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Characterization of metallothionein isoforms by reversed-phase high-performance liquid chromatography with on-line post-column acidification and electrospray mass spectrometric detection

Hubert Chassaigne, Ryszard Łobiński*

Laboratoire de Chimie Bio-inorganique et Environnement, CNRS EP132, Hélioparc, 2 Avenue Pr. Angot, 64000 Pau, France

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Abstract

Post-column acidification of the chromatographic effluent was developed to eliminate artefacts in investigations of the polymorphism of metallothionein (MT) by microbore reversed-phase HPLC with detection by pneumatically assisted electrospray mass spectrometry. Metallated species (Cd, Zn and mixed Cd–Zn complexes) were decomposed on-line to produce apo metallothioneins of which the molecular masses were determined by MS. Besides the simplification of the mass spectra taken at the apexes of the chromatographic peaks, the method resulted in a 10-fold improvement of the detection limit of metallothionein and allowed a more comprehensive and less ambiguous detection and identification of the iso- and subisoforms. The method was applied to the characterization of rabbit liver metallothioneins: rabbit liver MT (purified by size-exclusion chromatography only) and MT-1 and MT-2 isoform fractions purified additionally by anion-exchange chromatography.

Keywords: Metallothioneins; Proteins; Post-column derivatization

1. Introduction

There has been increasing interest recently in analytical techniques for the characterization of the polymorphism of metallothionein (MT) which is a group of non-enzymatic low-molecular-mass (6000–7000) metal-binding proteins involved in the metabolism of heavy metals [1–9]. The polymorphism occurs during the evolution of a species and consists of the variation of the primary structure of MT by the substitution of up to 15 amino acids, leading to the two major classes of MT isoforms, denoted on the basis of the elution order from an anion-exchange column, MT-1 and MT-2 [10]. Isoforms with minor differences (such as one amino acid residue) are considered as subisoforms of the main isoforms. Whereas sequences of the major isoforms have been decoded [10], the difficulties with the separation and identification of subisoforms are responsible for the lack of literature data regarding their identity. Investigation of the functional significance of the individual MT subisoforms and MT gene expression mechanisms requires analytical techniques that offer a high degree of resolution and the possibility of a rapid identification of the eluted subisoforms.

The majority of studies of the MT polymorphism have been based on the differences between the retention times of the individual subisoforms in

*Corresponding author.

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reversed-phase HPLC [11,12] or between their migration times in capillary zone electrophoresis (CZE) [2,3] with UV detection. These approaches have led to speculative, confusing and often contradictory data because of the virtual impossibility to know what species was detected. Indeed, chromatograms (electrophoregrams) reported show different morphologies, despite the use of the apparently identical analytical techniques and operating conditions (column, mobile phase) [4,11–13], and differ in terms of the number of peaks observed. This is likely to be due to the presence of ghost peaks in the chromatograms, possibly coming from products of oxidation (i.e. dimers or polymerized products), from different conformations, or from different metal composition (metalloforms). The absence of standards of sufficient and documented purity makes the unambiguous identification of an MT species in an HPLC eluate impossible without its tedious isolation and off-line sequencing.

Besides the time-consuming sequencing that requires a sample of high purity, the only technique able to identify the individual iso- and subisoforms has been shown to be electrospray mass spectrometry (ESI-MS). The latter allows a precise (± 1 u) determination of the molecular mass of polypeptide species. ESI-MS with pH control could provide analysis of metals in native and reconstituted metallothioneins showing what and how many cations were complexed by one MT molecule [14–17]. A deeper insight into the characterization of the polymorphism of MT was gained by reversed-phase HPLC with ESI-MS detection of the eluting species [16,17]. The fact that separation was carried out at neutral pH made the ESI-MS detection suffer from the lack of sensitivity, poor signal-to-noise ratio, and the numerous artefacts due to the formation of mixed metal complexes that eclipsed further the acquired mass spectra. Another disadvantage is that the molecular mass (M_r) of an MT subisoform was calculated back assuming a given stoichiometry of the metallated species which negatively affected the precision of the determination of the molecular mass.

The objective of this study was to evaluate on-line post-column acidification prior to pneumatically assisted ESI-MS as a means to improve the analytical selectivity, sensitivity, and reliability of the identification and characterization of MT subisoforms by reversed-phase HPLC–ESI-MS. Post-column acidification of the effluent was proposed to release the MT-bound metals in order to enable their subsequent determination by ESI-MS operated in source collision induced dissociation mode [18].

