Improved separation of rabbit liver metallothioneins by FPLC-ICP-MS: a comparison with the conventional anion-exchange chromatography

C. Ferrarello, M.R. Fernández de la Campa, H. Goenaga Infante, M.L. Fernández Sánchez and A. Sanz-Medel

Department of Physical and Analytical Chemistry, University of Oviedo, C/ Julián Clavería Nº 8, 33006, Oviedo, Spain

The analytical performance of the Fast - Protein Liquid Chromatography (FPLC) (on a Mono Q HR 5/5 column) and the conventional Anion-Exchange Chromatography (on a Protein Pak DEAE 5PW column) to realise rabbit liver metallothioneins separations and Cd speciation has been investigated and the results are compared. The Mono Q column was found to provide higher resolution and reproducibility in shorter separation times than Protein Pak DEAE 5PW, a conventional anion-exchange column. Thus, FPLC is proposed as a very useful tool for the study of the polymorphism of metallothioneins.

Introduction

Over the last decade, it has been documented that cadmium exposure can cause several health disorders including nephrotoxicity, hypertension, osteomalacia, lung disease and disturbed calcium metabolism [1,2]. Moreover, the International Agency for Research on Cancer (IARC) considers that cadmium and cadmium compounds are potentially carcinogenic to human (Group 1) [3,4].

Metallothioneins (MT) play an important role for heavy metal detoxification, zinc and copper homeostasis, protection against oxidative damage caused by free radicals and as part of the acute phase response to inflammation and stress.

The isoforms of MTs have structural similarity with the same number of cysteine residues and high metal binding affinity but differ in their total charge because of differences in certain amino acids other than cysteine.

The polymorphism occurs during the evolution of the species and consists of the variation of the primary structure by the substitution of 1 to 15 amino acids. Hence, isoforms differ in amino acid composition and consequently, have different isoelectric points and different hydrophobicities. Isoforms with minor differences, such as one amino acid residue, were detected as subgroups of the major isoforms and are termed sub-isoforms [5]. It has been demonstrated that the different isoforms may be regulated by different inducers [6]. Experiments performed on cultured rabbit kidney cell [7] and human cell lines [8] demonstrated that various MT isoforms can be synthesised by distinct inducing factors and may play different cellular roles.

Thus, the development of analytical methods for reliable measurement of the different isoforms of MTs, within a reasonable time, is nowadays urgently needed for many studies requiring MT isoforms information. Similarly, information about binding of toxic metals, *e.g.* Cd, to such MTs isoforms is desirable. That is, cadmium speciation studies in biological materials should be carried out [9]. Probably the coupling a powerful separation technique (*e.g.* chromatographic techniques) with an element specific detector (atomic detector) is the most popular approach for speciation purposes. For unknown species, electrospray ionisation MS studies are opening new exciting possibilities for a more detailed characterisation of metallothioneins in terms of their molecular mass [10].

The use of a quadrupole ICP-MS detector coupled on-line with liquid chromatography is one of the most powerful modern tools for studying protein binding biometals in biological fluids. Thus, in our laboratory the determination of Cd-MT in human urine has been carried out by using a Protein Pak DEAE 5PW anion-exchange column coupled to ICP-MS via a vesicular hydride generation interface [11]. This hybrid technique was assessed for the cadmium speciation in standards of rabbit liver MT. However, such methodology has shown to provide poor resolution and reproducibility and rather long separation time. Therefore, the main objective of the present study has been to investigate the potential use of Fast Protein Liquid Chromatography (FPLC) on a Mono Q HR 5/5, previously used for trace element speciation of proteins in biological material [12-15], for the separation of such cadmium binding proteins.

The performance of the two anion exchange columns (Protein Pak DEAE 5PW and Mono Q HR 5/5) for the

Article available at http://analusis.edpsciences.org or http://dx.doi.org/10.1051/analusis:2000280351

separation of closely related MT isoforms and speciation of cadmium, using an HPLC-ICP-MS set-up, is discussed in a comparative manner.

