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KRATKA NAVODILA
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Simona Murko

SPECIATION OF ALUMINIUM IN HUMAN SERUM BY CIM® (CONVECTIVE INTERACTION MEDIA) MONOLITHIC CHROMATOGRAPHY AND MASS SPECTROMETRY

Doctoral Dissertation

SPECIACIJA ALUMINIJA V SERUMU Z UPORABO CIM® (STACIONARNA FAZA S KONVEKTIVNIM PRENOSOM SNOVI) MONOLITNE KROMATOGRAFIJE IN MASNE SPEKTROMETRIJE

Doktorska disertacija

Supervisor: Assist. Prof. Dr. Janez Ščančar

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MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
JOŽEF STEFAN INTERNATIONAL POSTGRADUATE SCHOOL
Ljubljana, Slovenia

Index

Abstract

The toxicity, biological availability and mobility of aluminium (Al) depend on its chemical form. In order to understand toxicity and transport of Al in human body it is important to identify and quantify Al species present in human serum. This led to the development of sensitive and selective analytical methods for reliable speciation of Al in serum. The aim of my PhD research work was the development of analytical methods for the identification and quantification of high molecular mass (HMM-Al) and low molecular mass (LMM-Al) Al compounds in human serum. For the separation of above-mentioned Al complexes different anion-exchange monolithic and particled packed supports and size exclusion columns were used. Concentration of Al in separated species was determined on-line by inductively coupled plasma mass spectrometry (ICPMS). The characterisation of ligands which bind Al was performed by electrospray mass spectrometry and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Speciation of Al in spiked human serum (up to 300 ng mL^{-1} of Al) was performed using anion-exchange monolithic separation support based on Convective interaction media (CIM®) in combination with size exclusion chromatography (SEC) and ICPMS. The separation of proteins was performed on a weak anion-exchange CIM® diethylamine (DEAE) monolithic disk. It was experimentally proven that 90 ± 3 % of Al in spiked serum investigated was eluted under the transferrin (Tf) peak. The proposed speciation procedure removes LMM-Al species and enables reliable determination of the concentration and composition of Al bound to proteins when the concentration of Al in serum is higher than 5 ng mL^{-1}. In comparison to chromatographic columns, the CIM® disks enable faster separation and simpler manipulation during cleaning procedure. The developed analytical procedure enables reliable determination of the concentration and the composition of HMM-Al species in spiked human serum.

A new analytical procedure using a CIM® monolithic column 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® DEAE monolithic column. Separated Al species were detected on-line by ICPMS. It was experimentally found that 91 ± 7 % of Al in human serum was bound Tf. Tf was identified not only on the basis of the retention volume but also by acquity ultra performance liquid chromatography - electrospray ionization mass spectrometry (UPLC-ESI-MS) and SDS-PAGE. 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 3s and 10s of the blank) were 0.15 and 0.49 ng mL^{-1} of Al bound to Tf, respectively. To the best of our knowledge, this is the first report on quantitative and reliable speciation of Al in human serum at normal concentration levels.

A new analytical procedure for the efficient, reliable and fast separation of proteins from LMM compounds in serum was developed. A HiTrap desalting SEC column enables separation of HMM from LMM species present in serum in 10 min. The Tris-hydrochloric acid buffer (pH= 7.4) was used as eluent. In the first 5 min HMM-Al compounds were eluted followed by the elution LMM-Al species from 5 to 10 min. HMM and LMM species were collected in 5 mL fractions. The speciation of protein peak was performed by anion-exchange monolithic disk coupled to ICPMS, while the LMM fraction was injected onto the anion-exchange fast protein liquid chromatography column (FPLC) connected to ICPMS. It was experimentally confirmed that 93 ± 4 % of Al is bound to Tf and that the remaining Al corresponded to LMM-Al compounds. Citrate, one of the major LMM-Al species in serum was identified in the LMM fraction by UPLC-ESI-MS. The rapidness and reliability of the developed analytical procedure represent a promising tools for investigations of kinetics of Al binding to Tf.

The new-developed analytical procedures for speciation of HMM and LMM Al species in serum in combination with other experimental and theoretical techniques, could help to explain the role, distribution and toxicity of Al present in the human body.

Povzetek

Toksičnost, mobilnost in biološka dostopnost aluminija (Al) so odvisne od kemijskih zvrsti v kateri se Al nahaja. Da bi razumeli toksičnost in transport Al v človeškem telesu je potrebno identificirati in kvantificirati Al zvrsti (speciacije) prisotne v humanem serumu. To je vodilo do razvoja občutljivih in selektivnih analiznih metod za zanesljivo speciacijo Al v serumu. Namen mojega raziskovalnega dela je bil razvoj analiznih metod za identifikacijo visokomolekularnih (VM) in nizkomolekularnih (NM) Al zvrsti v humanem serumu. Za separacijo omenjenih Al kompleksov sem uporabila različne anionsko izmenjalne monolitne nosilce in nosilce s stacionarno fazo na osnovi polnil s krogljicami ter velikostno izključitvene kolone. Koncentracijo Al v ločenih zvrsteh sem določila z masno spektrometrijo z induktivno sklopljeno plazmo (ICPMS). Identifikacijo ligandov na katere se veže Al sem izvedla s tekočinsko kromatografijo ultra ločljivosti z masno spektrometrijo z ionizacijo na osnovi razprševanja v električnem polju (UPLC-ESI-MS) in nativno gelsko elektroforezo (SDS-PAGE).

Za speciacijo Al v serumu z znano dodano množino Al (dodatek do 300 ng mL^{-1} Al) sem uporabila anionsko-izmenjalne monolitne nosilce, ki temeljijo na konvektivnem prenosu snovi (CIM®) v kombinaciji z velikostno izključitveno kromatografijo (VIK) in ICPMS. Separacijo proteinov sem izvedla na šibkem anionskem-izmenjalnem CIM® dietilaminoetil (DEAE) monolitnem disku. Eksperimenti so pokazali, da se 90 ± 3 % Al v serumu z znano dodano množino Al eluira pod vrhom transferina (Tf). Predlagan postopek za speciacijo Al v serumu odstrani VM-Al zvrsti in omogoča zanesljivo določitev koncentracije in sestave Al vezanega na proteine, kadar je koncentracija Al v serumu večja od 5 ng mL^{-1}. V primerjavi z ostalimi kromatografskimi kolonami je CIM® monolitni DEAE disk omogočal hitrejšo separacijo in enostavnejše rokovanje med postopkom čiščenja. Razvit analizni postopek omogoča zanesljivo določitev koncentracije in sestave VM-Al zvrsti v vzorcu seruma z dodatkom Al.

Z uporabo CIM® monolitne kolone sem razvila analizni postopek za speciacijo Al v humanem serumu, ki vsebuje normalne koncentracije Al. Separacijo proteinov sem izvedla na šibko anionsko-izmenjalni CIM® DEAE monolitni koloni. Ločene Al zvrsti sem sledila z ICPMS. Ugotovila sem, da je 91 ± 7 % Al v humanem serumu vezanega na Tf. Tf sem identificirala ne samo na osnovi retenzijskega volumna temveč tudi z UPLC-ESI-MS in SDS-PAGE. Da bi rešili problem kontaminacije, moramo uporaba učinkovite postopke čiščenja eluentov in kromatografskih nosilcev. Učinkovito čiščenje je bistvenega pomena pri speciaciji Al na ekstremno nizkih koncentracijskih nivojih. Ponovljivost merjenja, ki sem jo testirala s šestimi zaporednimi separacijami seruma brez dodatka Al je bila ± 8.6 %. Meji zaznavnosti in kvantifikacije (3s in 10s slepega vzorca) sta bili 0.15 and 0.49 ng mL^{-1} Al vezanega na Tf. To je prvo poročanje o kvantitativni in zanesljivi speciaciji Al v humanem serumu na normalnih koncentracijskih nivojih.

Razvila sem nov analizni postopek za učinkovito, zanesljivo in hitro separacijo proteinov od NM zvrsti v serumu. HiTrap velikostno izključitvena kolona omogoča separacijo VM od NM zvrsti v 10 min. Za elucijo sem uporabila Tris-HCl pufer. VM-Al zvrsti so se eluirale v prvih 5 min, medtem ko so se NM-Al zvrsti eluirale med 5 in 10 min. VM and NM zvrsti sem zbrala v dve 5 mL frakciji. Speciacijo proteinskega vrha sem izvedla na anionsko-izmenjalnem monolitnem disku v direktni povezavi z ICPMS. NM frakcijo sem injicirala na anionsko-izmenjalno hitro proteinsko tekočinsko kolono (FPLC). Eksperimentalno sem dokazala, da je 93 ± 4 % Al v humanem serumu vezanega na Tf, medtem ko je preostali Al vezan na NM zvrsti. Citrat, ki je ena glavnih NM-Al zvrsti v serumu, sem identificirala v NMM frakciji z UPLC-ESI-MS. Predlagani analizni postopki za speciacijo VM-Al in NM-Al zvrsti v kombinaciji z ostalimi eksperimentalnimi in teoretičnimi tehnikami omogočajo razumevanje vloge, porazdelitve in toksičnosti Al v človeškem telesu. Hitrost in zanesljivost razvitega analiznega postopka sta bistveni prednosti, ki omogočata preučevanje kinetike vezave Al na serumski transferin.

Abbreviations

AC = alternating current
AD = Alzheimer disease
AFS = atomic fluorescence spectrometry
amu = atomic mass unit
Aβ = amyloid protein
BBB = blood-brain barier
CAPD = continuous ambulatory peritoneal dialysis
CDGSs = carbohydrate-deficient glycoprotein syndromes
CDT = carbohydrate-deficient transferrin
CE = capillary electrophoresis
CIM® = Convective Interaction Media
CQ = clioquinol
CRC = collision/reaction cell
CSF = cerebrospinal fluid
CZE = capillary zone electrophoresis
Da = dalton
DC = direct current
DEAE = diethylamine
DFO = desferrioxamine
EDTA = ethylene-diaminetetraacetic acid
EI = electron impact
EM = electron multiplier
ESI = electro spray ionization
ETAAS = electrothermal atomic absorbtion spectrometry
FAAS = flame atomic absorbtion spectrometry
FDA = Food and Drug Administration
FPLC = fast protein liquid chromatography
HMM-Al = high molecular mass Al species
HPLC = high pressure liquid chromatography
ICPAES = inductively coupled plasma atomic emission spectrometry
ICPMS = inductively coupled plasma mass spectrometry
ID = isotope dilution
LA = laser ablation
LFER = linear free-energy relationships
LMM-Al = low molecular mass Al species
MALDI = matrix-assisted laser desorption ionization
Mr = molecular permeation range
PEEK = polyether ether ketone
Q = quadrupole
QA = quaternary amine
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC = size exclusion chromatography
Tf = transferrin
TOF = time-of flight
UPLC = ultraperformance liquid chromatography
UV = ultraviolet

1 Introduction

1.1 General introduction

Aluminium (Al) is the most abundant metal and the third most abundant chemical element in the Earth crust (Milačič, 2005). Due to its reactivity Al never occurs as a free metal in nature but it exists predominantly in sparingly soluble oxides and alumosilicates. Because of the widespread distribution of Al compounds, human exposure to this metal is unavoidable (Ščančar and Milačič, 2006). In mammals Al has no demonstrated essential function (Milačič, 2005). The exact evaluation of the risks for humans is directly related to a correct assessment of Al bioavailability. In this respect, compounds of Al are absorbed poorly from the gastrointestinal tract, but excessive intake can create problems. A major pathway for the elimination of any absorbed or systematically administered Al is the kidneys. Under ordinary health conditions the kidneys appear to be able to eliminate all of the absorbed Al. Nevertheless, Al is related to many clinical disorders especially when renal insufficiency occurs (Sanz Medel et al., 2002). Numerous studies about the connection of Al with dementia disorders such as Alzheimer and Parkinson disease have been reported. However, the participation of Al^{3+} in the etiology and/or development of Alzheimer disease (AD) are still a subject of discussion (Kozłowski et al., 2006). In order to better understand Al transportation, distribution and fate in the human body it is necessary to know the chemical species in which Al is present in body fluids and tissues (Milačič, 2005).

Speciation analyses are an area of analytical chemistry occupied with separation, quantification and identification of chemical species of an element in a sample. Among the different approaches used to undertake speciation analysis in samples hyphenated techniques, where an efficient separation technique is coupled off-line or on-line to element-specific atomic detector, are preferred. High performance liquid chromatography (HPLC) is the most common technique used for such separations including size exclusion and/or ion exchange. Although atomic absorption spectrometry - flame (FAAS) or electrothermal (ETAAS), atomic emission spectrometry - inductively coupled plasma atomic emission spectrometry (ICP-AES) or atomic fluorescence spectrometry (AFS) can be used for specific detection, there sensitivities are not as adequate as that of inductively coupled plasma mass spectrometry (ICPMS) (Cornelis et al., 2003; Milačič, 2005). Since identification of the element-binding ligand on the basis of retention volume and element specific detection is not enough, it is necessary to provide more detailed information on the identity of the species eluted under the chromatographic peak. Al speciation in biological samples is beset with many difficulties. One major drawback is that basal Al concetrations in healthy people are very low. Moreover, Al is ubiquitous element in nature so external contamination problems are almost unavoidable. Particularly for speciation, those low levels and the high risk of exogenous contamination demands special analytical ability and care (Sanz Medel et al., 2002). Among a variety of biological samples, Al speciation was most frequently done in human serum. Progress has been made in the development of analytical techniques for the determination of the amount and composition of high molecular mass Al (HMM-Al) as well as low molecular mass Al (LMM-Al) species in human serum (Milačič, 2005). Nevertheless, speciation analysis of HMM-Al or LMM-Al species in serum was up till now performed only in spiked serum or in serum with elevated Al concentrations.

1.1.1 Toxicity of Al

The sparingly soluble nature of most Al compounds considerably decreases the probability of an Al body burden in humans (Milačič, 2005). The bioavailability of Al can be raised by environmental and industrial factors, such as acid rain, water purification treatment with Al compounds, the use of Al as food additives, and certain pharmacological and therapeutic treatments such as aspirin and Al-based antacids. Al is present in certain foods and beverages such as tea, herbs and spices, grain products and processed cheese (Kozłowski et al., 2006). In the environment and biological systems, Al exists only in Al^{3+} oxidation state (Milačič, 2005). The gastrointestinal tract is relatively impervious to Al, absorption is normally between 0.6 and 0.1 %. Some individuals absorb 2-3 times more Al than others (Walton, 2009; Kozłowski et al., 2006). About half of the absorbed Al is retained in tissues and the other half is excreted by urine. Elimination from tissues is slow and in the face of constant intake, tissues accumulate Al over time. Gastric acidity and citrate, as well as other LMM organic acids including ascorbic, oxalic, lactic and tartaric acids favour the absorption of Al. Al is absorbed by a mechanism related to that of Ca. However, the exact mechanism is not yet understood (Yokel, 2004; Kozłowski et al., 2006). The major route of Al elimination from human body is by kidneys, since a small amount of Al is excreted in bile. Therefore, patients with renal insufficiency are more susceptible to Al toxicity. Al overload in renal patients has been largely prevented by eliminating Al-based, phosphate-binding agents which control their serum phosphate. In addition, until the situation was recognized and improved many dialysis patients were exposed to increased risk due to high Al content in some dialysis fluids (Milačič, 2005; Shirley and Lote, 2005). Exposure to high amounts of Al or an increased concentration of Al in blood due to decrease renal functionality can lead to the accumulation of Al in the brain. Al and other metal ions abnormally accumulate in the brain with aging. It has been estimated that Al deposits in the brain at a rate of 6 μg per year of life (Kozłowski et al. 2006, Hedge et al. 2009). Al may enter the brain from blood, either through the choroid plexuses or the blood-brain barier (BBB). The primary site of Al entry into the brain seems to be the BBB. There are at least two different mechanism of Al transport into the brain. One could involve the receptor-mediated Al-Tf influx as it was described for iron. This mechanism is not well supported. Tf saturated with Al^{3+} does not interact effectively with the Tf receptor and even if Tf solubilizes Al^{3+} in the biological fluids it does imply that the Al transfer from the blood stream into the cell is the receptor-mediated process. The studies with Al-citrate strongly indicate that there is likely a second mechanism of the Al transport. Very quick appearance of Al in the brain after injection of Al-citrate suggested other than the receptor-mediated transport of Al^{3+} into the brain. The transporter of Al-citrate is still unknown, although it could be one of the monocarboxylate transporters, which is still uncharacterized, or one of the members of the organic anion transporters, which is expressed at the BBB. In the brain, the calculations predict about 90 % of Al^{3+} in the form of citrate complex and only 4 % as Tf-bound Al^{3+} (Kozłowski et al., 2006).

The toxicity of Al has been recognized as a risk factor in a number of disorders including encephalopathy, osteomalacia or aplastic bone disease, renal osteodystrophy, proximal myopathy and microcytic anemia (Regel, 2004; Milačič, 2005; Kozłowski et al., 2006). Al has also been associated with certain dementias, such as AD, Parkinsonism dementia and amyotrophic lateral sclerosis. Al was the first metal experimentally linked to AD. The “Al hypothesis” for the connection between Al and AD originated when it was found that the injection of Al^{3+} salts into the brain of rabbits produced neurofibrillary degeneration and that the Al^{3+} concentration is increased in the brain of AD patients. However, controversy developed regarding the role of Al^{3+} in the etiology of this disease because subsequent experiments produced conflicting results. The results remain controversial also due to the complexity of Al chemistry in biological systems. Al has been found in amyloid plaque cores of human brain tissue. On the other hand, Landsberg et al. reported that amyloid plaques lack Al and proposed that Al involvement in AD pathogenesis must be artifactual (Walton, 2009). The absence of Al in plaque cores, however, was not considered to contradict the Al hypothesis, since Al was shown to be more often associated with the neurofibrillary tangles than with plaques. The distribution of Al in the brain is not deeply known and limited studies have been done. However, it seems that Al mainly localizes in temporal cortex and in hippocampus, two regions that are also known to be significantly involved in AD. In pathologies such as AD and Parkinsonism dementia, increased amounts of Al^{3+} in the brain of post-mortem patients have been reported. Al^{3+} content was found to be 19- and 5-times higher in Parkinsonism dementia and in AD compared to healthy individuals, respectively. On the other hand, there actually seems to be no reliable evidence for any link between Al and AD. Furthermore, Al is also known to influence the aggregation and toxicity of the amyloid protein (Aβ). An increased burden of amyloid plaques is observed in patients with renal failure, which involves accumulation of Al in the brain. Al accumulated in the AD brain accelerates

the amyloidogenetic process of the amyloid precursor protein (APP). However, there is a general consensus that Al is not a major risk factor if a risk factor at all. Recent studies shift the focus from Al to Cu, Zn and Fe as key players in AD (Kozłowski et al., 2006). In order to reduce the abnormal accumulation of essential metals, such as Fe, Cu and Zn or nonessential or poisonous metals such as, Pb, Cd and Al, chelation therapy has been proposed. Typically, chelator binds to metal ions enhancing their urinary and fecal excretion and causing a progressive decrease of their body concentrations. However, chelator can also undesirably chelate other metals in various tissues leading to serious side effects including depletion of essential metal, neurotoxicity and neurological changes (Kozłowski et al., 2006; Baral et al., 2008). The use of currently available iron or other metals chelators is limited by their toxicity and/or poor transference across the BBB. The most common chelators that have been clinically tested were ethylene-diaminetetraacetic acid (EDTA), desferrioxamine (DFO), rasagline and clioquinol (CQ). DFO is the only drug approved by Food and Drug Administration (FDA) for clinical treatment of Al intoxication (Liu et al., 2005; Baral et al. 2008). DFO is a chelator of trivalent metals and is still used against Al overloading in chronic dialysis treatment and in the treatment of Fe overload conditions, but no longer being pursued clinically for AD (Hegde et al., 2009). The clinical use of DFO suffers from a few important drawbacks like high cost, lack of oral efficiency and major side effects in the long term (Baral et al., 2008). In recent studies nanoparticles made of natural or artificial polymers present a possible tool for transportation of chelation agents across BBB. The advantages of nanoparticles include reduced drug toxicity, improved biodistribution and theraupeutic efficacy (Liu et al., 2005). Despite the high pharmacological interest for the delivery of new commercial products based on chelating agents on the market, future studies are needed to define not only the optimal type and amounts of chelator but to demonstrate the protective efficiency of the chelator. Furthermore, evaluation of their toxicity and optimization of their capability to cross BBB are also important.

As the significance of Al toxicity has become apparent, considerable attention has been given to defining chemical species in which Al is present in body fluids and tissues. To achieve this goal the complete characterization of the serum constituents which bind and transport Al to its target organ, such as brain or bone, is an imperative task (Sanz Medel et al., 2002; Milačič, 2005).

1.1.2 High molecular mass Al-binding ligands

The majority of Al^{3+} in human serum is bound to proteins. This fraction is considered to be the high molecular mass Al^{3+} fraction (HMM-Al). Albumin with the molecular weight of 66000 Da is present in human serum at concentrations of about 40 g L^{-1}. Some literature data in the past reported that Al^{3+} is bind to albumin and Tf (Leung et al., 1988). The poor resolution between albumin and Tf was the main obstacle for the identification of Al-binding protein in serum by SEC. With the use of anion-exchange chromatography for the separation of serum proteins there is no doubt that Tf is the only serum protein that binds Al^{3+}. Albumin is too weak as a metal ion binder at physiological pH values (7.4) to be able to effectively compete for Al^{3+} with other much stronger Al^{3+} carriers such as Tf and citrate. These experimentally based conclusions are in agreement with theoretical predictions (Milačič, 2005).

1.1.2.1 Transferrin and Al-transferrin

Tf is one of the best characterized human glycoproteins with the average molecular weight of 79,573 Da. It is synthesized in the liver and to a little extent in the reticuloendothelial system and endocrine glands. It consists of a single polypeptide chain of 679 amino acids. The Tf molecule is formed of two symmetric and independent lobes, the N-terminal (amino acids 1-336) and C-terminal (amino acids 337-679) domains with the carbohydrate moities in the C-terminal domain at asparigines 413 and 611. Each globular domain can contain one metal binding center which can be free or occupied with iron or other metal ions such as Al^{3+}, Ga^{3+}, Cr^{3+}, Bi^{3+} (del Castillo Busto et al., 2009). The structure of Tf is presented in Figure 1. The most important role of Tf is the transport of iron from sites of absorption and storage to sites of utilization. Tf binds iron as Fe^{3+} very tightly but reversibly (Harris and Messori, 2002). When Tf becomes iron-loaded, it is recognized by the Tf receptor 1 (TfR) and is internalized in the cytoplasm by receptor-mediated endocytosis (Hémadi et al., 2003). Quantitative binding of two Fe^{3+} ions to each lobe of Tf requires the concominant attachment of the synergistic anion, normally carbonate or bicarbonate. In both N- and C-terminal binding sites, Fe^{3+} is found in a six–coordinate, distorted octahedral coordination environment. Two tyrosines, one histidines and one aspartatic acid provide four ligating groups to the metal. The fifth and sixth coordination sites are occupied by carbonate ligand. Essentially no metal-protein binding occurs in the absence of carbonate or some suitable substitute anion. This requirement of a synergistic carbonate

anion for metal binding is often considered to be the defining characteristic of the members of the Tf family (Harris and Messori, 2002). Blood contains about 37 μmol L^{-1} of serum Tf, but each Tf molecule has two metal binding sites which mean that the total binding capacity is about 74 μmol L^{-1}. In normal serum the Tf binding sites are only 30 % saturated with Fe^{3+}, which leaves of about 50 μmol L^{-1} of unoccupied metal binding sites (Harris, 1996). However, this percentage can be changed in the case of certain disorders related to iron deficiency (e.g. iron anaemia) or iron overload (e.g. hemochromatosis). Depending on the iron content, serum Tf can be iron free (apo-Tf or Fe_0-Tf), partially (Fe_{1N}-Tf and Tf-Fe_{1C}) or totally saturated (holo-Tf or Fe_2Tf) (del Castillo Busto et al., 2009).

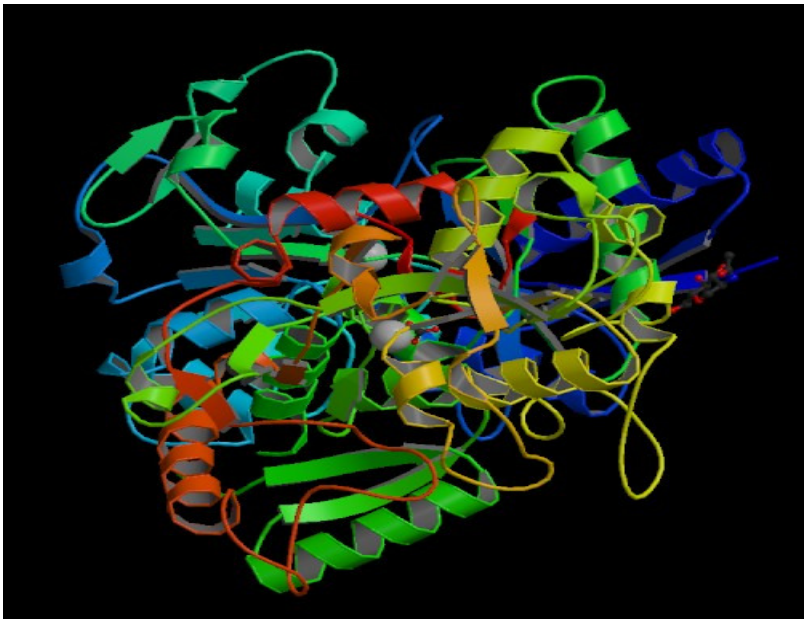

Figure 1: *The structure of Tf.*

Tf can be considered microheterogeneous due to both, the content of iron and the composition of the N-oligosaccharide chains (glycan). The two N-linked glycans attached to the protein can have different structures, containing four different carbohydrates such as N-acetyglucosamine, mannose, galactose and sialic acids. The glycans differ in their degree of branching showing bi, tri and tetra-antennary structures, each antenna terminated in a negatively charged sialic acid (N-acetylneuraminic acid) molecule. Attending at differences in sialic acid content, nine different Tf sialoforms or glycoforms can be distinguished. The most abundant form of Tf (65–80 % of total Tf) contains two biantennary N-glycans with four sialic acid residues and it is called tetrasialo-Tf (S_4). Minor glycoforms with two (disialo-Tf or S_2, < 2.5 %), three (trisialo-Tf or S_3, 5–9 %), five (pentasialo-Tf or S_5, 12–18 %), six (hexasialo-Tf or S_6, 1–3 %) and seven (heptasialo-Tf or S_7, < 1.5 %) sialic residues have also been found in the common serum. Also, in a healthy serum, only traces of asialo-Tf (S_0, <0.5 %) and monosialo-Tf (S_1, < 0.9 %) can be found. The normal distribution of Tf glycoforms in the human serum can be changed by several pathological conditions. The presence of some specific Tf glycoforms in serum in elevated concentrations can be used as a sensitive and specific biomarker for carbohydrate-deficient glycoprotein syndromes (CDGSs), chronic alcohol abuse and diagnosis of cerebrospinal fluid leakage (high proportion of S_0) (Durand and Seta, 2000; Arndt, 2001; del Castillo Busto et al., 2005; Castillo Busto et al., 2009). Congenital disorders of glycosylation (CDG), formerly named carbohydrate-deficient glycoprotein syndrome are rare hereditary disorders caused by mutations in the genes coding for enzymes involved in the biosynthesis of glycoproteins and other glycoconjugates. The clinical characteristics are variable, but often include psychomotor, growth and mental retardation from early childhood. CDG are the result of defects in the assembly and transfer (type I) or processing (type II) of the glycan moieties. The result is that carbohydrate chains are either completely missing (type I) or structurally abnormal (type II) (Delanghe and De Buyzere, 2009). Measurement of the Tf glycoform profile in serum, under the name carbohydrate-deficient Tf (CDT) is one of the most common diagnostic marker for detecting chronic alcohol abuse, exhibiting higher selectivity than other classical biomarkers (e.g. γ-glutamyl transferase, ethanol). Since the first report in 1976 (Stibler and Kjellin, 1976) many studies and critical reviews on CDT have been published (Arndt, 2001; Bortolotti et al., 2006;

Niemelä, 2007; Delanghe and De Buyzere, 2009). In the original definition, CDT includes the glycoforms with pIs ≥ 5.7 after complete iron saturation, corresponding to the sum of asialo-Tf, monosialo-Tf and disialo-Tf. More recent investigations have recognized disialo-Tf and asialo-Tf as being the main alcohol-related glycoforms (Oberrauch et al., 2008). Although thorough investigations in the past, the structure of human CDT is still known only partially and the role of glycosylation of Tf is still unclear. A number of analytical methods have been applied for the identification and quantification of CDT, including electrophoretic, chromatographic, immunometric and mass spectrometric methods. Each of these approaches has several advantages and limits in practice (Bortolotti et al., 2002; Bortolotti et al., 2006). For forensic and occupational medicine application, such as delegation to working sites where alcoholism may have fatal consequences or to obtain a driver's license, a highly selective, accurate, and precise method is needed. In general, HPLC and capillary zone electrophoresis (CZE) are to be preferred for CDT analysis in comparison to currently used immunoassay kits (Bortolotti et al., 2006; Delanghe and De Buyzere, 2009). More recent studies have applied on anion-exchange FPLC column in combination with ICPMS for the successful separations of Tf glycoforms before ESI-MS analysis (del Castillo Busto et al., 2005; del Castillo Busto et al., 2006). Despite the lack of consistency regarding CDT measurement, special attention on CDT standardization should be devoted. The aim is to define the analyte, select and validate a reference method and define the recommendations for the production of reference materials. Disialo-Tf was proposed as the primary target molecule for CDT measurement. The combination of HPLC and MS has been suggested as the method of choice for the development of a primary CDT reference method. Having access to a CDT reference material in a serum matrix will facilitate comparison of results between methods and aid in the standardization process (Jeppsson et al., 2007; Oberrauch et al., 2008).

