Anal Bioanal Chem (2013) 405:2301–2309
DOI 10.1007/s00216-012-6662-7

ORIGINAL PAPER

Manganese speciation in paired serum and CSF samples using SEC-DRC-ICP-MS and CE-ICP-DRC-MS

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Received: 25 September 2012 / Revised: 6 December 2012 / Accepted: 13 December 2012 / Published online: 17 January 2013

Abstract Occupational manganese (Mn) overexposure leads to accumulation in the brain and has been shown to cause progressive, permanent, neuro-degenerative 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 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 coupled to ICP-DRC-MS in a 2D approach for clear identification. For additional species verification, electrospray ionization–Fourier transform ion cyclotron resonance–mass spectrometry was used after SEC-ICP-DRC-MS (second 2D approach). The Mn species from the different sample types were interrelated and correlation coefficients were calculated. In serum protein-bound Mn species like Mn-transferrin/albumin (Mn-Tf/HSA) were dominant, which had the main influence on total Mn in serum if Mn_{total} was <1.5 μg/L. Above serum Mn_{total} concentration of 1.6 μg/L the serum Mn_{total} concentration was correlated with increasing Mn-citrate (Mn-Cit) concentration. In parallel Mn_{total} and Mn species in CSF were determined. It turned out that Mn_{total} from CSF was about half of Mn_{total} in serum; Mn-Tf/HSA was only about 10 % compared to serum. It turned out that above 1.6 μg/L Mn_{total} in serum Mn-Cit was not only the leading Mn species in serum but also was the main influencing factor of both Mn_{total} and Mn-Cit concentration in CSF. These results were further investigated using two statistical models (orthogonal partial least squares discriminant analysis, canonical discriminant analysis). Both models discriminated the samples in two groups where CSF samples were either correlated to Mn_{total} and Mn-Cit (samples with serum Mn_{total}>1,550 ng/L) or correlated to Mn-Tf/HSA (samples with serum Mn_{total}<1,550 ng/L). We conclude that elevated $Mn\text{-}Cit_{serum}$ could be a valuable marker for increased total Mn in CSF (and brain), i.e., it could be a marker for elevated risk of Mn-dependent neurological disorders such as manganism in occupational health.

Keywords Manganese · Speciation · Cerebrospinal fluid · Serum · SEC-ICP-DRC-MS · CE-ICP-DRC-MS

Introduction

(1) Aspects of Mn in biological function: Mn is an essential ubiquitous trace element required for normal growth, development and cellular homeostasis [1]. Mn is specifically important in bone formation, for enzymes in energy production influencing fat and carbohydrate metabolism or blood sugar regulation. In humans and animals, Mn function 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]. Despite its essential role in multiple metabolic functions, excessive Mn exposure can accumulate in the brain and it has been associated with dysfunction of the basal ganglia system that causes a severe neurological disorder

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similar to Parkinson's disease (PD) [4]. Increased Mn levels are known for damaging the central nervous system, resulting in motor abnormalities and psychotic disorder [5, 6]. Studies from occupational health with increased Mn exposure demonstrated 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 [7–9].

(2) Mn exposure, blood–brain barrier and manganese speciation: in a series of investigations, we previously showed that different Mn species are present in human serum and cerebrospinal fluid (CSF) [10–13]. In serum, Mn was found mainly in a size exclusion chromatography (SEC) fraction associated with transferrin and human serum albumin (Mn-Tf/HSA). But we found also small amounts of Mn-citrate. Contrarily, in CSF, mainly low molecular mass (LMM) Mn species were found, where Mn-citrate was identified as the major Mn species [12]. Our previous results indicated that total Mn and Mn-Tf/HSA were decreased across neural barriers (NB) but Mn-citrate was enriched by a factor of 4–5 [13]. These results from non-exposed humans could be confirmed with animal experiments from Diederich et al. [14], who applied a single i.v. low-dose exposure of inorganic Mn to experimental rats and analyzed rat serum 1 h or rat serum and organs 4 days after exposure. In this previous paper we found inorganic Mn and Mn-Tf/HSA strongly, Mn-citrate little increased in serum 1 h after inorganic Mn exposure. After 4 days, no difference in serum to control rats was seen, but in brain and kidney significantly increased Mn-citrate concentration but no increase of Mn-Tf/HSA was found. These findings are in accordance with others who suggested a LMM Mn-carrier to the brain, independent from transferrin [15, 16]. Also, Yokel et al. [6] and Aschner et al. [17] found Mn-citrate to be facilitated transported across NB when performing perfusion experiments in rat brains.

From these previous results, it is clear that Mn-citrate plays a paramount role in entering the brain after exposure. This gives inducement to check whether Mn speciation targeted to Mn-citrate in human serum could provide a Mn biomarker for Mn exposure. To date no validated biomarker for Mn exposure is available, as total Mn concentrations in blood or serum from non-exposed and exposed persons are not significantly different and Mn ranges of both groups widely overlap [18]. Recently, Zoni et al. published an epidemiologic interrelation between olfactory function and environmental exposure, where these authors conclude that after long-term environmental exposure, olfactory impairment could be an indicator for Mn-based impairment, but they consider further research to be imperative [19]. Fe/Mn ratios in serum were investigated, too, but evaluation of results was finally discouraging. This held true also for urine: renal Mn excretion is below 1 % of Mn excretion and is consequently not suitable to act as biomarker [20].

