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The chemical speciation of aluminium in human serum

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Contents

Abstract

Chemical speciation of aluminium in the low molecular mass (LMM) and high molecular mass (HMM) fractions of human serum is discussed. A critical review of the literature on different analytical procedures described for the speciation of aluminium in human serum samples is presented here. The methodologies, the experimental and instrumental requirements and the ability of the reported analytical procedures for identification of HMM and LMM aluminium species in human serum are examined in detail. Non-chromatographic separations coupled to electrothermal atomic absorption spectrometry for aluminium detection are compared with chromatographic techniques (size exclusion chromatography, anion exchange chromatography and fast protein liquid chromatography) coupled to ETAAS or inductively coupled plasma mass spectrometry (ICP-MS) detection for Al-HMM species investigations. Studies and techniques reported for Al-LMM compounds are also summarised, both for healthy volunteers and dialysis patients. On the basis of the knowledge obtained from the application of the developed analytical procedures to real serum analysis, it has been demonstrated that most of Al in human serum is bound to Al-transferrin, while the LMM-Al fraction (10–20% of total Al) mainly contains Al-citrate, Al-phosphate and ternary Al-citrate–phosphate complexes.

Keywords: Aluminium; Speciation; Al-transferrin; Al-citrate; Al-phosphate; Ternary Al-citrate–phosphate complex; Ultramicrofiltration; Liquid chromatography; ETAAS; ICP-MS; Electrospray ionisation MS–MS; Human serum of healthy volunteers and of dialysis patients

1. Introduction

Aluminium is one of the most abundant metals in the earth's crust (8.3% by weight); it is exceeded in abundance only by non-metals O (45.5%) and Si (25.7%), and only approached by the metals Fe (6.2%) and Ca (4.6%). Because of the widespread distribution of aluminium compounds, human exposure to this metal is unavoidable. Thus moderate amounts are inhaled in atmospheric dust and taken up in food and drink. Moreover, acid rain releases the metal from soils and so exposure of plants, fish and humans to dissolved aluminium is today a source of concern.

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Aluminium constitutes a potential threat for: (a) the whole earth population which have to cope with constantly increasing levels of bioavailable aluminium from the environment; and (b) more specifically, patients who ingest high doses of metal present in Al-containing drugs, specially those with renal insufficiency or peptic ulcer disease [1,2]. For all cases the exact evaluation of the risks is directly related to a correct assessment of aluminium bioavailability. In this respect, compounds of aluminium are absorbed poorly from the gastrointestinal tract, but excessive intake, such as may occur through over-medication can create problems [3]. The major pathway for the elimination of any absorbed or systematically administered aluminium is the kidneys and rarely has been reported to have increased tissue stores of aluminium in an individual with normal renal function. Under ordinary health conditions kidney appears to be able to eliminate all the absorbed aluminium [4,5].

In renal failure patients, aluminium overload has been documented to cause neurological, skeletal and hematopoietic toxicity [6]; origin of different diseases such as dialysis encephalopathy or dialysis dementia, osteomalacia, bone pain and pathological fractures and microcytic or hypochromic anaemia. Thus, aluminium overload in patients with chronic renal failure has been largely prevented by eliminating Al-containing phosphate-binding agents and by the use of high purity water for preparation of dialysis fluids [7]. Nevertheless, aluminium may still enter the body of dialysis patients via consumption of Al-based drugs and antacids [8].

In other words, the speciation of aluminium in serum is today mandatory to understand the toxicity of aluminium in humans [9,10]. To achieve this goal the complete characterization of the serum constituents which bind and transport this element to its target organs (e.g. brain and bone) is an imperative task [11].

Blood serum is a complicated and heterogeneous mixture containing high molecular mass compounds, small molecules and ions. Thus, trace element speciation studies in serum samples can be approached first by discriminating between high molecular mass (HMM) and low molecular mass (LMM) trace metal fractions.

Unfortunately, Al speciation analysis in biological samples is beset with many difficulties. One major drawback is that 'normal' Al tissue levels in healthy people (a concept that we will call, from now on, 'basal' level of the element) are very low. In fact it is believed today that 'basal' Al concentrations in blood serum are around or lower than 2 μg dm^{-3} [3,12,13]. Moreover, Al, as detailed above, is an ubiquitous element in Nature and so external contamination problems are almost unavoidable.

Particularly for speciation, where fractions of the sought element are analysed in each species in which it occurs, those extremely low levels and the high risk of exogenous contamination will demand special analytical ability and care [14–16]. These problems, the progress made on the development of analytical techniques for the identification and determination of Al-HMM and LMM species present in human serum and the final results obtained using such techniques to unveil Al compounds in human serum are reviewed in this paper.

2. Analytical techniques and chemical speciation of HMM-Al species in human serum

Hyphenated methodologies including a powerful separation technique coupled to an on- or off-line highly sensitive and selective detection system are preferred today for trace element speciation in biological materials [17,18]. According to their basic separation mechanism, they can be classified as non-chromatographic and chromatographic techniques.

A summary of such techniques, with the stationary and mobile phases proposed published so far for Al speciation in human serum is given in Table 1.

2.1. Non-chromatographic techniques

As stated before [18]; the complexity of the human serum samples calls for a knowledge and judicious choice of different principle based separations assisted by complementary selective detectors. In this vein, a most advisable first step is the fractionation of the aluminium biocompounds into two broad groups: (a) HMM; and (b) LMM type of species. This 'primary' or rough information can provide a very useful preliminary information [19].

