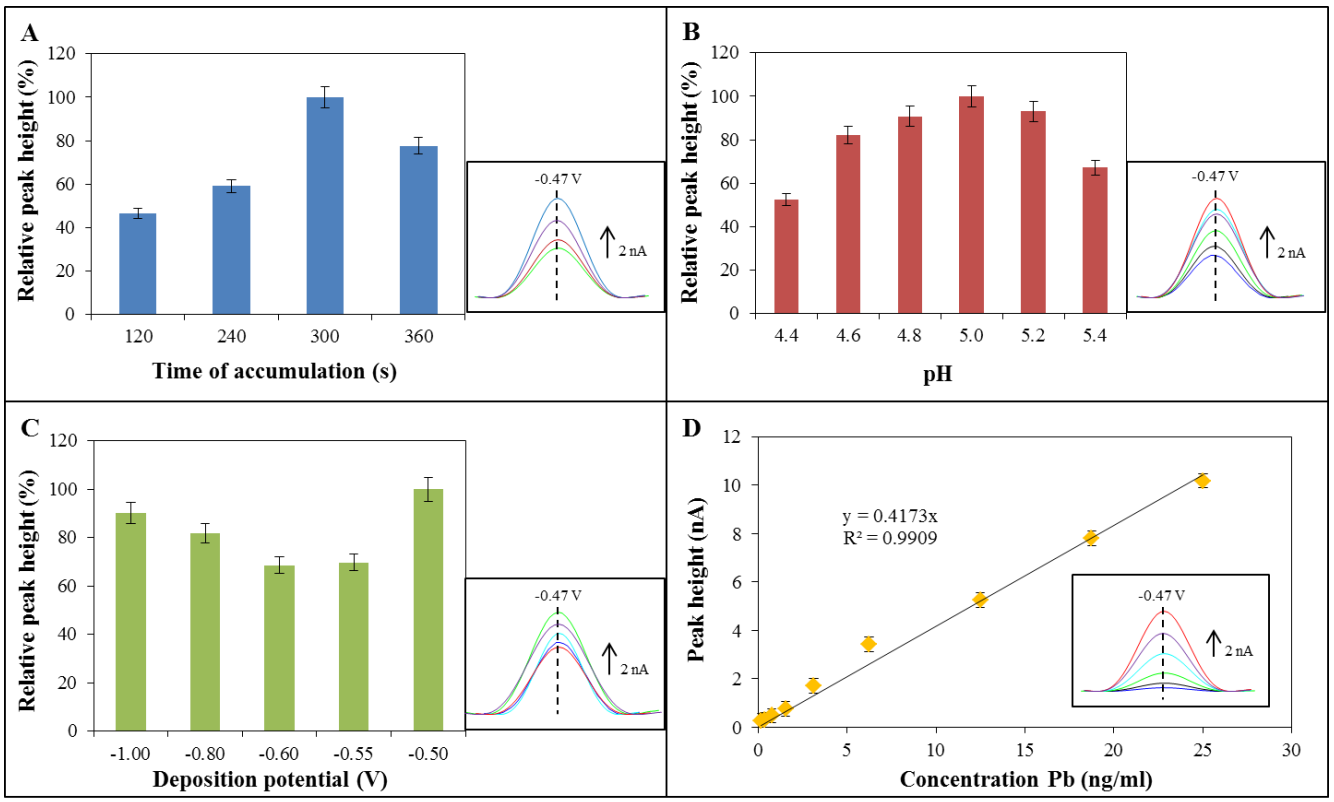

Figure 1. Optimization of electrochemical determination of lead. Lead was determined by DPV method. Optimization of (**A**) time of accumulation, (**B**) pH, and (**C**) deposition potential. The relative peak heights of lead are related to the highest value. (**D**) Calibration curve of lead determined by DPV method without mineralization. 0.2 M acetate buffer (pH=5) was used as an electrolyte. The parameters were chosen as follows: initial potential -0.6 V, end potential -0.2 V, deposition potential -0.5 V, accumulation time 300 s, pulse amplitude 25 mV, pulse time 0.04 s, voltage step 5.035 mV, voltage step time 0.3 s, sweep rate 0.0168 V/s.

3.2. Interaction of lead ions with EDTA

Due to the fact that blood sample of patients are often collected to tubes that contain various agents including EDTA, which is commonly used as an anticoagulant [48], the effect of this complex agent was investigated. EDTA concentration of 1.5 - 2 mg per 1 ml of blood has no significant effect on cell blood count thus it is ideal for use in haematology [49]. Part of this work was aimed to investigate the effect of EDTA on the electrochemical determination of lead and their interaction. Figure 2A shows an electrochemical signal of EDTA within the concentration range from 6.25 to 250 µM. The electrochemical signal of EDTA was composed of three peaks (E1, E2 and E3) with the potential at positions -0.15 V (E1), 0 V (E2) and 0.15 V (E3), which are shown in insets in Fig. 2A. Interaction of lead with EDTA is shown in Fig. 2B. There was used the same concentration of EDTA (25 µM) to which the gradually increasing concentration of lead within the range from 0 to 5.5 µg/ml was added. With the increasing concentrations of lead the individual peaks EDTA decreased. E3 peak was not detected since the addition of lead concentration 2.5 µg/ml. After the addition of lead concentration 5 µg/ml the signal of lead was detected at the potential -0.46 V and the signal of EDTA

was not detected. In Fig. 2b1, we can see the changes in relative lead peak height (related to the lead ions alone) affected by the increasing concentration of EDTA (0.125 – 6.25 μM) with the addition of lead 12.5 ng/ml. With increasing concentration of EDTA the lead signal decreased, which confirms the effectiveness of EDTA as a chelating agent. Decrease in the lead signal (comparing the lowest (0.125 μM) and highest (6.25 μM) added EDTA concentration) was 87 %. Lead signal was not detected when the EDTA concentration was higher than 6.25 μM. Figure 2b2 shows a decrease in the signal of lead with the addition of EDTA (25 μM) compared to the signal of lead without EDTA (dotted bars). The relative peak heights of lead are related to the highest value.

