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Convective Interaction Media Monolithic Chromatography with ICPMS and Ultraperformance Liquid Chromatography−Electrospray Ionization MS Detection: A Powerful Tool for Speciation of Aluminum in Human Serum at Normal Concentration Levels

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A new analytical procedure using separation support based on Convective Interaction Media (CIM) was developed for speciation of Al in human serum at normal concentration levels. The separation of proteins was performed on a weak anion exchange CIM diethylamine monolithic column. Isocratic elution with buffer A (0.05 M tris(hydroxymethyl)aminomethane−hydrochloric acid + 0.03 M sodium hydrogen carbonate) was applied for 5 min, followed by linear gradient elution from 100% buffer A to 100% buffer B (buffer A + 1 M ammonium chloride) for the next 40 min. Separation of proteins was followed by UV detection at 278 nm. Separated Al species were detected online by inductively coupled plasma mass spectrometry. It was experimentally proven that 91 ± 7% of Al in human serum was eluted under the transferrin peak. Transferrin was identified on the basis of the retention volume and by ACQUITY ultraperformance liquid chromatography−electrospray ionization mass spectrometry. The problem of extraneous contamination with Al was successfully overcome by using efficient cleaning procedures of eluents and chromatographic supports. The efficient cleaning was of paramount importance to perform Al speciation at extremely low concentration levels. The repeatability of measurement tested for six consecutive separations of unspiked serum was ±8.6%. The limits of detection and quantification (based on 3 and 10 s of the blank) were 0.15 and 0.49 ng mL^{-1} Al bound to transferrin, respectively. This is the first report on quantitative and reliable speciation of Al in human serum at normal concentration levels.

Elevated concentrations of Al can cause toxic effects to living organisms.[1] The main routes of entry of Al into the human body are through the diet, drinking water, and medications.[2] The reported normal serum Al concentrations are low, ranging from 0.5 to 8 ng mL^{-1}.[1] Patients with chronical renal failure may be subjected to higher Al concentrations via the contaminated dialysis fluids and consumption of Al-based drugs. Al overload in renal patients is related to many clinical disorders. Its accumulation in the brain and bone is associated with dialysis encelopathy[3] and osteomalacia.[4] The use of high-quality water for the preparation of dialysis fluids[5] and a lesser consumption of Al-based drugs prevent intoxication with Al. Several studies have demonstrated that Al is involved in the neurodegenerative processes in Alzheimer's disease.[6] Interest in Al bioavailability and toxicity resulted in intensive investigations that were carried out on speciation of Al by computer modeling and with the use of experimental analytical techniques.[1] These techniques identified and quantified particular groups of Al species. Speciation of low molecular mass (LMM) and high molecular mass (HMM) Al species in human serum and the serum of renal patients was usually performed by anion exchange or/and size exclusion chromatography (SEC) with electrothermal atomic absorption spectrometry (ETAAS)[7,8] or inductively coupled plasma mass spectrometry (ICPMS) detection.[9] The progress in Al speciation in biological samples has been achieved with the use of the robust anion exchange Mono Q fast protein liquid chromatography (FPLC) columns. These columns enable quantitative separation of either LMM-Al[7,10−13] or HMM-Al[7−9,13]

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species and are superior to silica-based columns on which the adsorption of Al on the column support is evident.[14] It was demonstrated by combining FPLC with ETAAS detection and ES-MS−MS techniques that LMM-Al species present in serum were aluminum citrate, aluminum phosphate, and ternary aluminum citrate phosphate complexes. Their composition and amount varied among particular individuals.[11,12] The results of the investigations on the HMM-Al species in serum in general suggested that at equilibrium about 90% of Al was bound to HMM proteins.[8,9,15] Aluminum binds to the two high-affinity iron binding sites of the serum iron transport protein transferrin (Tf).[16] It has been demonstrated that the favored binding site of Al in transferrin is the N-lobe site.[17,18] For accurate determinations of transferrin isoforms in human serum, combined chromatography techniques with ICPMS, MALDI-TOF, and ESI-Q-TOF were applied.[19,20]

The main problems in speciation of Al in the serum are low Al concentration and contamination by extraneous Al. The contamination from external sources may significantly contribute to nonreliable analytical data. Therefore, special attention must be devoted to efficient cleaning of chromatographic supports, eluents, and all devices used for the analysis. In addition, the analysis should be performed under clean room conditions. Several published studies have demonstrated different cleaning procedures of the eluents and chromatographic supports, which resulted in substantial lowering of the risk of contamination.[7−9,11,12,17,18,21] Efforts of researchers to perform Al speciation in human serum led to the use of robust and high-capacity chromatographic supports. Chromatography stationary phases based on monoliths were introduced in the early 1990s. Different types of monoliths differ in the method of their preparation, morphology, and chemistry.[22] Monolithic-based supports have properties that distinguish them fundamentally from the particle-based column. Their advantage properties are extremely high porosity, cheaper preparation, simple column filling, and high binding capacity.[23,24] Convective Interaction Media (CIM) monolithic supports (disks or columns) are based on polymethacrylate polymers, which are the largest and most examined class of monoliths.[22,24] Furthermore, CIM technology allows different types of chromatography (or its combinations) such as ion exchange, hydrophobic, reversed phase, and affinity. Several published studies have demonstrated their successful application for analytical or industrial purposes for the separation, purification, and isolation of peptides, organic acids, viruses, enzymes, and DNA molecules.[22,24−27] However, ion exchange chromatography of proteins still has the dominance among other applications of polymethacrylate-based monoliths.[23] In our group CIM disks were successfully applied for the speciation analysis of Zn in environmental samples[28] and Cr(VI) in samples from the workplace of plasma cutters.[29] Specifically, a CIM diethylamine (DEAE) disk was successfully applied for the speciation analysis of Al in spiked human serum.[15] To perform speciation of Al at normal concentration levels, a more powerful chromatographic tool is necessary. CIM monolithic columns with higher binding capacity than disks offer the potential for their use in speciation analysis of Al in human serum at normal concentration levels. A highly sensitive detector is required to determine low amounts of Al. In this regard, the coupling of a CIM monolithic column to an element-specific detector such as the ICPMS detector provides a sensitive and selective approach for the speciation analysis of Al in human serum at very low concentration levels. To the best of our knowledge, the reliable speciation of Al was until now done only in serum samples containing elevated Al concentrations.[9]