Experimental

Instrumentation

Gradient anion-exchange HPLC (AE-LC) was performed with two LKB (Browman, Sweden) high-pressure pumps equipped with a model 2152 gradient controller and a Rheodyne model 7125 sample injector valve (Berkeley, CA, USA) fitted with a 50 µL loop. Analytical anion–exchange columns were a Protein Pack DEAE 5PW (75 × 7.5 mm id.) (Waters, Milford, MA, USA) and a Mono Q HR 5/5 (50 × 5 mm id.) (Pharmacia Biotech, Uppsala, Sweden). A scavenger column (25 × 0.5 mm id.) was placed between the pumps and the injection valve to remove exogenous metal ions, coming from the mobile phases used. This column was laboratory packed with Kelex-100 impregnated silica C18 material, prepared as described previously [16]. A glass column (100 × 1 cm id.) packed with Sephadex G-75 (Pharmacia Fine Chemical) was used to perform Size-Exclusion Chromatography (SEC) on real sample analysis. A LKB model 2151 variable wavelength detector provided with a 10 µL flow cell was used for absorptiometric measurements and a LKB model 2221 recording integrator for signal processing and readout of absorptiometric measurements.

Discontinuous ETAAS determination of Cd in column fractions was carried out using a Perkin Elmer model 3030 Atomic Absorption Spectrophotometer (Norwalk, CT, USA) with a model HGA–500 Graphite Furnace, equipped with a model AS – 40 autosampler and a PR-10 printer.

For ICP-MS measurements, a Hewlett Packard model 4500 Instrument (Yokogawa Analytical, Tokyo, Japan) equipped with a Meinhard nebulizer for conventional measurements was used.

Reagents

Metallothionein 1 (MT-1) and metallothionein 2 (MT-2) standards (each nominally 5.7 % m/m Cd and 0.7 % m/m Zn, isolated from rabbit liver and essentially salt-free), Tris (hydroxymethyl - aminomethane), ammonium acetate and standard proteins used to the SEC column molecular calibration were purchased from Sigma (St. Louis, MO, USA).

Kelex-100 cationic exchanger (Schering España, Madrid, Spain) was used as received and the bonded silica reversed-phase sorbent with octadecyl functional groups (C18) was purchased from Waters (Milford, MA, USA). Metallothionein isoform solutions were prepared by dissolving standard rabbit liver MT-1 (1 mg.mL^{-1}) and MT-2 (1 mg.mL^{-1}) in 2 mM Tris/ HCl buffer (pH 7.4).

Mobile phases for the Anion-Exchange Liquid Chromatography experiments were detailed in table I (degassed with helium for 30 min before use in all cases).

All mineral acids and metal salts used were of analytical reagent grade and Milli-Q purified water (Millipore, Molsheim, France) was used throughout.

Procedures

Conventional anion-exchange chromatography procedure

A 50 µL of a rabbit liver MT-1 and MT-2 mixture (dissolved in 2 mM Tris/HCl at pH 7.4 to obtain 150 µg.L^{-1} of total Cd) was injected. The separation and elution of Cd species was achieved by using a concentration gradient 2mM – 200 mM of Tris/HCL buffer (optimum conditions for the chromatographic separations are given in table I). The effluent from the analytical column emerging at 1 mL.min^{-1} and was directly connected to the ICP-MS nebulizer and the corresponding on-line chromatograms were recorded. Assays were made by triplicate.

After each chromatographic analysis, buffer A was switched and passed through the column for 15 min for reconditioning before the next injection.

Optimum instrumental parameters for on-line Cd detection by ICP-MS are detailed in table I. Mass 111 was selected because the determination of the most abundant Cd isotope, ^{114}Cd can be overlapped by the presence of ^{114}Sn. Data acquisition in the "Time resolved analysis mode" of the HP 4500 instrument was selected with an integration time of 1.5 s per peak.

FPLC procedure

The separation and elution of Cd species by FPLC-ICP-MS was achieved by using a gradient concentration of 4 mM Tris/HCl, pH 7.4 (buffer A) and 10 mM Tris/HCl + 250 mM ammonium acetate, pH 7.4 (buffer B). Optimum conditions for this particular chromatographic separations are also given in table I. Of course, with the exception of column and mobile phases (with the gradient elution) the general procedure, number of assays and instrumental set-up were identical to those described above.

