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Characterization of metal complexes of pharmaceutical interest by capillary electrophoresis with element sensitive detection

H. Wittrisch[a], S. Conradi[a], E. Rohde[b], J. Vogt[c], C. Vogt[a,*]

[a]*University of Leipzig, Faculty of Chemistry and Mineralogy, Institute of Analytical Chemistry, Linnéstrasse 3, 04103 Leipzig, Germany*

[b]*University of Cincinnati, Department of Chemistry, Mail Location 172, Cincinnati, OH 45221, USA*

[c]*University of Leipzig, Faculty of Physics and Geosciences, Institute of Nuclear Solid State Physics, Linnéstrasse 5, 04103 Leipzig, Germany*

Abstract

The application of capillary electrophoresis to the separation and detection of compounds of medical interest is described. A proton-induced X-ray emission (PIXE) detector was used for metal-specific and sensitive detection. The detected X-rays are generated inside the capillary by an accelerated proton beam. The application of this novel detection mode in addition to common UV detectors is demonstrated for different complexes of gadolinium with polyaminopolyacetic acids. Using the PIXE detector lower limits of detection have been achieved which could be convincingly demonstrated by a comparison with UV detection in the direct and indirect detection mode. Furthermore, kinetic experiments investigating the decomposition of the Re(III)–HEDP (1-hydroxyethylidene diphosphonic acid) boon seeking agent were discussed. Finally, metallothioneins (MTs) from rabbit liver and horse kidney have been separated and up to six isoforms were completely or partially resolved. Copper, zinc and cadmium, attached to the isoforms, have been detected simultaneously. © 1997 Elsevier Science B.V.

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1. Introduction

At present metal and metal containing compounds are becoming more and more important in medicine and ecology. Metal containing proteins play important catalytic and structural roles within cells. They are essential to normal cellular processes such as respiration, metabolism, gene expression and metal homeostasis. Metal complexes are widely used in medicine, preferably as cancerfighting drugs or contrast medium in computer-assisted tomography. These substances of pharmaceutical interest have to undergo severe impurity and stability controls. Metal containing compounds also play an important role in the analysis of environmental samples, because uncommon concentrations of metal containing species in air or soil may have great influence on health and well-being of plants and animals. In all these cases not only information about the metal but also about the kind of bonding to different ligands is necessary. For the investigation of such samples high-performance separation systems, such as capillary electrophoresis (CE) [1,2] and high-performance liquid chromatography (HPLC) [3], are used. In most cases optical detectors in either the absorbance or fluorescence mode are applied. Unfortunately, many inorganic ions or metal complexes possess negligible absorbance at wavelengths accessible by

*Corresponding author.

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UV detectors. This requires derivatization of the species or the use of the indirect detection mode. But often derivatization is impossible and the indirect detection mode is unable to supply satisfactory information about the separated species.

In most cases information about the metal in a compound is not available either with direct or with indirect optical detection. Only the organic part of the structure or the bond to the metal ion could be detected. But often the metal in a compound is mainly responsible for the chemical and biochemical behavior of a substance. As alternatives to the optical detectors, atomspectroscopic, electrochemical and methods generating X-rays could be used. With the application of such detectors metal specific signals could be obtained, thus the information content of a separation increases significantly.

The application of voltametric detectors [4,5] for CE is affected by the limited range of elements, which provide appropriate electrochemical signals. In addition, only a few elements could be analyzed simultaneously. For a simultaneous detection of more elements an atomspectroscopic detector could be used. First results of the coupling of CE systems with miniaturized inductively coupled plasma-mass spectrometry (ICP-MS) detection systems have been published during the last few years [6,7]. For this technique the small amount of liquid flowing through the capillary represents a permanent problem. Therefore, special interfaces and nebulizers are needed [8]. In addition, the selection of electrophoretic buffer systems is restricted because of interferences in the mass spectrometer and effects in the nebulizer.

For generating of X-rays in a capillary high density energy radiation is necessary. Such a high energy is only provided by accelerated ions or synchrotron radiation. Among these excitation sources accelerated ions provided by a proton-induced X-ray emission (PIXE) system [9,10] are more common. This technique enables the simultaneous detection of elements with atomic numbers higher than $Z>14$.