2. Experimental

2.1. Instrumentation

HPLC was performed using an ABI 140C microbore syringe pump, an ABI Model 112A injection module and an ABI Model 785A absorbance detector equipped with a microbore cell (Applied Biosystems, Foster City, CA, USA). Electrospray MS experiments were performed using a PE-SCIEX API 300 pneumatically assisted electrospray (Ion-spray) triple-quadrupole mass spectrometer (Thornhill, ON, Canada). The BioToolBox software was used for the calculation of molecular masses and deconvoluting of protein mass spectra.

Separations with post-column acidification (PCA) were realized in a setup shown in Fig. 1. A flow of a mixture of methanol and formic acid at 4 μl min^{-1} supplied from a 500-μl Hamilton gas-tight syringe (Hamilton Company, Reno, NV, USA) by means of a Harvard Model 55111 syringe pump (Harvard Apparatus, Southnatick, MA, USA) was mixed with the chromatographic effluent via a zero-dead volume T-piece (Interchim, Montluçon, France). A mixing coil (25 cm×250 μm I.D.) made of PEEK (polyether ether ketone) was placed behind the T to accomplish the mixing process. The UV detector was placed between the column and the T in order to allow the detection of metallated MT species.

2.2. Reagents and standards

Methanol and acetonitrile (Sigma–Aldrich) were of LC grade. Water purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA) was used. Liquid nitrogen (99.996%) was evaporated in situ and supplied at 2.75×10^5 Pa. The buffer solution was prepared by dissolving 5 mmol l^{-1} of ammonium acetate in water or in water–acetonitrile (50:50, v/v), and adjusting the pH to 6.0 with acetic

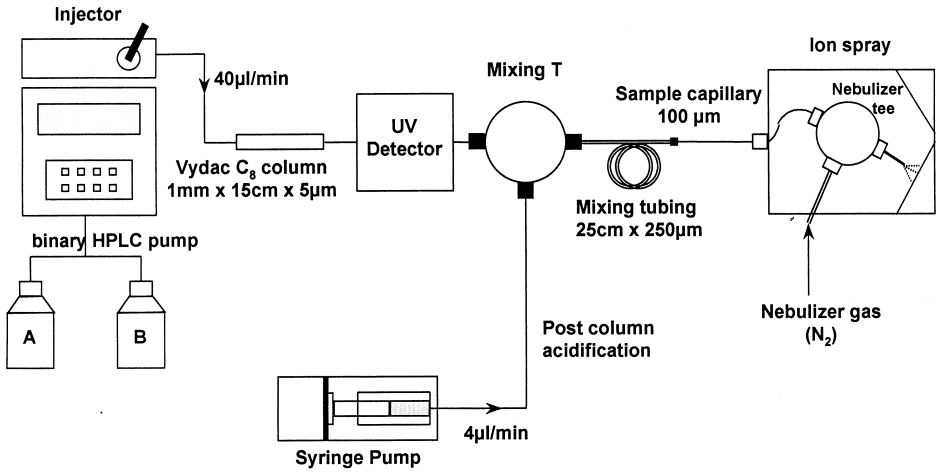

Fig. 1. Scheme of the instrumental setup.

acid. Buffers were sparged with helium to remove dissolved oxygen and thus to attain a non-oxidizing environment. This is an important consideration in view of the observed susceptibility of MTs to oxidation during isolation [1].

Metallothionein preparations MT-2 (34H95161), MT-1 (94H9504) and MT (56H9500) from rabbit liver were purchased from Sigma–Aldrich (Saint Quentin Fallavier, France). The preparations contained 6.0% Cd, 0.6% Zn, 0.5% Cu for MT-1, 5.3% Cd, 0.7% Zn, 0.5% Cu for MT-2, and 5.9% Cd, 0.5% Zn, 0.8% Cu for MT_{RL} as found by an independent inductively coupled Plasma (ICP)-MS analysis. Batch-to-batch variation can be significant; the preparations should be checked for purity and fully characterized prior to their use as standards [13].

The stock MT solution (1 mg ml^{-1}) was prepared by dissolving 1 mg of metallothionein in 1 ml of water. Working solutions were prepared by the dilution of the stock solution with water or buffer as required. The stock solution was kept in the fridge at 4°C in the dark.

2.3. HPLC conditions

Separations were carried out using a Vydac C_8 150×1 mm, 5 µm column with the pore size of 300 Å. The pump flow was set at 40 µl min^{-1} which corresponded to a pressure of 4.5 MPa. Injection volume was 5 µl. MT subisoforms were eluted with a linear gradient of acetonitrile (10–16% B) within 50 min. Buffer A was 5 m*M* acetate buffer in water (pH 6.0) and buffer B–5 m*M* acetate buffer (pH 6.0) in water–acetonitrile (50:50, v/v). The solutions were degassed by sparging with helium.

