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Analytical methodology

Aluminium (Al) speciation in serum and urine after subcutaneous venom immunotherapy with Al as adjuvant

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ABSTRACT

Background: Aluminium is associated with disorders and is the commonly used vaccine adjuvant. Understanding the mechanisms of how Al is transported, metabolized or of its toxicity depends on the knowledge of Al-interactions with bioligands, i.e. Al-species. Al-speciation in serum is difficult because of low concentration and the risk of exogenous Al contamination. Furthermore, Al-measurements may be hampered according to various interferences. This study aims for developing quality controlled protocols for reliable Al- and Al-species determination and for investigating probable differences in Al (-speciation) after Al-containing subcutaneous immunotherapy (SIT).

Methods: Sample donors were recruited either for the control group ("class-0", they never had been treated with SIT containing an Al-depot extract) or for the SIT-group ("class-1", they previously had been treated with SIT for insect venom allergy with an Al-depot extract). Blood was drawn for medical reasons and serum prepared. Additionally, some sample donors collected 24-h-urine. They had been informed (and they consented) about the scientific use of their samples. The study was approved by the ethic committee of the "Medical Association Westphalia-Lippe" and of the University of Münster, evaluating the study positively (No. 2013-667-f-S).

We applied quality controlled sample preparation and interference-free Al detection by ICP sectorfield-mass spectrometry. Al-species were analysed using size-exclusion-chromatography-ICP-qMS.

Findings: Al-concentrations or speciation in urine samples showed no differences between class-0 and class-1. Al-citrate was the main uric Al-species. In serum elevated Al-concentrations were found for both classes, with class-1 samples being significantly higher than class-0 ($p = 0.041$), but class-0 samples being approximately 10-fold too high compared to reference values from non-exposed persons. We identified gel-monovettes as contamination source. In contamination-free samples from HNO_3-prewashed gel-free monovettes ($n = 27$) there was no difference in the serum Al concentration between the two patient groups ($p = 0.669$)

Interpretation: Thorough cleaning of sample preparation ware and use of gel-free monovettes is decisive for an accurate Al analysis in serum. Without these steps, wrong analysis and wrong conclusions are likely. We conclude that gel-monovettes are unsuitable for blood sampling with subsequent Al-analysis. Whether Al in serum is elevated after SIT treatment containing an Al-depot extract, or not, remains inconclusive as the non-contaminated sample size was small.

1. Introduction

Repeated inquiries about a possible risk from aluminium in biomedical drugs had recently motivated the *Paul-Ehrlich-Institut* to provide the current state of knowledge on the safety of aluminium-containing adjuvants in extracts for allergen-specific immunotherapy [1]. Such actual inquiries are caused by reports where Al is associated with clinical disorders, e.g. in renal patients. Actually, high exposure of aluminium is recognized to be neurotoxic for more than a century and is discussed to be a toxic factor in several human diseases [2]. Its accumulation by patients with renal failure is a well-known hazard [3–5].

Despite similarities of Al-induced encephalopathy and Alzheimer's Disease (AD) in many symptoms, discussions about Al exposure and cognitive decline are still controversial. Specifically, because not all AD patients have elevated Al levels [6] while familial AD patients showed higher Al concentrations than all previous measurements of aluminium

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in brain [7], the discussion on Al being cause or consequence of AD is not decided. For complementing the data basis, reference [8] conducted speciation studies in serum to study the involvement of metals, including aluminium, in the progression of Alzheimer's disease. They found Al increased in demented patients but also evidence for a complex interdependency between different metals.

Notably, the aluminium load to humans is multi-factorial. Several routes for aluminium exposure to humans are known with Al from nutrition being generally accepted as the predominant source. The *European Food and Safety Agency* (EFSA) reports about 1.6–13 µg aluminium per day [9]. Another Al-source in discussion is the use of Al-containing anti-transpirants. Based on Al skin penetration rates from human studies [10] the German *Bundesintitut für Risikobewertung* (BfR) calculated an Al-uptake of 10.5 µg/d for a 60 kg person [11]. Additionally to the above every-day exposure, patients receiving Al-containing SIT can get up to 15 injections per year, each containing between 0.1–1.25 mg aluminium [12,13], which calculates as 4–51 µg Al on a daily basis. This amount is a relevant add-on-top to the general every-day-exposure.