The present work illustrates the quantitative and reliable speciation analysis of HMM-Al that was obtained for the first time in unspiked human serum at normal concentration levels. This was accomplished with the use of a CIM monolithic column in combination with sensitive and selective mass spectrometry-based techniques: ICPMS and ACQUITY ultraperformance liquid chromatography−electrospray ionization mass spectrometry (UPLC−ESI-MS). The problem of extraneous contamination with Al was successfully overcome by using efficient cleaning procedures for eluents and chromatographic supports.

EXPERIMENTAL SECTION

Apparatus. HPLC separations were carried out using a high-performance liquid chromatography pump, series 1100, from Agilent (Tokyo, Japan) equipped with a sample injection valve, Rheodyne, model 7725i (Cotati, CA), fitted with a 1 mL injection loop (for SEC and CIM DEAE). A UV−vis detector (Agilent 1100 series diode array and multiple-wavelength detector, DAD/MWD) was used online with HPLC equipment for absorption measurements at 278 nm. Two chromatographic columns were used. SEC was performed on a Superdex 75 HR 10/30 column (Amersham, Uppsala, Sweden) (column dimensions 10 × 300 mm, 13 μm beaded composite of cross-linked agarose and dextran, pH stability 3−12, molecular permeation range from 3000 to 100000). A CIM DEAE-8 monolithic column (Bia Separations, Ljubljana, Slovenia) (column dimensions 1.5 mm i.d. and length 45 mm, with matrix supports made of highly porous poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) bearing a weak anion exchange DEAE

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Table 1. Operating Conditions for SEC, CIM DEAE, ICPMS, and UPLC–ESI-MS

	SEC parameters	CIM DEAE parameters
mobile phase	(A) 50 mM Tris–HCl + 30 mM $NaHCO_3$	(A) 50 mM Tris–HCl + 30 mM $NaHCO_3$ (B) phase A + 1 M NH_4Cl
elution	isocratic	isocratic, 0–5 min, 100% phase A linear gradient, 0–100% phase B, 5–45 min
injection volume	1 mL	1 mL
flow rate	1 mL min^{-1}	1 mL min^{-1}

	ICPMS parameters
forward power	1500 W
outer gas flow	15.0 L min^{-1}
carrier gas flow	0.80 L min^{-1}
makeup gas flow	0.11 L min^{-1}
He gas flow	3.0 mL min^{-1}
isotope monitored	^{27}Al
QP bias	−15 V
OctP bias	−18 V
extract 1	−0.3 V
extract 2	−150 V

	UPLC–ESI-MS parameters
scan type	positive TOF-MS
ion spray voltage	3 kV
nebulizing gas	N_2
injection rate (UPLC)	50 μL min^{-1}
external calibration	CsI
scan range	m/z 1000–3500
spectrum deconvolution	maximum entropy method (MaxEnt)

functional group, pH stability 2–14) was used for speciation analysis.

Element-specific detection of Al after the chromatographic separation as well as the total concentration of Al in serum was performed using an inductively coupled plasma mass spectrometer, model 7500ce, from Agilent Technologies (Tokyo, Japan) equipped with a collision/reaction cell system (ICP-(ORS)-MS).

A WTW (Weilheim, Germany) 330 pH meter was employed to determine the pH.

Positive ion mode ESI-MS measurements of transferrin solutions[30,31] were performed on a Q-oa-TOF Premier mass spectrometer (Waters Corp., Manchester, U.K.). The transferrin fractions were analyzed by an ACQUITY UPLC chromatograph coupled online to an ESI-MS instrument (UPLC–ESI-MS). For ACQUITY UPLC chromatography a VanGuard precolumn (2.1 × 5 mm, ACQUITY UPLC BEH C18, 1.7 μm, Waters) was used.

Experimental working conditions are summarized in Table 1.