Hirokawa and coworkers first performed experiments of coupling an electrophoretic technique with PIXE [11–13]. For the determination of different metal complexes the application of an off-line PIXE detector in combination with isotachophoretic (ITP) separation was described. The disadvantage of this method could be the fractionation step, which partly destroys the previously achieved separation. The first on-line coupling of CE with PIXE detection has been published recently [14]. The possibility of detecting more than twenty elements simultaneously is the most important advantage of this detection technique. In addition, all measurements are performed independently of the buffer composition, because signals are generated across a 3–5 μm thick fused-silica wall. In comparison to CE–ICP-MS higher detection limits and higher uncertainties of the measured signals were observed.

This paper describes the application of PIXE detection to the electrophoretic separation of different polyaminopolyacetic acids. Direct and indirect UV detection modes are compared with PIXE detection with regard to the limits of detection and detectability. Secondly, separation of Re(III)–HEDP, a bone seeking agent, from its metabolites and kinetic studies of the decomposition are discussed. Finally the application of the PIXE detector for the determination of Zn, Cu and Cd in metallothioneins, (metal coupling proteins), is described in detail.

2. Experimental

2.1. Materials

$GdCl_3$, NTA (nitrilotriacetic acid), EDTA (ethylenediaminetetraacetic acid), DTPA (diethylenetriaminepentaacetic acid), phthalic acid and Na_2HPO_4 were obtained from Merck (Germany). HTAB and NH_4ReO_4 were purchased from Fluka (Buchs, Switzerland). The standard Cd, Zn metallothioneins (rabbit liver and horse kidney) were obtained from Sigma (St. Louis, USA). HEDP (1-hydroxyethylidene diphosphonic acid) was obtained from Norwich Eaton Pharma (USA).

TTHA (triethylenetetraaminehexaacetic acid) and TTAHA [N-tris(2-aminoethyl)amine-hexaacetic acid] were synthesized at the University of Leipzig, Department of Organic Chemistry and the Re^{3+}–HEDP agent at the University of Cincinnati. Buffers were prepared by dissolving appropriate amounts of the sodium salts in triply distilled water. All chemicals were of analytical-reagent grade.

Capillary material of 50 and 100 μm I.D.×360

μm O.D. was obtained from CS Chromatography Service (Langerwehe, Germany). The total length of the capillaries for the PIXE measurements was 120 cm, with an etched "ion window" at 100 cm. Only capillaries with 100 μm I.D. were used for the PIXE measurements. The preparation of the "ion window" was described earlier [14], capillaries with an "ion window" of 5 μm wall thickness were used in this work. For direct and indirect UV detection capillaries with 50 μm I.D. and a total length of 57 cm were used. The detection window was set at 50 cm.

2.2. Apparatus

The UV detection of the electrophoretic measurements was performed using P/ACE 5510 CE system from Beckman (Palo Alto, CA, USA) equipped with a diode array detector (DAD).

Electrophoretic separations with PIXE detection were performed with laboratory-made apparatus. For rinsing and injection a vacuum pump was used. The electric field was generated with an HCN 7E-35000 power supply (F.u.G. Elektronik, Germany).

For the PIXE experiments the 2 MeV Van de Graaff accelerator at the University of Leipzig was used with a proton beam of 400 μm diameter and an energy of 1.7 MeV. Passing the capillary wall the ion beam loses energy. Therefore, the proton energy inside the capillary was about 1.2 MeV. Due to the high energy the ion beam decomposes the water molecules of the buffer (radiolysis) inside the capillary. During this process oxygen and hydrogen are generated and these bubbles may interrupt the charge transport in the capillary. These effects make a real on-line separation and detection impossible. To overcome this problem the migration time of the first sample zone to the PIXE detector was calculated [14]. In addition, for the separation with PIXE detection lower voltages were used. Only in this way could destruction of the "ion window" due to the interaction between the ion beam and the electric field inside the separation unit be avoided. The separation was stopped some seconds before the first sample zone reaches the detector. Then the separated sample compounds were pushed to the "ion window" by a low pressure of 10 kPa. For the detection of the X-rays a low-energy Ge detector was used. Each generated X-ray spectrum was accumulated for 10 s to obtain a sufficiently high signal-to-noise ratio for the X-rays. These accumulated X-rays were shown as "counts". Because of peak widths of about 10 s in CE often only one data point for the peaks was obtained. Quantitative interpretation of the characteristic K_α and L_α signals can be carried out after mathematical handling of the peak areas. The X-rays were accumulated while the liquid was pushed through the capillary by low pressure. Therefore, the scale of the abscissa in the figures showing signals of the PIXE system is different from those of the UV detector because each point represents an accumulation time of 10 s.

