The coupling of size-exclusion HPLC with ICP-MS in bioinorganic analysis

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Size-exclusion chromatography (SEC) with on-line detection by ICP-MS appears to be the primary technique that allows the detection of the presence of metals bound to macromolecular ligands in an unknown sample. It enables the obtention of information on the distribution of the metal(s) present in the sample among the fractions of different molecular weight with detection limits down to 1 ng mL^{-1} for most elements. Some applications in the fields of the plant, animal and clinical biochemistry are presented.

The detection of a metal compound in a sample is the prerequisite of any further action concerning its identification, characterisation and the role in biochemistry. The actual problem can be reduced to proving that the analytical signal detected by the spectrometer is due to the presence of a metal compound and not to that of a simple ion. This can be done by preceding the detection by a separation technique that would differentiate between the free metal or metalloid ion and the same element bound with a larger, usually macromolecular structure.

The detection of the presence of metal complexes with biological macromolecules has commonly been realised by ultrafiltration using a filter with a small cut-off molecular mass, usually 500 − 5 000 Da. The concentration of the element of interest was determined in the initial sample and in the filtrate, usually by graphite furnace AAS; the metal retained was considered to be bound to a macromolecular species. A refinement of this technique by the successive ultrafiltration through membranes with molecular weight cut-offs of 500, 5 000 and 30 000 Da has widely been used to study the distribution of metal-species as a function of the molecular weight [1-4]. The method allows only a rough speciation and is time consuming.

An improvement in terms of the resolution can be obtained by the use of size-exclusion chromatography which also seems to be less cumbersome and faster allowing the on-line detection to be used. Because the resolution of SEC is insufficient for the discrimination of the small aminoacid heterogeneities, the coupling SEC-ICP-MS is the most popular technique for the first screening of an unknown sample in view of the presence of macromolecular species of elements. SEC using a short guard column with ICP-MS detection allows the rapid quantification of the bound metal fraction by comparison of the chromatographic signal with that, obtained in parallel, by flow-injection ICP-MS analysis of the sample.

This paper presents the technique, reviews its applications and shows some recently established potential in the fields of the plant, animal and clinical biochemistry.

Size-exclusion chromatography in bioinorganic analysis

Size-exclusion chromatography (SEC) is based on the molecular sieve effect and enables species to be separated according to their size, and to a lesser extent, shape. The average time a substance spends in the pores is determined by its size which for a given shape, can usually be related directly to its molecular weight. The following conditions have to be taken into consideration for separation of biological constituents containing metals: interactions between metals and buffers and chromatographic support material should be minimized, and the pH of the eluent should be weakly alkaline to prevent the metals from dissociating.

Packing

Separation by SEC should be independent of the analyte's charge but in practice the stationary phase surface displays charged properties so that a mixed mode separation is observed. This makes the choice of packing critical. The two categories of packing used included silica and organic polymers. At the nanogram metal levels, significant silanophilic effects are involved including metal losses in the presence of low ionic strength eluents on silica-based SEC supports [5-7]. A copolymeric styrene-divinylbenzene SEC support which provided a symmetrical peak with negligible losses of Cd during chromatography was proposed [5,6]. The average pore size of the packings used varies from 100 to 1 000 Å. Standard columns used include 80 kDa dead volume. For a detailed human milk speciation, especially for Se species, a separation range up to 250 kDa should be used [8]. The combination of two SEC columns provides a wider molecular range [8].

Mobile phase

The optimum eluent should assure the minimum competition between buffer and cytosolic ligands, and between these ligands and the gel. The separation by SEC with H_2O as mobile phase prevented structural changes, denaturation of proteins and destruction of protein-metal complexes [9]. In practice, various aqueous mobile phases of fairly high ionic strength have been used to avoid interactions with the packing material. Dilute buffers, in general, may cause adsorption of low molecular weight proteins by the column packing. When silica-based packings cannot be avoided, the addition of a non-complexing salt (e.g. 0.1 M NaCl) to the

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mobile phase was shown to suppress the residual silanol activity of the column packing [7]. With such conditions no significant exchange of Cd occured but occasional (for Hg) or appreciable losses (for Zn) were observed for other metals [7]. pH of the mobile phase should be close to that of a sample, e.g. 7.2 for tissue cytosols [6,11–18], 5.5 for tea infusions [19,20], and 5.8 for wine [21].

