Fresenius J Anal Chem (2001) 371 : 764–774
DOI 10.1007/s002160101019

SPECIAL ISSUE PAPER

Andreas Prange · Dirk Schaumlöffel · Peter Brätter
Andrea-Nicole Richarz · Christian Wolf

Species analysis of metallothionein isoforms in human brain cytosols by use of capillary electrophoresis hyphenated to inductively coupled plasma–sector field mass spectrometry

Received: 6 April 2001 / Revised: 17 June 2001 / Accepted: 20 June 2001 / Published online: 13 October 2001

Abstract A new approach for the speciation of metallothioneins (MT) in human brain cytosols is described. The analysis is performed by application of a newly developed coupling of capillary electrophoresis (CE) with inductively coupled plasma–sector field mass spectrometry (ICP–SFMS). Isoforms of metallothioneins are separated from 30–100 μL sample volumes by CE and the elements Cu, Zn, Cd, and S are detected by use of ICP–SFMS.

The extraction of cytosols is the first step in the analytical procedure. Tissue samples from human brain are homogenized in a buffer solution and submitted to ultra-centrifugation. The supernatant is defatted and the cytosol pre-treatment is optimized for CE separation by matrix reduction. The buffer concentration and pH used for capillary electrophoretic separation of metallothionein from rabbit liver were optimized. CE with ICP–MS detection is compared to UV detection. In the electropherograms obtained from the cytosols three peaks can be assigned to MT-1, MT-2, and MT-3. As an additional method, size-exclusion chromatography (SEC) is applied. Fractions from an SEC separation of the cytosol are collected, concentrated, and then injected into the CE.

The detection of sulfur by ICP–SFMS (medium resolution mode) and quantification by isotope dilution have also been investigated as a new method for the quantification of MT isoforms.

The analytical procedure developed has been used for the first time in comparative studies of the distributions of MT-1, MT-2, and MT-3 in brain samples taken from patients with Alzheimer's disease and from a control group.

Dedicated to Professor Dr. Bernd Neidhart on the occasion of his 60th birthday

A. Prange (✉) · D. Schaumlöffel
GKSS Forschungszentrum,
Max-Planck-Straße, 21502 Geesthacht, Germany
e-mail: andreas.prange@gkss.de

P. Brätter · A.-N. Richarz · C. Wolf
Hahn-Meitner-Institut,
Glienicker Straße 100, 14109 Berlin, Germany

Introduction

Living organisms are highly dependent on trace elements in different ways. Whereas metals such as Mo, Mn, Fe, Co, Cu, and Zn are essential, others such as Cd, Pb, and Hg or metalloids (As) are regarded as being highly toxic [1]. Some known human and animal diseases are related to the presence or absence of metals. Information about metals in body fluids or organ tissues can be relevant for distinguishing between a pathogenic and a healthy status and for making a diagnostic statement. To understand the metabolism, bioavailability, and toxicity of a metal or metalloid, information on the total element concentration is not sufficient. Additional information on the identities and quantities of the metal compounds, also called metal species, present in a biomedical sample such as a tissue is necessary [2].

A sensitive and specific method for the determination of metalloproteins in small volumes of animal or human samples is, therefore, an important prerequisite for the extension of clinical diagnosis and therapeutic methods of approach.

Metalloproteins with anti-oxidative properties, e.g. metallothioneins (MT) [3, 4, 5, 6], are of current interest. Metallothioneins are cysteine-rich proteins with a molecular mass of 6–7 kD that include 20 cysteines which complex mainly with the metals Cu, Zn, and Cd. They occur in several isoforms that differ slightly in amino acid sequence. MT-1 and MT-2 occur mainly in the liver and kidney and are formed by organisms as a specific ligand for the transport and storage of essential or toxic metals. Their formation is induced by heavy metals, hormones, cytotoxic agents, and physical or chemical stress. The functions of metallothioneins under discussion are:

- homeostasis of copper and zinc
- detoxification of heavy metals

- scavenging of radicals; and
- acute phase protein.

Another metallothionein isoform was first described in 1991, when the growth inhibition factor in human brain that inhibits the survival of cortical neurones in-vitro, was found to be a metallothionein-like protein with a sequence 70% similar to that of human MT-2 [7]. It has been classified as MT-3 [8]. The difference is a one-amino-acid insert in the NH_2-terminal region and a six-amino-acid insert in the COOH-terminal region. These additional residues cause the unusual acidity of MT-3. The net charge of the apo-protein is –4; those of apo-MT are +2 to +5 [7]. MT-3 is not induced by agents such as heavy metals as are the other metallothioneins [8].

The downregulation of MT-3 in brains affected by Alzheimer's disease [7], which has been the subject of some controversy [9], has recently been confirmed [10]. MT-3 is, therefore, believed to be implicated in the pathogenesis of Alzheimer's disease. Some aspects of Alzheimer's disease in which MT could play a role are disturbances in zinc metabolism with zinc contributing to the formation of β-amyloid aggregations [11] and oxidative stress.

The species analysis of metallothionein isoforms is an example of the special challenges of the development of microanalytical procedures for element species analysis [12, 13]. A strategy for the species analysis of metallothionein isoforms in human cytosols can be described as a multi-dimensional analytical procedure that is based on chromatographic and/or electrophoretic and mass spectrometric methods. An overview is given in Fig. 1. After preparation of the cytosols from brain samples, chromatographic methods such as high-performance liquid chromatography (HPLC) – especially size-exclusion chromatography (SEC) [14, 15] – are used as a first separation step, followed by capillary electrophoresis (CE). The use of CE with UV detection for the separation of MT isoforms has often been demonstrated [16, 17, 18]. The hyphenation of CE with inductively coupled plasma–mass spectrometry (ICP–MS) enables the metal-specific detection of metallothionein isoforms [19, 20, 21, 22, 23]. LC- or CE–electrospray tandem mass spectrometry (ESI–MS–MS) combinations can be used to obtain molecule-specific information [23]. The coupling of CE with inductively coupled plasma–sector field mass spectrometry (ICP–SFMS) is an approach for the quantification of metallothionein isoforms via the sulfur of the amino acids (cysteine and methionine) of the proteins by isotope dilution [24]. Altogether, this analytical information finally leads to more concrete information about the particular metallothionein isoforms under investigation.

This paper will focus on applications using CE–ICP–SFMS. Results obtained from investigations using CE–ESI–MS-MS will be published later.