The first studies reported on aluminium fractionation in human serum of normal and uremic subjects [20–26] using ultrafiltration showed controversial results. Such conflicting obtained data could be ascribed to the use of different analytical methods and procedures, for ultrafiltration and for eventual aluminium determination or most likely to the high contamination risks of such Al determinations.

The above mentioned problem was critically investigated by Parajón et al. [27]. They evaluated two ultrafiltration methodologies: conventional ultrafiltration and ultramicrofiltration, for fractionation in vitro of aluminium serum; the determination of the aluminium content of the serum was performed by ETAAS, following the procedure described by Sanz-Medel et al. [25]. Their results using ultrafiltration suggest that the majority of aluminium (about 95%) should be in the HMM fraction (bound to proteins), but contamination for extraneous aluminium reduces the reliability of the results. However, when ultramicrofiltration is employed, contamination risks are minimised and the proportion of ultrafiltrable aluminium in serum found was 8.3 ± 2.1,

Table 1
Aluminium speciation techniques for serum samples

Technique	Serum sample	Sample concentration (μg dm^{-3})	Authors and references
Ultrafiltration			
Amicon CF-50 A and YMT membranes, Spectropore pressurized membranes	Normal	20–75	Lundin et al. (Ref. [21])
YM10, DDS pressurized membranes and Amicon YMT	Normal + Al	65–108.2	Pérez Parajón et al. (Ref. [27])
Amicon YMT membranes	Normal + Al; uremic	32.1–262.5	Wróbel et al. (Ref. [31])
Centrifee Micropartition, Amicon YMT membranes	Normal and uremic	7.8–311	Leung et al. (Ref. [23])
Amicon YM10, pressurized membranes	Normal and uremic	6.4–235	Rahman et al. (Ref. [40])
Sartorius pressure filtration at 100 KPa	Normal + Al	200	Gardiner et al. (Ref. [41])
SEC			
Sephadex S-200 (sodium, chloride, calcium, magnesium, Tris)	Normal + Al	240–395	Bertholf et al. (Ref. [29])
Sephadex S-200 (NaCl, NaOH, NaN_3, $MgCl_2$, Tris)	Uremic	22.95–130.95	King et al. (Ref. [39])
Sephacryl S-300 (Tris–HCl, NaCl, $NaHCO_3$)	Uremic serum pool	378	Cochran et al. (Ref. [48])
Sephadex G-200 (Tris, KNO_3)	Normal + Al	500 μg cm^{-3}	Trapp (Ref. [54])
TSK Gel HWW-55S (NaCl, Tris–HCl, NaN_3)	Normal + Al, uremic	21.6–270	Favarato et al. (Ref. [46])
Anion exchange			
TSK DEAE 5PW (gradient of NaCl)	Serum + Al	187	Van Landeghem et al. (Ref. [16])
TSK DEAE 3SW (gradient of sodium acetate)	Normal + Al	300–1000	Blanco González et al. (Ref. [50])
Protein Pak DEAE 5 PW (gradient of sodium chloride)	Serum pool + Al	1000	Wróbel et al. (Ref. [56])
FPLC Mono Q HR 5/5 (gradient of sodium chloride)	Uremic	92	Soldado et al. (Ref. [15])
FPLC Mono Q HR 5/5 (gradient of ammonium acetate)	Uremic, normal	2.5–120	Soldado et al. (Ref. [57])
FPLC Mono Q HR5/5 (gradient of ammonium acetate)	Normal, normal-+Al	–	Nagaoka and Maitani (Ref. [61])

close to the value reported by Leung et al. [23] using a similar method, by Eliot et al. [20] and Khalil-Manesh et al. [28] using a conventional ultrafiltration, by Bertholf et al. [29] using gel chromatography, or by Graf et al. [22] using dialysis in vivo.

These studies were later confirmed by Wróbel et al. [30,31]. They observed that 11 ± 2% of the total aluminium in serum is ultrafiltrable and this value does not seem to be influenced by the total serum elemental concentration, storage conditions, or particular renal pathology of the patients or even by kidney transplantation (see Fig. 1).

From these studies, it can be concluded that ultramicrofiltration shows several important advantages when compared to conventional ultrafiltration: it is simpler, quicker and more efficient in minimizing the

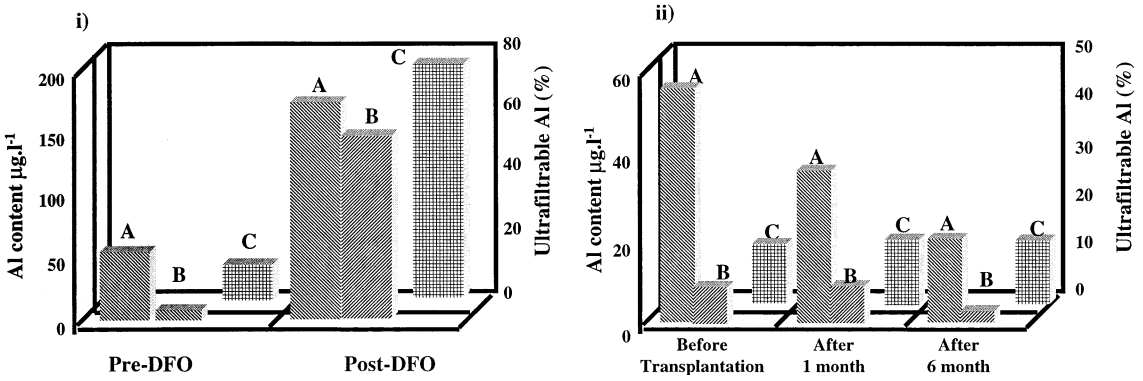

Fig. 1. Influence of: (i) administration of DFO on A, total and B ultrafiltrable levels of Al; (ii) kidney transplantation on A, total and B ultrafiltrable levels of Al. C shows the ultrafiltrable percent of the element [31]. (Reproduced by permission of The Royal Society of Chemistry.)