Tf forms stable complexes with more than 40 different metals. Such binding may play an important role in the transport and delivery of medical diagnostic radioisotopes such as $^{67}Ga^{3+}$, $^{60}Co^{3+}$ and $^{111}In^{3+}$, toxic elements such as Al^{3+} and therapeutic metal ions such as Ru^{3+} and Ti^{4+} (Ha-Duong et al., 2008). Metal ions binding to Tf involve the phenolic groups of tyrosine side chains. Each of the two metal ions which bind to Tf requires a carbonate ion (Martin et al., 1987). Because of the unique involvement of the synergistic bicarbonate anion in the formation of metal-Tf complexes, metals are only bound as a ternary complex between the metal, the protein and a carbonate anion. The mechanism of Al^{3+} uptake by Tf is still not known (Hémadi et al., 2003). The Al^{3+} binding constants to Tf are high; Log K1 is 12.9 and Log K2 12.3. Since the corresponding Fe^{3+} binding constants are much higher (Log K1 22.7 and Log K2 22.1) there is no competition between Al^{3+} and Fe^{3+} in binding to Tf (Milačič, 2005). Al^{3+} can bind to the both two binding sites of Tf. It was assumed that Al^{3+} uptake starts with the C-lobe site in interaction with bicarbonate followed by that at N-lobe site. This is in agreement with the other researcher who reported that the favoured binding site of Al in Tf is the N-lobe site (Dejong at al., 1995; Nagaoka and Maintani, 2000; Nagaoka and Maintani, 2005; Harris and Messori, 2002). The order of lobe loading for Al^{3+} is identical to that of Fe^{3+}, which first occurs with the C-lobe site. The first step in binding Al^{3+} to Tf is quick and releases one proton. The final slow step releases additional two protons. Many investigators reported that Al^{3+} uses the Tf receptor-mediated pathway for its transportation from biological fluids to the brain or other organs. Thus, a part of the Al toxicity may be explained by the fact that its transport competes with that of iron, which disturbs its metabolism in the cell. This explanation is illogical, because there is still a question whether Al^{3+} transport occurs by the iron acquisition pathway. Data from studies indicate that there is no interaction or it is too weak to be detected between TfR and Al-Tf complex (by TfR mediated endocytosis occur iron uptake). Extremely rapid interaction between TfR and saturated metal-Tf complexes was observed for Ga^{3+}, Bi^{3+} and Co^{3+} (Hémadi et al., 2003; Ha-Duong et al., 2008). Moreover, Tf-mediated iron acquisition pathway is one of many metal transport systems that prevail in a living organism. Divalent metals, such as Cu^{2+}, Zn^{2+} or Cr^{3+} are known to form stable complexes with Tf without Tf-mediated iron acquisition path (Hémadi et al., 2003). Binding of Al to Tf and Al-Tf complex is still of interest for researchers since no crystal structure has been reported for the Al-Tf complex and also the complex has not yet been identified in human serum (Kozłowski et al., 2006).

1.1.3 Low molecular mass Al-binding ligands

LMM species are the most active in terms of bioavailability. Al bioavailability firstly depends on the composition of its LMM fraction (Venturini and Berthon, 1989). The fact, that one of the possible proposed mechanisms of Al transport to the brain is as Al-citrate, is not negligible (Ackley and Yokel, 1997). These observations indicate the important role of LMM ligands, particularly citrate, in Al absorption as well as in Al distribution in the body. Concentrations of citrate in serum are 5.8-76.8 mg L^{-1} and in cerebrospinal fluid (CSF) 17.3-113.4 mg L^{-1}. Citrate forms complexes with many transition metals under physiological

conditions and is therefore considered an important low molecular mass ligand for metal transport in the human body (Hoffmann et al., 1993). Clinical and experimental studies have shown that the gastrointestinal absorption of Al is markedly increased by the presence of citrate and that the administration of Al-citrate causes the enhancement of Al concentrations in tissues (Bantan Polak et al., 2001). The Al citrate complex is formed through coordination binding of Al with the hydroxyl group and the two terminal carboxylates of citrate, thus leaving a free carboxylate and leading to dissociation at physiological pH. The complex is extremely stable (Nagasawa et al., 2006; Zhou et al., 2008). Although the majority of the citrate in serum is present as the Ca^{2+} salt, Al^{3+} easily displaces Ca^{2+} from citrate. Citrate solubilizes Al^{3+} from insoluble $Al(OH)_3$ and $AlPO_4$. Therefore it is strongly recommended that citrate compounds are avoided in the diet of dialysis patients (Milačič, 2005).

In the human serum the total concentration of phosphate is about 2 mmol L^{-1}. Al^{3+} readily forms sparingly soluble species with PO_4^{3-}, which may precipitate from body fluids as a mixed phosphato-hydroxy complex and under serum conditions may be described as $Al(PO_4)_{0.2}(OH)_{2.4}$. Due to precipitation reactions the solution chemistry of the phosphate system may be directly studied only at pH < 4, so it was scarcely investigated. In many investigations soluble Al-phosphate species were not involved in speciation models and citrate was considered as the prevailing LMM-Al specie in human serum. In order to estimate the binding constants of Al-phosphate species at physiological pH, linear free-energy relationships (LFER) have been used. Stability constants estimated by LFER calculations were then used in computer modeling of Al-phosphate at physiological pH. The prevailing LMM-Al species in human serum are deduced to be the mixed hydroxy complex $Al(PO_4)(OH)^-$ (Milačič, 2005).

Proposed models for biological speciation took into consideration only binary species of Al^{3+} with LMM ligands. Since biological systems contain various potential LMM-Al binding ligands, the formation of ternary Al complexes is also possible. In the group of Kiss (Lakatos et al., 2001) a comprehensive investigation was carried out on the main potential LMM-Al binders in blood serum. The species distribution of binary Al-citrate and Al-phosphate and ternary Al-citrate-phosphate systems was calculated in a pH range from 2 to 8. The data clearly demonstrated that at physiological pH, the LMM-Al species present in serum are binary citrate $(AlAH_{-2})^{2-}$ and phosphate $(AlBH_{-1})^-$ complexes, and ternary species $(AlAB)^{3-}$ and $(AlABH_{-1})^{4-}$ (Milačič 2005). The percentage of particular LMM-Al species varies with the total concentration of Al in serum. Further, Bantan and co-workers (Bantan et al., 1999; Bantan Polak et al., 2001) experimentally proved that the main LMM-Al species present in serum were Al-citrate, Al-phosphate and ternary Al-citrate-phosphate complexes. The study was performed on the spiked serum of eight healthy subjects in order to estimate individual variability in the percentage and composition of LMM-Al species. 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 complex or Al-citrate species alone. Individual variability observed in the percentage of LMM-Al species in the spiked serum ranged from 14 up to 55 %. These data are in agreement with the computer-aided speciation calculations in human serum performed in the group of Kiss (Milačič, 2005).

Other LMM constituents of blood serum like lactate, oxalate and amino acids have significantly lower affinities for Al^{3+} than citrate and phosphate and were therefore not considered in modeling calculations. The presence of $Al(OH)_4^-$ was predicted in human serum but at a very low concentration, representing only about 3 % of total LMM-Al species (Milačič, 2005).

1.1.4 Monolithic chromatography

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 (Barut et al., 2008). The separation support in monolithic chromatography is made of a single piece of a rigid porous material that is tightly sealed into the wall of a tube (Ščančar and Milačič, 2009a). Monoliths are formed from synthetic polymethacrylate, polyacrylamide and polystyrene polymers, natural agarose polymers or from inorganic silica, zirconium and hafnium and carbon based supports. The supports are available in disk, column and tube packings (Ščančar and Milačič, 2009b). Mass transfer in monoliths is mainly based on convection and that is the basis for naming one particular type of these supports Convection Interaction Media® (CIM®) (Forčič et al., 2005). CIM® monolithic supports are based on polymethacrylate polymer, which are the largest and most examined class of monoliths (Vlakh and Tennikova, 2009). Methacrylate monoliths are made through bulk polymerisation from glycidyl methacrylate that is crosslinked with ethylene glycol dimethacrylate, while as porogens cyclohexanol and dodecanol are used. The pore size distribution depends on the polymerisation temperature: at higher temperatures smaller pores are formed. Methacrylate based

monoliths possess excellent mechanical and chemical stability over a wide pH range and withstand the regeneration with NaOH (Ščančar and Milačič, 2009a). Furthermore, CIM® technology allows different types of chromatography (or its combinations) such as ion-exchange, reverse-phase, affinity, activated and hydrophobic/hydrophilic interaction chromatography (Štrancar et al., 1998).

Monoliths have properties that distinguish them fundamentally from the particle-based column (Vlakh and Tennikova, 2009). The advantage properties of monolithic supports are extremely high porosity, cheaper preparation, simple column filling and high binding capacity (Podgornik and Štrancar, 2005). Other advantages of monolithic based supports are related to very efficient mass transport at low back pressures, good separation efficiencies and operation of chromatographic separations at higher than usual flow rates (Ščančar and Milačič, 2009b). Another advantage in comparison with conventional chromatographic supports is that air bubbles do not lower the separation power or destroy the support (Štrancar et al., 1998). Besides, up to four disks may be put together into a housing, increasing the volume and hence the column capacity. Some important characteristics of ion-exchange FPLC and CIM® monolithic chromatographic supports are summarised in Table 1.

Table 1: *Some important characteristics of ion-exchange FPLC columns (Pharmacia, Uppsala, Sweden) and CIM® monolithic supports (Bia Separations, Ljubljana, Slovenia) reported by the producers.* (Adopted from Ščančar and Milačič, 2009a)

Parameter and characteristics	Mono Q HR 5/5 column	Mono S HR 5/5 column	CIM® QA disk	CIM® DEAE disk	CIM® DEAE 8 mL column
Chromatographic support	10 μm beaded hydrophilic polyether resin	10 μm beaded hydrophilic polyether resin	highly porous poly(glycidylmethacrylate-co-ethyleneglycol-dimethacrylate) monolith	highly porous poly(glycidylmethacrylate-co-ethyleneglycol-dimethacrylate) monolith	highly porous poly(glycidylmethacrylate-co-ethyleneglycol-dimethacrylate) monolith
Chemical characteristic	strong anion-exchanger, quaternary amine	strong cation-exchanger, methyl sulfonate	strong anion-exchanger, quaternary amine	weak anion-exchanger, diethylamino	weak anion-exchanger, diethylamino
Column/disk dimensions	i.d. 5 mm lenght 50 mm	i.d. 5 mm length 50 mm	i.d. 12 mm length 3 mm	i.d. 12 mm length 3 mm	i.d. 1.5 mm length 45 mm
Column/disk volume (mL)	1	1	0.34	0.34	8
Working flow rate (mL min^{-1})	0.5 - 2	0.5 - 2	0.5 - 10	0.5 - 10	1 - 40
Chemical pH stability	2-12	2-12	2-14	2-14	2-14
Maximal amount of NaOH recommended for cleaning	0.5 mL 2 mol L^{-1} NaOH (may stay on column support during the time of analysis)	0.5 mL 2 mol L^{-1} NaOH (may stay on column support during the time of analysis)	10 mL 1 mol L^{-1} NaOH (may stay on disk support up to 3 hours)	10 mL 1 mol L^{-1} NaOH (may stay on disk support up to 16 hours)	80 mL 1 mol L^{-1} NaOH (may stay on column support up to 16 hours)

It can be seen from Table 1, that regarding ion-exchange bearing groups, the same type of strong anion-exchanger (quaternary amine) is applied in Mono Q FPLC column and CIM® QA disk, while weak anion-exchanger (diethylamino) is used in CIM® DEAE monolithic supports. For the time being there are no weak anion-exchange FPLC columns available on the market. FPLC columns enable separations at flow rates from 0.5 up to 2 mL min^{-1}, while CIM® disks allow separations at flow rates from 0.5 up to 10 mL min^{-1}. The flow rate in CIM® column may be even higher, from 1 up to 40 mL min^{-1}. Operation at high flow rates and the ability of cleaning the chromatographic supports with aggressive reagents in speciation

analysis is of crucial importance. The appropriate cleaning procedures are one of the most important steps in the speciation of Al in human serum or in other biological samples (Murko, et al. 2009; Ščančar and Milačič, 2009a; Milačič et al., 2009). CIM® QA and CIM® DEAE columns and disks permit extremely rigorous cleaning of the chromatographic supports, since they have exhibited long-term stability even after being exposed to 1 mol L^{-1} NaOH for up to 3 months or even 1 year, respectively (Vidič et al., 2007). Nevertheless, the main advantage of the CIM® monolithic procedure lies in the speed of the chromatographic separation.

Widespread use of monolithic chromatography covers industrial separation of biomolecules, purification of proteins and DNA, separation of biomolecules like serum proteins, nucleosides, nucleic bases and sugar derivates, nucleic acids and peptides (Ščančar and Milačič, 2009a). They were also applied in the analysis of impurities in immunoglobulin concentrates, analysis of organic acids and in the isolation of tomato pectin methylestearase and polygalacturonase. Recently, the concept of monolithic columns has been transferred to the fast separation of inorganic anions like chloride, chlorate, iodide, iodate, bromide, bromate, nitrite, nitrate, sulphate and phosphate and also allowed the separation of hydroxide anion and thiocyanate and chromate from some other anions. However, only a limited number of applications were reported for the use of monolithic phases for rapid separation of metal cations and for speciation analysis (Ščančar and Milačič, 2009b). By the use of reversed phase silica-based monolithic column, As excretion was investigated after ingestion of rice. In this study five As species were determined in rice and in urine by ion-pairing monolithic silica-based chromatography coupled to ICPMS. Other applications were related to speciation of elements by the use of methacrylate based CIM® monolithic supports. Chelating CIM® monolithic disks were applied in a flow analysis system for fractionation of labile Cu species in environmental water samples (Ščančar and Milačič, 2009a). Svete et al. (Svete et al., 2001) investigated the possibilities of using CIM® disks for the speciation analysis of Zn in environmental samples. Ščančar and Milačič (Ščančar and Milačič, 2002) reported about the successful application of CIM® monolithic supports for speciation of Cr(VI) in samples from the workplace of plasma cutters. The main advantage of the CIM® monolithic procedure in comparison to FPLC column lay in the speed of the chromatographic separation. The chromatographic run for the separation of Cr(VI) was completed in 15 min by applying CIM® DEAE disk and in 30 min by using the FPLC column. Speciation methods require an efficient and rapid separation of element species. Fast analyses can help to preserve the original species present in the sample and prevent any species conversion. Monolithic chromatography has great potential and needs to be investigated further for the application in the field of elemental speciation and studies in metallomics (Ščančar and Milačič, 2009b). Based on the above findings the potential of monolithic chromatography was also investigated for Al speciation in human serum (Murko et al., 2007; Murko et al., 2009).

1.1.5 Analytical techniques for speciation of Al in human serum

Determination of total element concentration cannot usually provide the required information about mobility, bioavailability and the impact of elements on ecological systems or biological organisms (Milačič, 2005). Speciation analyses is an important sub-discipline of analytical chemistry and could contribute considerably to the understanding of processes in environmental and health sciences which have been up to now the most important areas of its application (Cornelis et al., 2003).
In order to understand the toxicity of Al in humans, it is essential to identify and quantify the chemical species in which Al is transported and stored in the body. For these reasons, speciation of Al in human serum has been intensively investigated (Milačič et al., 2009).

1.1.5.1 Fractionation and separation of Al species in human serum

First attempts in the speciation of Al in human serum were oriented to distinguish between the percentage of LMM-Al and HMM-Al species. For this purpose fractionation of serum samples using ultrafiltration and microultrafiltration procedures, SEC and HPLC TSK G 4000 SW column were performed (Milačič et al., 2009). Since the classical ultrafiltration procedure was more liable to contamination with extraneous Al, microultrafiltration which minimized contamination risk gives more reliable results. Data indicated that in spiked serum of healthy subjects about 8 % of total Al was ultrafiltrable, while in renal patients this percentage was about 13 %. Wróbel et al. (Wróbel et al., 1994) reported that about 11 % of total serum Al was ultrafiltrable. They found that the percentage was influenced neither by the individual renal pathology of the patients nor by kidney transplantation. In further investigations Wróbel et al. (Wróbel et al., 1995) reported that ultrafiltrable Al in spiked human serum was found to be 12 ± 5 %. Fractionation data obtained by other investigators indicated that in normal, non-exposed subjects, the percentage of microultrafiltrable

Al was about 20 %. It was also experimentally demonstrated that the percentage of LMM-Al in spiked serum was the same, regardless the total Al concentration in the spiked serum. However, it was found that the percentage of ultrafiltrable Al varied between different pooled serum samples and ranged from 15 to 19 %. From these data it was concluded that the majority of Al, about 90 %, is bound to the HMM protein fraction (Milačič, 2005). The same observations were reported by a study on the fractionation of Al by SEC with UV and ETAAS detection in spent continuous ambulatory peritoneal dialysis (CAPD) fluids (Milačič et al., 2009). The SEC analysis of protein bound Al in serum is rather complicated due to the critical role of bicarbonate in the binding of Al by Tf. In the absence of bicarbonate in the eluting buffer, more peaks and a greater proportion of LMM-Al fraction were observed. In contrast, when bicarbonate was added to the eluting buffer, fewer peaks were observed in the SEC chromatogram and the largest peak appeared in the Tf and albumin region. Due to the poor resolution between Tf and albumin it was not possible to identify the Al-binding protein in serum by SEC procedures (Milačič, 2005; Milačič et al., 2009).
In order to obtain higher resolution in separation of serum proteins anion-exchange chromatographic columns were applied. Blanco González et al. (Blanco González et al., 1989) and Garciá Alonso et al. (Garciá Alonso et al., 1989) used a TSK DEAE-3SW silica-based ion-exchange column and 0.05 mol L^{-1} (hydroxymethyl) aminomethane Tris-hydrochloric buffer. Their investigations indicated that Al was bound only to Tf, but a significant amount of Al was retained by the silica-based column support. To prevent Al adsorption on the column Wróbel et al. (Wróbel et al., 1995) used a polymeric anion-exchange (Protein Pak DEAE-5-PW) column for separation of serum proteins. Linear gradient elution was applied in 30 min, using (0-1.0 mol L^{-1}) NaCl in Tris-hydrochloric buffer (pH 7.4) containing 0.01 mol L^{-1} of $NaHCO_3$. The elution profile was detected with a UV detector at 280 nm. The results again confirmed that Tf was the only serum protein that binds Al. The significant progress in Al speciation in serum has been achieved by the use of the robust anion-exchange FPLC Mono Q HR 5/5 column. This polyether resin based column enabled quantitative separation of Al species and has been successfully applied in the further investigations of the percentage and the composition of both LMM-Al as well as HMM-Al species (Milačič et al., 2009). Soldado Cabezuelo et al. (Soldado Cabezuelo et al., 1998) used FPLC Mono Q column in speciation of spiked human serum and serum of uremic subjects. Linear gradient elution was applied in 20 min using (0-0.25 mol L^{-1}) NaCl in Tris- hydrochloric buffer (pH 7.4) containing 0.01 mol L^{-1} of $NaHCO_3$. The same FPLC column on-line coupled to ICPMS was used for the separation of proteins when linear gradient elution was applied in 15 min using (0-0.25 mol L^{-1}) ammonium acetate in Tris-hydrochloric buffer (pH 7.4) (Milačič, 2005). Nagaoka and Maitani (Nagaoka and Maitani, 2000) used the same FPLC Mono Q HR column and the same buffer as Soldado Cabezuleo et al. (Soldado Cabezuelo et al., 1998) but different gradient elution conditions in 50 min. By combining UV detection and ICPMS detection of Fe and Al they concluded, that Al is selectively bound to the N-lobe site of Tf.
The above experimental data obtained by chromatographic and various detection techniques demonstrated that the HMM-Al binding ligand in human serum is Tf. These conclusions agree with theoretical predictions that Tf is a potential protein binding ligand for Al^{3+} at physiological conditions (Milačič, 2005).

1.1.5.2 Quantification of Al species in human serum

ETAAS and ICPMS are instrumental techniques that have generally been used for quantification of Al in human serum.
In the past ETAAS was usually the method of choice for determination of separated Al species. Due to very low Al concentrations (few ng mL^{-1}) in healthy subjects and high limits of detection obtained by ETAAS speciation of Al was in general possible only in spiked serum sample. Another disadvantage of ETAAS was that after chromatographic separation the collection of eluted fractions was required. The off-line procedure was also time consuming. Moreover, in the case of Al the risk of contamination from extraneous Al becomes even higher. The problems with contamination led to controversial results reported by many previous investigators. Further, determination of Al or other elements by ETAAS in biological samples has some difficulties because of leakage of the sample from a graphite tube during drying or ashing steps. Ščančar and co-workers (Ščančar et al., 2000) reported that the use of nitric acid in the sample pretreatment and as modifier enable reliable determination of elements by ETAAS in liquid biological samples and in isolated DNA.

The progress in the field of speciation has been achieved by the use of ICPMS as a very sensitive detector. ICPMS is arguably the most versatile trace elemental analysis technique available today. Since its first commercial introduction in 1983 it has become applicable in various areas such as environmental, biological, food and agriculture, semiconductor, clinical and pharmaceutical, geological, nuclear, forensic and petrochemical (Nelms, 2005; Agilent Technologies, 2005). The advantages of ICPMS are high sensitivity, rapid multi-elemental analysis, wide dynamic range, good precision and accuracy, better

detection limits for the majority of elements and the ability for semi-quantitative analysis and isotope dilution (ID) quantification. Further, ICPMS is a robust detector that can (more or less easily) be coupled to any of the separation techniques such as liquid and gas chromatography and capillary electrophoresis (CE). However, ICPMS has some drawbacks such as destructive character of the technique, costly maintenance and the need for well skilled analysts. The required sample volume is typically about 2-5 mL which can sometimes be a problem in biological applications. The main components of a typical ICPMS instruments are sample introduction system, plasma, interface, ion focusing, collision/reaction cell, mass analyzer and detector (Figure 2).

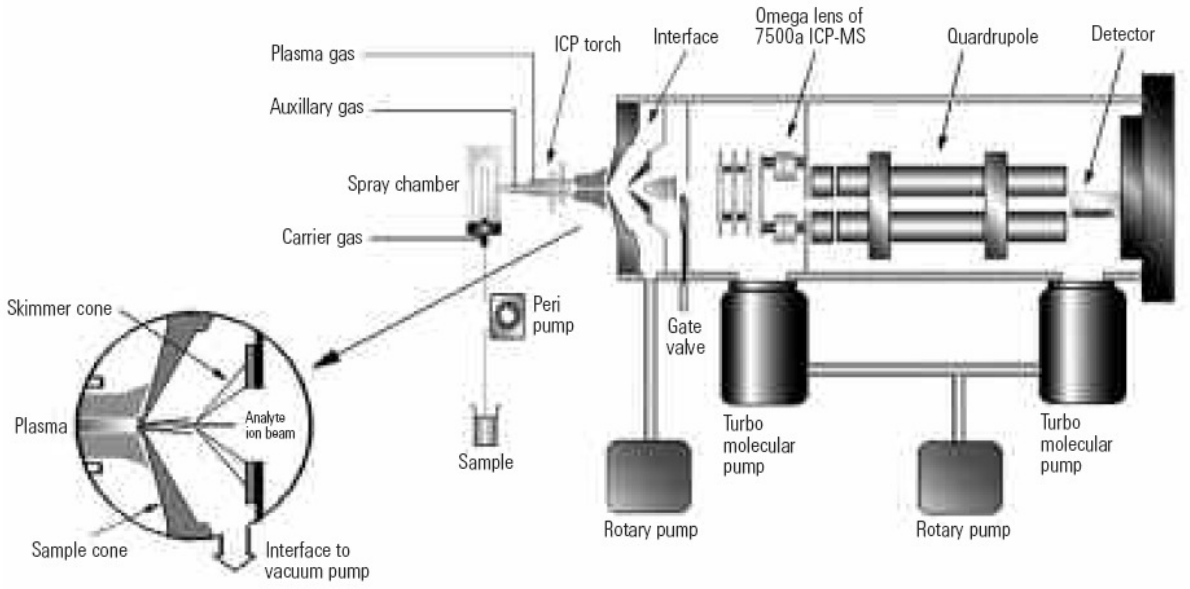

Figure 2: *Scheme of ICPMS instrument*. (Adopted from Agilent Technologies, 2005)

The sample introduction system is one of the most important components of the entire ICPMS system. The principal purpose of the sample introduction system is to convert a liquid sample into an aerosol and transport the smaller droplets efficiently in to the plasma. For nebulization different types of nebulizers are used. Each of them has its own merits. Different nebulizers optimize at different sample flow rates, vary in their tolerance of both suspended and dissolved solids, tolerate strong acids, bases or organic solvents differently and may different sample memory characteristics. Although there is a wide range of nebulizers available on the market, three nebulizers which commonly meet the application needs are Babington, MicroFlow and Concentric nebulizer (Agilent Technologies, 2005). The all three types of nebulizers are pneumatic nebulizers which have two basic configurations, concentric and crossflow type. Pneumatic and ultrasonic nebulizers are the most commonly used sample introduction devices for ICP spectrometry. Babinton nebulizer is a variant of crossflow nebulizer. The crossflow type has a liquid-carrying capillary set at a right angle to the tube carrying the high velocity gas stream. It is not prone to blockage by particulates or solutions of high salt content because the liquid emerges through a relatively large slot or hole in a surface (Montaser, 1998). For ICP applications it is constructed of polyether ether ketone (PEEK) and is resistant to most acids, bases and organic solvents. It optimizes at moderate flows between 0.4 and 1.0 mL min^{-1}, but does not self-aspirate (Agilent Technologies, 2005). The fine droplets of the aerosol which are generated by the nebulizer and represent only 1-2 % of the sample are separated from larger droplets by means of a spray chamber. The fine aerosol is then transported to plasma torch where desolvation, vaporization, atomization and ionization occur (Thomas, 2004). The positively charged ions that are produced in the plasma are extracted into the vacuum system, via a pair of interface cones. The cones, sample and skimmer cone, are essentially metal plates with central orifices through which the ions pass. Electrostatic lens, located within the intermediate stage of the vacuum system, focuses ions into a compact ion beam and separates positively charged ions from photons and neutral species. After the main ion lenses also in the intermediate stage of the vacuum system the collision/reaction cell (CRC) system are located. There are different configurations of CRC but fundamentally the device consists of an ion guide, which is enclosed in a cell that can be pressurized with gas. The gas interacts with the ion beam to remove interferences which are produced in the plasma from Ar or matrix constituents of the sample. Elimination or reduction of the interferences is the main purpose of the CRC. There are two types of interaction of gas with the ion beam in CRC, reaction or collision mode (Agilent Technologies, 2005). Despite all benefits

that CRC offers it is important to know that only thorough optimization of the type of cell gas and their flow rate allows efficient removal of interferences. In the final, analyzer stage, the low pressure allows effective transmission of the ions through a mass analyzer to the detector. Three different types of mass analyzers have been used with ICPMS, these are quadrupole, magnetic sector or double focusing and time-of flight (TOF). By far the most common mass analyzer used in ICPMS is the quadrupole (Q) (Agilent Technologies, 2005). Due to its ease of use, rapid scan across the mass range (2 to 260 amu), good linearity and relatively accessible price, Q represent approximately 95 % of all ICPMS used today (Thomas, 2004; Agilent Technologies, 2005). The Q is a sequential mass filter, which separates ions based on their mass to charge ratio (m/z). It consists of four cylindrical or hyperbolic metallic rods of the same length and diameter. One set of rods is at a positive electrical potential, and the other one at a negative potential. By varying the alternating current (AC) and direct current (DC) voltages, but keeping the ratio between them constant, different masses can be selectively allowed to pass through the filter (Thomas, 2004). Each ion exiting from the mass analyzer is detected by an electron multiplier (EM). EM is largely responsible for the characteristics of very high sensitivity, wide linear dynamic range and low random background, for which the technique is well known. The detector electronics count and store the total signal for each mass (m/z), creating a mass spectrum. The spectrum that is produced provides a simple and accurate qualitative representation of the sample. The magnitude of each peak is directly proportional to the concentration of an element in a sample (Agilent Technologies, 2005).

The combination of liquid chromatography with ICPMS provides a powerful and sensitive technique for on-line elemental speciation because of its simplicity, robustness and high reliability and reproducibility (Nelms, 2005). However, analysis of biological samples by ICPMS is not without problems. It is necessary to be aware that the proteins, other organic compounds and high concentration of inorganic salts in biological samples may block the sample introduction system of an ICPMS during an analysis run (Nelms, 2005). Lower limits of detection obtained by ICPMS enable speciation of Al in unspiked serum of normal subjects. Soldado Cabezuelo and co-workers (Soldado Cabezuelo et al., 1998) used anion-exchange Mono Q column on-line coupled to quadrupole ICPMS and high resolution ICPMS systems for detection of separated Al species. The detection limit (*3s* criterion) estimated in this work from the peak height of the Al signal was of the order of 0.6 ng mL^{-1}. There was no doubt that Al is bound only to Tf. However, no data about the column blanks and the behavior of LMM species on the column resin were reported. So, there is a need for a development of reliable, accurate and precise procedures for speciation of Al in human serum at basal concentration levels.

