Speciation of basal aluminium in human serum by fast protein liquid chromatography with inductively coupled plasma mass spectrometric detection

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ABSTRACT

The coupling of fast protein liquid chromatography (FPLC) with inductively coupled plasma mass spectrometry (ICP-MS) was evaluated as a technique for studying aluminium bound to proteins present in human serum. Separation of human serum proteins was achieved on a MonoQ (HR5/5) anion-exchange column using an ammonium acetate gradient (0–0.25 mol l^{-1}) at the physiological pH of 7.4 (0.05 mol l^{-1} TRIS-HCl buffer). Aluminium contamination was avoided with an on-line Al-chelating scavenger column. Proteins were detected spectrophotometrically at 295 nm and the Al detection was carried out on-line using both quadrupole ICP-MS and double-focusing ICP-MS systems. At metal basal levels in serum the latter detector proved to be adequate for this detection. Results obtained with the procedure developed confirmed clearly that transferrin is the only significant Al-binding protein in unspiked uraemic serum. In addition, a high-resolution ICP-MS instrument was applied successfully as an Al-specific detector allowing for the first time Al speciation studies in unspiked normal serum. The technique can also be used for studying the protein binding of elements other than Al.

Keywords: *aluminium; speciation; human serum; fast protein liquid chromatography; inductively coupled plasma mass spectrometry*

INTRODUCTION

Aluminium has been implicated as a toxic factor in a number of human diseases. In patients with chronic renal failure, Al accumulation in certain body tissues induces encephalopathy, osteomalacia and anaemia.[1,2] More controversially, aluminium also appears to play a role in the development of some neurodegenerative diseases in subjects with normal renal function.[3,4]

Clearly, both preventive measures and treatments for Al toxicity require the elucidation of the main factors that govern Al bioavailability, which, in turn, depends

on the Al chemical form(s) in human serum.[5] Therefore, aluminium speciation strategies are needed to identify and determine high molecular mass (HMM) and low molecular mass (LMM) biocompounds binding the metal.

Several separation techniques, such as ultrafiltration,[6–10] gel filtration chromatography,[11–17] anion-exchange high-performance liquid chromatography[18–21] and recently anion-exchange fast protein liquid chromatography (FPLC)[22] coupled with an element-specific detector such as an ETAAS instrument, have been widely used for studying protein binding of Al in human serum. However, most of the described methodologies are 'off- line' techniques and usually they lack selectivity, are prone to contamination or lack sensitivity for real unspiked serum samples. As a result, data published so far on Al speciation may be contradictory and should be interpreted with caution because they have usually been worked out by using 'spiked' Al serum samples.

The binding of Al to human serum proteins was studied recently in this laboratory using FPLC–ETAAS.[22] The results agreed with those of other workers[18] and indicated clearly that transferrin seems to be the only protein binding Al in unspiked serum from dialysis patients. However, the developed speciation procedure suffers from a lack of the necessary sensitivity required from unspiked non-uraemic serum samples. Moreover, the 'off-line' procedure was time consuming (since ETAAS requires fraction collection after elution) and chromatographic selectivity can be ruined by this total detection mode of analysing every 0.6 ml of eluent leaving the column.[22]

ICP-MS is an element-specific and very sensitive detection method which may be coupled on-line to liquid chromatography.[23] In this work, the potential of ICP-MS, as a detection system for Al, coupled with FPLC for protein separation, was investigated for basal Al speciation in human serum. The observed favourable analytical performance of the developed speciation procedure allowed us for the first time to study protein binding of Al in unspiked serum from non-uraemic patients.

EXPERIMENTAL

Instrumentation

The chromatographic system used consisted of a Shimadzu (Kyoto, Japan) LC-10A HPLC pump with a Rheodyne (Cotati, CA, USA) Model 7125 sample injection valve

fitted with a 100 ml loop. Separations were performed on a 10 mm anion- exchange Mono-Q HR 5/5 FPLC column (50 3 5 mm id) (Pharmacia Biotech, Uppsala, Sweden). Chromatograms were obtained using an LKB (Bromma, Sweden) Model 2151 UV/ VIS detector.

