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Attomole voltammetric determination of metallothionein

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Dedicated to Prof. Rudolf Brdicka.

Abstract

Number of authors have concerned with electrochemical analysis of metallothionein. Recently new electroanalytical techniques enabling determination of MT at picomole level has been suggested. The aim of the presented work was to show advantages and disadvantages of the different electrochemical procedures, which are commonly used for the detection of MT—(i) cyclic voltammetry, (ii) differential pulse voltammetry, and (iii) Brdicka reaction. Primarily we aimed on improvement of the mentioned techniques. Using of reducing agent (tris(2-carboxyethyl)phosphine) and combination of the mentioned method with adsorptive transfer stripping technique (AdTS) were the main improvements of the voltammetric method. The detection limits of metallothionein measured by cyclic voltammetry (CV), differential pulse voltammetry (DPV) and DPV Brdicka reaction were 0.5 pmol, 4 fmol and 10 amol, respectively. In addition AdTS DPV Brdicka reaction was used for the determination of metallothionein in human blood serum of 11-year-old girl, which were lead poisoned.

Keywords: Metallothionein; Mercury electrode; Differential pulse voltammetry; Cyclic voltammetry; Brdicka reaction; Adsorptive transfer stripping technique; Reducing agent; Tris(2-carboxyethyl)phosphine; Lead poisoning; Human blood serum

1. Introduction

Metallothionein (MT) belongs to group of intracellular, high molecular and cysteine-rich proteins with molecular weight from 6 to 10 kDa [1]. The MT was discovered in 1957, when Margoshes and Valee isolated it from horse kidney [2]. It is known that aromatic amino acids are not present in the MT molecule and twenty cysteines usually occur in its primary sequence at these repetitions: Cys-**X**-Cys, Cys-Cys-**X**-Cys-Cys, Cys-**X**-Cys-Cys, where **X** represents other aminoacid than cysteine [1,3,4]. MTs consist of two binding domains (α and β) that are assembled from cysteine clusters. Cysteine sulfhydryl groups participate in covalent bindings with heavy metals. The N-terminal part of the protein is marked as α-domain, which has three binding places for divalent ions. β-Domain (C-terminal part) has the ability to bind four divalent ions of heavy metals (Fig. 1A). In the case of univalent ions of heavy metals, MT is able to bind 12 metal ions [1,4]. In addition, presence of metallothioneins has not proved in plants tissues and organs except plant metallothionein-like proteins [5].

Number of analytical techniques including electrochemistry [6–14] is used for the determination of MT. Many authors have been giving attention to electrochemical determination and study of MT for more than 20 years [15–24]. For the basic study of electrochemical behaviour of MT, cyclic voltammetry and differential pulse polarography were usually used [25–29]. In addition, more than 70 years ago Brdicka discovered the catalytic evolution of hydrogen in the presence of cobalt(III) solution and

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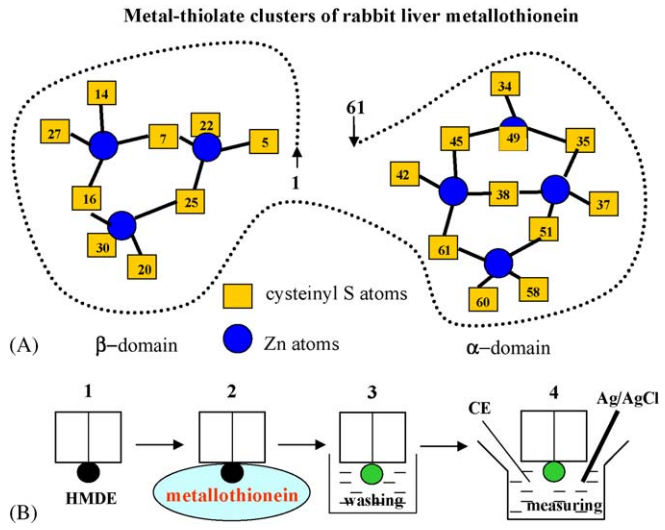