Therefore, as a new approach, this paper aimed to elucidate whether significant relationships between Mn species from serum and CSF, specifically regarding Mn-citrate, are seen in individual paired serum/CSF samples. Identification of Mn compounds, specifically of Mn-citrate, was provided using an orthogonal speciation scheme based on serial analysis of the samples first by size exclusion chromatography–inductively coupled plasma–dynamic reaction cell–mass spectrometry (SEC-ICP-DRC-MS), second by collecting SEC fractions and subsequent analysis with capillary electrophoresis (CE)-ICP-DRC-MS or by electrospray ionization–Fourier transform ion cyclotron resonance–mass spectrometry (ESI-FT-ICR-MS). Finally, after clear identification in both sample types, Pearson's relationship coefficients were calculated using Microsoft Excel and data were statistically evaluated using canonical discriminant analysis using Proc Candisc, SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and OPLS analysis SIMCA-P 12 (Umetrics, Umea, Sweden).

Experimental

Chemicals

Compounds for mass calibration of the SEC column and Mn species standard compounds for retention time determination were purchased from Sigma-Aldrich, Deisenhofen, Germany. The following compounds were used: Blue dextran, 2000 kDa; α-2-macroglobulin, 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*,*N*′-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 ordered from Merck, Darmstadt, Germany. HNO_3 was purified by sub-boiling distillation. $Argon_{liqu}$ and NH_3 were purchased from Air-Liquide, Gröbenzell, Germany. An Ar vaporizer at the tank provided Ar gas. The TSK SEC-gel (230–450 mesh) was purchased from Merck, Darmstadt, Germany.

Standards, samples, and sample preparation

Mn-protein stock standards (1 mg powder/ml) were prepared by dissolving the powder of 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 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 $MnCl_2$ solution (4+1, each), resulting in 1 mg Mn/L for each compound. Stock solutions were aliquoted and stored in the dark at −20 °C. No destabilization of standard compounds was observed using these conditions. Working solutions were prepared daily by appropriate dilution with Tris–HAc, 10 mM, pH7.4.

Single standards and the analysis of standard mixtures were used to achieve information on SEC retention times or migration times in CE separations.

Serum and CSF sample pairs, drawn at the Department of Neurology of the Technische Universität München, were obtained from 24 patients. Sample donors had unspecific neurological complaints, such as headache, dizziness and various sensory symptoms. CSF and serum samples were collected and handled as described previously [21]. In short terms: CSF was collected from each individual by standardized lumbar puncture, and serum was obtained from blood drawn from the cubital vein directly after the spinal tab. Thus, CSF and serum are referred to as "paired samples". In case of unremarkable CSF test results, CSF and serum samples were considered to origin from neurologically healthy individuals. After patients consented to the use of their samples for scientific investigations, the previously aliquoted, frozen-stored samples were thawed at 4 °C in the refrigerator, vortexed and were ready for further analysis.

Repetitive analysis of a sample showed no differences in size distribution pattern in analogy to our previous work [13].

SEC Parameters

Based on our previous experiences about Mn species stability during HPLC separation we chose size exclusion chromatography, as a minimum of species changes were expected. SEC of Mn species from paired serum and CSF samples was performed using a Knauer 1100 Smartline inert Series gradient HPLC system connected to an electronic valve with a 100 μl injection loop (Perkin Elmer, Rodgau-Jügesheim, Germany) and further to two serially installed SEC columns: Biobasic 300mesh column (300×8 mm ID, Thermo, separation range 700–5 kDa) serially connected to a 550×10 mm ID Kronlab column filled with TSK-HW40S (separation range 100–2,000 Da). This column combination provided separation of various Mn-proteins from each other and from Mn-citrate as well as the latter clearly from inorganic Mn. Tris–HAc (10 mM, pH7.4)+250 mM NH_4Ac was used as the eluent at a flowrate of 0.75 mL/min. In preliminary experiments, the eluent was optimized with respect to species stability and minimized sticking of Mn compounds to the stationary phase. For checking species stability, reinjection experiments were performed in analogy to [22]. Possible sticking of Mn compounds to the columns was checked by purging after each run with Tris–HCl (10 mM, pH2) and analyzing the eluate. It turned out that the mass balance typically was between 91 and 108 %. Mass balances for Mn species specifically investigated in this paper were: α-2-macroglobulin, 91 %; Mn-Tf, 98 %; Mn-HSA, 105 %; Mn-citrate, 108 %; inorganic Mn, 102 %.

An auxiliary UV detector was installed between the outlet of the second column and the nebulizer of the ICP-MS. UV was detected at 220 and 280 nm. Thus, element (ICP-MS) and UV detection were possible in parallel with a time shift (UV to ICP-MS) of approximately 15 s.