The acidifying reagent was formic acid–methanol (30:70, v/v). Supplied at 4 µl min^{-1}, it allowed a final pH of 1.9 to be obtained and the conversion of Cd, Zn and mixed Cd–Zn complexes to apo-MT subisoforms. Copper complexes, if present would not be completely demetallated under these conditions [19].

2.4. Electrospray-mass spectrometric conditions

An *m*/*z* range of 1200–1800 was monitored that allowed the observation of the +4 and +5 ionization states of the metallated subisoforms and of the apo-MTs (in the case of the post-column dilution). The mass spectra were acquired with a step size of 0.5 u, dwell time 2 ms, which required 2.8 s per scan.

3. Results and discussion

Rabbit liver metallothionein (MT_{RL}) is isolated by size-exclusion chromatography and further purified by anion-exchange chromatography that allows the fractions of isoforms, MT-1 and MT-2, to be ob-

tained. To date, six rabbit liver MT species have been isolated and characterized [20]. The number of putative subisoforms, observed as unidentified peaks in HPLC or CZE [4,6], or in the relatively poorly resolved direct infusion ESI mass spectra [16] is apparently much larger. The analytical selectivity can be improved by coupling ESI-MS with reversed-phase HPLC. The to-date efforts focussed exclusively on Cd_7–MT complexes [16,17] and suffered from the need to extrapolate the molecular mass data obtained for a metal complex to a demetallated (apo-MT) subisoform. This may lead to errors because of the non-precisely defined stoichiometry of the complex, due to, for example, the possible attachment of hydrogen radicals [14]. Since the separation of apo-MTs by reversed-phase HPLC is known to be long and to suffer from poor recoveries [1], it was attempted to follow the conventional separation in neutral media by cleaving the metal ions by on-line PCA in order to introduce the apo-MT in the electrospray ion source for a direct molecular mass measurement.

3.1. Post-column acidification (PCA)

First attempts were based on a simply mixing of a flow of an acid solution with the column effluent via a T-piece. Despite a wide range of concentrations of different acids (formic, acetic, hydrochloric) tested, the results obtained suffered from poor reproducibility while the acidification was seldom completed; mass spectra indicated that apo-MTs coexisted with Cd_4–MT complexes. The origin of this problem was judged to be the insufficient mixing of the column effluent with the acid stream. Therefore it was decided to employ a reaction coil the length of which was optimized in the range of 5–100 cm with a step of 20–25 cm in order to achieve a complete demetallation of the MT. The optimum solution found was a 25-cm mixing coil (0.25 mm I.D.) placed behind the T to accomplish the mixing process. Longer reaction coils ($\geq$50 cm) resulted in the chromatographic peak broadening and loss of resolution, whereas a shorter one did not allow the complete release of Cd from the complex. The optimum acidifying reagent was found to be a mixture of formic acid–methanol (30:70, v/v). Supplied at 4 μl min^{-1}, it allowed a final pH of 1.9 to be obtained and thus the conversion of Cd, Zn and mixed Cd–Zn complexes to apo-MT subisoforms. The setup developed did not cause any noticeable broadening of the chromatographic peaks, the dead volume of the system being controlled by that of the electrospray ionization source.

3.2. Characterization of MT-2 subisoforms

A reversed-phase HPLC chromatogram of an MT-2 preparation is shown in Fig. 2. Despite the identical operational conditions (column, mobile phase, and the origin of the sample) as described elsewhere [16], the chromatographic resolution is better for the major (>80%) putative subisoforms (peaks 4 and 5). The number of minor (<5%)

