DETERMINATION OF METALLOTHIONEINS IN TROUTS BY ADSORPTIVE STRIPPING VOLTAMMETRY

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Abstract

Metallothioneins are a group of low molecular weight proteins, existing virtually in all living organisms. These proteins are rich in cysteine clusters, which are known to have the ability to complex heavy metals through their thiol groups.

Metallothionein production and accumulation is induced by the presence of heavy metals; in the cell, metallotoneins act as protecting molecules by complexing the cations (avoiding them to be free). This (particular) characteristic allows these proteins to be used as biomarkers for heavy metal exposure of the living organisms in their biota.

In order to overcome some of the actual limitations of the existing methods for metallothionein determination, a square wave adsorptive stripping voltammetric method is proposed in the present work using a hanging mercury drop electrode.

The method development involved optimisation studies of instrumental and analytical conditions, and influence of interferences. The method was applied to samples extracted from fish liver, which was previously submitted to a controlled heavy metal exposure. The method presents a detection limit of 10^{-9}M of metallothionein and has a good linear range. The results obtained show that the method can be easily applied to metallothionein determination in trout liver samples submitted only to simple separation procedures.

Keywords: metallothionein, biomarker, biomonitorization, adsorptive voltammetry, polarography.

1. Introduction

Attention and concern of the effects of human activity in the environment have grown considerably throughout the last decades. In respect to chemical pollution, there is an increasing knowledge of the

effect that this type of pollution has on single living organisms, on population and even on ecosystems.

At the present time, the main information about the level of environmental pollution comes from conventional chemical analysis directly applied to detect and quantify the level of pollutants. There are, however, some drawbacks, because those methodologies are not capable to predict the effects of long-term exposure of the pollutants in living organisms at moderate levels. They must be complemented by biomonitorization studies on living organisms.

Biomonitorization is based on the study of biological reaction as response to a certain stress condition. The biological effects that chemical pollutants in the environment may cause in living organisms can be used as an indicator of the extent of pollution and its influence in biota [1].

Metallothioneins are a low-molecular-weight sulphydryl-rich proteins that is presumed to be involved in Cu and Zn homeostasis under normal metabolism. One of their most characteristic features is the fact that their hepatic synthesis and accumulation is also induced by the presence of other non-essential heavy metals like Cd and Hg in the cell or organism as consequence of their uptake from the environment. The living organisms synthetize these metalloproteins as a form to defend themselves from the harmful effects of exposure at heavy metal levels above critical values. This particular biological function of metallothioneins made them biomarkers for assess the exposition of animals to heavy metals [2,3,4,5].

Due to the inexistence of simple methods for determination of these proteins in biological samples, the development of reliable methodology for sensitive and specific determination of metallothioneins is very important, since the methods usually applied involve sophisticated determinations such as isotope incorporation, immunological methods, ELISA and radioimmunological assay. Electrochemical methods based on differential pulse polarography (DPP) have been developed for these determinations and their sensitivity and simplicity have attracted many interest [5,6,7]. In this work an adsorptive cathodic stripping square wave voltammetric method was developed for metallothionein determination using copper (II) in solution. The method proved to be more sensitive, faster and easier to perform than DPP.

The proposed adsorptive voltammetric method is based on the use of a hanging mercury drop electrode, as it was found that metallothioneins adsorb at the mercury surface. The optimised voltammetric method was applied to determination of metallothioneins in biological samples extracted from fish exposed to toxicological trials with cadmium (II).

2. Experimental

2.1. Instruments and apparatus

Voltammetric equipment

Voltammetric work was performed using an Autolab PGSTAT 10 Voltammetric System (Eco Chimie), controlled with a PC equipped with GPES® for Windows® - Version 4.4 software. With this software, current is sampled during the last 20 ms, if the pulse lasts more than 40ms or during the last half of the pulse if this is shorter. A Metrohm 663 VA Voltammetric Stand was used in the SMDE mode, set to drop size 3. The three-electrode potentiostatic system was completed with a glassy carbon auxiliary electrode and an AgCl/Ag (3M KCl) reference electrode. Measurements were made at room temperature.

2.2. Reagents, standards and solutions

All chemicals used were of analytical grade. Ultra-purified water (Millipore Simplicity System) was used for solutions preparation. Borate 0.02M buffer solution was prepared weekly, from boric acid (Panreac), and pH was adjusted to 8.10 with sodium hydroxide.

Standard solutions of metallothionein I, 2E-5M (assumed MW of 6500), obtained from rabbit liver (Sigma), were prepared in water. These standard solution were afterwards divided in 1ml aliquots and frozen. Each portion once melted should not be refrozen.