ETAAS was used as reference specific technique in order to validate the ICP-MS results obtained for Cd speciation using the Mono Q HR 5/5 column: 50 µL of aliquots of the respective rabbit liver MT isoform (400 µg.L^{-1} of Cd) were chromatographed (using the experimental conditions given in table I) while fractions of 500 µL of the effluent were collected with an automatic fraction collector LKB, FRAC-100 (Pharmacia, Browman, Sweden) in Cd-free autosampler cups. Then, each fraction was analysed in the graphite furnace AAS instrument using the operating conditions detailed in previous work [17]. Cd calibration graphs were constructed with aqueous standards as no matrix effects were observed for the determination of Cd-MT in 250 mM ammonium acetate + 10 mM Tris/HCl (corresponding to elution

Table I. Chromatographic conditions for cadmium speciation by Anion – Exchange Chromatography - ICP-MS.

Characteristics	*FPLC*		*Conventional anion-exhange*	
Column:	Mono Q HR 5/5		Protein Pack DEAE 5PW	
Injection loop:	50 µL		50 µL	
Flow rate:	1 mL.min^{-1}		1 mL.min^{-1}	
Eluents:	(A) 4 mM Tris/HCl (pH 7.4) (B) 250 mM ammonium acetate +10 mM Tris/HCl (pH 7.4)		(A) 2mM Tris/HCl (pH 7.4) (B) 200 mM Tris/HCl (pH 7.4)	
Gradient elution:	time (min)	B (%)	time (min)	B (%)
	0	0	0	0
	1.5	2	10	15
	2	3	15	25
	5.5	4	20	50
	6	10	21	100
	10	17	30	99
	11	18	35	0
	12	99		
	19	100		
	23	0		
	ICP-MS detection: Spray chamber temperature: 4 °C Rf power: 1300 W Carrier Ar flow rate: 1.2 L.min^{-1} Sampling depht: 5.9 mm Data adquisition: mass ^{111}Cd Points per peak: 1 Integration time: 1.5 s per peak			

with 100 % of mobile phase B). The total content of Cd in MT-1 and MT-2 had also been previously determined by conventional ETAAS in the original MT standard solutions.

Animals

A 50 specimens of mussel Mytilus edulis were obtained from a wild coast in Asturias, Spain. Fifteen mussels (blank) were directly subjected to cytosol preparation. The remaining mussels were transferred to an aquarium tank containing 50 L of continuously aerated seawater. Water was maintained at 20 ºC and changed every day. A spike of cadmium ($CdCl_2$; Merck, Darmstadt, Germany) to achieve a final concentration of 500 µg.L^{-1} was daily added in order to induce the metallothionein-like proteins (MLPs) synthesis. Eleven days later the specimens were removed from the aquarium, rinsed thoroughly with Milli-Q water and subjected to cytosol preparation.

Cytosol preparation

Mussel cytosolic extracts were obtained based on a modified protocol of the methods of Roesijadi and Fowler [18] and Lobinski *et al.* [19]. Firstly, the mussels were subjected to dissection of their hepatopancreas. From that pool of hepatopancreas, 5 g of such wet tissue were taken to be homogenised in 5 mL of buffer solution: 10 mM Tris/HCl, pH 7.4; 5 mM 2-mercaptoethanol – 2-MCE - (Merck, Darmstadt, Germany); 0.1 mM phenylmethylsulphonyl fluoride – PMSF - (Sigma, St. Louis, MO, USA); 25 mM NaCl (Suprapur Merck, Darmstadt, Germany) using an Ultraturrax T25 (Antrieb, Janke and Kinkel, Ika Labortechnik): 13.000 rpm; 5 min; 4 ºC. The tissue debris was removed by centrifugation with a Centrikon T-1180 (Kontron Instruments): 26.000 rpm; 30 min; 4 ºC. The supernatant liquid was heated at 60 ºC for 15 min and then centrifuged (same conditions as above). The supernatant (cytosol) was collected and stored at –20 ºC.