Reagents and Materials. All chemicals were of analytical reagent grade quality. Human serum apo-transferrin, albumin, and imunoglobulin G (IgG) were purchased from Sigma-Aldrich (Steinheim, Germany). Buffer A composed of 50 mM Tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany)–hydrochloric acid (Merck) (Tris–HCl) + 30 mM sodium hydrogen carbonate (Merck), pH 7.4, and buffer B composed of buffer A + 1 M ammonium chloride (Merck) were prepared by dissolution of salts with ultrapure water. Ultrapure 18.2 MΩ cm water was obtained by means of a Direct-Q 5 ultrapure water system (Millipore Watertown, MA). A stock Al^{3+} solution (100 μg of Al mL^{-1}) was prepared in a 100 mL calibration flask by dissolving 0.1388 g of aluminum nitrate 9-hydrate (Riedel-de Haën, Hannover, Germany) in water. It was used for the preparation of calibration standard solutions for the determination of the total concentration of Al in serum, for the spiking of serum samples and samples of standard proteins, and for the study of Al^{3+} behavior on a CIM DEAE column. Fresh working standard solutions were prepared daily by dilution of stock solutions with water. A stock aluminum citrate solution (100 μg of Al mL^{-1}) was made by mixing citric acid (Merck) and aluminum nitrate 9-hydrate in a 100:1 citric acid to Al molar ratio.[11]

The certified serum sample, Seronorm trace elements of serum L-1, was obtained from Sero AS (Billingstad, Norway).

Sample Preparation. Venous blood (venous puncture) from a transplanted renal patient was taken during clinical examination after informed consent was obtained. It was collected into Al-free Becton-Dickinson vacutainers without additives. The sample was centrifuged for 10 min at 855 g. Serum aliquots were transferred into 1 mL polyethylene tubes with a polyethylene pipet and stored in a freezer at −20 °C. Prior to analysis samples were equilibrated to room temperature.

Standard proteins (5 g L^{-1} albumin, 1 g L^{-1} IgG, and 0.5 g L^{-1} transferrin) used for the optimization of the analytical procedure for the separation of serum proteins at pH 7.4 were dissolved in buffer A. The certified serum sample was reconstituted following the producer's instructions.

For the determination of total Al concentrations, serum samples were diluted (1 + 4) with water. To study the speciation of HMM-Al compounds, spiked and unspiked human serum was used. The spiking of the serum was performed with 100 μL of Al^{3+} solution (aluminum nitrate salt) added to 2 mL of serum, so that the final concentration of Al in serum was 10 ng mL^{-1}. Spiked serum was left to equilibrate at room temperature for 5 h.[10,11] Serum samples were directly injected onto the SEC column. Five-fold dilution with buffer A was applied before the serum samples were injected onto the CIM DEAE column. Speciation of Al was then performed following the recommended analytical procedures. It was experimentally proven that freezing of samples did not influence the speciation of Al. The same results were obtained when fresh or frozen serum samples were analyzed.

For identification of Al binding protein, diluted (1 + 4) serum sample was injected onto the CIM DEAE column. The separation of serum proteins was performed, and a fraction at the elution time of transferrin was collected for a further UPLC–ESI-MS experiment. For this purpose, the fraction was processed through a Centricon YM-30 (30 000 Da cutoff) centrifugal filter device (5000*g*, 10 min at 4 °C) to exchange the elution buffer by an aqueous solution and to reduce the final sample volume to 0.1 mL.

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Recommended Procedures. Sample preparation, chromatographic separations, and determination of Al by ICPMS were carried out under clean room conditions (class 10000).

Anion Exchange CIM DEAE−ICPMS. A 1 mL portion of the sample was injected onto a column resin. Isocratic elution with 50 mM buffer A was applied for the first 5 min, followed by a linear gradient for the next 40 min from 0% to 100% buffer B. A 10 min equilibration with buffer A followed. The chromatographic run was performed at a flow rate of 1 mL min^{-1}. The eluate from the CIM DEAE column was passed through a UV detector (set at 278 nm) for protein monitoring and was coupled with a sample uptake inlet of the Babington nebulizer of the ICP-(ORS)-MS detector used for the quantification of separated aluminum species.

SEC. The SEC separations were carried out by injecting 1 mL of undiluted sample onto the SEC column. Isocratic elution with buffer A was applied for 15 min. From 15 to 16 min a linear gradient from 100% buffer A to 100% buffer B followed. Elution with 100% buffer B was kept up to 29 min. From 29 to 30 min a linear gradient from 100% buffer B to 100% buffer A continued. In the following 10 min the column was equilibrated with buffer A. The chromatographic run was performed at a flow rate of 1 mL min^{-1} and was followed by UV detection at 278 nm.

UPLC−ESI-MS. For ESI-MS analysis it was necessary to clean and concentrate the reconstituted transferrin fraction to obtain the adequate charge distribution profile of protonated tansferrin molecules and its molecular mass. The transferrin standard (concentration 10 μmol L^{-1}) and transferrin fraction were analyzed by UPLC−ESI-MS. A 4 μL vollume of the sample solution was injected onto the VanGuard precolumn. The flow rate of 50 μL min^{-1} of mobile phases A (water, 0.1% formic acid) and B (acetonitrile, 0.1% formic acid) was used in a linear gradient of 5% phase B to 85% phase B in 6 min. The elution time of transferrin was 0.7 min and was twice the dead time of the LC system.