Copper (II) solutions were prepared by dilution of a 1000mg/L standard copper (II) solution in 1M HNO_3 purchased from REAGECON, Ireland.

2.3. Voltammetry of metallothioneins at the mercury electrode

The adsorption and voltammetric behaviour of metallothioneins in a mercury electrode is not yet fully understood, although the behaviour of similar electroactive sulphur compounds at the mercury electrode are well known and could be used to propose mechanisms for metallothioneins [8,9,10, 11, 12,13, 14].

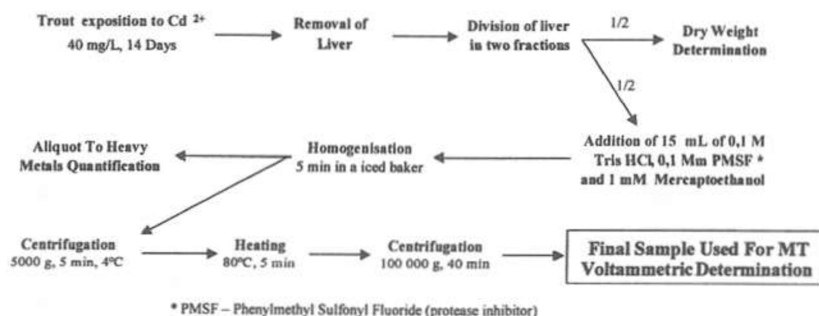
The electroactive groups of metallothioneins are the thiol groups of their cysteine residues, which might as well be involved in the pre-concentration process at the mercury electrode. When present in solution, copper (II) react via a redox process to form a cuprous metallothionein complex, which is also electroactive at the mercury electrode.

2.4. Exposure and preparation procedures to obtain fish samples

The animals used in these experiments were exposed to cadmium (II) for induced synthesis and accumulation of metallothioneins. Liver is known to be the most metabolic active organ in fish. Metallotoneins were extracted from liver, because the protein production levels in this organ are several times higher than in other parts of fish.

The experience was conducted in an experimental system (closed circuit) at the Aquaculture Station of the University of Tras-os-Montes and Alto Douro (UTAD), at a constant temperature 15° C and a controlled photoperiod of 12 hours in dark and 12 hours in light (12D:12L). The fish used, adult rainbow trout (*Oncorhynchus mykiss*) were exposed during a period of two weeks in dechlorinated water in 500-l tank containing 40μ/l of cadmium (II).

After exposure, livers were removed and divided in two portions, one for determination of dry weight, and the other for protein extraction according to the technique described by DeConto Cinier [4]. After being prepared and before being used samples were maintained frozen at −18°C.



Scheme 1- Protocol steps for sample preparation from trout liver, by DeConto Cinier [4]. (PMSF- Phenylmethyl sulfonyl fluoride).

3. Results and discussion

In the present work a method was developed for the analysis of metallothioneins, using adsorptive cathodic stripping voltammetry. The method was applied to the determination of metallothioneins in fish liver and the possibility of using these proteins as biomarkers for the heavy metal exposure of fish was investigated. The starting point was the work of Scarano and Morelli [15], in which the determination was made by adsorptive cathodic stripping voltammetry in the presence of copper (II) to complex metallotioneins.

3.1. Optimisation of variables

Cu^{2+} in solution

As already observed, the electrolyte (borate buffer 0.02M, pH 8.1) used in these studies was the same referred in a previous work [4]. The optimisation studies of the voltammetric parameters have been done both in the absence and in the presence of copper (II) ($2.5x10^{-6}$M), to assess the effect of using this metal ion in solution. In fact, several peaks can be observed, corresponding to different reduction potentials of complexes of metallotionein with different metal ions [16]. The presence of $2.5x10^{-6}$M Cu^{2+} in solution had a positive qualitative and quantitative effect in the analytical response of metallothioneins in solution. When copper (II) is in solution in a concentration larger than that of the other heavy metal cations, the different complexes and the free form of MT are replaced for MT-Cu complexes that are more stable. In those conditions a single voltammetric signal is obtained that corresponds to total MT in solution, avoiding the need to sum all individual results due to different voltammetric signals (at different potentials) of different MT complex forms. Furthermore, the signal of the MT-Cu complex is larger and sharper than the signal of the free form of MT alone, as it can be seen in figure1. The benefits of using copper (II) in solution, suggested by several authors, are thus confirmed and can be attributed to a better instrumental response and to a lower interference from other MT complexometric forms.