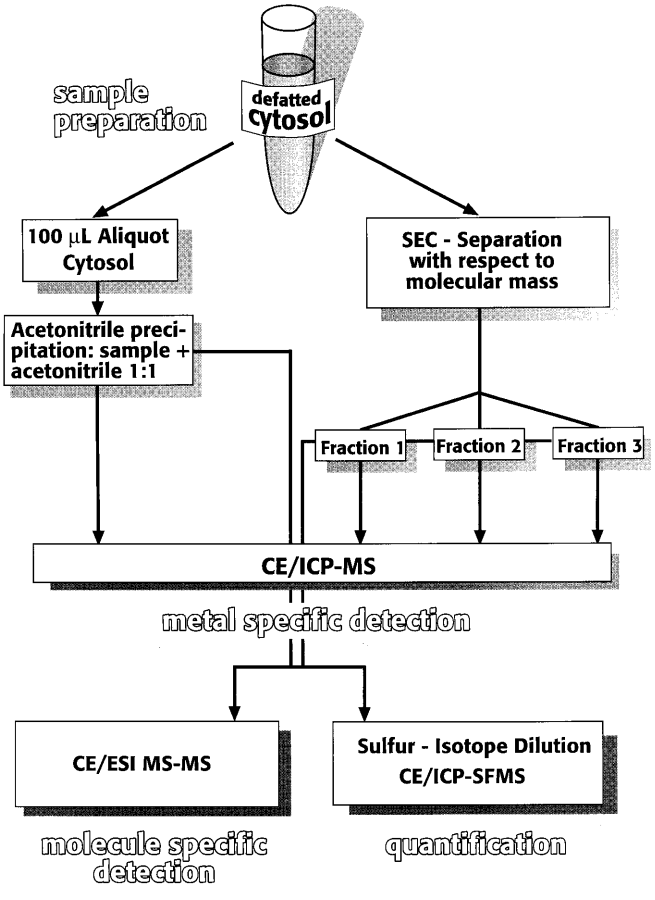

Fig. 1 Schematic diagram of the multi-dimensional framework of an integrated analytical procedure for species analysis of metalloproteins

Experimental

Chemicals and materials

Rabbit liver metallothionein (MT, Lot 19H7812) and metallothionein 1 (MT-1, Lot 127H7810) and metallothionein 2 (MT-2, Lot 49H7822) were purchased from Sigma (Deisenhofen, Germany). Human metallothionein 3 (MT-3) expressed in *E. coli* was provided by courtesy of Professor M. Vasak, University of Zürich, Switzerland. The metallothionein preparations were diluted with degassed water or buffer and stored under argon at –18 °C to prevent oxidation.

Tris buffer solution (20 mmol L^{-1}) was prepared by dissolving 1.21 g Tris(hydroxymethyl)aminomethane (p.A., Merck, Darmstadt, Germany) in 500 mL water and adjusting the solution to pH 7.4 with nitric acid. The buffer solution was further cleaned, to remove metal contamination (especially Cu and Zn) by use of Chelex 100 ion exchange resin (Fluka, Buchs, Switzerland). A solution of potassium hydroxide (0.1 mol L^{-1}, suprapur, Merck) was prepared for conditioning the CE capillaries.

Fused silica capillaries, 75 µm i.d. and 360 µm o.d., were purchased from Thermo Separation Products (TSP, Egelsbach, Germany) and pre-conditioned by rinsing with 1 mol L^{-1} KOH (suprapur, Merck) for 5 min before use.

An ammonium nitrate buffer solution (20 mmol L^{-1}) was used as make-up liquid for CE–ICP–MS. Ammonia solution (suprapur, Merck; 25%, 0.78 mL) was dissolved in 500 mL water and adjusted to pH 7.4 with nitric acid (suprapur, Merck). A spike of 1 µg Rb L^{-1} served for stability control of the nebulizer and as internal standard. Rb^+ was selected because, as an alkali metal ion, it forms no insoluble hydroxides at pH 7.4 and therefore the Rb^+ solution is stable. Nitric acid (suprapur, Merck) was cleaned by sub-boiling in a quartz apparatus and was diluted with ultra-pure water.

By using high analytical grade ammonia and nitric acid to prepare the make-up liquid, the background levels of Cu and Zn could

be kept as low as possible. This could not be achieved by using cleaned Tris buffer as make-up liquid.

Stock solutions of the isotopes ^{34}S, ^{65}Cu, ^{68}Zn, and ^{116}Cd were used for the isotope-dilution experiments. A species-unspecific spike solution was prepared by dilution of the isotopes to final concentrations of 300 μg L^{-1} ^{34}S, 90 μg L^{-1} ^{65}Cu, 60 μg L^{-1} ^{68}Zn and 280 μg L^{-1} ^{116}Cd with ultra-pure water. The metals were then stabilized with 2,6-diacetylpyridine-bis(*N*-methylenepyridiniohydrazone) dichloride (0.2 mmol L^{-1}) prepared as described elsewhere [25]. After this the solution was adjusted to pH 7.4 with an ammonia solution (25%, suprapur, Merck). This spike solution was used as make-up liquid for the isotope dilution measurements. Further details are described in a special publication dedicated to the use of isotope dilution for the quantification and characterization of metallothioneins [24].

Dithiothreitol (DTT) (Sigma) was used for stabilization of the metalloproteins during fraction collection after SEC separation. Frigen (Karl Schindler, Köln, Germany) was used to defat the cytosols and acetonitrile (Merck) for matrix reduction.

All dilutions were prepared with filtered 18 MΩ deionized water obtained from a Milli-Q system (Millipore, Milford, MA, USA)

Sample preparation

Brain samples

The human brain samples investigated in this work were obtained from the Referenzzentrum für neurodegenerative Erkrankungen c/o Klinikum Großhadern, München, Germany. The samples were from different brain regions, e.g. temporal, occipital, parietal, and cerebellum. For comparative studies samples were obtained from patients with Alzheimer's disease and from a control group. Both groups were age-matched.

Cytosol preparation

For cytosol preparation approximately 1 g brain tissue was homogenized with a Potter–Elvehjem homogenizer in 2 mL degassed Tris buffer (20 mmol L^{-1}, pH: 7.4). Whenever possible this procedure was performed with ice cooling and under an argon atmosphere to prevent degradation of the samples. The homogenate was then centrifuged at 100,000×g for 1 h (4 °C). The supernatant was stored under argon until analysis at –18 °C.

Cytosol pre-treatment before CE measurements

The cytosol was generally defatted with frigen. Four different pretreatment steps were compared: (i) pure defatted cytosol, (ii) diluted cytosol, (iii) heat denaturation, and (iv) acetonitrile precipitation.

Fractionation by size-exclusion chromatography

Size-exclusion chromatography (SEC) was performed as a separation step before CE. SEC separation was performed using a HiLoad 16/60 Superdex 75 PG column (Pharmacia Biotech, Upsala, Sweden) and an HPLC system (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled to an ICP–QMS (Agilent 4500) as element specific detector. The instrumental conditions are listed in Table 1.

Firstly, an SEC–ICP–QMS measurement was performed to obtain the retention times of the detected peaks from the resulting chromatogram. In a second run, 30 fractions of 2 mL each were taken from the time window between 1500–3200 s. The three fractions of the corresponding peaks in the chromatogram (see Fig. 13, below) were concentrated by ultrafiltration with a 3 kD membrane (Amicon/Millipore, Eschborn, Germany). An enrichment factor of approximately 15–30 was achieved.