Aluminium is commonly used in authorized vaccines and allergen preparations for SIT because it appears to boost or potentiate the immune response to the injected vaccine or allergen. However, despite about 90 years of widespread use of aluminium adjuvants, medical science's understanding about their mechanisms of action on a molecular level is still poor. There is also a concerning scarcity of data on toxicology and pharmacokinetics of these compounds.

Nowadays there is a growing acceptance of the fact that the understanding of the mechanisms of how Al is transported and excreted or the mechanism of aluminium toxicity is decisively dependent on the knowledge of the in-vivo Al interactions with bioligands at a molecular level [14]. This implies the determination of the Al speciation, i.e., the particular chemical forms and their concentrations at site in which the element is transported and deposited in the human body [14–17].

Speciation of Al in human serum is an extremely difficult task because the basal levels of this element in serum are typically below 3–5 µg/L and these low concentrations are even fractioned in speciation analysis [18]. Even worse, the risk of significant exogenous Al contamination to samples and used laboratory material is very high [14,17–19]. Consequently, inadvertent contamination during sampling, storage, sample preparation and analysis of serum or urine can introduce considerable uncertainty in the determination of aluminium [20,21]. During measurement of aluminium in biological materials, in particular in plasma or serum, the risk of contamination is the major factor. The sample must therefore be handled with as few as possible preparation steps. Analytical recommendations from validated Al determination methods provided by the German Research Foundation (DFG) recommend thorough rinsing of all disposable containers used for sample preparation with diluted nitric acid and Milli-Q water before use [20]. Aside from contamination, interfered Al determination, leading supposedly to wrong-elevated determinations, should be strictly prevented. To date, in clinical laboratories mostly graphite furnace atomic absorption spectrometry (GF-AAS) is applied for Al determination. Unfortunately, this method can suffer from serum Al concentrations being below methodical threshold value of detection in the necessary 1/5 or 1/10 dilutions for measurements [22], or from interferences by high chloride content in samples [20]. Inductively coupled plasma mass spectrometry (ICP-MS) thus is the more powerful method of choice. However, the $[^{11}B^{16}O]^+$ -cluster interferes the ^{27}Al mono-isotope and can cause wrong-elevated results [23] in boron-rich samples such as urine (up to 3000 µg boron/L urine of non-exposed, healthy sample donors) [24]. Therefore, high resolution ICP-sectorfield-mass spectrometry (ICP-sf-MS) was recommended for total Al determination [21]. With this instrument practically all interferences can be resolved from the ^{27}Al-signal [23]. It is further essential providing documented proof about adequate quality control and quality assurance measures during the study for which analytical results are reported [25,26].

Biological monitoring of human aluminium exposure has been conducted with determination of total Al in urine, which is thought to indicate recent exposure, and determination of total Al in plasma or serum, which is thought to better reflect the aluminium body burden and long-term exposure [25].

1.1. Study aims

According to the frequent use of aluminium in vaccination and SIT and the questions regarding its toxic effects we intended to monitor aluminium in urine and serum of controls (class-0) and patients who previously had received subcutaneous immune therapy with an insect venom extract containing Al as an adjuvant (class-1). The primary aims of the study had a special focus on developing quality controlled protocols, i.e. a pre-analytical protocol avoiding Al-contamination during sampling and sample preparation, an interference free, validated total Al and Al-species determination, and finally investigating whether differences in total Al or Al-speciation will be detected between class-0 and class-1 groups.