1.1.5.3 Identification of Al species in human serum

Multi-elemental specificity and accurate quantification are the key features of ICPMS, whereas molecular weight determination and structural information are those contributed by molecular mass spectrometry. Both atomic and molecular mass spectrometry supported by on-line or off-line coupling to chromatographic separation methods have created the basis for structural and/or quantitative insights in the interactions between metals and biomolecules (Meija et al., 2006). This approach is an unique analytical tool for speciation studies. Accurate-mass determination provided by molecular mass spectrometry enables almost unequal confirmation of the identity of the compound. Furthermore, by the use of molecular MS the efficiency of the precedent chromatographic separation may be examined. However, it is well known that the intensity of such MS signals is strongly affected by both the sample matrix and the composition of the species under study. The most common MS methods for analysis of proteins in human body fluids such as serum, plasma and cerebrospinal fluid are electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) mass spectrometry. MALDI produces singly-charged ions, which are advantages where there is a complex mixture accompanied by significant fragmentation (e.g. micro heterogenous mixture of glycopeptides). In the case of intact protein analysis, the calculated resolution is highly compromised at m/z above 25 kDa. Thus, the use of ESI where multiply charged ions are formed seems more adequate (del Castillo Busto et al., 2009). Also, MALDI is a solid-phase technique while ESI is a liquid technique compatible with on-line chromatographic separations and capillary electrophoresis. MALDI-TOF and ESI-Q-TOF have been compared for intact Tf isoform analysis (del Castillo Busto et al., 2005). The use of ESI-Q-TOF, although exhibiting about 5-fold lower sensitivity than MALDI-TOF, has provided the best results in terms of mass accuracy ($\Delta m \leq 0.05$ %) and peak width in the deconvoluted spectra (45-Da peak width vs 2000-Da peak width by MALDI).

Blood serum is a complicated matrix and heterogeneous mixture containing beside HMM and LMM compounds also a high concentration of salts. For chromatographic separation of HMM and LMM species in human serum high ionic strength eluents with high concentration of salts were used. Thus, prior to ESI-MS analysis, the serum fraction must be concentrated and desalted. For desalting on-line or off-line liquid

chromatography, membrane filtration or on-line microdialysis has been used (Pramanik et al., 2002). In speciation studies of Al in human serum identification of potential Al-binding ligands was performed by gel electrophoresis or mass spectrometry. Identification of Tf, as the major HMM-Al species in human serum, was performed by SDS-PAGE electrophoresis (Wrobel et al., 1995; Kralj et al., 2004). Kralj and co-workers (Kralj et al., 2004) identify Tf in serum and spent CAPD fluid samples. After separation of proteins on the anion-exchange FPLC column, Al-containing fractions were collected and combined together. Due to low concentrations of proteins in spent CAPD fluid, preconcentration by microultrafiltration was applied. Tandem mass spectrometry is a useful tool not only for identification of proteins but also for identification of LMM species present in body fluids. Bantan and co-workers (Bantan et al., 1999) characterized LMM-Al species by ESI-Q-Q technique. Identified LMM-Al species in human serum are Al-citrate, Al-phosphate and ternary Al-citrate-phosphate complex.

1.1.6 Extraneous contamination

Due to low Al concentration in body fluids and their high abundance in the nature the problem of contamination should not be overlooked. Contamination from extraneous sources is one of the most important obstacles in the field of speciation of Al. To obtain reliable analytical data it is of extreme importance to avoid all possible sources of contamination and to apply appropriate cleaning procedures for removing traces of Al from the eluents, reagents, chromatographic supports and all devices used in the speciation procedure. In order to prevent contamination by extraneous Al, high density polyethylene or Teflon ware, previously soaked with 10 % nitric acid for 48 h and rinsed with MiliQ water should be used (Fairman et al., 1994). The overall analytical procedure should be carried out under clean room conditions (at least class 10000). In order to remove Al from eluents used in chromatographic procedures (Van Landeghem et al., 1994) on-line silica-based C18 scavenger column, which has a strong affinity to adsorb Al, was used. Similarly, in Medel's group chelating Kelex 100-impregnated silica C18 scavenger column was used on-line to clean the eluents (Soldado Cabezuelo et al., 1998). Bantan and co-workers (Bantan et al., 1998) applied the chromatographic where eluents were first treated with the chelating resin Chelex 100 (batch procedure). Filtered eluents were then passed through a silica-based HPLC LiChrosorb RP-18 column to remove residual Al. Since the chromatographic supports used in the speciation procedure also contained trace amounts of Al, it was of great importance to efficiently clean the resin of chromatographic supports. There were not many reports that draw attention to this problem. The cleaning procedure for FPLC column used for separation of HMM-Al species in serum was reported (Nagaoka and Maitani, 2000). After rinsing the column with a buffer, 100 µL of 7 mmol L^{-1} sodium acetate was first injected, followed by seven subsequent injections of 0.25 mmol L^{-1} ammonium acetate in 50 mmol L^{-1} Tris buffer. After that a single injection of 2 mol L^{-1} sodium chloride, 2 mol L^{-1} sodium hydroxide and 75 % acetic acid was applied. Authors stated that by the use of the proposed cleaning procedure the blank was lowered to 0.1 ng mL^{-1} of Al. In our group, efficient cleaning procedures of SEC, FPLC, CIM® DEAE disk/column and HiTrap desalting SEC chromatographic supports used for speciation Al in human serum were also developed (Bantan et al., 1998; Bantan et al., 1999; Bantan Polak et al., 2001; Kralj et al., 2004; Murko et al., 2007; Murko et al., 2009).

2 Aims and Hypothesis

Speciation of Al in human serum has been intensively investigated owing to its participation in many clinical disorders. For the determination of the proportion of HMM-Al and LMM-Al species and for the identification of their composition various analytical techniques have been used. The combination of liquid chromatography with element specific detectors such as FAAS, ETAAS and AFS represents an appropriate tool for the speciation of Al. However, because of the low concentration of Al in biological fluids the sensitivity of FAAS, ETAAS and AFS is not as adequate as that of ICPMS. ICPMS offers not only unequall sensitivity, selectivity and better detection limits for majority of elements, but is also a robust detector that can be coupled to any of the chromatographic separation techniques. In general, the results obtained by different techniques, suggested that Tf is the major Al transport protein in serum. LMM-Al species present in serum were Al-citrate, Al-phosphate and ternary Al-citrate-phosphate complexes. Their composition and amount varied among particular individuals.

However, the main problem in Al speciation at trace concentrations remained contamination originating from the chromatographic supports and eluents. Therefore, special attention was devoted to the cleaning procedures of eluents and chromatographic supports in order to obtain low blanks.

To obtain reliable analytical data in speciation analysis it is important to apply and compare complementary analytical procedures. As an alternative to ion-exchange FPLC particle packed columns, ion-exchange separation supports based on CIM® were developed in the last decade. The matrix supports of poly(glycidylmethacrylate-co-ethyleneglycoldimethacrylate) substituted with strong or weak cation and anion exchangers offer very fast separation of biomolecules. The robustness, high binding capacity and fast separations of CIM® monolithic supports have great potential for their use in the field of speciation. CIM® monolithic chromatography in combination with ICPMS detection can offer unique analytical tools for speciation analysis at very low concentrations in human serum. To the best of our knowledge, the quantification of Al species in human serum was up till now performed only in spiked serum or in serum with elevated concentration of Al. Therefore, the aim of my PhD research was to develop new analytical procedures for the reliable speciation of HMM-Al compounds in spiked and unspiked human serum using size exclusion and CIM® monolithic chromatography with ICPMS and UV detection. Identification of the Al-binding protein was performed not only on the basis of the retention volume, but also by the mass spectrometry and SDS-PAGE electrophoresis. Citrate as one of the major LMM-Al binding ligands has also been identified by mass spectrometry.

In addition to experimental approaches for the speciation of Al bound by Tf, the application of computational methods was proposed to test whether 90 % of all Al is bound by Tf, or 90 % of Al is available to be bound by Tf. The idea is to contribute to the development of a model of Al being bound by Tf which will explain the binding, transport and fate of Al in serum. In order to fulfill this goal a new analytical procedure for the efficient, reliable and fast separation of proteins from LMM compounds in serum was developed. The developed analytical procedure for the fast separation of the HMM-Al species from LMM-Al species in serum may be useful for a kinetic study of the binding of Al to Tf and for further speciation studies of LMM-Al compounds in serum.

The new-developed analytical procedures for speciation of HMM and LMM Al species in human serum could be an important basis for computational biochemistry not only for Al but also for other essential and nonessential elements present in blood. Combination of experimental and mathematical/computational data could help to understand toxicity of elements in connections with many inexplicable diseases.

3 Materials and Methods

3.1 Apparatus

For chromatography five chromatographic supports were used. SEC was performed on a Superdex 75 10/30 GL column (Amersham, Uppsala, Sweden), column characteristics (dimensions 10 x 300 mm, matrix composite of cross-linked agarose and dextran, average particle size 13 µm, pH stability 3-12, exclusion limit Mr $1x10^5$) and on a HiTrap desalting 5 mL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), Sephadex G-25 Superfine, column characteristics (dimensions 1.6 x 2.5 cm, matrix cross-linked dextran, pH stability 2-13, bead size 15-70 µm, exclusion limit Mr 5000). Anion-exchange chromatography was performed on three chromatographic supports. For the separation of HMM-Al species a weak anion-exchange CIM® (Convective Interaction Media) DEAE monolithic disk or column (Bia Separations, Ljubljana, Slovenia) with matrix supports made of highly porous poly(glycidyl methacrylate-co-ethylene dimethacrylate) bearing weak anion exchange diethylamino (DEAE) functional group, (pH stability 2-14) was used. The CIM® DEAE disk dimensions were, diameter: 12 mm, thickness: 3 mm and bed volume: 0.34 mL. The dimensions of the CIM® DEAE column were, outer diameter: 15 mm, inner diameter: 1.5 mm, length: 45 mm and bed volume: 8 mL. A strong anion-exchange FPLC column of Mono Q HR 5/5 (Pharmacia, Uppsala, Sweden), column characteristics (dimensions 5 x 50 mm, matrix polystyrene/divinyl benzene, particle size 10 µm, pH stability 2-12) was used for the separation of LMM-Al compounds.

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, California, USA), fitted with a 1 mL injection loop for Superdex 75 10/30 GL, HiTrap desalting and CIM® DEAE column. A 0.1 and 0.5 mL injection loops were used for CIM® DEAE disk and FPLC column, respectively. A UV-vis detector (Agilent 1100 Series Diode Array and Multiple Wavelength Detector, DAD/MWD) was used on-line with HPLC equipment for absorption measurements at 278 nm.

Element-specific detection of Al after chromatographic separation as well as the total concentration of Al in serum were performed using inductively coupled plasma mass spectrometer model 7500ce from Agilent Technologies (Agilent, 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 for the identification of Tf (Kleinert et al., 2003; Wuhrer et al., 2005) were performed on Q-oa-ToF Premier mass spectrometer (Waters Corporation, Manchester, UK). The Tf fractions were analysed by ACQUITY UPLC chromatograph coupled on-line to ESI-MS (UPLC-ESI-MS). For ACQUITY UPLC chromatography VanGuardTM Pre-Column (2.1 x 5 mm, Acquity UPLC BEH C18, 1.7 µm, Waters) was used. The ACQUITY UPLC ESI-MS system was also used for the identification of citrate. The MS analyses of citrate were performed by scanning negative ions. For chromatography a ACQUITY UPLC column (2.1 x 30 mm, Acquity UPLC BEH C18, 1.7 µm, Waters) was used.

The identification of proteins after the separation of serum on the CIM® DEAE column was performed by SDS-PAGE on an invitrogen Xcell SureLock electrophoresis system.

To concentrate the LMM fraction of serum Eppendorf concentrator 5301 was used.

Experimental working conditions for ICPMS and UPLC-ESI-MS are summarized in Table 2.

Table 2: *Operating condition for ICPMS and UPLC-ESI-MS.*

ICPMS	
Parameter	Value
Forward power (W)	1500
Outer gas flow (L min^{-1})	15.0
Carrier gas flow (L min^{-1})	0.80
Makeup gas flow (L min^{-1})	0.11
He gas flow (mL min^{-1})	3.0
Isotop monitored	^{27}Al
QP bias (V)	-15
Oct bias (V)	-18
Extract 1 (V)	-0.3
Extract 2 (V)	-150
UPLC-ESI-MS	
Parameter	Value
Scan type	for Tf: positive TOF-MS for citrate: negative TOF-MS
Ion spray voltage (kV)	3
Nebulizing gas	N_2
Injection rate (µL min^{-1})	50
External calibration	CsI
Scan range	for Tf: m/z 1000-3500 for citrate: 50-1000
Spectrum deconvolution	for Tf: Maximum entropy method (MaxEnt)

3.2 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). Merck (Darmstadt, Germany) suprapur acids were used. For adjusting buffer solution to pH=7.4 50 mmol L^{-1} Tris (hydroxymethyl) aminomethane-hydrochloric acid was used that contained also 30 mmol L^{-1} sodium hydrogencarbonate (Merck) (buffer A). To achieve higher ionic strength various salts were dissolved in buffer A (buffer B). Tris, sodium hydrogencarbonate, sodium hydroxide and salts were purched from Merck. The 4 mol L^{-1} ammonium nitrate eluent was prepared by dissolving 320.16 g of ammonium nitrate in 1 L water. Ultrapure 18.2 MΩ cm water was obtained by means of a Direct-Q 5 Ultrapure water system (Millipore Watertown, MA, USA). A stock Al^{3+} solution (100 µg Al mL^{-1}) was prepared in a 100 mL calibration flask by dissolving 0.1388 g of aluminium 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 all chromatographic supports used for the separations. Fresh working standard solutions were prepared daily by dilution of stock solutions with water. A stock Al-citrat solution (100 µg Al mL^{-1}) was made by mixing citric acid (Merck) and aluminium nitrate-9-hydrate in 100:1 citric acid to Al molar ratio (Bantan et al., 1999). A formic acid and acetonitrile used for the identification of Tf and citrate were HPLC grade quality (J.T. Baker, Deventer, Holland).

Chelex 100 (Na+ form, 100-200 mesh) chelating ion-exchange resin (Sigma) and a silica based LiChrosorb RP-18 HPLC column (150 x 4.6 mm i.d.) were used for the purification of the eluents (Bantan et al., 2001). For purification and preconcentration of Tf solutions a Centricon 30 YM-30 (Amicon, Germany) 30 000 Da cut-off centrifugal filter device was used.

The certified serum sample, SeronormTM Trace Elements of Serum L-1, was obtained from Sero AS (Billingstad, Norway).

Centricon 30 concentrators (Amicon, Beverly, MA, USA) with nominal cut-off 30 000 Da were used to concentrate Tf fraction of serum before applying UPLC-ESI-MS.

3.3 Recommended cleaning procedures

3.3.1 Laboratory ware, tubes, eluents and ICPMS system

To avoid contamination by extraneous Al, polyethylene or Teflon laboratory ware and tubes were used. Teflon bottles were also used for rinsing solutions in the ICPMS system. Before use, all laboratory ware and tubes for chromatographic separations and ICP determinations were treated with 10 % nitric acid for 48 h, rinsed well with MilliQ water and dried at room temperature. In order to reduce memory effects from ICPMS system five rinses after each analysis of serum sample (the first rinse with 5 % nitric acid and the following four with water) were applied. All eluents used in the chromatographic separations were efficiently cleaned by the procedure that was previously optimised in our laboratory (Bantan et al., 1998; Bantan et al., 1999; Bantan et al., 2001). The eluents were first subjected to chelating ion-exchange chromatography (Chelex 100, Na+ form, batch procedure) and then passed through silica based reversed-phase HPLC column.

3.3.2 Chromatographic supports

3.3.2.1 Superdex 75 10/30 GL 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. Then 1 mL of 1 mol L^{-1} sodium hydroxide was injected and the column was rinsed with water for 15 min. A linear gradient elution from 100 % water to 100 % 2 mol L^{-1} citric acid was applied for 10 min, followed by 10 min of rinsing with 2 mol L^{-1} citric acid. Then, a linear gradient elution from 100 % 2 mol L^{-1} citric acid to 100 % water was applied for 10 min. Rinsing 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. It was experimentally found, that the cleaning of Superdex 75 10/30 GL column should be applied after five consecutive analysis of serum samples.

3.3.2.2 HiTrap desalting column

The cleaning of the column was usually performed at a flow rate of 5 mL min^{-1}. When 2 mol L^{-1} citric acid was used in the cleaning procedure, a flow rate of 2 mL min^{-1} was applied. The column was first rinsed with water for 10 min. Then, 1 mL of 1 mol L^{-1} sodium hydroxide was injected, followed by 10 min rinsing with water. After that, 5 min rinsing with 2 mol L^{-1} citric acid was used, followed by 20 min rinsing with water. The procedure with 2 mol L^{-1} citric acid was repeated three times. At the end, the equilibration of the column was performed by rinsing with buffer A for 10 min. It should be pointed out that by applying more rigorous cleaning traces of Al could be eluted from the column resin, resulting in higher blanks.

3.3.2.3 CIM® DEAE disk

The cleaning of the CIM® DEAE disk was performed at a flow rate of 5 mL min^{-1}. The disk was first rinsed with 0.1 mL of 1 mol L^{-1} sodium hydroxide for 0.5 min followed by 5 min of rinsing with 0.2 mol L^{-1} Tris-hydrochloric acid buffer (pH 7.4). After that the disk was rinsed with buffer A for 5 min. Then, 0.5 min of rinsing with 2 mol L^{-1} citric acid was applied. At the end, the equilibration of the disk was performed by rinsing with buffer A for 10 min.

3.3.2.4 CIM® DEAE column

The cleaning of the chromatographic support was carried out at a flow rate of 5 mL min^{-1}. 5 min rinsing with 1 mol L^{-1} sodium hydroxide was first applied. After application of sodium hydroxide more concentrated buffer that used in separation procedure is required in order to regenerate the column support. Then, 20 min of rinsing with 0.2 mol L^{-1} Tris-hydrochloric acid buffer (pH 7.4) followed. After that the column was rinsed with buffer A for 20 min. Then, 8 min of rinsing with 2 mol L^{-1} 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 (3.3.1). It was experimentally found that the cleaning of the CIM® DEAE column

should be applied after seven consecutive analyses of serum samples.

3.3.2.5 FPLC Mono Q column

The cleaning of the column was generally performed at a flow rate of 1 mL min^{-1}. For the cleaning with 1 mol L^{-1} sodium hydroxide a flow rate of 0.5 mL min^{-1} was applied.

The column was first rinsed with 1 mol L^{-1} sodium hydroxide for 8 min followed by 15 min of rinsing with water. The procedure was applied twice. After that 10 min of rinsing with 2 mol L^{-1} citric acid was used. At the end the column was rinsed with water for 30 min.

3.4 Sample preparation

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

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

In order to study the speciation of HMM-Al and LMM-Al compounds, spiked and unspiked serum samples were used. The spiking of the serum was performed with 100 μL of Al^{3+} solution (Al-nitrate salt) added to 3 mL of serum, so that the final concentration of Al in serum ranged from 10 to 300 ng mL^{-1}. Spiked serum was left to equilibrate at room temperature for 5 hours (Bantan et al., 1998; Bantan et al., 1999). 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 analysed.

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 eluted under the Tf peak 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) in order to exchange the elution buffer by an aqueous solution and to reduce the final sample volume to 0.1 mL.

In order to identify the citrate in serum, 1 mL of undiluted serum was injected onto the HiTrap desalting SEC column. The separation of proteins from LMM species was performed and the fraction from 5 min to 10 min was collected. After that the LMM fraction of serum was concentrated until the volume of the fraction was reduced to 0.1 mL. Concentration was performed at 30 °C and at 1400 rpm.

3.5 Recommended analytical procedures

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

3.5.1 Superdex 75 10/30 GL column procedure

The separations were carried out by injecting 1 mL of undiluted serum sample onto the column. Isocratic elution with buffer A was applied for 15 min. From 15 to 16 min linear gradient from 100 % buffer A to 100 % buffer B (buffer A+1 mol L^{-1} ammonium chloride) followed. Elution with 100 % buffer B was kept up to 29 min. From 29 to 30 min 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. The protein peak was collected from 7.5 to 13 min (5.5 mL) into a polyethylene sample cup and further subjected to anion-exchange chromatography. The concentration of total Al in protein fraction was determined by ICPMS.

3.5.2 HiTrap desalting column procedure

1 mL of undiluted serum sample was injected onto the column. Isocratic elution with 50 mmol L^{-1} Tris-hydrochloric acid buffer (pH 7.4) was applied for 10 min. The chromatographic run was performed at a flow rate of 1 mL min^{-1}. Two fractions were collected, protein peak from 0 to 5 min (5.0 mL) and the fraction of LMM species from 5 min to 10 min (5.0 mL).

3.5.3 CIM® DEAE disk procedure

The protein fraction from Superdex 75 10/30 GL column was subjected to anion-exchange chromatography. A 0.1 mL of the sample aliquot was injected onto a CIM® DEAE disk. The chromatographic run was carried out at a flow rate of 1 mL min^{-1}. Linear gradient elution from 100 % buffer A to 100 % buffer B was applied for 10 min. The disk was rinsed with 100 % buffer B for 0.5 min and from 10.5 to 15 min with buffer A. To follow the separation of proteins the disk was connected on-line to an UV detector. For quantification of separated Al species the disk was connected on-line to ICPMS.

3.5.4 CIM® DEAE column procedure

1 mL of serum sample was injected onto a column resin. Isocratic elution with buffer A containing 30 mmol L^{-1} sodium hydrogencarbonate was applied for the first 5 min, followed by a linear gradient for the next 40 min from 0-100 % of buffer B (buffer A+1 mol L^{-1} ammonium chloride). 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 an UV detector (set at 278 nm) for protein monitoring and was coupled with a sample uptake inlet of the Babington nebulizer of ICPMS used for the quantification of separated Al species.

3.5.5 FPLC Mono Q column procedure

0.5 mL of aliquot of the LMM fraction was injected onto the column. Aqueous (0-100 % 4 mol L^{-1} ammonium nitrate) linear gradient elution was applied for 10 min at a flow rate of 1 mL min^{-1}. The eluate from the column was connected on-line to ICPMS. After each separation the column was regenerated for 5 min with 4 mol L^{-1} ammonium nitrate at a flow rate of 1 mL min^{-1} and equilibrated with water firstly for 20 min at a flow rate of 2 mL $min^{-1}$and in the following 5 min at a flow rate of 1 mL min^{-1}.

3.5.6 UPLC-ESI-MS procedure

For the ESI-MS analysis of Tf it was necessary to clean and concentrate the reconstituted Tf fraction in order to obtain the adequate charge distribution profile of protonated Tf molecules and its molecular mass. The Tf standard (concentration 10 µmol L^{-1}) and Tf fraction was analysed by UPLC-ESI-MS. 4 µL of sample solution were injected on the VanGuardTM Pre-Column. 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 linear gradient B = 5 % to B = 85 % in 6 min. The elution time of Tf was 0.7 min and was twice as dead time of the LC system.

For the identification of citrate, a LMM fraction of serum sample was concentrated. A 10 µL of sample was injected onto the column of UPLC-ESI-MS system. Isocratic elution with mobile phase A (water, 0.1 % formic acid) was applied for 4 min. The chromatographic separation was performed at a flow rate of 200 µL min^{-1}. Citrate was eluted from 1.0 to 2.0 min.

4 Results and Discussion

4.1 Determination of the total Al in serum by ICPMS

Before analysis serum samples were diluted (1+4) with water and total Al concentrations were determined by ICPMS under the optimal operating conditions given in Table 2. The same analytical procedure was applied to determine Al concentrations in spiked standard serum proteins. The accuracy of the determination of total Al was checked by the analysis of 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. Serum of three renal patients was involved in the study. The concentration of total Al in serum samples investigated (mean of three parallel analysis) was determined by the standard addition method.

First serum sample was used for the development of the analytical procedure for speciation of HMM-Al compounds by anion-exchange CIM® DEAE disk. The concentration of Al was found to be 4.0 ± 0.2 ng mL^{-1}. This concentration was too low to perform speciation analysis. Therefore, samples were spiked with Al^{3+} solution, so that the final concentration of Al in spiked serum ranged between 50 and 300 ng mL^{-1} Al.

The concentration of Al in second serum sample investigated was found to be 5.7 ± 0.4 ng mL^{-1}. In order to optimize the parameters for the separation of serum proteins by the use of anion-exchange CIM® DEAE column samples were spiked with 10 ng mL^{-1} Al.

The third serum sample was used for the development of the analytical procedure for the reliable separation of HMM-Al species from LMM-Al compounds in serum by HiTrap desalting SEC column. The concentration of Al was found to be 2.6 ± 0.3 ng mL^{-1}. In this study serum samples were spiked with 150 and 300 ng mL^{-1} Al.

4.2 Development of the analytical procedure for speciation of HMM-Al compounds in spiked serum by combining SEC and anion-exchange CIM® DEAE disk

4.2.1 Optimization of the parameters for the separation of serum proteins by the use of CIM® DEAE disk-UV-ICPMS

To separate HMM-Al compounds from LMM-Al species in serum, SEC was first applied. Isocratic elution with buffer A, pH 7.4, as described in 3.5.1 was applied. Proteins were eluted from 7.5 to 13 min. The 5.5 mL protein peak was collected into a polyethylene cup (sample diluted 5.5 times).

In order to investigate the separation of serum proteins by anion-exchange CIM® DEAE disk synthetic solutions of the standard proteins IgG, Tf, albumin and their mixture were prepared (5 g L^{-1} of albumin, 1 g L^{-1} of IgG and 0.5 g L^{-1} of Tf). As an eluent ammonium chloride that enables both UV and ICPMS detection was chosen. Furthermore, ammonium chloride does not form any complexes with Al and therefore has no influence on Al speciation during the chromatographic run. First strong anion-exchange CIM® quaternary amine (QA) disk was used. 0.1 mL of standard proteins was injected onto the disk and linear gradient elution from 100 % buffer A to 100 % buffer B preformed in 10 min. Results indicated that separation was not selective enough since albumin and Tf were eluted under the same chromatographic peak. Therefore, the potential of a weak anion-exchange CIM® DEAE disk was further investigated. First the influence of ionic strength of the eluent (0.25, 0.5 and 1 mol L^{-1} ammonium chloride) was examined. 0.1 mL of standard proteins was injected onto the disk and linear gradient elution from 100 % buffer A to 100 % buffer B was applied in 10 min. Separation with 0.25 and 0.5 mol L^{-1} ammonium chloride resulted in the broadening of chromatographic peaks, while by the use of 1 mol L^{-1} ammonium chloride well resolved chromatographic peaks of IgG, Tf and albumin were obtained. The optimal concentration of

ammonium chloride was therefore 1 mol L^{-1}. In further experiments 0.1 mL of standard proteins was injected onto the disk and different times of gradient elution were examined (5, 7.5 and 10 min). Experimental data indicated that the best resolution of chromatographic peaks was obtained when gradient elution in 10 min was applied. Finally, the influence of a sample volume loaded on the disk was observed (0.02, 0.05, 0.1, 0.2 and 0.5 mL sample loops). Chromatographic peaks were well resolved using 0.02 up to 0.1 mL sample loop, while the application of higher sample volume resulted in broadening and overlapping of the chromatographic peaks. To obtain a better limit of detection, 0.1 mL sample loop was therefore used and separation of proteins performed under the optimal conditions described in 3.5.3.

A combined procedure including removal of LMM compounds from proteins on SEC column followed by separation of protein fraction on CIM® DEAE disk was then applied at optimal separation conditions. Chromatographic separations were followed by UV detection at 278 nm. The chromatograms for the mixture of standard proteins are presented in Figure 3 and for serum in Figure 4. Data of Figures 3 A and 4 A indicate that a protein peak is eluted from SEC column between 7.5 and 13 min in the mixture of standard proteins as well as in serum sample. It may be further seen from Figures 3 B and 4 B that good separation of IgG, Tf and albumin is obtained in protein fraction on CIM® disk. This is evident for a mixture of standard proteins (Figure 3 B) and also for a protein fraction of a serum sample (Figure 4 B). On the basis of data from Figures 3 and 4 it is evident that the proposed analytical procedure enables good separation of serum proteins.