Columns mass calibration

The mass calibration in the serially connected columns was performed using protein and LMM standards with well known molecular weight. As mobile phase Tris–HAc 10 mM, pH7.4+250 mM NH_4Ac was used. The retention times (RT) were determined by peak maxima in UV and for Mn/Fe-proteins additionally by ICP-MS detection. Retention times followed two calibration curves for the two columns [14]: From 28 min (void, >700 kDa) to 47 min (~5 kDa) according to the equation "$\ln(kDa) = -2384 \times RT + 14.888$ ($r^2 = 0.9953$)" and from 49 min (>2 kDa) to 60 min ($^{55}Mn^{2+}$, $^{56}Fe^{2+}$) according to the equation "$\ln(kDa) = -2612 \times RT + 13.148$ ($r^2 = 0.9974$)".

Fraction collection

For additional identification of Mn species by CE-ICP-DRC-MS and FT-ICR-MS, the column effluent was fractionated using a "Fraction Collector 100" (Pharmacia, Freiburg, Germany). Fractions were collected in 2.5-min intervals at regular flowrate of 0.75 mL/min. Three subsequent runs were collected and respective fractions pooled, frozen at −20 °C and subsequently freeze-dried (Heraeus-Christ, type "Beta", Osterode, Germany). The controlled sample temperature in the freeze dryer was adjusted to 4 °C to minimize the risk of species degradation or transformation during freeze drying. The freeze-dried fractions were re-dissolved in 300 μL Milli-Q water and kept frozen again until measurements with CE-ICP-DRC-MS or FT-ICR-MS analysis.

Capillary zone electrophoresis coupled to ICP-DRC-MS

A "Biofocus 3000" capillary electrophoresis system (BioRad, Munich, Germany) was used as the CE device. The temperature was set to 20 °C for sample/buffer carousels by air cooling and also 20 °C for the total capillary by liquid cooling.

The capillary (120 cm×50 μm ID, non-coated) was bought from CS-Chromatographie Service GmbH (Langerwehe, Germany).

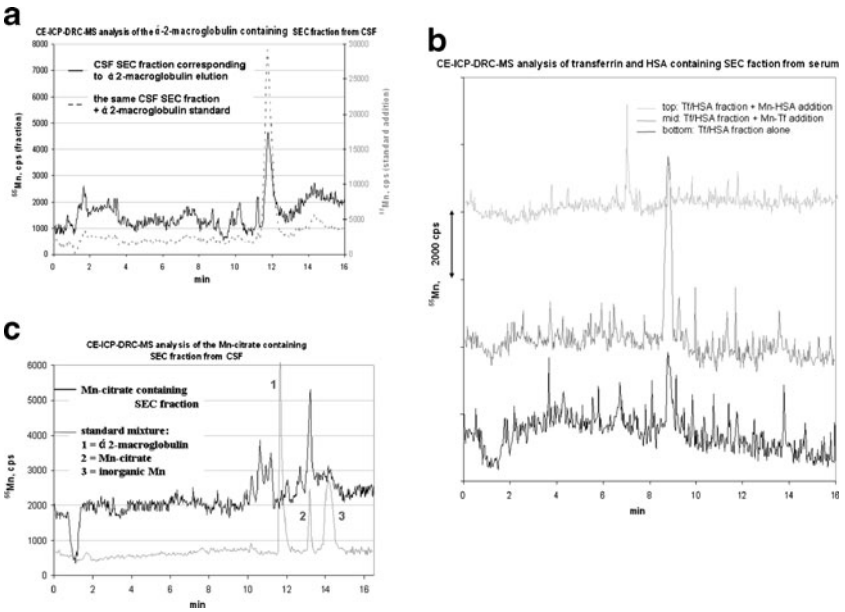

Fig. 1 **A** CE-ICP-DRC-MS analysis of the α-2-macroglobulin SEC fraction from CSF and the same fraction diluted with α-2-macroglobulin standard solution (*dotted gray line*). **B** CE-ICP-DRC-MS analysis of the Mn-transferrin/HSA SEC fraction from serum and the same fraction diluted with Mn-transferrin standard solution (*dotted gray line*) or Mn-HSA standard solution (*light gray line*). **C** CE-ICP-DRC-MS analysis of the Mn-citrate SEC fraction from CSF and three standard Mn species for comparison. Off-sets of electropherograms are made to facilitate the comparison

Before each run, the capillary was purged with Milli-Q H_2O (180 s, 8 bar) and background electrolyte (BE, 180 s, 8 bar).

The separation method used Tris (10 mM, adjusted to pH 8.0 with HAc) buffer as BE. For sample stacking a buffer sandwich was injected consisting of 160 nL Na-acetate (200 mM, high conductivity), acting as leading electrolyte (LE), 60 nL sample, and 235 nL terminating electrolyte, consisting of BE/H_2O (1:100; low conductivity). The inlet vial was filled with BE adjusted to pH6, the sheath flow at capillary end was BE/methanol (1:1). The applied voltage was set to +28 kV.

The hyphenation was based on a micromist nebulizer (50 μL/min optimal flowrate). A cross-piece just before the entrance to the nebulizer (at the first arm) provided the positioning of the CE capillary (at the third arm), the electrode (second arm) and the fourth arm was used as influx for the make-up flow responsible for electrical connection and flowrate adjustment. This make-up flow was provided by a Hamilton syringe pump at 50 μL/min. Positioning of the CE capillary was not critical with respect to signal response and stability. Inlet buffers were renewed after each run for avoiding a pH shift. Suction flow: The possible occurrence of a suction flow through the capillary was checked, although it was considered to be not very likely due to extended experience with 1.2 m capillaries in former studies [23]. No significant flow was seen.