3. Results and discussion

3.1. Separation of different complexes of gadolinium with polyaminopolyacetic acids

Metal complexes of polyaminopolycarboxylic acids with paramagnetic properties are utilized as relaxation reagents for ^{13}C-NMR and magnetic resonance imaging (MRI), because they are extremely useful in enhancement of contrast [15]. After the addition of these complexes to a sample the relaxation times of carbon nuclei are changed resulting in enhanced signal-to-noise ratios and rendering the collection of data faster and more effective. Paramagnetic relaxation reagents soluble in organic solvents have been studied quite extensively, however, only a few reports have been published dealing with water soluble relaxation reagents [16,17]. It turned out that paramagnetic gadolinium chelates are most effective MRI contrast reagents, e.g., the complexes of DTPA and DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) [18]. These ligands appear to encapsulate the metal ion quite effectively producing stable complexes over a wide pH range with saturated coordination sphere. This is important due to the acute toxicity of free gadolinium ions to human organism. Therefore, a strict control of impurities and intermediates of the synthesis, such as NTA and EDTA, is necessary.

Gadolinium complexes of NTA, EDTA, DTPA, TTHA and TTAHA were separated using CE with direct and indirect UV detection.

The separation with direct UV detection was

performed at a physiologically relevant pH of 6.5 in a 20 m*M* phosphate buffer (Fig. 1A). For the observed peaks in the electropherogram satisfactory resolution was obtained although Gd–TTAHA and Gd–TTHA migrate with similar velocities. Resolution enhancement of all complexes could be achieved by decreasing the pH of the buffer. Thereby the electroosmotic flow is decreased and slower migra-