Retention times and peak heights were obtained with a Shimadzu C-R6A recording integrator. A scavenger column (25 3 0.5 mm id) was placed between the pump and the injection valve to remove exogenous Al, a contaminant coming mainly from the mobile phase used.[22] This column was laboratory packed with Kelex-100 impregnated silica C_{18} material (20 mm particle size), prepared as described previously.[22]

Aluminium detection was performed using both a quadrupole (Q) (Model HP 4500, Hewlett-Packard, Yokogawa Analytical Systems, Tokyo, Japan) and a high-resolution (HR) magnetic sector field ICP-MS system (ELEMENT, Finnigan Mat, Thermo Instruments, Bremen, Germany). In both cases the instruments were fitted with a conventional Meinhard nebulizer and with a double-pass spray chamber, cooled to 2 °C in the case of the quadrupole ICP-MS system.

The ETAAS determination of aluminium concentrations in serum samples and in column fractions was carried out using a Perkin-Elmer (Norwalk, CT, USA) Model 3030 atomic absorp- tion spectrometer with a Model HGA-500 graphite furnace, equipped with a Model AS-40 deuterium-arc background corrector and a PR-10 printer.

All the instrumentation employed was housed in a clean room equipped with a filtered laminar flow air supply in order to avoid contamination of the aluminium by atmospheric dust.

Reagents

All the reagents used were of the highest purity available and were of analytical-reagent grade. Milli-Q-purified water (Milli- pore, Molsheim, France) was used throughout. A 1000 mg l^{-1} stock standard solution of Al and Suprapur nitric acid (65%) were obtained from Merck (Darmstadt, Germany). An inter- mediate working standard solution of 100 mg l^{-1} was prepared diluting 10 ml of the stock standard solution to 100 ml with diluted nitric acid (1 + 20).

Kelex 100 (Schering España, Madrid, Spain) was used as received and the bonded

silica reversed-phase sorbent with octadecyl functional groups (C_{18}) was purchased from Waters (Milford, MA, USA). Human protein standards (albumin, apotransferrin and immunoglobulin G) were obtained from Sigma (St. Louis, MO, USA) and tris(hydroxymethyl)amino- methane (TRIS) from Merck. Uraemic and normal serum samples were kindly provided by the Hospital Central of Asturias in Oviedo (Asturias, Spain)

Procedures

The risks of sample contamination with external Al during the sampling, transport, storage, separation and determination steps was minimised following the detailed procedures described in previous work.[9]

A standard solution of human proteins was prepared by dissolving apotransferrin (0.5 g l^{-1}), albumin (5 g l^{-1}) and immunoglobulin G (1 g l^{-1}) in 0.01 mol l^{-1} TRIS-HCl buffer (pH 7.4) containing 0.03 mol l^{-1} sodium hydrogencarbonate. The conditions used for the separation of serum proteins in both standard solution and serum samples by anion-exchange FPLC are detailed in Table 1. The separation was achieved using an ammonium acetate gradient (0–0.25 mol l^{-1}) in 0.05 mol l^{-1} TRIS adjusted to pH 7.4 with HCl.

The mobile phases were degassed with helium for 15 min before use and checked for their Al content by ETAAS. They were discarded when the level of Al was above 5 ng l^{-1}. The eluate from the FPLC column was passed through a UV detector, set at 295 nm for protein monitoring, and to a Q-ICP-MS or HR-ICP-MS detector for aluminium detection and the corresponding chromatogram was registered.

Both ICP-MS instruments were firstly optimised, independently of the chromatographic system, for nebulizer gas flow rate, ion lens voltages, plasma forward power and sampling depth (when possible) using a standard solution containing Li, Na, Sc, Co, Y, In, Ba, Tb, Tl and Th at 10 ng g^{-1} to obtain maximum sensitivity over the whole mass range. The optimisation was also achieved using a 10 ng g^{-1} Al standard solution to improve the optimisation further.

