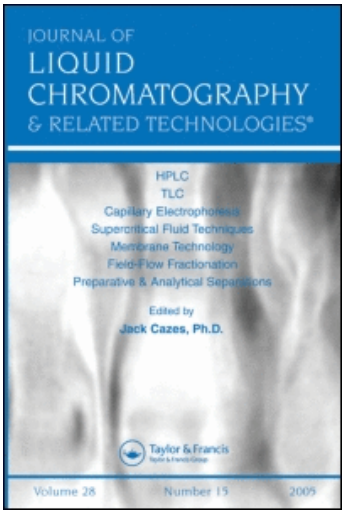


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# STUDY OF MAMMALIAN METALLOTHIONEIN POLYMORPHISM BY REVERSE PHASE HPLC WITH ON-LINE DIODE ARRAY AND ELECTROCHEMICAL DETECTION

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# STUDY OF MAMMALIAN METALLOTHIONEIN POLYMORPHISM BY REVERSE PHASE HPLC WITH ON-LINE DIODE ARRAY AND ELECTROCHEMICAL DETECTION

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## ABSTRACT

The polymorphism of rabbit liver metallothionein RL and of its two isoforms RL-1 and RL-2 has been studied by means of reverse phase HPLC with on-line diode array (DAD) and electrochemical (EC) detection. Separations have been carried out in an acidic mobile phase (TFA). Using the typical UV absorption characteristics of MT, as well as the electrochemical response of the various peaks and comparing the morphology of the chromatograms obtained with a gradient at pH 3 and at pH 2, we reached the following conclusions: RL-1 has three subisoMTs which are apoforms at pH 3 and four other Cu, Cd metalloforms; RL-2 has a less complex polymorphism with one major and one minor subisoMTs under apoform at pH 3; RL has four main subisoMTs which are apoforms at pH 3, coming either from RL-1 or from RL-2 and two main other metalloforms including a pure Cu form not found in the isoforms. In all three MTs, an extra peak has been identified by its non MT-type UV spectrum, its time-dependence increase, its total lack of electrochemical response, and its larger variation of retention time with increasing temperature. This could be a degradation product of MT.

## INTRODUCTION

Metallothioneins (MTs) form a category of ubiquitous low molecular weight metalloproteins, of which one of the main characteristics is the high cysteine content, about 30% in MTs from mammals. They display a strong capacity to bind metallic elements such as zinc, cadmium, copper, or mercury, resulting in a three-dimensional structure organized in two metal-thiolate clusters or domains, the $\alpha$ domain (carboxyl end) $M_4Cys_{11}$ and the $\beta$ domain (amino end) $M_3Cys_9$, where M is a divalent metal. The properties of metallothioneins have been extensively studied by means of a great variety of analytical methods and have been the subject of important monographs.[1-6] These metalloproteins also exhibit a specific polymorphism which will depend on the animal species and on the organ from which the MTs are extracted. This polymorphic character expresses itself as two major isoforms (isoMTs) MT-1 and MT-2 for mammalian MTs, being the product of two distinct genes. Isoforms will differ by the substitution of 1 to 15 amino acids in their primary structure, while minor differences within a given isoMT will result in the occurrence of subisoforms (subisoMTs).



Although most of the main biological functions of MTs which arise from their metal complex forming properties - as seen through their storage, transfer, regulation, and detoxification of metal ions - are quite well known,[7] this does not hold true for those at the isoform and even less at the subisoform level. The primary roles of individual isoforms and subisoforms may be quite distinctive and specific and remain, for the moment at least, rather unknown. Furthermore, it can easily be stated that the expression and functions of these forms will remain difficult to establish as long as efficient separation and quantification methods of isoMTs, but, above all, of subisoMTs are not routinely available. To be able to decode the sequences of individual subisoforms for which little has been published,[8] the development of analytical separation techniques is therefore of primary importance.

Fortunately this field of investigation is far from being uncharted and a great deal of progress has already been registered, exploring both liquid chromatographic and capillary electrophoretic techniques.[3,9] In previous papers,[10-11] we introduced the use of reverse phase HPLC with on-line UV and electrochemical (EC) detectors for the characterization of subisoMTs of mammalian origin. The electrochemical detection is based on that of thiol groups through their oxidation at a porous graphite electrode according to the reaction $2\ RSH \rightarrow RSSR + 2H^+ + 2e^-$, and was first applied to the characterization of a trithiolic hexapeptide intrinsic to the mouse liver MT structure.[12] Subisoforms of rabbit liver and horse kidney MTs were readily separated using a gradient of trifluoroacetic acid and acetonitrile. With this system, it was possible to discriminate in some MTs putative original (apo)subisoforms (equivalent UV and EC responses of individual peaks) from other somewhat different forms displaying relatively less EC than UV signals.

Although working with an acidic mobile phase means working with apoMTs, that is to say not in the same forms as they would have been found in organisms, the TFA medium eliminates peak coelution that can occur at neutral pH. Comparing different systems for the separation of isoMTs, some authors have reported that a TFA/acetonitrile gradient has been shown to be the most convenient.[13] However, the double detection we have used until now did not allow a full and unambiguous identification of the species separated as being subisoMTs or not. In the present work, the UV detector, set up at a fixed wavelength, has been replaced by a diode array detector (DAD) in order to obtain more information on the various peaks of the chromatograms.