Fig. 1. Scheme of the metallothionein clusters domains (A). Scheme of adsorptive transfer stripping technique used for determination of metallothionein (B); (1) renewing of the hanging mercury drop electrode (HMDE) surface; (2) adsorbing of MT in a drop solution onto the HMDE surface at open circuit; (3) washing electrode in a supporting electrolyte; (4) analysis of MT by CV, DPV and/or DPV Brdicka reaction in a supporting electrolyte.

of proteins [30–33]. This reaction has been intensively using for electrochemical determination of proteins and has been modified by number of authors [34–43]. The exchange of cobalt by nickel [44–48], rhodium [49] or platinum complexes was also used [50,51]. The sensitivity of the proteins determination was increased up to 0.3 μg/l through changing some parameters of original procedures (e.g. temperature, type and concentration of the catalytic metal, composition of the basic electrolyte) [50].

The aim of the presented work was to show advantages and disadvantages of the different electrochemical procedures, which are commonly used for the detection of MT—(i) cyclic voltammetry, (ii) differential pulse voltammetry, and (iii) Brdicka reaction. In addition we selected the most sensitive methods and used it for the determination of MT in a human blood serum sample.

2. Materials and methods

2.1. Chemicals

Rabbit liver MT (MW 7143), containing 5.9% Cd and 0.5% Zn, was purchased from Sigma-Aldrich (St. Louis, USA). Tris(2-carboxyethyl)phosphine (TCEP) was produced by Molecular Probes (Evgen, Oregon, USA). Sodium chloride and other used chemicals were purchased from Sigma-Aldrich. The stock standard solutions of MT at 10 μg/ml was prepared by ACS water (Sigma–Aldrich, USA) and stored in the dark at −20 °C. Working standard solutions were prepared daily by dilution of the stock solutions. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14/3 M KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany).

2.2. Human blood serum samples

Human blood serum samples were obtained from the Department of Clinical Biochemistry and Pathobiochemistry, 2nd Faculty of Medicine Charles University, Czech Republic. The sample was prepared by heat treatment and solvent precipitation. Briefly, the 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, 15 000 *g* for 30 min. (Eppendorf 5402, USA). Heat treatment and solvent precipitation effectively denature and remove high molecular weight proteins out from samples [37]. Determination of MT in the human blood serum samples was performed by optimised AdTS DPV Brdicka reaction, for other details see Section 3. Analysed sample volume was 5 μl.

2.3. Electrochemical measurements

Electrochemical measurements were performed with AUTOLAB Analyser (EcoChemie, Netherlands) connected to VA-Stand 663 (Metrohm, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl/3 M KCl electrode and the auxiliary electrode was a graphite electrode. The supporting electrolyte was prepared by mixing buffer components except sodium chloride. For smoothing and baseline correction the software GPES 4.4 supplied by EcoChemie was employed.

2.3.1. Adsorptive transfer stripping technique

Principle of the AdTS (Fig. 1B) is based on the strong adsorbing of the studied analyte on the electrode surface at an open electrode circuit (see Fig. 1B(2)). The excess of analyte is rinsed from the surface of the working electrode in the buffer (Fig. 1B(3)). The adsorbed analyte is finally detected in the presence of indifferent electrolyte (Fig. 1B(4)).

2.3.2. AdTS cyclic voltammetry (CV) and AdTS differential pulse voltammetry (DPV) of metallothionein

The amount of MT was measured using AdTS CV and/or AdTS DPV. The samples of the MT were reduced before each measurement by 1 mM tris(2-carboxyethyl)phosphine addition according to [52,53], for other details see Section 3. The supporting electrolyte was 0.5 M sodium chloride, pH 6.4. AdTS CV parameters were as follows: an initial potential of 0 V, a vertex potential −1.2 V, an end potential 0 V, a step potential 2 mV, a scan rate 20, 40, 80, 160, 320 or 640 mV/s, a time of accumulation of MT 120 s, E_{ads} = 0 V. AdTS DPV parameters were as follows: an initial potential of −1.2 V, an end potential −0.3 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV, E_{ads} = 0 V. All experiments were carried out at room temperature (22–24 °C). The CV and DPV samples analyzed were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 240 s.

2.3.3. AdTS DPV Brdicka reaction of MT

In our studies, 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; surface-active agent was not added. 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/s, a modulation amplitude of 250 mV, E_{ads} = 0 V. Temperature of supporting electrolyte was tested.