The chromatographic system was then coupled to the ICP- MS instrument using PTFE capillary tubing (0.5 mm id) from the column outlet to the inlet of the standard Meinhard nebulizer.

Chromatographic data were collected using the time-resolved analysis mode with an integration time of 0.3 s per point. Instrumental operating conditions for both ICP-MS systems are summarised in Table 2.

RESULTS AND DISCUSSION

Anion-exchange FPLC separation of human serum proteins

A very satisfactory separation of human serum proteins was achieved in previous studies[22] using a Mono Q (HR 5/5) anion-exchange column and a mobile phase with a sodium chloride gradient (0–0.25 mol l^{-1}). These levels of sodium chloride in the mobile phase were found to produce salt deposits and clogging of the sampling orifice in the ICP-MS detector. Experiments were carried out using lithium chloride instead for the separation, but without success, whereas a final ammonium acetate gradient provided the solution to this clogging problem. In order to prevent changes of *in vivo* speciation of aluminium, the separation was carried out at the serum physiological pH of

7.4 (attained with 0.05 mol l^{-1} TRIS-HCl buffer). The effect of flow rate and elution gradient on the separation of serum proteins was evaluated by using a standard solution of 0.5 g l^{-1} apotransferrin, 5 g l^{-1} albumin and 1 g l^{-1} immunoglobulin G. Using the chromatographic conditions detailed in Table 1 these three human serum proteins were easily separated, eluting at 1.2, 10.4 and 18.4 min, respectively, as shown in Fig. 1. An acceptable resolution of the proteins was also obtained in undiluted real serum samples (Fig. 1). As can be seen in Fig. 2, transferrin was eluted as two incompletely separated peaks at

9.4 and 10.8 min. The relative height of these peaks varied between different serum samples. These two peaks could be explained by the fact that depending on the individual plasma iron levels, four forms of transferrin have been identified: apotransferrin, two monoferric transferrin species (iron bound at the *N*- or *C*-terminal domain) and one diferric transferrin, as demonstrated by anion-exchange HPLC techniques previously.[24] It is likely that the two transferrin peaks in Fig. 1 can be ascribed to two different Fe-transferrin molecular forms.

Aluminium elution profile

Fig. 2 shows the chromatogram obtained with on-line quadrupole ICP-MS aluminium

detection for an undiluted serum sample from a dialysis patient (total Al content 120 mg l^{-1}). As can be seen, the mass flow chromatogram of ^{27}Al contains two clear peaks at t_r = 9.4 and 10.8 min, which clearly overlap the transferrin peaks (see Fig. 1), confirming the previously reported association of the metal with this protein.[22] However, owing to the high noise observed in the Q-ICP-MS signal at *m/z* 27, the sensitivity of this detector was insufficient to detect Al in unspiked serum samples from a healthy person (total Al content 2.5 mg l^{-1}), as shown graphically by the results in Fig.
2. This high background could be attributed to trace Al contamination originating from the chromatographic system and reagents. However, this contamination problem was avoided in further experiments by using an on-line chelating Al scavenger column described previously.[22] Therefore, we be- lieve that most of the background signal at *m/z* 27 is probably due to interference caused by the formation of $^{13}C^{14}N$ and $^{12}C^{15}N$[25] mainly from the mobile phase reagents used (ammonium acetate).
Since Al has only one natural isotope, this problem cannot be overcome by selecting another isotope. Hence an effective approach to eliminate this interference should resort to using an HR-ICP-MS instrument. The HR-ICP-MS instrument used in this work (ELEMENT, Finnigan Mat) can be used in three different operating modes (300, 3000 and 7500 *m*/D*m*, 10% valley definition). In all mass spectrometers there is an inverse relationship between sensitivity and resolution, *R*. For instance, the sensitivity at a medium resolution (*R* = 3000) is about 10% of that at *R* = 300. In any case this instrument exhibits extremely low instrumental background levels even at low *R* (less than 1 count s^{-1}). Therefore, this HR-ICP-MS detector even in the medium resolution mode can still compete in terms of detection limits with quadrupole instruments.