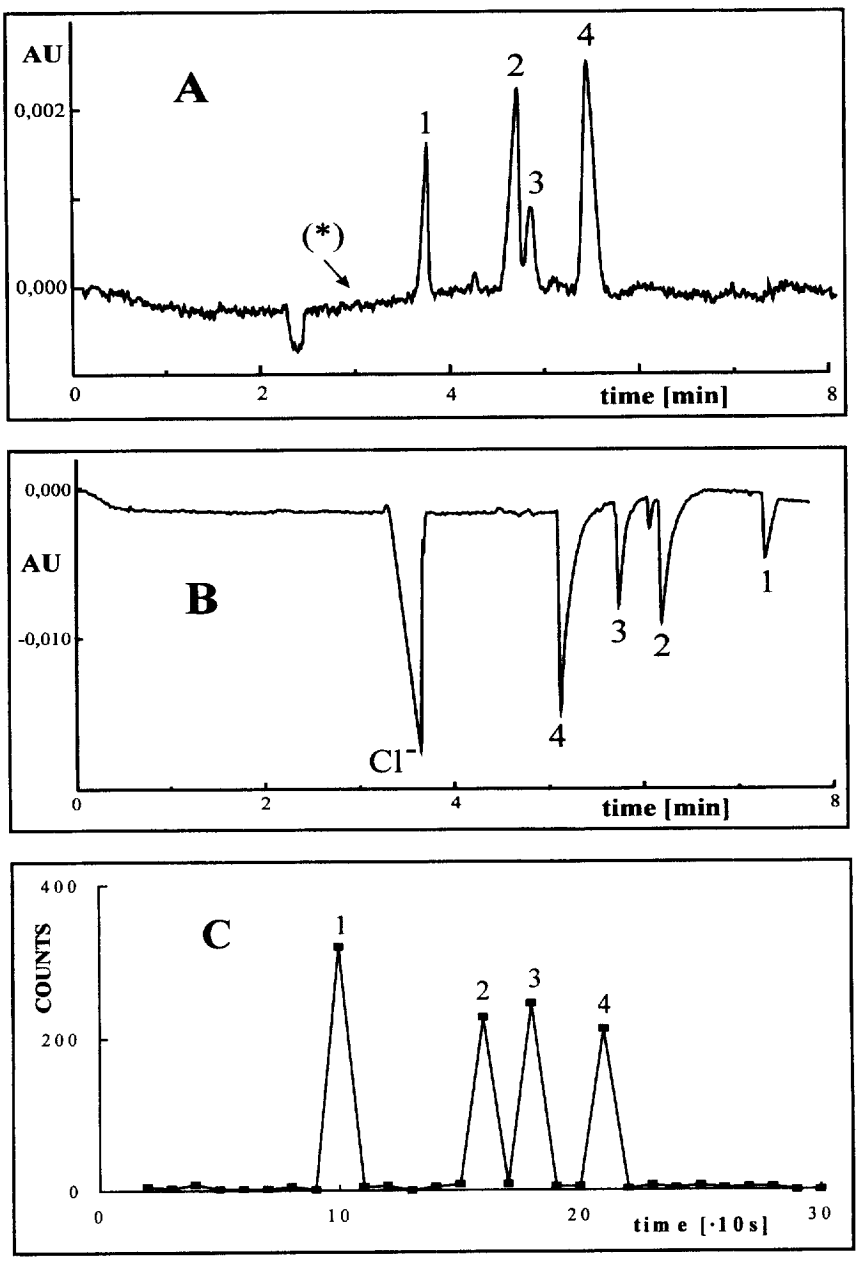

Fig. 1. Separation of Gd–EDTA (1), Gd–TTAHA (2), Gd–TTHA (3) and Gd–DTPA (4) (0.1 m*M* each complex) in (A) 20 m*M* phosphate pH 6.5, 25 kV, UV detection at 200 nm; (B) 10 m*M* phthalate–0.1 m*M* HTAB pH 6.5, −15 kV, UV detection at 214 nm; and (C) 20 m*M* phosphate pH 6.5, 10.5 kV (12 min), PIXE detection.

tion of the complexes towards the detector results. But at pH below 5.5 no satisfactory separation could be carried out because complex formation was impaired due to alteration of dissociation degree of the ligands.

Due to the relatively low UV absorbance of the ligands and bonds of the complex detection limits between $7.5 \cdot 10^{-4}$ *M* for Gd–TTHA and $5 \cdot 10^{-4}$ *M* for the other complexes have been obtained for the complexes shown in Fig. 1. Unfortunately, the UV absorption of the Gd–NTA complex, one of the most common intermediates, is lower than for all the other complexes. Therefore only a concentration of 10^{-3} *M* could be detected under these conditions. In the case of supplementary injection of Gd–NTA (not shown) the complex migrates faster than the other complexes due to the weaker bonding of the nitrilotriacetic acid ligand and slight changes of the charge of the complex. In Fig. 1A the migration time of Gd–NTA is marked with an asterisk (*), since the complex could not be detected at the applied concentration and for that reason it was not considered during the sample preparation.

To improve the limits of detection the indirect detection mode was used. Separation was performed in a 10 m*M* phthalate buffer pH 6.5. To lower the electroosmotic flow and to improve the resolution, 0.1 m*M* HTAB was added to the buffer. The pH was again chosen with respect to the physiological conditions. A significantly better resolution of Gd–TTAH and Gd–TTAHA could be observed (Fig. 1B). The reversal of the migration behavior of all sample components is caused by the negative power applied. Under these conditions limits of detection have been determined between $5 \cdot 10^{-5}$ *M* for Gd–EDTA and $1 \cdot 10^{-5}$ *M* for Gd–DTPA. All peaks are characterized by a tailing. This indicates, that the complexes migrate with higher electrophoretic velocities than the phthalate ions, providing the background signal. For a better peakshape a background electrolyte with a higher specific electrophoretic velocity should be chosen. With indirect detection mode it was also possible to determine the Gd–NTA complex down to a concentration of $5 \cdot 10^{-5}$ *M* (not shown).