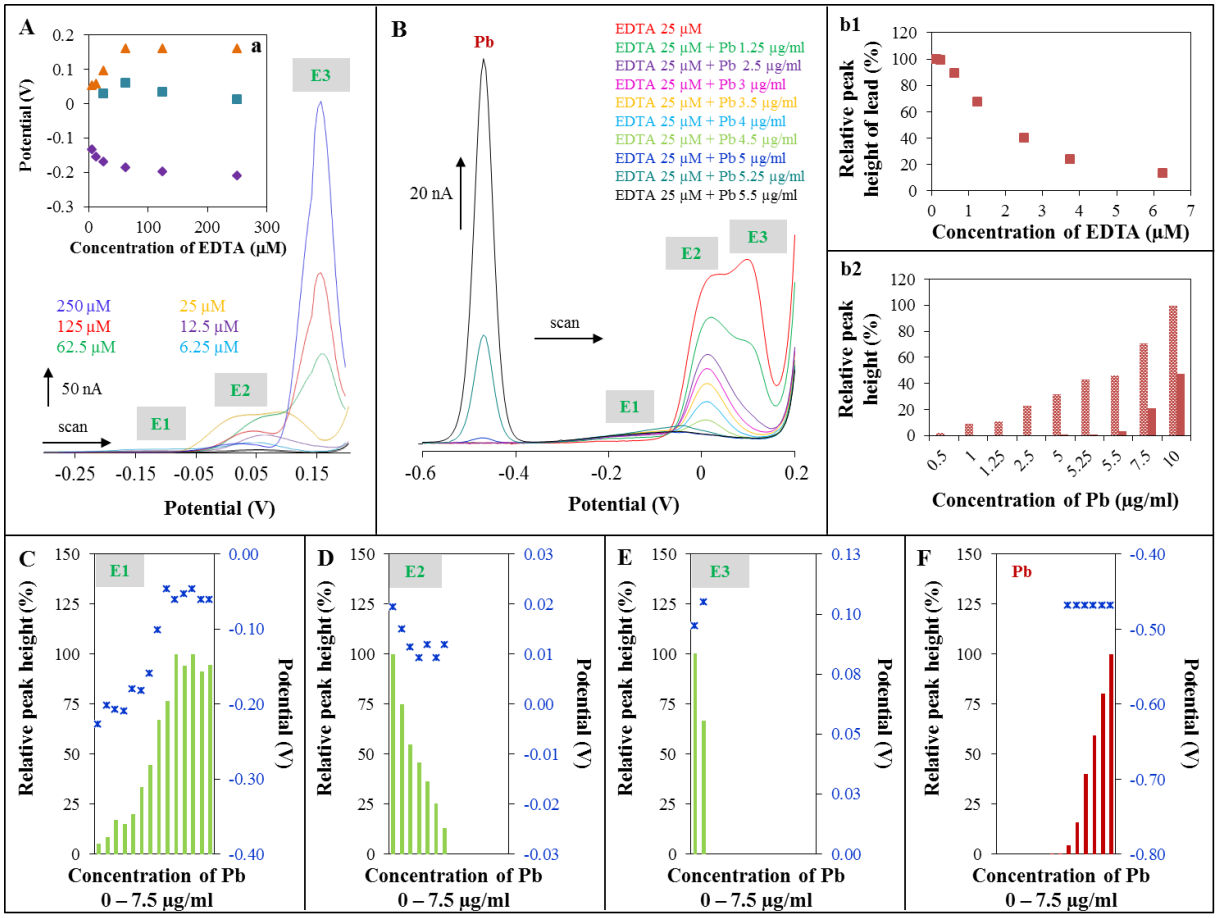

Figure 2. Interaction of lead ions with EDTA. (**A**) Typical DP voltammograms related to the mixture of lead ions and EDTA. Concentration of EDTA vary from 6.25 to 250 μM. There were detected three peaks E1, E2 and E3. Inset: dependence of individual peak potentials on varying concentration of EDTA. (**B**) Real voltammograms measured by interactions of EDTA with various concentrations of lead ions. (**b1**) Dependence of relative lead peak height (related to the maximum value) on concentration of EDTA. (**b2**) Dependence of relative lead peak height (related to the maximum value) on concentration of lead. Changes of various relative peaks heights and potentials with various concentration of lead ions in mixtures of EDTA and lead as follows: (**C**) E1 peak, (**D**) E2 peak, (**E**) E3 peak and (**F**) lead peak. 0.2 M acetate buffer (pH=5) was used as an electrolyte. The parameters were chosen as follows: initial potential -0.6 V, end potential 0.2 V, deposition potential -0.5 V, accumulation time 300 s, pulse amplitude 25 mV, pulse time 0.04 s, voltage step 5.035 mV, voltage step time 0.3 s, sweep rate 0.0168 V/s.

Table 1. Analytical parameters of electrochemical determination of Cd(II), Zn(II), Pb(II) and Cu(II).

Substance	Regression equation	Linear dynamic range (μM)	Linear dynamic range (ng/ml)	R^{2a}	LOD[b] (μM)	LOD (ng/ml)	LOQ[c] (μM)	LOQ (ng/ml)	RSD[d] (%)
Cd	y = 97.792x	0.007 – 15.00	0.8 – 1686	0.999	0.002	0.2	0.01	1	2.4
Zn	y = 108.08x	0.007 – 15.00	0.5 – 981	1.00	0.01	1	0.03	2	6.5
Pb	y = 108.52x	0.007 – 15.00	1.5 – 3108	0.999	0.001	0.3	0.01	1	6.6
Cu	y = 107.65x	0.007 – 15.00	0.4 – 953	0.999	0.01	1	0.04	2	2.5

a…regression coefficients

b…limits of detection of detector (3 S/N)

c… limits of quantification of detector (10 S/N)

d…relative standard deviations

Figures 2C-F indicate the relative peak heights and changes of potentials for individual peaks of EDTA and lead in their mutual interaction. Figure 2C shows the peak E1 of EDTA. With the increasing concentrations of lead the peak E1 increased and the potential value was shifted from -0.23 V to -0.06 V. The peak E2 (Fig. 2D) decreased with the increasing concentration of lead and there was also a shift of the potential value from 0.02 to 0.01 V. Figure 2E describes the third peak of EDTA, which disappeared after the second added lead concentrations (2.5 μg/ml). The shift of the potential value was from 0.09 V to 0.1 V. The signal of lead was detected from the added concentration of 4.5 μg/ml with a constant potential value of -0.469 V (Fig. 2F). The disappearance of the peak E3 corresponds to detection of the signal of lead. From the obtained results the significant interaction of lead with EDTA is evident. Nowadays, EDTA is among the widely used anticoagulants [24] and its ability to act as a chelating agent should not be neglected especially in the case of a potential impact to the uptake of metals during the blood collection and then getting the distorted results of the actual metals amount in the body.