An addition of EDTA proposed by some authors to minimize metal ion-gel interactions [10] was found unsuitable by others [22] because of the occasional presence of anomalous Cd peaks in subsequent runs. Polymeric supports suffer from the deposition of excess free metal ion which interacts with the analytes, often causing severe degradation in peak resolution [6]. Because the weak complexing character of Tris is not sufficient to compete with the polymeric support for metal, the use of a stronger complexing agent, e.g. b-mercaptoethanol for Cd^{2+} was advised [6]. An addition of 0.03% NaN_3 as a retardant of bacterial activity is advised to protect the column from damage resulted from bacterial growth when real-world samples are analysed. The coinjection of a complexing agent was essential to suppress free Cd^{2+}- support interactions that otherwise induced an undesirable metal-affinity retention mechanism [6].

An extensive column clean-up procedure and a new guard column for each injection may be necessary [22]. Adsorption of organic material from tea infusions on the column required a cleaning step after each injection [19]. The interpretation of data from SEC is limited as molecular markers are primarily globular proteins. As the column elutes on the basis of size rather than M_r, species with a non-globular shape elute earlier than equivalent mass globular proteins [22].

Analysis time

This is a function of the column size and the flow rate. Although the columns up to 120 cm were occasionally used the standard 30 cm column is a good choice. At a flow rate of 1 mL min^{-1}, the separation requires c.a. 20 min to complete. The separation of MT-bound and unbound cadmium within 3 min on a 4.0 cm SEC column was reported [17].

The coupling of size-exclusion chromatography with ICP-MS

Advantages of the ICP-MS detection, besides its high sensitivity, include the detection of several elements in one run and the possibility of the use of stable isotopes instead of radioactive ones.

For multielement SEC-HPLC-ICP-MS, to ensure acceptable sensitivity the elements of interest were divided into groups, within each group the replicate time, dwell time and number of sweeps per reading were optimized, a separate chromatographic injection was made for each group [19].

Eluents should not contain elements that give polyatomic interfering ions in an ICP. The wide variety of buffers reported in the literature makes it relatively easy to chose one readily tolerated by an ICP. Up to 30 mM Tris-HCl formate or acetate buffer is the most common choice. The standard flow rate of 0.7 − 1.0 mL/min is compatible as well with most of the sample introduction systems of the ICP-MS instruments. The interface based on a low flow rate direct injection nebulizer has been also reported [23].

In many applications SEC-HPLC-ICP-MS is used as a semi-quantitative technique used to monitor relative changes of analytical signals in a well defined series of samples. Quantification of signal obtained is usually done by a peak area calibration either by changing the measurement system into the flow-injection mode after completing the chromatographic run [21,24] or using a calibration graph if standards are available [6,17].

One of major problems in the analysis of real world samples is the unavailability of standards. Only a very restricted number of standards (e.g. serum proteins, metallothioneins) can be used for peak identification. In most applications further signal characterization by orthogonal (complementary) chromatographic techniques is necessary.

Areas of applications

Applications of size-exclusion chromatography coupled to ICP-MS to the analysis of metal and metalloid complexes in biological samples are summarized in table I.

Biomedical

Metalloproteins binding essential and heavy metals in serum [23,26] and in erythrocytes [26,27] have been the most widely studied targets. Availability of many serum proteins standards makes peak identification possible for some elements (e.g. Fe, Cu). However, full identification of Se peaks was not possible, several of them were assigned from their molecular sizes in the Se profiles [11–14]. Understanding the Al neurotoxicity makes the identification of Al species in neuroblastoma model cells important (see Zatta et al., this issue). A low molecular compound containing Al was found after long term incubation with inorganic aluminium (Fig.1). The speciation of some non-metals in serum samples, e.g. iodine (Fig. 2) is also possible with ICP-MS.