2. Experimental

2.1. Sample donors

Sample donors were selected from patients receiving SIT with insect venoms who visited the Clinic for Skin Diseases – General Dermatology and Venereology of the University Hospital in Münster for a control examination during two 2-months recruitment periods. We recruited patients for the control group ("class-0", n = 23), who never had been treated with SIT containing an Al-depot extract, and for the SIT-group ("class-1", n = 18) from patients, who previously had been treated with insect venom extracts containing Al as $Al(OH)_3$. Each injection contained 1.13 mg Al as adjuvant. Blood samples were sent blinded to the laboratory in Munich for Al-analysis and Al-speciation. Additionally, patients collected 24-h-urine. Patient's consent for the participation on this observational study was obtained. The study protocol was approved by the ethical commission of the "Medical Association Westphalia-Lippe" and of the University of Münster.

2.2. Samples and sample preparation

Urine (24 h urine) samples were collected into HNO_3-precleaned containers (Nalgene®, VWR, Ismaning, Germany). Blood samples were drawn using gel-monovettes for the first sampling and gel-free monovettes from Sarstedt (Nümbrecht, Germany) for later sampling. Serum was prepared subsequently.

All plastic containers for sample preparation or sample storage were pre-cleaned by incubation in HNO_3 (2%) for 1 h and subsequent three-times flushing with double-distilled water.

Serum and urine samples were stored at −70 °C and were blinded sent on dry-ice to the analytical laboratory in Munich. Before use, samples slowly thawed overnight at 4 °C and were diluted 1:10 in Milli-Q water directly before measurement. The entire sample preparation was performed under laminar flow benches (clean room condition).

2.3. Chemicals

Aluminium and rhodium single standards for ICP-MS were from Spex CertiPrep (Stanmore, UK). NH_4Ac, HAc and HNO_3 (65%) were purchased from Merck (Darmstadt, Germany). HNO_3 was purified by subboiling destillation. $Argon_{liqu}$ was purchased from Air-Liquide, Düsseldorf, Germany. An Argon vaporizer at the tank provided Ar gas for ICP-MS systems.

2.4. Sample analysis

2.4.1. Determination of aluminium concentrations

Total Al was analyzed with ICP-sector field -mass spectrometry (ICP-sf-MS).

An inductively coupled plasma- sectorfield- mass spectrometer (ICP-sf-MS, *"Element II"*, Thermo Fisher Scientific, Germany) was employed for ^{27}Al determination in medium resolution. The medium resolution mode guaranteed Al determination free from typical interferences, specifically from $[^{11}B^{16}O]^+$ [23], which could be relevant due to high boron concentration in urine [24]. Sample introduction was carried out using a prepFast system (ESI, Elemental Scientific, Mainz, Germany), connected to a Seaspray nebulizer with a cyclon spray chamber. Rhodium (^{103}Rh) was used as internal standard, which was continuously introduced and mixed with the sample solution before introduction into the spray chamber. The ICP-radio frequency (RF) power was set to 1260 W, the plasma gas was 15 L Ar/min, the auxiliary gas was 1.45 L Ar/min, whereas the nebulizer gas was approximately 0.95 L Ar/min after daily optimization. Calibration was carried out by a five-point calibration at concentrations of 0 (blank), 0.25, 0.50, 1.00 and 10 µg/L by appropriate dilution of a certified Al-standard (Spex CertiPrep, Stanmore, UK). For quality control certified reference materials (aluminium in serum, BCR-637, BCR-638 and BCR-639, purchased from Institute for Reference Materials and Measurement, Joint Research Center of the EU) were measured together with samples. The determined concentrations corresponded with certified values (c.f. results on quality control).

2.5. Al speciation analysis

Aluminium speciation was performed by an established coupling of size exclusion chromatography (SEC) hyphenated to ICP-qMS [27].