Size-Exclusion Chromatography procedure

Preparative scale Size-Exclusion Chromatography using Sephadex G-75 was carried out with 10 mM Tris/HCl, pH 7.4, 5mM 2-MCE, 0.1 mM PMSF and 25 mM NaCl (gassed with N_2) at a flow rate of 0.2 mL.min^{-1}.

The column calibration was carried out using Cytochrome C (12.4 KDa), Lysozyme (14.4 KDa), Carbonic anhidrase (29 KDa) and Albumin (66 KDa).

An 1 mL aliquot of cytosol was applied directly to the Size-Exclusion column. Forty fractions of 3 mL were collected with the automatic fraction collector. Cd-thiolate bonds molecular absorbance (254 nm) and cadmium levels were measured on each fraction using UV-Vis Spectrophotometry and ICP-MS detection, respectively. The chromatographic fraction with highest values for Cd and

Cd-thiolate was subjected to speciation by FPLC with ICP-MS and UV-Vis molecular absorption detection.

Results and discussion

For metal binding protein speciation studies, the different equilibrium processes, the possible presence of other metal ions or ligands, microbial contamination and non-biotic parameters (pH, temperature, etc.) must be taken into account in order to prevent changes in the *in vivo* speciation of Cd. In this line, all these variables were controlled and optimised. A special emphasis was put on the pH regulation because of its dramatic influence on the separation of protein isoforms. Therefore, we studied the separation of the two rabbit liver MT isoforms at various pH values between 7.2 – 8.0 (at such pH values the expected order of elution from both anion – exchange columns should be MT-1, charge –2 followed by MT-2, charge –3). A physiological pH of 7.4 was selected for their final speciation.

Cd speciation in standard rabbit liver MTs by FPLC-ICP-MS

A standard mixture of rabbit liver MT-1 and MT-2 isoforms (150 μg.L^{-1} of total Cd) was used to investigate the Cd-MT speciation by FPLC-ICP-MS applying different gradients and the aid of different salts used in the mobile phase. As previously noticed [12], ammonium acetate was found to be the most appropriate eluent for ICP-MS detection. It presents low levels of metal ion impurities and prevents salt deposition and clogging in the sampling cone of the ICP-MS. An adequate separation of MT-1 and MT-2 was achieved under the selected conditions described in table I. The observed elution profile of ^{111}Cd (for 50 μL of a MT-1 and MT-2 mixture containing 150 μg.L^{-1} of total Cd) is given in figure 1. The chromatogram shows that at least four main Cd-MT fractions were observed for MT-1 (retention times (t_R) = 5.8; 8.17; 8.85; 10.3 min), while three fractions are apparent for MT-2 (t_R = 11.5; 12.8; 15.2 min) A small peak appearing at t_R = 1.3 min was traced back to inorganic cadmium (not bonded to the protein).

Cd speciation in standard rabbit liver MTs by conventional anion-exchange chromatography-ICP-MS

A standard protein mixture of rabbit liver MT-1 and MT-2 isoforms (150 μg.L^{-1} of total cadmium) was used to investigate Cd speciation under different elution gradients. MT-1 was efficiently separated of MT-2 under the conditions described in table I. The observed elution profile of ^{111}Cd is given in figure 2. Three fractions were observed for MT-1 (retention times of 3.2¸ 5.6 and 9.3 min) and only two fractions for MT-2 (t_R = 16.7 and 24 min). A broad Cd elution peak at t_R = 26 min was also observed (see Fig. 3b). This last fraction might represent inorganic cadmium (not bound to proteins). In order to verify the identity of this peak, aliquots of 50 μL of 5, 10, 15, and 20 μg.L^{-1} Cd (II) aqueous standard were injected. It was observed that there was a proportional increase of the Cd ICP-MS signal at t_R = 26 min with increasing cadmium concentration. Therefore, the observed peak could be explained by exogenous free cadmium contamination of the mobile phases [11].