Table 1 Experimental conditions used for SEC–ICP–QMS

Size-exclusion chromatography	
Column	HiLoad 16/60 Superdex 75 PG (16 mm×600 mm)
Buffer solution	20 mmol L^{-1} Tris+5 mmol DTT, pH 7.4;
Flow rate	2 mL min^{-1}
Injection volume	500 μL
ICP-QMS	
Cool gas	15.2 L min^{-1}
Auxiliary gas	1.0 L min^{-1}
Nebulizer gas	0.95 L min^{-1}
Power	1250 W
Nebulizer	Babington
Isotopes monitored	^{63}Cu; ^{64}Zn; ^{114}Cd

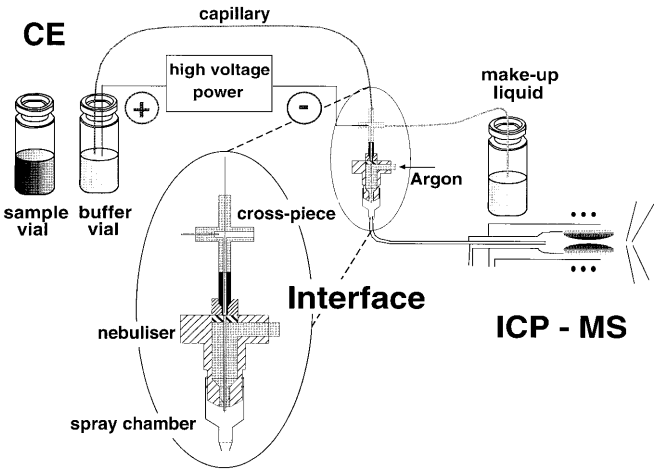

Fig. 2 Schematic diagram of the CE–ICP–MS system

Instrumentation

A schematic view of the instrumental set-up illustrating the CE, the interface and the ICP–MS is shown in Fig. 2. An Agilent 3D CE system containing fused-silica capillaries of length 70 cm and inner diameter (i.d.) 75 μm was used in this work. The new interface (CEI-100, CETAC, Omaha, USA), described in detail in previous publications [26, 27], was used to couple the CE to an inductively coupled plasma–sector field mass spectrometer (Element, Finnigan MAT, Bremen, Germany) as element-specific detector. The instrumental conditions are summarized in Table 2.

CE

The sample is injected hydrodynamically at 20 kPa s (5 kPa for 4 s), which corresponds to a sample volume of 22 nL. To condition the capillary it is rinsed with 0.1 mol L^{-1} KOH for 5 min and with buffer for 15 min at 0.1 kPa before each run. To prevent buffer depletion and to reduce background levels of copper and zinc, the buffer is changed completely every run. The MT separations are performed under optimized conditions at 30 kV and 15 °C.

Interface

One important component of the coupling interface is a specially designed nebulizer that works in the self-aspiration mode at a stable flow rate of approximately 8.0 μL min^{-1}. This ensures that the make-up liquid (NH_4NO_3 buffer, 15 mmol L^{-1}, pH 4) is aspirated continuously, which provides the electrical connection and adapts

Table 2 Experimental conditions used for CE–ICP–SFMS

Capillary electrophoresis	
Capillary	Fused silica, 70 cm length, 75 μm i.d.
Buffer solution	20 mmol L^{-1} Tris, pH 7.4;
Temperature	15 °C
Voltage	30 kV
Injection	Hydrodynamic, 2 kPa s: MT preparations 20 kPa s: cytosols
CE–ICP–MS interface	
Liquid flow rate	8.0 μL min^{-1}
Buffer/make-up liquid	15 mmol L^{-1} ammonium nitrate, pH 7.4;
Spike	1 μg Rb L^{-1}
ICP–SFMS	
Cool gas	15 L min^{-1}
Auxiliary gas	0.9 L min^{-1}
Nebulizer gas	1.0–1.1 L min^{-1}
Power	1075–1100 W
Mass resolution	300 resp. 3000
Scan duration	600 ms (low res.) or 880 ms (med. res.)
Isotopes monitored	^{63}Cu; ^{64}Zn; ^{85}Rb; ^{114}Cd

the flow rate of the electroosmotic flow inside the CE capillary to the flow rate of the nebulizer [26].

ICP–MS

The ICP–SFMS normally runs in the low-resolution mode (R=300). Only for simultaneous sulfur measurements and metal determinations is the medium resolution (R=3000) mode used. For this mode the acquisition method is optimized to a scan duration of 880 ms [24].

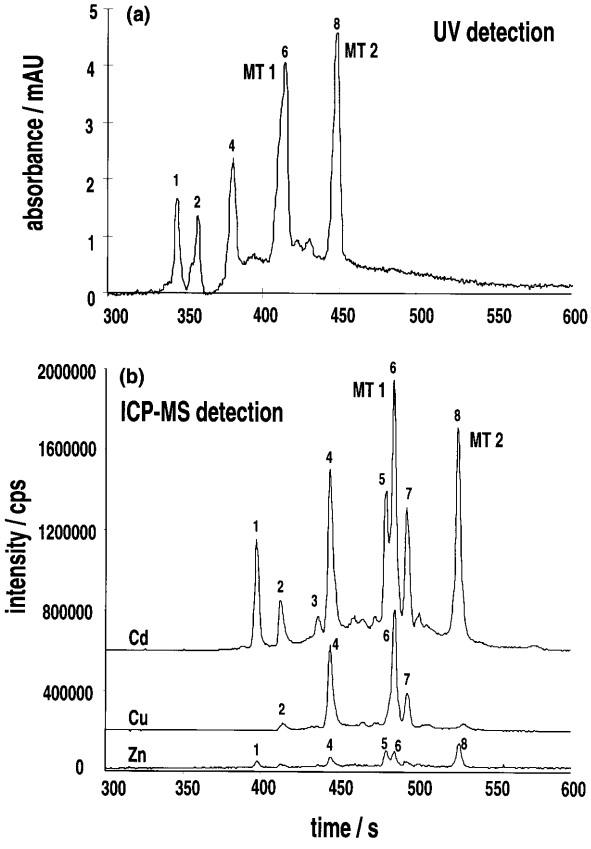

Fig. 3 Comparison of electropherograms obtained from rabbit liver MT (Lot 19H7812) by (**a**) UV-detection (λ 250 nm; 1 g MT L^{-1}; 22 nL injection, 20 kPa s) and (**b**) ICP–MS detection (0.1 g MT L^{-1}; 2.2 nL injection, 2 kPa s)

Results and discussion

Before application of the CE–ICP–MS system to cytosol samples, systematic investigations of metallothionein preparations from rabbit liver and on the sample preparation procedure had to be performed, to find optimum conditions for CE–ICP–MS.

Systematic investigations of MT preparations from rabbit liver

Optimization of CE separation of MT isoforms

Separations of the MT-isoforms performed by CE–ICP–MS and by CE–UV system were compared using commercially available MT (rabbit liver). The resulting electropherograms are compared in Fig. 3. Although the peak pattern is similar, it is evident that more analytical information is provided by the CE–ICP–MS system. The shift in migration times between UV detection and ICP–MS detection is because of the shorter effective length in UV detection. In both techniques the capillary lengths are 70 cm, but the UV detection window is located 8.5 cm before the end of the capillary, so the effective separation length is 61.5 cm. In addition to clearly improved sensitivity, metal-specific detection becomes possible with ICP–MS. Because of the greater sensitivity of ICP–MS detection a hundred-times smaller amount of sample can be injected into the CE capillary. This results in better separation compared with UV detection. At least eight peaks can be detected (Fig. 3b), and MT-1 and MT-2 can be identified by comparison of the migration times of relatively pure preparations of rabbit liver MT-1 and MT-2 in Fig. 4 with the migration times of peaks 5, 6, 7, and 8 in Fig. 3b. In comparison with UV detection, MT-1 seems to be split into peaks 5, 6 and 7 with different metal loadings of Cu, Zn, and Cd. For further detailed classification of these peaks more investigations must be performed to clarify their identity. Cd is present in all peaks detected, together with Zn at a lower level, but Cu is present in peaks 4, 6, and 7 only.