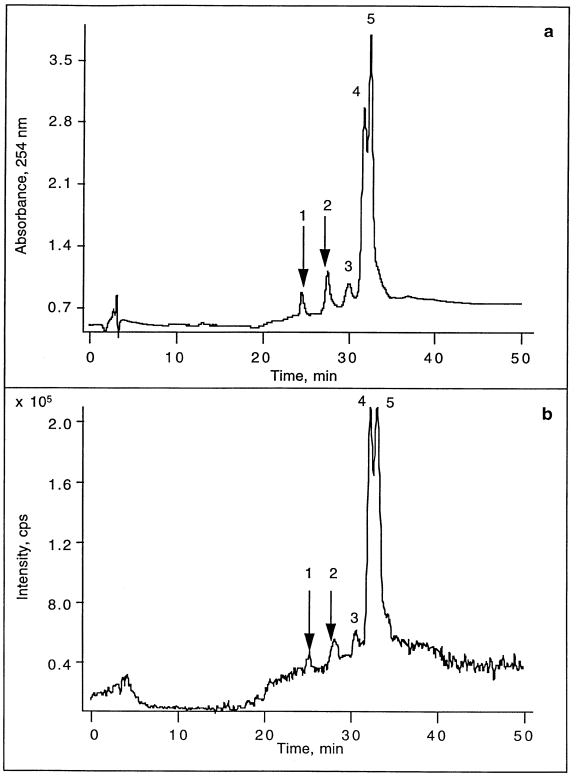

Fig. 2. Chromatogram obtained for the separation of MT-2 putative isoforms by microbore RP HPLC: 1.0 μg (5 μl) of MT-2 at pH 6.0. Gradient: 0–50 min, 5–8% CH_3CN. (a) UV detection; (b) ESI-MS detection after post-column acidification–total ion current (TIC) chromatogram. For the identification of the peaks see Fig. 3.

subisoforms is smaller (3 in place of 6). The UV detection allows neither the identification of the peaks, nor even their unambiguous attribution to 'true' (having amino acid heterogeneities and not simply being a differently metallated species) subisoforms. This issue can be approached by the use of ESI-MS for detection that allows the acquisition of a mass spectrum at the apex of each of the chromatographic peaks. Such mass spectra are shown in Fig. 3a.

The interpretation of MS data in Fig. 3a is relatively difficult because of the complexity of the observed clusters of peaks (Cd, Cd–Zn, and Cd–Cu may co-exist) and the poor signal-to-noise ratio (ESI-MS response of metal complexes is 10-times lower in comparison with apo-MTs). These drawbacks can be eliminated by PCA as it can be seen from the spectra in Fig. 3b. Despite the fact that the TIC chromatogram after PCA (Fig. 2b) is almost identical in terms of signal-to-noise ratio with the one (not shown) before PCA, mass spectra corresponding to the chromatographic signals obtained after PCA are simpler in terms of the number of peaks and show a much better signal-to-noise ratio (S/N). The latter is particularly important in the case of peaks 1–3, for which the analytical signals observed for Cd_7 complexes hardly exceed the noise level, rendering identification of the MT subisoforms speculative.

MS data obtained after PCA confirm that the complexity of the ESI-MS spectra of HPLC signals reported earlier [16,17] is caused by the presence of differently metallated species that eclipse the true polymorphism (the occurrence of species with different amino acid composition/sequences). The advantage of the PCA is clearly seen for the identification of the major (split) peak in Fig. 2. The mass spectra corresponding to peaks 4 and 5 by direct MS are relatively complex and indicate the presence of a number of co-eluting species. The PCA leads to the identical and simple spectra for these peaks. The major compound can be clearly identified as belonging to the MT-2a subisoform (M_r=6124.5±0.5 found, 6125.3 theoretical). It is accompanied by a minor species that, on the basis of the molecular mass, can be attributed to MT-2c (M_r=6156.0±0.0 found; 6155.3 theoretical). This leads not only to the conclusion that peaks 4 and 5 correspond to different mixed Cd–Zn–MT complexes, but also that the different composition in terms of metal may modify the retention of the complex on a reversed-phase column leading to the formation of artefacts observed when the UV detection is applied. Taking this assumption the peaks in mass spectra by direct ESI-MS of peaks 4 and 5 can be identified as belonging to the mixed Cd–Zn complexes: Cd_7, Cd_6Zn, Cd_5Zn_2, Cd_4Zn_3, etc. Whereas the complexes Cd_6Zn–MT-2a and Cd_7–MT-2a have a slightly different retention time, the Cd_7–MT-2a and Cd_7–MT-2c co-elute which makes the separation of the MT-2a and MT-2c as Cd complexes on a reversed-phase column impossible. It should be noted that the unit m/z resolution of the ESI mass spectrometer does not allow to distinguish between Zn and Cu in their mixed complexes with Cd. The PCA seems to destroy the species present completely since, with one exception (second panel in Fig. 3b), no signals with m/z>1550 are present; this exceptional signal is unlikely to contain copper since no possible match in terms of molecular mass could be found, and its intensity is too important taken into account the amount of copper present in the sample. The M_r of this peak corresponds to a species denoted elsewhere as apo-$MT_{RL}\beta$ [16].

Regarding the origin of the minor peaks (1–3) in the chromatogram of the MT-2 preparation (Fig. 2), the mass spectra after PCA indicate that a previously unaccounted for subisoform (M_r=6140.0±0.0) is eluted as peak 1. Peak 2 would correspond to the MT-2b isoform (M_r=6144.5±0.5 found; 6143.3 u theoretical) contaminated by a trace of an unidentified species referred to as $MT_{RL}\beta$ [16]. Peak 3 (M_r=6084.5±0.5) corresponds to the subisoform tentatively named MT-2α in the direct infusion spectrum of the preparation [16].