The use of the DAD provides a UV spectrum for each peak which should then give the possibility of a further characterization of the corresponding chemical species. Due to the occurrence of the metal-thiolates, the UV spectra of MTs are very typical, either at neutral or at acidic pH. The present paper will present the results obtained for rabbit liver MT and for its two isoforms MT-1 and MT-2, studied by RP-HPLC-DAD-EC with an acidic mobile phase.



## EXPERIMENTAL

### Instrumentation and Procedure

The system consisted of a Kontron chromatograph (Zürich, CH) equipped with a dual piston pump Model 420, a gradient-former GF 425, and a HPLC 360 autosampler with an injection loop of 100 µL. The separations were performed on a reverse phase column Hi-Pore RP 318 (250 x 4.6 mm), 300 Å pore size and 5 µm particle size (Bio-Rad Laboratories), with the following elution programme: linear gradient 10-25 % B in 50 minutes, at a flow rate of 1 $mL.min^{-1}$, where A was 0.1 % (v/v) trifluoroacetic acid (TFA) in water (pH = 3 unless otherwise mentioned) and B was 0.1 % (v/v) TFA in acetonitrile. The column was kept in an Alltech 330 Column heater at 35°C.[11] The detection system included a Diode array detector Model 440 (Kontron) followed by a Coulochem Model 5100 A electrochemical detector (ESA Inc., Bedford, USA) set at a potential of $E_2$ = 0.6 V used with a Model 5020 guard cell (E = 0.65 V) and a Model 5011 analytical cell containing two porous graphite working electrodes. The mobile phases were filtered through 0.22 µm Millipore membranes and continually purged with helium to remove dissolved oxygen in order to avoid oxidation during separation.

Fractions were collected for metal analysis carried out by Electrothermal atomic absorption using a Perkin Elmer model 5100 PC equipped with a Zeeman furnace model 5100 ZL with L'vov platform pyrolytically coated tubes.

**Chemicals**

Metallothioneins of rabbit liver Cd, Zn-MT, (RL, lot 56H9500), and its purified isoforms MT-1 (RL-1, lot 74H9528) and MT-2 (RL-2, lot 90H9605 and 13H95481), 2-mercaptoethanol and TFA (1 mL ampoules) were purchased from Sigma (St Louis, MO, USA). Acetonitrile was HPLC grade (Super gradient grade, Lab-Scan, Dublin, IRL). Ultra pure water was obtained from a Millipore Mill-RO 10 Plus deionisation system followed by a Milli-Quater system (18 MΩcm resistivity) and a sub-boiling quartz distillation unit (Quartex SA, Paris, F). All metallothionein solutions were prepared in water at a concentration of 600 $mg.L^{-1}$ and kept in the refrigerator at 4°C in the dark.

Titrisol (Merck) standards (1000 µg/mL individual solutions of Cd, Cu, and Zn) were used for the establishment of the calibration curves for metal AAS analysis.

In this article, peak numbering matches increasing retention time $t_R$ and labelling such as $H_2$ stands for the height of peak 2.

## RESULTS

### Polymorphism of Rabbit Liver MT-1 (RL-1) and MT-2 (RL-2)

#### *RP-HPLC Chromatograms*

Chromatograms of RL-1 and RL-2 samples eluted immediately after preparation are shown in Figures 1 and 2, respectively. For RL-1, the trace recorded at $\lambda$ = 214 nm (a) shows a very good separation of eight main peaks organized in two groups, $\alpha$, $\beta$, $\gamma$ and $\omega$ and 1, 2, 3, and 4. This chromatographic series confirms the results we had obtained previously with other lots of the same RL-1 metallothionein.[10-11] The trace at $\lambda$ = 240 nm (b) reveals some interesting new features: the $\alpha$-$\omega$ peaks have decreased but are still important; the 1-4 peaks have almost disappeared while a new peak, labelled X, has now emerged as one of the dominant peaks between the two former groups of peaks. At $\lambda$ = 280 nm (c), only the $\alpha$-$\omega$ peaks still show a small but significant absorbance. The electrochemical record (d) shows the $\alpha$-$\omega$ and 1-4 peaks but not the X peak which appears not to be electroactive.

For RL-2 (Figure 2), at $\lambda$ = 214 nm (trace a) and by EC, one dominant peak, labelled 3, is detected along with a small but well defined preceeding peak (peak 3') and with several other minor peaks, some of them (1, 2, 4) probably being residues of RL-1 as remains of an incomplete anion-exchange chromatographic separation of the two isoforms. Keeping on the same track, peak 3 observed in RL-1 is a remainder of RL-2. The separation of RL-2 also shows