3.3. Optimization of microwave digestion

Further, we aimed our attention at sample preparation, which is another key for lead(II) determination. The most common procedure of sample preparation for metal determination is its dissolving in various acids or their mixtures [50]. Determination of elements is often realized by wet chemistry step coupled with a suitable detection technique. Wet chemistry includes decomposition of the sample with typical inorganic acids (HNO_3, HCl, HF, H_2SO_4, $HClO_4$). Conventional heating of samples was replaced by the use of microwaves [51]. Microwave heating ensures elimination of contamination, and it improves the speed and efficiency of digestion for some types of samples, which cannot be easily solved. The process is not only dependent on the used mineral acid and its volume, but also on the time and power of microwaves therefore the optimization is necessary [52,53]. In our case microwave digestion was applied for preparation of small amount of sample according to protocol mentioned in the cited papers [27,39]. Parameters of this procedure were optimized for obtaining the

best results for electrochemical detection of metals. They were primarily the ratio of digestion mixture and also the digestion power.

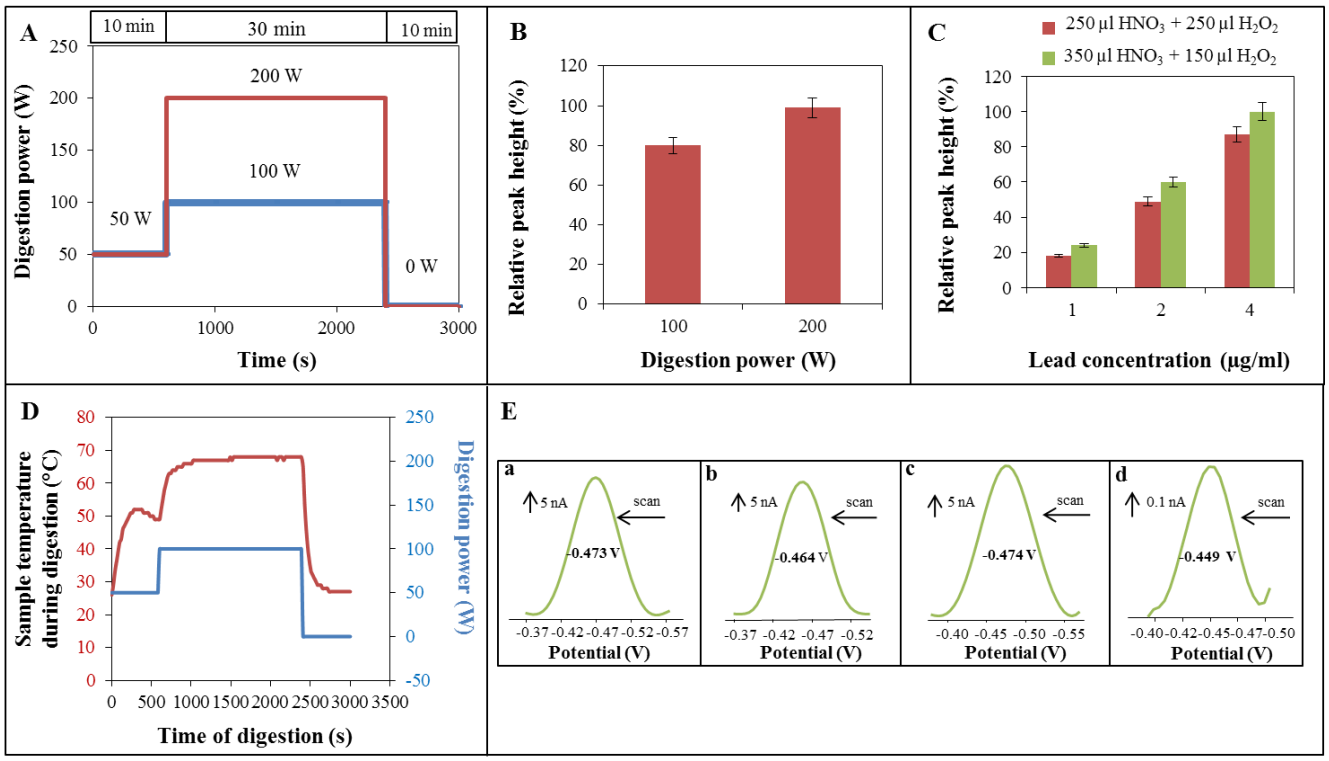

Figure 3. Optimization of microwave digestion. (**A**) Two digestion power with 30 min. long main period. (**B**) Lead(II) ions peak height as a function of digestion power for two digestion programmes. Peak height is presented as percentage of maximum value. (**C**) Changes in the height of lead(II) ions peak after their addition to 250 µl (65%, *w*/*w*) HNO_3 + 250 µl (30%, *w*/*w*) H_2O_2 and 350 µl (65%, *w*/*w*) HNO_3 + 150 µl (30%, *w*/*w*) H_2O_2. The highest response was used as 100 %. Concentrations of lead(II) ions were 1, 2 and 4 µg/ml. (**D**) Stages of mineralization plasma sample. (**E**) Electrochemical signals of lead. Lead was determined by DPV method. 0.2 M acetate buffer (pH=5) was used as an electrolyte. (**Ea**) Voltammograms of metals standards with concentration of 13 µg/ml, (**Eb**) voltammogram of lead standard after digestion with a concentration of 10 µg/ml, (**Ec**) voltammogram of lead standard added to the digestion mixture after digestion with 10 µg/ml concentrations of lead and (**Ed**) signals of lead detected in the plasma of paediatric patients with cancer after previous digestion.