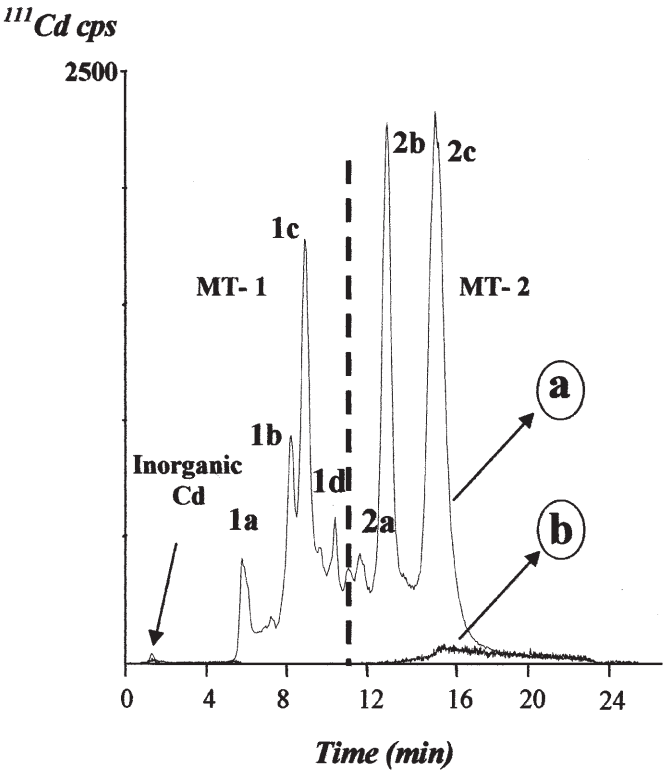

Figure 1. Mono Q HR 5/5–ICP–MS profiles obtained for 50 μL of (a) MT-1 and MT-2 isoforms mixture (150 μg.L^{-1} of total cadmium); (b) mobile phase (blank).

Analytical performance of both anion-exchange columns

Following our objective of comparing the performance of both columns for the separation of MT isoforms we have resumed their characteristics in tables II and III. Results show that, as compared to Protein Pak DEAE 5PW (*e.g.* Cd-MT elution profiles in figures 1 and 2), the Mono Q column was able to separate more fractions of each isoform, with narrower peaks and in shorter time. Table II summarises the retention times observed for the different rabbit liver Cd-MT fractions obtained by FPLC-ICP-MS and conventional AE-LC–ICP-MS. As it can be observed, the retention times obtained with Mono Q HR column exhibit slightly better reproducibility than that obtained with Protein Pack DEAE 5 PW column for MT-2 isoforms. Moreover, figure 2a shows clearly that with Protein Pack DEAE 5 PW column the baseline was not recovered. This may be attributed to gradual adsorption of the mobile phase impurities on the column, which were eluted by the increasing ionic strength of the mobile phase in the gradient. In this way, the ^{111}Cd signal should increase with increasing buffer B

Table III. Comparative retention times of the rabbit liver MT fractions obtained by using two different anion-exchange columns coupled to ICP-MS detection.

MT isoform	*Mono Q HR 5/5* Fraction	t_R (min)[a]	*Protein Pak DEAE 5PW* Fraction	t_R (min)[a]
MT-1	1 a	5.8 ± 0.09	1 a	3.2 ± 0.01
	1 b	8.1 ± 0.07	1 b	5.6 ± 0.06
	1 c	8.8 ± 0.08	1 c	9.3 ± 0.06
	1 d	10.3 ± 0.05	---	---
MT-2	2 a	11.5 ± 0.06	2 a	16.7 ± 0.09
	2 b	12.8 ± 0.04	2 b	24.0 ± 0.1
	2 c	15.2 ± 0.03	---	---

(a) Precisions calculated for three independent chromatograms.

concentration. In contrast, baseline was always recovered when using the Mono Q column and the t_R of inorganic cadmium was coincident with the void time (Fig. 1a). Furthermore, ammonium acetate was proved to contain low levels of Cd impurities (see the buffer profile in figure 1b).

