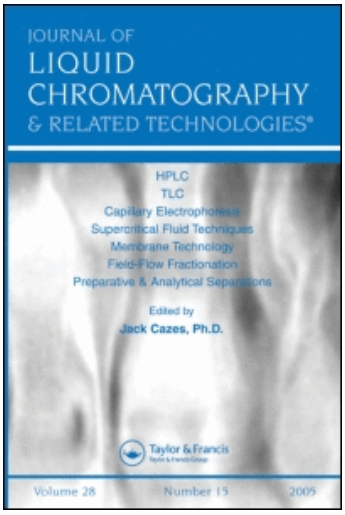

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CHARACTERIZATION OF METALLOTHIONEIN ISOFORMS BY REVERSE PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE UV AND ELECTROCHEMICAL DETECTION

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ABSTRACT

Four metallothioneins (MTs), rabbit liver MT (RL), MT-1 (RL 1) and MT-2 (RL 2) and horse kidney MT (HK), were subjected to reverse phase HPLC with on-line UV and electrochemical (EC) detection, the latter comprising of a graphite electrode. The EC detection is based on the direct oxidation of the thiols contained in the MT chains, into disulphides. MT samples were prepared at different pH values and eluted with a gradient of TFA and acetonitrile. The MTs are found to exhibit a different polymorphism and the various peaks differ in their detectability depending on the detection mode. RL 2 (one major peak) and HK (three peaks) have peak patterns which are stable with time and varying pH, all peaks being detected by both EC and UV modes. The two other MTs also exhibit peaks which are eluted within the same retention time range as the previously mentioned MTs and which give EC and UV signals (three peaks for RL 1 and four for RL), but which

also show some other peaks - eluted earlier - which are perfectly detected in UV but not or very little in EC. This latter information means that these species contain none, or very few, thiol groups. Stable with time at neutral and basic pH, the RL 1 and RL chromatograms are highly evolutional in acid pH with the formation of more of the non-EC detected species and a decrease in the doubly detected ones. We can therefore assume that the peaks detected by both EC and UV modes correspond to the original thiol containing isoforms, while those less hydrophobic of RL 1 and RL correspond to modified species, probably containing disulphide bonds which explains the lack of EC response at the graphite electrode. Since many MTs are highly reactive, this UV/EC combination appears to be a very good tool for a quick identification of reduced (thiolic) isoforms from oxidised (with disulphides) species.

INTRODUCTION

Metallothioneins (MTs) constitute a class of ubiquitous low molecular weight (6000-7000 Da) proteins, characterised by a high cysteine content (~ 30%), the lack of aromatic amino acids and the ability of binding metals such as zinc, cadmium, copper and mercury. The characteristics and properties of these molecules have been the subject of several monographies.[1-4] These metalloproteins probably play important roles in, at least, the homeostasis of some essential metals (Zn, Cu) and the detoxification of harmful (Cd, Pb) and excessive essential metals.[5] The metals are bound to the cysteine residues of the MT chains through thiolate complexes which are organised into two separate metal-binding clusters, α and β.[6,7] In most cases, MTs exist under various isoforms (isoMTs) that arise from genetic polymorphism encountered in many species.[5]

Based on their electrophoretic properties, two major isoforms of MT have been identified in mammals, MT-1 and MT-2, named after their order of elution by anion-exchange chromatography. They differ slightly in amino acid content and exhibit a single charge difference at neutral pH.[8] These two isoMTs are the products of two distinct genes.[9] Furthermore, many animal species generate various sub-isoforms of MT-1 and MT-2, therefore displaying significant microheterogeneity. However, the biological functions of individual isoforms remain unknown. Progress in this field is largely dependent on efficient separation and quantification of each isoMT, and therefore, on the development of analytical techniques providing a high degree of resolution.

Reverse phase high performance liquid chromatography (RP-HPLC) which separates compounds on the basis of their difference in hydrophobicity, has shown its significant ability to resolve additional isoforms of MT not previously separated by conventional techniques.[10-12]

Classically, RP-HPLC has been employed with UV detection at 214, 220 or 254 nm[10,12,13] and/or in combination with atomic absorption spectrometry (AAS) for quantification of metals.[11,14,15] Although sensitive, the HPLC-AAS technique is accurate only when the metal occupation is complete and homogeneous, which is not always the case.[13] Furthermore, applying this procedure obviously involves the need of a neutral buffer for both sample preparation and elution. However, in some situations, only acidic buffers are suitable for efficient separations, hence forbidding the AAS mode of detection. For instance, it has been shown that copper containing isoMTs cannot be adequately separated and detected by RP-HPLC at neutral pH.[15,16]