The standard samples were divided into two groups according to ratio of digestion mixture and also the digestion power. In generally, the increasing digestion power led to the increase of electrochemical signal (Figs. 3A and B). During monitoring the effect of the ratio of the substances contained in the digestion mixture to an electrochemical signal of lead there was found that the highest and the most stable electrochemical signal is obtained in the ratio of the substances of digestion mixture 7:3 (350 µl 65% (*v*/*v*) HNO_3 and 150 µl 30% (*v*/*v*) H_2O_2) (Fig. 3C). All values were related to the maximum value. Figure 3D shows a real record of the changes in temperature and power during the digestion depending on the time.

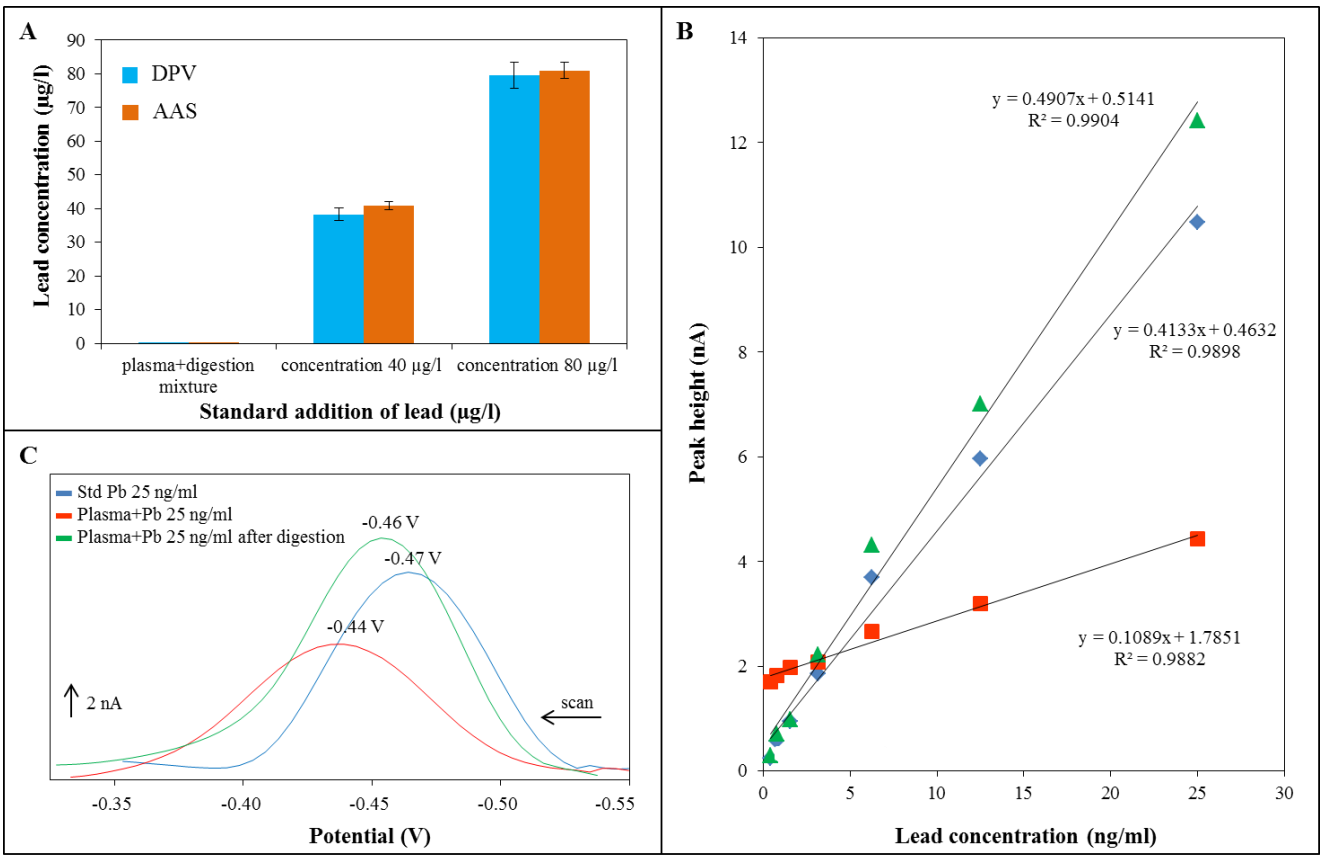

Figure 4. (**A**) Comparison of lead concentrations determined by electrochemical method (DPV) and atomic absorption spectrometry (AAS). Signals of lead after addition of standard and digestion with concentration 40 µg/l and/or 80 µg/l. (**B**) Determination of lead ions in (♦) standard, (■) in the mixture of lead and plasma, and (▲) in the mixture of lead and plasma after digestion. Determination of lead using DPV method. 0.2 M acetate buffer (pH=5) was used as an electrolyte. (**C**) Real voltammograms of lead peaks for lead standard (lead nitrate), mixture of lead and plasma, and mixture of lead and plasma after digestion.

Figure 3E shows a change in the electrochemical signal of lead, depending on the added lead standard before and after digestion. In Figure 3E the potential changes of lead standards depending on sample preparation (Figs. 3E(a), 3E(b), 3E(c)) are indicated too. In the DP voltammogram Figs. 3E(a) it is possible to observe the electrochemical signal of lead ions standard without mineralization, of which the characteristic peak for lead was recorded in the potential of -0.473 V. Voltammogram Fig. 3E(b) shows the electrochemical signal of lead after digestion, where we observed a shift of the peak to the potential -0.464 V. In the third case, where the standard of lead (concentration of 10 µg/ml) was added to the digested mixture of nitric acid and hydrogen peroxide (Fig. 3E(c)), the peak of lead was detected at potential -0.474 V, which is comparable with the case of indigested standard of Pb(II). Fig. 3E(d) shows voltammogram of real sample (plasma of the patient with a tumour disease) after digestion process. In comparison with lead standard in Figs. 3E(a), 3E(b), 3E(c) there was a significant shift of the peak potential of lead ions to -0.449 V.