Preliminary experiments at *R* = 3000 and 300 indicated that for the speciation of Al at basal levels with the described FPLC system combination with HR-ICP-MS is mandatory. Using the working conditions given in Tables 1 and 2 and *R* = 3000, a serum sample from a dialysis patient (total Al content 120 mg l^{-1}) was analysed and results are plotted in Fig. 3(a). The information provided is similar to that observed at this Al level using Q-ICP-MS detection (Fig. 2).

Our previous findings using 'non-spiked' aluminium uraemic serum samples for this type of FPLC (mobile phases: buffer A consisted of an aqueous solution containing 0.01 mol l^{-1} TRIS adjusted to pH 7.4 with HCl, plus 0.01 mol l^{-1} sodium

hydrogencarbonate, and buffer B consisted of an aqueous solution containing buffer A plus 0.25 mol l^{-1} sodium chloride) with ETAAS detection[22] also showed that the metal is bound only to transferrin, as shown in Fig. 3(b). In other words, as expected from previous experiments,[20–22] most of the serum Al co-elutes with transferrin (two molecular forms, t_r = 9.4 and 10.8 min) from the Mono Q (HR 5/5) anion-exchange column with the eluents used (Table 1).

Fig. 3(a) also shows the presence of a small Al peak at a retention time of about 15 min. Previous results for spiked serum samples obtained by HPLC–ETAAS[21] indicated the presence of LMM Al biocompounds, which elute between transferrin and albumin with anion-exchange chromatographic separation.[21,22] Therefore, this small Al peak at 15 min in Fig. 3(a) could probably be ascribed to an aluminium–citrate complex as it appears to be the predominant LMM-Al species in human serum[17,21,26,27]

Fig. 3(a) demonstrates that CN spectral interferences from the mobile phase were avoided by using a resolution of 3000 (allowing a low baseline of approximately 1000 counts) and the sensitivity of the proposed FPLC–HR-ICP-MS hybrid tech- nique offers the possibility of speciating Al at the concentra- tions that occur in normal serum samples (basal levels).

Speciation of basal Al in serum

Having demonstrated the ability of such a hybrid strategy for the basal level speciation of Al in human serum, serum samples from non-uraemic subjects were analysed by the proposed procedure. Fig. 4 shows typical observed results, demonstrating clearly for the first time that the results for spiked serum[21] and non-spiked uraemic sera[22] are very similar with regard to the identity of the protein transporting Al in blood: it seems that transferrin is also the protein that binds very low levels of Al in normal human serum.

Association between aluminium and citrate in these serum samples could not be confirmed, probably owing to a lack of the necessary sensitivity. This is consistent with the fact that we injected 100 ml of unspiked and undiluted serum with a total aluminium content of about 2.5 mg l^{-1}. Therefore, if about 90% of the total aluminium is bound to transferrin,[21,22] only 10% of the total Al would remain to be bound to citrate. This means that the Al concentration corresponding to the citrate complex will be less than 0.25 mg l^{-1}, which is below the detection limit of the HR-ICP-MS system used here. This detection limit ($3ss_B$ criterion), estimated from the peak height of

the aluminium signal in Fig. 4, was of the order of 0.6 mg l^{-1}.

CONCLUSIONS

Speciation of Al in human serum is an extremely difficult task because the basal levels of this element in serum are below 5 mg l^{-1} and these minute amounts are fractioned in the speciation process. To make matters worse, the risk of significant exogenous Al contamination is very high.

The potential of coupling anion-exchange FPLC separation with ICP-MS has been demonstrated here for the speciation of aluminium in human serum. Results obtained with the quadru- pole-ICP-MS system are similar to those obtained off-line by ETAAS previously[22] for the determination of relatively ele- vated metal levels in serum. However, there are two important advantages of using ICP-MS detection: first, the total analysis time for the chromatographic run is considerably shorter (typically 25 min) than the 2 h required in ETAAS, where fractions are collected and analysed individually; second, ICP- MS gives time-resolved profiles with better resolution than profiles obtained by analysing 0.5 ml fractions by ETAAS. Transient individual peaks can be identified provided that they reside longer than the integration times used in the data acquisition processes. In fact, the single broad peak for Al- transferrin [Fig. 3(b)] identified with ETAAS detection can be resolved into two distinct peaks with retention times of 9.4 and
10.8 min using ICP-MS and a mobile phase of ammonium acetate.