The measurement of UV absorbance is, by far, the most widely used mode of detection. It is a universal detector and, consequently, some commonly used elution buffers such as tris(hydroxymethyl)aminomethane (TRIS) can contribute significantly to the absorbance in the 200-220 nm range.[17]

Compared to this, the use of more selective detectors such as those based on electrochemistry could be more advantageous for applications that involve complex matrices such as those of biological origin. Electrochemical detectors (EC) are naturally highly selective because only the compounds, or functional groups, that undergo an electrochemical reaction at a particular potential will be detected. In the case of MT, this would bring information on the redox state of the sulphydryl groups contained in the structure. Until now, electrochemistry has been used to either quantify the total concentration of MTs in biological fluids through differential pulse polarographic (DPP) assays of thiol groups, based on the Brdicka reaction [18-20] or to study, in a more fundamental manner, the electrochemical behaviour of MTs (complexing properties of thiol groups, speciation)[21,22] However, to our knowledge, no study on electrochemical detection of MT isoforms via an on-line RP-HPLC-EC combination has ever been published. In a previous work, we studied the chromatographic behaviour of the trithiolic hexapeptide Lys-Cys-Thr-Cys-Cys-Ala (56-61) MT-1 intrinsic to the mouse liver MT structure, employing electrochemical detection.[23] We showed the advantage of using a coulometric mode in order to detect thiol groups at a porous graphite electrode according to the reaction $2\ R\text{-}SH \rightarrow R\text{-}SS\text{-}R + 2\ H^+ + 2\ e^-$ with a medium voltage of only 0.6 V. It was then possible to separate the reduced and oxidised forms of the peptide and to follow the evolution of the formation of intra- and intermolecular disulphide bonds.

In the present work, our main objective was therefore the study of the applicability of the same electrochemical system, along with UV detection, to identify and quantify the isoforms of four MTs, three from rabbit liver and one from horse kidney.

EXPERIMENTAL

HPLC Intrumentation and Procedure

A Kontron (Zurich, Switzerland) chromatograph was used, 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 reverse phase column was a Hi-Pore RP 318 (250 x 4.6 mm), 300 Å pore size, 5 μm particle size (Bio-Rad Laboratories). The detection system includes a Coulochem Model 5100A electrochemical detector (ESA Inc., Bedford, MA, USA) set at a potential of 0.6 V, used with a Model 5011 analytical cell containing two coulometrically efficient porous graphite working electrodes and a Model 5020 guard cell. A UV capillary detector Model 433 (Kontron), set at λ = 230 nm, was also connected in series, before the electrochemical detector.

Buffer A was 0.1 % (v/v) trifluoroacetic acid, TFA, (pH = 3) and buffer B consisted of 0.1 % TFA in acetonitrile. MTs were eluted with a linear gradient of 10-30 % B in 70 minutes, at a flow rate of 1 $ml.min^{-1}$.

Chemical

Rabbit liver Cd, Zn MT (RL) and its purified isoforms MT-1 (RL 1) and MT-2 (RL 2), horse kidney Cd, Zn MT (HK), TFA (1 mL ampoules) were purchased from Sigma (St Louis, MO, USA). Acetonitrile was HPLC grade (Super gradient grade from Lab-Scan, Dublin, Eire). Ultra pure water was obtained from a Millipore Milli-Ro 10 Plus deionisation system followed by a Milli-Quater system (18 MΩ·cm resistivity) and a sub-boiling quartz distillation unit (Quartex SA, Paris, France).

All mobile phases were filtered through 0.22 μm Millipore membranes and continually purged with helium to remove dissolved oxygen. All experiments were carried out at room temperature with, unless otherwise stated, freshly prepared solutions.

RESULTS

MT Samples Prepared at Neutral and Basic pH

The MTs have been first prepared at neutral pH. The four proteins exhibit a different polymorphism, as shown in Figure 1. More stable baselines are generally obtained with the electrochemical detection where no shifting is oberved when the proportion of organic phase increases, which is sometimes the case for the UV detection. On the whole, UV and EC chromatograms present comparable morphologies in the cases of RL2 (Figure 1 b-b') and of HK (Figure 1 d-d'), while they display some different aspects for RL 1 (Figure 1 a-a') and RL (Figure 1 c-c'). *Nota bene:* in this text, peak numbering goes in the direction of increasing retention times, and labelling like H_2 stands for the height of peak 2.

-RL2 appears to be the least complex case, comprising one highly dominant peak (3 in Figure 1 b-b') eluted at a retention time (t_R) of about 41 minutes (Table 1), surrounded by several minor peaks (1, 2, 4). The height ratios of the various peaks are comparable on both EC and UV chromatograms (Table 2). One small UV peak, γ which is eluted earlier (t_R = 30 min), is not detected electrochemically.