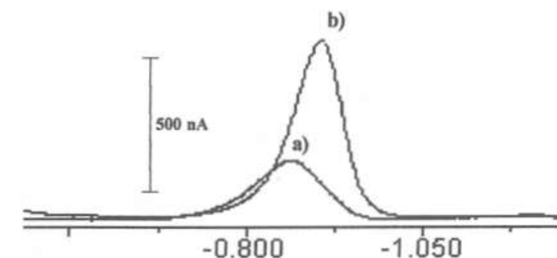


Fig. 1- SW voltammetric analysis of a solution of $1x10^{-7}$ M metallothionein in borate buffer 0.02M, NaCl 0.5M, pH 8.1: a) no Cu^{2+} added; b) in the presence of Cu^{2+} $2.5x10^{-6}$M. Results obtained with a SW frequency of 100 Hz and an accumulation time of 120s at the hanging mercury drop electrode.

Voltammetric technique

Initially two different voltammetric techniques, differential pulse voltammetry (DPV) and square-wave voltammetry (SWV) were investigated for metallothioneins analysis. SWV was found to be more suitable than DPV due to a higher sensitivity, a lower scanning time and a wider linear calibration range. Another very important advantage was the possibility of using higher frequencies

(shorter sampling times), at which oxygen interference is drastically reduced. The voltammetric technique selected for further studies was, then, square-wave voltammetry.

Square wave voltammetric parameters and adsorption time

In order to optimise the voltammetric signal and the analytical response of metallothioneins, SWV studies have been conducted taking into consideration the adsorption time at the mercury drop electrode.

Optimum values of 25 mV for E_{ampl} and of 2 mV for E_{step} were established, as the best compromise between a good definition of the voltammetric peaks and the sensitivity of the analytical response. When necessary the purge time for oxygen removal was 300s before the first scan and 30s before the following ones. A deposition potential ($E_{dep.}$) of –0,4V was used in the adsorption step at the mercury drop for the concentration of metallothionein, as indicated in reference [15]. The voltammetric scan was performed from –0.4 V to –1.3 V.

The influence of square-wave (SW) frequency and adsorption time on the metallothionein signal can be seen in figure 2. As expected the peak intensity increases with the accumulation time and with the frequency, although becoming less defined for higher frequencies. An accumulation time of 120 s was considered convenient to obtain a high sensitivity and a wide calibration range without a significant increase of the time analysis.

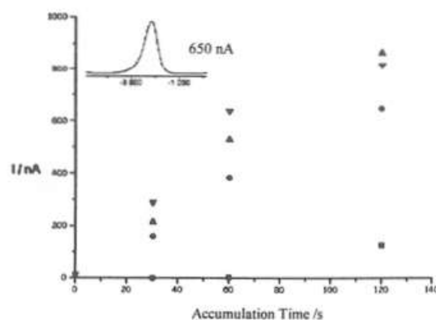


Fig 2 – Current intensity as function of accumulation time in the SW voltammetric analysis of a $1x10^{-7}$M metallothionein solution in borate buffer 0.02 M, NaCl 0.5 M, pH 8.1, in the presence of $2.5x10^{-6}$M copper (II). SWV frequencies (Hz): ■ - 25, ● - 100, ▲ - 200, ▼ - 400. The voltammogram obtained with a frequency of 100 Hz and an accumulation time of 120s is represented on the upper left corner.

In figure 3 we can see calibration curves of metallothionein at different SW frequencies in borate buffer 0.02M, pH 8.1, NaCl 0.5M, in the presence of Cu (II) and with 120s of accumulation time. Although the analytical response was linear at all frequencies used, 200 Hz was considered a good

compromise between a lower sensitivity obtained for lower frequencies and a worse definition of the base line and of the peak obtained for higher frequencies. For lower frequencies, besides poor sensitivity the need for a longer deoxygenation step is another drawback to be considered.

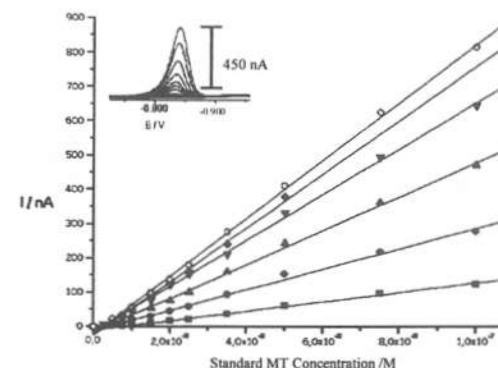


Fig 3 – Calibration curves for metallothionein using an accumulation time of 120 s at different square-wave frequencies, in borate buffer 0.02M, NaCl 0.5 M, pH 8.1 in the presence of $2.5x10^{-6}$M Cu^{2+}. SWV frequencies (Hz): ■ - 10, ● - 25, ▲ -100, ▼ - 200, ◆ - 300, ○ - 400. The voltammograms obtained with a frequency of 100 Hz are represented on the upper left corner.