Cleaning Procedures. *Laboratory Ware, Tubes, and Eluents.* To avoid contamination by extraneous Al, polyethylene or Teflon laboratory ware and tubes were used. Before use, all laboratory ware and tubes for chromatographic separations and ICP determinations were treated with 10% HNO_3 for 24 h, rinsed well with Milli-Q water, and dried at room temperature. All eluents used in the chromatographic separations were efficiently cleaned by the procedure that was previously optimized in our laboratory.[10−12] The eluent was first subjected to chelating ion exchange chromatography (Chelex 100, Na^+ form, batch procedure) and then passed through a silica-based reversed-phase HPLC column.

Chromatographic Supports. SEC Column. The cleaning of the chromatographic support was performed at a flow rate of 1 mL min^{-1}. The column was first rinsed with water for 15 min. A 1 mL sample of 1 M NaOH was injected, and the column was rinsed with water for 15 min. A linear gradient elution from 100% water to 100% 2 M citric acid was applied for 10 min, followed by 10 min of rinsing with 2 M citric acid. Then a linear gradient elution from 100% 2 M citric acid to 100% water was applied for 10 min. The rinsing of the column with water followed for the next 15 min. Finally, the equilibration of the column was performed by rinsing with buffer A for 15 min.

CIM DEAE Column. The cleaning of the chromatographic support was carried out at a flow rate of 5 mL min^{-1}. A 5 min rinsing with 1 M NaOH was first applied. A 20 min rinsing with 0.2 M Tris−hydrochloric acid buffer (pH 7.4) followed. It is important to note that, after cleaning with 1 M NaOH, a more concentrated buffer for the regeneration of the column support was applied. After that the column was rinsed with buffer A for 20 min. Then 8 min of rinsing with 2 M citric acid was applied. At the end, the equilibration of the column was performed by rinsing with buffer A for 30 min. It should be stressed that buffer A used in the cleaning procedures was cleaned as described in the recommended cleaning procedures. It was experimentally found that the cleaning of the SEC and CIM DEAE columns should be applied after five and seven consecutive analyses of serum samples, respectively.

RESULTS AND DISCUSSION

Determination of the Total Al Concentration in Serum. The total Al concentrations in the serum samples were determined by ICPMS under the optimal operating conditions given in Table 1. To avoid contamination, Teflon bottles were used for rinsing solutions in the ICPMS system. To reduce memory effects, five rinses after each analysis of the serum sample (the first rinse with 5% HNO_3 and the following four with water) were applied. Before analysis the serum samples were diluted (1 + 4) with water. The accuracy of the determination of total Al was checked by the analysis of the reference serum sample. The obtained results of the determination of Al (7.1 ± 0.6 ng mL^{-1}) showed good agreement with the certified value (7.6 ± 0.7 ng mL^{-1}), confirming the accuracy of the analytical procedure applied. The concentration of Al in the serum investigated (mean of three parallel analyses) was determined by the standard addition method and was found to be 5.7 ± 0.4 ng mL^{-1}.

Development of the Analytical Procedure for the Determination of HMM-Al in Human Serum. *Optimization of the Parameters for the Separation of Serum Proteins by the Use of CIM DEAE−UV/ICPMS.* To optimize the analytical procedure for the separation of serum proteins, a synthetic solution of standard proteins IgG, transferrin, and albumin (5 g L^{-1} albumin, 1 g L^{-1} IgG, and 0.5 g L^{-1} transferrin) was prepared in buffer A. The concentration of standard serum proteins was similar to that in the 5-fold diluted human serum. Our previous investigations on separation of HMM-Al species in human serum demonstrated that the main advantages of the use of a CIM DEAE monolithic disk[15] in comparison to a Mono Q FPLC column[8] are its robustness and speed of the analysis. Besides disks, CIM DEAE monolithic supports are also available in column packings with higher binding capacity, which enables the injection of higher sample volumes. Therefore, the potential for the use of a CIM DEAE column was investigated to lower the detection limit for speciation of HMM-Al in human serum. It was experimentally proven that the maximal sample volume injected onto the CIM monolithic column that enables efficient separation of serum proteins was 1 mL of diluted serum sample (1 + 4). This amount is 2 times higher than the maximal serum sample volume injected onto the FPLC particle-packed columns[7−9,17] and 10 times higher than the volume injected on the CIM disk.[15] For the separation of standard serum proteins the following procedure