The use of a quadrupole ICP-MS system for detection, however, proved to be problematic in this application owing to interference (probably from CN^+ ions formed from the mobile phase) occurring at *m/z* 27 of aluminium, which would explain the high background observed at different retention times in the corresponding chromatogram (see Fig. 2).

However, coupling of FPLC with ICP-HR-MS detection, allowing for elimination of this spectral interference at resolution 3000, offers the possibility of Al speciation studies in non-uraemic human serum samples. In other words, speciation is possible without the need for spiking the metal to raise its serum level artificially. In this way, Al species in serum could be preserved in its naturally occurring state and studied at basal levels in non-uraemic subjects (less than 5 mg l^2 total Al concentration) for the first time.

1. **References**

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TABLES

Table 1 Instrumental operating conditions for FPLC

Column	Mono Q HR5/5 (50 3 5 mm id, 10 mm particle size)	
Injection loop	100 ml	
Flow rate	1 ml min^{-1}	
Eluents	(A) 0.05 mol l^{-1} TRIS-HCl (pH 7.4)	
	(B) A + 0.25 mol l^{-1} ammonium acetate	
Gradient elution	Time	B (%)
	0	0
	15	100
Detection (proteins)	UV absorption at 295 nm	

Table 2 Instrumental operating conditions for aluminium detection

Quadrupole ICP-MS—	
Instrument	HP 4500
Rf power	1300 W
Sampling depth	6.2 mm
Carrier gas flow rate	1.10 l min^{-1}
Intermediate gas flow rate	1.0 l min^{-1}
Outer gas flow rate	15.0 l min^{-1}
Spray chamber	Double pass/Peltier cooled (2 °C)
m/z monitored	27
Double-focusing ICP-MS—	
Instrument	ELEMENT
Rf power	1300 W
Sampling depth	Fixed
Carrier gas flow rate	1.15 l min^{-1}
Intermediate gas flow rate	0.9 l min^{-1}
Outer gas flow rate	14.5 l min^{-1}
Spray chamber	Double pass/room temperature Resolution, *m*/D*m* 3000
m/z monitored	26.982 ± 0.004

FIGURES

Legend of Figures

Figure 1.- Anion-exchange FPLC of human serum proteins in (dotted line) a standard solution [0.5 g l^{-1} apotransferrin (Tf), 5 g l^{-1} albumin (A) and 1 g l^{-1} immunoglobulin G (Ig G)] and (solid line) an undiluted serum sample. UV absorptiometric detection at 295 nm.

Figure 2. Chromatogram of unspiked human serum, after FPLC separation of proteins using quadrupole ICP-MS detection of Al: solid line, uraemic serum (Al content 120 mg l^{-1}), and dashed line, normal serum (Al content 2.5 mg l^{-1}).

Figure 3. Chromatograms of unspiked uraemic serum (Al content 120 mg l^{-1}) after FPLC separation of proteins using (a) on-line HR-ICP-MS (R = 3000) and (b) off-line ETAAS detection of Al. The UV detection of proteins at 295 nm is also shown (note that the mobile phase used here is different, *i.e.*, different retention times for transferrin)

Figure 4. Chromatogram of normal unspiked human serum (Al content 2.5 mg l^{-1}) after FPLC separation of proteins using HR-ICP-MS (R = 3000) detection of Al.

