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# **A new manganese biomonitoring concept for Mn exposure assessment based on Mn speciation**

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Running title: Mn-speciation for Mn-biomonitoring

## **Abstract**

For humans Mn is an essential trace element, but at higher doses a neuro-toxic metal. Chronic Mn exposure is affecting the central nervous system. Occupational Mn overexposure leads to an accumulation in the brain and has been shown to cause progressive, permanent, neurodegenerative damage with syndromes similar to idiopathic Parkinsonism. Mn is transported by an active mechanism across neural barriers (NB) finally into the brain, but to date modes of Mn neurotoxic action are poorly understood. This paper investigates the relevant Mn-carrier species which are responsible for a widely uncontrolled transport across NB. Mn speciation in paired serum/cerebrospinal fluid (CSF) samples was performed by size exclusion chromatography – inductively coupled plasma – dynamic reaction cell – mass spectrometry (SEC-ICP-DRC-MS) and capillary zone

electrophoresis (CZE) coupled to ICP-DRC-MS in an 2D approach for clear identification. The Mn-species from the different sample types were interrelated, and correlation coefficients were calculated.

In serum, protein-bound Mn-species like Mn-transferrin (Mn-Tf) had important influence on total Mn in serum if Mn<sub>total</sub> was less than 1.5 µg/L, but above 1.6 µg/L the serum-Mn<sub>total</sub> concentration was correlated with increasing Mn-citrate (Mn-Cit) concentration. Correlations between serum Mn species and CSF showed that Mn<sub>total</sub> from CSF was correlated to Mn-Cit from serum above 1.6 µg/L Mn<sub>total</sub> in serum.

Statistical models discriminated the samples in two groups where CSF samples were either correlated to Mn<sub>total</sub> and Mn-Cit (samples with serum Mn<sub>total</sub> > 1.5 µg/L) or correlated to Mn-TF (samples with serum Mn<sub>total</sub> < 1.5 µg/L).

We conclude that elevated Mn-Cit<sub>serum</sub> could be a valuable marker for increased total Mn in CSF (and brain). It could be used as a marker for elevated risk of Mn-dependent neurological disorders in occupational health.

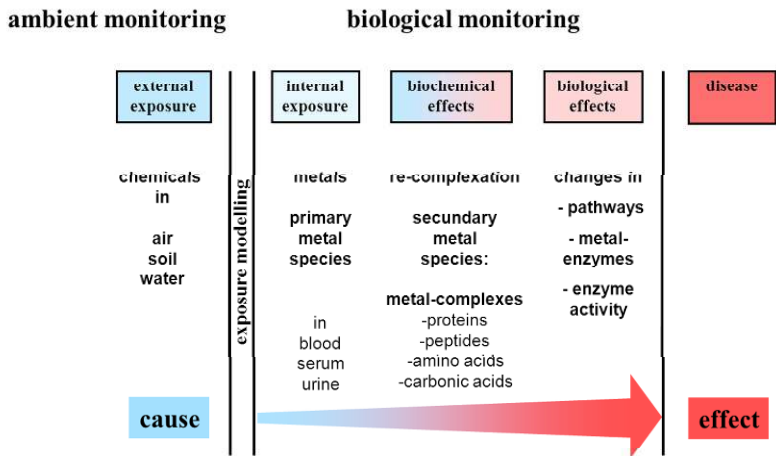
## Introduction

Mn is an essential trace element required for normal growth, development and cellular homeostasis [1]. Mn is a required cofactor of several enzymes necessary for neuronal and glial cell function, as well as enzymes involved in neurotransmitter synthesis and metabolism [2, 3, 4]. However, excessive Mn exposure can accumulate in the brain causing severe neurological disorder similar to Parkinson's disease (PD) [5]. Studies from occupational health after Mn exposure showed a limitation of mental ability, proceeding to psychotic phase with reduction of psychomotoric coordination, damage of the extrapyramidal nervous system and finally leading to symptoms similar to Parkinson's disease [6, 7, 8].