3.3. Characterization of MT-1 subisoforms

A reversed-phase HPLC chromatogram of an MT-1 preparation is shown in Fig. 4. Contrary to the case of MT-2, the chromatographic resolution seems to be worse than reported elsewhere despite, again, the apparently identical operational conditions (column, mobile phase, and the origin of the sample) [16]. Not only is the number of observed signals smaller, but MTs are retained on the column less strongly since

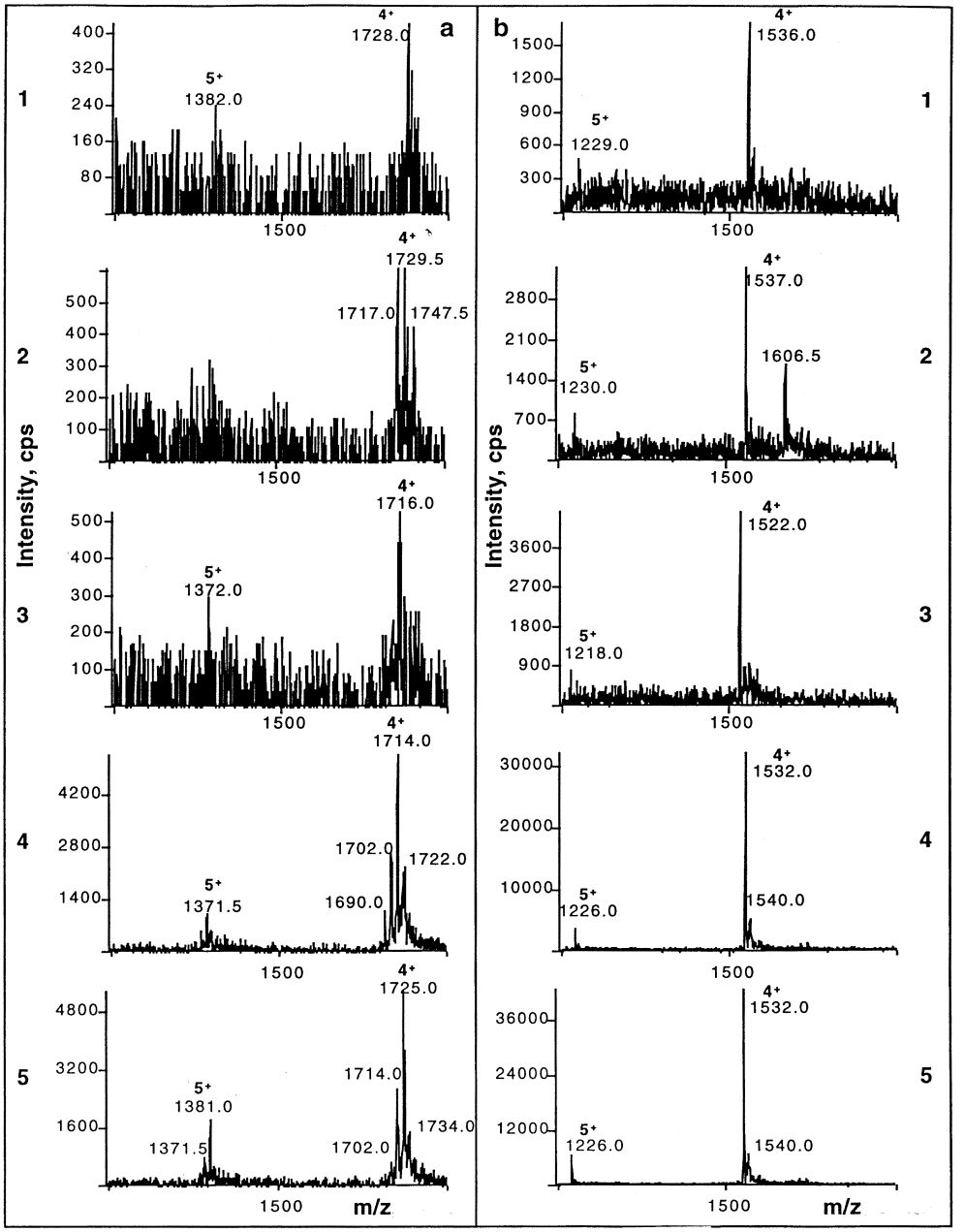

Fig. 3. ESI mass spectra taken in the vicinity of apexes of the HPLC peaks observed in the chromatogram for MT-2 (Fig. 2). (a) Spectra obtained without post-column acidification. (1) M_r=6906±1 (unidentified); (2) M_{r1}=6864, Cd_4–$MT_{RL}\beta$; M_{r2}=6914, Cd_7–MT-2b; M_{r3}=6986 (unidentified); (3) M_r=6857±2, Cd_7–MT-2α; (4) M_{r1}=6756, Cd_4Zn_3–MT-2a; M_{r2}=6796, Cd_5Zn_2–MT-2a; M_{r3}=6852.2±0.2, Cd_6Zn–MT-2a; M_{r4}=6884, Cd_6Zn–MT-2c; (5) M_{r1}=6804, Cd_5Zn_2–MT-2a; M_{r2}=6852.2±0.2, Cd_6Zn–MT-2a; M_{r3}=6898±2, Cd_7–MT-2a; M_{r4}=6932, Cd_7–MT-2c. (b) Spectra obtained after post-column acidification. (1) M_r=6140±0 (unidentified); (2) M_{r1}=6144.5±0.5, apo-MT-2b; M_{r2}=6422, apo-$MT_{RL}\beta$; (3) M_r=6804.5±0.5, apo-MT-2α; (4) M_{r1}=6124.5±0.5, apo-MT-2a; M_{r2}=6156, apo-MT-2c; (5) M_{r1}=6124.5±0.5, apo-MT-2a; M_{r2}=6156, apo-MT-2c.

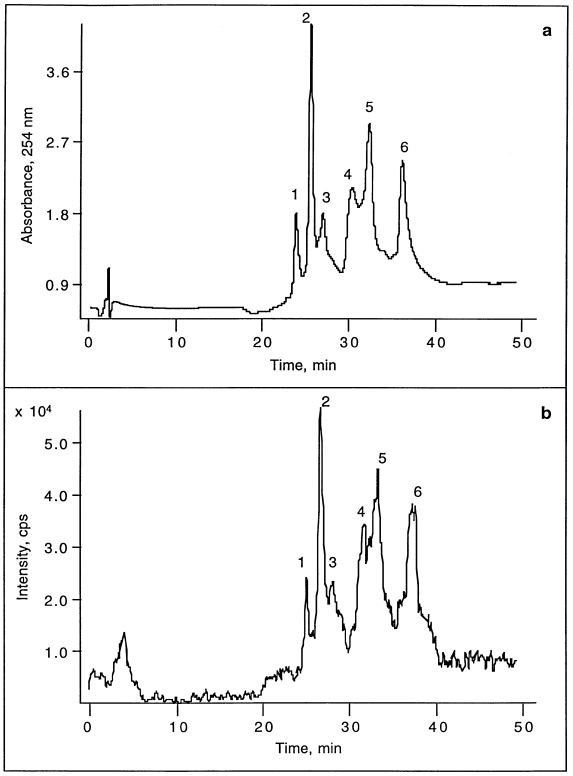

Fig. 4. Chromatogram obtained for the separation of MT-1 putative isoforms by microbore RP HPLC: 1.0 μg (5 μl) of MT-1 at pH 6.0. Gradient: 0–50 min, 5–8% CH_3CN. (a) UV detection; (b) ESI-MS detection after post-column acidification–total ion current (TIC) chromatogram. For the identification of the peaks see Table 1.