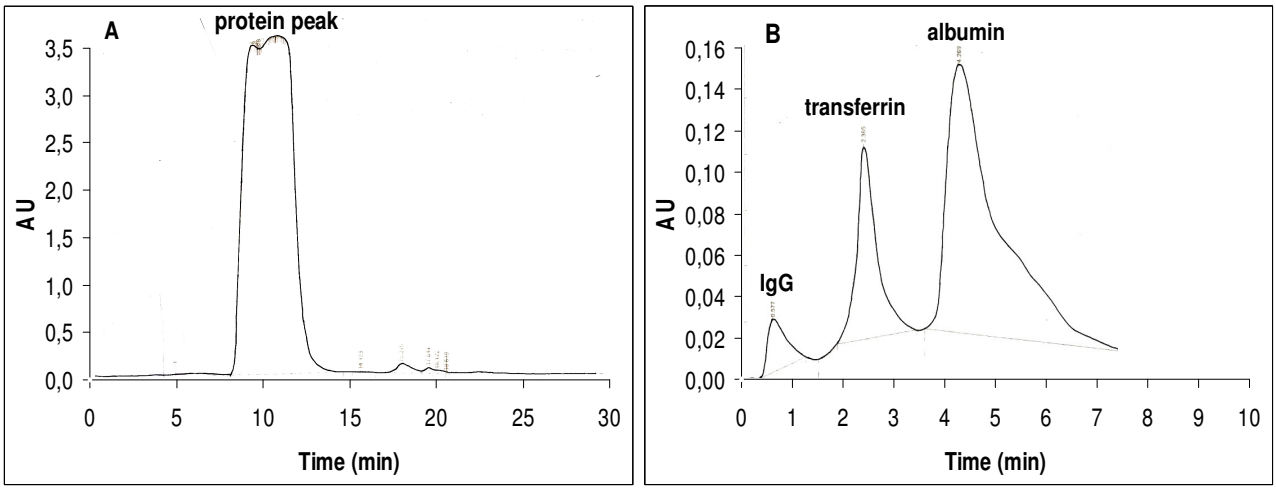

Figure 3: *Separation of a standard solution of proteins at a flow rate of 1 mL min^{-1} followed by UV detection at 278 nm. 1 mL of sample containing 25 g L^{-1} of albumin, 5 g L^{-1} of IgG and 2.5 g L^{-1} of Tf was first injected onto the SEC column (A). A protein peak was collected from 7.5 to 13 min (5.5 mL). 0.1 mL aliquot of a protein peak was then injected onto the CIM® DEAE disk (B).*

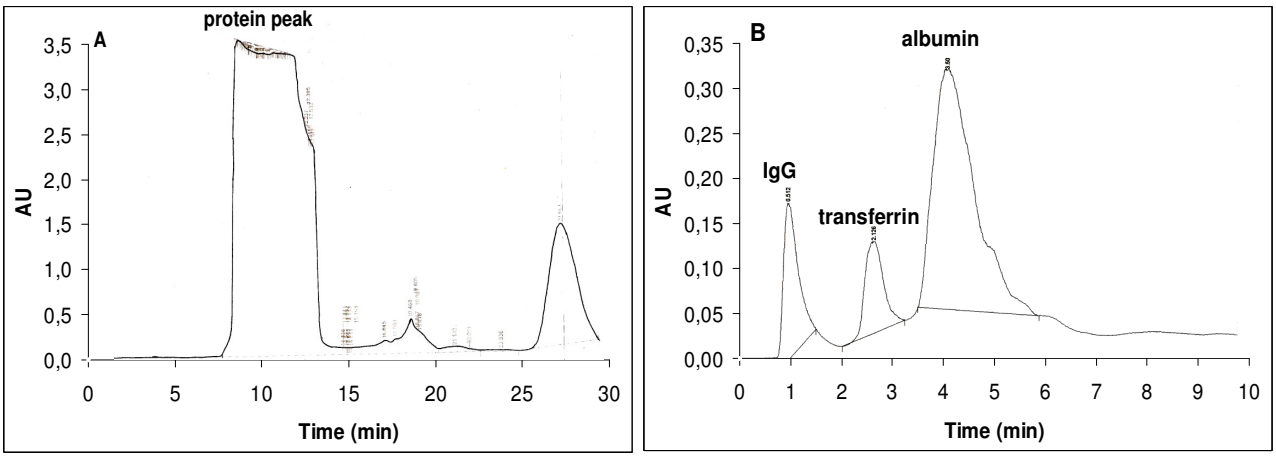

Figure 4: *Separation of a serum at a flow rate of 1 mL min^{-1} followed by UV detection at 278 nm. 1 mL of sample was first injected onto the SEC column (A). A protein peak was collected from 7.5 to 13 min (5.5 mL). 0.1 mL aliquot of a protein peak was then injected onto the CIM® DEAE disk (B).*

4.3 Speciation of HMM-Al species in spiked serum by combining SEC and CIM® DEAE disk-ICPMS

Once the separation of serum proteins was optimised, speciation of Al in spiked serum was investigated by combining SEC and CIM® DEAE-ICPMS. To obtain on-line ICPMS detection, the outlet of the CIM® disk was directly connected to the Babington nebuliser of ICPMS. The advantage of the use of Babington nebuliser is its ability to operate at a flow rate of 1 mL min^{-1} that is the same as applied in the separation of proteins on CIM® disk. In addition, Babington nebuliser is capable of introducing samples with high salt contents into ICPMS. The study was first performed by the use of standard serum proteins (5 g L^{-1} of albumin, 1 g L^{-1} of IgG and 0.5 g L^{-1} of Tf). Standard serum Tf as well as the mixture of standard serum proteins were spiked with 100 ng mL^{-1} of Al. 0.1 mL of spiked samples were injected onto the CIM® DEAE disk coupled on-line with ICPMS. Results are presented in Figure 5. It is evident that the shape and the elution profile of Al are the same in the Tf-spiked sample (Figure 5 A) and in the mixture of spiked standard proteins (Figure 5 B). In both chromatograms Al was eluted under the chromatographic peak of Tf. These observations confirmed again that Tf is the only serum protein that binds Al. For calibration in speciation analysis of Al, standard serum Tf (0.5 g L^{-1}) was spiked with 10, 20, 50, 100 and 150 ng mL^{-1} of Al. 0.1 mL of spiked samples were then injected onto the CIM® DEAE disk coupled on-line with ICPMS.

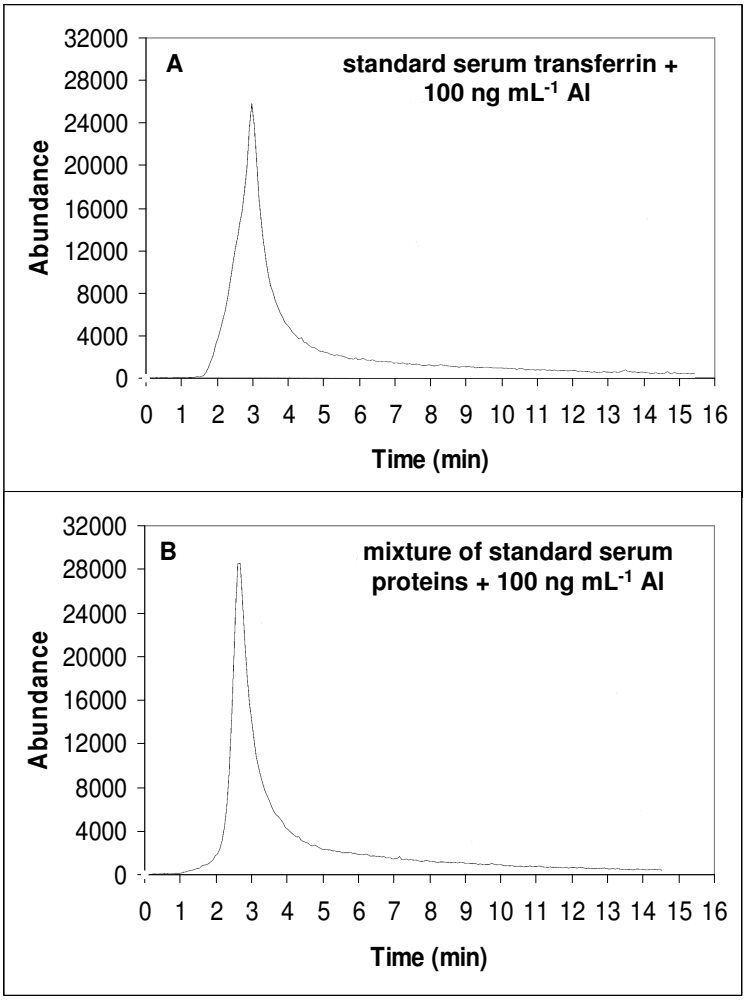

Figure 5: *Separation of standard serum Tf (0.5 g L^{-1}) spiked with 100 ng mL^{-1} Al (A) and a mixture of standard serum proteins (5 g L^{-1} of albumin, 1 g L^{-1} of IgG and 0.5 g L^{-1} of Tf) spiked with 100 ng mL^{-1} Al (B) on a CIM® DEAE disk followed by ICPMS detection.*

In order to check the linearity of developed analytical procedure the standard serum Tf was spiked with 10, 20, 50, 100 and 150 ng mL^{-1} Al. Figure 6 represents overlay chromatograms of spiked standard serum Tf. A calibration curve was done on the basis of the peak area. A linear response of the calibration curve was obtained with correlation coefficient, $R^2 = 0.998$.

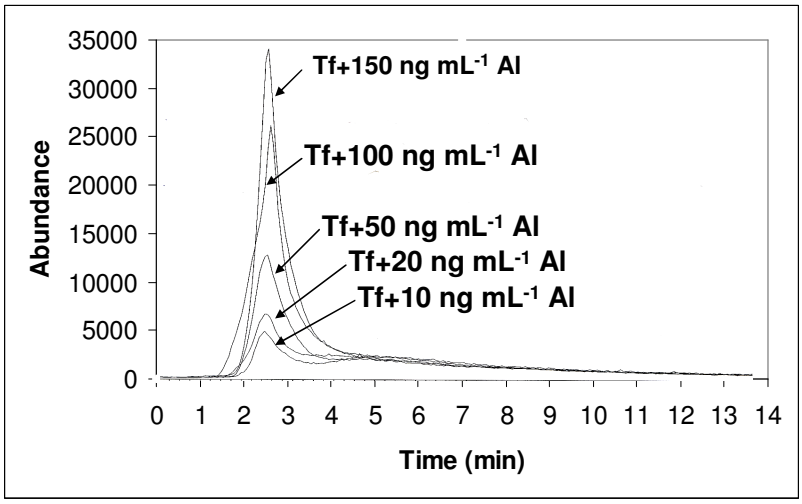

Figure 6: *Overlay chromatograms of standard serum Tf (0.5 g L^{-1}) spiked with 10, 20, 50, 100 and 150 ng mL^{-1} of Al. Speciation was performed by CIM® DEAE-ICPMS procedure.*

In Figure 7 three consecutive speciation analysis of a standard serum Tf (0.5 g L^{-1}) spiked with 20 ng mL^{-1} of Al are presented. The speciation was carried out by CIM® DEAE-ICPMS. Data indicate that consecutive chromatographic separations are repeatable.

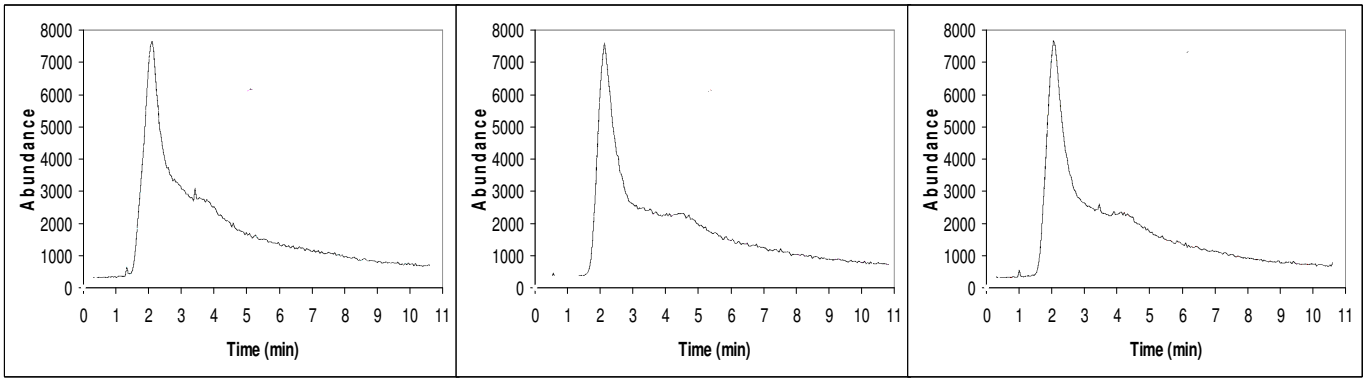

Figure 7: *Consecutive speciation analysis of standard serum Tf (0.5 g L^{-1}) spiked with 20 ng mL^{-1} of Al. Speciation was carried out by CIM® DEAE-ICPMS procedure.*

A combined procedure including SEC pre-separation of proteins and speciation of Al in the protein fraction by CIM® DEAE-ICPMS was then performed on a spiked serum. Analyses were done under analytical procedures described in 3.5.1 and 3.5.3. As an example of these analyses overlay chromatograms of spiked serum with 50, 100 and 300 ng mL^{-1} of Al are shown in Figure 8. The chromatograms in Figure 8 represent speciation of Al by CIM® DEAE-ICPMS procedure after pre-separation of LMM-Al complexes by SEC. On the basis of the elution time between 2 and 3.5 min it is evident that Al is eluted under the Tf peak.

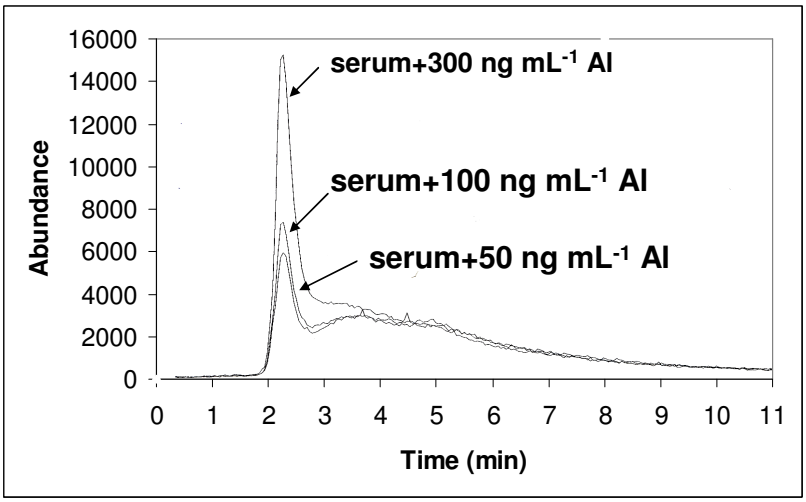

Figure 8: *Speciation of Al by CIM DEAE-ICPMS in spiked serum after pre-separation of LMM-Al complexes by SEC. Overlay chromatograms represent spiked serum with 50, 100 and 300 ng mL^{-1} Al.*

For quantification of the separated Al species by SEC in combination with CIM® DEAE-ICPMS, the following procedure was applied. A serum sample was spiked with different concentrations of Al. Total Al in spiked serum was determined by ICPMS. Then 1 mL of serum was subjected to SEC to separate LMM-Al species from proteins. Protein fraction was collected between 7.5 to 13 min and the concentration of Al in the protein peak was determined by ICPMS. After that 0.1 mL sample aliquot of the protein peak was injected onto CIM® DEAE disk coupled to ICPMS and speciation analysis was performed. Al species bound to Tf were quantified on the basis of the peak area. In order to prepare a calibration curve, standard serum Tf was spiked with Al and CIM® DEAE-ICPMS speciation procedure was applied. The results are presented in Table 3.

Table 3: *Quantification of Al species in spiked serum samples.*

ICPMS concentrations of Al in spiked serum (ng mL^{-1})	SEC, ICPMS concentration of HMM-Al species (ng mL^{-1})	HMM-Al species (%)	CIM® DEAE-ICPMS concentration of Al bound to Tf (ng mL^{-1})	HMM-Al species bound to Tf (%)
49.8 ± 0.9	45.1 ± 0.9	91 ± 2	44.2 ± 0.8	98 ± 2
50.2 ± 0.9	45.2 ± 0.9	90 ± 2	43.4 ± 0.8	96 ± 2
98 ± 2	91 ± 2	93 ± 2	88 ± 2	97 ± 2
97 ± 2	85 ± 2	88 ± 2	83 ± 2	96 ± 2
256 ± 5	233 ± 5	91 ± 2	226 ± 6	97 ± 2
285 ± 6	254 ± 6	89 ± 2	246 ± 6	97 ± 2

Data of Table 3 indicate that, on the SEC column, in all spiked samples investigated 90 ± 3 % of Al is eluted as HMM-Al species (Al bound to proteins). Speciation analysis of the protein fraction by CIM® DEAE-ICPMS furthermore confirmed that 97 ± 2 % of Al of the protein fraction is eluted exclusively under the elution volume of the Tf peak. This findings are in agreement with the reported literature data (Soldado Cabezuelo et al., 1997; Soldado Cabezuelo et al., 1998; Nagaoka et al., 2000; Kralj et al., 2004). The proposed speciation procedure removes LMM-Al species and enables reliable determination of the concentration and composition of Al bound to proteins by CIM® DEAE-ICPMS when the concentration of Al in serum is higher than 5 ng mL^{-1}.

4.4 Development of the analytical procedure for speciation of HMM-Al species in unspiked serum by anion-exchange CIM® DEAE column

4.4.1 Optimization of the parameters for the separation of serum proteins by the use of CIM® DEAE column-UV-ICPMS

In order to optimize the analytical procedure for the separation of serum proteins, a synthetic solution of standard proteins IgG, Tf and albumin (5 g L^{-1} of albumin, 1 g L^{-1} of IgG and 0.5 g L^{-1} of Tf) was prepared in buffer A. The concentration of standard serum proteins was similar as in the five times diluted human serum. Our previous investigations on separation of HMM-Al species in human serum demonstrated that the main advantages of the use of CIM® DEAE monolithic disk (Murko et al., 2007) in comparison to Mono Q FPLC column (Kralj et al., 2004) are its robustness and speed of the analysis. Beside 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 CIM® DEAE column was investigated in order to lower the detection limit for speciation of HMM-Al in human serum. It was experimentally proven that the maximal sample volumen 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 two times higher than the maximal serum sample volume injected onto the FPLC particle packed columns (Soldado Cabezuelo et al., 1997; Soldado Cabezuelo et al., 1998; Nagaoka and Maitani, 2000; Kralj et al., 2004) and ten times higher than the volume injected on the CIM® disk (Murko et al., 2007). For the separation of standard serum proteins the following procedure was applied. 1 mL of synthetic solution of standard serum proteins was injected onto the CIM® DEAE column. As eluent ammonium chloride (1M) 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 on-line by UV detection at 278 nm, while Al was detected by ICPMS. Figure 9 shows the separation of standard serum proteins on CIM® DEAE column under the optimised chromatographic procedures. As it can be seen from Figure 9 A, a good resolution among IgG, Tf and albumin was obtained on CIM® DEAE column. It is evident from the elution profile of Al (Figure 9 B), that Al was eluted at the elution time of Tf. In order to determine the concentration of Al bound to Tf, standard serum Tf was spiked with 1, 2, 5, 10, 20 ng mL^{-1} of Al. Linearity of measurement, within this concentration range, was obtained with a correlation coefficient better than 0.998. The concentration of Al in sample from Figure 9 B was calculated on the basis of the peak area and was found to be 3.0 ± 0.2 ng mL^{-1}.

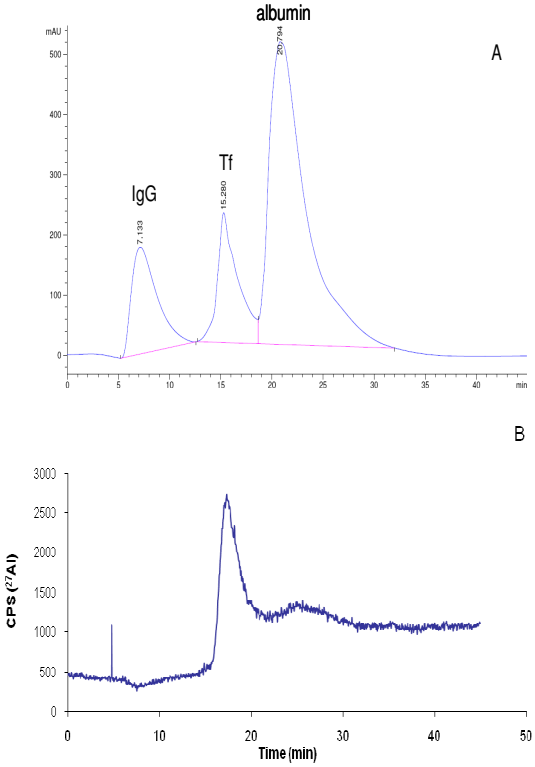

Figure 9: *Separation of standard serum proteins by anion-exchange CIM® DEAE column using UV (278 nm) and ICPMS detection. (A) UV chromatogram and (B) Al elution profile.*

4.4.2 Distribution of Al-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 (Milačič, 2005). At physiological pH it is present as negatively charged complex that is strongly retained by the anion-exchange chromatographic supports (Bantan et al., 1998; Bantan et al., 1999). In serum samples exists the possibility of co-elution of negatively charged LMM-Al species (Bantan et al., 1999) with serum proteins. Therefore, it is necessary to examine the behaviour of Al-citrate on the CIM® DEAE column prior to the speciation analysis of Al bound to proteins. It is also important to know the behaviour of ionic Al that may be present as a contaminant in eluents and chromatographic supports. For this purpose synthetic solutions of Al-citrate and Al-nitrate (10 ng mL^{-1} of Al) were prepared in buffer A at physiological pH of 7.4. The separation was performed under the optimized CIM® DEAE chromatographic procedure. Al elution profiles are presented in Figure 10. Data from Figure 10 indicate that under the chromatographic conditions applied Al-citrate and ionic Al (Al-nitrate) are eluted as broaden peaks from 25 to 45 min and do not overlap with the elution profile of Al bound to Tf. It was experimentally proven that the shape of chromatographic peaks was the same after several consecutive injections of Al-citrate or Al-nitrate. Based on these observations it was presumed that pre-separation of LMM-Al complexes prior to speciation analysis of HMM-Al was not necessary.

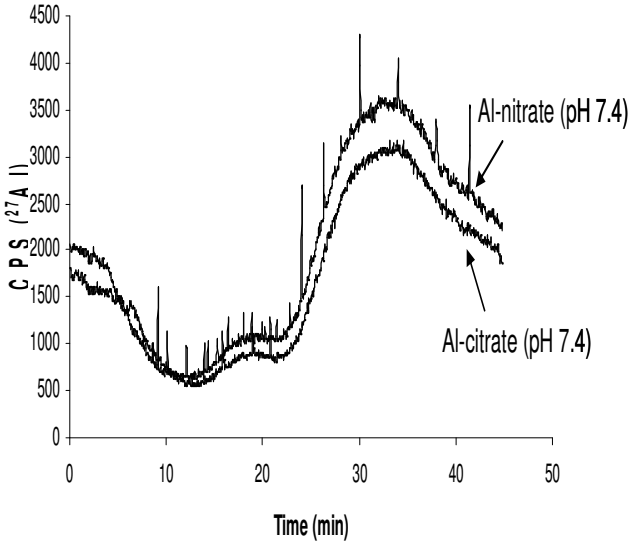

Figure 10: *The Al elution profiles obtained for the separation of Al-citrate and Al-nitrate (10 ng mL^{-1} of Al) on the CIM® DEAE column.*

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 was performed following the Recommended analytical procedure described in 3.5.4. Proteins were eluted in 5 mL chromatographic peak (Kralj et al., 2004; Murko et al., 2007) from 7.5 to 12.5 min and were separated from LMM-Al species. The protein peak was collected (sample diluted five times) and further processed for protein separation using 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 CIM® DEAE procedure. The Al elution profiles obtained by ICPMS are presented in Figure 11. 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 pre-separation of LMM-Al complexes by SEC (Figure 11 A) or when the speciation of Al was performed only by CIM® DEAE-ICPMS (Figure 11 B). Furthermore, on the basis of the elution time Al species separated corresponded to Al bound to Tf. 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 ng mL^{-1} 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 ng mL^{-1} and 15.3 ± 0.6 ng mL^{-1}, respectively. These data proves that when the Recommended analytical procedure (3.5.4) for speciation of HMM-Al in serum by CIM® DEAE is applied, the pre-separation of LMM-Al complexes by SEC is not necessary.

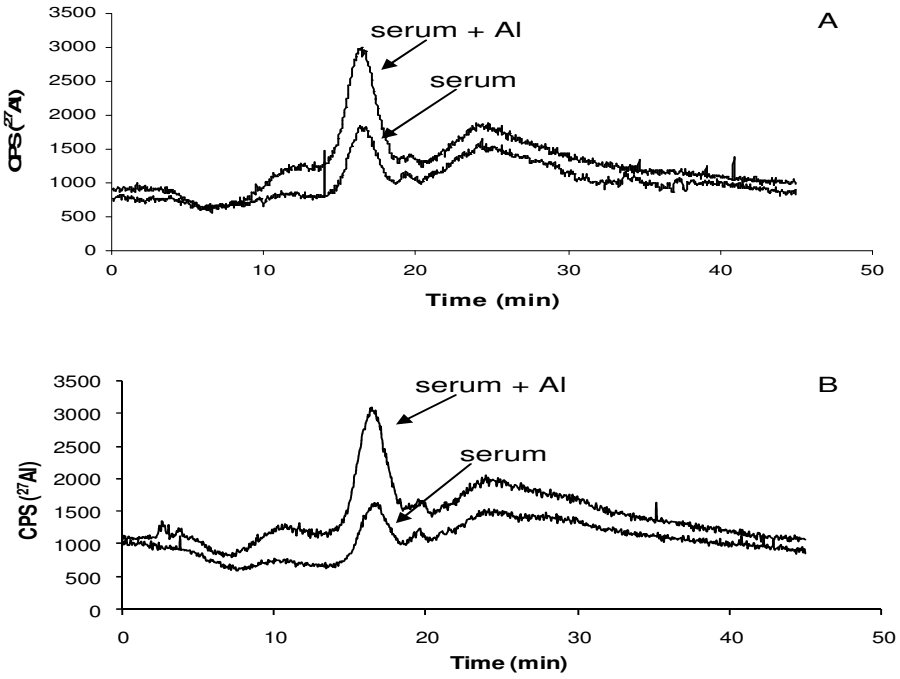

Figure 11: *The Al elution profiles for the speciation of Al in unspiked (1 + 4) and spiked serum samples (1 + 4, spike after dilution 2 ng mL^{-1} Al) when (A) CIM® DEAE-ICPMS was applied after pre-separation of LMM-Al species by SEC or (B) when only CIM® DEAE-ICPMS was used.*

4.4.3 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 3.3 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 (3.3), extremely low blanks were obtained during the chromatographic separation. The Al elution profiles for serum (diluted 1+4) and blank sample that are presented in Figure 12 clearly demonstrate that the overall cleaning procedure is extremely efficient. It was experimentally found that after each cleaning of CIM® DEAE column, three blank samples should first be injected to obtain a reproducible and low blank chromatogram as presented in Figure 12. Al that contributes to the blank value of the overall analytical procedure is eluted as a broaden peak from 25 to 45 min and does not overlap with the elution profile of Al bound to Tf. Based on data from Figure 10 it may be presumed that Al impurities corresponded to ionic Al species. It should be stressed that the chemical and chromatographic stability of methacrylate-based monolithic anion-exchange DEAE column (Vidič et al., 2007), enabled a rigorous cleaning of the chromatographic supports. Low blank as presented in Figure 12 are essential to perform speciation analysis of Al in human serum at normal concentration levels.

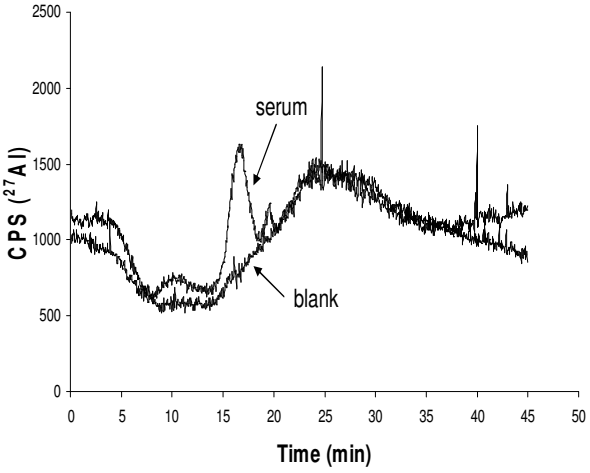

Figure 12: *The Al elution profiles for the separation of unspiked serum (1 + 4) and blank sample after overall cleaning procedure. The peak of Al in unspiked serum sample (1 + 4) corresponds to 1.04 ng mL^{-1} of Al.*

4.5 Speciation of HMM-Al species in unspiked serum by CIM® DEAE column-ICPMS

Serum sample that contained 5.7 ± 0.4 ng mL^{-1} of 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 Tf was determined by the standard addition method. Al species bound to Tf 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 CIM® DEAE-ICPMS procedure confirmed that 91 ± 7 % of Al is bound to Tf. 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 CIM® DEAE column that allowed efficient cleaning of the chromatographic support, enabled in combination with ICPMS reliable quantification of Al in serum bound to Tf. This study confirmes that the percentage of Al bound to Tf at normal serum concentration levels is the same as found for spiked serum samples by Medel's (Soldado Cabezuelo et al., 1997) and by our group (Kralj et al., 2004; Murko et al., 2007). Similar confirmation of Al binding to Tf in unspiked serum of renal patients with elevated Al concentrations was reported by Medel's group (Soldado Cabezuelo et al., 1997; Soldado Cabezuelo et al., 1998) as well. The data on the speciation of Al at normal concentration levels may also serve as a basis for computational investigations (Exley et al., 2007; Beardmore and Exley, 2009).

4.5.1 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 13 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 Tf 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} of 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 3s of the blank sample in the chromatogram. It was found to be 0.15 ng mL^{-1} of Al bound to Tf.