- HK mainly exhibits three peaks (1, 2 and 3 between 36 and 44 minutes) on both chromatograms with a good resolution (R_{2-3} = 1.1). For this MT also, the peak height ratios are similar for both EC and UV modes (Table 2).

-RL I is more complex than the previous proteins (Figure la-a'). Four peaks (1-4) are clearly detected (37 min < t_R < 46 min) by both EC and UV modes with a good resolution (R_{1-2} = 0.85) and comparable height ratios except for the minor peak 3 (Table 2). Furthermore, the UV chromatogram exhibits, between 25 and 35 minutes, some smaller peaks (α,β,γ...) which are not or only slightly obvious on the EC picture.

- Both chromatograms of RL show two groups of peaks, group A (peaks 1, 2 and 3 at t_R < 35 min) and group B (one major peak, 6, and three minor peaks at t_R > 35 min, Table 1).

Considering A and B independently of one another, the peak height ratios in each group are comparable in both EC and UV (Table 2). On the contrary, when group A peaks are compared to those of group B, large differences occur between the two types of detection. Group A peaks clearly give much less intense EC than UV responses than those of group B.

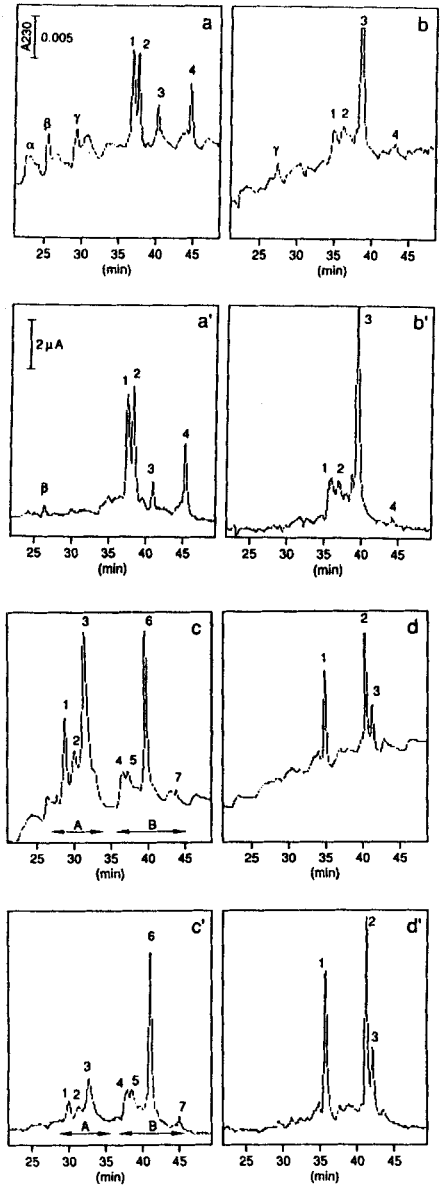

Figure 1. Reverse phase chromatograms of four metallothioneins prepared at neutral pH. a, b, c, d: UV detection (λ= 230 nm; 0.005 AUFS). a', b', c', d': EC detection (E = 0.6 V, gain = 700). a-a': RL 1 b-b': RL2 c-c': RL d-d': HK (C=500mg.l^{-1}). Elution: from 90% A (0.1%TFA)-10% B (0.1% TFA in acetonitrile) to 70% A-30% B in 70 min.

Table 1

Retention Times (Minutes) of all MT Peaks Detected by both UV and EC Detectors for Samples Prepared in Neutral and Acid Solutions*

	Peaks						
	α	β	γ	1	2	3	4
HK							
Acid pH	--	--	--	36.60±0.58	42.36±0.63	43.41±0.57	--
Neutral pH	--	--	--	36.77±0.72	42.36±0.71	43.47±0.70	--
RL 1							
Acid pH	23.260.83	26.11±0.84	29.94±0.78	37.70±0.63	38.66±0.60	41.14±0.56	45.60±0.57
Neutral pH	23.92±0.64	26.66±0.59	30.28±0.44	37.88±0.58	38.87±0.57	41.54±0.43	45.67±0.60
RL2							
Acid pH	--	--	29.71±0.58	37.60±0.48	38.58±0.40	40.72±0.58	44.33±0.31
Neutral pH	--	--	30.48±0.86	37.94±0.76	39.31±0.87	41.30±0.73	44.23±0.25

	Peaks						
	1	2	3	4	5	6	7
RL							
Acid pH	29.87±0.89	31.55±1.02	32.96±1.00	38.37±1.06	39.16±1.10	40.96±0.79	44.50±0.77
Neutral pH	29.81±1.06	30.76±0.90	32.49±1.04	37.88±1.38	39.44±1.14	40.93±1.00	45.32±0.97

* Respective peak numbering according to Figure 1.