ICP-MS parameters

A NexIon ICP-MS, Perkin Elmer (Sciex, Toronto, Canada), with dynamic reaction cell capability was employed for on-line determination of ^{55}Mn in the graphic mode.

For SEC coupling, the column effluent was passing the UV detector and then was directed to a Meinhard nebulizer (which was mounted to a cyclone spray chamber) using a PEEK transfer tube (ID 100 μm), while for CE coupling a Micromist nebulizer was installed (see above). The RF power was set to 1,250 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 DRC was operated using NH_3 as DRC gas, finally at a flowrate of 0.58 ml/min. The DRC band pass (q) was set to 0.45. These parameters were the optimal conditions for this instrument. LoD for Mn-peaks in SEC-ICP-DRC-MS were calculated as 3 σ-criterion. They were: α-2-macroglobuline, 13 ng/L; Mn-Tf, 15 ng/L; Mn-HSA, 15 ng/L; Mn-citrate, 10 ng/L; inorganic Mn, 10 ng/L and were set to 15 ng/L uniformly.

ICP-OES

For quality control in total Mn determination, additionally, an ICP-OES "Optima 7300" (Perkin Elmer) was used in parallel to ICP-DRC-MS. Sample introduction was performed by the instruments peristaltic pump at 1.0 mL/min and a Meinhard nebulizer which was fitting into a cyclon spray chamber. The measured spectral element line was: Mn 257.610 nm.

The RF power was set to 1000 W, the plasma gas was 15 L Ar /min, whereas the nebulizer gas was 600 mL Ar/min. Regularly, after ten measurements, three blank determinations and a control determination of a certified Mn standard were performed.

Comparability between the ICP-OES and -MS was recently also checked by analyzing identical rat brain and liver extracts (n=3) with both detectors [14, 24].

ESI-FT-ICR-MS

A Solarix FT-ICR mass spectrometer (Bruker Daltonics, Bremen, Germany) coupled to a 12-T magnet (Magnex, UK) was used. The FTMS consists of an octopole–

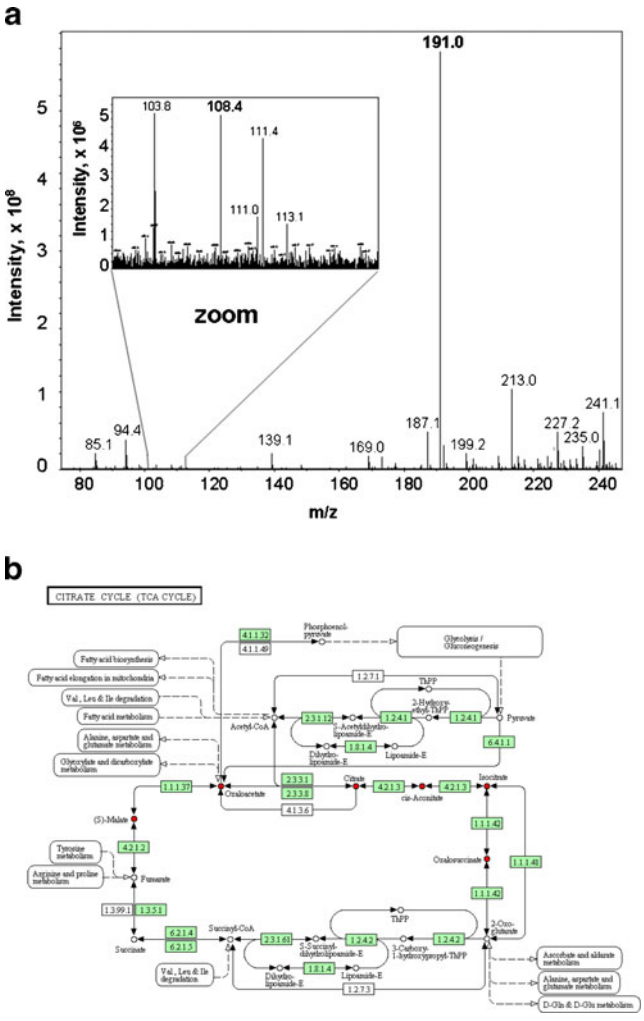

Fig. 2 **a** The ESI-FT-ICR-MS spectrum of the Mn-citrate containing SEC fraction from CSF. The main figure shows the lower *m/z* part of the measured spectrum with deprotonated citric acid $(C_6H_7O_7)^-$ at *m/z*=191 being the predominant peak. The *insert window* shows the signal of the $[Mn(C_6H_5O_7)_2]^{4-}$ complex at *m/z*=108.4, which is the Mn-citrate complex appearing at neutral pH [25]. **b** Metabolites identified (*full circles*) by FT-ICR-MS in the SEC fraction assigned to Mn-citrate. The high NH_4Ac concentration from the SEC eluent prohibited a very sensitive ES ionization. Therefore, only high concentrated metabolites were ionized sufficiently and detected in this SEC fraction

quadrupole–hexapole ion guide, coupled to an infinity ICR cell. All ion excitations were performed in broadband mode (frequency sweep radial ion excitation); 200 scans were accumulated for each mass spectrum.