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

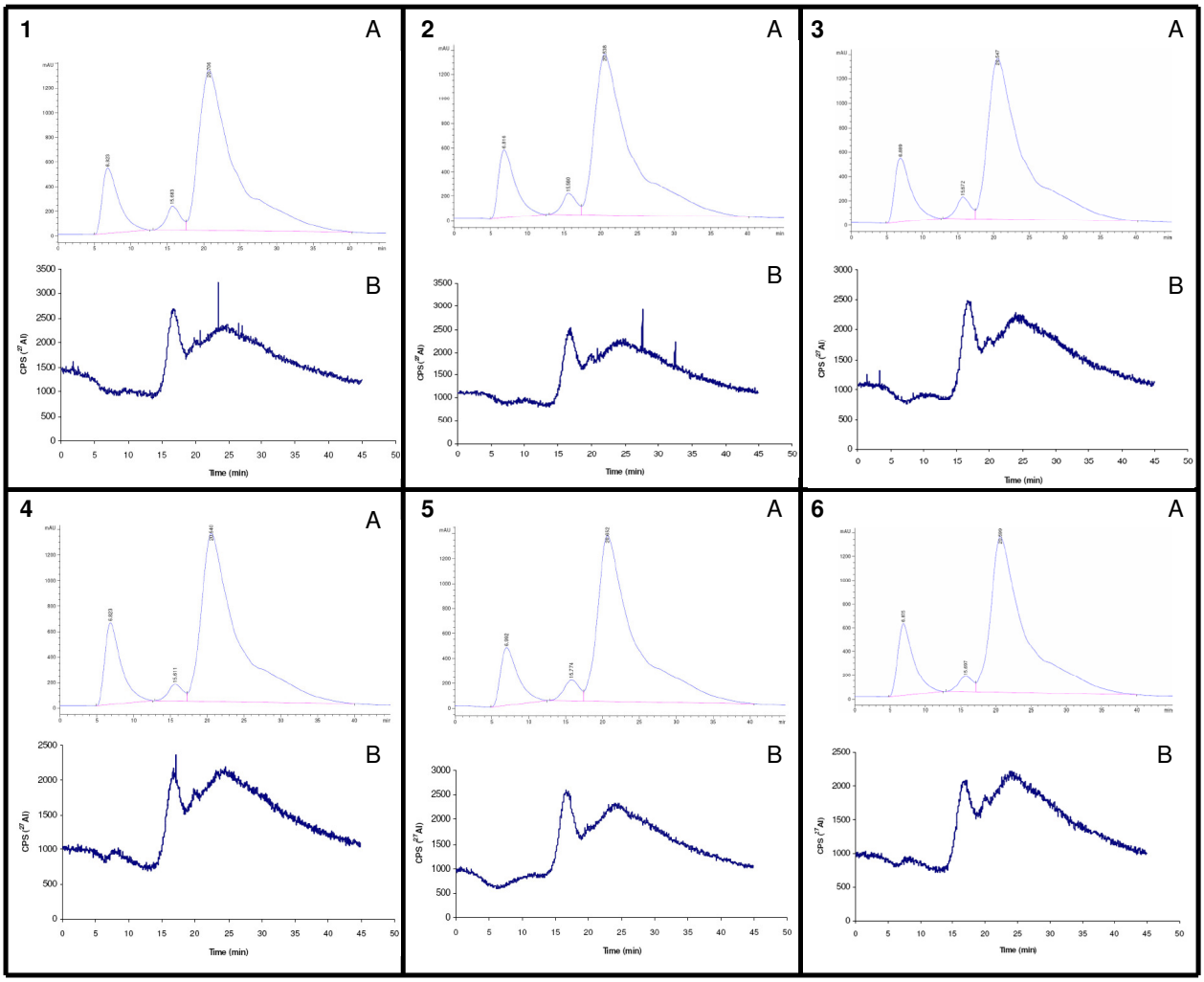

Figure 13: *Six consecutive separations of unspiked human serum (1 + 4) on CIM® DEAE column with UV and ICPMS detection. (A) UV chromatogram and (B) Al elution profile.*

4.6 Identification of Al binding protein

In order to prove that HMM-Al species in serum corresponded exclusively to Al-Tf the separation of serum sample was performed by CIM® DEAE procedure. The fraction eluted under the retention volume of Tf was collected, cleaned/preconcentrated and Tf in reconstituted solution was identified by UPLC-ESI-MS. Tf standard (1 A and 1 B) and serum Tf (2 A and 2 B) are presented in Figure 14. Figure 1 A represents ESI-MS peaks of serum Tf 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 1 B deconvoluted mass spectra of Tf standard (M=79756 Da) is presented. Figures 2 A and 2 B represent the ESI-MS data of serum Tf fraction collected from CIM® DEAE column at the same elution volume as Al detected by ICPMS. Data presented in Figure 14 confirmed that Tf is the only protein that binds Al in human serum. This confirmation is in agreement with previously reported data obtained by SDS-PAGE (Wróbel et al., 1995; Kralj et al., 2004).

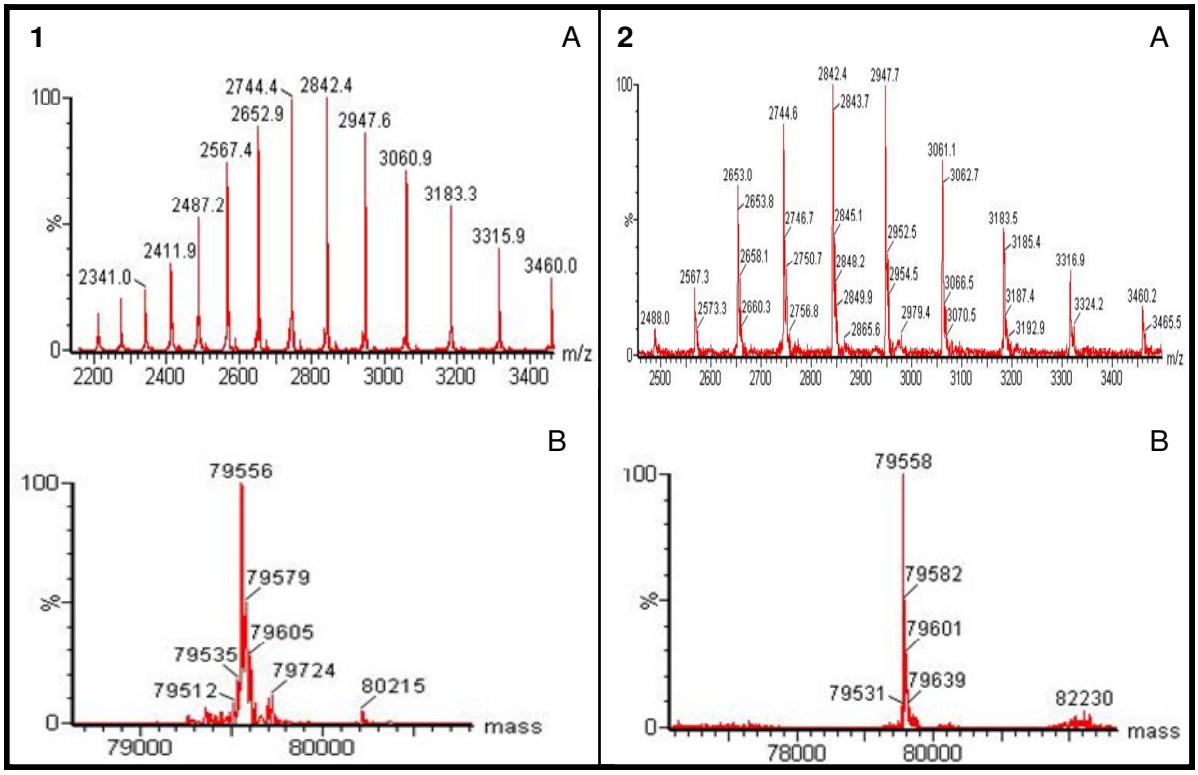

Figure 14: *UPLC-ESI-MS analysis of Tf standard (1 A and 1 B) and human serum Tf after separation on CIM® DEAE column (2 A and 2 B). (A) ESI-mass spectrum, (B) deconvoluted mass spectrum.*

Identification of Al-binding protein was also performed by SDS-PAGE electrophoresis. The same as for identification by UPLC-ESI-MS the separation of serum sample was performed by the CIM® DEAE procedure. The fractions eluted under the retention volume of IgG, Tf and albumin were collected and subjected to the SDS-PAGE electrophoresis. Data from these investigations are presented in Figure 15. From left to right IgG fraction, IgG standard, four fractions of Tf, albumin fraction and albumin standard are presented. As it can be seen from Al elution profiles obtained for serum sample (Figure 9 B) Al bound to proteins is eluted at the elution time corresponding to the Tf. It is clear that in the 4 fractions collected under the peak of Tf exclusively Tf was present. The results confirmed again that Tf is the only HMM-Al species present in human serum. Further, these data also confirmed efficient separation of Tf from other serum proteins on the CIM® DEAE column.

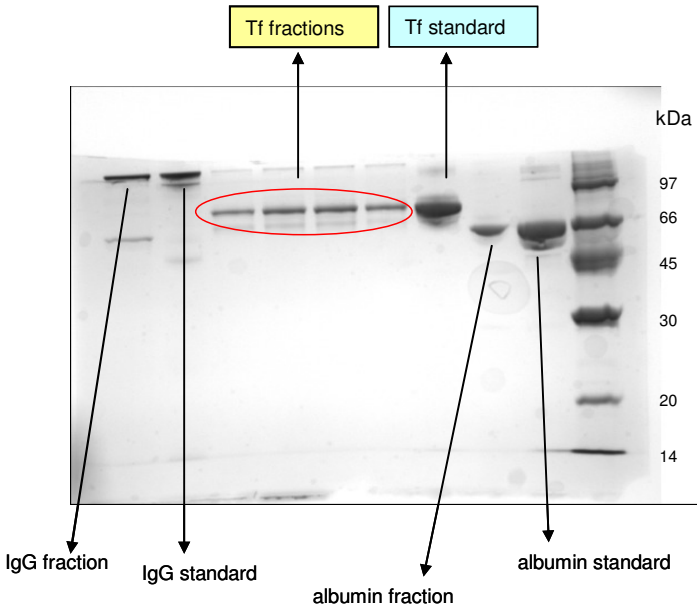

Figure 15: *SDS-PAGE electrophoresis of standard serum proteins (IgG, Tf, albumin) and selected fractions of a human serum. Fractions of serum sample were collected under the retention volume of IgG, Tf and albumin after separation at anion-exchange CIM® DEAE column.*

4.7 Development of the analytical procedure for the reliable separation of HMM-Al species from LMM-Al compounds in serum

4.7.1 Optimization of the analytical procedure for the separations of serum proteins from LMM compounds by the use of HiTrap desalting SEC column and FPLC-UV-ICPMS

To separate serum proteins from LMM species SEC using the HiTrap desalting column was applied. 1 mL of a synthetic solution of standard proteins IgG, Tf and albumin (25 g L^{-1} of albumin, 5 g L^{-1} of IgG and 2.5 g L^{-1} of Tf) prepared in buffer A was injected onto the column. The chromatographic run was carried out at a flow rate of 1 mL min^{-1}. Isocratic elution using 50 mmol L^{-1} Tris-hydrochloric acid buffer was applied for 10 min (3.5.2). The separation of proteins was followed on-line by UV detection at 278 nm, while Al was detected by ICPMS. The UV chromatogram of a synthetic solution of standard proteins IgG, Tf and albumin separated on the HiTrap desalting SEC column is presented in Figure 16 A. It is evident that proteins were eluted in one peak from 1.5 to 4 min. The same chromatographic conditions were used for the separation of undiluted serum. The UV chromatogram is presented in Figure 16 B. As it can be seen from Figure 16 B proteins were separated from 1.5 min to 5 min, while LMM species present in serum were eluted from 7 to 10 min. Figure 16 C shows Al elution profiles obtained for the synthetic solution of standard proteins (IgG, Tf and albumin) and undiluted serum. It can be seen from the elution profiles that the majority of Al in serum was eluted at the elution time corresponding to HMM compounds. In order to prove that LMM compounds present in serum did not co-elute with proteins it was necessary to examine the behaviour of Al-citrate on the HiTrap desalting SEC column. For this purpose a synthetic solution of Al-citrate (10 ng mL^{-1} Al) was prepared in 50 mmol L^{-1} Tris-hydrochloric acid buffer (pH=7.4). The separation of Al species was performed following the Recommended analytical procedure (3.5.2). The elution profile for Al-citrate is presented in Figure 16 C. It can be seen from Figure 16 C that Al-citrate was eluted from the HiTrap desalting SEC column as LMM species from 5 to 8 min. Data from Figure 16 confirmed that the proposed chromatographic separation efficiently separates proteins from LMM compounds in serum.

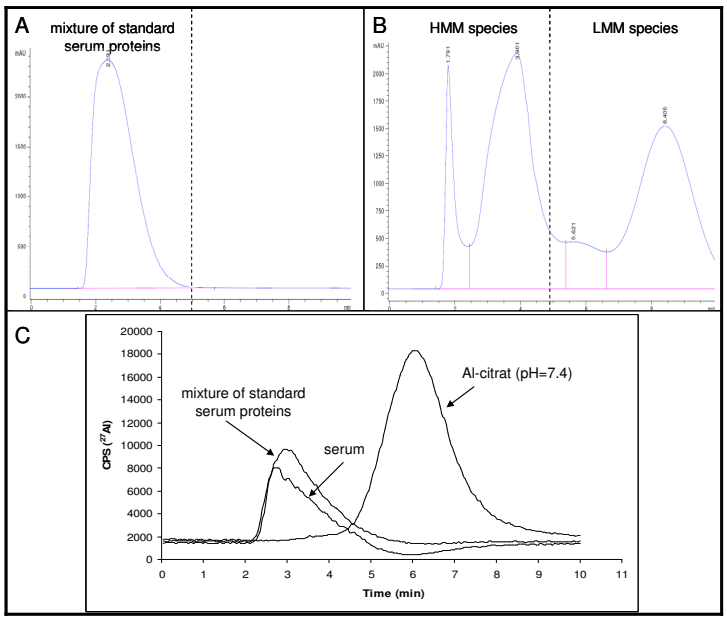

Figure 16: *Separation of the mixture of standard serum proteins, undiluted serum and Al-citrate (10 ng mL^{-1} Al) on a HiTrap desalting SEC column with UV and ICPMS detection: (A) UV chromatogram of the mixture of standard serum proteins, (B) UV chromatogram of undiluted serum, (C) Al elution profiles for the separation of the mixture of standard serum proteins, undiluted serum and Al-citrate.*

In order to ensure that the separation of serum on the HiTrap desalting SEC column does not have an influence on the further speciation of HMM-Al and LMM-Al species, serum spiked with 300 ng mL^{-1} Al was injected onto the HiTrap desalting SEC column and separation was performed following the Recommended analytical procedure (3.5.2) by the use of 50 mmol L^{-1} Tris-hydrochloric acid buffer (pH=7.4). On the basis of previous observations (Figure 16), the protein peak was collected from 0 to 5 min, while the fraction of LMM compounds was collected from 5 to 10 min. Since 1 mL of serum was injected onto the HiTrap desalting SEC column HMM and LMM fractions after separation were diluted five times. Speciation of HMM-Al species was examined by applying a 0.1 mL aliquot of protein fraction onto the CIM® DEAE disk. The chromatographic procedure was performed as described under the Recommended analytical procedure (3.5.3). The elution profiles of separated proteins followed by UV and Al by ICPMS detection are presented in Figure 17. As expected, Al was eluted under the Tf peak. These data are in agreement with our previous observations (Kralj et al., 2004; Murko et al., 2007; Murko et al., 2009). In addition, experimental data obtained by CIM® DEAE disk-ICPMS procedure (Figure 17) proved that the separation of serum on the HiTrap desalting SEC column did not influence the speciation of HMM-Al species.

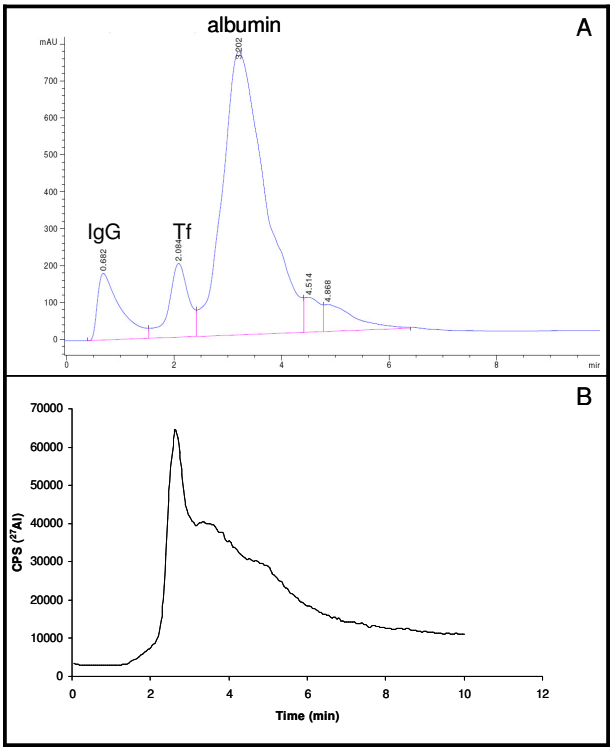

Figure 17: *Separation of spiked serum (300 ng mL^{-1} of Al) by anion-exchange CIM® DEAE disk using UV (278 nm) and ICPMS detection. (A) UV chromatogram and (B) Al elution profile.*

In the LMM serum fraction eluted from HiTrap desalting SEC column (collected from 5 to 10 min) the speciation of LMM-Al compounds was performed by the use of anion-exchange FPLC. A 0.5 mL aliquot of the LMM fraction was injected onto the column and chromatographic separation was accomplished following the Recommended analytical procedure (3.5.5). The elution profile of Al obtained by ICPMS is presented in Figure 18. In the same figure elution profile of synthetic solution of Al-citrate (10 ng mL^{-1} Al, pH=7.4) is overlied. It can be seen from Figure 18 that the majority of LMM-Al species in serum were eluted at the elution time corresponding to Al-citrate. The small LMM-Al peak from serum fraction can also be observed at around 2 min, presumably corresponding to Al-phosphate (Bantan et al., 1999; Bantan et al., 2001). Experimental results obtained by FPLC-ICPMS procedure (Figure 18) proved that the separation of serum on the HiTrap desalting SEC column did not influence the speciation of LMM-Al species.

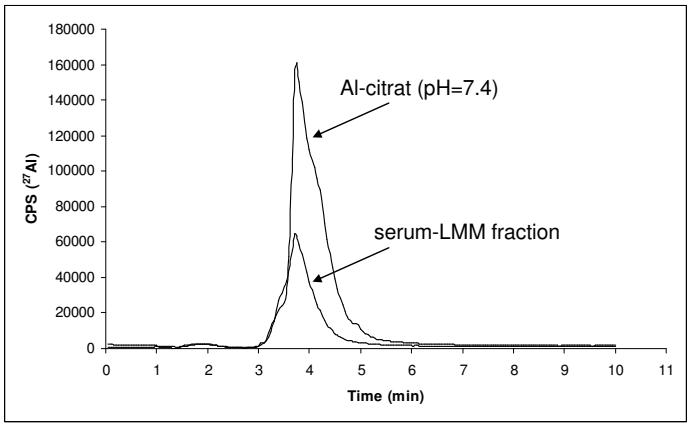

Figure 18: *Al elution profiles obtained for the speciation of LMM-Al compounds in serum and for separation of Al-citrate (10 ng mL^{-1} Al) on the anion-exchange FPLC column.*

In order to study the distribution between HMM-Al and LMM-Al species in serum, a serum sample that contained 2.6 ± 0.3 ng mL^{-1} of total Al was spiked with Al^{3+} solution (150 and 300 ng mL^{-1} Al). Unspiked and spiked serum were injected onto the HiTrap desalting SEC column and the separation of HMM from LMM compounds was performed following the Recommended analytical procedure (3.5.2). Total Al and concentrations of Al in separated HMM and LMM fractions were determined by ICPMS. The results of determination of Al are presented in Table 4.

Table 4: *Distribution of Al species in unspiked and spiked serum samples analysed by combination of SEC separation and ICPMS detection.*

Sample	Total Al (ng mL^{-1})	HMM-Al fraction (ng mL^{-1})	LMM-Al fraction (ng mL^{-1})	HMM-Al species (%)
serum	2.6 ± 0.2	2.4 ± 0.2	0.2 ± 0.03	92 ± 8
serum + 150 ng mL^{-1} Al	157 ± 6	149 ± 5	7.8 ± 0.4	95 ± 3
serum + 300 ng mL^{-1} Al	305 ± 6	284 ± 6	22.6 ± 0.8	93 ± 2

Data in Table 4 indicate that in all samples investigated 93 ± 4 % of Al is eluted as HMM-Al species (Al bound to proteins) and that the remaining Al corresponds to LMM-Al compounds. These findings are in agreement with previous reported literature data (Soldado Cabezuelo et al., 1997; Kralj et al., 2004; Murko et al., 2007; Murko et al., 2009).

Since there was enough evidence that HMM-Al was exclusively bound to Tf (Soldado Cabezuelo et al., 1997; Kralj et al., 2004; Murko et al., 2007; Murko et al., 2009), identification of Al species was further performed in a LMM serum fraction. For this purpose, the UPLC-ESI-MS technique was applied. Due to low concentration of citrate in the collected serum fraction, vacuum assisted concentration was used before UPLC-ESI-MS analysis. UPLC-ESI-MS was performed as described in the Recommended analytical procedure (3.5.6). The same procedure was used for the analysis of matrix matched citrate solution. For this purpose the LMM fraction of serum was spiked with a synthetic solution of citric acid (30 ng μL^{-1}). Figure 19 shows ESI-mass spectra for the matrix matched citrate solution (30 ng μL^{-1}) and for the concentrated LMM fraction of serum. In the mass spectrum of matrix matched citrate solution (19 A) a peak with m/z 191 which corresponds to deprotonated citric acid was present. The same peak with m/z 191 was observed in the mass spectrum of concentrated serum LMM fraction (19 B). These data confirmed the presence of citrate as a LMM binding ligand.

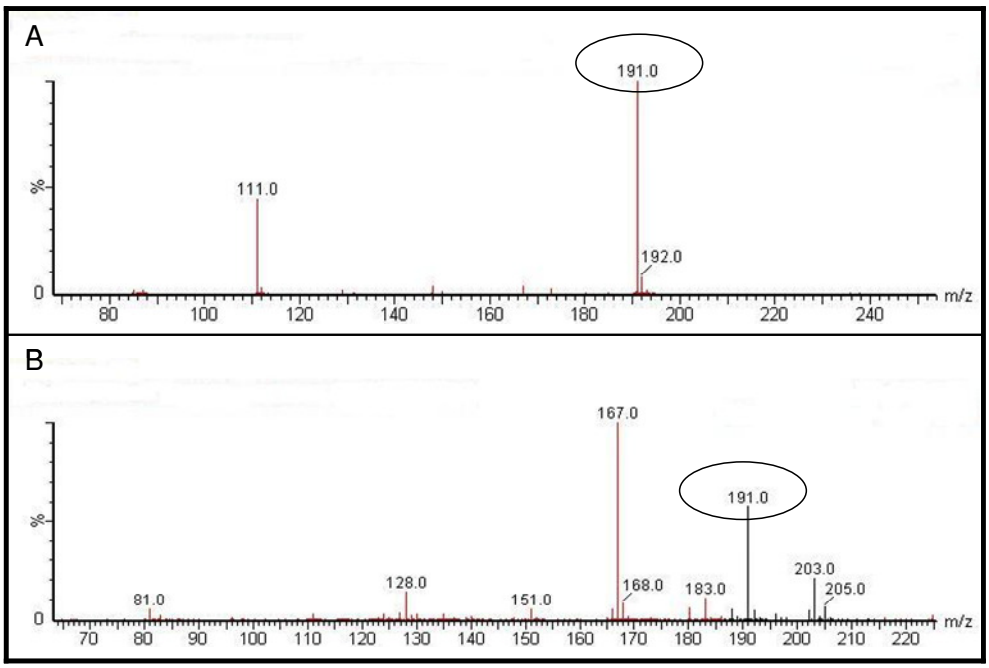

Figure 19: *ESI-mass spectra for (A) matrix matched citrate solution (30 ng μL^{-1}) and (B) concentrated LMM fraction of serum.*

After confirming that citrate, as a major serum LMM compound that binds Al was present in the LMM serum fraction, speciation of unspiked and spiked serum (150 and 300 ng mL^{-1} Al) was performed by the FPLC-ICPMS procedure as described in (3.5.5). Overlays of separated Al species are presented in Figure 20. It should be considered that concentrations of Al in the LMM fraction were low, since only about 10 % of total Al in unspiked and spiked serum corresponded to LMM species (see data in Table 4). In addition, the LMM fraction represented five times diluted serum. Thus the elution profile of LMM-Al in unspiked serum represents about 0.05 ng mL^{-1} of Al and is almost overlapped with the column blank. Elution profiles of LMM-Al in spiked serum (150 and 300 ng mL^{-1} Al) represent about 1.5 and 4.5 ng mL^{-1} Al, respectively. It may be seen from Figure 20 that Al is eluted in two peaks (from 1.5 to 2.8 min and from 2.8 to 4.8 min). Based on the data of present research and the outcomes of our previous investigations (Bantan et al., 1999; Bantan Polak et al., 2001) as well as on the theoretical computational data in the group of Kiss (Lakatos et al., 2001) it may be presumed that the peaks eluted from 1.5 to 2.8 min correspond to Al-phosphate, while the peak from 2.8 to 4.8 min to Al-citrate and ternary Al-citrate-phosphate species. The rapidness and reliability of the developed analytical procedure represent promising tools for the investigations of kinetics of Al binding to Tf.

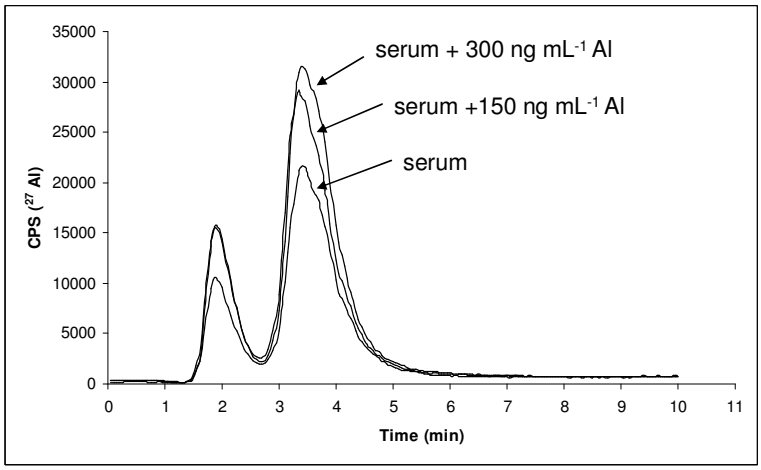

Figure 20: *Speciation of LMM-Al species by anion-exchange FPLC-ICPMS in unspiked and spiked (150 and 300 ng mL^{-1} of Al) serum after separation of HMM-Al species from LMM-Al species by SEC.*

5 Conclusions

Al is not considered to be an essential element in humans. It has been implicated as a toxic factor in a number of human diseases especially in subjects with renal failure. Al bioavailability depends on its chemical forms present in human serum. Speciation analysis may contribute important information for understanding of the mechanism of Al toxicity, its transport through human body and its accumulation in target organs. Therefore, reliable speciation procedures are needed to quantify and identify HMM and LMM Al species present in human serum.

The developed analytical procedure combining SEC and anion-exchange CIM® DEAE disk with ICPMS and UV detection enables reliable determination of the concentration and the composition of the HMM-Al species in spiked human serum. The use of ammonium chloride as an eluent in gradient elution enables fast and selective separation of IgG, Tf and albumin in 10 min. The results confirmed that about 90 % of Al in spiked serum was eluted under the Tf peak. This is in agreement with observations of other researchers and previous work in our group where anion-exchange FPLC column was applied. Therefore, CIM® monolithic disks can be used as a complementary separation support to FPLC columns for the speciation of Al-binding proteins in human serum. In comparison to FPLC chromatographic columns CIM® disks enable faster separation and simpler manipulation during the cleaning procedure as well as simpler coupling to ICPMS. An additional advantage is also that the price of disks is much lower than that of the FPLC columns. The novelty in separation represents the application of ammonium chloride as an eluent that enables both UV and ICPMS detection. Furthermore, ammonium chloride does not form any complexes with Al and therefore has no influence on Al speciation during the chromatographic run.

Combination of 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} of 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 chromatographic support and effective cleaning of eluents considerably lowered the blanks. High capacity and good selectivity of CIM® monolithic column enabled, in combination with ICPMS, reliable quantification of Al in serum bound to Tf. Tf was identified on the basis of the retention volume and also by the UPLC-ESI-MS. The present study confirmed that the percentage of Al bound to Tf 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 Al distribution in human body.

Separation of proteins from LMM species has to be fast, efficient and reliable. By the use of a HiTrap desalting SEC column the separation of HMM from LMM compounds in serum is possible in 10 min. In order to determine the distribution of Al between HMM and LMM species the developed analytical procedure for speciation of HMM-Al species and LMM-Al compounds was used. Once more, results confirmed that in serum around 90 % of Al is bound to Tf. Identification of citrate, one of the main LMM-Al species in serum, was performed in the LMM serum fraction by the UPLC-ESI-MS. The proposed analytical procedure for the separation of HMM from LMM species in serum represents a powerful tool for kinetic studies of Al binding to Tf. The rapidness and reliability of the developed analytical procedure represent promising tools for investigations of kinetics of Al binding to Tf.

The developed analytical procedures for speciation of HMM-Al compounds and for fractionation of Al species in serum represent progress in the field of speciation studies of Al in human body. As the exact mechanism of Al binding to Tf is still not known, the results obtained in speciation and kinetic studies could be an important basis for construction of an Al-Tf model. A computational model could describe the binding and transport of Al by Tf both at equilibrium and as equilibrium for Al-Tf was being approached. The combination of experimental data and the application of mathematical/computational modelling

provide insight into a complex biological system such as blood and also improve our understanding of Al toxicity in humans.

The results of the PhD research work were published in two papers and one review paper in renowned international journals with SCI impact factors (Appendix 1). An additional paper is in preparation. The work has been presented at national and international scientific conferences with oral or poster presentations (Appendix 2).

6 Acknowledgements / Zahvala

Ta stran bi lahko bila prazna, ker ne velja le tisto, kar je na papirju, ampak le tisto, kar nosiš v srcu.

A vendarle.