Speciation of rabbit liver MT-1 and MT-2 by FPLC with off-line ETAAS determination

Several fractions of 0.5 mL were automatically collected from the FPLC system, under conditions of table I and were analysed by ETAAS in a clean room in order to check cadmium fractions binding MT-1 and MT-2. Assays were made by triplicate. Measurements of Cd on each chromatographic fraction were carried out by ETAAS following conditions given in previous work [17], as a robust, well established technique for such determination. As the analyte is diluted by the mobile phase, higher Cd concentration (400 $\mu g.L^{-1}$) were used to guarantee reliable Cd detection by ETAAS. Recoveries of 99.7 ± 0.9 % and 100.3 ± 1.1 % were obtained for Cd-MT-1 and Cd-MT-2, respectively. Similar quantitative recoveries of cadmium present were found using the conventional anion-exchange column, 95.3 ± 4.9 % for Cd-MT-1 and 109 ± 5.1 % for Cd-MT-2. These data indicate that

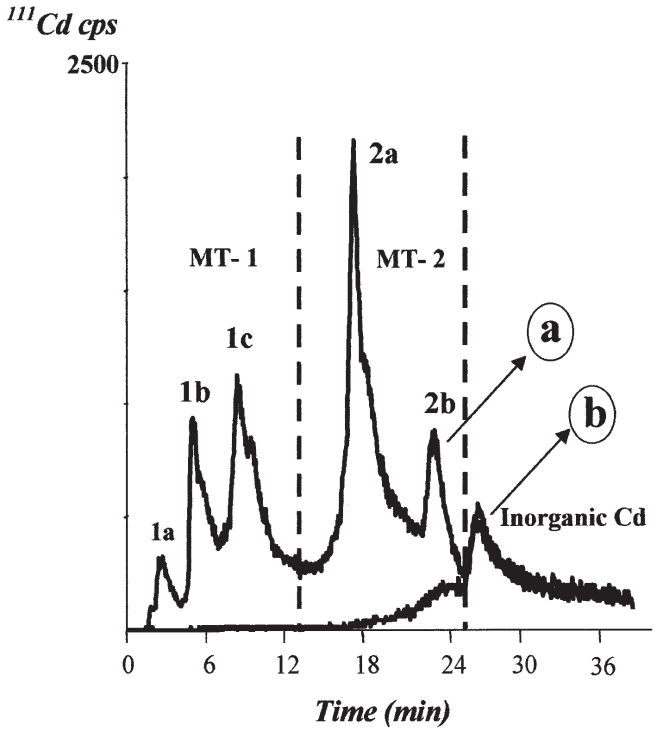

Figure 2. Protein Pack DEAE 5 PW–ICP–MS profiles obtained for 50 μL of (a) MT-1 and MT-2 isoforms mixture (150 $\mu g.L^{-1}$ of total cadmium); (b) mobile phase (blank).

Cd is completely bound to the proteins in the standards and that it is not adsorbed by the columns.

Real sample analysis

In the light of the results, Mono Q HR 5/5 column provided better resolution, reproducibility and lower analysis time than the Protein Pack DEAE 5 PW. Thereafter, FPLC (coupled to ICP-MS or UV-Vis Spectrophotometer) was selected to be applied to the analysis of real sample.

To do so, specimens of the mussel Mytilus edulis were exposed to cadmium chloride in an aquarium tank, in order

Table II. Comparative features of Protein Pak DEAE 5PW and Mono Q HR 5/5 anion-exchange columns.

Properties	*Mono Q HR 5/5*	*Protein Pak DEAE 5PW*
Column-size	5 cm × 5 mm (id)	7.5 cm × 7.5 mm (id)
Particle size	10 μm	10 μm
Support material	Beaded hydrophilic resin	Polymethyl metacrylate
Charged group	$-CH_2N^+(CH_3)$	$-CH_2-CH_2-N(CH_2-CH_3)$
Maximum flow rate	2.0 $mL.min^{-1}$	1.2 $mL.min^{-1}$
Protein capacity	20 – 50 mg/ column	50 – 75 mg/ column