2.4. Graphite furnace atomic absorption spectrometry of lead

The Pb content of the human blood serum was determined by graphite furnace atomic absorption spectrometry (GFAAS) with Zeman-effect background correction (220 Z, Varian Australia), using graphite tubes. The matrix modifier for whole blood samples was prepared from 0.2 ml concentrated nitric acid, 0.5 ml Triton X-100 and 0.2 g diammonium hydrogen phosphate, then the volume was filled up to 100 ml with deionised water. For sample preparation 100 μl of sample was added to 1900 μl of matrix modifier, then the mixture was analysed by GFAAS.

2.5. Statistical analysis

STATGRAPHICS® (Statistical Graphics Corp®, USA) was used for statistical analyses. Results are expressed as mean ± S.D. unless noted otherwise. Value of $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Cyclic voltammetry

Number of authors studied electrochemical behaviour of metallothionein (MT) containing cadmium and zinc on the surface of hanging mercury drop electrode (HMDE) by cyclic voltammetry [19,22,54,55]. Three MT signals, which were called as peaks A, B and C, were observed [55]. In our experiments we have proved that maintenance of the redox state of the protein represents serious problem at the electrochemical analysis [53]. Recently, we found out via cyclic voltammetry that we could use for the maintenance of the protein redox state the reducing agent – phosphine (TCEP), which does not contain sulphur [53]. Here, we studied 10 μM MT (5 μl drop) by AdTS CV in the presence of 0.5 M NaCl (pH 6.4). We were interested in the issue how does the CV voltammogram look like, if we reduced the MT by TCEP before measurements. We observed four signals of MT – peaks A, B, C and D (Fig. 2A) but the height of the peaks were higher in comparison with non-reduced ones. Peak A appeared at potential −0.2 V and corresponds to reduction of the MT clustered cadmium(II): $CdT + 2e^- = Cd(0) + T^{2-}$. At the anodic scan, it was possible to observe the back forming of the Cd-complex (peak B at potential about −0.73 V): $Cd(0) + T^{2-} - 2e^- = CdT$. Peak C (about potential of −0.7 V at anodic scan) represents dissolution of mercury electrode in presence of CdT complex; $CdT + Hg(0) - 2\ e^- = Cd^{2+} + Hg(II)T$ [55]. The last peak exists on the anodic scan at −0.2 V which is quoted as peak D and that seems to be the reverse peak of A reduction. This concern relates to the fact that the voltammogram