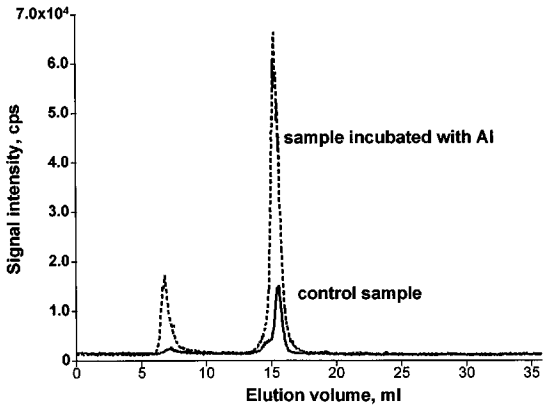

Figure 1. SEC-HPLC-ICP-MS chromatogram of Al in cytosol of neuroblastoma cells. Superdex-75 HR (30 cm × 10 mm × 13 μm), eluent: 30 mM Tris-HCl buffer, pH 7.2.

ICP-MS is able to analyse individual isotopes which enables the use of enriched stable isotopes as tracers in metabolic studies, HPLC-ICP-MS with enriched stable isotopes is a unique analytical method by which speciation of both endogenous elements and external tracers can be achieved in a single experiment, this method has been used with a ^{82}Se tracer; it allows the simultaneous differentiation of the fate of injected and endogenous Se and if it was influenced by the previous Se burden [11,13,14].

SEC-HPLC-ICP-MS is a fast method allowing the evaluation of the kinetics of metallodrug binding to individual proteins and serum. This is required for: (i) estimation of desactivation of the drug (e.g. cisplatin in serum, Fig. 3), (ii) estimation of transport efficiency (e.g. complex of Ru(III) drugs with apotransferrin), (iii) finding suitable "release agents" for metallodrugs bound to serum proteins (e.g. citrate, EDTA, tartrate). The biologically active (e.g. anticancer) species is usually an unchanged metallodrug (e.g. cisplatin or carboplatin) that shows no significant antitumour activity after being bound to a plasma protein (see Pieper and Keppler, this issue). However, some ruthenium-based antitumour drugs (e.g. trans-HInd($RuInd_2Cl_4$)) do not lose their antitumour activity and the transferrin-bound species exerts a considerably higher inhibitory effect compared to a free complex. To show an antitumor activity, the complexes must be released inside the cells. The addition of chelating agents such as citrate, EDTA, ATP to the metallodrug-plasma protein adduct leads to a new "free" metallodrug [28].

Foodstuffs

Research in this area has been triggered by health concerns, applications published so far have concerned the bioavailability of toxic elements such as lead, aluminium and cadmium. Some of these studies involve the use of simulated gastro-intestinal digestion procedures [20,22], cooking [22] or enzymolysis [29] to monitor the changes in the chemical speciation of cadmium [22,30], zinc [31], aluminium [20] and lead and REE [29]. Speciation study of 24 elements in tea leaves and tea infusion [19] was presented.

The relatively high concentration of Al in tea has been the subject of concern, since for many people tea is the major source of Al in the diet; the exctretion of Al is fairly effective, however, toxic effects can occur for patients with chronic renal failure [19,20]. It was assumed that the metal binding ligands in tea infusion are large polyphenolic compounds occuring widely in tea and other plants [19].

Speciation of lead in plants, vegetables and wine; lead was found to be associated with one major macromolecular (5 − 15 kDa) species and with one to three minor compounds depending on the wine sample. The dominant species which accounted for 40 − 85% of lead was identified as the lead complex with the dimer of a pectic polysaccharide (Rhamnogalactorunan II) (see Pellerin and O'Neill, this issue). Other lead species found in wine which showed the molecular weight in the range 1 000 − 3 000 Da were not identified [21]. Figure 4 shows a typical multielemental chromatogram of a wine sample.