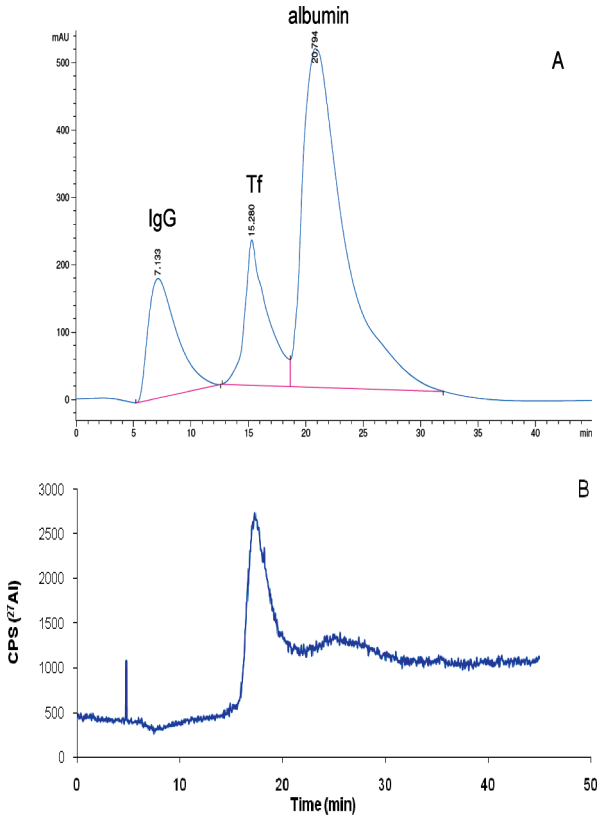

Figure 1. Separation of standard serum proteins by an anion exchange CIM DEAE column using UV (278 nm) and ICPMS detection: (A) UV chromatogram and (B) Al elution profile.

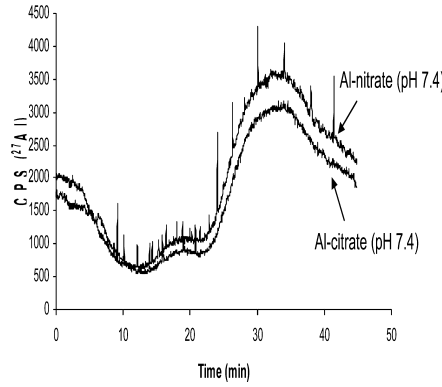

Figure 2. Al elution profiles obtained for the separation of aluminum citrate and aluminum nitrate (10 ng mL^{-1} Al) on the CIM DEAE column.

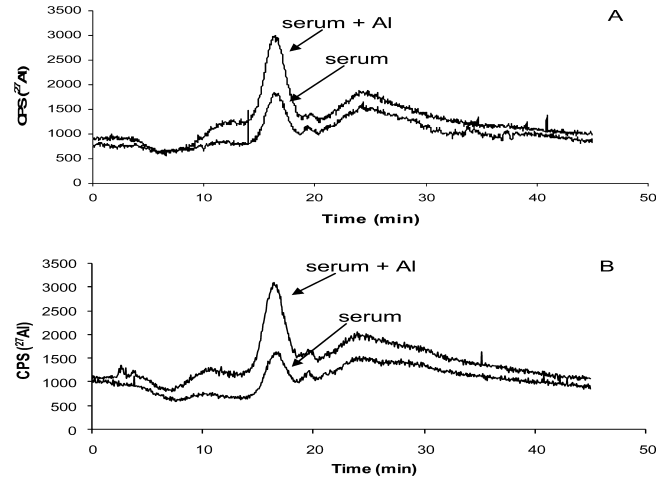

Figure 3. Al elution profiles for the speciation of Al in unspiked (1 + 4) and spiked (1 + 4, spike after dilution to 2 ng mL^{-1} Al) serum samples when (A) CIM DEAE−ICPMS was applied after preseparation of LMM-Al species by SEC or (B) when only CIM DEAE−ICPMS was used.

was applied. A 1 mL volume of a synthetic solution of standard serum proteins was injected onto the CIM DEAE column. As the eluent ammonium chloride (1 M) at pH 7.4 (buffer B) was applied. Optimization of chromatographic parameters indicated that the best resolution among standard serum proteins was achieved when after 5 min of isocratic elution with buffer A, a linear gradient elution from 100% buffer A to 100% buffer B in 40 min at a flow rate of 1 mL min^{-1} was applied. The separation of proteins was followed online by UV detection at 278 nm, while Al was detected by ICPMS. Figure 1 shows the separation of standard serum proteins on a CIM DEAE column under the optimized chromatographic procedures. As can be seen from Figure 1A, a good resolution among IgG, transferrrin, and albumin was obtained on the CIM DEAE column. It is evident from the elution profile of Al (Figure 1B) that Al was eluted at the elution time of transferrin. To determine the concentration of Al bound to transferrin, standard serum transferrin was spiked with 1, 2, 5, 10, and 20 ng mL^{-1} Al. Linearity of the measurement, within this concentration range, was obtained with a correlation coefficient better than 0.998. The concentration of Al in the sample from Figure 1B was calculated on the basis of the peak area and was found to be 3.0 ± 0.2 ng mL^{-1}.

Distribution of Aluminum Citrate and Ionic Al on the CIM DEAE Column at pH 7.4. Citrate occurs in human serum at a concentration of about 0.1 mmol L^{-1} and is considered to be one of the major LMM-Al binding ligands in human serum.[1] At physiological pH it is present as a negatively charged complex that is strongly retained by the anion-exchanged chromatographic supports.[10,11] In serum samples exists the possibility of coelution of negatively charged LMM-Al species[11] with serum proteins. Therefore, it is necessary to examine the behavior of aluminum citrate on the CIM DEAE column prior to the speciation analysis of Al bound to proteins. It is also important to know the behavior of ionic Al that may be present as a contaminant in eluents and chromatographic supports. For this purpose synthetic solutions of aluminum citrate and aluminum nitrate (10 ng mL^{-1} Al) were prepared in buffer A at a physiological pH of 7.4. The separation was performed under the optimized CIM DEAE chromatographic procedure. Al elution profiles are presented in Figure 2. Data from Figure 2 indicate that under the chromatographic conditions applied aluminum citrate and ionic Al (aluminum nitrate) are eluted as broad peaks from 25 to 45 min and do not overlap with the elution profile of Al bound to transferrin. It was experimentally proven that the shape of the chromatographic peaks was the same after several consecutive injections of Aaluminum citrate or aluminum nitrate. On the basis of these observations, it was presumed that preseparation of LMM-Al complexes prior to speciation analysis of HMM-Al was not necessary. However, to confirm this presumption, unspiked and spiked (10 ng mL^{-1} Al) serum samples were injected onto the SEC column and the separation of Al species