to induce the metallothionein-like proteins (MLPs) synthesis. Cd-exposed mussels and control unpolluted mussels "blank" were processed to obtain the cytosol (see protocol described above). Separate 1 mL aliquots of such cytosolic extracts were directly injected into the SEC column. A single broad peak of cadmium for Cd-exposed mussels, with a metal concentration 32-times higher than that of control mussels was observed. Subsequently, a 50 µL aliquot of the fraction (of such peak) showing the highest ICP-MS values for cadmium and UV-Vis molecular absorbance (254 nm) was used for speciation by FPLC with ICP-MS and UV-Vis Spectrophotometric detection, respectively. Figure 3 shows the profiles observed of Cd-MLP ICP-MS and the corresponding UV absorbance at 254 and 280 nm. Two sharp Cd peaks appearing at retention times of 15.6 ± 0.03 and 16.1 ± 0.02 min should probably indicate the presence of MLP-1 and MLP-2 mussel isoforms, respectively. The observed absorbance profile at 254 nm (due to the Cd-thiolate complex) was coincident with that of Cd-MLP by ICP-MS. Moreover, no absorption at 280 nm (see Fig. 3c) was noticed for those retention times. This is also supporting the MT-like identity of the mussel protein isoforms separated by FPLC (as such proteins do not have aromatic aminoacids).

Our results are similar to those previously found in Zebra mussels by High *et al*. who used a TSK-Gel DEAE 5PW, a conventional anion-exchange column, except that in their work MLP-1 and MLP-2 isoforms were found to appear at retention times longer than 30 min [20].

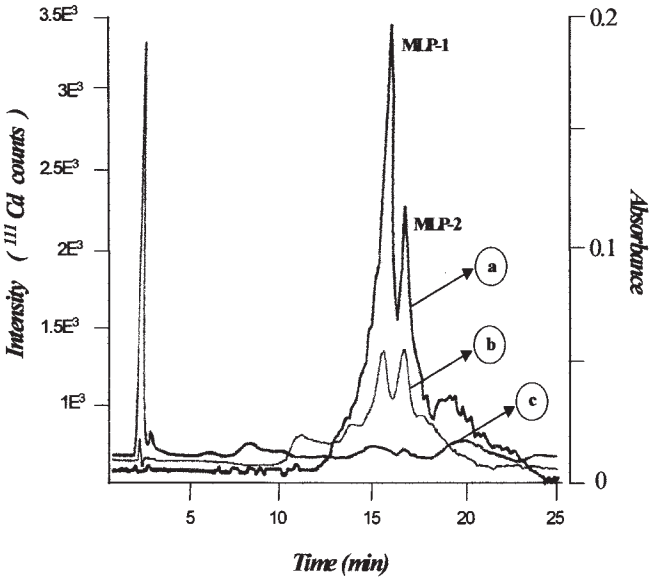

Figure 3. Composite chromatograms of the FPLC separation of mussel MLPs isolated by SEC. (a) ICP-MS signal of ^{111}Cd obtained by on-line FPLC-ICP-MS. (b) and (c) molecular absorbance at 254 and 280 nm, respectively, performed by on-line FPLC-UV-Vis Spectrophotometer.

Conclusions

Results demonstrate that the Mono Q HR 5/5 column is superior to the Protein Pak DEAE 5PW column in terms of better resolution, reproducibility and analysis time for the separation of MT isoforms. FPLC has proved to be suitable for studying speciation of metal-proteins in biological fluids [12-15]. However, this column has been scarcely used for speciation studies of metallothioneins [21], in spite of the fact that it provides narrow sharp-pointed peaks in shorter time and better resolution than conventional anion-exchange columns. These advantages are usually explained by the nature of its hydrophilic resin packing which has one of the narrowest particle size distribution available. The bead monodispersity and the absence of fines gives the packed column large void volume (40 %) and, therefore, low back-pressure and faster chromatographic runs.

Moreover, FPLC on a Mono Q HR 5/5 anion-exchange column coupled to ICP-MS has proved to be an excellent tool for studying Cd-MT speciation. It has been shown that this column allows rapid separation and reproducible profiling of the main isoforms of rabbit liver Cd-MT and mussel hepatopancreas Cd-MLP at a physiological pH of 7.4. Therefore, FPLC can be considered more than a simple alternative to conventional anion-exchange chromatography. It could prove to be a helpful separative tool to be coupled to atomic detectors in order to investigate problems requiring efficient separation of MTs isoforms. FPLC could become a powerful allied of reverse-phase chromatography [6,22-24] for the study of the polymorphism of metallothioneins.