For the experiments with PIXE detection the same buffer as for direct UV detection was used. Fig. 1C shows the separation of all four Gd complexes. Although the sample zones were pushed by low pressure to the detector, a satisfactory resolution was obtained. Each compound was identified by standard addition, which could be followed by an increase of the corresponding X-ray signal.

To demonstrate the advantages of the PIXE detection mode, a mixture of three Gd complexes including Gd–NTA was investigated (Fig. 2). Corresponding to the same content of gadolinium in each complex, there are only slight differences in the number of counts for all three peaks and additionally a high signal is generated for Gd–NTA. The limits of detection (signal$>3 \cdot \sqrt{\text{noise}}$) for these three complexes were determined to be $5 \cdot 10^{-6}$ *M*. Relative standard deviations between 4.0 and 6.8% have been determined for three replicate runs with $2.5 \cdot 10^{-5}$ *M* of each complex during the calibration measurements. Because the obtained X-ray signals depend on the homogeneity and the position of the ion beam standard deviations of about 5% represent satisfactory results. The calibration graphs are strictly linear with regression coefficients (r^2) between 0.9939 for Gd–EDTA and 0.9997 for Gd–NTA. The obtained detection limits were in most cases two orders of magnitude better than with direct UV detection and one order of magnitude lower than with indirect UV detection.

3.2. Kinetic studies of the decomposition of Re(III)–HEDP

Complexes labeled with ^{186}Re and ^{188}Re have

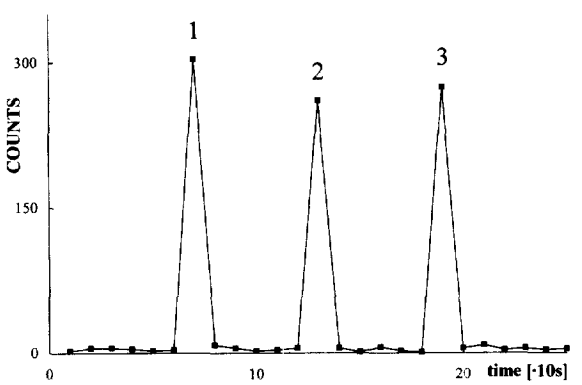

Fig. 2. Separation of Gd–NTA (1), Gd–EDTA (2), Gd–DTPA (3), each 0.1 m*M*, in 20 m*M* phosphate pH 6.5, 10.5 kV (12 min), PIXE detection.

been synthesized for the radiotherapeutic treatment of humans. Medicine utilizes the nuclear properties of these radioisotopes [19,20] and similarities of the chemical properties of rhenium to those of technetium (^{99m}Tc), a well established diagnostic agent. Diphosphonic acid derivatives of Tc, such as complexes with 1-hydroxyethylidene diphosphonic acid (HEDP), hydroxymethylene diphosphonic acid (HMDP) and methylene diphosphonic acid (MDP), have been widely used as imaging agents for bone disease. Studies of diphosphonates, labeled with ^{186}Re, indicate, that Re compounds are as effective as the group of boon seeker agents ^{99m}Tc-diphosphonates. The ^{186}Re–HEDP complex has proved to be an effective agent for palliation of bone pain caused by metastases of primary carcinomas [21,22]. Nevertheless, the instability of Re^{3+} in the HEDP-complex is still an unsolved problem, because Re^{3+} will be oxidized to ReO_4^- in contact with air.