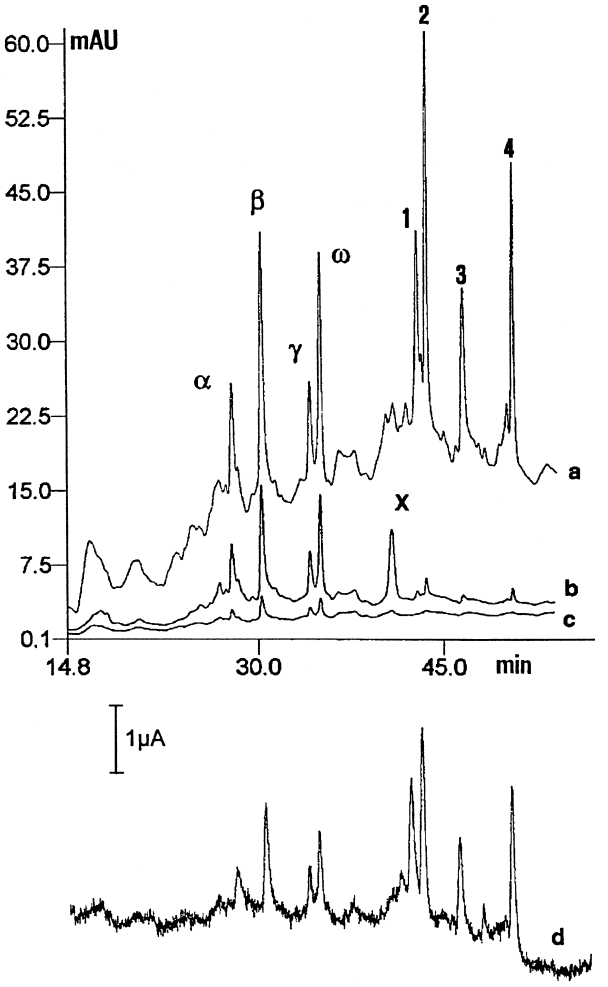


**Figure 1**. Reverse phase chromatogram of a rabbit liver MT-1 (RL-1) sample in water (600 $mg.L^{-1}$). Elution: from 10 to 25 % B (0.1 % TFA in acetonitrile) in 50 min; pH = 3, 35°C. Diode array (traces a, b and c) and electrochemical (trace d) detections: a: $\lambda$ = 214 nm; b: $\lambda$ = 240 nm; c: $\lambda$ = 280 nm; d: E = 0.60 V.

the presence of the small $\gamma$ and $\omega$ peaks in UV and to a lesser extent in EC, while peaks $\alpha$ and $\beta$ are never observed. The trace at $\lambda$ = 240 nm (b) shows a very strong decrease of the main peak 3, much more moderate decrease of the $\omega$ peak and the emergence of the new peak X. At $\lambda$ = 280 nm (c), the sole notable peak is that of $\omega$.

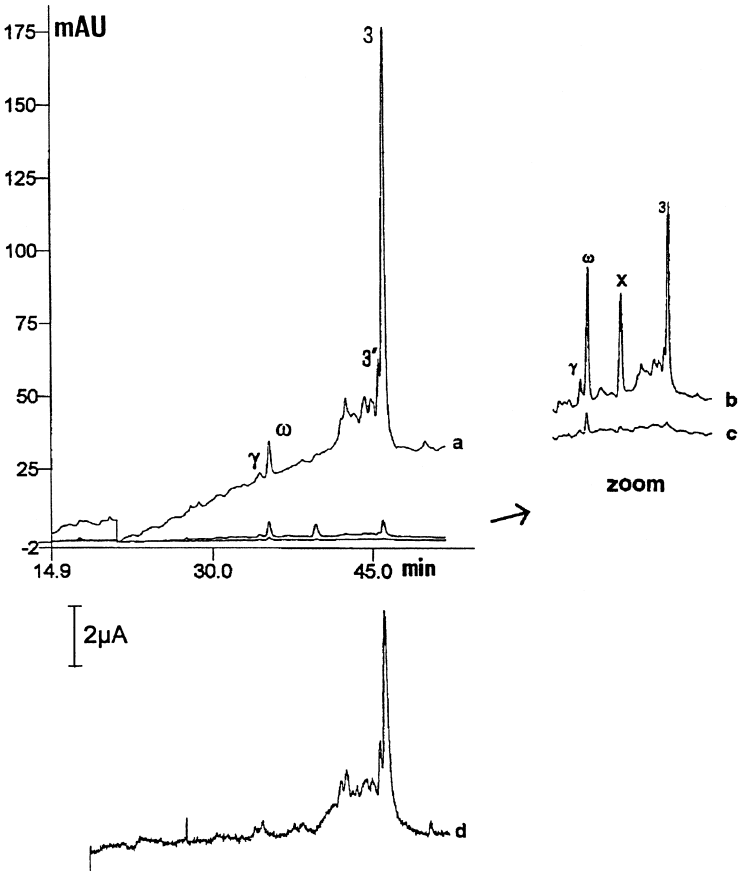


**Figure 2**. Reverse phase chromatogram of a rabbit liver MT-2 (RL-2) sample. All conditions as in Figure 1.

Peaks 1-4, α-ω and X have respective different chemical natures, as illustrated by figures in Table 1. Peaks 1, 2, 3, and 4 have obviously equivalent UV and EC responses as shown by the example of the $H_2/H_4$ ratio which gives the same value using either mode of detection ($H_2/H_4 \approx 1.30$). On the contrary, this does not hold true for the peaks α, β, γ and ω: for instance, the $H_2/H_w$ ratio is much higher in EC (= 2.80 on average) than in UV (≈ 1.60 at λ = 214 nm). All this means that the α-ω peaks give lower electrochemical responses relative to the peaks 1-4.