they elute within 15 instead of 20 min. The identification of the peaks was attempted by ESI-MS. Data for mass spectra taken at the apexes of the chromatographic peaks directly and after PCA is summarized in Table 1.

Table 1 confirms that apo-MT subisoforms can be detected in the chromatographic eluent at levels 10 times lower owing to PCA. Before PCA the S/N ratio exceeds seldom the value of 4 which makes the M_r determinations of limited reliability. Mass spectra of the chromatographic peaks after PCA are relatively simple and usually correspond to not more than two species. The principal subisoforms: MT-1δ, MT-1α (MT-1a) and MT-1ϵ (see Ref. [16] for nomenclature) elute as Cd_7–MT complexes as peaks 4–6. Whereas the identification of these signals on the basis of mass spectra acquired in the direct mode is relatively difficult because of the poor S/N, it is largely simplified by PCA. Peak 4 contains MT-1δ, whereas peak 5 MT-1a, which are well separated from each other. Their poor resolution in the chromatogram is probably due to the presence of an impurity after the anion-exchange purification being the major isoform of MT-2 (MT-2a). This species co-elutes with MT-1δ and MT-1a and eclipses the mass spectrum. This information is practically impossible to be deduced from the appropriate mass spectra obtained without PCA, since not only is it difficult to distinguish the signals from the noise, but the complexity of the system is also increased by the occurrence of the mixed metal species. Peak 6 seems

