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An approach for manganese biomonitoring using a manganese carrier switch in serum from transferrin to citrate at slightly elevated manganese concentration

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ABSTRACT

After high-dose-short-term exposure (usually from occupational exposure) and even more under low-dose long term exposure (mainly environmental) manganese (Mn) biomonitoring is still problematic since these exposure scenarios are not necessarily reflected by a significant increase of total Mn in blood or serum. Usually, Mn concentrations of exposed and unexposed persons overlap and individual differentiation is often not possible. In this paper Mn speciation on a large sample size ($n = 180$) was used in order to be able to differentiate between highly Mn-exposed or low or unexposed individuals at low total Mn concentration in serum (Mn(S)). The whole sample set consisted of three subsets from Munich, Emilia Romagna region in Italy and from Sweden. It turned out that also at low total Mn(S) concentrations a change in major Mn carriers in serum takes place from Mn-transferrin (Mn-Tf(S)) towards Mn-citrate (Mn-Cit(S)) with high statistical significance ($p < 0.000002$). This carrier switch from Mn-Tf(S) to Mn-Cit(S) was observed between Mn(S) concentrations of 1.5 μg/L to ca. 1.7 μg/L. Parallel to this carrier change, for sample donors from Munich where serum and cerebrospinal fluid were available, the concentration of Mn beyond neural barriers – analysed as Mn in cerebrospinal fluid (Mn(C)) – positively correlates to Mn-Cit(S) when Mn(S) concentration was above 1.7 μg/L. The correlation between Mn-Cit(S) and Mn(C) reflects the facilitated Mn transport through neural barrier by means of Mn-citrate. Regional differences in switch points from Mn-Tf(S) to Mn-Cit(S) were observed for the three sample subsets. It is currently unknown whether these differences are due to differences in location, occupation, health status or other aspects. Based on our results, Mn-Cit(S) determination was considered as a potential means for estimating the Mn load in brain and CSF, i.e., it could be used as a biomarker for Mn beyond neural barrier. For a simpler Mn-Cit(S) determination than size exclusion chromatography inductively coupled plasma mass spectrometry (SEC-ICP-MS), ultrafiltration (UF) of serum samples was tested for suitability, the latter possibly being a preferred choice for routine occupational medicine laboratories. Our results revealed that UF could be an alternative if methodical prerequisites and limitations are carefully considered. These prerequisites were determined to be a thorough cleaning procedure at a minimum Mn(S) concentration >1.5 μg/L, as at lower concentrations a wide scattering of the measured concentrations in comparison to the standardized SEC-ICP-MS results were observed.

Abbreviations: CSF, cerebrospinal fluid; CE, capillary electrophoresis; CNS, central nervous system; ESI-FT-ICR-MS, electrospray ionization fourier transform ion cyclotron resonance mass spectrometry; ICP-DRC-MS, inductively coupled plasma–dynamic reaction cell–mass spectrometry; ICP-OES, inductively coupled plasma optical emission spectrometry; LMM, low molecular mass; LoD, Limit of detection; Milli-Q-water, ultra pure water with resistivity (typically 18.2 MΩ cm at 25 °C); MMT, methyl-cyclopentadienyl-manganese-tricarbonyl; Mn, manganese; Mn(C), total Mn concentration in cerebrospinal fluid; Mn-Cit(S), Mn-citrate in serum; Mn(S), total Mn in serum; Mn-Tf(S), Mn-transferrin concentration in serum; NB, neural barrier; PD, Parkinson disease; SEC, size exclusion chromatography; UF, ultrafiltration.

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1. Introduction

Manganese (Mn) is an essential element for humans. It has been widely reported in literature that in general its concentration is low. However after increased exposure at elevated concentration it causes detrimental health effects. Following (excessive) exposure Mn can accumulate in basal ganglia which may cause severe neurological disorders, one of the worst being manganism, a disease with symptoms similar but different to Parkinsonís disease (PD) [1–3]. However, neurological effects have been observed at exposures considerably lower than those in the past being associated with manganism [4–9]. A long series of studies with many participants have unravelled the neurotoxic mechanisms of Mn which have been partly summarized in several reviews (e.g., [5,7,10]).

Mn concentrations in plasma and serum, but also in saliva, erythrocytes, urine and hair have been extensively evaluated for use in Mn exposure assessment with only limited success [11,12]. Total Mn levels were serving as an indicator of recent Mn exposure only on a group comparison basis. This was explained by homeostatic mechanisms regulating levels within a narrow range and precluding a direct relationship between external exposure and levels within the body [12]. Another approach was considered in differentiated distribution and speciation of manganese.

In a series of investigations it was previously shown that the distribution of Mn-species differ between serum and cerebrospinal fluid (CSF) [13–17]. In serum Mn was found mainly associated to proteins like transferrin and only at minor amounts to small carriers like citrate whereas contrarily in CSF mainly low molecular mass (LMM) Mn-species were found of which Mn-citrate was identified as the most important Mn-species. These findings were in accordance with others who suggested a LMM Mn-carrier into the brain, independent from transferrin [18,19]. Further, Yokel et al. [9] and Aschner et al. [7] reported about Mn-citrate to be transported at increased rates across neural barriers (NB) in rats.