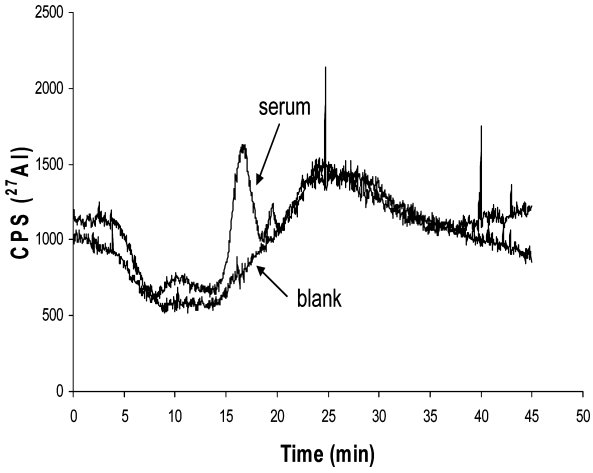

Figure 4. Al elution profiles for the separation of unspiked serum (1 + 4) and the blank sample after the overall cleaning procedure. The peak of Al in the unspiked serum sample (1 + 4) corresponds to 1.04 ng mL^{-1} Al.

was performed following the recommended procedure. Proteins were eluted in a 5 mL chromatographic peak[8,15] from 7.5 to 12.5 min and were separated from LMM-Al species. The protein peak was collected (sample diluted 5-fold) and further processed for protein separation using the CIM DEAE procedure. The same experiment was performed on unspiked and spiked serum samples without prior separation of LMM-Al from HMM-Al species, using only the CIM DEAE procedure. The Al elution profiles obtained by ICPMS are presented in Figure 3. It can be seen that the elution profiles for unspiked and spiked serum samples are the same for the two procedures when the speciation of Al by CIM DEAE−ICPMS was applied after preseparation of LMM-Al complexes by SEC (Figure 3A) or when the speciation of Al was performed only by CIM DEAE−ICPMS (Figure 3B). Furthermore, on the basis of the elution time, Al species separated corresponded to Al bound to transferrin. Their concentrations determined on the basis of the peak area also indicate that there are no differences between the two procedures applied. Al concentrations in unspiked and spiked serum applying SEC and CIM DEAE were 5.1 ± 0.4 and 15.4 ± 0.6 ng mL^{-1}, while those obtained by the use of CIM DEAE only were found to be 5.2 ± 0.4 and 15.3 ± 0.6 ng mL^{-1}, respectively. These data prove that when the recommended analytical procedure for speciation of HMM-Al in serum by CIM DEAE is applied, the preseparation of LMM-Al complexes by SEC is not necessary.

Elimination of Extraneous Contamination with Al. Due to the very low concentrations of Al in serum and the high environmental abundance of Al, there is a high risk of contamination during all steps of the analytical procedure. Appropriate handling of samples and efficient cleaning procedures (see the recommended cleaning procedures) should be therefore applied to perform reliable speciation analysis of Al in human serum at normal concentration levels. After the use of appropriate cleaning of the laboratory ware, tubes, and eluents, the concentrations of Al in all eluents determined by ICPMS were below 0.01 ng mL^{-1}. Furthermore, it is of paramount importance to efficiently clean the chromatographic supports as well. When the cleaning of chromatographic supports exactly followed the recommended cleaning procedures, extremely low blanks were obtained during the chromatographic separation. The Al elution profiles for the serum (diluted 1 + 4) and blank sample that are presented in Figure 4 clearly demonstrate that the overall cleaning procedure is extremely efficient. It was experimentally found that, after each cleaning of the CIM DEAE column, three blank samples should first be injected to obtain a reproducible and low blank chromatogram as presented in Figure 4. Al that contributes to the blank value of the overall analytical procedure is eluted as a broad peak from 25 to 45 min and does not overlap with the elution profile of Al bound to transferrin. On the basis of data from Figure 2 it may be presumed that Al impurities corresponded to ionic Al species. It should be stressed that the chemical and chromatographic stability of the methacrylate-based monolithic anion exchange DEAE column[32] enabled a rigorous cleaning of the chromato-