In the following experiments, we investigated the effect of plasma on the electrochemical determination of lead. A comparison of concentrations of lead determined by electrochemical method

(DPV) and atomic absorption spectrometry (AAS) is shown in Fig. 4A. With the increasing concentration and addition of the standard, the values of measured lead are not significantly different and are comparable for both methods. AAS is suitable comparative method for the electrochemical determination of metals because it is not affected by the complexation of the individual components of the sample and determines the absolute value of the analyte in the sample [54-56]. Calibration curves of lead standard, digested plasma sample with a lead standard and indigested plasma sample with a lead standard are shown in Fig. 4B. The results show that the plasma has a great influence on electrochemical detection. For indigested plasma sample with a standard of lead the values of the standard curve ($y = 0.1089x + 1.7851$) significantly decreased and was shifted on the y-axis to more positive values (from 0.5141 to 1.7851) compared to digested plasma sample with a standard of lead ($y = 0.4907x + 0.5141$). For electrochemical detection of lead in the plasma there is always important to digest the sample before detection because of the higher sensitivity. From electrochemical signals it is clear that there is a potential shift of the lead signal and these changes are shown in Fig. 4C. Characteristic peak of lead standard was found at potential of -0.47 V. The use of a mixture of lead standard with plasma leads to the shift of peak potential, whereas for digested mixture of plasma and lead standard shift in the electrochemical signal to the value of -0.46 V was observed, for indigested mixture to -0.44 V.

3.4. Determination of metal levels in plasma at patients with tumour diseases

After that we optimized determination of lead ions in various types and modifications of blood serum including those based on EDTA using, we decided to use this technique to determine content of these ions in blood serum of patients with tumour disease. To lead ions, we also determined zinc, cadmium and copper ions as well as markers of the oxidative stress as metallothionein GSH, GSSG and their ratio. For determination of metal ions, we utilized both electrochemical and spectrometric methods. The electrochemical determination was done on mercury electrode, for which the microwave digestion of liquid sample in acids mixture was chosen. The total content of metals determined in a sample of patients with tumour disease is shown in Fig. 5A. Electrochemically determined zinc levels ranged from 1.9 – 22.6 μg/ml, levels of cadmium ranged from 0.001 – 0.006 μg/ml, lead levels ranged from 0.05 – 0.9 μg/ml and copper levels ranged from 0.8 – 7.5 μg/ml in blood plasma (Fig. 5A). Values of the metals in the plasma of paediatric patients with tumour disease determined by AAS are ranged as follows: zinc 1.5 – 19.4 μg/ml, cadmium 0.006 – 0.048 μg/ml, lead 0.16 – 0.76 μg/ml and copper 0.66 – 4.13 μg/ml (Fig. 5B). The measured levels of metals in the plasma of patients with tumour disease are comparable for both monitored methods. Ions of zinc and copper are usually present in the blood plasma. Physiological values of zinc in serum are 0.6 – 0.9 μg/ml and in the case of copper 0.9 – 1.9 μg/ml for children 6 – 12 years. Other determined metals, cadmium and lead, are in very low concentration levels in the human body. Physiological values of cadmium are in the range 0.0003 – 0.0012 μg/ml and lead < 0.25 μg/ml (for children 0.1 – 0.15 μg/ml) in blood. In comparison with physiological values, the determined concentrations of all metal ions were higher in the plasma of child patients. These results suggest that tumour diseases cause significant changes in metal ions level.

Highest levels of Zn(II) were detected in neuroblastoma and hepatoblastoma, Cd(II) in the non-Hodgkin lymphoma, Pb(II) for nephroblastoma and testicular germ cell tumour and Cu(II) in testicular germ cell tumour and hepatoblastoma.