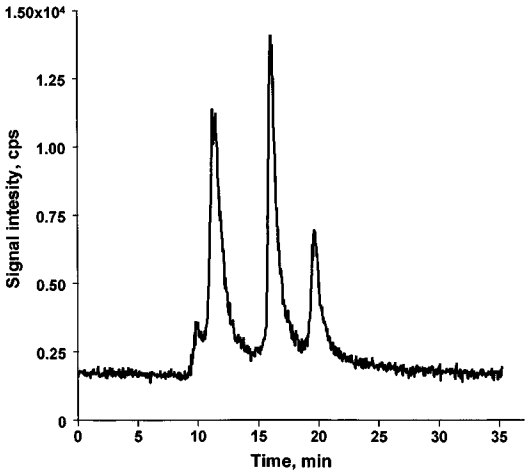

Figure 2. SEC-HPLC-ICP-MS chromatogram of iodine in human serum, Progel TSK (30 cm × 7.8 mm × 4 μm), eluent: 30 mM Tris-HCl buffer, pH 7.2.

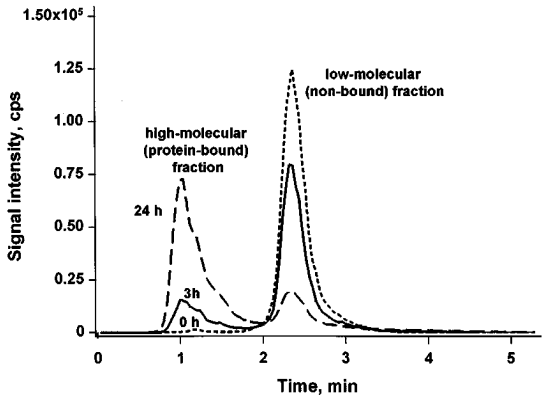

Figure 3. Kinetics of binding of cisplatin to serum proteins, Progel TSK (4 cm × 6 mm × 6 μm), eluent: 30 mM Tris-HCl buffer, pH 7.2.

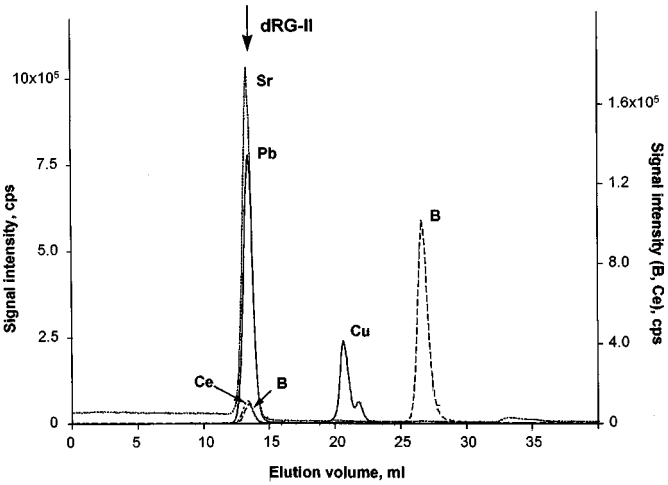

Figure 4. Multielement SEC-HPLC-ICP-MS speciation in wine (red Touraine wine, France). Experimental conditions: Superdex-75 HR (30cm × 10 mm × 13 μm), eluent: 30 mM formate buffer, pH 5.8. dRG-II denotes a dimer of ramnogalacturonan-II.

Table I. Applications of size-exclusion chromatography with ICP-MS detection.