Al contamination risk, which seems to be the most probable cause for the inconsistency of previous analytical data [27].

This fractionation of aluminium in serum samples was very useful in order to investigate the effect of chelation therapy with chelators on the protein binding of aluminium and its distribution in serum [31–33]. Two known chelators, 1,2-dimethyl-3-hydroxypyridin-4-one (L1) and desferrioxamine (DFO), independently or combined has been studied as therapeutical drugs to remove Al from the body [34–37]. The data in Fig. 1A [31] show that chelation therapy with DFO increases considerably (ca. 70%) Aluminium content in the ultrafiltrable fraction of total serum. A most straightforward explanation of this observation could be that DFO is able to release bound aluminium from its stores within the body (e.g. bones), and it also could release at least some of the protein-bound aluminium from within the serum [23,35,36,38] as DFO forms a stable six-coordinate 1:1 Al–DFO complex (having a molecular mass of 587, a stability constant of 10^{22} and so it could cross the filtration and dialysis membranes, appearing in the ultrafiltrable fraction of serum).

2.2. Chromatographic techniques

Thus, using non-chromatographic techniques it seems that about 10% of aluminium in human serum is ultrafiltrable [20,22,23,28–31]; so, about 90% of aluminium should be bound to non-ultrafiltrable HMM proteins. The question is now which protein(s) binds aluminium in human serum. In order to answer this question chromatographic techniques coupled to Al-specific detectors are the most powerful analytical tools.

However, at this stage a new controversy arose on the type of chromatography to be applied: early workers in this field used size exclusion chromatographic techniques (SEC) for the separation of human serum proteins [23,29,39–41]. Several problems were identified along such studies on serum aluminium: quite often an exchange of aluminium between the serum sample and the stationary phase of the column was noticed, an excess of spiked Al could elute from the column [26,40] and, indeed, there was a consistent difference between the size exclusion chromatography profiles of aluminium in serum depending on whether or not bicarbonate was added to the mobile phase [42]; as a matter of fact, more peaks and a greater proportion of LMM aluminium species were observed when bicarbonate was not present in the buffer; in contrast, results with bicarbonate added to the eluting buffer showed fewer aluminium chromatographic peaks.

SEC Al speciation techniques showed that the largest Al peaks in the region where associated to transferrin and albumin. The peak in the transferrin/albumin region seems to be for some authors the only aluminium peak in the chromatogram [43]. However, King et al. [44] using SEC also reported multiple interactions, including Al with the proteins alpha-2-macroglobulin, IgM, haptoglobin or orosomucoide. Bertholf et al. [35], Khalil-Manesh et al. [45] and Favarato et al. [46] discussed also the possibilities of Al interaction with LMM proteins in human serum.

It seems accepted today that SEC separations experiments do not allow an unequivocal identification of the aluminium binding protein, mainly owing to the poor resolution between albumin and transferrin achieved using this separation technique [26,40,47,48].

These drawbacks prompted researchers to look for alternative chromatographic techniques offering higher resolution in the separation of serum proteins. In particular, anion exchange HPLC has been successfully used to separate the proteins for trace metal speciation in serum. Literature indicates that such separation mechanism could offer important advantages compared with traditional SEC [49–52].

Studies carried out by Blanco González et al. [50] using a TSK DEAE-3SW ion-exchange column with a buffer containing 0.05 mol l^{-1} Tris(hydroxymethyl)-aminomethane (Tris) allowed an efficient separation of albumin and transferrin in serum. The eluate from the column was recollected (in fractions of 500 μl each one) in autosampler cups, and immediately analysed off-line by ETAAS in a clean room for aluminium in the transferrin fraction. Such studies demonstrated that transferrin seems to be the only protein binding aluminium in human serum. Identical results were obtained by the same group [53] using the same column but with an on-line aluminium derivatization with 8-hydroxiquinoline-5-sulphonic acid in a micellar medium and a final aluminium detection using molecular fluorimetry of the formed complex [53].

These HPLC results agree with the reports of other research groups [16,54,55]. The main problem, however, was the silica based column used in those studies because a significant amount of Al was retained on the column. In order to solve this problem Wróbel et al. [56] used a polymeric anion-exchange column (Protein Pak DEAE-5-PW) and a sodium chloride gradient (0–0.25 mol l^{-1}) in Tris–HCl buffer pH 7.4, in the presence of 0.01 mol l^{-1} of sodium hydrogen carbonate to separate proteins in human serum. Proteins were detected at 280 nm with an UV detector, and aluminium was determined by ETAAS in the fractions of 600 μl collected at the exit of the column. Again these results pointed to transferrin as the aluminium binding protein in spiked serum samples.

At this point the main problem in aluminium speciation (specially in human serum with an Al content of a few ppb) was clearly identified: it was exogenous Al contamination. The problem of Al contamination during chromatography was addressed firstly by Van

Landeghem et al. [16] using an on line silica based C_{18} scavenger column, and later by Soldado et al. [15] using an aluminium scavenger column containing the chelating agent 7-(4-ethyl-1-methyl-octyl-quinolin-8-ol) (Kelex 100) adsorbed on C_{18} bonded silica and placed between the chromatographic pumps and the injection valve, as can be seen in Fig. 2. In this way, an Al speciation procedure was proposed allowing the investigation of Al in unspiked serum from dialysis patients [15].