In a series of investigations we previously showed that different Mn-species are present in human serum and cerebrospinal fluid (CSF) [9, 10, 11, 12]. In serum mainly Mn-transferrin (Mn-Tf) was found, aside from

small amounts of Mn-citrate. Contrary, in CSF mainly low molecular mass (LMM) Mn-species were found, where Mn-citrate was identified as the major Mn-species [11]. Nischwitz et al. investigated paired serum and CSF samples [12] showing that total Mn and Mn-Tf were downgraded across neural barriers (NB) but Mn-citrate was enriched. Also Yokel et al. [13] and Aschner et al. [14] found Mn-citrate to be facilitated transported across NB when performing perfusion experiments in rat brains.

Thus, Mn-speciation targeted to Mn-citrate in human serum could provide a Mn-biomarker for Mn-exposure. Up to now a validated biomarker for Mn exposure is not available: Total Mn determination in blood or serum often cannot distinguish between non-exposed and exposed persons [15], and renal Mn excretion being below 1% of total Mn excretion is consequently not suitable as biomarker [16]. Therefore, in this paper significant relationships between Mn-species from serum and CSF were elucidated. Identification of Mn-compounds was provided using an orthogonal speciation scheme based on analysis of the samples by SEC ICP-DRC-MS, followed from analysis of SEC characterized fractions by CE-ICP-DRC-MS. Pearson's relationships between serum and CSF Mn species were calculated.



monitoring scheme for metals in environmental and occupational medicine

Figure 1: Scheme of environmental- and bio-monitoring: For avoiding exposure effects (diseases) monitoring should be as early as possible (left side of the scheme). In case of Mn, simple metal analysis in bodyfluids does not result in clear differentiation between exposed and non-exposed persons. Therefore, biomonitoring must include metabolized Mn species (secondary Mn species) and their specific pathways: Mn speciation may help to improve efficiency of differentiation of exposed from non-exposed persons (from Angerer, modified).

# Experimental

## Chemicals

From Sigma-Aldrich, Deisenhofen, Germany: Blue dextran: 2000 kDa,  $\alpha$ -2-macroglobuline: 609 kDa, arginase: 107 kDa, transferrin: 78 kDa, albumin: 68,5 kDa,  $\beta$ -lactoglobuline: 36,5 kDa, lysozyme: 14,3 kDa, Metallothionein: 7 kDa, L-thyroxine: 777 Da, N,N'-bis(t-BOC)-l-cystine: 440,5 Da, citric acid: 192,5 Da, inorganic  $MnCl_2$ .

From Merck, Darmstadt, Germany: TRIS,  $\text{HNO}_3$ ,  $\text{HCl}$  (suprapure),  $\text{NH}_4$ -acetate ( $\text{NH}_4\text{Ac}$ ), acetic acid ( $\text{HAc}$ ) and TSK SEC-gel (230 – 450 mesh). From Air-Liquide, Gröbenzell, Germany:  $\text{Argon}_{\text{liq}}$  and  $\text{NH}_3$ .

## Standards, samples and sample preparation

Mn-protein stock standards were prepared by dissolving the powder of each compound in 10 mL TRIS-HCl buffer (10 mM, pH 7.4). Stock solution of  $\text{MnCl}_2$  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  $\text{MnCl}_2$  solution (5 mg/L) using a ratio of 4+1 (v:v), resulting in a Mn-citrate stock concentration of 1 mg Mn/L. Mn-albumin and Mn-transferrin stock solutions were prepared in analogy by mixing 1 g/L protein solution with 5 mg/L  $\text{MnCl}_2$  solution (4+1, each), resulting in 1 mg Mn/L for each compound. Stock solutions were aliquoted and stored in the dark at  $-20^\circ\text{C}$ . Working solutions were prepared daily by appropriate dilution with TRIS-HCl, 10 mM, pH 7.4.