Ions were accumulated in the hexapole for 300 ms prior to ICR ion detection. The base pressure in the ICR vacuum chamber was 1×10^{-9} mbar and in the quadrupole and hexapole regions 3×10^{-6} mbar. The mass range *m/z* 76–1,000 amu was scanned.

The electrospray ionization source (Apollo II, Bruker Daltonics, Bremen, Germany) was used in the negative ionization mode to ionize the studied analytes in 50 % methanolic solution (Lichrosolv, Sigma-Aldrich, Schnelldorf, Germany). The sample solutions were injected directly to the ionization source by the use of a microliter pump at a flowrate of 2 μL/min. A source heater temperature of 200 °C was maintained and no nozzle–skimmer fragmentation was performed in the ionization source. The instrument was previously calibrated by the use of Arginine negative cluster ions starting from a methanolic arginine solution of 5 mg/L.

Statistics

Microsoft Excel was used for calculation of Pearson's relationship coefficients. The discriminant analysis was performed by Proc Candisc, SAS version 9.2 (SAS Institute Inc., Cary, NC, USA) and OPLS analysis SIMCA-P 12 (Umetrics, Umea, Sweden).

Results and discussion

SEC-separation of Mn species in serum and CSF and two-dimensional Mn species identification

The first aim of this work was a sufficient separation of various Mn-proteins from different LMM Mn compounds in serum and CSF samples and their identification by a 2D approach. The identification was based on CE-ICP-DRC-MS and ESI-FT-ICR-MS as additional determination systems in previously SEC-separated fractions. Peak resolving was performed by Peakfit™ software.

Figure 1 demonstrates the Mn-electropherograms of specific SEC fractions from serum (Mn-Tf/HSA) or CSF (α-2-macroglobuline and Mn-citrate). Figure 1A shows an electropherogram of the SEC fraction, collected at the elution time of α-2-macroglobulin, and for comparison the electropherogram of α-2-macroglobulin standard. Figure 1B shows the Mn-electropherogram of the serum-SEC fraction collected at the elution time of Mn-TF/HSA, which is compared to the same fraction with Mn-Tf or Mn-HSA addition. Finally, Fig. 1C shows the Mn-electropherogram of the CSF-SEC fraction collected at the elution time of Mn-citrate. This electropherogram is compared to a standard solution containing α-2-macroglobulin, Mn-citrate and inorganic Mn. Co-elutions of standards and main Mn species in fractions show α-2-macroglobulin, transferrin or Mn-citrate to be the predominant Mn species in their respective SEC fractions. Mn-HSA cannot doubtlessly be assigned to a peak in the respective electropherogram. This result can be explained with respect to a considerable amount of highly competing divalent metals for HSA binding sites (e.g., Cu, Zn, both having by far higher binding constants for HSA than Mn) and the high binding coefficient of Mn to Tf.

Table 1 Median values of Mn compounds in serum and CSF differentiated into two groups, where total Mn in serum was (a) <1,550 ng/L and (b) >1,550 ng/L. Mn species assigned according to standard match in SEC and subsequent match in CE-ICP-DRC-MS and FT-ICR-MS. LoD values were: α-2-macroglobulin: 13 ng/L, Mn-Tf: 15 ng/L Mn-HSA: 15 ng/L Mn-citrate: 10 ng/L inorganic Mn: 10 ng/L and were set to 15 ng/L uniformly

Fraction assignment	Total Mn	Void Fraction 27–29 min	α 2-macroglobulin Fraction 30–34 min	Fraction 36–38 min	Arginase Fraction 42–44 min	Mn-transferrin/albumin Fraction 45–47 min	Fraction 49–53 min	Mn-citrate Fraction 55–57 min	Inorg. Mn Fraction 58–62 min
CSF									
Median (serum <1,550 ng/L)	457.00	<LoD	22.57	<LoD	67.16	103.58	23.49	169.26	<LoD
Median (serum >1,550 ng/L)	548.00	<LoD	31.45	<LoD	39.14	<LoD	<LoD	330.85	<LoD
Serum									
Median (serum <1,550 ng/L)	1161.00	85.19	211.47	31.05	70.49	549.78	57.22	60.85	<LoD
Median (serum >1,550 ng/L)	1641.00	229.44	313.43	54.75	233.02	698.25	39.75	101.74	<LoD

The ESI-FT-ICR-MS analysis of the citrate-SEC fraction from CSF confirmed the presence of Mn-citrate aside from free citrate, too. The FT-ICR-MS spectrum of the Mn-citrate SEC fraction is shown in Fig. 2a. Matzepatkis et al. [25], who investigated Mn-citrate chemistry, report about a $[Mn(C_6H_5O_7)_2]^{4-}$ complex as the predominant Mn-citrate species around neutral pH. The respective *m*/*z* signal should be found at *m*/*z*=108.3 which matches our signal at 108.4. Aside from the comparatively small Mn-citrate signal also the prominent signal of citrate (without metal binding, negatively charged by –H at *m*/*z*=191)) is found. This is the first time that Mn-citrate was monitored with ESI-MS in a native sample at such a low Mn concentration of 1 μg/L. Nischwitz et al. [26] reported a limit of detection (LoD) for Mn-citrate of 250 μg Mn/L by ESI-MS/MS.