Another potential problem in aluminium speciation studies using an anion exchange chromatographic separation is the displacement risk of aluminium from the protein. That is, stationary and mobile phases used, could compete and displace the metal from their natural protein sites. In this vein, the faster the separation process the lower the displacement risks. Thus, a Mono Q HR 5/5 (fast protein liquid chromatography FPLC) column could minimise these problems [15,53]. Using a Kelex-100 scavenger column to avoid contamination and a FPLC column, Sanz-Medel's group developed a methodology for aluminium speciation at clinically relevant levels in human serum with ETAAS detection [15]. The protein detection was accomplished by ultraviolet (UV) absorption spectroscopy detection at 280 nm; the eluate was collected in fractions of 500 μl to analyse aluminium by ETAAS. The type of chromatogram and protein separation from these studies are illustrated in Fig. 3 [15]; it shows that both aluminium and transferrin, coelute with a migration time of 6.5 min.

The observed recovery of total aluminium in such system was of $95 \pm 10\%$, indicating the reliability of the separation and the absence of typical column absorption of the analyte.

In any case, this speciation procedure suffers from a lack of the necessary sensitivity. To investigate required from unspiked non-uraemic serum samples aluminium detection limits below 2 μg dm^{-3}. Moreover, the 'off-line' ETAAS detection was time consuming (it requires fraction collection after elution) and chromatographic selectivity could be ruined as fraction are collected

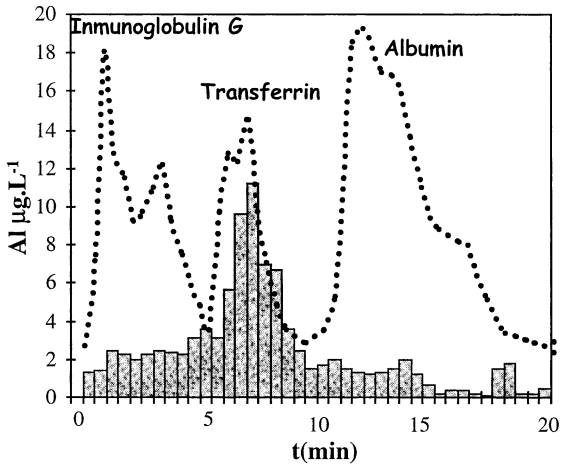

Fig. 3. Aluminium elution profiles (fraction volume 0.5 ml) of unspiked human uremic serum (93 μg dm^{-3} Al) [15,57]. (Reproduced by permission of The Royal Society of Chemistry.)

[15,56]. Thus, the potential of ICP-MS as an element-specific and very sensitive detector for aluminium and the possibilities of coupling on-line FPLC with ICP-MS were studied after adequated comparison of the performance of quadrupole (Q) and double focusing (DF) ICP-MS for Al detection in serum [57–59].

Therefore, separation of serum proteins was accomplished with the Mono-Q HR 5/5 anion exchange column [57] and the mobile phase was changed to an ammonium acetate gradient (0–0.25 mol l^{-1}) more compatible with the ICP-MS detector used. Fig. 4 shows the Al elution profile obtained using a Quadrupole-ICP-MS for undiluted serum sample of an uremic patient; while Fig. 4B shows the profile observed using a (DF) ICP-MS detector to especiate Al in a normal serum sample (around or lower than 2 μg dm^{-3} of the metal). As can be seen in the figure, the use of (DF)-ICP-MS at resolving power of 3000 was necessary to avoid CN^+ polyatomics producing serious interferences. These results demonstrated for the first time very low levels of Al in non-uremic human serum (that is, 'basal' Al levels in normal population) behave similarly to higher

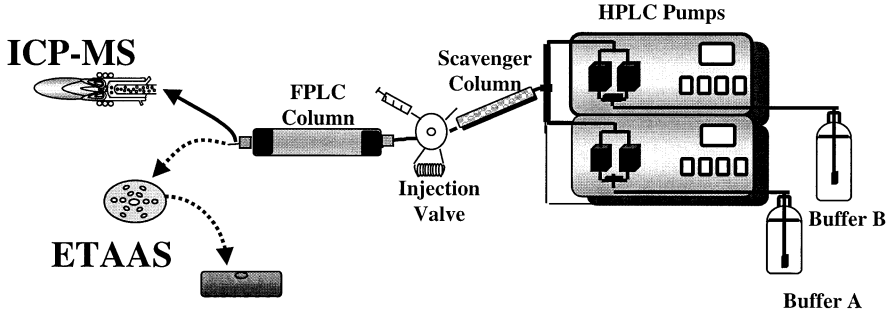

Fig. 2. Schematic diagram of the experimental setup used in the coupling of FPLC to ETAAS and ICPMS detection for Al-speciation studies.