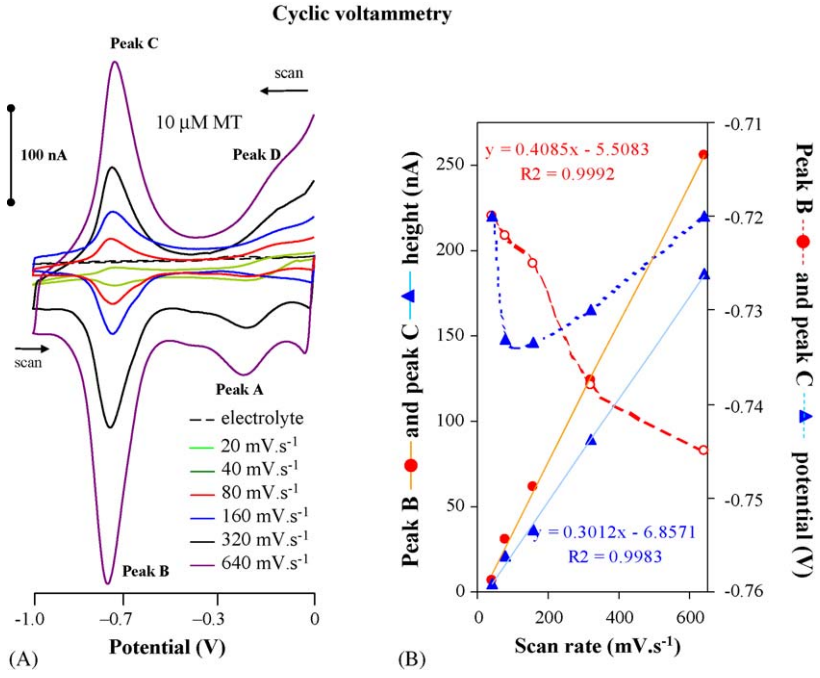

Fig. 2. Cyclic voltammetry. CV voltammograms of basic electrolyte (0.5 M NaCl, pH 6.4) and 10 μM MT at different scan rates of 40, 80, 160, 320 and 640 mV/s (A). Dependence of height and potential of signals B and C on scan rate (B). The supporting electrolyte was sodium chloride: 0.5 M NaCl, pH 6.4. AdTS CV parameters were as follows: an initial potential of 0 V, a vertex potential −1.2 V, an end potential 0 V, a step potential 2 mV, a time of accumulation of MT 120 s, E_{ads} = 0 V. For other details see Section 2.

Table 1
Detection limits of metallothionein ($n = 5$) estimated by different electroanalytical methods

Method (%)	Limit of detection[a]		R.S.D.[c]
CV	120 nM	0.5 pmol[b]	9.5
DPV	0.8 nM	4 fmol[b]	7.9
Brdicka reaction	2 pM	10 amol[b]	5.7

[a] Limits of detection (3 *S*/*N*).
[b] Limits of detection per 5 μl.
[c] Relative standard deviations.

integrates two pairs of peaks (B/C and A/D, the second being less reversible. Moreover we studied the influence of different scan rate values (40, 80, 160, 320 or 640 mV/s) on the observed MT signals. The obtained voltammograms are shown in Fig. 2A. The dependences of the heights of the peaks B and C on different scan rates are strictly linear ($R^2_{\text{peak A}} = 0.9992$ and $R^2_{\text{peak B}} = 0.9983$; Fig. 2B). The potentials of the peaks are shifted about 25 mV according to different scan rates (Fig. 2B). Sensitivity of the metallothionein determination by AdTS cyclic voltammetry was low—AdTS CV detection limit (3 *S*/*N*) of MT was about 120 nM (0.5 pmol in 5 μl drop, scan rate was 640 mV/s, Table 1).

3.2. Differential pulse voltammetry

It is already known that differential pulse voltammetry is suitable for a sensitive determination of heavy metals especially [56,57]. Moreover, this electrochemical technique has been intensively used for the study of electrochemical metallothionein behaviour too [27–29,58,59]. On the base of the abovementioned results with reducing agent (TCEP), we primarily studied the effect of the TCEP on AdTS DPV signals of MT (time of MT accumulation 120 s). The voltammogram of 10 μM MT without TCEP is shown in Fig. 3Aa. MT gives a few electrochemical signals of complexes of MT with metals (Cd and Zn) MT(Cd); MT(Zn), CdT′, ZnT′, CdT and ZnT. Except the mentioned signals, the voltammograms usually contain the redox signals of free ions Cd(II) and Zn(II). We observed all assumed electrochemical signals of MT complexes with Cd(II) and Zn(II)—ZnT: −0.99 V; ZnT′: −0.87 V; CdT′: −0.71 V; CdT: −0.65 V; MT(Zn): −0.49 V; MT(Cd): −0.42 V but none of the free metals ions signals. In our previous experiments, we proved that free ions of heavy metals are not able to be adsorbed which makes detection of the free ions impossible [52,60].

In case that we reduced MT by 1 mM TCEP, we observed significant decrease of ZnT′ signal height. On the other hand, heights of the signals of CdT and MT(Cd) increased about 30–40% compared to non-TCEP signals. In addition, the signal CdT′ disappeared. The observed changes could be caused by reduction of MT oxidised clusters. Thereafter we always used 1 mM TCEP for reduction of MT. The detailed description of the signals will be published elsewhere.

Dependence of the MT (125 fmol in 5 μl drop) current response (signal CdT) on the accumulation time is shown in inset in Fig. 3B. Perfect coverage of the electrode surface – forming the surface assembled monolayer – was probably reached at longer time than 180 s (inset in Fig. 3B). Furthermore, we studied the dependence of the CdT peak height on different MT concentrations (Fig. 3B). The dependence was strictly linear in the concentration range 15.6–250 fmol ($y = 0.0098x - 0.0784$; $R^2 = 0.9965$). The detection limit (3 *S*/*N*) was about 4 fmol per 5 μl drop (Table 1).