Serum samples were collected from healthy persons as described in [12] and stored at  $4^\circ\text{C}$  in a refrigerator. CSF samples were taken from non Mn exposed persons at the Dept. of Neurology, Technical University of Munich, following standard clinical procedures. The CSF samples were aliquoted and stored at  $-20^\circ\text{C}$ . Before analysis, the CSF samples were thawed slowly at  $4^\circ\text{C}$  and analyzed immediately.

## SEC Parameters

SEC was performed with a Knauer 1100 Smartline inert Series gradient HPLC system and two serially installed SEC columns: Biobasic 300 mesh column (300x8mm ID, Thermo, separation range 700 - 5 kDa) serially connected to a 550x10 mm ID Kronlab ECO column filled with TSK-HW40S (separation range 100 - 2000 Da). Tris-HAc (10 mM, pH 7.4) + 250 mM  $\text{NH}_4\text{Ac}$  was used as the eluent at a flow-rate of 0.75 mL/min.

For additional identification of Mn-species by CE-ICP-DRC-MS, the SEC column effluent was fractionated in 2.5 min intervals with a “Fraction Collector 100” (Pharmacia, Freiburg, Germany).

## Capillary zone electrophoresis coupled to ICP-DRC-MS

A “Biofocus 3000” capillary electrophoresis system (BioRad, Munich, Germany) was used as the CE device at a temperature of 20°C. The capillary (120 cm x 50 µm ID, non-coated) was from CS-Chromatographie Service GmbH (Langerwehe, Germany).

TRIS (10 mM, adjusted to pH 8.0 with HAc) buffer was the background electrolyte (BE). For sample stacking a buffer sandwich was injected consisting of 160 nL Na-acetate as leading electrolyte (LE), 60 nL sample, and 235 nL terminating electrolyte (TE, = BE/H<sub>2</sub>O (1:100)). The applied voltage was +28 kV. The hyphenation was described earlier [11].

## ICP-MS parameters

A Nexlon ICP-MS, Perkin Elmer (Sciex, Toronto, Canada) with dynamic reaction cell capability was employed for on-line determination of <sup>55</sup>Mn in the graphic mode.

For SEC coupling the ICP-introduction was managed with a Meinhard nebulizer whilst for CE-coupling a Micromist nebulizer was installed. The RF power was 1250 W, the plasma gas was 15 L Ar/min. The nebulizer gas was optimized and finally set to 0.98 (Meinhard) or 1.02 (Micromist) mL Ar/min. The dwell time was 500 ms for SEC coupling but 100 ms for CE coupling. The dynamic reaction cell (DRC) was operated using NH<sub>3</sub> for the DRC gas, finally at a flow rate of 0.58 ml/min. DRC band pass (q) was 0.45.

## Results and Discussion

### SEC-separation of Mn-species in serum and CSF

Mn-proteins were separated from LMM Mn-compounds in serum and CSF samples. The main part of Mn in serum is associated with proteins, whilst in CSF the main Mn fraction is associated with LMM Mn-species, predominantly with the citrate fraction. This was confirming our previous findings [10, 11].

Mn species were additionally investigated with CE-ICP-DRC-MS in 2.5 min fractions from SEC separation (two-dimensional analysis). For example figure 2 demonstrates the Mn-electropherogram of the citrate SEC fraction from CSF. The comparison with standard solutions containing  $\alpha$ -2-macroglobulin, Mn-citrate and inorganic Mn, standard match is found for Mn-citrate but no match was seen for  $\alpha$ -2-macroglobulin. However, the latter was not suspected in this citrate SEC fraction.