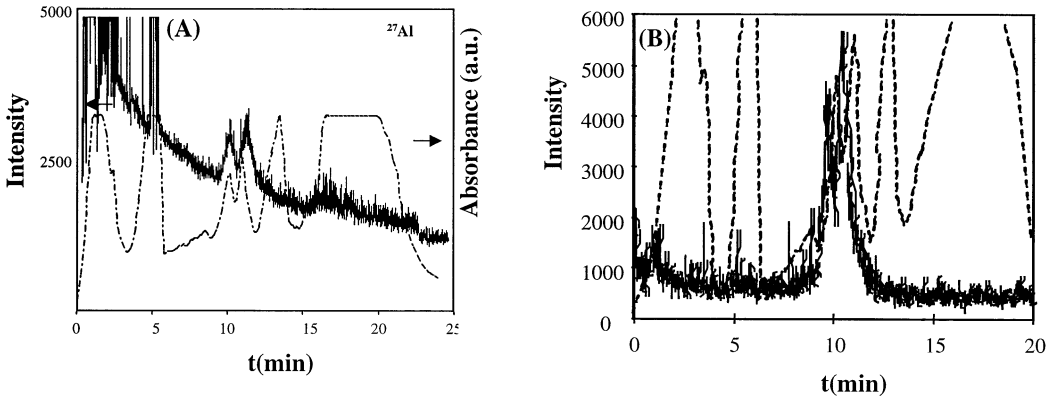

Fig. 4. Chromatogram of unspiked human serum, after FPLC separation of proteins using: (A) quadrupole-ICP-MS (Al content 120 μg dm^{-3}); (B) double focusing-ICP-MS (Al content 2.5 μg dm^{-3}) [57]. (Reproduced by permission of The Royal Society of Chemistry.)

levels of the metal studied before in spiked samples or Al-elevated uremic sera [57].

Fig. 4 shows an Al elution profile coeluting with transferrin. However, with this on-line detection two Al-transferrin peaks are apparent [57] indicating these Al-transferrin complexes [57,60–62]. It is known by now that Fe^{3+} has two metal binding sites [63], while Ga^{3+} [64] and Al^{3+} [62] are also capable of binding at these sites. Direct metal competition experiments have indicated that Al^{3+} competes for the same protein sites [62,63] and in all cases the binding of the trivalent ion requires simultaneous binding of bicarbonate or carbonate ion [65]. Thus, the types of Al-transferrin complexes should be similar to those of Fe-transferrin [60,61]. That is, one could expect monoaluminium-transferrin (Al-Tf) and dialuminium-transferrin (Al_2-Tf). Moreover, Al-Tf can produce two complexes: Al_N-Tf (Al bound to the N terminal lobe of transferrin) and Al_C-Tf (bound to the C terminal lobe of transferrin). Also Al, Fe-Tf multielemental complexes are possible.

In order to investigate the actual presence of such Al-transferrin chemical forms Nagaoka and Maitani [61] coupled a FPLC (Mono Q HR 5/5) separation on line with a DF-ICP-MS, as previously described by Sanz-Medel's group [57] but with a different gradient elution; Fig. 5 (from Ref. [58]) summarizes in a graphical way the type of results observed: it appears that the preferred binding site of Al in transferrin is the N-lobe (peak that appears at 32 min); confirming results previously established by Kubal et al. [66] using ^{1}H- and 2D-NMR and working with solution at pH 8.8. The second peak observed (19 min) can be ascribed to Al_2-Tf or Al_N, Fe_C-Tf, or both [61,67]. Since Tf could not be saturated with Al completely even by adding an excess amount of Al, the assignment of the $metal_2$-Tf peak to Al,Fe-Tf seems more probable [61]. The Al peak with the lowest height (11 min) could be assigned to Al bonded to transferring in the C lobe. It should be noted that the HPLC–UV chromatogram of the human serum showed four peaks that were associated to transferrin.

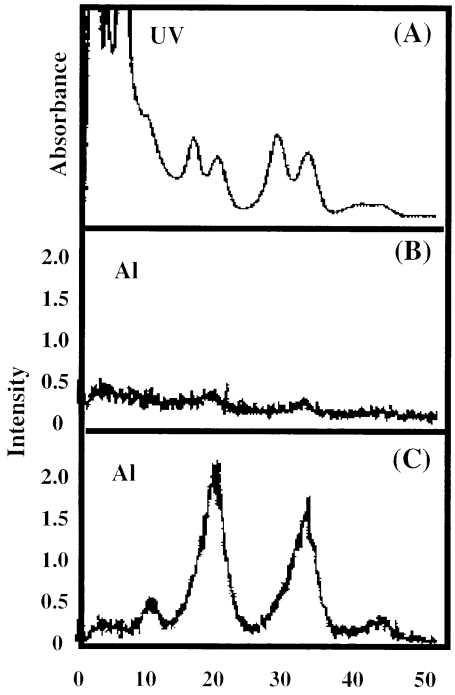

Fig. 5. HPLC-(DF)-ICP-MS chromatogram of a healthy people serum: (A) UV absorption at 280 nm; (B) unspiked human serum; and (C) human serum spiked with Al-citrate (with permission from Ref. [61]). (Reproduced by permission of The Royal Society of Chemistry.)

Since no Al peak was observed at 28 min, the peak at 28 min was assigned to apo-Tf.

3. Analytical techniques and chemical speciation of LMM-Al species in human serum

The distribution of LMW-Al species, playing an important role in the toxicity of aluminium, has been intensively investigated by computer simulation. On the basis of known thermodynamic equilibrium stability constants the LMW-Al species were predicted in human

serum [68–72]. Al-citrate, Al-phosphate, Al-hydroxide and mixed ternary complexes of Al-citrate–phosphate, Al-phosphate–hydroxide and Al-citrate–hydroxide were reported, but quantitative data on distribution of these species in human serum were rather controversial. In order to get a complete picture of aluminium speciation in humans, it is therefore necessary to experimentally determine the composition of LMM-Al complexes in human serum. This requirement has been clearly highlighted in investigations of the aluminium toxicity to humans.