**Table 1**

**UV and Electrochemical Peak Height Ratios in Rabbit Liver MT-1 (RL-1) Dissolved in Water***

| Peak Ratio in RL-1 | UV Detection $\lambda = 214$ nm | UV Detection $\lambda = 240$ nm | EC Detection |
|---|---|---|---|
| $H_2/H_4$ | 1.33 ± 0.18 | 1.28 ± 0.22 | 1.27 ± 0.20 |
| $H_2/H_\omega$ | 1.56 ± 0.34 | 0.18 ± 0.05 | 2.80 ± 0.68 |
| $H_2/H_X$ | 26.6 ± 7.9 | 0.40 ± 0.10 | No EC signal for X |

* Labeling of peaks in Figure 1; n =13.



Peak X behaves in yet a third manner: when the sample is injected just after preparation, it is, in most cases, not measurable at 214 nm ($H_2/H_X$ is very high), often already significant at 240 nm ($H_2/H_X < 1$) and undetectable in EC. However, it tends to increase with time, to such an extent that it can sometimes already be observable at $\lambda = 214$ nm. Similar trends are observed with RL-2.

***UV Absorption Spectra Analysis***

The three types of peaks give three kinds of UV spectra as shown in Figure 3. Trace (a) represents the spectrum of peaks 1, 2, 3, 3', or 4 and corresponds to that of metallothionein totally lacking a cation-thiol bond: it is typical of an apothionein form.

Working with an acidic mobile phase will decomplex the metal cations at some point during the elution, probably immediately after injection, and therefore lead to the separation of aposubisoforms. Trace (b) is that of the spectrum of peaks $\alpha$, $\beta$, $\gamma$, or $\omega$. This case is opposite to what occurs with apoMT, although there is no absorption maxima or shoulder between 220 and 280 nm, the absorbance increases continuously when the wavelength goes from 300 to 200 nm. Therefore, unlike peaks 1-4, the $\alpha$-$\omega$ species cannot be aposubisoforms.

Finally trace (c) displays the UV spectrum of the species X: it shows a clear maximum of absorbance at 240 nm and a minimum at 220-225 nm.

***Metal Concentration Determination in the Chromatographic Fractions***

Cd, Cu, and Zn were analysed in discrete chromatographic fractions corresponding to the peaks of interest of RL-1. Results are given in Table 2. In all

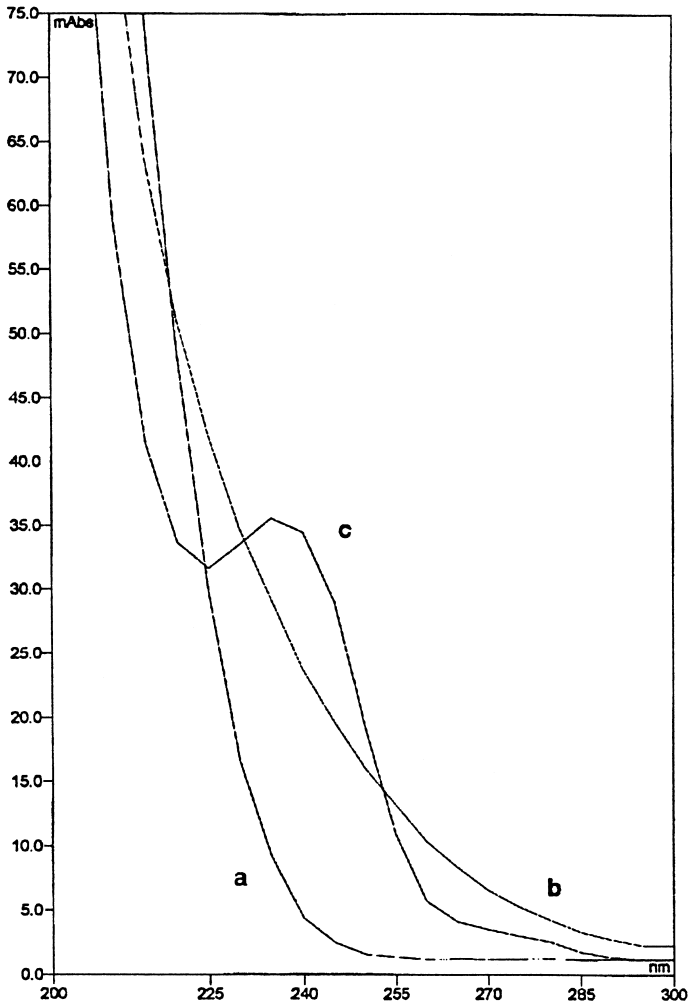


**Figure 3**. Absorption spectra of the RL-1 peaks: a: type of spectrum of peaks 1-4. b: type of spectrum of peaks α-ω. c: spectrum of peak X.

cases, Zn concentrations were found negligible. The metal characteristics of the fractions agree well with the respective UV spectra: peaks 1-4 contain almost no more metals, explaining the typical apothionein behavior. On the other hand, peaks α-ω still contain appreciable amount of Cd and Cu: they are not apoforms and explain the UV trace (b) in Figure 3.

**Table 2**

**Metal Concentrations in Chromatographic Fractions from Rabbit Liver MT-1 Dissolved in Water***

| Peaks | Cd (μg/L) | Cu (μg/L) | Zn (μg/L) |
|---|---|---|---|
| α | 36.5 ± 0.65 | 35.2 ± 3.1 | <dl |
| β | 51.9 ± 0.66 | 48.5 ± 3.0 | <dl |
| ω | 42.7 ± 0.68 | 58.5 ± 3.0 | <dl |
| 1 | 1.2 ± 0.58 | 35.2 ± 2.8 | <dl |
| 3 | 1.1 ± 0.59 | 27.9 ± 2.8 | <dl |
| 4 | 1.2 ± 0.58 | 30.4 ± 2.8 | <dl |

* Labeling of peaks in Figure 1; n = 3. <dl means under detection limit.