Conditions such as buffer pH and buffer concentration play an important role in the optimization of the separation; Figs 5 and 6 illustrate their effect. It was found that Tris buffer at pH 7.4 is well suited for application to cytosol samples of physiological pH 7.4. Buffer concentrations of 20 mmol L^{-1} are better than 50 mmol L^{-1}. At higher concentrations the increasing Joule heating of the capillary impaired the separation.

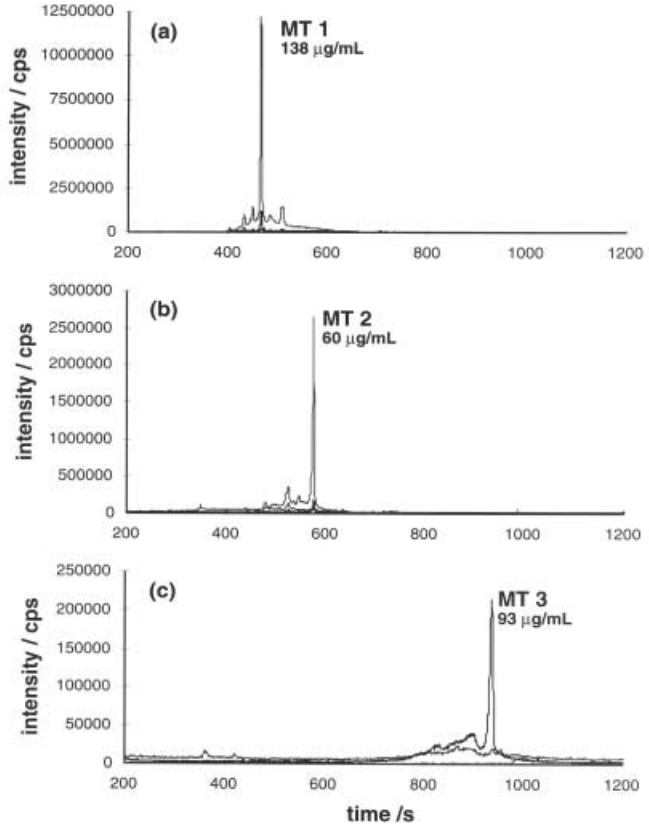

Fig. 4 Electropherograms of MT-1 (**a**), MT-2 (**b**) (both Cd) and MT-3 (**c**) (Zn)

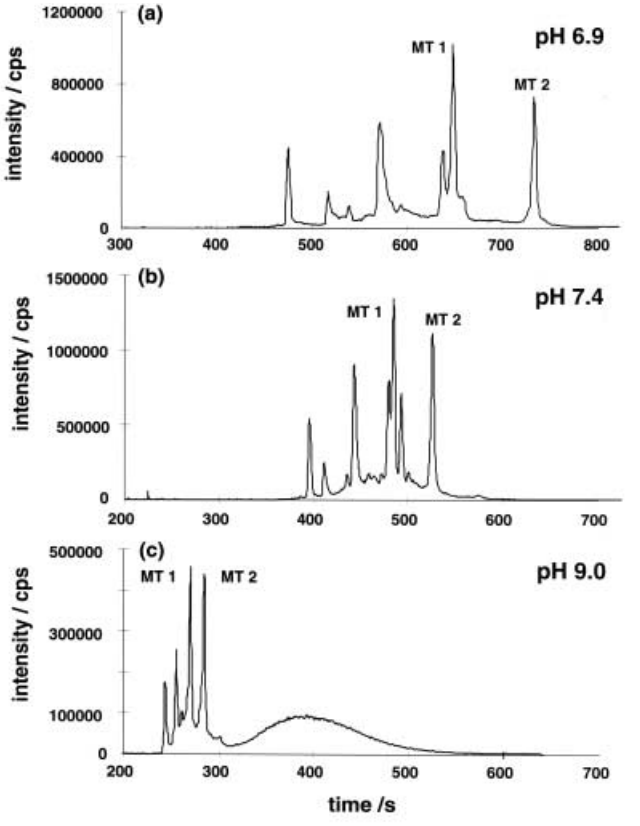

Fig. 5 Effect of pH on the separation of metallothionein isoforms from rabbit liver (Lot 19H7812). Electropherograms measured at the ^{114}Cd isotope

Detection limits and measurement precision

For analytical characterization of the CE–ICP–MS system, detection limits and precision were estimated from preparations of MT-1, MT-2, and MT-3. Each solution was prepared to a concentration of 0.1 mg mL^{-1} MT. Because of the impurity of the available MT preparations the concentration was checked by an independent method. The concentrations of the MT shown in Fig. 4 were, therefore, determined by use of total reflection X-ray fluorescence (TXRF), a method enabling determination of total element concentrations from a 2-µL sample. Each of the electropherograms obtained from MT-1, MT-2, and MT-3 (Fig. 4) contains one major peak from the corresponding isoform and some small peaks from other components. The approximate MT concentration can be calculated from a total sulfur determination in these MT preparations. Figure 7 shows an example of a TXRF spectrum from MT-2 (1 mg mL^{-1}). On the basis of a molar mass of 6870 Da (average value obtained from Ref. [23]), and via the sulfur of the cysteine and methionine, the MT-2 concentration was found to be 600 µg mL^{-1} for the example shown. Table 3 gives the estimated detection limits of the CE–ICP–MS system for MT-1, MT-2 (based on ^{114}Cd or ^{64}Zn measurements), and MT-3 (^{64}Zn measurement). In addition the relative and absolute detection limits of Cd and Zn in MT are given. Table 4 gives the precision of the

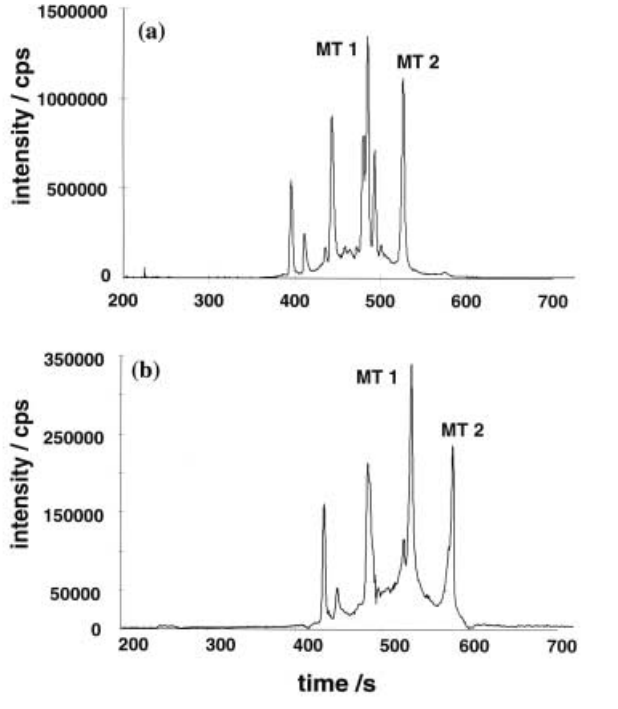

Fig. 6 Effect of buffer concentration on the separation of metallothionein isoforms from rabbit liver (Lot 19H7812). (**a**) 20 mmol L^{-1}, (**b**) 50 mmol L^{-1}. Electropherograms measured at the ^{114}Cd isotope