For quantitative determination of LMM-Al species in human serum, microultrafiltration has been used to fractionate high molecular mass from LMM-Al complexes. Reported data indicated that 8–20% of total Al in spiked pooled serum of healthy volunteers corresponded to ultrafiltrable LMM-Al species [27,73]. Keirsse et al. [26] applied SEC with electrothermal atomic absorption (ETAAS) detection for speciation of Al species in spiked serum of healthy volunteer and spiked hemofiltrate of uremic patients. Beside HMM fraction, which represented 40% of total aluminium in spiked serum, two unidentified LMM fractions of aluminium were reported on P10 Bio-gel column. Two unidentified LMM fractions of aluminium were also found in spiked hemofiltrate when separated on P4 gel Favarato et al. [46] employed TSK-GEL HW 55S SEC column for separation of Al species present in the serum of normal and occupationally exposed subjects. Aluminium in the separated species was determined by ETAAS. Three to five separated fractions, which contained aluminium but were not identified were found. It was concluded that in order to determine the composition of LMM-Al species present in human serum the development of more powerful Al speciation techniques was required. Since Al-citrate has been theoretically predicted as the main LMM-Al binding ligand in human serum [68,69] efforts were first oriented to the development of reliable analytical procedures for the speciation of this biologically important complex.

3.1. Speciation of Al-citrate and some other LMM-Al species

An extensive study on the possibilities of Al-citrate speciation by high performance liquid chromatography with ETAAS final Al detection was reported by Datta et al. [74]. In order to examine the potential for Al-citrate separation from other Al species, various normal, reversed phase and mixed phase (ODS/NH_2) columns and different mobile phases were investigated. The best results for synthetic standard solutions of Al-citrate were obtained when cyclobond and cyanobonded phase columns were employed using a mobile phase MeOH–H_2O (1:1 v/v) and pH 4.0. However, the retention characteristics were not reproducible and Al-citrate recoveries were moderate and did not exceed 65%, probably because of adsorption of aluminium to the silica based columns. Due to that reason the technique was not recommended for routine quantification of Al-citrate in biological samples. Leung et al. [75] examined in vitro the effect of citrate on aluminium binding to plasma proteins, desferrioxamine and other constituents by gel filtration chormatography on Bio-Gel P-2 or FPLC on Superose-6 HR10/30 and HR 15/50 columns in the aluminium separated species was determined by ETAAS. A serum of a chronic renal failure patient was investigated. It was found that after addition of citric acid excess to serum, the chromatographic peak which was presumed to correspond to Al-citrate was significantly increased. In the above mentioned studies the LMM-Al species were not completely characterised or quantified. In the group of Milačič [76] a systematic study was carried out in order to develop a reliable analytical procedure for quantitative determination of LMM-Al complexes. A FPLC separation on Mono Q HR 5/5 strong anion-exchange column with ICP-AES detection was used with an aqueous—4 mol dm^{-3} $NaNO_3$ gradient elution. The hydrophilic polyether resin based column enabled quantitative determination of synthetic solutions of Al-citrate over a wide pH range from 3.5 to 11.0. At pH values higher than 8.0 $Al(OH)_4^-$ was identified. Due to its high tendency to be adsorbed strongly on the column resin, this species was quantitatively determined only at pH of 11.0. Although the repeatability of measurement was very good (RSD 2%), the applicability of the developed technique to most of the biological samples was limited due to its moderate sensitivity (LOD 0.1 mg dm^{-3} of the separated aluminium species).

3.2. Problems and progress on the determination of LMM-Al species in human serum: the analytical approach

The total concentration of Al in human serum of healthy subjects reported by Caroli et al. [12] ranged from 0.5 to 8 μg dm^{-3}, while a recent report from the Sanz-Medel's group indicated even lower normal aluminium concentration (in general below 0.35 μg dm^{-3}) [13]. Due to such very low concentrations, speciation of aluminium in healthy subjects had been possible only in spiked samples. Most of the investigators used spiked serum in such a way that total serum aluminium, after spiking, ranged between 100 and 200 μg dm^{-3} matching high concentrations that could be found in the serum of some dialysis patients. Since reported concentrations of ultrafiltrable aluminium in serum represented only 10–20% of total aluminium [27,73], it was necessary to apply very sensitive analytical procedures in order to identify and quantify the LMM-Al complexes present

even in spiked serum and in high aluminium level sera of dialysis patients.