### *Effect of Temperature*

In our previous article,[11] we have shown that above 50°C, the α-ω peaks were drastically altered, while peaks 1-4 remained rather unchanged. To complete our first observations, the previous separations carried out at 35°C were repeated at 60°C. It could be noticed that in RL-1 at high temperature, the α-ω peaks remain only as residues of former peaks and the resolution between peaks 1 and 2 has decreased. In both RL-1 and RL-2, X is still present at 60°C with the same responses but its retention time $t_R$ is more affected than those of other peaks. For instance, the difference of retention times between peak 3 and peak X in RL-1 at 35°C is 6.05 ± 0.24 min, but it doubles at 60°C at 11.90 ± 0.60 min. The corresponding values in RL-2 are equal. The UV spectrum is of great help for following the displacement of peak X. For comparison purposes, all other peaks see their retention time decrease of about 7 min between 35°C and 60°C.

## Polymorphism of Rabbit Liver MT (RL)

### *RP-HPLC Chromatograms and UV Spectra*

Since the rabbit liver MT is theoretically a mixture of both rabbit liver MT-1 and MT-2, although in unequal proportions with much more MT-2 than MT-1,[14-15] we should logically observe all the peaks of RL-1 and RL-2 in the chromatogram of a RL sample. However things happen to be more complicated than expected as depicted in Figure 4. Chromatogram (a) represents the UV detection at λ = 214 nm. Some peaks, those labelled as group B peaks, corre-

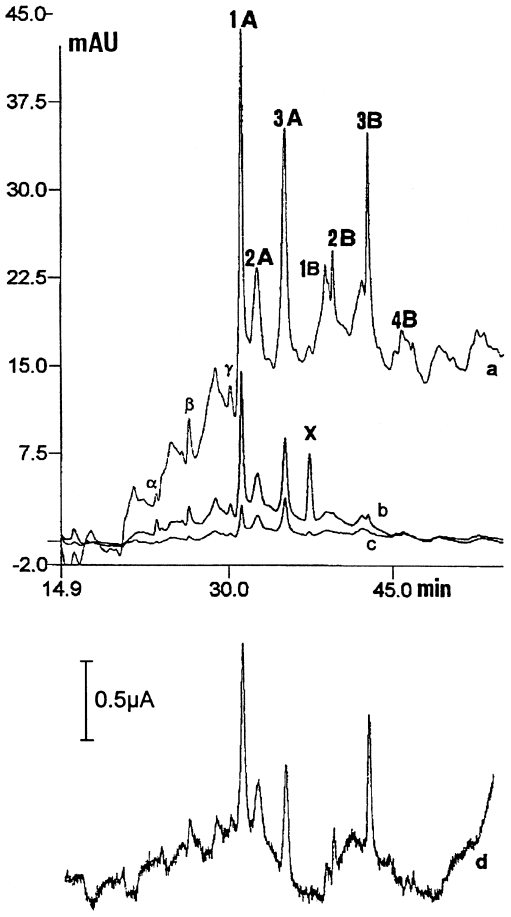


**Figure 4**. Reverse phase chromatogram of a rabbit liver MT (RL) sample. All conditions as in Figure 1.

spond to the peaks 1-4 of RL-1 and RL-2. They also display UV spectra typical of apoforms (Figure 5, trace a).

We can also see several less hydrophobic peaks, labelled group A peaks, the highest of which (1A) is equivalent in retention time and in UV spectrum (Figure 5, trace b) to the ω peak of RL-1 and of RL-2. On the other hand, peak 3A appears to be specific to RL: there is no peak with the same retention time