Acknowledgements

The authors thank the Instituto de Cooperación Internacional, Madrid (Spain) for awarding a doctoral grant to H. Goenaga and C. N. Ferrarello and they also acknowledge financial support from DGICYT, Spain, through project number PB – 94 –1331.

References

1. Nordberg, G.F. *Environmental Sciences* **1996**, *4*(3), 133-147.
2. Nordberg, M. *Talanta* **1998**, *46*, 243-254.
3. Nordberg, M.; Nordberg, G.F. *Environmental Sciences* **1996**, *4*(3), 187-197.
4. Nordberg, G.F.; Kjellstrom, T.; Nordberg, M. In *Kinetics and Metabolisms Cadmium and Health: A toxicological and Epidemiological Appraisal;* Friberg, L; Elinder, C.G.; Kjellstrom, T. and Nordberg, G.F., Ed.; Boca Ratón: CRC Press, Fl, 1985; pp 103-178.

5. Stillman, M.J.; Shaw, C.F. Suzuki, K.T. In: *Metallothiuoneins: Synthesis, Structure and Properties of Metallothioneins, Phytochelatins and Metalthiolate Complexes;* Stillman, M.J.; Frank Shaw, C.; Suzuki, K.I., Ed. ; New York: VCH, 1992.
6. Karin, M; Richards, R.I. *Environ. Health Perspect.* **1984**, *54*, 111-116.
7. Wan, M.; Hunzinker, P.E.; Kägi, J.H.R. *Biochem. J.* **1993**, *292*, 609-615.
8. Hammer, D.H. *Ann. Rev. Biochem.* **1986**, *55*, 913-917.
9. Sanz-Medel, A. *Spectrochim. Acta, part B* **1998**, *53*, 197-211.
10. Chassaigne, H.; Lobinski, R. *Fresenius J. Anal. Chem.* **1998**, *361*, 267-273.
11. Goenaga Infante, I.; Fernández Sanchez, M.L.; Sanz-Medel, A. *J. Anal. At. Spectrom.* **1999**, *14*, 1343-1348.
12. Soldado Cabezuelo, A.B.; Montes Bayón, M.; Blanco González, E.; García Alonso, J.I.; Sanz-Medel, A. *Analyst* **1998**, *123*, 865-869
13. Tomono, T.; Ikeda, H.; Tokunaga, E. *J. of Chromatography* **1983**, *266*, 39-47.
14. Soldado Cabezuelo, A.B.; Blanco González, E.; Sanz-Medel, A. *Analyst* **1997**, *122*, 573-577.
15. Montes Bayón, M.; Soldado Cabezuelo, A.B.; Blanco González, E.; García Alonso, J.I.; Sanz-Medel, A. *Analyst* **1999**, *14*, 947-951.
16. López García, A.; Blanco González, E.; Sanz-Medel, A. *Mikrochim. Acta* **1993**, *112*, 19-29
17. Goenaga Infante, I.; Fernández Sanchez, M.L.; Sanz-Medel, A. *J. Anal. At. Spectrom.* **1996**, *11*, 571-575
18. Roesijadi, G.; Fowler, B.A. In *Methods in Enzymology*, Riordan, J.F.; Vallee, B.L. Ed.; San Diego: Academic Press, 1992. Vol. 205.
19. Lobinski, R; Chassaigne, H.; Szpunar, J. *Talanta* **1998**, *46*, 271-289.
20. High, K.A.; Barthet, K.J.; McLaren, J.W.; Blais, J.S. *Environ. Toxicol. and Chem.* **1997**, *16* (6), 1111-1118.
21. Olsson, P.E.; Hogstrand, C. *J. of Chromatography* **1987**, *402*, 293-299.
22. Chassaigne, H.; Lobinski, R. *Analusis* **1998**, *26* (6), 48-51.
23. Chassaigne, H.; Lobinski, R. *Analusis* **1998**, *26* (6), 65-67.
24. Chassaigne, H.; Lobinski, R. *Fresenius J. Anal. Chem.* **1999**, *363*, 522-525.