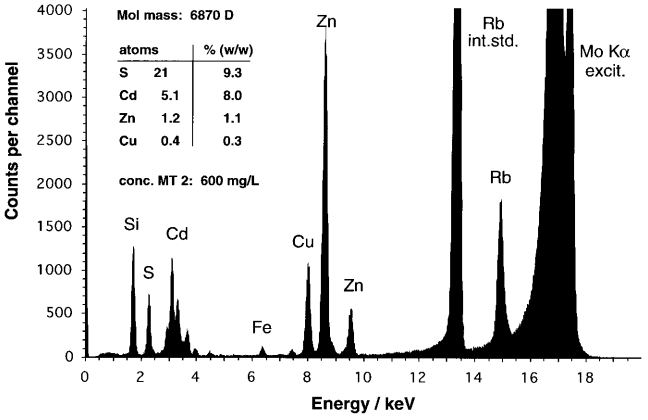

Fig. 7 TXRF spectrum of MT-2 using Mo-excitation and Rb as internal element standard (Si signal from the sample plate). The MT-2 concentration, molar sulfur/metal ratios, and the amounts (%) of the considered elements in MT-2 are obtained from this TXRF measurement

Table 3 Estimated limits of detection (LOD) for MT-1, MT-2, MT-3, and the related metals (3σ criterion)

	Cd-114	Zn-64
MT-1		
MT conc. LOD (μg mL^{-1})	0.011	2.02
Metal conc. LOD (ng mL^{-1})	0.8	21.2
Metal absolute LOD (fg)	1.9	46.6
MT-2		
MT conc. LOD (μg mL^{-1})	0.023	5.45
Metal conc. LOD (ng mL^{-1})	1.8	55
Metal absolute LOD (fg)	4.0	125
MT-3		
MT conc. LOD (μg mL^{-1})	–	7.6
Metal conc. LOD (ng mL^{-1})	–	420
Metal absolute LOD (pg)	–	9.4

Table 4 Precision of MT measurements

	MT-1	MT-2	MT-3
Obtained from metallothionein preparations RSD (%) (n=5)			
Migration time	1.1	0.7	n.d.
Peak area	9.9	16.5	n.d.
Peak height	6.5	16.0	n.d.
Obtained from MT in human brain cytosol RSD (%) (n=5)			
Migration time	1.2	1.3	4.2
Peak area	4.8	18.2	26.1
Peak height	4.7	9.8	34.7

migration time, peak area and peak height obtained from the MT-preparations considered and from cytosol samples (see below).

Quantification of MT using CE–ICP–IDMS

By use of MT preparations from rabbit liver an on-line CE–ICP–IDMS method was developed for quantification of MT isoforms via the determination of sulfur isotope ratios [24]. In contrast with TXRF measurements, CE–ICP–IDMS enables the quantification of the isoforms after CE separation. The isotopes ^{32}S, ^{34}S, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{68}Zn, ^{114}Cd, and ^{116}Cd were detected simultaneously by use of ICP–SFMS in medium-resolution mode. On-line isotope dilution was performed by continuous introduction of an isotopically enriched species-unspecific spike solution as make-up liquid after the separation step. Figure 8 shows the results obtained from CE–ICP–IDMS measurement of an MT sample, the same MT preparation as was used to obtain the results shown in Fig. 3b. In addition the isotope ^{32}S, the enriched isotope ^{34}S and the calculated ^{32}S/^{34}S ratio are shown. A mass-flow electropherogram can be derived from the isotope-ratio electropherogram and by peak-area integration; the amount of sulfur can be obtained. Considering the molar ratio of 21 sulfur atoms per molecule the quantities of the MT isoforms are calculated. As an example, the results from quantification of MT-1 and MT-2 are presented in Fig. 9.

After metal quantification, the molar ratios of sulfur to metals can be used to characterize the composition of the metalloprotein complex of the MT isoforms. Thus cautious suggestions for their stoichiometric formulae can be given. For example, the composition of the MT-2 metallo-

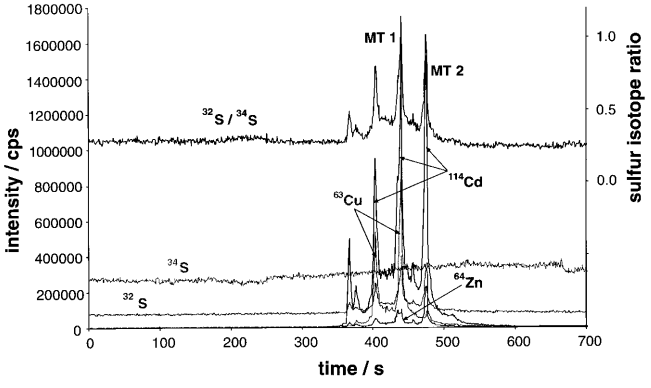

Fig. 8 Electropherogram obtained from rabbit liver MT with medium mass resolution. Measurement of ^{32}S and ^{34}S and calculation of the ^{32}S/^{34}S isotope ratio

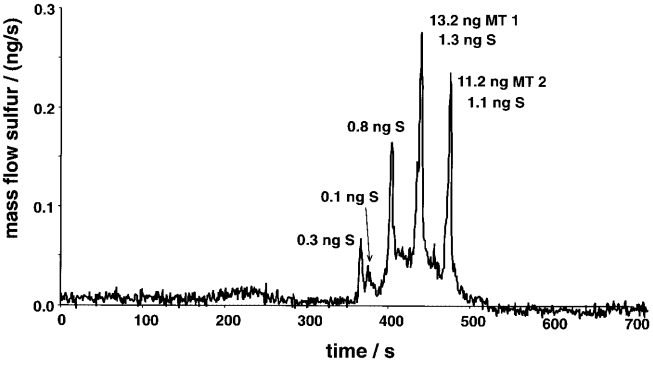

Fig. 9 Resulting sulfur mass flow derived from Fig. 8 and the amount of sulfur obtained by peak-area integration