Table 2

Electrochemical and UV Peak Height Ratios of the Four Metallothioneins Prepared at Neutral pH*

MT	Peak Height Ratios	EC	UV
	H_1/H_2	0.93	1.04
RL 1	H_1/H_4	1.57	1.64
	H_4/H_3	2.03	1.34
	H_3/H_1	4.05	3.86
RL 2	H_3/H_2	4.59	4.87
	H_3/H_1 (A-A)	1.73	1.82
	H_3H_2 (A-A)	2.3	2.73
RL	H_6/H_5 (B-B)	4.12	4.36
	H_6/H_3 (B-A)	3.28	0.97
	H_1/H_2	0.75	0.81
HK	H_1/H_3	1.95	1.95

* Respective peak numbering according to Figure 1.

Table 3

Ratios Between UV and EC Responses of Peaks of MTs Prepared at Neutral pH for the Given Detection Conditions*

Peaks	HK	RL 1	RL 2	Peaks	RL
--	--	--	--	1	13.1
--	--	--	--	2	10.3
--	--	--	--	3	13.8
1	2.7	3.6	3.5	4	3.9
2	2.7	3.2	3.9	5	3.8
3	2.7	5.1	3.3	6	4.1
4	--	3.4	4.1	7	4.1

* UV: λ = 230 nm, 0.005 AUFS; EC: E = 0.6V, gain = 700. MT respective peak numbering according to Figure 1.

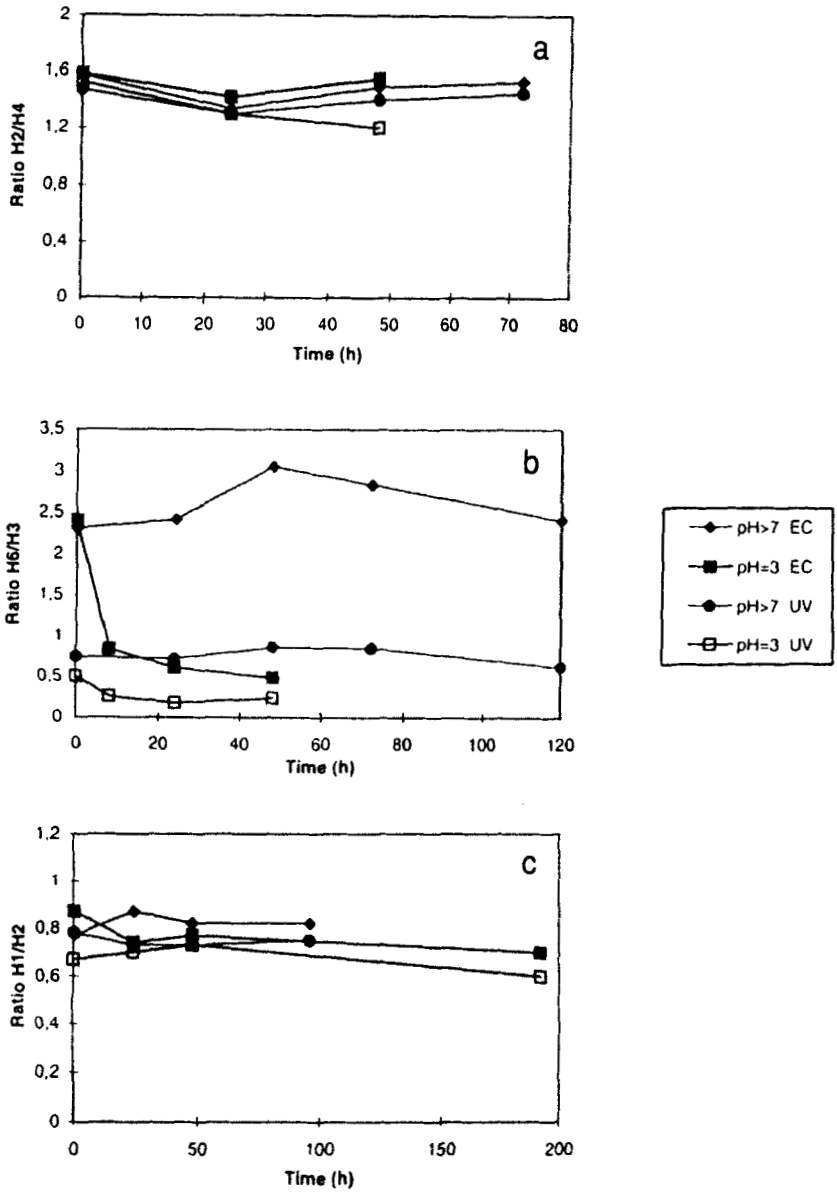

Figure 2. Variations in time of the UV and EC peak height ratios H_2/H_4 of the metallothionein RL 1 (a), H_6/H_3 of the metallothionein RL (b) and H_1/H_2 of the metallothionein HK (c) prepared at neutral and acid pH (respective peak numbering according to Figure 1).