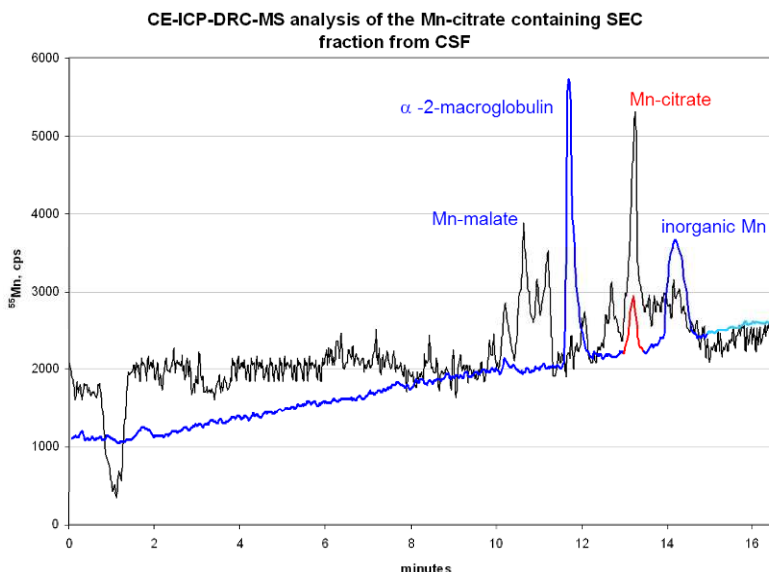


Figure 2: CE-ICP-DRC-MS analysis of the Mn-citrate SEC fraction from CSF and three standard Mn-species for comparison. Standard matches are observed for Mn-citrate and traces of inorganic Mn. The peak at 10.5 min relates to Mn-malate (standard match not shown).

Based on the above described 2D approach the Mn-peaks in SEC chromatograms were assigned to the main Mn-species eluting at respective retention times for quantification [17] and calculating Pearson's relationships.

## Pearson's relationships

Mn-species concentrations from serum and CSF were interrelated and Pearson's correlation coefficients were calculated. Only few relationships had reasonable  $r^2$  values indicating correlation, which were dependent on the total Mn concentration in serum.

The total Mn concentration in serum was correlated to the serum Mn-Tf fraction when total Mn was  $< 1.5 \mu\text{g/L}$ . Above  $1.5 \mu\text{g/L}$  correlation was

excluded. Contrary, no correlation was seen for Mn-citrate vs. total Mn in serum below 1.5  $\mu\text{g/L}$ , but a pronounced correlation was found above 1.5  $\mu\text{g/L}$  total  $\text{Mn}_{\text{serum}}$ .

Therefore we conclude that above a total  $\text{Mn}_{\text{serum}}$  concentration of 1.5  $\mu\text{g/L}$  Mn-citrate seems to be the most important Mn-species in serum, being responsible for the elevated  $\text{Mn}_{\text{total}}$  concentration. The findings from serum are also reflected in correlations between serum vs. CSF Mn species: for total  $\text{Mn}_{\text{serum}}$  concentrations below 1.5  $\mu\text{g/L}$ , total  $\text{Mn}_{\text{CSF}}$  concentrations were correlated mainly with Mn-Tf from serum, but above 1.5  $\mu\text{g/L}$  (total  $\text{Mn}_{\text{serum}}$ ) the total  $\text{Mn}_{\text{CSF}}$  concentration was predominantly correlated to Mn-citrate in serum.

This result is specifically important because it indicates that Mn-citrate is the main Mn-species in CSF when total  $\text{Mn}_{\text{CSF}}$  or total  $\text{Mn}_{\text{serum}}$  concentration is elevated. Furthermore, elevated  $\text{Mn-citrate}_{\text{serum}}$  and  $\text{Mn-citrate}_{\text{CSF}}$  concentrations are directly correlated. This can be used for estimating the  $\text{Mn-citrate}_{\text{CSF}}$  concentration from the  $\text{Mn-citrate}_{\text{serum}}$  concentration. This fact could be used for biomonitoring purposes, as CSF usually is not simply available (only after strict neurological indication at the hospital) in contrast to serum.

## Statistical evaluation

Two statistical techniques have been applied in order to retrieve useful information whether there is a differentiation between sample pairs.