Established SEC separation methods were applied, using a Knauer 1100 Smartline inert series HPLC system with two serially installed SEC columns: (650 × 10 mm ID) from YMC-Europe GmbH (Dinslaken, Germany) was packed with Toyopearl TSK HW 55S (TosoHaas, Stuttgart, Germany; separation range 700–5 kDa) and a 250 × 10 mm ID Kronlab column filled with TSK-HW40S (separation range 100–2000 Da). This column combination provided separation of various Al-proteins from each other and from Al-citrate as well as the latter from inorganic Al compounds. The sample loop had 100 µL injection volume. NH_4Ac (250 mM, pH 7.4) served as the eluent at a flow-rate of 0.7 mL/min.

An auxiliary UV detector was installed between the column outlet and the nebulizer at ICP-MS. UV was detected at 280 nm for predominantly monitoring proteins and other organic ligands.

2.6. ICP-qMS parameters

A NexIon 300D ICP-qMS system from Perkin Elmer (Sciex, Toronto, Canada) was employed for on-line determination of the isotope ^{27}Al in SEC-chromatograms.

The HPLC column outlet was passing through the UV detector to a Meinhard nebulizer (which was mounted to a cyclone spray chamber) using a PEEK transfer tube (ID 100 µm). The RF power was set to 1250 W, the plasma gas was 15 L Ar/min. The nebulizer gas was optimized and finally set to 0.93 mL Ar/min. The dwell time was 500 ms.

Post-column flow injection (FI) to ICP-qMS was used for quantification with a 5 point calibration at concentrations of 0, 0.50, 1.00, 5.0 and 10 µg/L by appropriate dilution of a certified Al-standard (Spex CertiPrep, Stanmore, UK). Peak areas from SEC-ICP-qMS chromatograms were evaluated by Peakfit ™ software and compared to FI-ICP-qMS peak areas of calibration curve for quantification.

Mass balances during SEC-ICP-qMS: The sum of eluted and quantified Al peaks per sample from SEC-ICP-qMS was related to the previously determined total Al concentration (= 100%) by ICP-sf-MS.

A probable $[^{11}B^{16}O]^+$ interference at the ^{27}Al chromatogram – mainly relevant for urine samples – was checked by injecting 2000 µg/L B-standard solution into SEC-ICP-qMS.

2.7. Statistics

All statistical calculations were performed using SPSS version 25 (IBM SPSS Statistics). Medians were calculated and compared by the use of Mann-Whitney-*U*-test (non-parametric test for unrelated samples) to analyze the significance of differences between patient groups. Difference were considered as significant at a p value of < 0.05.

3. Results and discussion

3.1. Analytical quality control

Blank determinations from pre-washed sample and storage containers or sample preparation devices showed Al-values below the limit of detection at < 0.01 µg/L. This proofed that the cleaning protocols and pre-analytical steps avoided successfully Al contamination. Recoveries of Al-compounds during speciation analysis in serum or in urine were calculated by comparing the sum of quantified Al-peaks from SEC-ICP-qMS with total Al in respective samples. Recoveries were 97–102% for serum and 84–90% for urine samples. Recoveries were sufficient for the purpose.

Spectral interferences, specifically a probable $[^{11}B^{16}O]^+$ interference on the ^{27}Al isotope was excluded during total Al determination by use of the ICP-sf-MS instrument in medium resolution. This was demonstrated by meeting the reference values of certified reference materials: We determined the following concentrations: 13.5 ± 0.7 µg/L (reference value BCR-637: 12.5 ± 3 µg/L), 57 ± 1.7 µg/L (reference value BCR-638: 55 ± 7 µg/L), 189 ± 2.1 µg/L (reference value BCR-639: 194 ± 14 µg/L).

For checking the $[^{11}B^{16}O]^+$ interference on ^{27}Al-chromatograms during SEC-ICP-qMS, a boron standard was analysed at concentration typical for native urine (2000 µg/L). Fig. 1 compares three chromatograms, from the interference, from urine (1/5 diluted, measurement total Al concentration 7 µg/L) and from serum (1/3 diluted, measurement total Al concentration: 1 µg/L). Fig. 1 demonstrates that the boron standard produced just baseline signal at around 20 cps signal height without an interfering peak during the elution of Al-peaks from urine or serum. Thus, sample chromatograms were considered to be unaffected by this interference.