The FT-ICR-MS data were analyzed subsequently with MassTrix software (© Helmholtz Zentrum München) [27], where the found metabolites were assigned to their metabolic pathways. The most important pathway found, having six consecutive metabolic hits, was the citrate cycle. Six compounds from this cycle were found in the SEC-citrate fraction from CSF, such as malate, oxalo-acetate, citrate, isocitrate, *cis*-aconitate, and oxalo-succinate. This is shown in Fig. 2b.

So far, these findings confirm our results from 2007 where these Mn species were found by CE-ICP-MS, showing Mn-citrate to be the most prominent one [12] in CSF.

Based on the species identification approaches, the Mn species concentrations were quantified and used for calculating Pearson's relationships. Table 1 shows the median values of Mn species in serum and CSF, each differentiated into two groups, where (a) total Mn in serum was <1,550 ng/L and (b) total Mn in serum was >1,550 ng/L.

With respect to total Mn concentrations, the results found here are in agreement with the range published earlier. Santamaria reported about 1–2 μg/L Mn for serum of non-exposed persons [28] and of ca. 0.5–1 μg/L in CSF. Nischwitz et al., too, reported values of 1.5 μg/L in serum and 0.7 μg/L in CSF as well as the factor 2:1 of Mn_{serum} to Mn_{CSF} [13], which was found also in our work.

Pearson's relationships

When relating serum Mn species to that of CSF for each individual and calculating Pearson's correlation coefficients it gets obvious that only for very few Mn species relationships r^2 values were gained which indicate a correlation. For most Mn species, no correlation or dependence was found. Importantly, it appeared that such correlations are dependent on the total Mn concentration in serum. Neither the existence of such a switching value nor its amount had been published before.

The total Mn concentration in serum is best correlated to the serum Mn-Tf/Mn-HSA fraction when total Mn is below 1,550 ng/L. Above 1,550 ng/L, clearly no significant influence of Mn-Tf/Mn-HSA on total Mn is proven, as is shown in Fig. 3A. Contrarily, no correlation is seen for Mn-citrate vs. total Mn in serum below 1,550 ng/L, but a pronounced correlation is found above 1,550 ng/L total Mn in serum (Fig. 3B).

This means that above a total Mn_{serum} concentration of 1.6 μg/L, Mn-citrate seems to be the most important Mn species in serum, being responsible for the elevated Mn_{total} concentration. The results confirm our previous observation [29], where two individual sera were compared, having 1.52 or 2.32 μg/L total Mn: the 2.32 μg/L serum showed an elevated fraction assigned to Mn-citrate complexes but no difference in the Mn-protein fraction was observed. Subsequent investigations from Nischwitz et al. [26] assigned this fraction to a $[Mn(H_2Cit)_3H_2]^+$ complex species. Matzepetakis, too, investigated Mn-citrate chemistry and found several

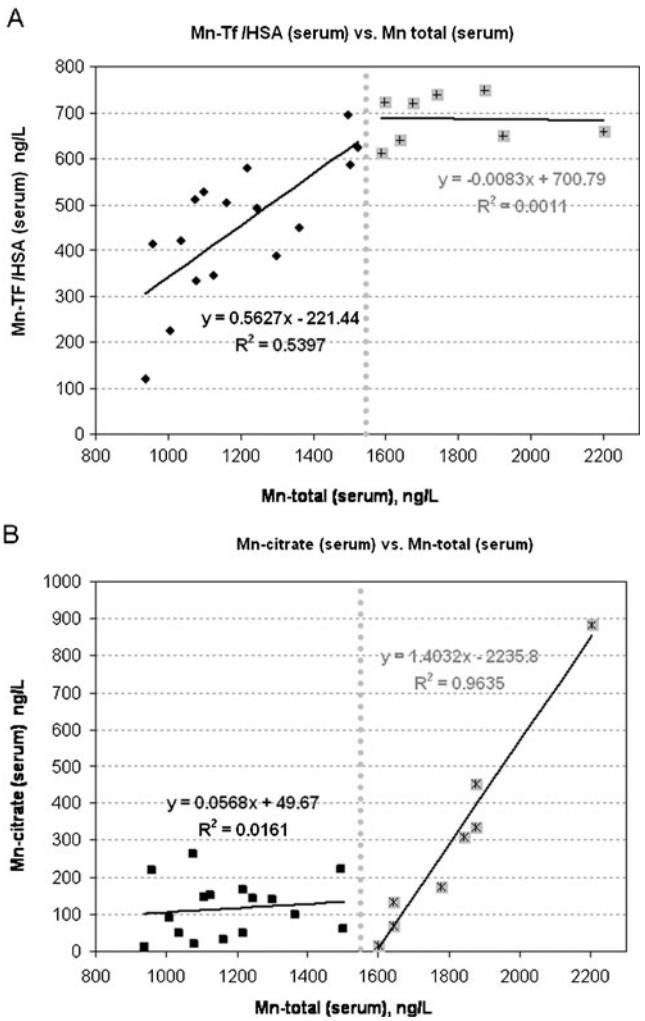

Fig. 3 The correlations of Mn_{total} in serum to Mn-Tf/HSA (**A**) or Mn-Cit (**B**, both from serum). Below Mn_{total} of 1,550 ng/L Mn_{total} serum is mainly correlated to Mn-Tf/HSA but above 1,550 ng/L it is influenced by Mn-Cit