Sample	*Column*	*Eluent (flow rate)*	*Ref.*
Serum (Pb, Cd, Cu, Zn, Fe, Ba, Na)	Synchropak GPC 300 (250 mm × 2 mm × 5 µm)	0.1 M Tris - HCl (pH 6.9)	23
Pb, Fe, Cu, Zn and Mg in serum and red blood cells	Progel TSK G 3000 SW (30 cm × 7.8 mm)	0.1 M Tris - HCl (pH 7.2)	26
Erythrocytes	Superdex 200 HR 10/30		27
Metalloproteins	Spherogel SW 2000 (60 cm × 7.5 mm × 10 µm)	0.06 M Tris - HCl (pH 7.5) or 0.25 M NaCl - 0.06 M Tris - HCl (pH 7.5) (+ 0.05% of NaN_3) (1 ml min^{-1})	25
Metals in tea	Superdex 75 HR 10/30	0.1 M CH_3COONH_4 (pH 5.5) (1 ml min^{-1})	19
B in radish roots	YMC-Pack Diol–120 (30 cm × 8 mm)	0.2 M $HCOONH_4$ (pH 6.5) (1 ml min^{-1})	32
Cu, Se, Zn, Fe and S in mice liver	Asahipak GS 520 (50 cm × 7.6 mm × 9 mm)	0.05 M Tris-HCl buffer; pH 7.4 (1.0 ml min^{-1})	11
Plasma, red blood cells, liver and kidney of rats, Fe, Cu, Zn, Se	Asahipak GS 520 (50 cm × 7.6 mm × 9 µm)	0.05 M Tris-HCl buffer; pH 7.4 (1 ml/min)	12–14
Ru- and Pt- based metallodrugs in serum	Progel TSK G 3000 PWxL (6 cm × 7.8 mm)	0.03 M Tris-HCl; pH 7.2 (0.9 ml min^{-1})	28
Al in neuroblastoma cells	Superdex-75 HR (30 cm × 10 mm)	0.03 M Tris-HCl; pH 7.2 (0.9 ml min^{-1})	10
Pb, Ba, Sr, REE in plants	Superdex-75 HR (30 cm × 10 mm)	0.03M formate buffer; pH 5.8	29
rat urine	Asahipak GS-320 (50 cm × 7.6 mm i.d.)	0.05 M Tris-HCl buffer; pH 7.4 (1 ml/min)	12,14
Cu, Zn and Al in the femur, brain and kidney of guinea pigs	Progel TSK-HW55S (25-40 µm)	0.12M Tris-HCl; pH 8.2 (0.75 ml min^{-1})	15
chicken meat	Pharmacia Superose–12 HR 10/30	0.12 M Tris - HCl; pH 7.3	31
Cd in pig kidney	Pharmacia Superose–12 (2 cm × 5 mm)	0.12 M Tris-HCl; pH 7.5	22,30
Zn isotope chicken meat simulated gastro-intestinal digest	Pharmacia Superose–12 (30 cm × 10 mm)	0.1 M CH_3OONH_4 0.1% TFA; pH 6.0	31
Anacystis nidulans	Asahipak GFA-30F (30 cm × 7.6 mm)	0.05 M Tris-HCl; pH 7.5 and 0.2 M $(NH_4)_2SO_4$ with 0.1 mM EDTA (0.8 ml min^{-1}).	16
Cd in *M. galloprovincialis*	Progel TSK G 3000 PWxL (30 cm × 7.8 mm)	0.03 M Tris-HCl; pH 7.2 (0.75 ml min^{-1})	17
Cd in mussels and haemolysed osprey blood	Progel TSK G3000 PWxL (30 cm × 7.8 mm)	0.03 M Tris-HCl; pH 8.6 (0.8 ml min^{-1})	6
wine	Superdex-75 HR (30 cm × 10 mm)	0.03M formate buffer; pH 5.8	21
Cu,Zn, Cd in cyanobacterium	DuPont GF-250 (250 mm × 9.4 mm)	0.05 M Tris-HCl; pH 7.5 and 0.2 M $(NH_4)_2SO_4$	18

Ecotoxicological

Rapid differentiation between metallothionein-bound and non-bound cadmium is essential to evaluate the content of toxic cadmium in edible resources and foodstuffs. The interest in the determination and characterisation of metallothioneins comes from their role in metabolism and detoxification of a number of essential (Zn, Cu) and toxic (Cd, Hg) trace metals.

Metallothioneins in mussels have widely been studied [6,17]. Individual and synergistic effects of Cu, Zn and Cd ions on the induction of metallothionein in cyanobacterium (the origin of trophic chains in aquatic systems) were investigated [24]. The *in vivo* selenium association with cyanobacterial metallothioneins following the coadministration of Zn and selenite or selenate was examined [16].