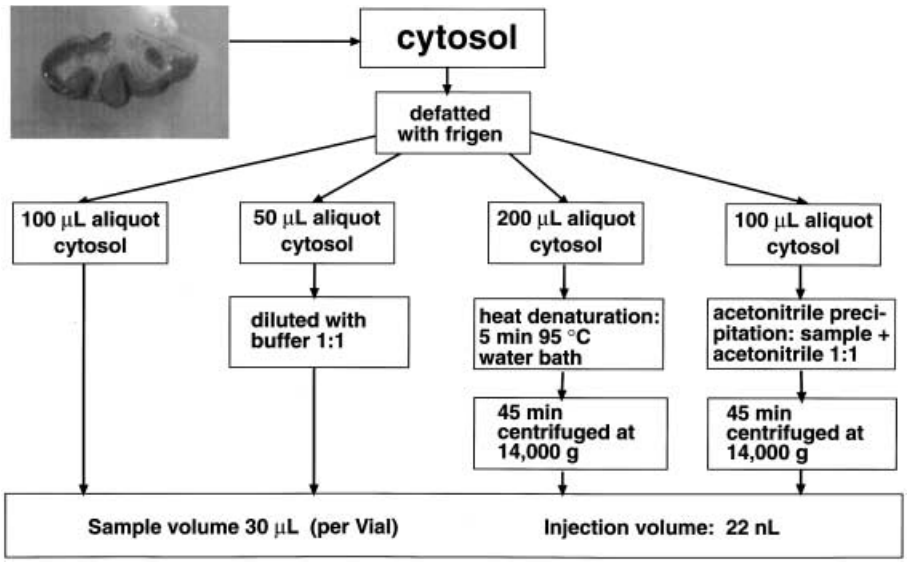

Fig. 10 Flow chart for cytosol preparation for subsequent measurements

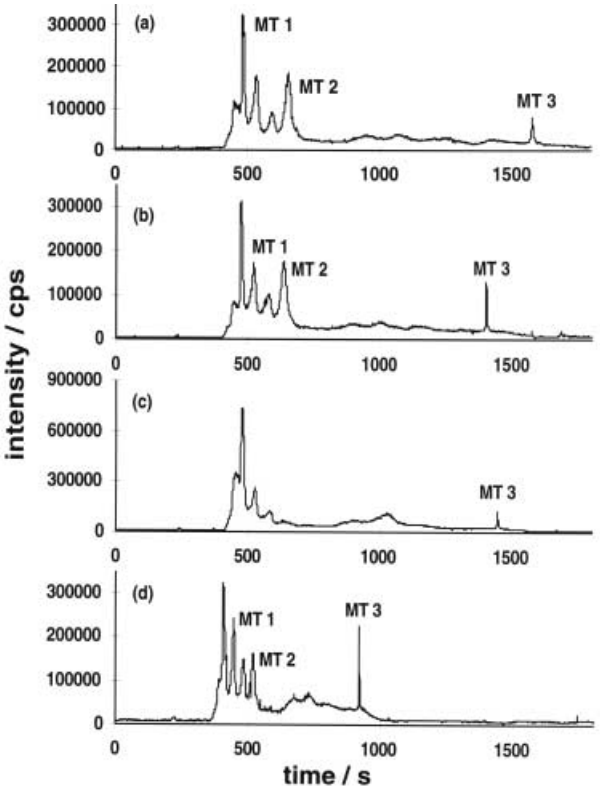

Fig. 11 Effect of cytosol pre-treatment on CE separation: (**a**) cytosol, (**b**) diluted cytosol, (**c**) heat denaturation, (**d**) acetonitrile precipitation. Matrix separation results in a change in sample viscosity which results in different amounts of sample injected. Peak intensities in (**a**), (**b**), (**c**) and (**d**) are, therefore, not comparable

protein complex in the sample investigated can be characterized as Cd_6Zn_1-MT-2. Further details of this method and its application to the characterization and quantification of MT isoforms will be published separately [24].

Application of the method to human cytosol samples

Optimization of sample pre-treatment

Because of the high protein content of cytosol samples, reduction of this interfering matrix is recommended before chromatographic separation, especially when using capillary electrophoresis. The effect on the separation of the MT-isoforms of different sample-preparation methods for the cytosols from human brains – dilution of the cytosol, heat denaturation, and acetonitrile precipitation – was investigated:. Figure 10 summarizes the different approaches in a flow chart. Figure 11 shows the corresponding results. To simplify the comparison only the copper electropherograms are shown. The electropherograms in Figs 11a, 11b, and 11d have the same pattern but migration times decrease in the order (a)>(b)>(d), because of the removal of interfering proteins by the pretreatment procedure. These matrix proteins are not detected by ICP–MS but are still present in the cytosol and influence the migration times as a result of their adsorption on the capillary wall. As a result it can be stated that for optimum separations acetonitrile precipitation gives the best results with regard to separation and speed. By this treatment most of the interfering proteins are removed without changing the MT. In contrast, heat denaturation seems to alter the metal loading of the MT-species (Fig. 11c).

Assignment of the peaks detected

Figure 12 shows the electropherogram obtained from a cerebellum cytosol containing the elements Cu, Zn, and Cd after acetonitrile precipitation. Two indications support the hypothesis that the signals monitored are metallothioneins:

1. the compounds observed are stable against acetonitrile; and
2. Cu, Zn, and Cd peaks can be observed simultaneously.

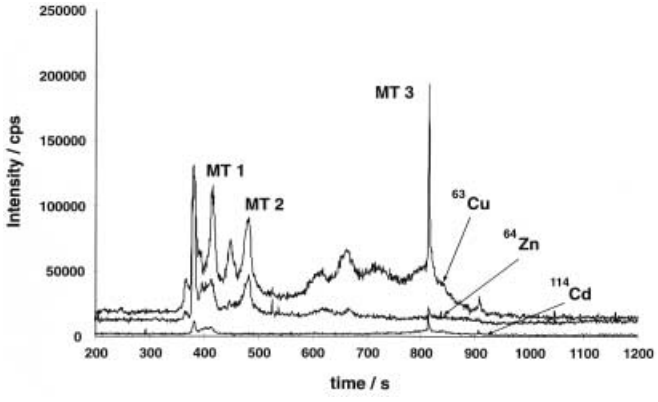

Fig. 12 Electropherogram from a human brain cytosol (cerebellum) for the elements Cu, Zn, and Cd

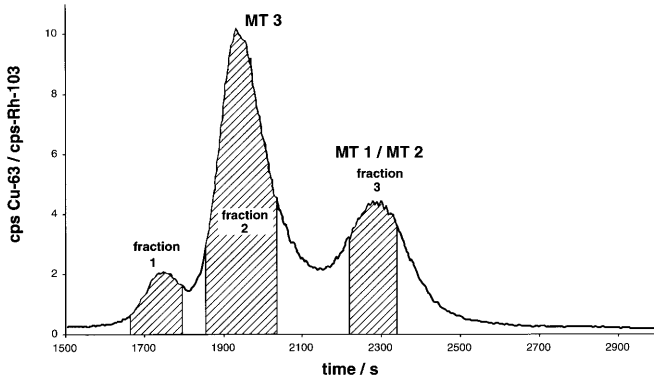

Fig. 13 SEC chromatogram from a cerebellum cytosol detected on ^{63}Cu. Three fractions are cut, concentrated and investigated further

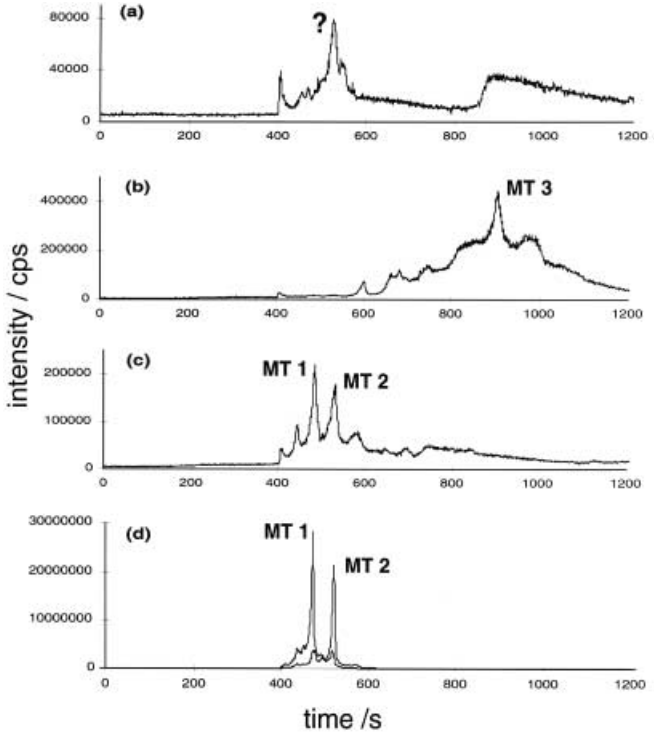

Fig. 14 CE–ICP–MS electropherograms obtained from the SEC fraction from Fig. 13. (**a**) fraction 1 (^{63}Cu); (**b**) fraction 2 (^{63}Cu); (**c**) fraction 3 (^{63}Cu); (**d**) Comparison with MT-1 and MT-2 from rabbit liver (detected on ^{114}Cd and overlay of two measurements)

An additional comparison with the migration times of the MT preparations shown in Fig. 4 leads to the tentative suggestion that three of the observed peaks correspond to MT-1, MT-2, and MT-3, as indicated in Fig. 12. A problem is, of course, comparison of MT from rabbit liver or gene expression with human brain cytosols. The conformity of the amino acid sequences of human MT and rabbit MT is approximately 90%. MT-1 human [28] differs from MT-1 rabbit [29] in seven of 61 amino acids, whereas MT-2 human [30] and MT-2 rabbit [31] differ in five only. Being conscious of the uncertainty, we made the assumption that human MT and rabbit MT have the same electrophoretic migration behaviour.