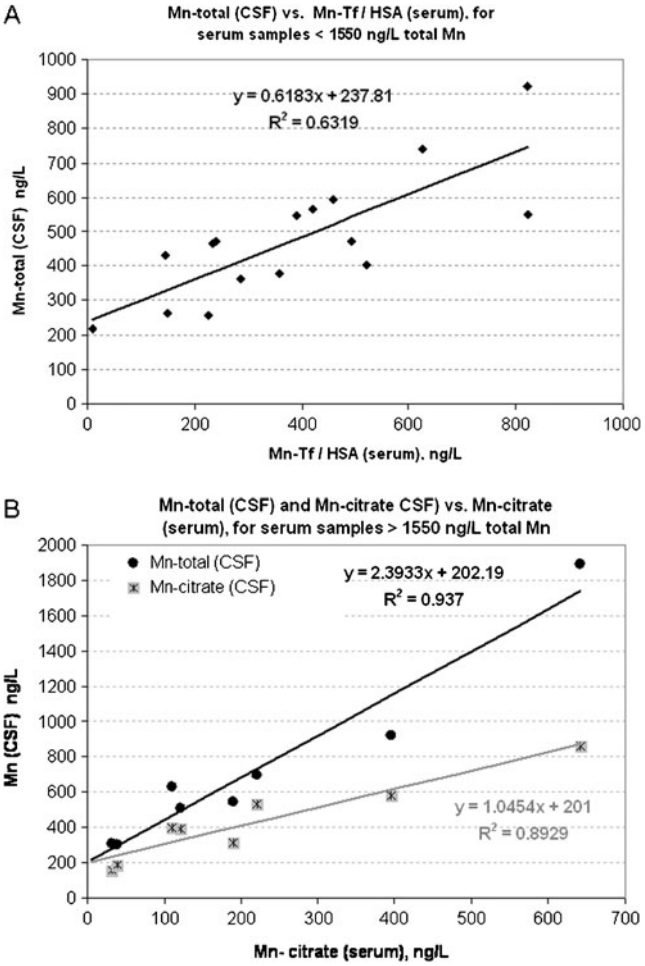

Fig. 4 **A** Mn_{total} from CSF is correlated to Mn-Tf/HSA in serum when Mn_{total} in serum is below 1,550 ng/L. **B** Mn_{total} and Mn-Cit, both from CSF, are correlated to Mn-Cit from serum when Mn_{total} (serum) is above 1,550 ng/L

Mn-citrate complexes specifically confirming our findings from ESI-FT-ICR-MS shown in this paper [25]. The findings from serum are also reflected in correlations between serum vs. CSF Mn species: For total Mn_{serum} concentrations below 1,550 ng/L, total Mn_{CSF} concentrations are correlated mainly with Mn-Tf/Mn-HSA from serum, but above 1,550 ng/L (total Mn_{serum}) the total Mn_{CSF} concentration is predominantly correlated to Mn-citrate in serum. Total Mn_{CSF} vs. Mn-$citrate_{serum}$ has r^2=0.9371 and Mn-$citrate_{CSF}$ vs. Mn-$citrate_{serum}$ has r^2=0.8929. The lower r^2-value for the latter relationship is explained by higher measurement uncertainty due to the lower concentrations of Mn-$citrate_{CSF}$ compared to total Mn_{CSF}. These findings are shown in Fig. 4A and B.

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

Statistical evaluation

Different 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=total Mn, Y2=Mn-Tf/HSA fraction (peak 45–47) and Y3=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