Table 1
Electrospray mass spectra taken in the vicinity of apeces of the HPLC peaks observed in the chromatogram of MT-1

Peak in Fig. 4	Before acidification				After acidification			
	M_r	S/N	Abundance (%)	Tentative formula	M_r	S/N	Abundance (%)	Tentative formula
1					6162.2±0.2	3.5	36	apo-MT-2c
	6953±1	2	100		6513±1	9.5	100	
2					6147.7±0.7	4.5	13	apo-MT-1a
	6887±2	8	100		6441±1	35	100	
3					6176	5	28	apo-MT-1β
	6980±0	4.5	100		6536.7±0.7	17.5	100	
4					6144	9	22	apo-MT-1a
	6991±1	4	100	Cd_7–MT-1δ	6125.5±0.5	40	100	apo-MT-1δ
5	6900	2	64	Cd_7–MT-2a	6126	25	50	apo-MT-2a
	6915.5±0.5	3.5	100	Cd_7–MT-1a	6145±1	50	100	apo-MT-1a
6	6967.7±0.2	2.5	71	Cd_6Cu–MT-1ϵ	6636.7±0.7	5	25	Cd_3Cu–MT-1ϵ
	7011±1	3.5	100	Cd_7–MT-1ϵ	6241±1	20	100	apo-MT-1ϵ

to contain two isoforms referred to elsewhere as MT-1ϵ (M_r=6241±1). The impossibility of the separation between MT-2a and MT-1a on a reversed-phase column leads to the hypothesis that the works that claim that MT-2 elutes before MT-1 on a reversed-phase column compare MT-2a with MT-1ϵ rather than with MT-1a.

Except for the peaks corresponding to the apo-subisoforms of MT-1, the mass spectra of peaks 1–3 and 6 show the presence of compounds with M_r 300–400 u higher. In the case of peaks 1–3 these are the most intense signals. M_r values in this region may indicate incomplete demetallation, the reason for which may be the presence of copper in an MT complex. Whereas this hypothesis can be readily defended by mathematical calculations in the case of peak 6 (Cd_6Cu–MT-1ϵ is transformed by PCA into a minor Cd_3Cu–MT-1ϵ species), the case of peaks 1–3 appears to be more complex. HPLC–ICP-MS data [16] show that the Cu/Cd ratio in these peaks is 10-fold higher than in the case of all other chromatographic peaks which indicates the possibility of the existence of less hydrophobic Cu–MT complexes. In the case of peaks 1 and 2, the difference between the molecular mass calculated on the basis of the mass spectrum obtained without and with acidification corresponds to four Cd atoms. It is not clear, however, what the original species can be. Mathematical matches for mixed Cd–Cu complexes can be found but isolation of the species would be necessary for their verification.

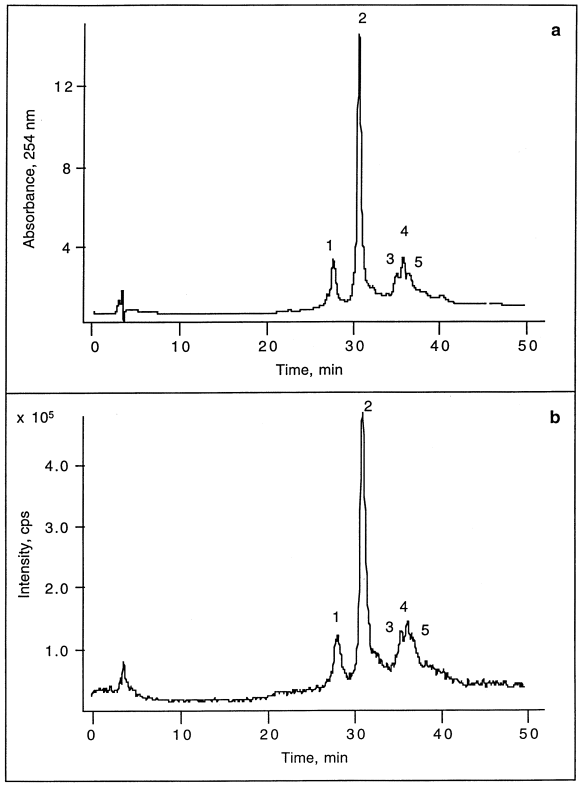

Fig. 5. Chromatogram obtained for the separation of MT_{RL} putative isoforms by microbore RP HPLC: 1.0 μg (5 μl) of MT at pH 6.0. Gradient: 0–50 min, 5–8% CH_3CN. (a) UV detection; (b) ESI-MS detection after post-column acidification–total ion current (TIC) chromatogram. For the identification of the peaks see Table 2.