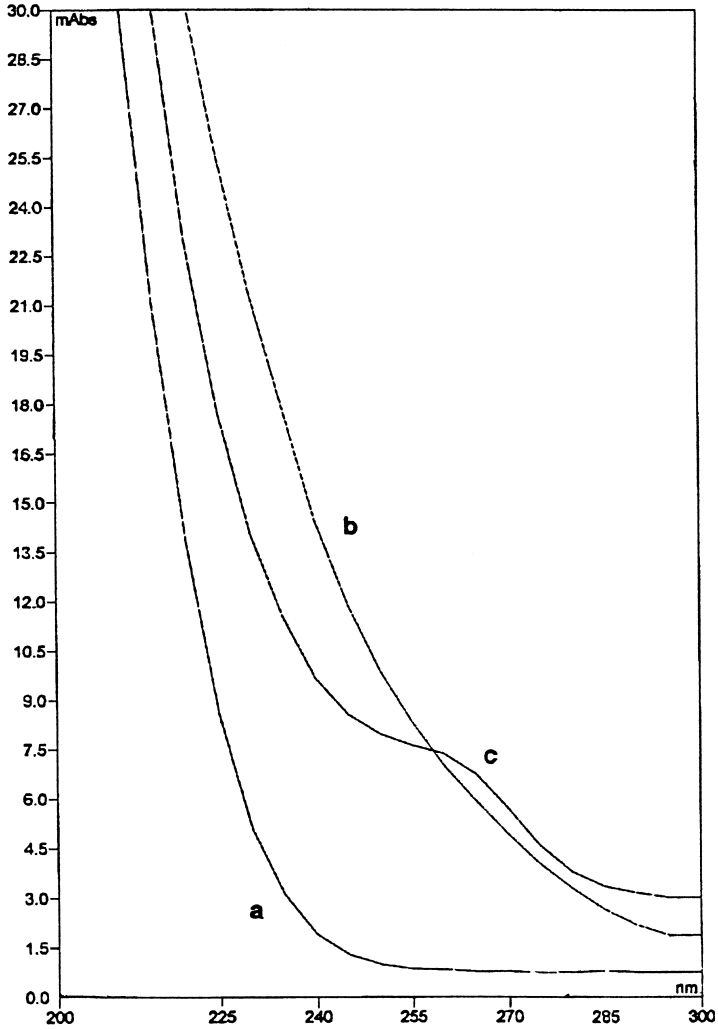


**Figure 5**. Absorption spectra of the RL peaks: a: type of spectrum of peaks 1B-4B. b: spectrum of peak 1A. c: spectrum of peak 3A.

and UV spectrum (Figure 5, trace c) in either RL-1 or RL-2. Its UV spectrum shows a clear maximum around 260-265 nm. Peak 2A is not always well resolved and often appears as a shoulder of the major peak 1A; its UV spectrum is quite similar to that of 3A.

The EC chromatograms exhibit similar results but with group A peaks being relatively less electroactive than group B peaks as shown by the value of the $H_{3B}/H_{3A}$ ratio: 1.13 ± 0.32 in EC versus 0.91 ± 0.25 in UV at 214 nm. The peak previously labelled X in the two isoforms is also observed in RL: at t =0, it is very small at 214 nm and undetectable in EC, but already quite large at 240 nm (Figure 4, trace b). At 280 nm, only peaks A are still significant. After a rather short time, 1-2 days, peak X increases and becomes important, even at 214 nm.

### *Metal Concentration Determination in the Chromatographic Fractions*

All Zn concentrations were found insignificant (Table 3). Peaks B (here 3B) contain no more Cd and very little Cu, displaying logically the typical apoMT UV spectrum. Peak 1A still contains notable amount of Cd and especially of Cu, explaining the trace (b) in Figure 5, while peak 3A contains only remaining Cu displaying therefore the characteristic trace (c) in Figure 5.

### *Effect of Temperature*

At 60°C, the A peaks are strongly reduced in size, confirming our previous results.[11] Similarly to what occurred with the isoforms, peak X has a retention time which is doubly affected compared to the other peaks by the increase of temperature.

To summarize the temperature effect on the variation of the retention times, Table 4 gathers the $\Delta t_R$ between peaks 3 and X for the three MTs on the one hand and between all other peaks represented here by peaks 3 and ω in RL-1 and RL-2 and peaks 3B and 1A, their equivalents in RL, on the other hand. The singularity of peak X appears clearly.



**Table 3**

**Metal Concentrations in Chromatographic Fractions from Rabbit Liver MT Dissolved in Water***

| Peaks | Cd (µg/L) | Cu (µg/L) | Zn (µg/L) |
|---|---|---|---|
| 1A | 79.6 ± 0.95 | 116.4 ± 2.86 | <dl |
| 3A | 36.6 ± 0.53 | 108.2 ± 3.52 | <dl |
| 3B | 1.23 ± 0.58 | 32.0 ± 2.78 | <dl |

* Labeling of peaks in Figure 4; n = 3. <dl means under detection limit.

**Table 4**

**Differences of Retention Times $\Delta t_R$ Between Peak 3 and Peak X and Between Peak 3 and Peak ω at 35 and 60° in the Three Rabbit Liver MTs**

| MT | $\Delta t_R$ (Peak 3-Peak X) min 35°C | 60°C | $\Delta t_R$ (Peak 3-Peak ω) min 35°C | 60°C |
|---|---|---|---|---|
| RL | 5.85 ± 0.28 | 12.87 ± 1.00 | 11.42 ± 0.31 | 10.10 ± 0.80 |
| RL-1 | 6.05 ± 0.24 | 11.91 ± 0.60 | 11.25 ± 0.43 | 10.48 ± 0.37 |
| RL-2 | 5.97 ± 0.33 | 11.69 ± 0.27 | 11.45 ± 0.88 | 11.29 ± 0.79 |



## DISCUSSION

In the comments on all the results and observations presented above, we will first consider what is seen from the UV detection at 214 nm and from the electrochemical detection, before looking at the UV detection at 240 nm.

### Detection by UV at λ = 214 nm and in EC

#### *Isoforms RL-2 and RL-1*

The morphology of the RL-2 chromatogram is the most simple of all three MTs and is quite similar to that of most results obtained for this metallothionein, with one highly dominant peak and several other minor forms, using either an acidic mobile phase[16-17] or a neutral mobile phase.[15,18] This peak 3 probably corresponds to the main rabbit liver subisoform MT-2a, the sequence of which is reported in the literature.[8] Peak 3' is distinct from peak 3, but they have very close retention times. This means that their primary structures might be very alike. Knowing that threonine is slightly less hydrophobic than alanine which results in retention times for T being shorter than for A,[19] peak 3' could therefore be attributed to the A/T heterogeneity of the rabbit liver subisoform labelled MT-2c.[8] At pH 3, the two peaks display logically typical absorption spectra of apoMT, being completely demetalated. Zn is known to be fully decomplexed from the thionein at pH 5 while Cd, although, is largely, on average, not completely dissociated at pH 3.[20-22] Cd is, however, totally decomplexed from subisoMTs 3 and 3'.