3.3. Catalytic signals—Brdicka procedure

It has been known more than 70 years that it is suitable to use the catalytic signal of hydrogen evolution in the presence of ammonium buffer (1 M $NH_4Cl + NH_4OH$) containing cobalt solution $Co(NH_3)_6Cl_3$ for the determination of the proteins containing cysteine amino acids, e.g. metallothionein (see in Fig. 1) [31–33,37,41]. A number of authors have concerned

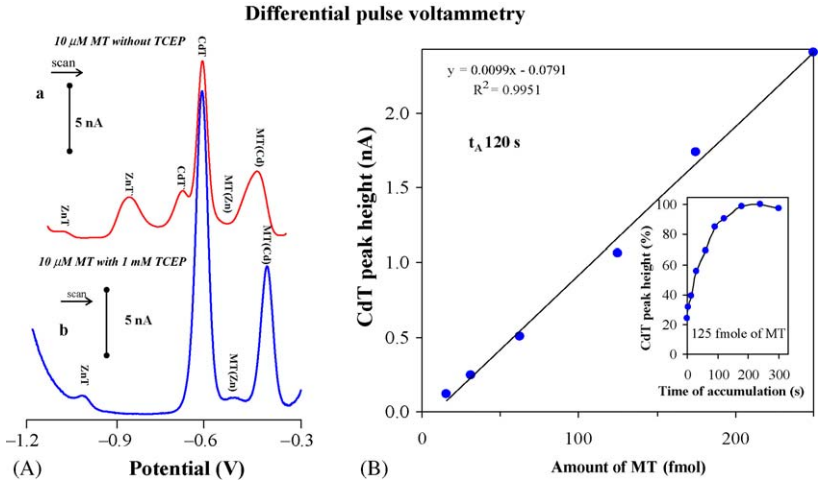

Fig. 3. Differential pulse voltammetry. Typical DPV voltammograms of 10 μM MT without (a) or with (b) 1 mM TCEP measured in 0.5 M NaCl, pH 6.4 (A). Dependence of CdT peak height on different MT concentration (B); bottom inset: dependence of CdT peak height on accumulation time. Peak height of 1.1 nA corresponds to 100%. The supporting electrolyte was sodium chloride: 0.5 M NaCl, pH 6.4. AdTS DPV parameters were as follows: an initial potential of −1.2 V, an end potential −0.3 V, a modulation time 0.057 s, a time interval 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV, time of accumulation of MT 120 s, $E_{ads} = 0$ V. For other details see Section 2.