First we applied an orthogonal partial least square discriminant analysis (OPLS/O2PLS-DA) dividing the dataset in two classes and studying the dependence of the variables  $Y1 = \text{total Mn}$ ,  $Y2 = \text{Mn-Tf fraction (peak 45-47)}$  and  $Y3 = \text{Mn-citrate fraction (peak 55-57)}$  and studying which are the responsible variables for the groups' separation. The goodness of the fit and the prediction were expressed by these two indexes respectively,  $R^2(Y)$  was 0.85 and  $Q^2(\text{cum}) = 0.54$  (the maximum value for both is 1), as seen in figure 3.

The loading Biplot expresses this relation between the variables "total Mn in CSF", "Mn-TF in CSF", "Mn-citrate in CSF" and the observation in the

dataset of paired samples. Here it is seen that “Mn-Tf” is more related to “class 1 members”, i.e. members of the violet group which have total  $Mn_{\text{serum}} < 1.5 \mu\text{g/L}$  whilst “total  $Mn_{\text{CSF}}$ ” and “Mn-citrate<sub>CSF</sub>” are positively related to each other and with the “class 2 members”, i.e. members of the orange group having total  $Mn_{\text{serum}} > 1.5 \mu\text{g/L}$ .

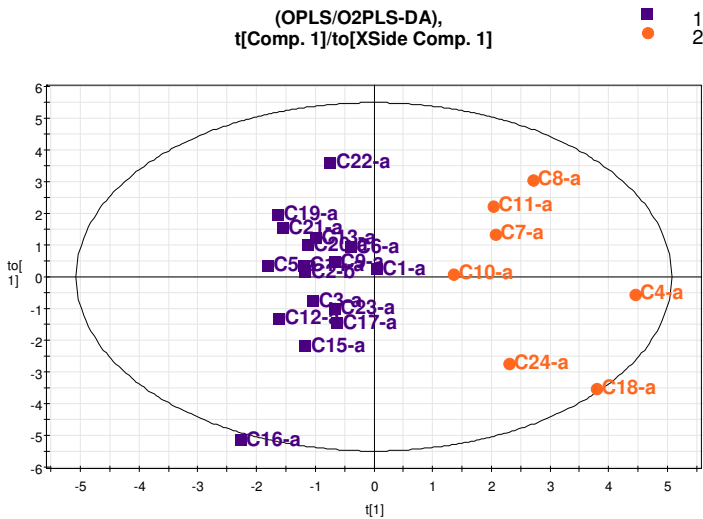


Figure 3: OPLS/O2PLS-DA model: It visualizes the two groups differences. Here it is shown that Mn-Tf is more related with the violet group having total Mn in serum  $< 1.5 \mu\text{g/L}$  whilst total  $Mn_{\text{CSF}}$  and Mn-citrate<sub>CSF</sub> are positively related to each other and with the orange group, having total Mn in serum  $> 1.5 \mu\text{g/L}$ .

We applied also canonical discriminant analysis. The class variable has been set up in the canonical discriminant analysis as dependent variable.

Summarized from both statistical evaluations we get a confirmation of Pearson’s relationships calculated above: When total Mn in serum is above  $1.5 \mu\text{g/L}$ , then “total Mn in CSF” and “Mn-citrate in CSF” are higher and CSF samples out of these sample pairs are differentiated from CSF samples from sample pairs where total  $Mn_{\text{serum}}$  is below  $1.5 \mu\text{g/L}$ .

## Conclusion

A set of paired serum and CSF samples has been investigated with two 2D approaches for Mn speciation. The established SEC-ICP-DRC-MS method allowed smooth separation of important Mn species, whilst CE-ICP-DRC-MS provided improved species identification. The interrelation of Mn-species from both sample types revealed correlations from serum Mn-species and CSF Mn<sub>total</sub> or CSF Mn-species. As the most important result we found CSF Mn<sub>total</sub> or CSF Mn-Cit to be correlated with serum Mn-Cit when serum Mn<sub>total</sub> was (slightly elevated) > 1.5 µg/L, but serum Mn<sub>total</sub> and CSF Mn<sub>total</sub> were more correlated to serum Mn-Tf when serum Mn<sub>total</sub> was < 1.5 µg/L.

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