Fractionation of the cytosols by SEC before CE–ICP–MS

To improve the separation efficiency a combination of two separation methods, SEC and CE, was employed – fractions were collected after size-exclusion chromatography before CE separation. Figure 13 shows the SEC chromatogram obtained from a brain sample from the cerebellum. Three peaks can be observed, peak 2 includes MT-3 and peak 3 MT-1 and MT-2, according to Ref. [14]. A fraction was cut from each peak and the proteins were concentrated in the fractions. Finally, separation by capillary electrophoresis and metal-specific detection was performed for each fraction. Figure 14a–d shows the results from the electrophoretic separations compared with the MT-1 and MT-2 preparations (Fig. 14d). It was confirmed that fraction 2 contains MT-3 (by comparing the migration time with the MT-3 preparation in Fig. 4) and some unidentified isoforms but no MT-1 or MT-2, whereas the last two isoforms are contained in fraction 3. The peak in fraction 1 has not yet been identified. Further details of this work, dealing with preparative chromatographic separation before capillary electrophoresis, will be published elsewhere.

Application to patients with Alzheimer's disease

Human brain samples from the temporal, occipital, and parietal regions were analysed for a comparative study between brains affected by Alzheimer's disease and control samples. From each group three samples of the occipital and parietal and one of the temporal region were investigated. CE–ICP–MS was applied to cytosols treated with acetonitrile to reduce the protein matrix by precipitation. The resulting electropherograms are summarized in Figs 15 16 17. For better comparison all intensities are normalized to the intensity of ^{85}Rb, which was added to the make-up liquid as internal standard. Figure 15 shows the results from the temporal samples for the elements Cu,

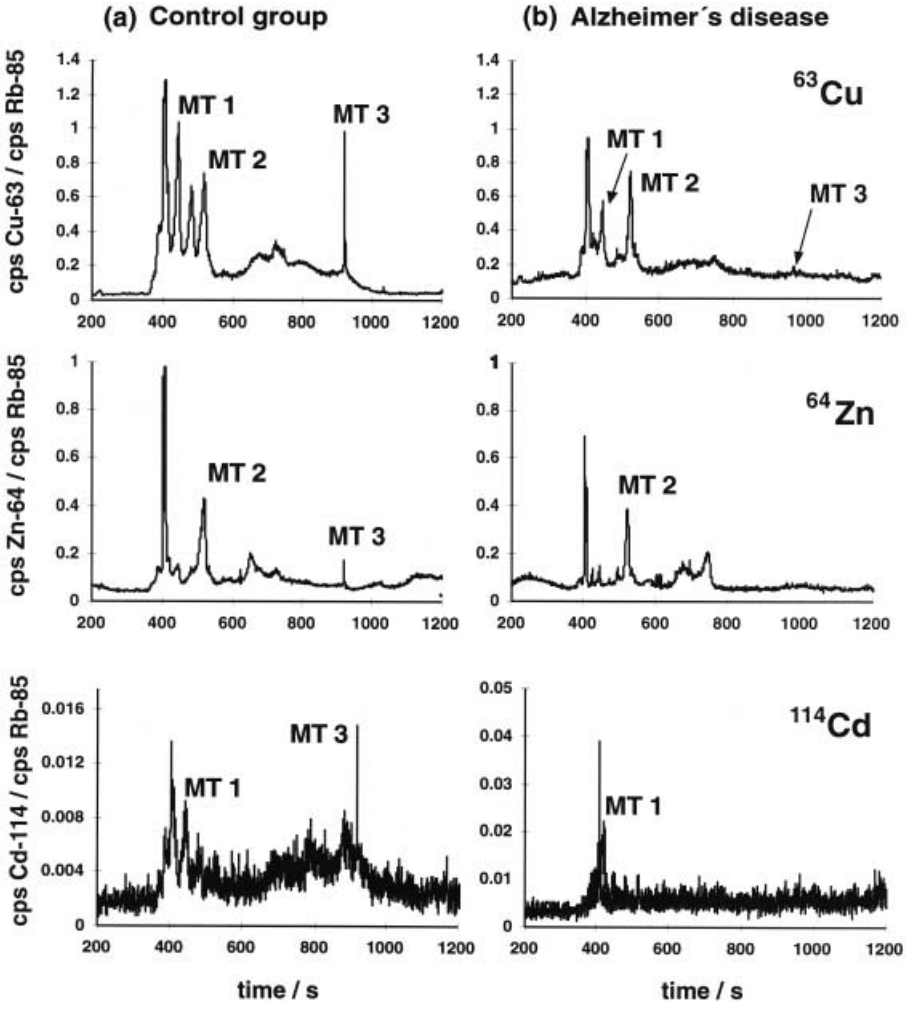

Fig. 15 Comparison of electropherograms from a brain affected by Alzheimer's disease (**b**) and those from a control brain (**a**) detected on ^{63}Cu, ^{64}Zn and ^{114}Cd (temporal region)

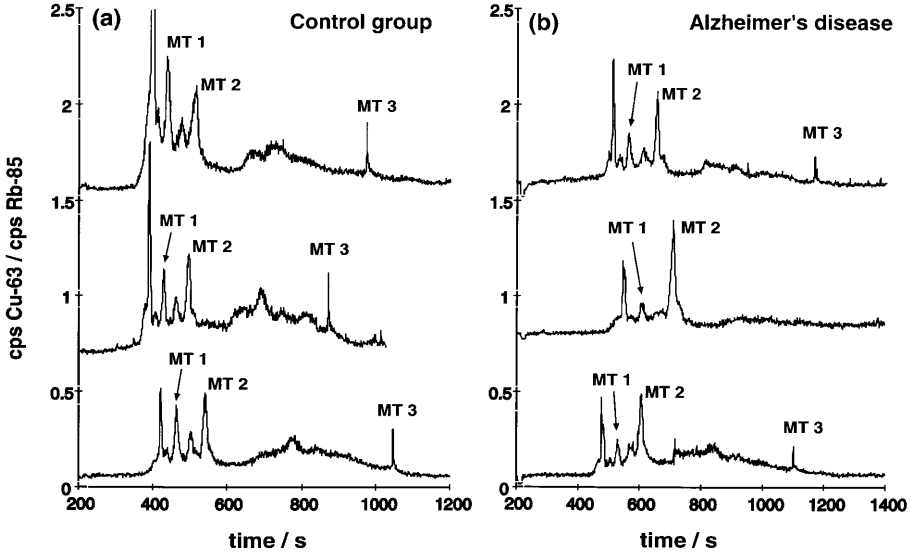

Fig. 16 Comparison of electropherograms from a brain affected by Alzheimer's disease (**b**) and those from a control brain (**a**) detected on ^{63}Cu (occipital region)

Zn, and Cd. To simplify the comparison only the copper electropherograms are shown in Figs 16 and 17. Because of the same signal pattern assignment of the MT-1, MT-2, and MT-3 peaks are adopted from Fig. 12.