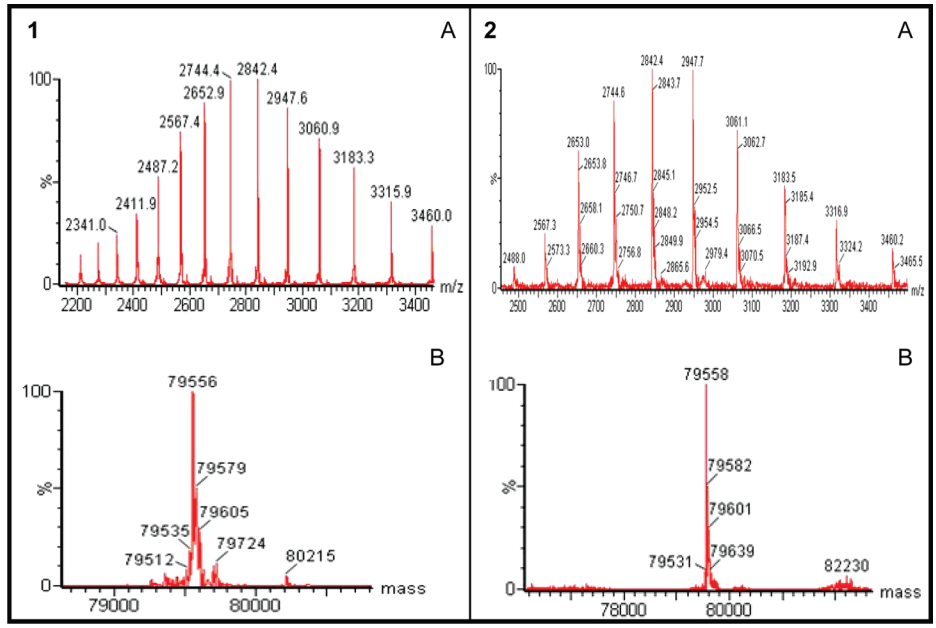

Figure 5. UPLC−ESI-MS analysis of transferrin standard (1A and 1B) and human serum transferrin after separation on a CIM DEAE column (2A and 2B): (A) ESI mass spectrum, (B) deconvoluted mass spectrum.

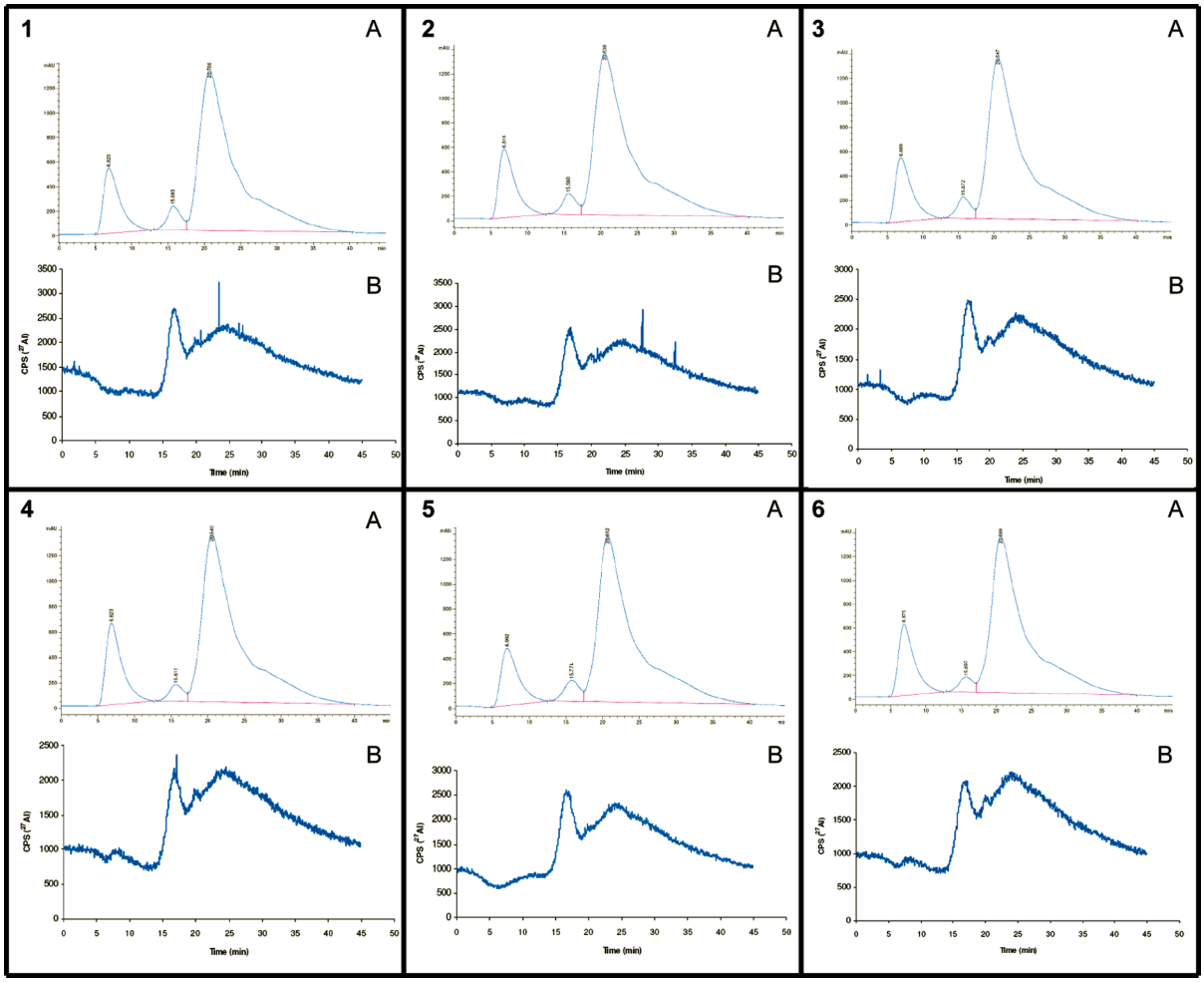

Figure 6. Six consecutive separations of unspiked human serum (1 + 4) on a CIM DEAE column with UV and ICPMS detection: (A) UV chromatogram and (B) Al elution profile.

graphic support. A low blank as presented in Figure 4 is essential to perform speciation analysis of Al in human serum at normal concentration levels.