Summarizing results from analytical quality control experiments, the pre-analytical cleaning protocols and steps reduced contamination and a probable interference on aluminium determination analysis could be excluded.

3.2. Al determination and Al speciation in urine

In occupational health driven biomonitoring studies, Al exposure appeared typically in urine samples but practically not in serum or plasma samples. Therefore, we started analysing urine samples from controls (= class-0, n = 20) and from patients after SIT with Al as adjuvant (= class-1, n = 16). The partly low Al values and concentrations being coincident with literature data suggested that no contamination during sampling or from storage containers appeared. HNO_3 and subsequent Milli-Q water pre-cleaning were sufficient for contamination prevention. This result matched findings from [20]. In our study, median concentrations of total aluminium were 16.7 µg/L for class-0 (range: 1.1–109 µg/L) and 14.8 µg/L for class-1 (range: 3.8–102 µg/L) in urine. The difference between groups was insignificant (p = 0.96 – Mann-Whitney-*U*-test). Remarkably, controls tended to show even higher values than class-1 samples, but both median values were below the accepted value for environmental background level being < 30 µg/L [28]. Median values were within known concentrations either from

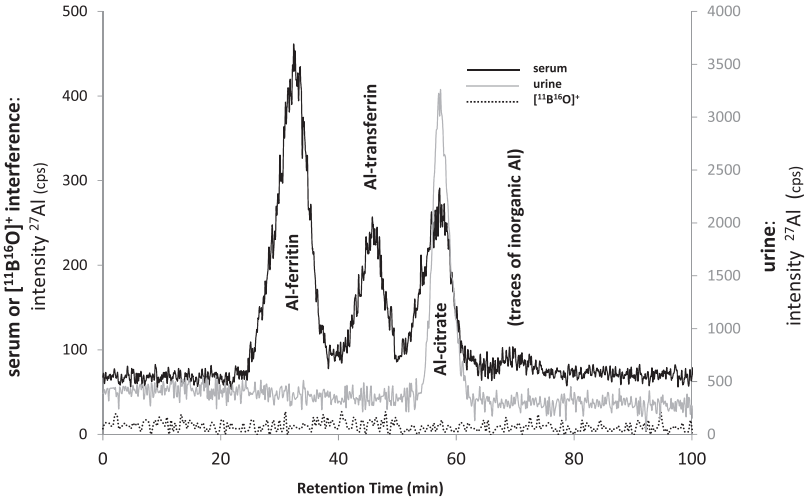

Fig. 1. $[^{11}B^{16}O]^+$ did not interfere serum or urine chromatograms. Urine or serum chromatograms are apparently not affected by this interference.

non-exposed persons (15 µg/L [22]) or from workers after only one-day shift exposure (23 µg/L) and did not reach values of workers being exposed for a longer period (up to 500 µg/L [22]). Al-speciation consequently showed no significant differences, too. We found aluminium nearly completely bound to low molecular weight compounds with the Al-citrate fraction being the most abundant one in both classes (class-0: median (Al-citrate) = 17.8 µg/L, class-1: median (Al-citrate) = 11.8 µg/L, p = 0.46). The regression coefficient r = 0.9979 between total Al and Al-citrate proved Al-citrate to be the dominant Al compound in urine. When we investigated a probable correlation between SIT injection number and total Al or Al-species we could not find any interrelation between injection number and aluminium in urine. The results of controls being higher than class-1 samples suggested that Al-biomonitoring in urine rather reflects the actual, incidental Al-exposure from every-day activities than probably that from SIT. A limitation of our sample set, however, was the small sample size (n = 20 or 16) and that the period since last injection was not exactly know.