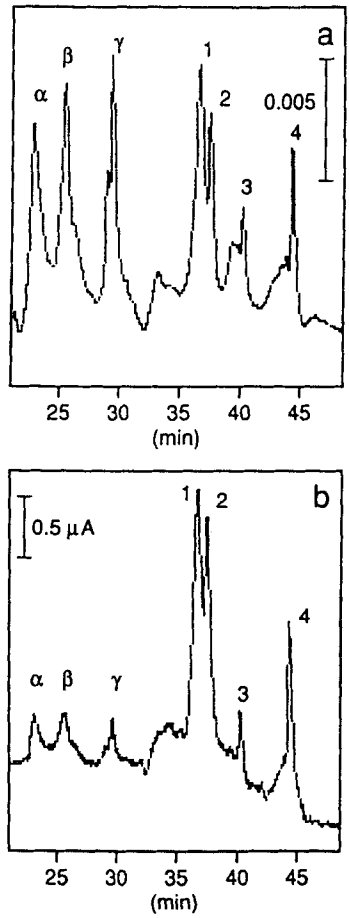

Figure 3. Reverse phase chromatogram of RL 1 prepared at acid pH. All other conditions as in Figure 1. a: UV detection b: EC detection.

Table 3 provides the ratios, for all MTs, between UV and EC heights for each peak under the given detection conditions. Peaks of RL 1, RL 2 and group B of RL obviously have the same UV/EC ratios comprised of between ~ 3.5 and 4. while group A peaks of RL show much higher values (> 10). As for HK, it has lower UV/EC ratios than the three rabbit liver MTs.

Prepared at pH 9, no major difference occurs in the morphology of any of the chromatograms. Retention times and peak ratios remain largely unchanged compared to neutral pH. Whatever the pH, neutral or basic, these separation

features are very stable with time. MT samples which had first been eluted immediately after preparation (t = 0, all results presented until now), have been re-subjected to separation at regular time intervals thereafter. No significant modification of the chromatograms of any of the MTs is observed. To illustrate this point, Figure 2 shows the evolution of the peak ratios H_2/H_4 of RL 1, H_6/H_3 of RL and H_1/H_2 of HK respectively. They remain perfectly constant even after three days at room temperature.

MT Samples Prepared at Acidic pH

At pH 3 and compared to what happened at pH 7, two types of behaviour occur: no appreciable changes are noticed for RL 2 and for HK (same number of peaks, retention times and peak ratios), while some significant changes other than that of the retention times, which do not vary (Table 1), are observed for RL 1 and for RL on both EC and UV chromatograms:

- for RL 1 (Figure 3a and 1a), the UV picture is characterised by a high increase of the α, β and γ peaks (t_R < 35 min) relative to the "main" peaks 1, 2, 3 and 4. In EC, the α, β, γ peaks remain rather negligible whatever the pH (Figure 3b and 1a'). However, despite the UV changes of the early peaks (α,β, γ), the height ratios of the main peaks (H_2/H_4 in Figure 2a), in both EC and UV modes, remain unchanged with time and are equal to the values obtained in neutral media .

- for RL, both EC and UV chromatograms, registered just after preparation, are rather similar to the neutral pH situation with quite stable group B/group A peak ratios (time = 0, Figure 2b). However, after a short time, one can observe a decrease of both EC and UV group B peaks (peak 6, Figure 4 a-b) and an increase of group A peaks (peaks 1 and 3, Figure 4a) in UV with a slight decrease in EC after 24 hours (Figure 4b). This results in an overall decrease of H_6/H_3 ratios in acid pH (Figure 2b). An example of a RL chromatogram after 24 hours in acid pH is shown in Figure 5.

Thus, in order to facilitate the attribution of the various peaks of RL, the elution of a mixture of RL 1 and RL 2 in an approximately 50/50 proportion (47% RL 1) has been carried out. This resulted in a chromatogram which was quite similar to the superimposition of RL 1 and RL 2 individual chromatograms (Figure 6), but which nevertheless showed an unexpected overincrease of the γ peak.