The RL-1 chromatogram is much more complex than the previous one. Out of a total of eight-nine peaks, the species 1, 2, 3, and 4 - equally detected in UV and in EC - correspond to aposubisoforms. Since peak 3 is a contamination of RL-2, RL-1 contains three putative subisoforms of its own which have

lost their Cd and Zn during the elution by the acidic mobile phase. Richards and Beattie[17] observed the presence of two major peaks in rabbit liver MT-1 at pH 2.5, while Klauser et al.[16] had also detected three peaks in a RL-1 preparation eluted by a TFA/acetonitrile gradient. In both cases, these peaks were the only ones observed.

Differently, the first peaks α-ω are less electroactive relative to the other peaks and absorb in UV already at $\lambda = 280$ nm. Consequently these species are obviously not aposubisoforms. It is known that, contrary to Zn and Cd, when Cu is also found in a MT, it is not totally decomplexed in acidic solution, even at pH 2.[23] While a Cu-MT exhibits a marked shoulder at 260-265 nm in the absorption spectrum, the presence of minor levels of copper in a Cd, Zn-MT should instead give rise to a continuous increase of absorbance between 280 and 200 nm, but without a shoulder.[24] The UV spectra of peaks α-ω in RL-1 and in RL-2 are similar to such a case. Our samples of Sigma MT are theoretically pure Cd, Zn-MT, but we saw by ETAAS that all the lots of the three MTs we had contain a significant amount of Cu, between 0.5 and 0.8 %, thus, far from being negligible.

By ICP-MS, Chassaigne and Lobinski[15] found identical Cu concentrations in the same (56H9500) or in other lots of the same Sigma MTs. We have, therefore, good evidence to say that the peaks α-ω correspond to Cu, Cd containing forms, still partially complexed at pH 3. Furthermore, we have seen that peaks α-ω give relatively lower EC responses than peaks 1-4, which is probably consistent with the fact that these peaks α-ω would be partially metalated compared to the apoforms 1-4. At a given potential, it would be easier to oxidise the protonated thiol groups into disulphides than the thiols complexed with metals, especially Cu.

For both isoMTs, our results agree with those of Chassaigne and Lobinski[15] although their mobile phase was at pH 6. Their chromatographic results were compared with those obtained by direct ion-spray MS of the MT samples. For instance, out of seven HPLC peaks in RL-2 (one major), three, including the two first eluted forms, could not be attributed to any Cd-MT subform. In RL-1 they attributed the first three peaks out of the nine separated to mixed Cu, Cd forms of variable stoichiometry.

***Metallothionein RL***

The RL chromatogram has two regions: one with the aposubisoforms attributed to contributions of RL-1 (peaks 1, 2 and 4B) or of RL-2 (peaks 3 and 3'B). In the less hydrophobic A region, peak 1A has the same characteristics as peak ω of RL-1 and RL-2: it is a mixed Cu-Cd form. It is important to mention that this peak is always the biggest one of an RL chromatogram in our conditions, which is not the case for ω either in RL-1 or in RL-2. Peak 3A is spe-

cific to this metallothionein and it is rather astonishing that although it is a significant peak of RL (1+2), it is not found in, at least, one of the two isoforms. The absorption spectrum of 3A is different from all others and corresponds to a "pure" Cu form.

It is not surprising to recover Cu forms since we have observed that the RL metallothionein contains a rather high Cu concentration, 0.8 % in lot 56H9500 and 0.82 % in lot 125H9512 as measured by ETAAS, values higher than in RL-1 and RL-2 (≈ 0.5 %), and also higher than the Zn concentration (≈ 0.5 %). In their recent work, Chassaigne and Lobinski[15] found two groups of peaks in their ion-spray MS spectrum at pH 1.8 of MT RL, one group corresponding to contributions from either MT-1 or MT-2, and one group of peaks which do not exist in the spectra of the isoforms. By RP-HPLC of the same sample, they obtain a morphology in which one major peak cannot be attributed to either MT-1 or MT-2: it could therefore well correspond to our Cu containing form 3A.



Our study shows that RL contains (metallo)forms which are no longer found once the two constitutive isoforms RL-1 and RL-2 have been separated. Indeed, the chromatograms of a mixture of RL-1 + RL-2 (20-30 % / 70-80 %)[10,14,15] display important differences from those of original RL (1 + 2) chromatograms (Figure 6). Two major facts should be noted, the absence of 3A in the artificial mixture and the inversion of response intensity between 1A and 3B: $H_{1A(RL)} > H_{1A(RL\text{-}1+RL\text{-}2)}$ while $H_{3B(RL)} < H_{3B(RL\text{-}1+RL\text{-}2)}$. These observations are obviously not due to the proportions of each isoform in the artificial mixture: increasing the concentration of RL-1 would increase peaks 1B, 2B and 4B and $\alpha$, $\beta$, and $\gamma$ to values which are never encountered in original RL.