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Kdor išče cilj, bo ostal prazen, ko ga bo dosegel, kdor pa najde pot, bo cilj vedno nosil v sebi.
(Nejc Zaplotnik)

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Index of Figures

Index of Tables

Appendix 1: Publications

Scientific paper: Speciation of Al in human serum by convective-interaction media fast-monolithic chromatography with inductively coupled plasma mass spectrometric detection

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Speciation of Al in human serum by convective-interaction media fast-monolithic chromatography with inductively coupled plasma mass spectrometric detection

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Abstract

A new analytical procedure using anion-exchange separation support based on convective-interaction media (CIM) was developed for the speciation of Al in human serum. The separation of proteins was performed on a weak anion-exchange CIM diethylamine (DEAE) fast-monolithic disk. To prevent co-elution of low molecular mass (LMM) Al species with high molecular mass (HMM) Al compounds on CIM disk serum proteins were first separated from LMM-Al species by the use of size exclusion chromatography (SEC). For this purpose 1 mL of serum was injected onto SEC (Superdex 75 HR 10/30) column. Isocratic elution using 0.05 M TRIS–HCl + 0.03 M $NaHCO_3$ was applied and separation of proteins was followed by UV detection at 278 nm. It was experimentally proven that proteins were eluted in 5.5 mL peak that was collected into a polyethylene cup. A 0.1 mL of the sample aliquot was then injected onto the CIM DEAE disk. The separation of serum proteins was obtained in 10 min by applying linear gradient elution from 100% buffer A (0.05 M TRIS–HCl + 0.03 M $NaHCO_3$) to 100% buffer B (A + 1 M NH_4Cl) and followed by UV detection at 278 nm. Separated Al species were detected on-line by inductively coupled plasma mass spectrometry (ICP-MS). Well-resolved protein peaks were obtained. It was experimentally proven that 90 ± 3% of Al in spiked serum of renal patient was eluted under the transferrin peak. The proposed speciation procedure removes LMM-Al species and enables reliable determination of the concentration and composition of Al bound to proteins by CIM DEAE-ICP-MS when the concentration of Al in serum is higher than 5 ng mL^{-1}. In comparison to chromatographic columns CIM disks enable faster separation and simpler manipulation during cleaning procedure and coupling to ICP-MS.

Keywords: High molecular mass Al species; Human serum; Size exclusion chromatography; Convective-interaction media diethylamine fast-monolithic chromatography; UV detection; ICP-MS

1. Introduction

Although Al is the most abundant metal in the environment, the sparingly soluble nature of most Al compounds considerably decreases the probability of an Al overload in humans from environmental sources. Absorption via the diet, drinking water and medications, represent the main pathways of Al absorption and accumulation in human body [1]. Al is known neurotoxic element [2,3]. Al accumulation in the brain and bones of renal patients is associated with dialysis encephalopathy [4–6] and osteomalacia [7]. Al accumulation in brain has also been related to the neurodegenerative process in Alzheimer's disease [8,9]. To prevent intoxication of dialysis patients with Al the quality of water used for dialysis has been significantly improved [10]. However, the absorption and accumulation of Al, particularly via consumption of Al-based drugs [6], is still an important source for Al overload in renal patients. In order to understand the toxicity of Al in humans, it is essential to identify and quantify the chemical species in which Al is transported and stored in the body [11]. Due to this, speciation of Al in human serum has been intensively investigated. For

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determination of the proportion of high molecular mass (HMM) and low molecular mass (LMM) Al species and for the identification of their composition various analytical techniques have been used [12,13]. By applying ultrafiltration and microultrafiltration the percentage of Al bound to HMM and LMM species [14–17] was estimated. In general, the results suggested that about 90% of Al was bound to HMM proteins. Further efforts of researchers were oriented towards identifying and quantifying LMM-Al and HMM-Al species. Speciation of LMM-Al complexes in spiked human serum and serum of renal patients was performed by the use of anion-exchange fast protein chromatography (FPLC) with ETAAS detection [18–20]. Serum samples were microultrafiltered prior to FPLC analysis to remove HMM-Al compounds. Fractions of separated Al species were collected and Al was determined by electrothermal atomic absorption spectrometry (ETAAS), while Al-binding ligands were identified by electrospray ionisation tandem mass spectrometry [19,20]. LMM-Al species present in serum were Al-citrate, Al-phosphate and ternary Al-citrate–phosphate complexes. Their composition and amount varied among particular individuals [19,20]. Since the major part of Al in serum is bound to proteins, investigations were also oriented towards identifying and quantifying Al-binding protein. Fractionation of Al by size exclusion chromatography (SEC) [21,22] was not selective enough to separate serum proteins. Al was eluted under the chromatographic peak of albumin and transferrin. In addition, the excess Al was eluted from the column [21,22], most probably due to the contamination of the column support. More selective separation of proteins was obtained by an anion-exchange HPLC using Protein-Pak DEAE-5PW [23] or TSK DEAE-3SW columns [24]. In combination with SDS–PAGE and element detection of Al by ETAAS, it has been demonstrated that transferrin is the only binding Al protein in human serum. However, the main problem in Al speciation remained contamination originating from the chromatographic columns and eluents [23]. The progress in speciation of HMM-Al compounds has been achieved by the use of anion-exchange FPLC columns [25–29]. Different cleaning procedures of the eluents resulted in substantial lowering of the risk of contamination with extraneous Al [18–20,25–29]. Efforts were also oriented towards efficiently cleaning the chromatographic support of the column resins [18–20,27–29]. For speciation of Al bound to serum proteins by FPLC different eluents and various types of gradient elution were applied. Separation of proteins were obtained in 20 min [25,26,29] or in 50 min [27,28]. The chromatographic peaks were identified by UV detection and Al was determined "off-line" by ETAAS [25,29] or "on-line" by high resolution ICP-MS (HR-ICP-MS) [26–28]. These investigations further verified that about 90% of Al was eluted under the elution volume of transferrin. It has been also demonstrated that the favoured binding site of Al in transferrin is the N-lobe site [27,28]. In addition to experimental approaches for speciation of Al bound by transferrin, application of computational methods was proposed to test whether 90% of all Al is bound by transferrin, or 90% of Al is available to be bound by transferrin [30].

To obtain reliable analytical data in speciation analysis it is important to apply and compare complementary analytical procedures. As an alternative to FPLC columns containing ion-exchange resins, ion-exchange separation supports based on convective-interaction media (CIM) were developed in the last decade. The matrix supports of poly(glycidylmethacrylate-co-ethyleneglycoldimethacrylate) substituted with strong or weak cation and anion exchangers [31] offer very fast separation of biomolecules [32–34] and are also used for fast separation of organic acids [35] as well as for the analysis of impurities of immunoglobulin concentrates [36]. In our group CIM disks were successfully applied in the speciation of Zn in environmental samples [37] and Cr(VI) at the workplace of plasma cutters [38].

The aim of the present work was to develop a new analytical procedure for the reliable speciation of HMM-Al compounds in spiked serum of a renal patient by the use of anion-exchange CIM fast-monolithic chromatography with ICP-MS and UV detection. To overcome the risk of co-elution of LMM-Al species with the HMM-Al compounds, pre-separation of LMM-Al complexes on SEC column was first performed [29]. Protein peak eluted from SEC column was collected and an aliquot used for the speciation procedure by CIM-ICP-MS. Special attention was devoted to the cleaning procedure of eluents and monolithic disks in order to obtain low blanks. Furthermore, the choice of the eluent that was compatible with ICP-MS and allowed also UV detection of the chromatographic runs was carefully examined.

2. Experimental

2.1. Instrumentation

The chromatographic system for UV detection consisted of a Varian (Mulgrave, Victoria, Australia) Model 9010 HPLC inert Star Gradient Solvent Delivery System, equipped with a Varian (Mulgrave, Victoria, Australia) Polychrom Model 9065 UV diode array detector and a Rheodyne (Cotati, California, USA) Model 7161 injector using 1 mL (SEC) or 0.1 mL loop (CIM DEAE). 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 100,000). A weak anion-exchange CIM DEAE disk (Bia Separations, Ljubljana, Slovenia) (disk dimensions 12×3 mm, poly(glycidylmethacrylate-co-ethyleneglycoldimethacrylate) matrix support substituted with diethylamine (DEAE) groups, pH stability 1–13) was used.

The chromatographic system for ICP-MS on-line detection consisted of a high performance liquid chromatography pump Series 1100 from Agilent (Tokyo, Japan) equipped with a Rheodyne (Cotati, California, USA)

Table 1
ICP-MS operating conditions for determination of Al

Parameter	Value
RF power (W)	1500
Sampling depth (mm)	8.0
Outer gas flow rate (L min^{-1})	15.0
Carrier gas flow rate (L min^{-1})	0.80
Make-up gas flow rate (L min^{-1})	0.17
He gas flow rate (mL min^{-1})	4.0
m/z monitored	27
Integration time (s)	3.0
Total acquisition time (s)	900
Repetition	1

Model 7725i injector using 0.1 mL loop. The outlet of the CIM monolithic DEAE disk was directly connected to the Babington nebuliser and a Scott-type spray chamber of ICP-MS (Agilent 7500ce, Tokyo, Japan). A nickel sampler and skimmer with 1.0 and 0.4 mm cone orifices, respectively, were used. Treatment of data was performed with the Agilent ChemStation software. Data processing was based on the peak area. ICP-MS operating conditions for determination of Al are listed in Table 1.

The total concentration of Al in serum as well as the total concentration of Al in protein peak eluted from SEC column was determined off-line by ICP-MS under operating conditions given in Table 1.

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

2.2. Reagents

Merck (Darmstadt, Germany) suprapur acids and Milli-Q water (Direct-Q 5 Ultrapure water system, Millipore Watertown, MA, USA) were used for the preparation of samples and standard solutions. All other reagents were of analytical reagent grade.

A stock standard solution of Al (1000 ± 0.002 mg L^{-1} in 5% HNO_3) used for calibration in ICP-MS determinations was obtained from Merck. A stock Al^{3+} solution (100 μg Al mL^{-1}) used for the spiking of serum sample and standard proteins was prepared in a 100 mL calibration flask by dissolving 0.1388 g of $Al(NO_3)_3 \cdot 9H_2O$ (Riedel-de Haën, Hannover, Germany) in water. Fresh working standard solutions were prepared daily by dilution of stock solutions with water.

Buffer A consisted of 0.05 M TRIS–HCl buffer (Merck) + 0.03 M $NaHCO_3$ (Merck). The pH of buffer A was adjusted to 7.4 with an appropriate amount of 1 M of hydrochloric acid. Buffer B contained 1 M of ammonium chloride (Merck) and was prepared by dissolving 53.49 g NH_4Cl in 1 L of buffer A.

Standard proteins (Sigma–Aldrich, Steinheim, Germany) of known molecular masses: albumin (66,000 Da), transferrin (77,000 Da), Immunoglobulin G (IgG) (150,000 Da) (0.5–5 mg mL^{-1}) were used for calibration of the CIM DEAE disks. Blue dextran with a molecular mass 200,000 Da (1 mg L^{-1}) was used to determine the void volume of the size exclusion chromatographic column.

Chelex 100 (Na^+ form, 100–200 mesh) chelating ion-exchange resin (Sigma) and a silica-based LiChrosorb RP-18 HPLC column (150 × 4.6 mm i.d.) were used for purification of the eluents [18].

2.3. Sample preparation

In order to study the separation of proteins at pH 7.4 standard proteins were dissolved in buffer A (albumin 25 mg L^{-1}, IgG 5 mg L^{-1} and transferrin 2.5 mg L^{-1}) and first injected onto the SEC column. After that the aliquot of the protein peak was injected onto the CIM DEAE disk.

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. Sample was centrifuged for 10 min at 855*g*. Serum aliquots were transferred into 5 mL polyethylene tubes with polyethylene pipette and stored in a freezer at −20 °C. Before analysis samples were equilibrated to room temperature. Total Al was first determined. In order to study the speciation of HMM-Al compounds, 3 mL of serum was spiked with 0.05 mL of Al^{3+} solution (Al-nitrate salt), so that the final concentration of Al in the spiked serum ranged from 50 to 300 ng mL^{-1}. Spiked serum was left to equilibrate at room temperature for 5 h [18,19]. After that it was injected onto the SEC column (1 mL loop). 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 sample were analysed.

2.4. Recommended procedures

Sample preparation, chromatographic separations and determination of Al by ICP-MS were carried out under clean-room conditions (class 10000).

2.4.1. SEC procedure

One milliliter of spiked serum was injected onto the column. The chromatographic run was carried out at a flow rate of 1 mL min^{-1}. Isocratic elution with buffer A was applied for 15 min. From 15 to 16 min linear gradient elution 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 linear gradient elution from 100% buffer B to 100% buffer A continued and from 30 to 40 min the column was rinsed with 100% buffer A. The protein peak was collected from 7.5 to 13 min (5.5 mL) into a polyethylene sample cup and the concentration of total Al determined by ICP-MS.

2.4.2. CIM DEAE-ICP-MS procedure

The protein fraction from SEC column was subjected to anion-exchange chromatography. 0.1 mL of the sample aliquot was injected onto a CIM DEAE fast-monolithic disk. The chromatographic run was carried out at a flow rate of 1 mL min^{-1}. Linear gradient elution from 100% buffer A

to 100% buffer B was applied for 10 min. The disk was rinsed with 100% buffer B for 0.5 min and from 10.5 to 15 min with buffer A. To follow the separation of proteins the disk was connected on-line to UV detector. For quantification of separated Al species the disk was connected on-line to ICP-MS.

2.4.3. Cleaning procedures

To avoid contamination by extraneous Al, polyethylene or Teflon ware was treated with 10% HNO_3 for 24 h, rinsed well with water and dried at room temperature. By combination of chelating ion-exchange chromatography (Chelex 100, Na^+ form, batch procedure) and silica-based reversed-phase HPLC [18] effective cleaning of the eluents was obtained. The cleaning of SEC column and CIM disk was performed at a flow rate of 1 mL min^{-1} under the same procedure with the exception that SEC column was cleaned in the opposite direction of the chromatographic separation. Rinsing with 15 mL of water was first applied. Then 1 M NaOH was injected (1 mL onto the SEC column and 0.1 mL onto the CIM disk, respectively). Rinsing of the chromatographic supports with 15 mL of water followed. The procedure was repeated twice. After that 10 mL of 2 M citric acid was applied and chromatographic supports were then rinsed with 15 mL of water. Cleaning with NaOH as described above was applied again. Finally, the chromatographic supports were rinsed with 15 mL of water and 15 mL of buffer A before the next chromatographic separation. All the above described cleaning procedures lowered the blanks in chromatographic separations down to 0.5 ng Al mL^{-1}. It was experimentally proven that cleaning procedure should be applied after five subsequent SEC and 10 subsequent CIM separations.

3. Results and discussion

3.1. Determination of total Al concentration by ICP-MS

Before analysis serum samples were diluted five times with water and total Al concentration was determined by ICP-MS under the optimal operating conditions (Table 1). The same analytical procedure was applied to determine Al concentrations in spiked standard serum proteins. The accuracy of determination of total Al was checked by the analysis of Seronom™ Trace Elements Serum standard reference material obtained from Nycomed Pharma AS (Oslo, Norway). Good agreement between determined Al (61 ± 1 ng mL^{-1}) and the reported certified value (63 ± 4 ng mL^{-1}) was obtained. The concentration of total Al in serum (mean of three parallel analysis) was found to be 4.0 ± 0.2 ng mL^{-1}. This concentration was too low to perform speciation analysis. Therefore, samples were spiked with Al^{3+} solution, so that the final concentration of Al in spiked serum ranged between 50 and 300 ng mL^{-1} Al.

3.2. Development of the analytical procedure for separation of serum proteins by combining SEC and anion-exchange CIM DEAE

To separate HMM-Al compounds from LMM-Al species in serum, SEC was first applied. Isocratic elution with 0.05 M TRIS–HCl + 0.03 M $NaHCO_3$ (buffer A, pH 7.4) as described in Section 2.4 was applied. Proteins were eluted from 7.5 to 13 min. The 5.5 mL protein peak was collected into a polyethylene cup (sample diluted 5.5 times).

In order to investigate the separation of serum proteins by anion-exchange CIM fast-monolithic chromatography synthetic solutions of the standard proteins IgG, transferrin, albumin and their mixture were prepared (5 mg L^{-1} of albumin, 1 mg L^{-1} of IgG and 0.5 mg L^{-1} of transferrin). As an eluent NH_4Cl that enables both UV and ICP-MS detection was chosen. Furthermore, NH_4Cl does not form any complexes with Al and therefore has no influence on Al speciation during the chromatographic run. First strong anion-exchange CIM quaternary amine (QA) disk was used. 0.1 mL of standard proteins was injected onto the disk and linear gradient elution from 100% buffer A to 100% buffer B preformed in 10 min. Results indicated that separation was not selective enough since albumin

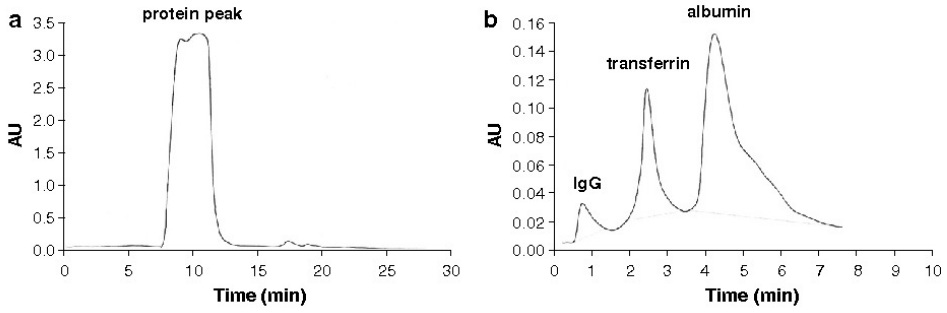

Fig. 1. Separation of a standard solution of proteins at a flow rate of 1 mL min^{-1} followed by UV detection at 278 nm. One milliliter of sample containing 25 g L^{-1} of albumin, 5 g L^{-1} of IgG and 2.5 g L^{-1} of transferrin was first injected onto the SEC column (a). A protein peak was collected from 7.5 to 13 min (5.5 mL). 0.1 mL aliquot of a protein peak was then injected onto the CIM DEAE disk (b).

and transferrin were eluted under the same chromatographic peak. Therefore, the potential of a weak anion-exchange CIM DEAE disk was further investigated. First the influence of ionic strength of the eluent (buffer B) (0.25, 0.5 and 1 M NH_4Cl) was examined. 0.1 mL of standard proteins was injected onto the disk and linear gradient elution from 100% buffer A to 100% buffer B was applied in 10 min. Separation with 0.25 and 0.5 M NH_4Cl resulted in the broadening of chromatographic peaks, while by the use of 1 M NH_4Cl well-resolved chromatographic peaks of IgG, transferrin and albumin were obtained. The optimal concentration of NH_4Cl in buffer B was therefore 1 M. In further experiments 0.1 mL of standard proteins was injected onto the disk and different times of gradient elution were examined (5, 7.5 and 10 min). Experimental data indicated that the best resolution of chromatographic peaks was obtained when gradient elution in 10 min was applied. Finally, the influence of a sample volume loaded on the disk was observed (0.02, 0.05, 0.1, 0.2 and 0.5 mL sample loops). Chromatographic peaks were well resolved using 0.02 up to 0.1 mL sample loop, while the application of higher sample volume resulted in broadening and overlapping of the chromatographic peaks. To obtain a better limit of detection, 0.1 mL sample loop was therefore used and separation of proteins performed under the optimal conditions described in Section 2.4.

A combined procedure including removal of LMM compounds from proteins on SEC column followed by separation of protein fraction on CIM DEAE disk was then applied at optimal separation conditions. Chromatographic separations were followed by UV detection at 278 nm. The chromatograms for the mixture of standard proteins are presented in Fig. 1 and for human serum of a renal patient in Fig. 2. Data of Figs. 1a and 2a indicate that a protein peak is eluted from SEC column between 7.5 and 13 min in the mixture of standard proteins as well as in serum sample. It may be further seen from Figs. 1b and 2b that good separation of IgG, transferrin and albumin is obtained in protein fraction on CIM disk. This is evident for a mixture of standard proteins (Fig. 1b) and also for a protein fraction of a serum sample (Fig. 2b). On the basis of data from Figs. 1 and 2 it is evident that the proposed analytical procedure enables good separation of serum proteins.

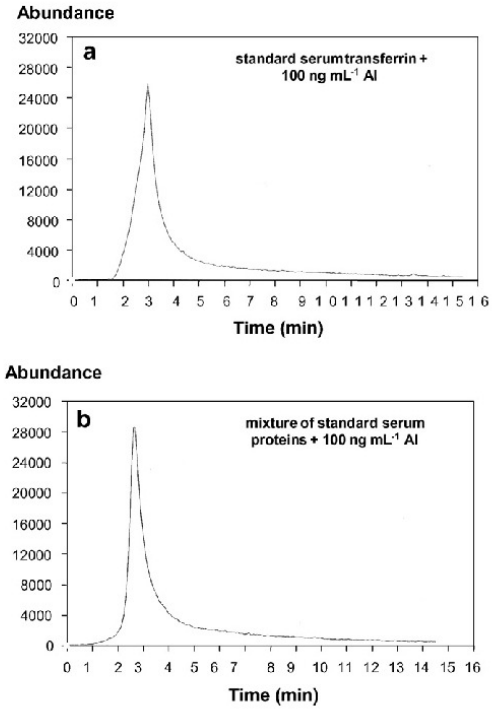

Fig. 3. Separation of standard serum transferrin (0.5 g L^{-1}) spiked with 100 ng mL^{-1} Al (a) and a mixture of standard serum proteins (5 g L^{-1} of albumin, 1 g L^{-1} of IgG and 0.5 g L^{-1} of transferrin) spiked with 100 ng mL^{-1} Al (b) on a CIM DEAE disk followed by ICP-MS detection.

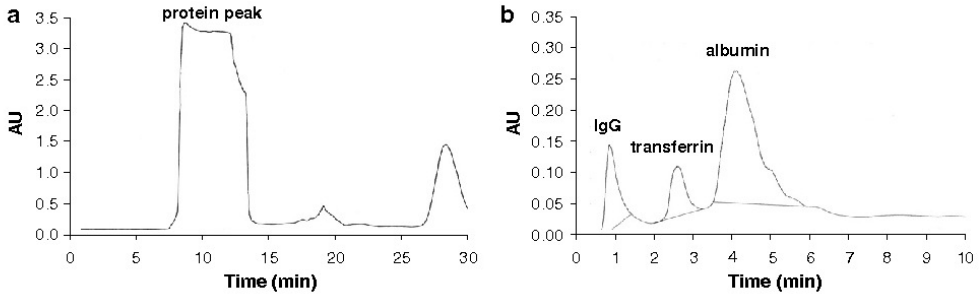

Fig. 2. Separation of a human serum of a renal patient at a flow rate of 1 mL min^{-1} followed by UV detection at 278 nm. One milliliter of sample was first injected onto the SEC column (a). A protein peak was collected from 7.5 to 13 min (5.5 mL). 0.1 mL aliquot of a protein peak was then injected onto the CIM DEAE disk (b).

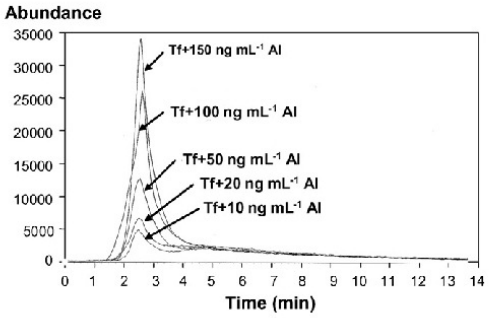

Fig. 4. Overlay chromatograms of standard serum transferrin (0.5 g L^{-1}) spiked with 10, 20, 50, 100 and 150 ng mL^{-1} of Al. Speciation was performed by CIM DEAE-ICP-MS procedure.

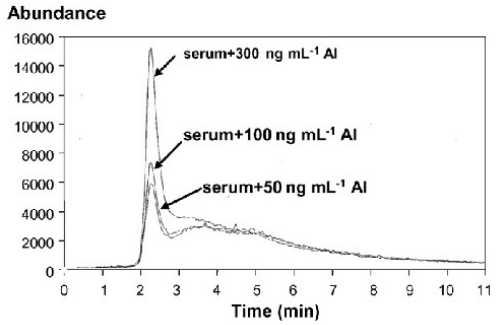

Fig. 6. Speciation of Al by CIM DEAE-ICP-MS in spiked serum of a renal patient after pre-separation of LMM-Al complexes by SEC. Overlay chromatograms represent spiked serum with 50, 100 and 300 ng mL^{-1} Al.

3.3. Speciation of Al in spiked serum of a renal patient by combining SEC and CIM DEAE-ICP-MS

Once the separation of serum proteins was optimised, speciation of Al in spiked serum was investigated by combining SEC and CIM DEAE-ICP-MS. To obtain on-line ICP-MS detection, the outlet of the CIM disk was directly connected to the Babington nebuliser of ICP-MS. The advantage of the use of Babington nebuliser is its ability to operate at a flow rate of 1 mL min^{-1} that is the same as applied in the separation of proteins on CIM disk. In addition, Babington nebuliser is capable of introducing samples with high salt contents into ICP-MS. The study was first performed by the use of standard serum proteins (5 mg L^{-1} of albumin, 1 mg L^{-1} of IgG and 0.5 mg L^{-1} of transferrin). Standard serum transferrin as well as the mixture of standard serum proteins were spiked with 100 ng mL^{-1} of Al. 0.1 mL of spiked samples were injected onto the CIM DEAE disk coupled on-line with ICP-MS. Results are presented in Fig. 3. It is evident that the shape and the elution profile of Al are the same in the transferrin-spiked sample (Fig. 3a) and in the mixture of spiked standard proteins (Fig. 3b). In both chromatograms Al was eluted under the chromatographic peak of transferrin. The corresponding peak areas for Al were 7,008,329 and 6,826,870, respectively. These observations confirmed again that transferrin is the only serum protein that binds Al. For calibration in speciation analysis of Al, standard serum transferrin (0.5 mg L^{-1}) was spiked with 10, 20, 50, 100 and 150 ng mL^{-1} of Al. 0.1 mL of spiked samples were then injected onto the CIM DEAE disk coupled on-line with ICP-MS. Fig. 4 represents overlay chromatograms of spiked standard serum transferrin. On the basis of the peak area, a calibration curve was done. A linear response of the calibration curve was obtained with correlation coefficient $R^2 = 0.9985$. In Fig. 5 three consecutive speciation analysis of a standard serum transferrin (0.5 g L^{-1}) spiked with 20 ng mL^{-1} of Al are presented. The speciation was carried out by CIM DEAE-ICP-MS. Data indicate that consecutive chromatographic separations are repeatable. The corresponding peak areas for Al were 1,129,170, 1,125,555 and 1,126,018, respectively.

A combined procedure including SEC pre-separation of proteins and speciation of Al in the protein fraction by CIM DEAE-ICP-MS was then performed on a spiked serum of a renal patient. Analyses were done under analytical

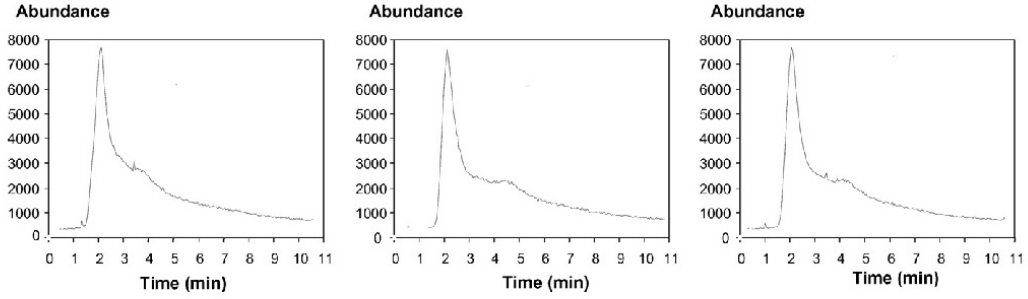

Fig. 5. Consecutive speciation analysis of standard serum transferrin (0.5 g L^{-1}) spiked with 20 ng mL^{-1} of Al. Speciation was carried out by CIM DEAE-ICP-MS procedure.

Table 2
Quantification and identification of Al species in spiked serum of a renal patient

ICP-MS Concentration of Al in spiked serum ($ng\,mL^{-1}$)	SEC, ICP-MS Concentration of HMM-Al species ($ng\,mL^{-1}$)	HMM-Al species (%)	CIM DEAE-ICP-MS Concentration of Al bound to transferrin ($ng\,mL^{-1}$)	HMM-Al species bound to transferrin (%)
49.8 ± 0.9	45.1 ± 0.9	91 ± 2	44.2 ± 0.8	98 ± 2
50.2 ± 0.9	45.2 ± 0.9	90 ± 2	43.4 ± 0.8	96 ± 2
98 ± 2	91 ± 2	93 ± 2	88 ± 2	97 ± 2
97 ± 2	85 ± 2	88 ± 2	83 ± 2	96 ± 2
256 ± 5	233 ± 5	91 ± 2	226 ± 6	97 ± 2
285 ± 6	254 ± 6	89 ± 2	246 ± 6	97 ± 2

procedures described in Section 2.4. As an example of these analyses overlay chromatograms of spiked serum with 50, 100 and 300 $ng\,mL^{-1}$ of Al are shown in Fig. 6. The chromatograms in Fig. 6 represent speciation of Al by CIM DEAE-ICP-MS procedure after pre-separation of LMM-Al complexes by SEC. On the basis of the elution time between 2 and 3.5 min it is evident that Al is eluted under the transferrin peak.

In order to quantify the separated Al species by SEC in combination with CIM DEAE-ICP-MS, the following procedure was applied. A serum sample of a renal patient was spiked with different concentrations of Al. Total Al in spiked serum was determined by ICP-MS. Then 1 mL of serum was subjected to SEC to separate LMM-Al species from proteins. Protein fraction was collected between 7.5 and 13 min and the concentration of Al in the protein peak was determined by ICP-MS. After that 0.1 mL sample aliquot of the protein peak was injected onto CIM DEAE disk coupled to ICP-MS and speciation analysis was performed. Al species bound to transferrin were quantified on the basis of the peak area. In order to prepare a calibration curve, standard serum transferrin was spiked with Al and CIM DEAE-ICP-MS speciation procedure was applied. The results are presented in Table 2.