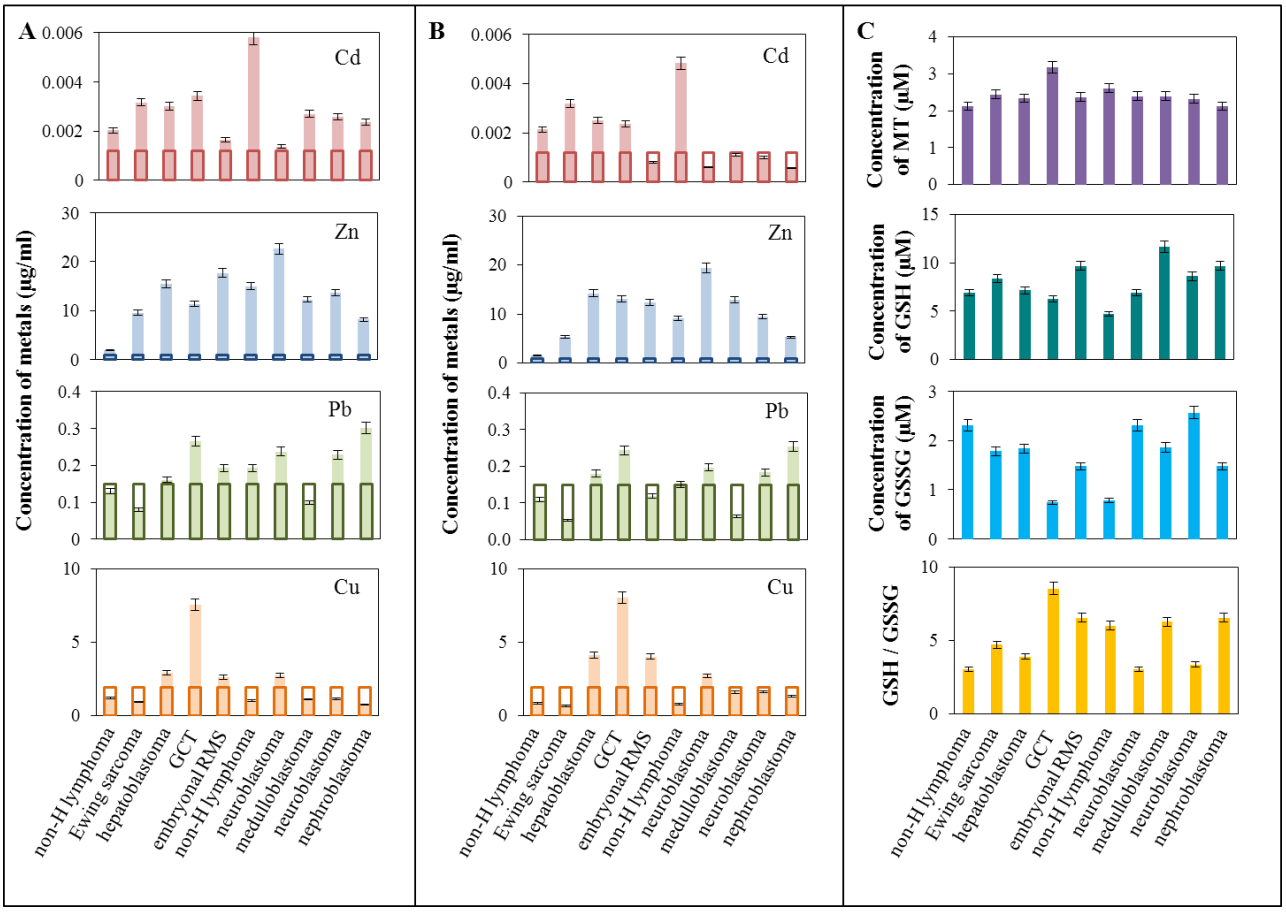

Figure 5. Determination of metal ions of Cd(II), Zn(II), Pb(II) and Cu(II) in plasma at patient with tumour diseases as follows: non-Hodgkin lymphoma (non-H lymphoma), Ewing sarcoma, hepatoblastoma, testicular germ cell tumour (GCT), embryonal rhabdomyosarcoma (embryonal RMS), neuroblastoma, medulloblastoma, and nephroblastoma. Determination of metal concentrations was proofed by (**A**) DPV and (**B**) AAS. The bold highlighted box shows the physiological value of the metal to humans. (**C**) Characterisation of biochemical parameters (MT, GSH, GSSG, and GSH/GSSG) in plasma of patient with tumour disease.

It was found that the loss of the protective effects of MT (against the action of heavy metals, maintaining intracellular homeostasis of zinc) leads to an escalation of disease processes [57]. MT has also the antioxidant effects. When the organism is intoxicated by heavy metals (Cd, Hg, Pb) MT can bind and deactivated them [58-60]. The detoxification is probably taken place in kidneys. However, the main functions of MT in the body are maintaining redox conditions, the transport of metal ions and the regulation of expression. Nowadays, the role in a cancer occurrence and development is also discussed. Therefore, the attention was also focused on the level of metallothionein (MT) in paediatric patients with tumour diseases. Metallothionein was already associated with cell proliferation [61],

wherein it is probably the transmitter and reservoir of Zn(II). Although the use of the MT as a tumour marker is not yet widespread due to ambiguous interpretation, it is known that the expression of MT is dependent on the degree of tumour differentiation, stage of disease and other characteristics of cancer cells [62]. Due to MT-based metal homeostasis in the cell and maintaining the level of free radicals, these proteins can significantly reduce the effectiveness of anticancer therapy [63,64]. MT relation to tumour proliferation is not completely understood and is currently the main subject of many research groups [40,65-72]. Values of metallothionein in children with cancer ranged from 2.11 to 3.18 μM (Fig. 5C). The highest values of metallothionein were found for GN tumour testes (3.18 μM) and VKS lymphadenopathy (2.61 μM). The MT can be viewed also as a proliferation marker in human patients. This area is studied in detail and first publications show a link between tumour diseases and the value of MT. Krizkova et al. [66] studied blood samples of paediatric patients and confirmed the suitability of MT as a tumour marker for early diagnostics and the most appropriate treatment setting.

Furthermore, in the plasma of patients with tumour diseases there were monitored additional biochemical parameters (GSH, GSSG and GSH/GSSG). Reduced glutathione (GSH), a ubiquitous thiol-containing tripeptide, is unanimously recognized to play a central role in cell biology [73]. It is highly implicated in the cellular defines against xenobiotics and naturally occurring toxic compounds such as free radicals and hydroperoxides. Consequently, GSH is an essential actor in several human diseases including cancer and cardio-vascular diseases [73]. Attention was focused on the determination GSH and GSSG, which were expressed as GSH/GSSG (Fig. 5C). Glutathione is found almost exclusively in its reduced form, since the enzyme, which reverts it from its oxidized form (GSSG) called glutathione reductase, is constitutively active and inducible upon oxidative stress. In the fact, the ratio of reduced to oxidized glutathione within cells is often used as a marker of cytotoxicity and also as a marker of considerable oxidative stress [73-75]. Measured values of the reduced (GSH) and oxidized (GSSG) forms of glutathione in patients with tumour diseases are shown in Fig. 5C. The lowest values of GSH and GSSG were detected in patients with GN tumour testis and VKS lymphadenopathy. The value of the ratio of GSH/GSSG is app. 10 at the healthy population. Our results show a reduction in the ratio of GSH/GSSG compared to healthy individuals, indicating a slowdown in the reduction of glutathione, an antioxidant defences slowdown and subsequently increased risk of oxidative stress. The lowest values of the ratio of GSH/GSSG were detected in patients with neuroblastoma and NH lymphoma.

4. CONCLUSION

In this study, we employed the electrochemical and spectrometric methods for analysis of samples obtained from children suffering from a tumour disease. The multi-instrumental approach opens new possibilities for studying of the development of a disease.

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Conflict of interest:
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References