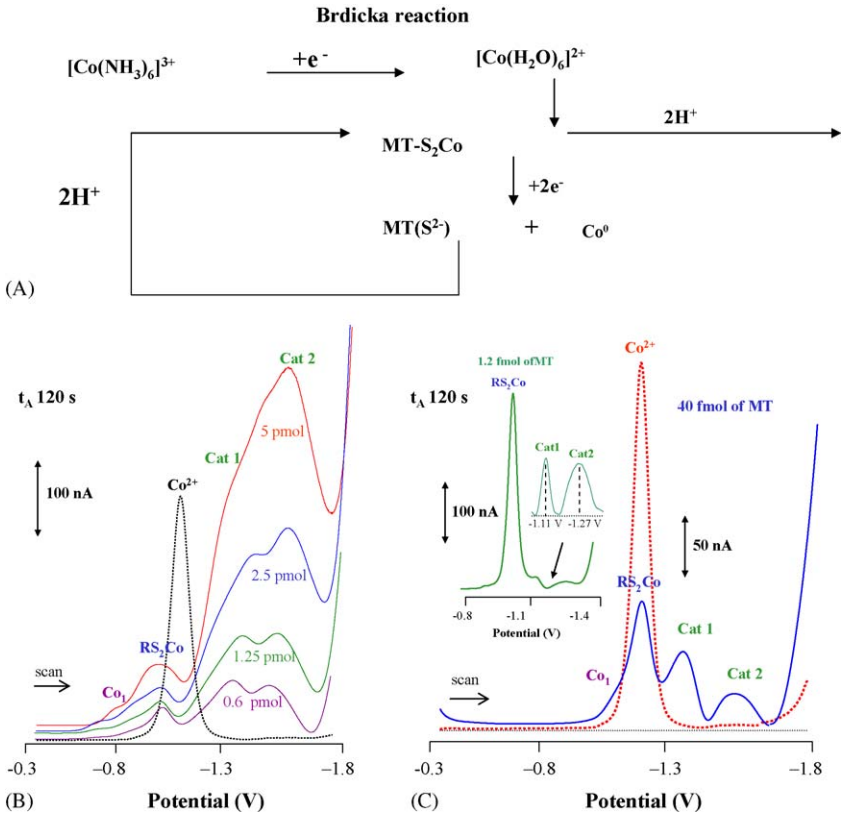

Fig. 4. Scheme of Brdicka reaction (A). AdTS DPV Brdicka reaction. Typical DPV voltammograms of different MT concentrations measured in the presence of supporting electrolyte contained 1 mM $Co(NH_3)_6Cl_3$ and 1 M $NH_3(aq) + NH_4Cl$, pH = 9.6; dotted line: voltammogram of supporting electrolyte without MT (B). DPV voltammograms of 40 fmol MT and 1.2 fmol MT (inset) measured in the presence of supporting electrolyte contained 1 mM $Co(NH_3)_6Cl_3$ and 1 M $NH_3(aq) + NH_4Cl$), pH = 9.6; dotted line: voltammogram of supporting electrolyte (C). 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/s, a modulation amplitude of 250 mV, the time of accumulation of MT 120 s, E_{ads} = 0 V. All measurements were carried at temperature of 5 °C.

with explanations of the processes, which proceed on the surface of working mercury electrode during Brdicka reaction. Due to this, there has been suggested a few possible explanations of hydrogen evolution from supporting electrolyte in the presence of a protein (Fig. 4A), see in Refs. [15,16,35,40], but the exact mechanism is not still clear.

On the base of the abovementioned results, we reduced MT before each measurement by Brdicka reaction. The voltammograms of 0.6, 1.25, 2.5 and 5 pmol MT are shown in Fig. 4B. We observed during modified Brdicka reaction by AdTS DPV analysis four MT signals—Co_1, RS_2Co, Cat1 and Cat2 (Fig. 4B). Signals of Cat1 a Cat2 correspond to the reduction of hydrogen at the mercury electrode [40]. Another signal, which is appeared at the potential about −1.0 V, relates with the reduction of the RS_2Co complex [40]. In addition the signal called Co_1 could result from reduction of $[Co(H_2O)_6]^{2+}$ [40]. It clearly follows from the figure that character of the mentioned MT signals change with different MT amount. Signal Co_1 decreased and shifted to more negative potential with decreasing MT amount. The signal is almost un-detectable at MT amount under 0.6 pmol. The others mentioned MT signals of Brdicka reaction (RS_2Co, Cat1 and Cat2) are getting well developed and separated with decreasing MT concentration. Typical voltammogram of 40 fmol MT is shown in Fig. 4C. If the concentration of MT still decreased, the signal RS_2Co markedly increased (inset in Fig. 4C). In addition RS_2Co and Cat signals decreased and slowly shifted to more positive potential according to decreasing MT concentration (see in inset Fig. 4C).

Recently it was published that analysis of MT by Brdicka reaction is effective to perform at temperatures in the range of 5–10 °C [41,50]. Therefore, we studied the influence of the different temperatures on MT determination, to be specific on height of Cat2 signal. MT (40 fmol) was adsorbed for 120 s on the surface of HMDE, the electrode was subsequently washed and the analysis was performed in the presence of Brdicka solution at different temperatures (5, 10, 18, 25 and 30 °C). We observed decrease of Cat2 signal about 0.6% per unit of Celsius degree increase (Fig. 5A). That is why it is effective to perform the MT determination by modified Brdicka reaction AdTS DPV technique at low temperature of supporting electrolyte. That is why we selected 5 °C as the most suitable temperature for the following MT determination by Brdicka reaction. On the base of the obtained results it is possible to estimate that adsorptive controlled reaction probably run on the electrode surface [41].