Data of Table 2 indicate that, on the SEC column, in all spiked samples investigated 90 ± 3% of Al is eluted as HMM-Al species (Al bound to proteins). Speciation analysis of the protein fraction by CIM DEAE-ICP-MS furthermore confirmed that 97 ± 2% of Al of the protein fraction is eluted exclusively under the elution volume of the transferrin peak. This findings are in agreement with the reported literature data [25–27,29].

The proposed speciation procedure removes LMM-Al species and enables reliable determination of the concentration and composition of Al bound to proteins by CIM DEAE-ICP-MS when the concentration of Al in serum is higher than 5 $ng\,mL^{-1}$.

4. Conclusions

The developed analytical procedure combining SEC and anion-exchange CIM DEAE fast-monolithic chromatography with ICP-MS and UV detection enables reliable determination of the concentration and the composition of the HMM-Al species in spiked human serum. Pre-separation of proteins from LMM-Al species by SEC, prior to speciation procedure on a weak anion-exchange CIM DEAE fast-monolithic disk, prevents co-elution of LMM-Al species with HMM-Al species on a CIM disk. The use of NH_4Cl as an eluent in gradient elution enables fast and selective separation of IgG, transferrin and albumin in 10 min. The results of the present work demonstrated that about 90% of Al in spiked serum of renal patient was eluted under the transferrin peak. This is in agreement with observations of other researchers and our previous work where anion-exchange FPLC column was applied. Therefore, CIM monolithic disks can be used as a complementary separation support to FPLC columns for the speciation of Al-binding proteins in human serum. In comparison to FPLC chromatographic columns CIM disks enable faster separation and simpler manipulation during the cleaning procedure as well as simpler coupling to ICP-MS. An additional advantage is also that the price of disks is much lower than that of the FPLC columns. The novelty in separation represents the application of NH_4Cl as an eluent that enables both UV and ICP-MS detection. Furthermore, NH_4Cl does not form any complexes with Al and therefore has no influence on Al speciation during the chromatographic run.

Acknowledgements

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Scientific paper: 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

Anal. Chem. **2009**, *81*, 4929–4936

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 (5000g, 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

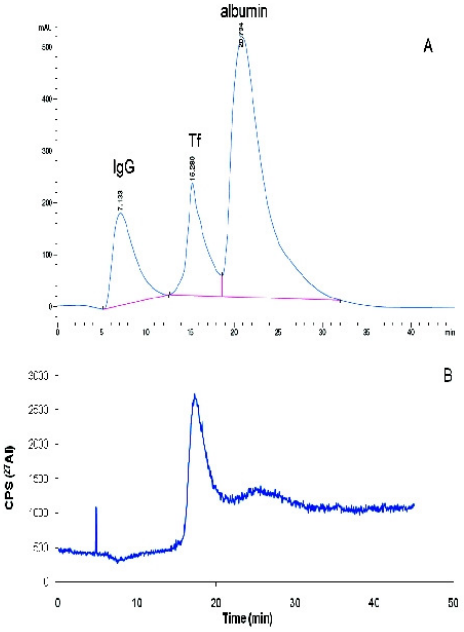

Figure 1. Separation of standard serum proteins by an anion exchange CIM DEAE column using UV (278 nm) and ICPMS detection: (A) UV chromatogram and (B) Al elution profile.

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

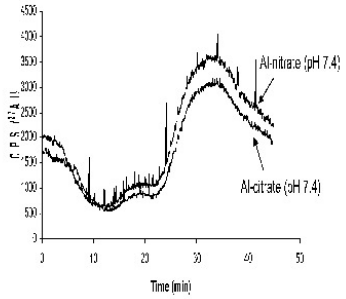

Figure 2. Al elution profiles obtained for the separation of aluminum citrate and aluminum nitrate (10 ng mL^{-1} Al) on the CIM DEAE column.

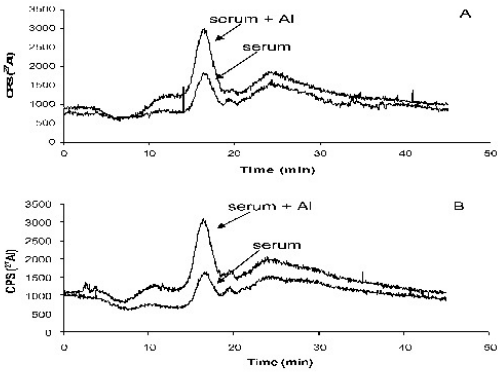

Figure 3. Al elution profiles for the speciation of Al in unspiked (1 + 4) and spiked (1 + 4, spike after dilution to 2 ng mL^{-1} Al) serum samples when (A) CIM DEAE−ICPMS was applied after preseparation of LMM-Al species by SEC or (B) when only CIM DEAE−ICPMS was used.

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

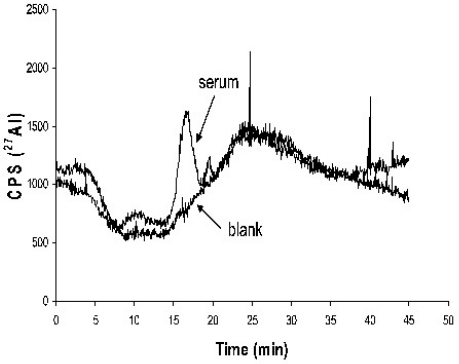

Figure 4. Al elution profiles for the separation of unspiked serum (1 + 4) and the blank sample after the overall cleaning procedure. The peak of Al in the unspiked serum sample (1 + 4) corresponds to 1.04 ng mL^{-1} Al.

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-

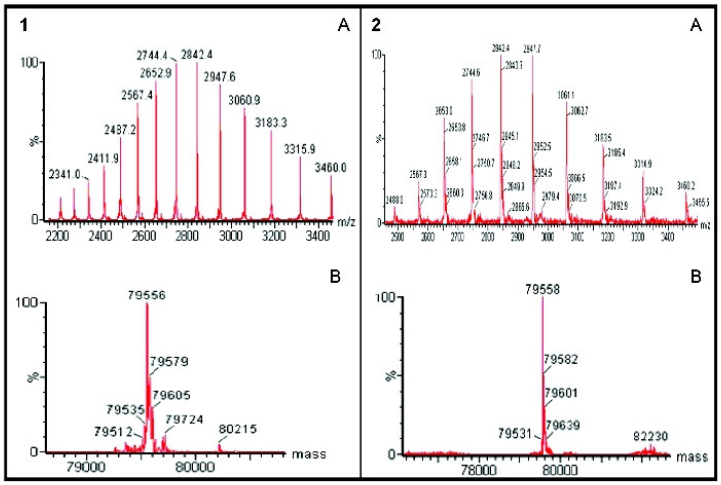

Figure 5. UPLC−ESI-MS analysis of transferrin standard (1A and 1B) and human serum transferrin after separation on a CIM DEAE column (2A and 2B): (A) ESI mass spectrum, (B) deconvoluted mass spectrum.

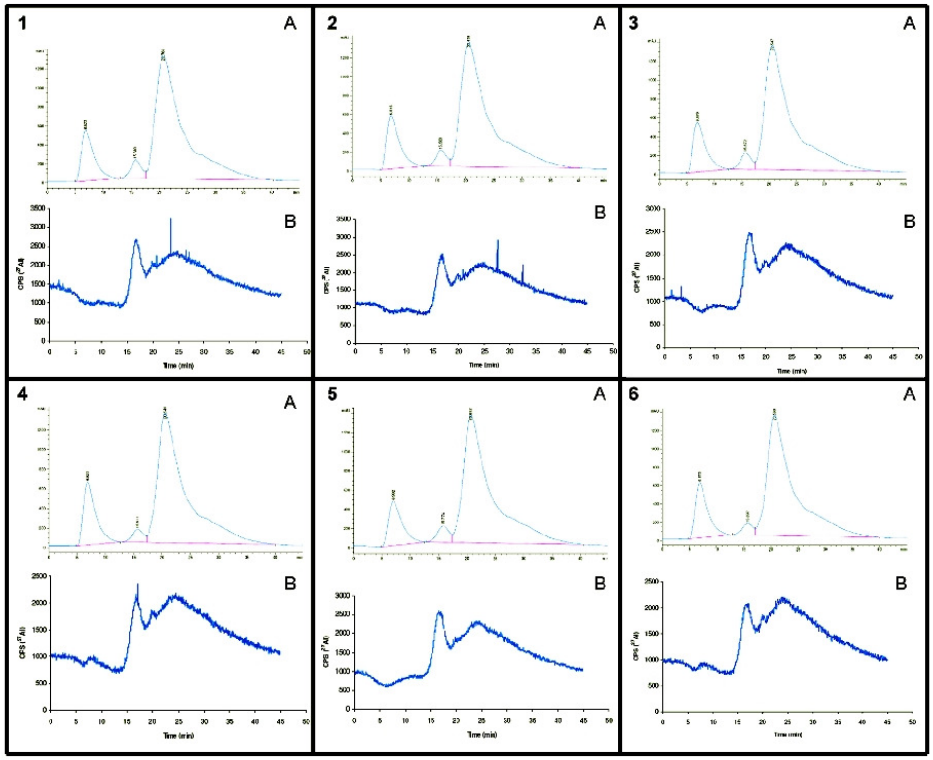

Figure 6. Six consecutive separations of unspiked human serum (1 + 4) on a CIM DEAE column with UV and ICPMS detection: (A) UV chromatogram and (B) Al elution profile.

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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Problems and progresses in speciation of Al in human serum: An overview

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ABSTRACT

Aluminium (Al) is associated with many clinical disorders in renal patients. Al accumulation in brain has also been related to the neurodegenerative processes in Alzheimer's disease. In order to better understand Al transport in the human body, it is necessary to identify and quantify chemical species in which Al is present in body fluids and tissues. Among a variety of biological samples, Al speciation was the most frequently investigated in human serum. Improvements were made in the development of analytical techniques for the determination of the amount and composition of high molecular mass Al (HMM-Al) and low molecular mass Al (LMM-Al) species in human serum. However, due to the complex chemistry of Al in serum, its low total concentration and the high risk of contamination, speciation of Al in biological samples is still a difficult task for analytical chemists. In this work, problems related to speciation of Al in human serum are critically discussed. An overview of the progress that was made by the use of different analytical procedures, in order to propose analytical protocols for reliable speciation of Al in serum at low ng mL^{-1} concentration range, is presented.

1. Introduction

Aluminium (Al) is the most abundant metal in the environment. However, the sparingly soluble nature of most Al compounds significantly decreases the probability of an Al body burden in humans from environmental sources. Al is not considered to be an essential element in humans. Its toxic effects are known particularly in patients with chronic renal failure [1–3]. Al accumulation in brain has also been related to the neurodegenerative process in Alzheimer's disease [4,5]. Concern over maintaining the quality of water and fluids used for dialysis considerably prevented intoxication of dialysis patients with Al [1]. Nevertheless, the absorption of Al via consumption of Al-based drugs [3] and its accumulation in target organs is still a possible source of Al overload in renal patients. In order to understand the toxicity of Al in humans, it is essential to identify and quantify the chemical species in which Al is transported and stored in the body [6–8]. For these reasons, speciation of Al in human serum has been intensively investigated. First attempts in speciation of Al in human serum were oriented to distinguish between the percentage of low molecular mass Al (LMM-Al) and high molecular mass Al (HMM-Al) species. For this purpose fractionation of serum samples using ultrafiltration and microultrafiltration procedures [9–11], size exclusion chromatography (SEC) [12,13] and HPLC TSK G4000 SW column [14] were performed. The results in general suggested that about 90% of Al was bound to HMM compounds. The same observations were reported from study on the fractionation of Al by SEC with UV and ETAAS detection in spent continuous ambulatory peritoneal dialysis (CAPD) fluids [15]. However, Keirsse et al. [12] reported, that an excess of Al was eluted from the SEC coulmn, most probably due to contamination from the column support. By SEC techniques it was demonstrated that Al was eluted under the elution profile of transferrin and albumin. More selective separation of proteins was obtained by an anion-exchange high performance liquid chromatography using Protein-Pak DEAE-5PW column [16]. In combination with SDS–PAGE and final element detection of Al by ETAAS, researchers in the Medel's group demonstrated that the transferrin is the only binding Al protein in human serum [16]. The significant progress in Al speciation in serum has been achieved by the use of the robust anion-exchange Mono Q fast protein liquid chromatographic column (FPLC). This polyether resin based column enabled quantitative separation of Al species and has been successfully applied in the further investigations of the percentage and the composition of both LMM-Al [17–19] as well as HMM-Al species [20–23]. Lower detection limits were obtained when ICP-MS was introduced as element specific detector in determination of separated Al species [21,22]. Recently, methacrylate based convective-interaction media® (CIM®) monolithic chromatography has been applied as a complementary separation support to FPLC columns for the speciation of Al-binding proteins in human serum [24]. Using DEAE CIM® monolithic disk in combination with UV and ICP-MS detection it has been demonstrated again, that HMM-Al is quantitatively eluted under the chromatographic peak of transferrin. Also, the percentage of Al bound to transferrin was found to be about 90%. All the above mentioned investigations

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were performed on spiked human serum (100–300 ng Al mL^{-1}) or serum of dialysis patient that contained elevated concentrations of Al (about 100 ng mL^{-1}). Research was oriented also to investigate the composition of LMM-Al-binding ligands in serum. Microultrafiltration (cut off 30,000 Da) was first applied to separate ultrafiltrable Al from HMM-Al species [17]. Aliquot of an ultrafiltrable fraction of Al was then injected onto the FPLC column. On the basis of the elution time and identification of the Al-binding ligands by ES-MS-MS, it has been demonstrated that the main LMM-Al species were Al-citrate, Al-phosphate and ternary Al-citrate–phosphate complexes [18,19]. These data agreed with the modeling calculations performed in group of Kiss [25]. In speciation analysis of Al in human serum, it was of crucial importance to prevent contamination. For this purpose the analyses were performed under the clean room conditions. Various cleaning procedures of eluents and the column resin were applied [17–24], that all resulted in substantial lowering of the risk of contamination with extraneous Al.

In 2002, a critical review on analytical methodologies for Al speciation in human serum was reported by the group of Medel and our group [26]. Although analytical procedures were appreciably improved in the last decade [27], speciation of Al in serum and other biological samples is still being developed. In the present work, the most important analytical approaches for speciation of Al in human serum are overviewed. Special attention is given to the analytical achievements of the last five years.

2. Analytical procedures for speciation of Al

Blood serum is a very complex matrix containing HMM and LMM compounds and high concentration of salts. Normal total concentrations of Al in serum range between 0.5 and 8 ng mL^{-1} [28], while recently, even lower normal Al concentrations, around 2 ng mL^{-1} or less were reported [29]. Due to the complexity of the serum constituents, low total concentration of Al and the high risk of contamination, speciation of Al in biological samples is still a difficult task for analytical chemists. In the following paragraphs drawbacks related to speciation of Al in human serum are presented and progress that was made through the development of the analytical procedures is discussed.

2.1. Contamination: problems and solutions

Because of low Al concentration the speciation of Al in serum of healthy subjects was in general performed in spiked samples or in serum of renal patients with elevated Al concentrations. To obtain reliable analytical data it is of extreme importance to avoid all possible sources of contamination and to apply appropriate cleaning procedures for removing traces of Al from the eluents, reagents, columns and filtering devices used in the speciation procedure. In order to prevent contamination by extraneous Al, high density polyethylene or Teflon ware, previously soaked with 10% HNO_3 for 48 h and rinsed with high purity MilliQ water should be used [30]. Overall analytical procedure should be carried out under clean room conditions (at least class 10,000). It is also important to clean microultrafiltration membranes used in fractionation of serum samples. For this purpose 0.1 mol L^{-1} NaOH was used in three successive filtrations followed by sufficient rinsing of membranes (by filtration) with ultrapure water [9–11]. In order to remove Al from eluents used in chromatographic procedures Van Landeghem et al. [31] applied an on-line silica-based C_{18} scavenger column which had a strong affinity to adsorb Al. Similarly, in Medel's group chelating Kelex 100-impregnated silica C_{18} scavenger column was used "on-line" to clean the eluents [21]. In our group [17] the chromatographic eluents were first treated with the chelating resin Chelex 100 (batch procedure). Filtered eluents were then passed through a silica-based HPLC LiChrosorb RP-18 column to remove residual Al. Since the chromatographic supports used in the speciation procedure also contained trace amounts of Al, it was of great importance to efficiently clean the column resin. For this purpose fast protein liquid chromatography (FPLC) columns used in the speciation of LMM-Al compounds were cleaned by passing 10 mL of 5 mol L^{-1} citric acid through the column resin, so that the column blanks were below 1 ng Al mL^{-1} [17]. A similar cleaning procedure was also applied to efficiently remove traces of Al from the microultrafiltration membranes Centricon 30 [17]. In speciation of HMM-Al in serum, cleaning of the column resin is even more important. Traces of Al and the retained serum constitutents should be efficiently removed from the chromatographic supports. There were not many reports that draw attention to this problem. Nagaoka and Maitani [22] reported the cleaning procedure for FPLC coulmn used for separation of HMM-Al species in serum. Separated Al species were determined "on-line" by HR ICP-MS. After rinsing the column with buffer, 100 μL of 7 m mol L^{-1} sodium citrate was first injected, followed by seven subsequent injections of 0.25 m mol L^{-1} ammonium acetate in 50 m mol L^{-1} TRIS buffer. After serum analysis one single injection of 2 mol L^{-1} NaCl, 2 mol L^{-1} NaOH and 75% acetic acid was applied. Authors stated that by the use of the proposed cleaning procedure the blank was lowered to 0.1 ng Al mL^{-1}. In our group, efficient cleaning procedures of SEC and FPLC columns used for speciation of HMM-Al in human serum were also developed [23]. Columns were first rinsed with 15 ml of water. Then 1 mL of 1 mol L^{-1} NaOH was injected onto the SEC column, while 0.5 mL of 1 mol L^{-1} NaOH was applied onto FPLC column, followed by rinsing with 15 mL of water. After that 10 min linear gradient elution at 1 mL min^{-1} from 100% water to 100% 2 mol L^{-1} citric acid was applied, followed by rinsing with 10 mL of 2 mol L^{-1} citric acid. 10 min linear gradient elution at 1 mL min^{-1} from 100% 2 mol L^{-1} citric acid to 100% water followed. Finally, the columns were rinsed with 15 mL of water and 15 mL of 0.05 mol L^{-1} TRIS–HCl buffer (pH 7.4). By applying the above described cleaning procedure low blanks from the column resin (below 1 ng Al mL^{-1}) were obtained. When CIM® DEAE fast-monolithic disk was later used in the separation of HMM-Al species, similar cleaning procedure as for FPLC column was applied [24], with exception that 100 μL of NaOH was injected onto the disk. The blanks from the disk support were lowered to below 0.5 ng mL^{-1} Al. In order to obtain reliable analytical data in speciation of Al in serum samples, the overall cleaning procedure and careful handling of samples to prevent contamination is of crucial importance.

2.2. Fractionation procedures

Pérez Parajón et al. [9] critically evaluated the speciation of Al in serum by ultrafiltration with ETAAS detection. The authors highlighted the problems of contamination that led to controversial results reported by previous investigators. Microultrafiltration which minimized contamination risk gave more reliable results than classical ultrafiltration. Data indicated that in spiked serum of healthy subjects about 8% of total Al was ultrafiltrable, while in renal patients this percentage was about 13%. The same researchers [9] also found that after desferrioxamine (DFO) chelation therapy in renal patients, total serum Al and the ultrafiltrable fraction significantly increased due to mobilization of Al by desferrioxamine from body tissues and serum proteins. Wróbel et al. [11] reported that the percentage of ultrafiltrable Al was influenced neither by the individual renal pathology of the patients nor by kidney transplantation. In patients undergoing DFO chelation therapy, the total Al was significantly increased. The ultrafiltrable fraction which represented about 11% of total Al before DFO treatment was in-

creased to almost 80% of total serum Al after DFO treatment. Fractionation of serum by the microultrafiltration procedure gives information on the ratio between HMM-Al and LMM-Al in human serum. It can also provide a useful analytical tool for estimation of the efficiency of the chelation therapy [32].

Further attempts of researchers were oriented to find out on which serum protein Al is bound. For this purpose size exclusion chromatography (SEC) was first applied. In SEC analysis bicarbonate plays a critical role in the binding of Al by transferrin [33,34]. In the absence of bicarbonate in the eluting buffer, more peaks and a greater proportion of LMM-Al fraction were observed [35]. In contrast, when bicarbonate was added to the eluting buffer, fewer peaks were observed in the SEC chromatogram and the largest peak appeared in the transferrin/albumin region [36]. Due to the poor resolution between transferrin and albumin it was not possible to identify the Al-binding protein in serum by SEC. SEC procedures enabled only to distinguish between the percentage of HMM-Al and LMM-Al species.

2.3. Development of chromatographic procedures for speciation of LMM-Al species in human serum

The distribution of LMM-Al species in serum has been intensively investigated by computer simulation. Based on known thermodynamic equilibrium stability constants the following LMM-Al species were predicted in human serum: Al-citrate, Al-phosphate, Al-hydroxide and mixed ternary complexes of Al-citrate–phosphate, Al-phosphate–hydroxide and Al-citrate–hydroxide species [37–40,25]. However, data on distribution of these species in human serum were rather controversial. So, there was a need to experimentally determine the composition and content of LMM-Al species in human serum. Favarato et. al. [41] applied a TSK-GEL HW 55S SEC column for separation of Al species present in the serum of normal and occupationally exposed subjects. Separated Al species were determined by ETAAS. In addition to one HMM-Al fraction three to five separated LMM fractions which contained Al were also found, but were not identified. In order to determine the amount and composition of LMM-Al species present in human serum there was a need for more powerful speciation techniques. Since Al-citrate has been theoretically predicted as one of the most important LMM-Al-binding ligands in human serum [25,38], efforts were oriented to the development of a reliable analytical procedure for speciation of this biologically important molecule. Datta et al. [42] used normal, reversed phase and mixed phase (ODS/NH_2) columns and various mobile phases for separation of Al-citrate. The best results for synthetic standard solutions of Al-citrate were obtained when cyclobonded and cyanobonded phase columns were used. However, the retention times were not reproducible and Al-citrate recoveries were moderate (less than 65%), presumably due to adsorption of Al on the silica-based columns. Therefore, it was not possible to use this technique for quantification of Al-citrate in biological samples. In our group first data on quantitative determination of Al-citrate [43] and its application to speciation in serum samples [17–19] were reported by the use of strong anion-exchange Mono Q fast protein liquid chromatography (FPLC) column. This polyether based column resin bearing quaternary amine functional groups did not adsorb negatively charged Al species and exhibited the wide pH range stability (2–12). For speciation of LMM-Al species at low ng mL^{-1} concentration level, there were several important requirements. The column resin should not adsorb Al species of interest, eluents used for chromatographic separation should enable quantitative separation of Al species and should not form complexes with Al. In addition, eluent should be compatible with the element specific detector used for the determination of separated Al species. By the use of strong anion-exchange Mono Q FPLC 5/5 column and aqueous 0–4 mol L^{-1} NH_4NO_3 gradient elution in 10 min, negatively charged Al-citrate species was quantitatively separated in the pH range 6.5–7.4. Separated Al species were then determined "off-line" in 0.5 mL fractions by ETAAS [17]. The main advantage of NH_4NO_3 eluent lied in its ability to decompose in the graphite tube during the ashing step, enabling quantitative and reproducible (RSD 2%) determinations of separated Al species by ETAAS. Al-citrate was quantitatively eluted from 4.5 to 5.5 min. The low limit of detection for separated species (2 ng Al-citrate mL^{-1}) offered the application of the developed procedure for determination of LMM-Al species in spiked human serum. Nitrate salt of Al (50–150 ng mL^{-1} Al^{3+}) was used for spiking of samples (equilibration at room temperature for 4 h). To separate HMM-Al from LMM-Al compounds samples were first microultrafiltered. All the necessary steps to avoid contamination as discussed in Section 2.1. were considered, including the cleaning of the column resin and ultrafiltration membranes with 5 mol L^{-1} citric acid that lowered the blank of the overall analytical procedure to below 1 ng Al mL^{-1} [17]. Speciation analysis of the microultrafiltrable fraction representing 15–19% of total Al indicated that LMM-Al species were quantitatively eluted under the elution time of Al-citrate. On the basis of these observations it was presumed that LMM-Al corresponded to Al-citrate. In addition to citrate [37,38,25] computational calculations predicted also phosphate [39,40,25] and ternary citrate–phosphate complexes [25] as important LMM-Al species present in human serum. Therefore, more detailed identification and quantification of the Al species eluted under the chromatographic peak [17] was necessary. For this purpose Bantan et al. verified also the behaviour of Al-phosphate on the anion-exchange Mono Q FPLC column [18,19]. Typical chromatograms for separation of Al-citrate and Al-phosphate at pH 7.4 is presented in Fig. 1. In addition, LMM-Al species in serum were characterized not only on the basis of the retention time but also by electrospray (ES)-MS-MS analysis of the LMM ligands eluted under the chromatographic peak. The reproducibility of measurement (RSD), tested for six consecutive separations of Al-citrate and Al-phosphate (100 ng mL^{-1} Al, pH = 7.4) was found to be 2% for Al-citrate and 15% for Al-phosphate. The limit of detection (LOD) for determination of separated Al species in serum by FPLC-ETAAS was 5.0 ng mL^{-1}. Investigations were performed on the spiked serums of healthy subjects [18] and spiked and unspiked serum of dialysis patients [19]. The same speciation procedure as described previously was applied [17], with exception that for better selectivity, 0.2 mL fractions were collected throughout the chromatographic run. In fractions that contained Al, ES-MS-MS analyses were performed. Based on the mass spectra and the corresponding daughter ion spectra the presence of citrate (peak m/z 191 and the corresponding daughter ion spectra with m/z 111, 87 and 85), phosphate (peak m/z 97 and the corresponding daughter ion spectra with m/z 97 and 79) and both phosphate and citrate binding ligands were confirmed in separated serum fractions. An example of ES-MS-MS spectra in separated serum fractions containing Al is presented in Fig. 2. By combining data from speciation of Al by FPLC-ETAAS procedure and further identification of the Al-binding ligands, it was experimentally proven that Al-citrate, Al-phosphate and ternary Al-citrate–phosphate species were present in serum samples. These data were in agreement with the computional calculations in human serum performed in the group of Kiss [25]. The experimental data [18,19] further indicate that the distribution of LMM-Al species varied between particular individuals. Individual variability was also observed in the percentage of LMM-Al species in spiked serum, which in general ranged from 14 up to 50%. It should be considered that health status of individuals and medical treatment may play a role in the distribution and amount of LMM-Al species in serum. However, this presumption should be further investigated.

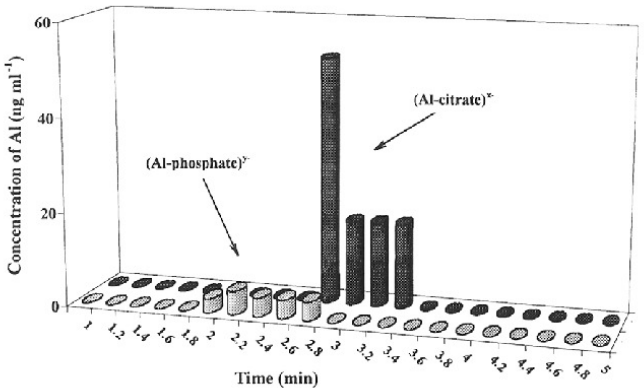

Fig. 1. Typical chromatograms of Al-citrate and Al-phosphate (100 ng mL^{-1} Al) at pH 7.4. Separation was performed on a Mono Q HR 5/5 anion-exchange FPLC column and separated species were detected by ETAAS. Reproduced from Ref. [19], with permissions from Elsevier.

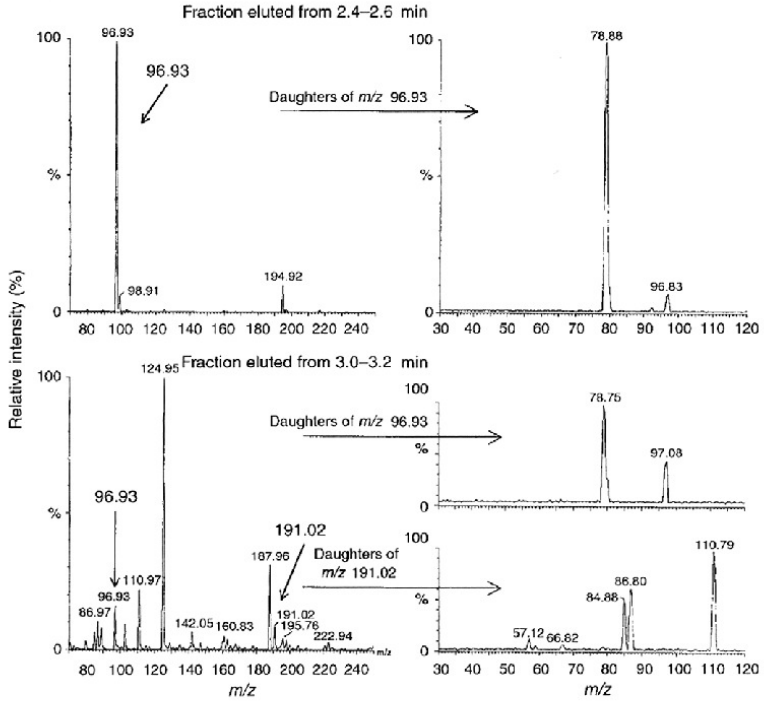

Fig. 2. ES-mass spectra and corresponding daughter ion mass spectra of *m/z* 191 and 97 for eluted fractions from 2.4 to 2.6 and from 3 to 3.2 min, respectively, on an anion-exchange FPLC column for serum sample No. IV. Reproduced from Ref. [18], with permissions from The Royal Society of Chemistry.