1. P. B. Kurt-Karakus, *Environ. Int.*, 50 (2012) 47.
2. J. O. Nriagu, *Environ. Pollut.*, 50 (1988) 139.
3. R. B. Hayes, *Cancer Causes Control*, 8 (1997) 371.
4. M. A. Fuertes, C. Alonso and J. M. Perez, *Chem. Rev.*, 103 (2003) 645.
5. D. Kriebel, N. L. Sprince, E. A. Eisen, I. A. Greaves, H. A. Feldman and R. E. Greene, *Br. J. Ind. Med.*, 45 (1988) 167.
6. D. Bellinger, *Epidemiology*, 6 (1995) 101.
7. G. T. Shi, Z. L. Chen, S. Y. Xu, J. Zhang, L. Wang, C. J. Bi and J. Y. Teng, *Environ. Pollut.*, 156 (2008) 251.
8. G. T. Shi, Z. L. Chen, C. J. Bi, L. Wang, J. Y. Teng, Y. S. Li and S. Y. Xu, *Atmos. Environ.*, 45 (2011) 764.
9. S. Maas, R. Scheifler, M. Benslama, N. Crini, E. Lucot, Z. Brahmia, S. Benyacoub and P. Giraudoux, *Environ. Pollut.*, 158 (2010) 2294.
10. J. A. Acosta, A. F. Cano, J. M. Arocena, F. Debela and S. Martinez-Martinez, *Geoderma*, 149 (2009) 101.
11. H. I. Afridi, T. G. Kazi, M. B. Arain, M. K. Jamali, G. H. Kazi and N. Jalbani, *J. AOAC Int.*, 90 (2007) 470.
12. H. I. Afridi, T. G. Kazi, A. G. Kazi, F. Shah, S. K. Wadhwa, N. F. Kolachi, A. Q. Shah, J. A. Baig and N. Kazi, *Biol. Trace Elem. Res.*, 144 (2011) 164.
13. A. Blazewicz, G. Orlicz-Szczesna, A. Prystupa and P. Szczesny, *J. Trace Elem. Med. Biol.*, 24 (2010) 14.
14. P. J. Parsons, *Environ. Res.*, 57 (1992) 149.
15. M. Ghaedi, A. Shokrollahi, K. Niknam, E. Niknam, S. Derki and M. Soylak, *J. AOAC Int.*, 92 (2009) 907.
16. J. Kruusma, C. E. Banks, L. Nei and R. G. Compton, *Anal. Chim. Acta*, 510 (2004) 85.
17. C. S. Muniz, J. M. Larchante-Gayon, J. I. G. Alonso and A. Sanz-Medel, *J. Anal. At. Spectrom.*, 14 (1999) 193.
18. S. K. Wadhwa, T. G. Kazi, A. A. Chandio, H. I. Afridi, N. F. Kolachi, S. Khan, G. A. Kandhro, S. Nasreen, A. Q. Shah and J. A. Baig, *Biol. Trace Elem. Res.*, 144 (2011) 86.
19. F. Shah, T. G. Kazi, H. I. Afridi, N. Kazi, J. A. Baig, A. Q. Shah, S. Khan, N. F. Kolachi and S. K. Wadhwa, *Biol. Trace Elem. Res.*, 141 (2011) 131.
20. R. H. Barton, D. Waterman, F. W. Bonner, E. Holmes, R. Clarke, J. K. Nicholson and J. C. Lindon, *Mol. Biosyst.*, 6 (2010) 215.
21. C. Coch, C. Luck, A. Schwickart, B. Putschli, M. Renn, T. Holler, W. Barchet, G. Hartmann and M. Schlee, *PLoS ONE*, 8 (2013) 1.
22. A. Cornall, L. Sharma, A. Solomon, P. R. Gorry, S. M. Crowe, P. U. Cameron and S. R. Lewin, *J. Virol. Methods*, 165 (2010) 90.
23. M. J. Evans, J. H. Livesey, M. J. Ellis and T. G. Yandle, *Clin. Biochem.*, 34 (2001) 107.
24. C. K. Chuang, S. P. Lin, Y. T. Lin and F. Y. Huang, *Clin. Chem.*, 44 (1998) 1052.
25. R. Patil, S. Shukre, R. Paranjape and M. Thakar, *Scand. J. Clin. Lab. Invest.*, 73 (2013) 452.
26. V. Gonzalez-Covarrubias, A. Dane, T. Hankemeier and R. J. Vreeken, *Metabolomics*, 9 (2013) 337.
27. D. Hynek, J. Prasek, J. Pikula, V. Adam, P. Hajkova, L. Krejcova, L. Trnkova, J. Sochor, M. Pohanka, J. Hubalek, M. Beklova, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 5980.