Prior to the investigations of the real Re(III)–HEDP agent, Re(III)–HEDP, generated in batch by mixing of Re^{3+} and HEDP, and ReO_4^- were separated in a 50 m*M* phosphate buffer. The pH of the separation buffer have to be close to the physiological conditions to obtain information about the chemical behavior under these conditions. However high electroosmotic flow was also necessary, to allow the separation of analytes with significant differences in charge and to obtain short migration times. Therefore, a pH of 8 was chosen. For the stabilization of the Re(III)–HEDP complex 20 m*M* HEDP was added to the buffer. Fig. 3A shows the separation of a mixture of Re(III)–HEDP and ReO_4^- under these conditions with direct UV detection. Due to the poor

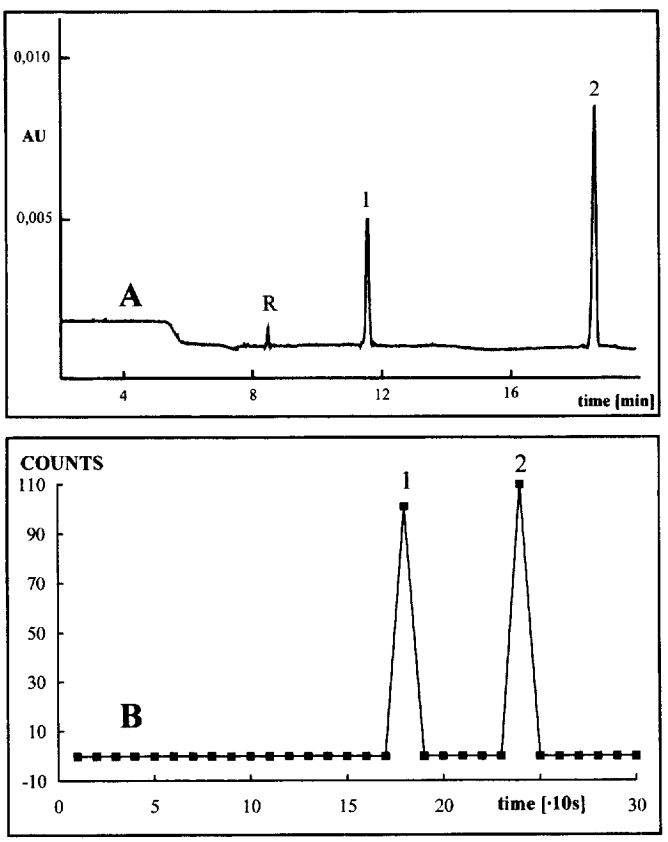

Fig. 3. Separation of Re(III)–HEDP (1) and ReO_4^- (2) in (A) 50 m*M* phosphate–20 m*M* HEDP pH 8.0, −20 kV, UV detection at 200 nm and (B) 50 m*M* phosphate–20 m*M* HEDP pH 8.0, −10.5 kV (10 min), PIXE detection.

absorbance of the analytes very high concentrations (10^{-3} *M* range) are needed to allow direct UV detection. Although Re(III)–HEDP was injected in a higher concentration than ReO_4^- the peak intensity for the Re(III)–HEDP complex was significantly lower than the ReO_4^- peak. In Fig. 3B the same separation using the PIXE detector is shown. Both rhenium compounds are injected at the same concentration and consequently nearly the same peak height could be observed. The concentration for each compound was 10^{-3} *M*, but corresponding to the peak intensity, a significant lower concentration could be used. Next, the decomposition of the Re(III)–HEDP agent to ReO_4^- was investigated with the PIXE detector. The time dependent alteration of the Re^{3+} and ReO_4^- X-ray signals is shown in Fig. 4. After dissolving the agent in triply distilled water, no signal for ReO_4^- was observed for up to 0.5 h. After 1 h a ReO_4^- signal was detected for the first time and after that the signal intensity increased with time. In contrast to this behavior the concentration of Re(III)–HEDP was decreased and consequently the signal intensity of the complex decreased also. These results fit with a rate law of first order. To calculate accurate kinetic parameters and to draw conclusions for the behavior of such complexes in the organism, more experimental data have to be obtained and the dependence of the results on the separation conditions have to be examined. For all that, this example impressively demonstrates the special ability of element selective detectors, like the PIXE detector, for the investigation of kinetic processes of metal containing compounds with poor absorbance properties.