**Detection by UV at $\lambda$ = 240 nm**

Recording the UV spectrum at 240 nm shows the presence of an extra peak X in all three freshly prepared MTs. Eluted between A and B peaks, X tends to increase with the aging of the sample. After some days, especially in RL-2 and in RL, X is often already observable at 214 nm and has become very large at 240 nm. It is not at all electroactive, a unique case of all the species separated from the three MTs. This peak is also more affected by the increase of temperature of the column, its retention time decreasing more than that of all other peaks.

The addition of the reducing agent 2-mercaptoethanol to a MT solution containing a notable X peak does not change the morphology of the chromatogram at all: none of the peaks are affected. Consequently peak X cannot be an oxidised form of MT containing disulphide bonds, which could well have explained the lack of electrochemical response. The exact nature of this "degra-

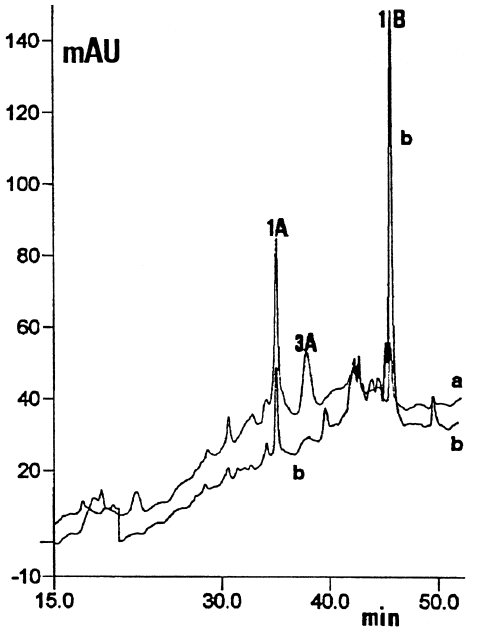


**Figure 6**. Comparison of the reverse phase chromatogram of a RL sample (trace a) with that of a mixture 20 % RL-1 + 80 % RL-2 (trace b). $\lambda$ = 214 nm. Conditions as in Figure 1.



dation" species will be clarified on further characterization, but the hypothesis that it is a product of hydrolysis of MT should probably be given priority.

### Elution with a Mobile Phase at pH 2

To bring even more evidence on the existence of the remaining metallo (Cu, Cd) forms, we have carried out several experiments with a mobile phase at pH 2. Only a limited number of tests have been done since pH 2 is the lowest pH limit compatible with the type of reverse phase column used. In any case, very interesting observations have been made. Peaks 1-3B in RL and 1-4 in RL-1 are still correctly resolved, while there are no clear peaks left in the region of the peaks A nor in that of the $\alpha$-$\omega$ peaks, only some remains being observed displaying UV spectra with the typical Cu shoulder. This means that at this more acidic pH 2, Cd is now totally decomplexed and only some Cu is still bound forming very small peaks.

## CONCLUSION

The combination of reverse phase HPLC with on-line diode array and electrochemical detection appears to be a very good tool for a first characterization of various subisoforms of metallothionein samples. Replacing the former UV detector at fixed wavelength by the DAD has allowed us to identify the first eluted peaks of rabbit liver MTs as subisoforms containing some Cu and Cd and not as disulphide containing oxidized forms as we concluded in our previous works.[10-11]

Table 5 gives a qualitative presentation of the polymorphism of the rabbit liver metallothioneins at pH 3. From the present study, we can state that:

- RL-2 has a major and a minor subisoforms which are under apoforms at pH 3, and has kept low levels of Cu, Cd forms which are completely lost at pH 2.

- RL-1 has a more complex polymorphism with three subisoforms which are apoforms at pH 3, and four other Cu, Cd metalloforms the peaks of which decrease strongly at pH 2.

- RL has four main subisoforms which are apoforms at pH 3, coming either from RL-1 (three subisoMT) or from RL-2 (one main subisoMT), and two main other metalloforms, one being a mixed Cu, Cd form also found in RL-1 and RL-2 and the other being a pure Cu form not found in the isoforms. At pH 2, only very low levels of Cu-MT remain.



**Table 5**

**Qualitative Distribution of Species in Rabbit Liver RL-1, RL-2, and RL Metallothioneins***

| | Group A Peaks ($UV_{214}$ > EC) | | | | | No EC | B Peaks (UV ≈ EC) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| MT | Cu, Cd subisoMT at pH 3 | | | | Cu-sub isoMT | Degrad. Peak | AposubisoMT at pH 3 | | | | |
| RL-1 | α | β | γ | ω | --- | X | 1 | 2 | --- | --- | 4 |
| RL-2 | --- | --- | γ | ω | --- | X | --- | -- | 3' | 3 | --- |
| RL | α | β | γ | ω=1A | 3A | X | 1 | 2 | 3' | 3 | 4 |

Increasing Retention Time →

*Peak labeling in Figures 1, 2, and 4.

• In all three MTs, an extra peak X has been identified by its different UV spectrum, its time-dependence increase, its total lack of electrochemical response and its larger variation of retention time with temperature: this could be a degradation product of MT.

This work clearly shows that the study of MT polymorphism is far from being an obvious task and that even for commonly used samples such as those extracted from rabbit liver, there is still a lot of information lacking. Coupled with different modes of detection, HPLC remains a method of choice for such investigations.

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