28. S. Krizkova, O. Zitka, V. Adam, M. Beklova, A. Horna, Z. Svobodova, B. Sures, L. Trnkova, L. Zeman and R. Kizek, *Czech J. Anim. Sci.*, 52 (2007) 143.
29. J. Sochor, P. Majzlik, P. Salas, V. Adam, L. Trnkova, J. Hubalek and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 414.
30. O. Krystofova, L. Trnkova, V. Adam, J. Zehnalek, J. Hubalek, P. Babula and R. Kizek, *Sensors*, 10 (2010) 5308.
31. P. Sobrova, L. Vyslouzilova, O. Stepankova, M. Ryvolova, J. Anyz, L. Trnkova, V. Adam, J. Hubalek and R. Kizek, *PLoS ONE*, 7 (2012) 1.
32. P. Majzlik, A. Strasky, V. Adam, M. Nemec, L. Trnkova, J. Zehnalek, J. Hubalek, I. Provaznik and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 2171.
33. A. Kleckerova, P. Sobrova, O. Krystofova, J. Sochor, O. Zitka, P. Babula, V. Adam, H. Docekalova and R. Kizek, *Int. J. Electrochem. Sci.*, 6 (2011) 6011.
34. J. Petrlova, D. Potesil, R. Mikelova, O. Blastik, V. Adam, L. Trnkova, F. Jelen, R. Prusa, J. Kukacka and R. Kizek, *Electrochim. Acta*, 51 (2006) 5112.
35. G. L. Long and J. D. Winefordner, *Anal. Chem.*, 55 (1983) A712.
36. V. Adam, O. Zitka, D. Huska, S. Krizkova, J. Strnadel, V. Horak, T. Vaculovic, K. Novotny, V. Kanicky, J. Kaiser and R. Kizek, *Febs J.*, 276 (2009) 96.
37. G. Aragay and A. Merkoci, *Electrochim. Acta*, 84 (2012) 49.
38. D. Huska, O. Zitka, V. Adam, M. Beklova, S. Krizkova, L. Zeman, A. Horna, L. Havel, J. Zehnalek and R. Kizek, *Czech J. Anim. Sci.*, 52 (2007) 37.
39. D. Hynek, L. Krejcova, S. Krizkova, B. Ruttkay-Nedecky, J. Pikula, V. Adam, P. Hajkova, L. Trnkova, J. Sochor, M. Pohanka, J. Hubalek, M. Beklova, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 943.
40. M. Kremplova, L. Krejcova, D. Hynek, P. Barath, P. Majzlik, V. Horak, V. Adam, J. Sochor, N. Cernei, J. Hubalek, R. Vrba and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 5893.
41. S. Krizkova, O. Krystofova, L. Trnkova, J. Hubalek, V. Adam, M. Beklova, A. Horna, L. Havel and R. Kizek, *Sensors*, 9 (2009) 6934.
42. S. Krizkova, P. Ryant, O. Krystofova, V. Adam, M. Galiova, M. Beklova, P. Babula, J. Kaiser, K. Novotny, J. Novotny, M. Liska, R. Malina, J. Zehnalek, J. Hubalek, L. Havel and R. Kizek, *Sensors*, 8 (2008) 445.
43. P. Majzlik, J. Prasek, L. Trnkova, J. Zehnalek, V. Adam, L. Havel, J. Hubalek and R. Kizek, *Lis. Cukrov. Repar.*, 126 (2010) 413.
44. J. F. Ping, J. Wu, Y. B. Ying, M. H. Wang, G. Liu and M. Zhang, *J. Agric. Food Chem.*, 59 (2011) 4418.
45. I. Svancara, K. Vytras, K. Kalcher, A. Walcarius and J. Wang, *Electroanalysis*, 21 (2009) 7.
46. I. Svancara, A. Walcarius, K. Kalcher and K. Vytras, *Cent. Eur. J. Chem.*, 7 (2009) 598.
47. J. Zima, I. Svancara, J. Barek and K. Vytras, *Crit. Rev. Anal. Chem.*, 39 (2009) 204.
48. H. G. Gordan and N. L. Larson, *Am. J. Clin. Pathol.*, 23 (1955) 613.
49. S. M. Lewis and C. T. Stoddart, *Lab. Pract.*, 20 (1971) 787.
50. D. Carroll and H. C. Starkey, *Clay Clay Min.*, 19 (1971) 321.
51. S. Garcia-Salgado, M. A. Quijano and M. M. Bonilla, *Anal. Chim. Acta*, 714 (2012) 38.
52. C. Bettiol, L. Stievano, M. Bertelle, F. Delfino and E. Argese, *Appl. Geochem.*, 23 (2008) 1140.
53. V. Sandroni and C. M. M. Smith, *Anal. Chim. Acta*, 468 (2002) 335.
54. D. Fialova, M. Kremplova, D. Hynek, M. Konecna, J. Kaiser, R. Malina, J. Kynicky, O. Krystofova, R. Kizek and V. Adam, *Int. J. Electrochem. Sci.*, 8 (2013) 7853.
55. M. Konecna and J. Komarek, *Spectroc. Acta Pt. B-Atom. Spectr.*, 62 (2007) 283.
56. M. Konecna, J. Komarek and L. Trnkova, *Spectroc. Acta Pt. B-Atom. Spectr.*, 63 (2008) 700.
57. W. H. Yu, W. J. Lukiw, C. Bergeron, H. B. Niznik and P. E. Fraser, *Brain Res.*, 894 (2001) 37.
58. T. Eckschlager, V. Adam, J. Hrabeta, K. Figova and R. Kizek, *Curr. Protein Pept. Sci.*, 10 (2009) 360.

59. P. Babula, M. Masarik, V. Adam, T. Eckschlager, M. Stiborova, L. Trnkova, H. Skutkova, I. Provaznik, J. Hubalek and R. Kizek, *Metallomics*, 4 (2012) 739.
60. S. Krizkova, M. Ryvolova, J. Hrabeta, V. Adam, M. Stiborova, T. Eckschlager and R. Kizek, *Drug Metab. Rev.*, 44 (2012) 287.
61. W. W. Nagel and B. L. Vallee, *Proc. Natl. Acad. Sci. U. S. A.*, 92 (1995) 579.
62. S. E. Theocharis, A. P. Margeli, J. T. Klijanienko and G. P. Kouraklis, *Histopathology*, 45 (2004) 103.
63. G. Chu, *J. Biol. Chem.*, 269 (1994) 787.
64. R. P. Perez, *Eur. J. Cancer*, 34 (1998) 1535.
65. L. Krejcova, I. Fabrik, D. Hynek, S. Krizkova, J. Gumulec, M. Ryvolova, V. Adam, P. Babula, L. Trnkova, M. Stiborova, J. Hubalek, M. Masarik, H. Binkova, T. Eckschlager and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 1767.
66. S. Krizkova, M. Masarik, P. Majzlik, J. Kukacka, J. Kruseova, V. Adam, R. Prusa, T. Eckschlager, M. Stiborova and R. Kizek, *Acta Biochim. Pol.*, 57 (2010) 561.
67. M. Namdarghanbari, W. Wobig, S. Krezoski, N. M. Tabatabai and D. H. Petering, *J. Biol. Inorg. Chem.*, 16 (2011) 1087.
68. J. Sochor, D. Hynek, L. Krejcova, I. Fabrik, S. Krizkova, J. Gumulec, V. Adam, P. Babula, L. Trnkova, M. Stiborova, J. Hubalek, M. Masarik, H. Binkova, T. Eckschlager and R. Kizek, *Int. J. Electrochem. Sci.*, 7 (2012) 2136.
69. J. Sochor, M. Pohanka, B. Ruttkay-Nedecky, O. Zitka, D. Hynek, P. Mares, L. Zeman, V. Adam and R. Kizek, *Cent. Eur. J. Chem.*, 10 (2012) 1442.
70. S. Takahashi, *J. Hematol. Oncol.*, 5 (2012) 1.
71. A. Vasatkova, S. Krizova, O. Krystofova, V. Adam, L. Zeman, M. Beklova and R. Kizek, *Neuroendocrinol. Lett.*, 30 (2009) 163.
72. A. Zamirska, L. Matusiak, P. Dziegiel, G. Szybejko-Machaj and J. C. Szepietowski, *Pathol. Oncol. Res.*, 18 (2012) 849.
73. R. Locigno and V. Castronovo, *Int. J. Oncol.*, 19 (2001) 221.
74. S. Carelli, A. Ceriotti, A. Cabibbo, G. Fassina, M. Ruvo and R. Sitia, *Science*, 277 (1997) 1681.
75. D. M. Townsend, K. D. Tew and H. Tapiero, *Biomed. Pharmacother.*, 57 (2003) 145.