In addition, we studied the influence of accumulation time on determination of MT (40 fmol, Fig. 5B). The height of Cat2 signal increased with rising accumulation time. On the other hand

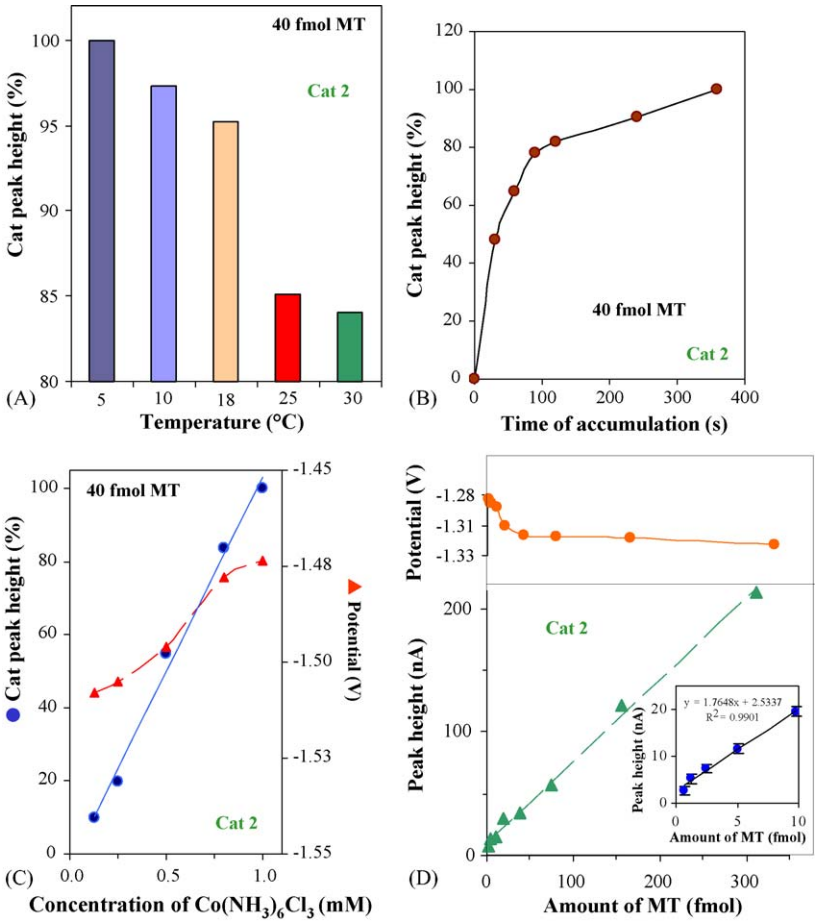

Fig. 5. AdTS DPV Brdicka reaction. Influence of temperature on Cat2 peak height (A). Dependence of Cat2 peak height on accumulation time (B). Dependence of Cat peak height and/or potential on different concentrations of $Co(NH_3)_6Cl_3$ (C). Peak height of 44 nA corresponds to 100%. Influence of different MT concentration on peak height and potential of Cat2 signal (D bellow and up, respectively); in inset in D: dependence of Cat2 on amount of MT. 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/s, a modulation amplitude of 250 mV, E_{ads} = 0 V, the temperature of supporting electrolyte is 5 °C (except A), the time of accumulation of MT is 120 s (except B), the concentration of $Co(NH_3)_6Cl_3$ is 1 mM (except C). For other details see Section 2 and Fig. 4.

we wanted to perform both quick and sensitive determination of MT. That is why we selected the accumulation time of 120 s in the following experiments. Moreover, we studied the influence of different concentrations (0.12, 0.25, 0.5, 0.8 and 1 mM) of cobalt solution $Co(NH_3)_6Cl_3$ on the observed AdTS DPV Cat2 signal of MT. It is clearly follows from the obtained results that height of Cat2 signal increased with increasing concentration of the cobalt solution (Fig. 5C). In addition, the Cat2 signal shifted to more positive potential with the increasing concentration of the cobalt solution (Fig. 5C).