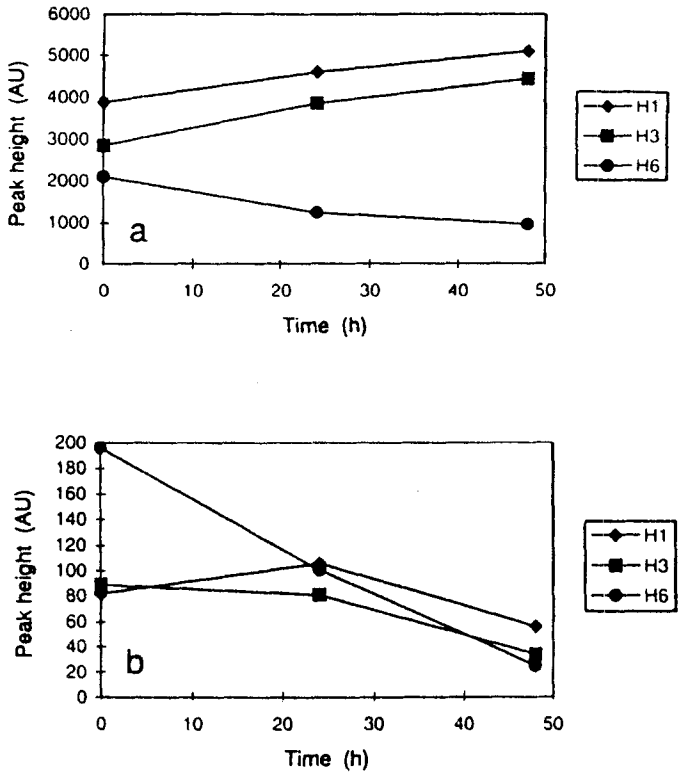

Figure 4. Variation in time of the height of the peaks 1, 3 and 6 (arbitrary units, AU) of RL prepared at acid pH. a: UV detection b: EC detection.

Variation of the EC and UV Responses with Concentration

Most of the previous experiments have been carried out with MT concentration of 500 $mg.l^{-1}$. We then checked that, for each MT, every EC and UV peak is linearly proportional to the MT concentration in the studied range of 20-700 $mg.l^{-1}$, with most of the correlation coefficients r being higher than 0.99. From these data, it becomes clear that the EC detector is capable, for instance, of detecting as low a content as about 0.5 μg of RL 1 (Table 4). It is possible to detect, in the absolute sense of the word, a much lower MT quantity in order to get a signal-to-noise ratio of at least 3, but we consider the values presented in the table as being more realistic limits of detection. In the working configuration presented here, the EC detector offers a sensitivity of 2 to 10 times higher than the UV.

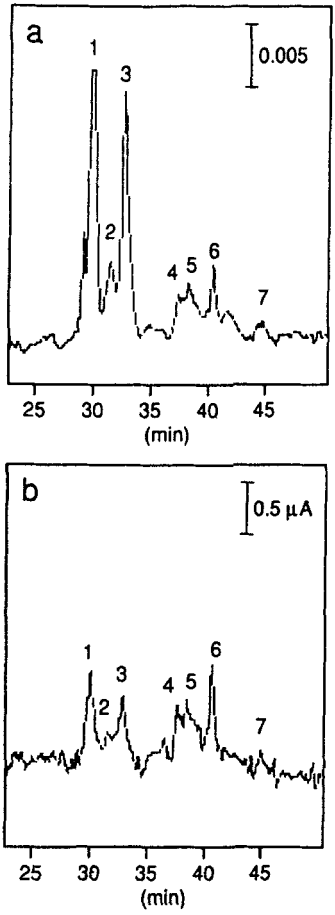

Figure 5. Reverse phase chromatogram of RL prepared at acid pH and kept at room temperature for 24 hours. All other conditions as in Figure 1. a UV detection b: EC detection.

DISCUSSION

The various metallothioneins submitted to a reverse phase HPLC separation show different peak patterns such as a variable number of peaks and a variable response depending on the detection mode (EC or UV). For all MTs which were studied, certain peaks are equally detected by both EC and UV with similar peak proportions.

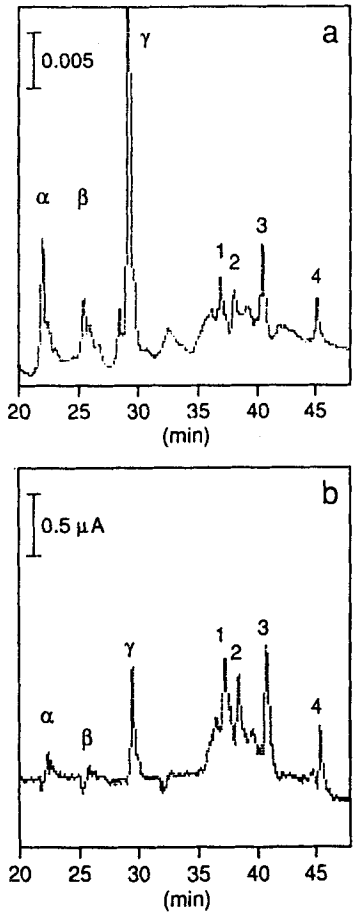

Figure 6. Reverse phase chromatogram of a mixture of RL 1 (47%) and RL 2 prepared at acid pH. All other conditions as in Figure 1.a: UV detection b: EC detection.