References

1. Lange-Hesse, K.; Dunemann, L.; Schwedt, G. *Fresenius' J. Anal. Chem*. **1991**, *339*, 240.
2. Lange-Hesse, K.; Dunemann, L.; Schwedt, G. *Fresenius' J. Anal. Chem.* **1994**, *34*, 460-464.
3. Blanco-Gonzalez, E.; Perez-Parajon, J.; Garcia-Alonso, J. I.; Sanz-Medel, A. *J. Anal. At. Spectrom*. **1989**, *4*, 175-179.
4. Messerschmidt, J.; Alt, F.; Tölg. G. *Electrophoresis* **1995**, *16*, 800.
5. High, K. A.; Blais, J. S.; Methven, B. A. J.; McLaren, J. W. *Analyst*. **1995**, *120*, 629.
6. High, K. A.; Methven. B. A.; McLaren, J. W.; Siu, K. W. M.; Wang, J.; Klaverkamp, J. F.; Blais, J. S.; *Fresenius' J. Anal. Chem*. **1995**, *351*, 393.
7. Micallef, S.; Couillard, Y.; Campbell, P. G. C.; Tessier, A. *Talanta* **1992**, *39*, 1073-1079.
8. Makino, Y.; Nishimura, S. *J. Chromatogr. Biomed. Appl*. **1992**, *579*, 346-349.
9. Michalke, B.; Schramel. P. *J. Trace Elem. Electrolytes Health Dis*. **1990**, *4*, 163.
10. Zatta, P.; Szpunar, J.; Lobinski, R. (in preparation).
11. Suzuki, K. T.; Yoneda, S.; Itoh, M.; Ohmichi, M. *J. Chromatogr. Biomed. Appl*. **1995**, *670*, 63-71.
12. Suzuki, K. T.; Itoh, M.; Ohmichi, M. *J. Chromatogr. Biomed. Appl*. **1995**, *666*, 13-19.
13. Suzuki, K. T. *Tohoku J. Exp. Med*. **1996**, *178*, 27.
14. Suzuki, K. T.; Itoh, M.; Ohmichi, M. *Toxicology* **1995**, *103*, 157-165.
15. Owen, L. M. W.; Crews, H. M.; Massey, R. C.; Bishop, N. J. *Analyst*. **1995**, *120*, 705-712.
16. Takatera, K.; Osaki, N.; Yamaguchi, H.; Watanabe, T. *Anal. Sci*. **1994**, *10*, 567-572.
17. Szpunar, J.; Chassaigne, H.; Donard, O.; Bettmer, J.; Lobinski, R. in: Holland, G. Ed., Applications of Inductively Coupled Plasma Mass Spectrometry, RSC, 1997.
18. Takatera, K.; Watanabe, T. *Anal. Sci.* **1992**, *8*, 469.

19. Oedegard, K. E.; Lund, W. *J. Anal. At. Spectrom*. **1997**, *12*, 403-408.
20. Owen, L. M. W.; Crews, H. M.; Massey, R. C. *Chem. Speciat. Bioavail*. **1992**, *4*, 89.
21. Szpunar, J.; Pellerin, P.; Makarov, A.; Doco, T.; Williams, P.; Medina, B.; Lobinski, B. *J. Anal. At. Spectrom.* **1998**, *13* (in press).
22. Crews, H. M.; Dean, J. R.; Ebdon, L.; Massey, R. C. *Analyst*. **1989**, *114*, 895.
23. Shum, S. C. K.; Houk, R. S. *Anal. Chem*. **1993**, *65*, 2972-2976.
24. Takatera, K.; Watanabe, T. *Anal. Sci.* **1993**, *9*, 19.
25. Mason, A. Z.; Storms, S. D.; Jenkins, K. D. *Anal. Biochem*. **1990**, *186*, 187-201.
26. Gercken, B.; Barnes, R. M. *Anal. Chem*. **1991**, *63*, 283-287.
27. Bergdahl, I. A.; Schütz, A.; Grubb, A. *J. Anal. At. Spectrom*. **1996**, *11*, 735-738.
28. Pieper, T.; Szpunar, J.; Keppler, B.; Lobinski, R. (in preparation).
29. Pellerin, P.; Szpunar, J. (in preparation).
30. Dean, J. R.; Munro, S.; Ebdon, L.; Crews, H. M.; Massey, R. C. *J. Anal. At. Spectrom*. **1987**, *2*, 607.
31. Owen, L. M.; Crews, H. M.; Hutton, R. C.; Walsh, A. *Analyst*. **1992**, *117*, 649.
32. Matsunaga, T.; Ishii, T.; Watanabe, H. *Anal. Sci*. **1996**, *12*, 673-675.