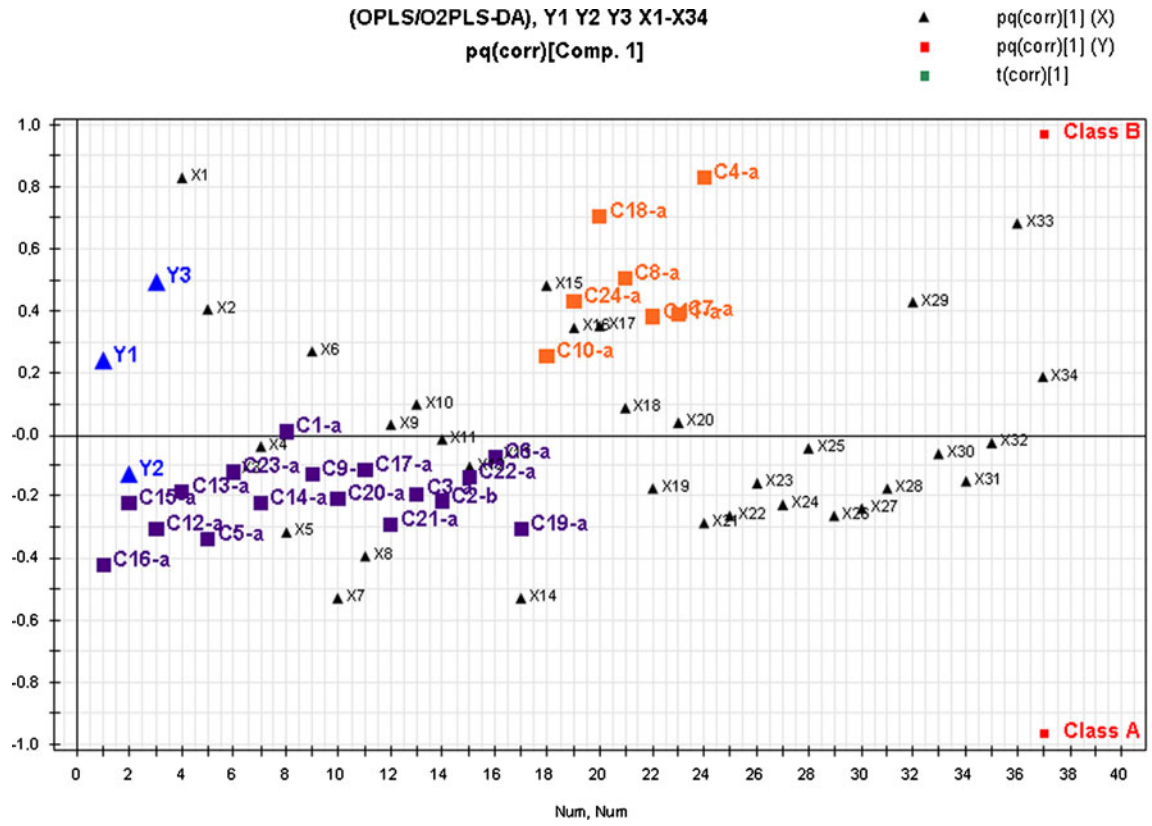

Fig. 5 Loading biplot has been calculated from our data using the OPLS/O2PLS-DA model. It visualizes the two groups differences and the relation between variables (dependent and independent) and groups (variables nearby are positively correlated). It is shown that Mn-Tf/HSA (=Y2) is more related to the (*violet*) group having total Mn in serum <1,550 ng/L while total Mn_{CSF} (=Y1) and Mn-$citrate_{CSF}$ (=Y3) are positively related to each other and to the (*orange*) group having total Mn in serum >1,550 ng/L (*X* standing for elements other than Mn)

these two indexes respectively, $r^2(Y)=0.85$ and $q^2(\text{cum})=0.54$ (the maximum value for both is 1).

In Fig. 5, the relation between the variables "Y1=total Mn in CSF", "Y2=Mn-Tf/HSA in CSF", "Y3=Mn-citrate in CSF" and the dataset of paired samples is shown. It is seen that Y2 (Mn-Tf/HSA has closer relation to class A members, i.e., members of the violet group which have total Mn_{serum}<1,550 ng/L while total Mn_{CSF} (Y1) and Mn-$citrate_{CSF}$ (Y3) are positively related to each other and to the class B members, i.e., members of the orange group having total Mn_{serum}>1,550 ng/L.

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

From the output of both models we drew up the VIP variables list (the list of variables that contributed mostly in the projection). Based on the canonical discriminant analysis we calculated the linear combination of the variables which has the highest possible multiple correlation with the two groups. Comparison between the two lists reveals the common variables which are important for the group determination. These variables are tabulated in Table 2.

Summarized from this statistical evaluation, we get a confirmation of above calculated Pearson's relationships: When total Mn in serum is above 1,550 ng/L, then Y1 (=total Mn in CSF) and Y3 (=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_{serum} is below 1,550 ng/L. The latter CSF samples are more correlated with Y2 (Mn-transferrin/HSA).

Table 2 The most important variables (Mn species) coming out from the two different multivariate analyses. The selection has been based on the VIP value (greater than 1) and based on the highest value of the canonical weights determined with the first canonical component. For both analyses the total Mn_{CSF} is predominant as well as the Mn-Tf/HSA fraction (45–47 min)

Variables	VIP (OPLS/O2PLS-DA)	Can1 (Canonical DA)
Y1=total Mn	2.77	0.79
Y2=Mn-transferrin	2.08	0.59
Y3=Mn-citrate	1.44	0.41

In both statistical models, the same variables were found to be important for differentiation between the sample groups.

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 to smoothly separate important Mn species from these biological samples, while CE-ICP-DRC-MS and ESI-FT-ICR-MS provided improved species identification. The interrelation of Mn species from both sample types revealed correlations from serum Mn species and CSF Mn_{total} or CSF Mn species. As the most important result, we found CSF Mn_{total} or CSF Mn-Cit to be correlated with serum Mn-Cit when serum Mn_{total} was (slightly elevated) >1.550 ng/L, but serum Mn_{total} and CSF Mn_{total} were more correlated to serum Mn-Tf/HSA when serum Mn_{total} was <1.550 ng/L. Statistical evaluation with two different models confirmed these findings. Elevated $Mn\text{-}Cit_{serum}$ could be a valuable marker for increased total Mn in CSF (and brain), i.e., a marker for elevated risk of Mn-dependent neurological disorders such as manganism in occupational health.

Acknowledgments The authors thank Katharina Fernsebner for reading the manuscript.

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