In summary there are at least three common effects.

- All the brain regions of the different samples investigated afford a similar signal pattern in the electropherograms of ^{63}Cu.

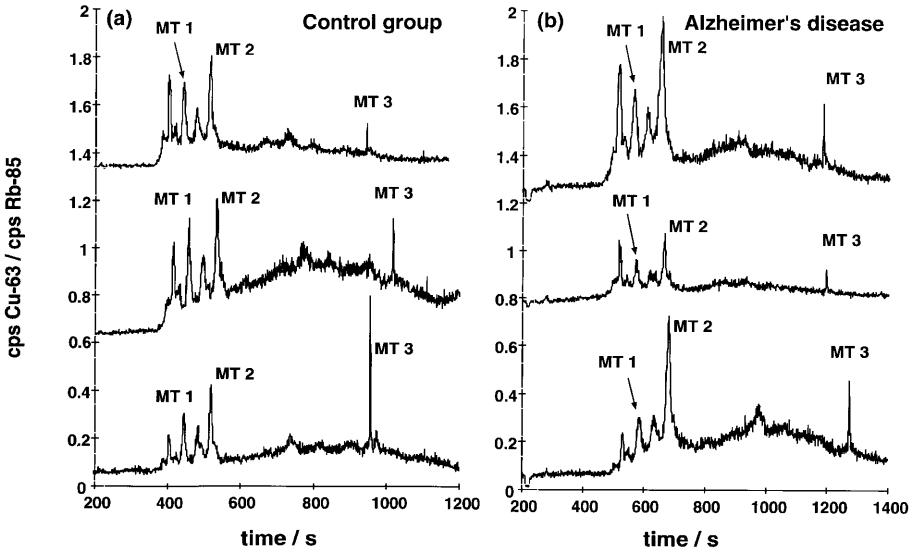

Fig. 17 Comparison of electropherograms from a brain affected by Alzheimer's disease (**b**) and those from a control brain (**a**) detected on ^{63}Cu (parietal region)

- Levels of MT-3 (determined by metal-specific detection) are lower in the temporal and occipital samples from patients with Alzheimer's disease compared with the control group.
- Levels of MT-1 (determined by metal specific detection) are also lower in all temporal and occipital samples from patients with Alzheimer's disease compared with the control group.

The second and third effects were not observed in the parietal samples.

The results indicate that metal-specific detection of metalloproteins gives additional information that should be considered in general discussions on metallothionein and Alzheimer's disease. To obtain medical relevant information it will be necessary to investigate more samples for statistical confirmation. Understanding more about the fundamentals of the role of metallothioneins in the human brain is a real challenge for future analytical methodological developments and their application in the biomedical field.

Conclusions

For the first time it has been shown that the CE–ICP–MS technique is suitable for the separation and identification of metals bound to metalloproteins in real-world samples such as human cell cytosols. The hyphenated system developed is capable of:

1. stable and reproducible separations with high separation efficiency
2. short analysis times (usually under 20 min, incl. MT-3); and
3. requires very small sample quantities (as low as 30 µL) which might be especially important in biomedical applications. In addition, very good detection limits for MT and good migration time precision have been achieved.

This study shows that for successful speciation of metallothionein isoforms in human cytosols a multi-dimensional analytical procedure, based on appropriate sample-preparation techniques, chromatographic and/or electrophoretic and mass spectrometric methods is necessary. The quantification of metallothioneins via sulfur isotope dilution is an elegant approach.

Further investigations using an ESI–quadrupole time-of-flight MS for molecule-specific detection will be performed in the near future. Thus the molecular mass and even the amino acid sequence of the separated proteins could be investigated to clarify their identity.

References

1. Marquard H, Schäfer SG (1994) Lehrbuch der Toxikologie. BI.-Wiss.-Verlag, Mannheim Leipzig Wien Zürich, pp 504–549
2. Lobinski R (2001) Fresenius J Anal Chem 369:113–114
3. Kägi JHR (1991) Methods Enzymol 205:613–626
4. Vallee BL (1995) Neurochem Int 27:23–33
5. Stillman MJ (1995) Coord Chem Rev 144:461
6. Nordberg M (1998) Talanta 46:243–254
7. Uchida Y, Takio K, Titani K, Ihara Y, Tomonaga M (1991) Neuron 7:337–347
8. Palmiter RD, Findley SD, Whitmore TE, Durnam DM (1992) Proc Natl Acad Sci USA 89:6333–6337
9. Amoureux M, Van Gool D, Herrero M, Dom R, Colpaert FC, Pauwels PJ (1997) Mol Chem Neuropathol 32:101–121
10. Yu WH, Lukiw WJ, Bergeron C, Niznik HB, Fraser PE (2001) Brain Res 894:37–45
11. Weiss JH, Hartley DM, Koh JY, Choi DW (1993) Neuron 10:1–20
12. Lobinski R, Szpunar J (1999) Anal Chim Acta 400:321–332
13. Szpunar J (2000) Analyst 125:963–988
14. Brätter P, Raab A, Richarz AN (2000) Trace element speciation in human body fluids. In: Roussel AM, Anderson RA, Favier AE (eds) Trace elements in man and animals 10 (Proc 10th Int Symp Trace Elements in Man and Animals, May 2–7, 1999, Evian, France). Kluwer Academic/Plenum Publishers, New York, 145–152
15. Wolf C, Rösick U, Brätter P (2000) Fresenius J Anal Chem 368:839–843

16. Beattie JH, Richards MP, Self R (1993) J Chromatogr 632: 127–135
17. Richards MP, Beattie JH (1995) J Chromatogr B 669:27–37
18. Beattie JH (1998) Talanta 46:255–270
19. Lu Q, Bird SM, Barnes RM (1995) Anal Chem 67:2949–2956
20. Lu Q, Barnes RM (1996) Microchem J 54:129–143
21. Taylor KA, Sharp BL, Lewis DJ, Crews HM (1998) J Anal At Spectrom 13:1095–1100
22. Majidi V, Miller-Ihli NJ (1998) Analyst 123:803–808
23. Mounicou S, Polec K, Chassaigne H, Potin-Gautier M, Lobinski R (2000) J Anal At Spectrom 15:635
24. Schaumlöffel D, Prange A, Marx G, Heumann KG, Brätter P (2002) Anal Bioanal Chem 372:
25. Main MV, Fritz JS (1989) Anal Chem 61:1272
26. Schaumlöffel D, Prange A (1999) Fresenius J Anal Chem 364:452
27. Prange A, Schaumlöffel D (1999) J Anal At Spectrom 14:1329
28. Richards RI, Heguy A, Karin M (1984) Cell 37:263–272
29. Hunziker PE, Kaur P, Wan M, Kaenzig A (1995) Biochem J 306:265–270
30. Karin M, Richards RI (1982) Nature 299:797–802
31. Hunziker PE (1991) Methods Enzymol 205:421–426