Specifically our investigation from 2013 [20] revealed results which possibly could be an appropriate means for Mn biomonitoring: Both, total Mn concentration of serum (Mn(S)) and total Mn concentration in CSF (Mn(C)) showed a very strong positive correlation to the concentration of Mn-transferrin in serum as long as total Mn(S) remained at a physiological, low concentration range, i.e., below ca. 1.6 μg/L. In turn when total Mn(S) exceeds ca. 1.6 μg/L, both, total Mn(S) and Mn(C) disclose a positive linear relationship to Mn-citrate in serum (Mn-Cit(S)). This link between total Mn(C) and Mn-Cit(S) at increased Mn concentration in serum is specifically important since – in contrast to serum – CSF samples usually are not available (and can only be obtained after neurological indication drawn from neurologists). Therefore the above reported correlation between Mn(C) and Mn-Cit(S) at elevated Mn(S) could be used to estimate an increased risk for internal Mn exposure of the brain beyond NB, in this case via Mn in CSF, by analysing Mn-Cit(S). But for these previous experiments only a limited number of samples was available. Consequently a confirmation of our findings from [20] with an increased sample size was desirable before Mn-Cit(S) could be proposed as a biomarker.

The previous experiments were conducted mainly by size exclusion chromatography (SEC) coupled to inductively coupled plasma mass spectrometry (ICP-MS), with a 2-D identification by CE-ICP-MS and ESI-FT-ICR-MS [20].

However, routine application of SEC-ICP-MS has drawbacks, such as time-consuming column cleaning. This is limiting sample throughput and increasing sample storage time prior analysis with elevated risk of sample degradation. These disadvantages, aside from high operating costs that are caused by prolonged runtimes, make SEC-ICP-MS less applicable for occupational routine laboratories. The use of ultrafiltration units at low cutoff (e.g., 5 kDa) and subsequent total Mn determination in the filtrate could be a practical alternative where the LMM ultrafiltrate (<5 kDa) from serum roughly represents the Mn-Cit(S) fraction [16]. A mandatory prerequisite however was a rigorous pre-cleaning of the UF-tubes and filters by a seven-step procedure and overall very careful handling to avoid any Mn contamination. Preliminary investigations however revealed low reproducibility and low statistical power at serum Mn concentrations below 1.2 μg/L. Therefore, the aim of this work was in a first step to confirm the linear relationships previously found only on a limited set of samples from the Munich area now with a significantly increase of the data set up to 180 samples from three different areas (from Munich, Germany, from the Emilia Romagna region in Italy, and from Sweden). In a second step the reliability of ultrafiltration versus SEC under practical conditions for occupational health laboratories was elucidated. Specific questions to be answered were “are the same results gained in SEC and UF?”, and if so, “under which conditions”? (i.e., simpler procedure of pre-cleaning of tubes, or concentration dependence of performance).

2. Materials and methods

2.1. Chemicals

Standard compounds for retention time determination as well as for mass calibration of the SEC column were purchased from Sigma–Aldrich, Deisenhofen, Germany, as: Blue dextran: 2000 kDa, α-2-macroglobuline: 609 kDa, arginase: 107 kDa, transferrin: 78 kDa, albumin: 68.5 kDa, β-lactoglobuline: 36.5 kDa, lysozyme: 14.3 kDa, Metallothionein: 7 kDa, L-thyroxine: 777 Da, *N*,*Ní*-bis(t-BOC)-L-cystine: 440.5 Da, citric acid: 192.5 Da, inorganic $MnCl_2$.

TRIS, HNO_3, HCl (suprapure), NH_4-acetate (NH_4Ac) and acetic acid (HAc) were from Merck, Darmstadt, Germany. HNO_3 was purified by subboiling destillation. $Argon_{liqu}$ and NH_3 were purchased from Air–Liquide, Krefeld, Germany. $Argon_{liqu}$ was vaporized at the tank providing Ar gas. The TSK SEC-gel (230–450 mesh) was purchased from Tosoh Bioscience GmbH, Stuttgart, Germany.

2.2. Standards, samples and sample preparation

Mn-protein stock standards (1 mg powder/ml) were prepared by dissolving each compound in 10 mL TRIS-HAc buffer (10 mM, pH 7.4). Stock solution of $MnCl_2$ was prepared by dissolving 100 mg/L (related to Mn). Mn – citrate stock solution was prepared by mixing a solution of 1 g/L citrate with a $MnCl_2$ solution (5 mg/L) using a volumetric ratio of 4 + 1 (v:v), resulting in a Mn-citrate stock concentration of 1 mg Mn/L. Analogous, Mn-albumin and Mn-transferrin stock solutions were prepared by mixing 1 g/L protein solution with 5 mg/L $MnCl_2$ solution (4 + 1, each), resulting in 1 mg Mn/L for each compound. Stock solutions were aliquoted before being stored in the dark at −20 °C. No destabilization of standard compounds was observed using these conditions. Working solutions were prepared daily by appropriate dilution with NH_4Ac, 10 mM, pH 7.4.

Single standards and the analysis of standard mixtures were used to achieve information on SEC retention times.

2.3. Samples

A set of paired serum and CSF samples was drawn from patients at the Department of Neurology of the Technical University Munich. These sample donors had unspecific neurological complaints, like headache, dizziness or various sensory symptoms. CSF and serum samples were collected for diagnostic purpose and handled as described previously [20]. In short terms: CSF was collected from each individual by standardized lumbar puncture and serum was