Previously developed anion-exchange FPLC-ICP-AES procedure [76], using $NaNO_3$ as eluent was not sensitive enough. To lower the detection limits for speciation of Al-citrate to the low μg dm^{-3} concentration level, the choice of an appropriate eluent which would enable quantitative separation of Al-citrate on a Mono Q HR 5/5 strong anion-exchange column and also reliable determination of aluminium in separated species by ETAAS played a critical role. A systematic research of Bantan et al. [77] was performed examining the capability of various eluent solutions. Aqueous—4 mol dm^{-3} NH_4NO_3 gradient elution was found to separate quantitatively Al-citrate on the strong anion-exchange Mono Q HR 5/5 FPLC column in the pH range 3.5–8.0. The main advantage of NH_4NO_3 eluent lied in its ability to decompose quantitatively in the graphite tube during the ashing step, what enabled quantitative and very reproducible (RSD 2%) determinations of separated aluminium species 'off line' (0.5 cm^3 fractions) by ETAAS at the elution time between 4.5 and 5.5 min. Low limits of detection (2 μg Al-citrate dm^{-3}) provided good basis for the technique to be applied in analysis of LMM-Al species in spiked human serum. Investigation was performed on a pooled serum of healthy volunteers spiked to 50–150 μg dm^{-3} of Al^{3+} (nitrate salt) [77]. Special attention was paid to avoid contamination with exogenous aluminium. Analyses were carried out under clean-room conditions (class 10 000) using polyethylene or Teflon ware and recommended cleaning procedures. It was also important to lower the blank in FPLC separations. The NH_4NO_3 solutions, which were used as eluent was treated first with Chelex 100 (Na^+ form, 100–200 mesh, batch procedure), decanted and filtered. To remove traces of aluminium, additional step of cleaning, similar to that of D'Haese et al. [78], was applied. The reagent was passed through a silica based LiChrosorb RP-18 HPLC column which had strong affinity to adsorb aluminium. Since the FPLC Mono Q HR 5/5 column support also contained trace amounts of aluminium, the cleaning of the column was performed by the use of 5 mol dm^{-3} citric acid. Similar cleaning procedure was applied to remove traces of aluminium from the ultramicrofiltration membranes of Centricon 30 concentrators, which were used to separate HMM from LMM aluminium species in serum. It was found that the percentage of this ultramicrofiltrable aluminium in spiked pooled serum of healthy volunteers ranged from 15 to 19%. Speciation analysis of the ultramicrofiltrable fraction indicated that LMM-Al species were quantitatively eluted at the retention time typical for Al-citrate. On the basis of these observations it was presumed that LMM-Al corresponded to Al-citrate.

Since, in addition to citrate, phosphate was also considered to be an important and possible LMM ligand to bind aluminium in serum [71,72], there was a need to provide more detailed information on the aluminium species eluted under the corresponding chromatographic peak [77]. For this purpose Bantan et al. [79] characterised LMM-Al species in spiked serum not only on the basis of their retention times, but also by electrospray (ES)-MS–MS analysis of the LMM ligands eluted under the Al chromatographic peak. The study was performed on spiked serum of eight healthy volunteers in order to estimate also individual variability in the percentage and composition of LMM-Al species. Separation and determination of aluminium was done according to the previously developed analytical procedure [77]. To gain selectivity 0.2 cm^3 fractions were collected now throughout the chromatographic run and in each separated fraction, containing aluminium, ES-MS–MS analysis was performed. An example of results observed in such analysis is presented in Fig. 6.

Mass spectra, as shown in Fig. 6 indicated that in the fraction eluting at 2.4–2.6 min phosphate ligand (peak m/z 97 and corresponding daughter ion spectra with m/z 97 and 79) was present, while in fraction eluted at 3.0–3.2 min the presence of citrate was confirmed (peak m/z 191 and corresponding daughter ion spectra with m/z 111, 87 and 85). These data confirm that citrate and phosphate are associated with binding of LMM-Al species in this particular individual's serum sample. However, on the basis of FPLC-ETAAS and ES-MS–MS analysis of other serum samples [79] it was found, that LMM-Al species present in the serum were Al-citrate, Al-phosphate and ternary Al-citrate–phosphate complexes. These data were in agreement with the computer-aided speciation calculations performed in the group of Kiss [72]. The distribution of LMM-Al species varied among particular individuals. In some of them Al-citrate and Al-phosphate were the main LMM-Al species in serum, while in others the ternary Al-citrate–phosphate complex was also present. The serum of some other individuals did not contain Al-phosphate and the main LMM-Al species were either Al-citrate and Al-citrate–phosphate complexes or Al-citrate species alone. The individual variability was observed also in the percentage of LMM-Al species in spiked serum, ranging from 14 to 55% depending on the individual testing.

The FPLC-ETAAS procedure in combination with ES-MS–MS technique was also applied by the same group to speciation of LMM-Al species in the serum of six continuous peritoneal dialysis patients (CAPD) [80]. Three serum samples with low total aluminium concentration (below 12 μg dm^{-3}) were spiked before Al speciation analysis. Moreover, high total serum aluminium concentration (80–130 μg dm^{-3} Al) of three