We can reasonably think of these peaks as corresponding to putative original (French: *originel)* isoforms of the metalloproteins.

From this point of view, horse kidney MT (HK) and rabbit liver MT-2 (RL 2) can be considered together, being relatively simple cases. HK exhibits three well resolved main isoforms and RL 2 one single dominant isoform (Figure 1), whatever the pH of the sample. Our HK results agree with those of Richards and Beattie,[24] also working on Sigma MT, who found three isoforms by capillary zone electrophoresis (CZE), and they improve their HPLC findings

Table 4

Detection Limits of the Four MTs According to the Mode of Detection, EC and UV

MT	Detection Limits (μg MT/100 μL) EC	UV
RL 1	0.5	1
RL 2	1	10
RL	2.5	15
HK	3	5

of two to three incompletely resolved peaks under acidic conditions. They also confirm our own CZE results which gave three largely dominant isoforms out of five peaks,[25] and they constitute a clear improvement of other works in which only two major isoforms were separated.[14,26] Our findings for RL 2 are also in agreement with most of the works published on this MT,[10,12,24] as well as with our CZE results.[25] Wan et al.[13] also found that MT-2 from rabbit kidney was composed of a single isoform. The separation features of these two MTs are stable with time at room temperature, being independent of the sample pH (Figure 2).

For the two other MTs, only the more hydrophobic species (peaks 1 to 4 of RL 1 and group B peaks 4 to 7 of RL, Figure 1) behave in an identical manner to those of RL 2 and HK: they are eluted by the same range of organic phase proportions and are detected by both EC and UV modes with similar peak signal ratios. The comparison of the retention times of the peaks of RL 1, RL 2 and RL (Table 1), together with that of their respective UV/EC ratios (Table 3), show that group B peaks of RL, peaks 4, 5 and 7 on one hand and peak 6 on the other hand correspond to isoforms of RL 1 and of RL 2 respectively. On the contrary, group A peaks (1, 2, 3) can hardly result directly from a simple contribution of either RL 1 or RL 2 (Table 1). Although not equal, we had found similar results in our work by CZE with UV detection in which one major peak of RL could not, indisputably, be attributed to either RL 1 or RL 2.

Taking into account the intensity of the peaks of RL group B, it clearly appears that the contribution of RL 2 to total RL is much higher (~70%) than that of RL 1 (~30%), in agreement with our previous studies on the same MTs using size-exclusion and reverse phase HPLC, where we found RL being composed of 20-30% RL 1 and 70-80% RL 2.[27] The present results also agree with those we obtained by anion-exchange chromatography (TSK DEAE-650

S, data not shown). Our experiments carried out with mixtures made of RL 1 and RL 2 reinforce these conclusions, showing what could be, for instance, the features of a RL composed of 50% of each isoform 1 and 2 (peaks 1, 2, 3, 4 in Figure 6).

It is also to be noted that peak 3 of RL 1 and the small peaks 1, 2 and 4 of RL 2 (Figure 1) probably result from a cross contamination due to imperfect separation of RL 1 and of RL 2 from the original RL (1+2).