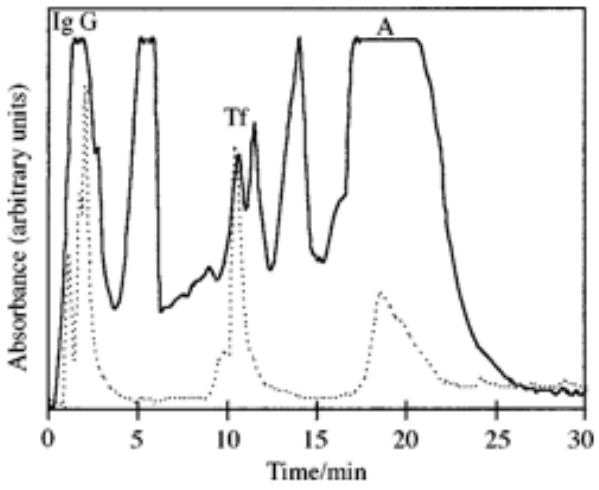

Figure 1

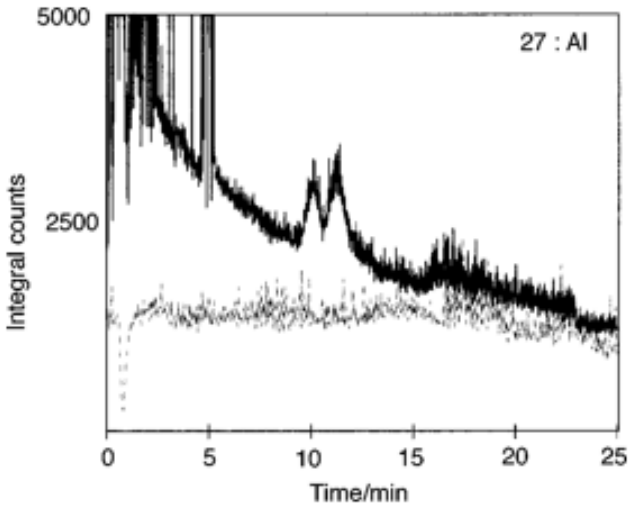

Figure 2

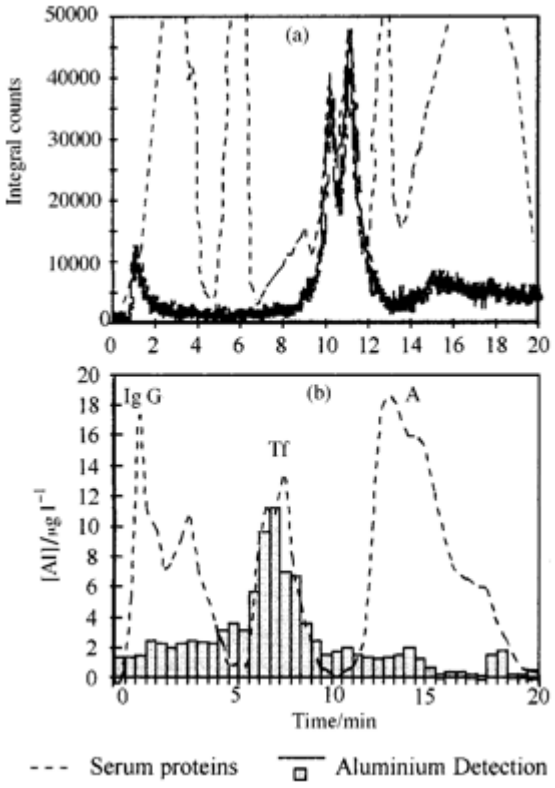

Figure 3

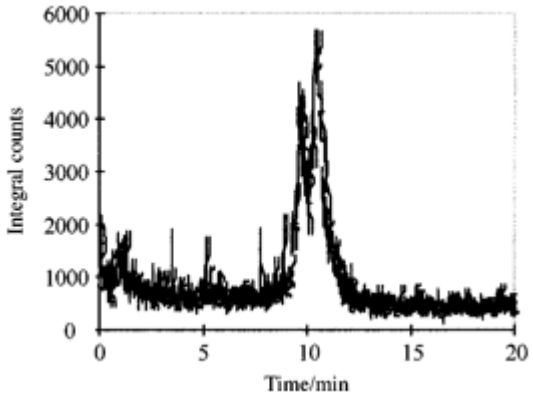

Figure 4