3.2. Analytical behaviour of metallothionein in solution.

Effect of Cu^{2+}

Copper (II) concentration in solution is an important factor to consider as it affects the intensity of the peak current. It was observed that the signal of the Cu-thionate clusters grows with the Cu^{2+} concentration until a certain extent after witch it actually decreases. This means that the concentration of the metal ion has to be carefully controlled; results can only be compared if they are obtained in solutions having the same concentration in Cu^{2+}.

Stability of mettalothionein in solution and effect of 2-mercaptoethanol

The stability of an analyte in solution is essential for its accurate determination. In the case of methalotioneins, although they are generally considered as highly stable peptides some precautions have to be taken. For instance, phenylmethyl sulfonyl fluoride (PMSF), which is a protease inhibitor, needs to be used during sample preparation to avoid enzymatic degradation; 2-mercaptoethanol needs also to be added at this stage to avoid metallotionein oxidation. As 2-mercaptoethanol is also a

thiol its interference in the determination of metallotionein was investigated. As it can be seen in figure 4, 2 - mercaptoethanol is in fact electroactive, but it does not interfere in the determination of metallotionein because its reduction potential is sufficiently apart from that of the protein.

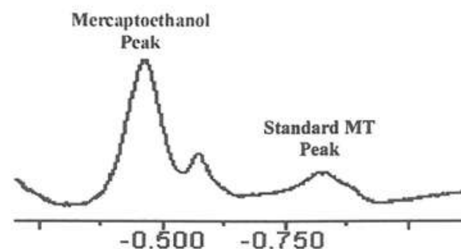


Fig 4 – Voltammetric analysis of a solution $2.5x10^{-8}$ M in metallothionein and $2x10^{-8}$ M in mercaptoethanol, in borate buffer 0.02M, NaCl 0.5 M, pH 8.1, in the presence of $2.5x10^{-6}$ M Cu^{2+}. Results were obtained using a SW frequency of 200 Hz and an accumulation time of 120s at the hanging mercury drop electrode.

The stability of metallothionein solutions was investigated comparing the voltammetric signals obtained at different times after the preparation of the solutions. As it can be seen in table 1, metallothionein-Cu complexes are fairly stable in solution during the first 85 min, a period of time more than sufficient to perform the analysis.

Table 1. Variation of the voltammetric signal of a metallotionein solution with the time elapsed after its preparation. Metallotionein solution in borate buffer 0.02M, NaCl 0.5 M, pH 8.1, in the presence of $2.5x10^{-6}$M Cu^{2+}. Results were obtained using a SW frequency of 200 Hz and an accumulation time of 120s at the hanging mercury drop electrode.

Time /s	0	5	10	15	20	25	35	45	65	85	100	115	145	175	205	235
% Signal	100,0	99,0	100,4	100,8	102,4	99,7	96,7	91,4	94,0	95,0	89,8	84,1	79,8	82,0	86,2	79,1

3.3. Application of the method to fish samples.

3.3.1. Features of the method

In the determination of the concentration of metallotioneins in fish samples both methods, calibration curve and standard additions, could be used in principle. However the calibration curve method can only be applied if there is no matrix interference, a situation in which the slopes of the calibration curve and of the standard additions curve are not significantly different. In figure 5 we can see the calibration curve of metallotionein in borate buffer 0.02M, pH 8.1, NaCl 0.5M and Cu^{2+} $2.5x10^{-6}$M. A linear range between $1x10^{-9}$M and up to $1X10^{-7}$M of metallothionein was obtained, although these limits may vary depending on the nature of the sample matrix.

The reproducibility/repeatability was found to be excellent, as well as the regression coefficients provided by this method. Up to 12 samples can be analysed per hour with precise results (RSD < 5%) being obtained.

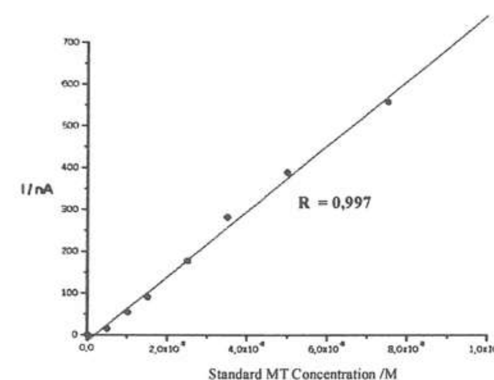


Fig 5 – Metallothionein calibration curve in borate buffer 0.02M, NaCl 0.5 M, pH 8.1 in the presence of $2.5x10^{-6}$ M Cu^{2+}. Voltammetric analysis was performed using a SW frequency of 200 Hz and an accumulation time of 120s at the hanging mercury drop electrode.