The first peaks of RL 1 (α, β, γ) and those of RL (group A: 1, 2, 3) pose more problems since they are well detected by UV but not at all, or with only very low response, by EC. Based on the retention times, we can observe that, while the first peak of RL (t_R= 29.8 min) coincides with the γ peak of RL 1 (t_R= 30.1 min), RL's third peak (t_R = 33 min) appears isolated, with no corresponding peaks on neither RL 1 nor RL 2 pictures (Table 1). Quite stable at neutral and basic pH, an acidic medium enhances the formation of the species α, β and γ of RL 1 (Figure 3) and of 1, (2) and 3 of RL (group A peaks, Figure 5). The formation of intra- or intermolecular disulphide containing compounds from the original reduced isoforms (all thiols being either complexed by metals when pH > 5, or protonated when pH < 3) could maybe explain these observations. Since the electrochemical detection is based on that of the thiols according to their oxidation in disulphides at the graphite electrode, thiols which were previously chemically oxidised will not be able to give an EC response anymore. Our UV-EC on-line detection system allows the registration of this chemical evolution. Let us consider the RL case. Initially, the UV chromatogram exhibits six to seven peaks, organised in two groups A and B, being independent of pH, of which three are dominant: 1-A, 3-A and 6-B. In EC, only the B peaks (6-B) give equivalent responses as in UV (Fig. 1c-c'). The A peaks might therefore correspond to species containing very few reduced thiols, giving low EC signals but "normal" UV absorbance. In acidic pH and at room temperature, the effect of time (air oxidation) is very striking: there is a decrease, both in UV and in EC, of the B peaks (disappearance of the SH containing isoforms) and an increase of A peaks in UV but not in EC (Figure 5): this being due to the formation of more disulphide containing molecules. The same occurs for RL 1 with its α, β and γ peaks, probably also corresponding to species with few remaining reduced thiols. Compared to RL 2 which does not show any significant changes with pH, time and temperature, RL 1 clearly appears to be much more unstable. A similar observation has also been made previously.[28] The fact that the oxidised forms are eluted earlier than the reduced ones is as expected. It is commonly observed on a reverse phase that, for a reduced (SH)/oxidised (SS) peptide pair, the capacity factor is larger for the reduced form of the peptide. This has been described for small peptides[23,29,30] and for a much larger thiol containing protein like

interleukin-2.[31] This could be attributed to a worse accessibility of the different amino acid residues to the solid phase when the peptide is oxidised (loop formation), therefore decreasing the overall hydrophobicity.

The original polymorphism we found for RL 1 (three isoforms - peaks 1 and 2 with close retention times and 4, more distant, Figure la-a') is in excellent agreement with the results of Klauser et al.[10] for the same MT and with those of Wan et al.[13] for rabbit kidney MT-1. Some other authors also working on rabbit liver MT-1 had found only one major isoform by RP-HPLC at neutral pH[12] or resolved two isoforms only by CZE and by RP-HPLC at acidic pH.[24] We had also previously stated that RL 1 was composed of two unequally abundant isoforms only, but this was found using a neutral buffer and a much shorter RP column.[27]

The chromatogram of the mixture RL1+RL2 (Figure 6) shows a major difference with that of the original RL (Figure 1 c-c')). The discrepancy does not arise from what we consider as being the putative original isoforms of RL (peaks 4 to 7), but from the chemically formed or modified ones, especially peak 3. This peak, which was previously qualified as isolated, has no corresponding peak on the chromatogram of RL1+ RL 2. This means that, from the point of view of the possibility of the various reduced isoforms to form disulphide bonds, there is no perfect reconstruction of RL by adding RL1 to RL 2, once these two have been separated. The primary structure determination of the reduced and oxidised species might provide a key to a more precise comprehension of this behaviour.

It is also important to note that RP-HPLC is capable of directly resolving more than the two main isoforms RL 1 and RL 2 of RL without their prior separation by anion-exchange chromatography, as has been reported.[14,24]

On the whole, this study demonstrates the differences in the degree of redox reactivity of the various metallothioneins, two of them, RL and RL1, being quite sensitive to pH change (neutral to acid).

From an operational point of view, two interesting conclusions may be drawn from this work. Firstly, the trifluoroacetic acid (TFA), although not yet widely used,[10,13,32] proves itself to be an excellent medium for MT isoform separation by RP-HPLC, eliminating peak co-elution that often occurs at neutral pH. Because of the acid pH, there is naturally a substantial decrease of the UV absorbance in the 200-220 nm range due to the dissociation of the metal-thiolate complexes.[33] Nevertheless, working at a detection wavelength of 230 nm, we still achieve UV limits of detection similar to those published by authors using a neutral buffer at 214 nm.[12] Furthermore, TFA is a volatile

buffer, which means that the various MT isoforms (original + modified) can be recovered, for further characterisation, by evaporation of the eluent with no salt formation, therefore, avoiding the problems encountered with phosphate. Secondly, the direct detection of thiols on the type of electrode that we have used does not require as high a potential as 1.0 V, with its inherent disadvantages, as has been stated several times:[34,35] a potential of about 0.6 V gives, in fact, an optimum signal-to-noise ratio, with a baseline reaching stability in a few minutes and a consistently low background current.

Overall, the use of the on-line WV-EC detection system constitutes a very good tool for MT isoform identification. In some cases, like that of RL 1 for instance, the UV mode used alone could overestimate the number of originally reduced isoforms. On the contrary, the electrochemical detection, employed alone, would provide the correct number of thiol containing isoforms (putative real polymorphism), but would not allow one to properly follow the chemical transformations of these species. Only the combination of the two detection modes, the universal UV and the more selective EC, can give access to the double information. To our knowledge, this work is the first one in which this type of electrochemical - coulometric- detection has been employed for MT isoform characterisation.

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