2.4. Development of chromatographic procedures for speciation of HMM-Al species in human serum

Since SEC procedures [33–36] were not selective enough to separate albumin and transferrin, more powerful chromatographic procedures in combination with various specific detection techniques were needed to characterize and quantify the Al-binding proteins in serum. To obtain higher resolution in separation of serum proteins anion-exchange chromatographic columns were applied. First attempts were made by TSK DEAE-3SW silica-based

ion-exchange column [44]. Since Al was significantly retained on this column resin, it was not appropriate for quantitative analysis of Al-binding protein. To prevent Al adsorption on the column support a polymeric anion-exchange (Protein-Pak DEAE-5PW) column for separation of serum proteins was used [16]. Separated proteins were followed at 280 nm by UV detection, while Al in separated fractions was determined by ETAAS. Linear gradient elution was applied in 30 min, using (0–1.0 mol L^{-1}) NaCl in TRIS–HCl buffer (pH 7.4) containing 0.01 mol L^{-1} of $NaHCO_3$. UV and Al elution profile of spiked serum (1000 ng Al mL^{-1}, diluted 1 + 4 with water) is presented in Fig. 3. The selected column fractions were also characterized by SDS–PAGE (Fig. 4). The results demonstrated that Al was eluted under the transferrin peak. Since light and heavy chains of immunoglobulin were also present in fractions where transferrin was eluted, additional experiment was done. The total protein content and the content of particular protein were determined in selected separated fractions and a correlation was made to the concentration of Al. Since excellent correlation was obtained for Al and transferrin concentrations ($r = 0.997$) and no correlation was found for Al and immunoglobulin or albumin, it was concluded that the transferrin was the only serum protein that binds Al. To exclude the risk of displacement of Al from the protein during the chromatographic procedure, Soldado Cabezuelo et al. [20] used faster separation columns. An anion-exchange FPLC Mono Q HR 5/5 column was applied in speciation of spiked human serum and serum of uremic subjects. 0.2 mL of diluted serum sample (1 + 4) was injected onto the column. Linear gradient elution was applied in 20 min using (0–0.25 mol dm^{-3}) NaCl in TRIS–HCl buffer (pH 7.4) containing 0.01 mol L^{-1} of $NaHCO_3$. A Kelex 100 scavenger column was used "on-line" to clean up the eluents, lowering the blank of eluents to below 1.5 ng Al mL^{-1}. Serum proteins were detected at 280 nm. Al was determined in 500 μl fractions "off-line" by ETAAS. It was experimentally proven that Al was eluted under the peak of transferrin. To lower the limits of detection the same chromatographic column was further applied, and the same cleaning procedures performed [20], while for detection of separated Al species "on-line" quadrupole ICP-MS and high resolution (HR)-ICP-MS instruments were used [21]. Elution profile of proteins was followed by UV spectrophotometry at 295 nm. Ammonium acetate that was compatible with UV as well as with ICP-MS detection was used for separation of proteins. 0.1 mL of sample was injected onto the column. Linear gradient elution was applied in 15 min using (0–0.25 mol L^{-1}) ammonium acetate in TRIS–HCl buffer (pH 7.4). The HR-ICP-MS detection enabled "on-line" speciation of unspiked human serum containing about 2.5 ng mL^{-1} of total Al. The elution profile of normal unspiked serum sample with HR-ICP-MS detection is presented in Fig. 5. It is evident, that in the eluting volume typical of transferrin two distinct peaks were obtained, presumably indicating two binding sites of Al in transferrin [34]. Authors presumed that 90% of total Al was bound to transferrin. The Al elution profile under the transferrin peak was not quantified. There were also no data about the column blank reported. Later, the same separation system using 0.5 mol L^{-1} ammonium acetate in linear gradient elution coupled to double focusing (DF)-ICP-MS enabled multielemental speciation of eleven ele-

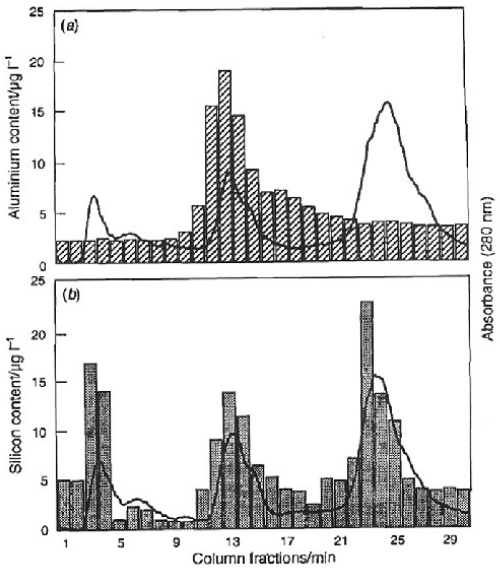

Fig. 3. Elution profiles of proteins (detection at 280 nm) and (a) aluminium and (b) silicon (ETAAS) in diluted (1 + 4) serum sample (1.0 μg mL^{-1} Al, 0.1 mmol L^{-1} citric acid, 5.0 μg mL^{-1} Si). Reproduced from Ref. [16], with permissions from The Royal Society of Chemistry.

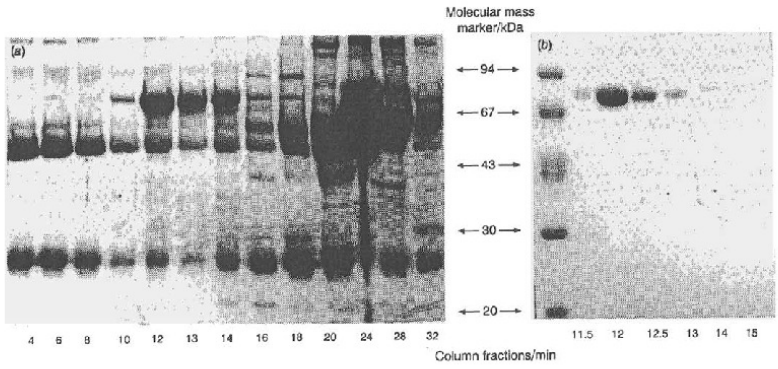

Fig. 4. SDS–PAGE of selected column fraction collected for elution of 500 μL of: (a) diluted spiked serum sample, and (b) transferrin standard solution. Molecular mass markers are given in the centre. Reproduced from Ref. [16], with permissions from The Royal Society of Chemistry.

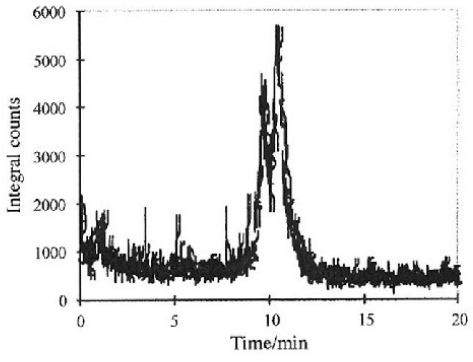

Fig. 5. Chromatogram of normal unspiked human serum (Al content 2.5 μg L^{-1}) after FPLC separation of proteins using HR-ICP-MS (R = 3000) detection of Al. Reproduced from Ref. [21], with permissions from The Royal Society of Chemistry.

ments, including Al, in human serum [45]. Nagaoka and Maitani [22] used the same FPLC Mono Q HR 5/5 column and the same buffer as Soldado Cabezuleo et al. [21], but different gradient elution conditions in 50 min. 0.1 mL of sample was injected onto the column resin. Authors reported the cleaning protocol of the column that lower the column blank to background levels of Al (see Section 2.1,[22]). They compared the elution profiles of nonspiked human serum using 15 and 50 min gradient elution and followed Fe and Al chromatograms by HR-ICP-MS and protein profiles by UV detection at 280 nm. The results presented in Fig. 6 indicated that by the use of shorter gradient two Al and two Fe peaks were detected (left), while with prolongation of the elution gradient, three main Fe and two Al peaks were recorded (right). Based on previous experiments with Fe and desferroxiamine (DFO) that removed Fe preferentially from the C-lobe site [46], and considering data from Fig. 6 (right) Nagaoka and Maitani presumed the binding sites of Fe to transferrin. By further spiking of transferrin with Al-citrate (Fig. 7) and/or by spiking of serum with Al-citrate (equilibration for 24 h), the peak assigned as Al bound to the transferrin N-lobe site was significantly increased, whereas the slight increase in Al peak, presumably forming the complex Al_NFe_C-Tf, was also observed. Based on these experiments authors concluded that the preferring binding site of Al to transferrin is N-lobe site. In the continuation of their research Nagaoka and Maitani studied the effect of sialic acid residues of transferrin on the binding with Al and Fe using FPLC-HR-ICP-MS [47] and later reviewed their work [48]. Again it was demonstrated that Al-citrate added to transferrin at Al/Tf ratio = 1 was preferentially bind to N-lobe site, while the binding affinity was higher to asialo-Tf than to native-Tf.

On the basis of knowledge that was gained in our group on the elution profiles of LMM-Al complexes in human serum on Mono Q

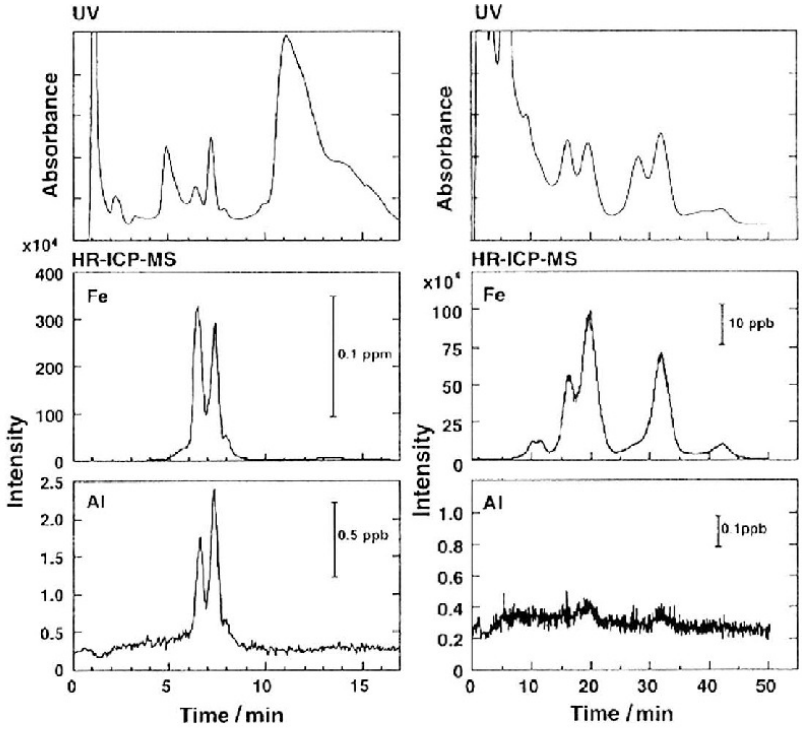

Fig. 6. Comparison of HPLC chromatograms under two gradient conditions. A 100 μL portion of serum from a healthy person without any Al spike was applied to the HPLC-HR-ICP-MS system, and UV absorption at 280 nm (HPLC-UV chromatogram) and the levels of ^{56}Fe and ^{27}Al (HPLC-HRICP-MS chromatograms) in the eluate were monitored. Left, conditions of Ref. [21]; right, modified conditions. Reproduced from Ref. [22], with permissions from The Royal Society of Chemistry.

FPLC column [18,19] and data reported for HMM-Al species separated on the same column [20–22], the possibility existed on the co-elution of LMM-Al species (Al-citrate, Al-phosphate, ternary Al-citrate–phosphate complexes) with the Al-transferrin complex, although for separation of HMM-Al species lower ionic strength of eluent was applied [20–22]. In order to obtain the reliable data, without risk of overestimation of the concentration of Al bound to transferrin, there was a need for pre-separation of LMM-Al complexes before using an anion-exchange FPLC in speciation of HMM-Al compounds. For this purpose an analytical procedure was developed using SEC (Superdex 75 HR 10/30) prior to an anion-exchange FPLC (Mono Q HR 5/5) separation [23]. Pre-separation and separation procedures were followed by the UV detection at 278 nm and Al determined "off-line" by ETAAS. All the necessary steps to avoid contamination as discussed in Section 2.1. were considered, including the cleaning of the SEC and FPLC column with 1 mol L^{-1} NaOH and 2 mol L^{-1} citric acid, that lowered the blank of the overall analytical procedure to below 1 ng Al mL^{-1} [23]. 1 mL of spiked serum (up to 300 ng Al mL^{-1}, Al-nitrate salt) was injected onto the SEC column. Isocratic elution using 0.05 mol L^{-1} TRIS–HCl + 0.03 mol L^{-1} $NaHCO_3$ was applied. It was experimentally proven that proteins were eluted in 5 mL peak that was collected into a polyethylene cup. A 0.5 mL of the sample aliquot was then injected onto the anion-exchange FPLC column. The separation of serum proteins was obtained applying linear gradient elution from 100% buffer A (0.05 mol L^{-1} TRIS–HCl + 0.03 mol L^{-1} $NaHCO_3$) to 100% buffer B (A + 0.25 mol L^{-1} NaCl). Well-resolved protein peaks were obtained. 0.25 mL fractions were collected during the chromatographic run and Al determined by ETAAS. It was experimentally proven that 90 ± 5% of Al in spiked serum (containing 300 ng Al mL^{-1} of total Al) was eluted under the transferrin peak (Fig. 8), which was identified not only on the basis of the retention volume, but also by the SDS–PAGE (Fig. 9). To study the repeatability of the analytical procedure, five individual spikes of a serum of a dialysis patient were successively analysed by combining SEC and anion-exchange FPLC separation with ETAAS detection. It was experimentally proven that on the SEC column, in all spiked samples investigated 90 ± 5% of Al was eluted as HMM-Al species (Al bound to proteins). Speciation analysis of the HMM-Al fraction on the anion-exchange FPLC column furthermore confirmed that 97 ± 3% of Al of the protein fraction was eluted exclusively under the elution volume of the transferrin peak. These findings were in agreement with the reported literature data [20–22]. There was no evident difference between the reported literature data when only anion-exchange FPLC separation without the pre-separation of LMM-Al species was applied [20–22]. However, to eliminate the possibility of overlapping of HMM-Al and LMM-Al species, the pre-separation of LMM-Al species is strongly recommended

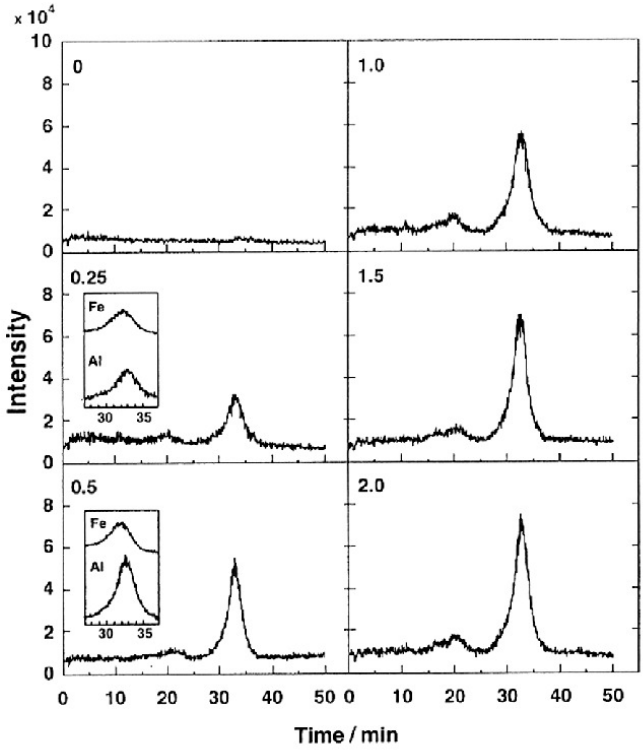

Fig. 7. HPLC-HR-ICP-MS chromatograms (^{27}Al level) for the apo-Tf solution supplemented stepwise with Al-citrate in the presence of bicarbonate. Al-citrate was added to the apo-Tf solution stepwise at the Al/Tf molar ratios of 0, 0.25, 0.5, 1.0, 1.5 and 2.0, and the respective solutions were allowed to stand for 24 h before analysis (final Tf concentration was 2 mg mL^{-1}). A 100 μL portion of the mixed solution was applied to the HPLC-HR-ICP-MS system, and UV absorption at 280 nm and the levels of ^{27}Al and ^{56}Fe in the eluate were monitored. The insert shows the comparison of retention times between the Al and Fe peaks. Reproduced from Ref. [22], with permissions from The Royal Society of Chemistry.

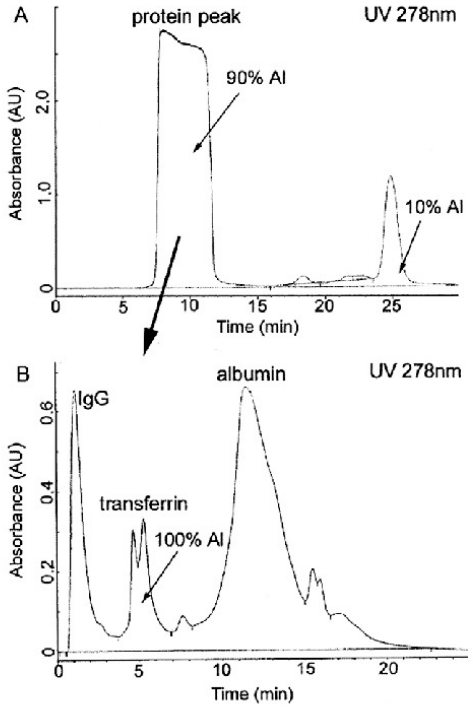

Fig. 8. Separation of a spiked human serum (spike No. 1) of a dialysis patient (300 ng Al mL^{-1}) at a flow rate of 1 mL min^{-1} followed by UV detection at 278 nm. 1 mL of spiked sample was first injected onto the SEC column (A). A protein peak was collected from 7 to 12 min (5 mL). 0.5 mL aliquot of a protein peak was then injected onto the anion-exchange FPLC column (B). Reproduced from Ref. [23], with permissions from The Royal Society of Chemistry.

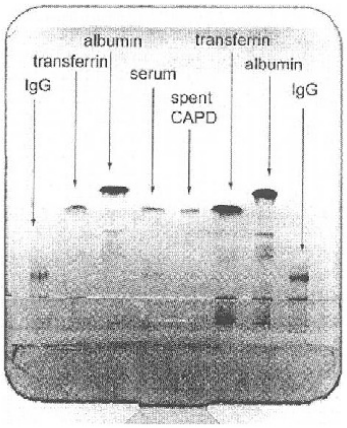

Fig. 9. SDS–PAGE electrophoresis of standard serum proteins (IgG, transferrin, albumin) and selected fractions of spiked human serum of a dialysis patient and spent CAPD fluid. Serum sample and spent CAPD fluid represent fractions 5.25–6.0 min collected from an anion-exchange FPLC column after pre-separation of proteins on SEC column. Spent CAPD fluid fraction was concentrated 100 times before being subjected to SDS–PAGE. Reproduced from Ref. [23], with permissions from The Royal Society of Chemistry.

before the anion-exchange FPLC separation of proteins is performed. Otherwise, careful investigation on the behaviour of LMM-Al species on the column resin under the same chromatographic conditions, as applied in separation of HMM-Al species is necessary.

Since there are no reference materials for speciation analysis of Al, the use of complimentary analytical procedures is desirable. As an alternative to ion-exchange FPLC columns, ion-exchange separation supports based on convective-interaction media (CIM®) were developed in the last decade. The matrix supports of poly(glycidylmethacrylate-co-ethyleneglycoldimethacrylate) substituted with strong or weak cation and anion exchangers offer very fast separation of biomolecules. Therefore, in our group a new analytical procedure was developed for speciation of HMM-Al compounds in spiked serum of a renal patient by the use of weak anion-exchange diethylamine (DEAE) CIM® fast-monolithic disk with ICP-MS and UV detection [24]. To overcome the risk of co-elution of LMM-Al species with the HMM-Al compounds, pre-separation of LMM-Al complexes on SEC column was first performed, as described previously [23]. Protein peak was collected and 0.1 mL of the sample aliquot was then used for the speciation procedure by CIM®-ICP-MS. Special attention, as described in Section 2.1 [24], was devoted to the cleaning procedure of eluents and monolithic disks. By the use of 1 mol L^{-1} NaOH and 2 mol L^{-1} citric acid for the cleaning of the disk support, the blank of the overall analytical procedure was lowered to below 0.5 ng Al mL^{-1}. The separation of serum proteins was obtained in 10 min by applying linear gradient elution from 100% buffer A (0.05 mol L^{-1} TRIS–HCl + 0.03 mol L^{-1} $NaHCO_3$) to 100% buffer B (A + 1 mol L^{-1} NH_4Cl) and followed by UV detection at 278 nm. NH_4Cl eluent did not form complexes with Al and was compatible with ICP-MS and UV detection. Separated Al species were detected on-line by ICP-MS. Well-resolved protein peaks were obtained. It was experimentally proven that 90 ± 3% of Al in spiked serum (spikes ranged from 50 to 250 ng Al mL^{-1}, equilibration time 4 h) of renal patient were eluted under the transferrin peak, confirming again the previous findings [20–23]. The investigation demonstrated that CIM® monolithic disks can be used as a complementary separation support to FPLC columns for the speciation of Al-binding proteins in human serum, when the concentration of Al in serum was higher than 5 ng mL^{-1}. In comparison to FPLC chromatographic columns CIM® disks enabled faster separation and simpler manipulation during the cleaning procedure as well as simpler coupling to ICP-MS.

Recent investigations were oriented to develop a reliable analytical procedure that would enable to identify and quantify HMM-Al species in unspiked human serum at normal concentration levels. For this purpose the potential of weak anion-exchange CIM® DEAE monolithic column coupled "on-line" to UV and ICP-MS detection was investigated [49]. The efficient cleaning of eluents and chromatographic supports that is of great importance to perform Al speciation at extremely low concentration levels enabled very low limits of detection and quantification to be achieved (LOD 0.15 ng mL^{-1} of Al, LOQ 0.49 ng mL^{-1} of Al). Pre-separation of proteins from LMM-Al species by SEC, prior to speciation procedure on a weak anion-exchange CIM® DEAE fast-monolithic column was applied. The same experiment was also performed using only CIM® DEAE procedure without prior separation of LMM-Al from HMM-Al species. Unspiked (total Al concentration 5.7 ± 0.4 ng mL^{-1}) and spiked serum (spike 10 ng mL^{-1} Al) were analysed. 1 mL of sample (serum diluted five times) was injected on the CIM® column. As can be seen from Fig. 10 the elution pro-

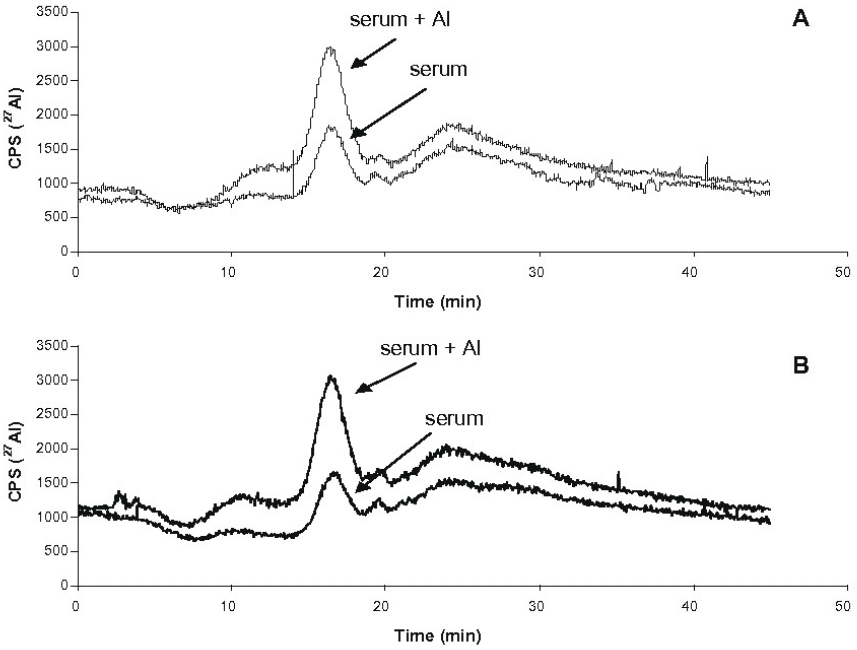

Fig. 10. Al elution profiles for the speciation of Al in unspiked (1 + 4) and spiked serum samples (1 + 4, spike after dilution 2 ng mL^{-1} Al) when (A) CIM DEAE-ICP-MS was applied after pre-separation of LMM-Al species by SEC or (B) when only CIM DEAE-ICP-MS was used. Reproduced from Ref. [49], with permissions from American Chemical Society.

files for unspiked and spiked serum samples are the same for the two procedures when the speciation of Al by CIM® DEAE-ICP-MS was applied after pre-separation of LMM-Al complexes by SEC (Fig. 10A) or when the speciation of Al was performed only by CIM® DEAE-ICP-MS (Fig. 10B). On the basis of the elution time and by the ACQUITY UPLC-SI-MS analysis it was confirmed that Al species separated corresponded to Al bound to transferrin. It was also experimentally proven that 91 ± 7% of Al in unspiked human serum was bound to transferrin, confirming the findings of previous investigators [16,20–24]. Since the experimental data from Fig. 10 demonstrated that under the chromatographic conditions applied LMM-Al species did not elute with HMM-Al species, the pre-separation of LMM-Al complexes by SEC was not necessary. However, if different chromatographic conditions are used, it is first necessary to prove that LMM-Al species did not elute with HMM-Al species. The data on the speciation of Al at normal concentration levels represent an important basis for computational studies of Al distribution [50] and its fate in human body.

3. Conclusions

Al is involved in many health disorders. To understand Al transport through the human body and its accumulation in target organs, identification and quantification of chemical species of Al in human serum is of great importance. In last two decades analytical techniques for the determination of the amount and composition of LMM-Al and HMM-Al species in human serum were progressively developed. Due to the very low concentrations of Al in serum of normal subjects and the environmental abundance of Al, there is a high risk of contamination during speciation analysis. Appropriate handling of samples and efficient cleaning procedures should be applied in order to avoid contamination by extraneous Al. In addition, analysis should be performed under clean room conditions. Since ultrafiltration and SEC procedures enabled only fractionation of Al in serum to distinguish between the amount of LMM-Al and HMM-Al species, more powerful analytical procedures were required. Anion-exchange FPLC columns in combination with element specific detectors, mass spectrometry based techniques, UV and SDS–PAGE, were found to be powerful analytical tools for speciation both LMM-Al as well as HMM-Al species. The combination of microultrafiltration and speciation analysis of ultrafiltrable Al by anion-exchange FPLC and ES-MS-MS techniques demonstrated that the LMM-Al fraction is composed of Al-citrate, Al-phosphate and ternary Al-citrate–phosphate complexes. The percentage and the composition of LMM-Al species is individually variable and presumably depend on the medical treatment and health status of individuals. Anion-exchange FPLC columns and anion-exchange CIM® monolithic chromatographic supports enabled selective separation of Al bind to serum proteins. By the use of ETAAS and more sensitive ICP-MS technique in combination with UV detection it has been demonstrated that about 90% of Al in serum is bound to transferrin. The Al eluted under the elution volume of transferrin was also identified by SDS–PAGE. By lowering the blanks of the chromatographic procedures, including efficient cleaning of the eluents and chromatographic supports, Al was identified in unspiked human serum at normal concentration levels. The composition of HMM-Al species and LMM-Al species determined by the analytical techniques developed agreed with the data calculated by computer modeling reported in the litera-

ture. To further support the computer modeling studies and to better understand the role of Al in health and disease, there is a need also to quantify Al species in human serum at normal concentrations levels. The latest achievements by the use of anion-exchange CIM® monolithic columns in combination with ICP-MS detection provide promising analytical tools to achieve this goal.

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Appendix 2: Personal bibliography for the period 2005-2009

SIMONA MURKO [26462]

ARTICLES AND OTHER COMPONENT PARTS

1.01 Original scientific article

1. MURKO, Simona, MILAČIČ, Radmila, ŠČANČAR, Janez. Speciation of Al in human serum by convective-interaction media fast-monolithic chromatography with inductively coupled plasma mass spectrometric detection. *J. inorg. biochem.*. [Print ed.], 2007, issue 9, vol. 101, str. 1234-1241. [COBISS.SI-ID 20803111]
tipologija 1.08 -> 1.01

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3. MURKO, Simona, MILAČIČ, Radmila, KRALJ, Bogdan, ŠČANČAR, Janez. 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. *Anal. chem. (Wash.)*. [Print ed.], 2009, vol. 81, no. 12, str. 4929-4936. [COBISS.SI-ID 22605351]

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tipologija 1.08 -> 1.01

1.06 Published scientific conference contribution (invited lecture)

7. MILAČIČ, Radmila, MURKO, Simona, ZULIANI, Tea, ŠČANČAR, Janez. Analizni pristopi k speciaciji elementov v biološki vzorcih. V: GLAVIČ, Peter (ur.), BRODNJAK-VONČINA, Darinka (ur.). *Slovenski kemijski dnevi 2009, Maribor, 24. in 25. september 2009*. Maribor: FKKT, [2009], 8 str. [COBISS.SI-ID 22943783]

1.08 Published scientific conference contribution

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