3.3. Al concentration in serum: contamination from gel-monovettes

The median total Al concentration in the serum samples of the 23 patients without Al containing SIT (class 0) was 44.4 ± 13.5 µg/L and 59.31 ± 22.96 µg/L for the 18 patients with Al containing SIT (class-1); the difference was statistically significant (p = 0.028–Mann-Withney *U* test). A statistically significant correlation was observed between the number of injections and aluminium concentration in the serum samples (Pearson correlation coefficient 0.387, p = 0.012). However, the striking high values specifically for class-0 samples, being about ten-fold the concentration reported for the reference value of non-exposed persons (< 3–5 µg/L [22]), motivated us to search for a contamination source, since in general the risk of significant exogenous Al contamination during sampling is known to be very high [14,18,22]. Re-evaluation of all processes in sampling, sample handling and analysis regarding contamination prevention revealed only one issue outside the cleaning protocol during the previous approach: At hospital routinely gel-monovettes were in use for blood draw, which could not be washed with HNO_3. Therefore, in the second, follow-up approach monovettes without gel were used for blood sampling. Those gel-free monovettes were pre-cleaned with HNO_3 and Milli-Q water, as described for all other containers and sample handling material.

3.4. Al determination in serum: no contamination from gel-free monovettes

Blood samples, using the decontaminated gel free monovettes, were taken from 7 patients of the control group (class 0) and 8 patients having a SIT with a depot venom preparation (class 1) were in the range between 0.27 and 10.1 µg/L (class 0 median: 2.86 µg/L ± 3.39 µg/L; class 1 median: 1.79 µg/L ± 1.74 µg/L); the difference was not statistically significant (p = 0.46–Mann-Withney-*U*-test)

For further confirmation whether gel-monovettes were the source of contamination during our 1st approach, a direct comparison of sampling modes with the gel monovettes and the pre-washed gel free monovettes was performed. Blood was drawn from 12 patients in parallel. A further sample donor provided samples with gel-monovettes, gel-free monovettes and gel-free HNO_3-washed monovettes (n = 3 each). As in Fig. 2 shown, there was a significant difference of the aluminium concentration in dependence of the used serum monovettes. The median aluminium concentration in blood samples taken with gel monovettes was 43.1 ± 28.1 µg/L and in serum samples using decontaminated gel free monovettes 2.6 ± 2.6 µg/L. The difference was highly statistically significant (p < 0.000077).

Therefore, we can clearly demonstrate, that the use of the commercially available gel monovettes caused a considerable aluminium contamination resulting in values averaged ca. 10-fold (range 5–45 fold) above the non-contaminated values from gel free monovettes.

Summarizing results from Al-analysis in serum, the most relevant finding was that, aside from cleaning of sample handling containers, specifically avoiding gel-monovettes and the thorough cleaning of the

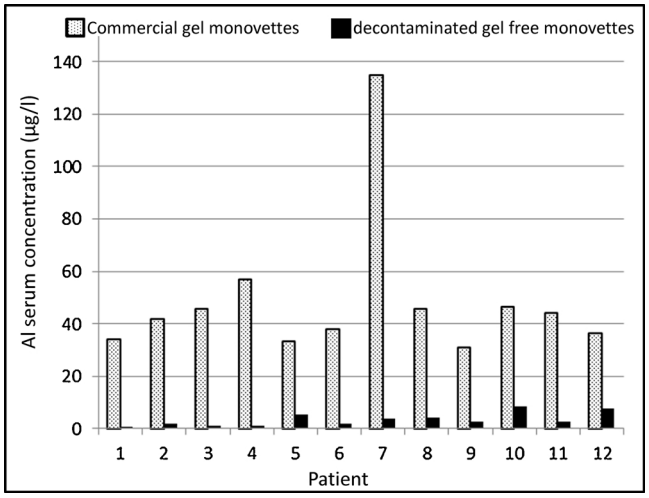

Fig. 2. Comparison of Al concentration in serum withdrawn in parallel by the use of a commercially available gel containing monovettes or decontaminated gel free monovettes in 12 patients.