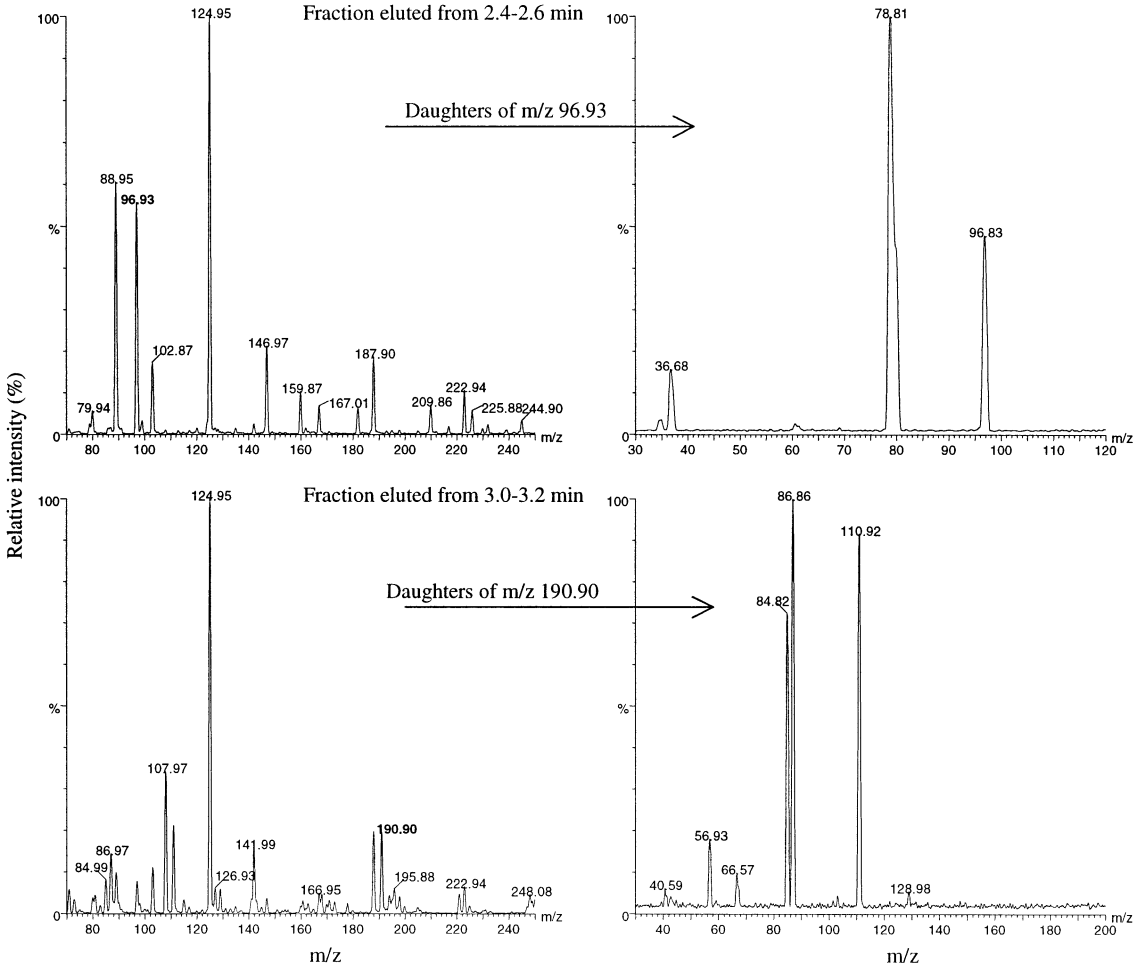

Fig. 6. ES-mass spectra and corresponding daughter ion mass spectra of *m/z* 191 and 97 for eluted fractions from 2.4 to 2.6 min and 3.0 to 3.2 min, respectively, on an anion-exchange FPLC column for spiked serum sample of healthy volunteer.

patients, who consumed Al-based drugs enabled the determination of the ultrafiltrable aluminium percentage and, for the first time, also its distribution of LMM-Al complexes in non-spiked samples. Data of that study indicated that the percentage of LMM-Al species in the serum of CAPD patients, in spiked and non-spiked samples, ranged from 24 to 53% and in one non-spiked sample was as high as 100% (no reason for such high content of LMM-Al species in this particular sample was found). LMM-Al species in spiked and non-spiked samples corresponded to Al-phosphate, Al-citrate and ternary Al-citrate–phosphate complexes. Data of this study also demonstrated that spiking of human serum with Al^{3+} did not influence the relative distribution of LMM-Al species. Al spiking of serum, therefore, seems appropriate to be applied in the investigations of the distribution of LMM-Al complexes in serum when the natural concentration of Al is too low to be detected reliably.

4. Conclusions

Nowadays, there is no doubt that analytical techniques for Al speciation in human serum are needed to appropriately address of the biomedical problems still awaiting solution. The vitality of the research work on the development of new analytical methodologies for Al speciation in human serum is obvious from the number of published papers during the last decade. However, it is clear that the speciation of Al in biological fluids has been fraught with difficulties in the past as is still in a state of development that has to surmount serious problems for its extensive application.

Although earlier work seems to have been plugged with serious contamination problems, some sort of consensus on the chemical speciation of serum aluminium has emerged in recent years based on results of some work carried out first by ultramicrofiltration, which demonstrates that usually 90% of total serum

Al^{3+} is not ultrafiltrable (i.e. the metal is bound to HMM biocompounds).

Speciation of Al in human serum is an extremely difficult task because the basal levels of this element in serum are bellow 2 μg 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. After revision of methods for speciation of aluminium in serum, the recommended analytical technique is the coupling of a powerful HPLC separation to a extremely high sensitive and selective DF-ICP-MS detector. This combination allows on-line Al speciation in human serum at levels typically found in such biological samples from healthy people.

HPLC separations coupled first 'off-line' with ETAAS and more recently 'on-line' with ICP-MS have shown that transferrin is the biocompound which binds Al^{3+} in serum. Thus Al-Tf complexes should be responsible for the transport of the metal in blood. Moreover, Al-Tf could be recognised by the transferrin cell receptors, e.g. brain (that is, a most likely pathway of Al^{3+} cellular uptake should be 'via' transferrin receptors, as it is the case for Fe^{3+}).

The percentage of ultrafiltrable aluminium in spiked pool sera ranged from 15 to 19% and increased (up to 53%) in the serum of CAPD patients. FPLC separation followed by ETAAS and also by ES-MS–MS studies showed that Al-LMM biocompounds (both in Al spiked and non-spiked uremic samples) correspond to Al-phosphate, Al-citrate and ternary Al-citrate–phosphate complexes. Moreover, chelation therapy of aluminium intoxication with the drug DFO seems to be based on formation of ultrafiltrable Al-DFO.

Finally, it was shown in different studies performed during the last decade that physiological behaviour and pathways in the organism of the metal aluminium tend to mimic the behaviour of Fe^{3+}.

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