3.3.2. Determination of metallothioneins in fish liver

The main objective of the work was the investigation of the possibility of using voltammetry in the determination of the level of metallotionein in fish. In case of success this would open new possibilities for the use of the protein as a biomarker of fish exposure to heavy metal. The feasibility of the method was tested comparing the results obtained in the analysis of the liver of fish submitted to a sub-lethal toxicological trial with cadmium (II) with the results obtained in the analysis of the liver of control fish, not submitted to any environmental stress.

Analysis of trouts submitted to sub-lethal toxicological trials with cadmium (II)

Samples obtained from the liver of trouts were prepared using the protocol of scheme 1 and metallotioneins were determined by the voltammetric method previously described. Samples were injected directly into the voltammetric cell without any further treatment. The signal due to metallothionein was readily identified by its peak potential and was subsequently confirmed by addition of standard metallothionein.

The results obtained, using the method of standard additions, in the determination of metallotionein in two different trouts submitted to ambient stress with cadmium (II) can be seen in figure 6A. There is a good agreement between the slopes obtained in the two cases. Correlation coefficients are good and the method proves to be applicable to metallotionein determinations in the range $1x10^{-9}$M to

$1x10^{-7}M$ in the voltammetric cell. Table 2 shows the concentrations of metallotionein found in the 2 trout samples and the different values obtained can be probably explained due to a different physiological response to the heavy metal exposure.

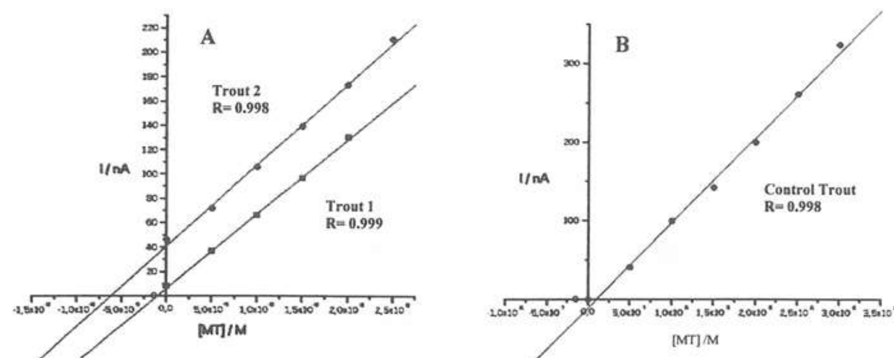


Fig 6 – Voltammetric determination of metallotionein in trouts using the method of standard additions. **A** – trouts submitted to toxicological trials with cadmium (II); **B** - control trouts. In the voltammetric cell, extracts of trout livers (20 μL in **A** and 25 μL in **B**) were diluted with 25 mL of borate buffer 0.02M, NaCl 0.5 M, pH 8.1 in the presence of $2.5x10^{-6}M$ Cu^{2+}. Voltammetric analysis was performed using a SW frequency of 200 Hz and an accumulation time of 120s at the hanging mercury drop electrode.

Table 2 – Concentration of metallotionein in the voltammetric cell and in liver (per fresh weight).

Trout	[MT] in the voltammetric cell/ M	[MT] in liver (fresh weight) / $M.g^{-1}$
1	$1,14 \times 10^{-9}$	$2,74 \times 10^{-6}$
2	$6,28 \times 10^{-9}$	$1,88 \times 10^{-5}$

Control trouts

To confirm that the formation of metallotionein was really induced by the presence of cadmium (II), a group of trouts of the same original batch culture but not submitted to the toxicological trial with cadmium (II) was also analysed. Results can be seen in figure 6B, and it is clear that in this case there is no measurable accumulation of metallotionein at the liver of the trouts.

4. Conclusions

In this work a voltammetric method was developed for the determination of metallotioneins. The method proves to have advantages over other methods currently used, namely in terms of sensitivity, simplicity and analysis time. The voltammetric detection limit is $\sim 1x10^{-9}$ M and there is an analytical linear response within the concentration range of $1x10^{-9}M$ to $1x10^{-7}M$ with a good correlation coefficient

The method was successfully applied to the determination of MT level in trouts, by direct analysis of the extracts of their liver, using the method of standard additions. In face of the results, it is possible that the method can be used to establish a correlation between metallothionein biomonitorisation and environmental heavy metal stress in fish. This type of studies is now being developed for application to fish living in Douro River.

5. Aknowledgements

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