Speciation of HMM-Al Species in Human Serum at Normal Concentration Levels. A serum sample that contained 5.7 ± 0.4 ng mL^{-1} total Al was diluted (1 + 4) with buffer A, and the speciation of HMM-Al species was performed by CIM DEAE−ICPMS. The concentration of Al bound to transferrin was determined by the standard addition method. The Al species bound to transferrin was quantified on the basis of the peak area and was found to be 5.2 ± 0.4 ng mL^{-1}. Speciation analysis of serum by the CIM DEAE−ICPMS procedure confirmed that 91 ± 7% of Al is bound to transferrin. To the best of our knowledge, this is the first time that quantitative determination of HMM-Al was performed in unspiked human serum at normal concentration levels. Excellent selectivity, high capacity, and extreme robustness of the CIM DEAE column allowed efficient cleaning of the chromatographic support, enabled in combination with ICPMS reliable quantification of Al in serum bound to transferrin. This study confirms that the percentage of Al bound to transferrin at normal serum concentration levels is the same as found for spiked serum samples by Medel's group[7] and by our group.[8,15] A imilar confirmation of Al binding to transferrin in unspiked serum of renal patients with elevated Al concentrations was reported by Medel's group[7,9] as well. The data on the speciation of Al at normal concentration levels may also serve as a basis for computational investigations.[33−35]

UPLC−ESI-MS Identification of Al Binding Protein. To prove that HMM-Al species in serum corresponded exclusively to Al−transferrin, the separation of the serum sample was performed by the CIM DEAE procedure. The fraction eluted under the retention volume of transferrin was collected and cleaned/preconcentrated, and transferrin in reconstituted solution was identified by UPLC−ESI-MS. Transferrin standard (1A and 1B) and serum transferrin (2A and 2B) are presented in Figure 5. Figure 1A represents ESI-MS peaks of serum transferrin standard from m/z 2211 ($[M + 36H]^{36+}$) to m/z 3460 ($[M + 23H]^{23+}$) with the charge distribution having the maximum at $[M + 28H]^{28+}$ (m/z 2842.4), while in Figure 1B deconvoluted mass spectra of transferrin standard (M = 79 756 Da) are presented. Figure 2 represents the ESI-MS data of the serum transferrin fraction collected from the CIM DEAE column at the same elution volume as Al detected by ICPMS. Data presented

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in Figure 5 confirmed that transferrin is the only protein that binds Al in human serum. This confirmation is in agreement with previously reported data obtained by SDS−PAGE.[8,36]

Repeatability and Limits of Detection and Quantification. The repeatability of the developed CIM DEAE−UV/ICPMS procedure was tested for six consecutive separations of unspiked serum. Figure 6 shows UV chromatograms and Al elution profiles for six consecutive injections of unspiked serum. Good repeatability of consecutive chromatographic separations was obtained. The concentration of Al bound to transferrin was determined by the standard addition method on the peak area basis. The average concentration of six consecutive separations was found to be 5.2 ± 0.4 ng mL^{-1} Al. The RSD of 8.6% for such low Al concentrations is also a great benefit of the developed analytical procedure.

The limit of detection was calculated as the concentration that provides a signal (peak area) equal to 3 s of the blank sample in the chromatogram. It was found to be 0.15 ng mL^{-1} Al bound to transferrin.

The limit of quantification was calculated as the concentration that provides a signal (peak area) equal to 10 s of the blank sample in the chromatogram. It was found to be 0.49 ng mL^{-1} Al bound to transferrin.

CONCLUSIONS

The developed analytical procedure combining an anion exchange CIM DEAE monolithic column with UV and ICPMS detection enabled quantitative and reliable determination of the composition and content of HMM-Al species in human serum at very low concentrations (LOQ = 0.49 ng mL^{-1} Al). To the best of our knowledge, this is the first report on quantitative determination of HMM-Al in unspiked human serum at normal concentration levels. Extreme robustness of the CIM DEAE column that allowed efficient cleaning of the chromatographic support and effective cleaning of eluents considerably lowered the blanks. The high capacity and good selectivity of the CIM monolithic column enabled, in combination with ICPMS, reliable quantification of Al in serum bound to transferrin. Transferrin was identified on the basis of the retention volume and also by ACQUITY UPLC−ESI-MS. The present study confirmed that the percentage of Al bound to transferrin at normal serum concentration levels is about 90% and is the same as previously reported for spiked and unspiked human serum with elevated Al concentrations. The data on the speciation of Al at normal concentration levels represent an important basis for computational studies of the Al distribution and fate in human body.

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