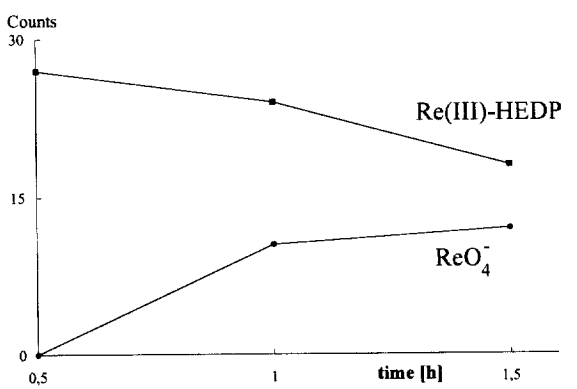

Fig. 4. Decomposition of Re(III)–HEDP to ReO_4^-. PIXE detection; buffer: 50 m*M* phosphate–20 m*M* HEDP pH 8.0, −10.5 kV (10 min).

3.3. Separation of metallothioneins (MTs)

Metallothionein (MT) is a metalloprotein with sixty-one amino acids, among them twenty conserved cysteine residues. They bind up to seven divalent transition metal atoms, such as Zn, Cd and Cu, by association with the cysteine thiol groups [23]. The binding of the metals to MT isoforms is crucial for the stabilization of the protein secondary structure. MT consists of a family of protein isoforms, which are formed from different genes. All have distinctive characteristics through substitution of as little as one amino acid up to fifteen residues. In most animal species MT has two major isoforms (MT-1 and MT-2). MT-1 carries two and MT-2 three negative charges at neutral pH [24]. MTs have been intensively studied over the last few years with respect to their reported roles in the zinc and copper homeostasis and heavy metal detoxification. Also the mechanism of metal mediated expression of MT genes is the focus of interest.

CE has been successfully applied to the study of different metalloproteins [25]. For the separation of MTs untreated [26,27] and coated capillaries [28] have been used. Micellar electrokinetic capillary chromatography (MECC) was also applied [29,30], to allow the separation of different isoforms of MT-1 and MT-2.

A metal specific detector is especially useful for the determination of the metals which bind to concrete isoforms. In Fig. 5A the separation of rabbit liver MT in a 20 m*M* phosphate buffer pH 6.0 is shown. Six different isoforms are clearly separated. The main isoforms are MT-1 (4) and MT-2 (5). With the PIXE detector the metals Zn, Cu and Cd have been detected. Comparing the UV with the PIXE detection mode, different metals could be assigned to distinct peaks or MT-isoforms. So Cu and Cd binds preferably to MT-1 and Zn to MT-2. For horse kidney MTs the separation of the main components in a phosphate buffer with pH 6.0 was incomplete. In this buffer the MT-1 and MT-2 isoforms migrate at the end of the electropherogram. Better resolution was obtained in a strong alkaline phosphate buffer