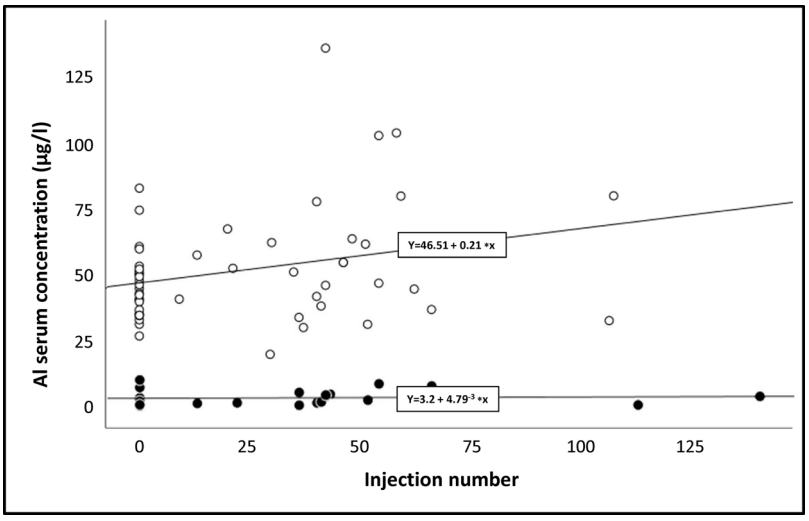

Fig. 3. Correlation between aluminium concentration in serum samples and the number of injections. ○ Serum samples taken by the use of aluminium contaminated gel monovettes. • Serum samples taken by the use of gel-free monovettes.

appropriate sampling device (gel-free monovettes) is mandatory for an accurate Al analysis in serum samples. Missing this pre-analytical quality control step leads to significantly elevated, wrong concentration determination and possibly to wrong conclusions. We conclude that the commercially available gel-monovette is unsuitable for blood sampling for the analyses of Al in serum samples.

In the samples from Fig. 2, we found a significant correlation between the serum aluminium concentration and the number of SIT injections only in those serum samples withdrawn by use of the aluminium contaminated commercially available gel monovettes (Pearson correlation coefficient 0.273, $p = 0.048$). The correlation was not significant using the prewashed, aluminium decontaminated monovettes (Pearson correlation coefficient 0.068, $p = 0.736$) (Fig. 3).

4. Summary

We applied quality controlled pre-analytical sample preparation steps and interference free Al detection by ICP mass spectrometry techniques. When analysing urine samples from controls and patients after Al-containing SIT no differences in Al concentration or Al speciation were seen. Al-citrate was the main Al-species in urine in both sample sets.

In the first determination we observed a significantly elevated Al concentration in serum samples from patients with aluminium exposure due to SIT with a depot venom extract (class-1 samples) but also an unexpected high aluminium concentration in control sera (class-0) indicating aluminium contamination. Commercially gel-monovettes were identified as contamination source in both sub-samples. A direct comparison of sampling devices regarding Al contamination confirmed gel-monovettes as cause for contamination. The serum results from first investigations had to be discarded. We therefore conclude that gel-monovettes are unsuitable for blood sampling with subsequent Al analysis. Patients, treated for several years with increased doses of aluminium containing allergen extracts for allergen specific immunotherapy, did not have elevated serum aluminium concentration, indicating that the use of depot extracts seem not to affect the aluminium body burden. Aluminium salts used as depot adjuvants are poorly soluble and are known to remain at the injection site for very long; whether aluminium is excreted or still remains at injection sites is unclear.

Conflict of interest

R. Brehler reports grants from Bencard, during the conduct of the study; personal fees from ALK, personal fees from Allergopharma, personal fees from Bencard, personal fees from HAL, personal fees from Stallergenes, personal fees from Astra Zeneca, personal fees from Gesellschaft zur Förderung der Dermatol. Forschung u. Fortbildung, personal fees from GSK, personal fees from D. Pfleger, personal fees from Merck, personal fees from Novartis, personal fees from Thermo Fischer, outside the submitted work.

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