The dependence of the height and potential of Cat2 signals on the different MT concentration (from 2.44 to 312 fmol MT in 5 μl) was studied. The obtained calibration curve is shown in Fig. 5D. The signals strictly linearly increased with rising MT concentration ($y_{Cat2} = 0.6616x + 10.211$; $R^2 = 0.9955$). Moreover we also tested influence of amounts of MT below 10 fmol on Cat2 signal height. We obtained linear dependence of Cat2 signal on MT concentration in the range from 0.65 to 9.75 fmol MT in 5 μl (inset in Fig. 5D) The detection limit of MT was about 10 amol in 5 μl drop (2 pM, Table 1).

3.4. Determination of MT in a human blood serum

Finally, we wanted to determine the MT level in a human blood serum. For this purpose we selected AdTS DPV Brdicka reaction as the most sensitive technique for determination of MT. To be specific, a 11-year-old girl was referred to the hospital by her general practitioner because of abdominal pain, vomiting, dark colour of tongue, low intake of fluid and food, and abnormal laboratory results (bilirubin 55 μmol/l, AST 2.94 μkat/l, ALT 3.46 μkat/l, haemoglobin 86 g/l). Blood film exhibited basophilic stippling is prompting for investigation of lead poisoning. Blood lead levels were measured by GF AAS and plasma metallothionein levels AdTS DPV Brdicka reaction, as we mentioned above. In time of admission, the blood lead (B-Pb) was 648 μg/l, and plasma metallothionein (P-MT) 153 μmol/l (normal values below 10 μmol/l) (Fig. 6). The source of 6 months lead exposure was identified as tea from a ceramic tea pot with insufficient glasing (lead concentration in tea after 30 min was 45332 μg/l). After hospitalisation of the girl, chelation therapy by EDTA was administered for five consecutive days. During the course of therapy the B-Pb decreased to 360 μg/l,

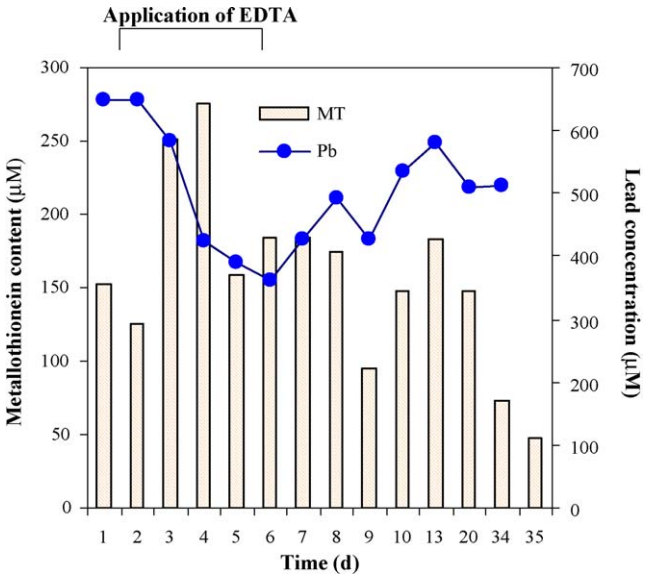

Fig. 6. Human blood serum. Dependence of plasma metallothionein content and blood lead concentration on time. Lead was determined by GF AAS and metallothionein by AdTS DPV Brdicka reaction. For other details see Section 2 and Fig. 5.

P-MT increased to 276 μmol/l (Fig. 6). After 5 days of chelation treatment, the B-Pb increased (535 μg/l), P-metallothionen decreased (147 μmol/l). It clearly follows from the figure that since the end of chelation therapy of EDTA the concentration of blood lead has been well correlated with the amounts of plasma metallothionein.

4. Conclusion

Number of authors have concerned with electrochemical analysis of metallothionein. Recently new electroanalytical techniques enabling determination of MT at picomole level has been suggested. Here we optimised the different technique for determination of MT such as cyclic voltammetry, differential pulse voltammetry and Brdicka reaction. The detection limits of the mentioned techniques were at picomole, femtomole and attomole level, respectively. In addition, we were able to determine MT in a human blood serum samples due the high sensitivity of AdTS DPV Brdicka reaction.

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