3.4. Characterization of the non-purified rabbit liver metallothionein

A reversed-phase HPLC chromatogram of a rabbit liver metallothionein preparation that had not been purified by anion-exchange chromatography is shown in Fig. 5. The morphology of the chromatogram is similar to that reported elsewhere [16]. The difference concerns the appearance of an additional peak in front of the eluting major subisoform and the drastically reduced intensity of the cluster of peaks 3–5. As in the case of the MT-1 and MT-2 preparations, the identification of the signals was attempted by ESI-MS in the chromatographic effluent without and after PCA. MS data are summarized in Table 2.

An analysis of the mass spectra of the cluster of peaks 3–5 after post-column acidification demonstrates that they correspond to the metallated (each peak differently) major subisoform of MT-2–MT-2a. This is accompanied by a small peak belonging to MT-2c which, as indicated earlier (cf. Fig. 3), cannot be separated from MT-2a on a reversed-phase column. As observed earlier, the introduction of a Zn (or Cu) atom into the structure of a MT complex shifts slightly the retention time leading to the formation of artefacts that eclipse the original subisoform.

The identification of peaks 1 and 2 is less straightforward. The acidification of the compound eluting as peak 2 leads to the formation of the major $MT_{RL}\beta$ subisoform observed earlier in an ESI-MS spectrum acquired by the infusion of a solution of the MT_{RL}

Table 2
Electrospray mass spectra taken in the vicinity of apeces of the HPLC peaks observed in the chromatogram of MT_{RL}

Peak in Fig. 5	Before acidification				After acidification			
	M_r	S/N	Abundance (%)	Tentative formula	M_r	S/N	Abundance (%)	Tentative formula
1					6140	10	9.5	apo-MT-2b
	6882±2	13	100		6442±2	105	100	
	6815±1	25	31	Cd_4–$MT_{RL}\alpha$	6372	25	3.5	apo-$MT_{RL}\alpha$
2	6860±2	80	100	Cd_4–$MT_{RL}\beta$	6421±1	700	100	apo-$MT_{RL}\beta$
	6902	17	22	Cd_4–$MT_{RL}\gamma$	6456	75	11	apo-$MT_{RL}\gamma$
3	6808	6	40	Cd_5Zn_2–MT-2a	6125.5±0.5	110	100	apo-MT-2a
	6852±2	15	100	Cd_6Zn–MT-2a				
					6156	20	18	apo-MT-2c
	6808	3.5	28	Cd_5Zn_2–MT-2a	6125.5±0.5	175	100	apo-MT-2a
4	6852±2	7.5	60	Cd_6Zn–MT-2a				
	6898±1	12.5	100	Cd_7–MT-2a				
	6932	6.5	52	Cd_7–MT-2c	6156	25	14	apo-MT-2c
					6125.5±0.5	65	100	apo-MT-2a
5					6372	10	15	apo-$MT_{RL}\alpha$
	6870	80	100		6421±1	62	96	apo-$MT_{RL}\beta$
	6912	75	93		6470	32		apo-$MT_{RL}\gamma$

preparation into an electrospray ion source under acidic conditions [16]. The $MT_{RL}\beta$ isoform is also observed after PCA of the compound eluting as peak 5.

The high intensity of the peak denoted as $MT_{RL}\beta$ and the fact that it cannot be further demetallated even at more acidic pH suggests that it is an apo-MT species. It is difficult to understand, however, why it coordinates only four Cd atoms (instead of seven) under the mobile-phase pH conditions. This issue needs to be further investigated.

It is worth noting that no MT-1 contribution to the MT_{RL} is detected in the preparation investigated. This is probably due to the large excess of MT-2 present and the lower response of MT-1 in comparison with MT-2 as reported elsewhere [16].

4. Conclusions

Despite the apparently identical chromatographic conditions (column, mobile phase, and the origin of the sample) the morphology of a chromatogram of a metallothionein preparation may differ. This requires the determination of the molecular mass of the eluting species, corresponding to each chromatographic peak, in order to enable the comparison of data on the characterization of the polymorphism of metallothionein obtained by different research groups. On-line post-column acidification offers an attractive possibility to eliminate artefacts due to the differently metallated species detected by ESI-MS and allows the direct detection of the original apo-MT subisoform associated with a given chromatographic peak. The post-column acidification also offers the possibility of a 10-fold increase in the detection limits of metallothionein in HPLC effluents.

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