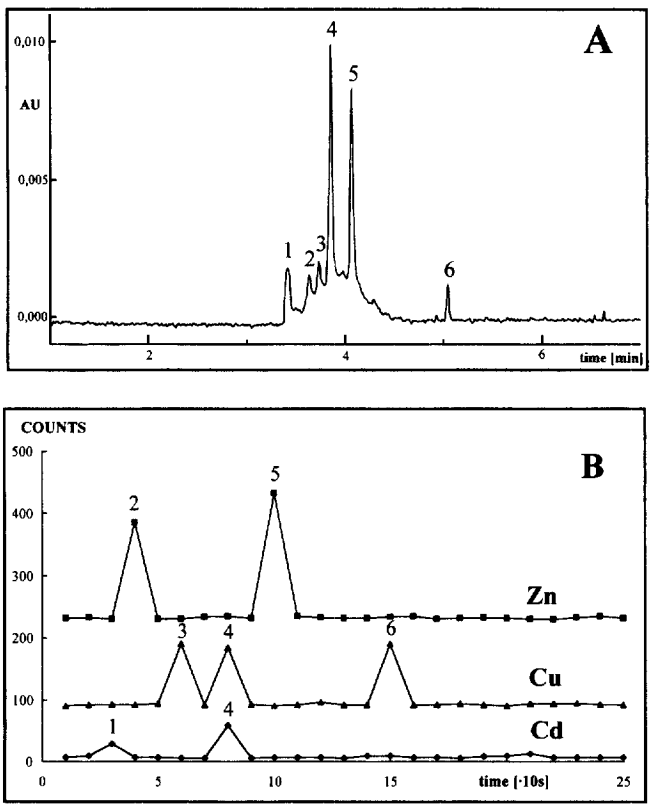

Fig. 5. Separation of rabbit liver metallothionein in (A) 20 m*M* phosphate pH 6.0, 20 kV, UV detection at 200 nm and (B) 20 m*M* phosphate pH 6.0, 10.5 kV, PIXE detection; metallothionein concentration 0.5 mg/ml.

with pH 11.0 (Fig. 6A). In this case five main compounds could be separated. Due to comparable peak intensities the main isoforms of MT-1 and MT-2 could not be committed, but a correspondence with peaks 1 and 2 is unlikely. Fig. 6B shows the corresponding PIXE detection. Zn is preferably bound to the components in peak 5 and 3, Cu to those in 4 and 2. Again both elements show clearly distinct behavior in bonding to the metallothioneins.

4. Conclusions

The element selective PIXE detector is a very useful completion to common optical detectors. For elements with $Z>14$ simultaneous detection of more than twenty elements is possible. Assignments between the signals of successively applied UV and PIXE detectors allow an improved characterization of the separated species and quantification of the metals independently of the compound to which the metals are bound. Neither the separation conditions nor the buffer composition interfere with the generation of the X-ray signals. In some cases lower limits of detection than for UV detection could be observed.

Further investigations have to be concentrated on a simultaneous detection with UV and PIXE detectors, which could help to improve the interpretation of the separation.

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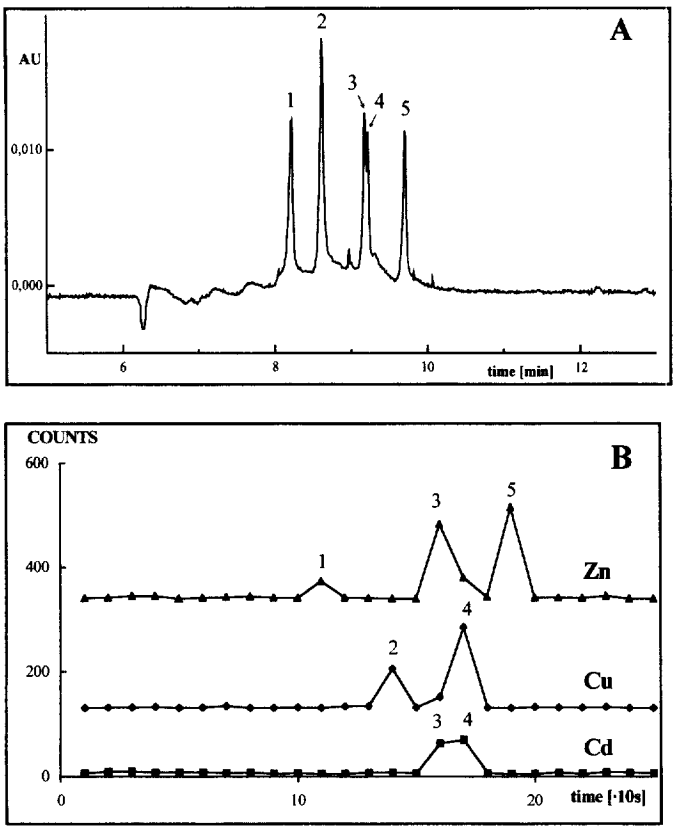

Fig. 6. Separation of horse kidney metallothionein in (A) 20 m*M* phosphate pH 11.0, 20 kV, UV detection at 200 nm and (B) 20 m*M* phosphate pH 11.0, 10.5 kV, PIXE detection; metallothionein concentration 0.5 mg/ml.

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