Determination of Manganese in Biological Materials by Electrothermal Atomic Absorption Spectrometry: a Review

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The great diversity of methods for measuring manganese in biological materials (serum, plasma, whole blood, urine, spinal fluid, and hair) reflects the difficulty in measuring extremely small quantities of this element. Detailed examination of these methods demonstrates that the one most used is flameless atomic absorption spectrometry. In this review we report the different instrument settings for wavelength, slit width, protection gases, graphite furnaces, type of background correction, amounts measured, and thermal programs. We give detailed recommendations by various authors for collecting samples. A thorough description of the preliminary steps and the handling of the specimen samples is also included: direct determination with or without dilution, addition of a matrix modifier or determination after ashing, with or without chelation-extraction steps. The preparation of the standards, procedures used, analytical criteria (accuracy, precision, specificity, detection limit, linearity), problems (interferences, matrix effects), and reference values and their physiological variations are also described. We give a consensus of recommendations concerning the choice of a method.

Manganese (atomic number 25, atomic mass 54.938 Da) is the most ubiquitous transition metal after iron and tin. Although widespread in nature, it is never found in the metallic state. The most frequently encountered valences for manganese-containing compounds are +2, +3, and +7. Manganese shares with certain other metals the property of being essential to life, but large doses are toxic (1, 2). The estimated daily requirement for adults is 2 to 3 mg. However, exactly how the body assimilates Mn still remains unknown. Furthermore, manganese metabolism apparently varies from individual to individual. Manganese is found in the body mainly in mitochondria-rich tissues.

Like most transitional metals, Mn is found in various complex enzymatic compounds (1–3): phosphoenolpyruvate carboxykinase (EC 4.1.1.32), pyruvate kinase (EC 2.7.1.40), pyruvate carboxylase (EC 6.4.1.1), superoxide dismutase (EC 1.15.1.1), and arginase (EC 3.5.3.1). It is recognized as a nonspecific activator of enzymes that require the presence of a divalent ion. The ion Mn2+ is analogous to Mg2+, which it may replace in numerous biological molecules.

Mn is mainly associated with the formation of connective and bony tissues, with growth and reproductive functions, and with carbohydrate and lipid metabolism (4). Manganese concentrations are controlled by the liver, most of it being eliminated in bile (4), with a small proportion eliminated through the urinary tract and superficial body growth such as hair (5).

Determination of manganese is of particular interest with regard to detecting excessive exposure to it in the workplace. Chronic intoxication from inhaling Mn at work is characterized primarily by psychological and neurological manifestations (Parkinson-like syndrome (6)).

Although frequently observed in the animal kingdom, no manganese deficiency is apparent among human beings. Nevertheless, a prolidase deficiency, which becomes evident in certain cases of chronic ulcers, is accompanied by a decreased concentration of manganese in serum and urine (7).

Pathologically, the manganese content of serum increases among patients with liver diseases of whatever etiology (8), and among hemodialysis patients when the Mn content of the dialysis liquid is increased (9). For patients with acute myocardial infarction, the mean serum Mn values do not significantly differ from the controls (10, 11).

These deficits and overloadings studies have led analysts to develop methods for Mn determination in biological fluids. The analytical difficulties and the very low concentrations to be measured lead to an important dispersion of values and to a great diversity of methods. The most fully developed analytical procedures are in the EAAS field.5

Methodology

The numerous analytical techniques include flame atomic absorption spectrometry (AAS), electrothermal atomic absorption spectrometry (EAAS), neutron activation analysis (NAA), inductively coupled plasma (ICP), photometry, spectrochemical emission, spectrophotometry, and fluorometry.

In the AAS method, air–acetylene is the most frequently used mixture (12–23).

Determination of Mn in serum, whole blood, urine, hair, tissue, or spinal fluid by NAA is reported in numerous publications (24–32).

5 Nonstandard abbreviations: AAS, flame atomic absorption spectrometry; EAAS, electrothermal atomic absorption spectrometry; NAA, neutron activation analysis; ICP, inductively coupled plasma; and ns, not specified (in the literature).
Sometimes ICP is coupled with EAAS. Aziz et al. (33-34) used this double system for determining Mn in serum and body tissues: after the sample is dried, mineralized, and atomized in a furnace, the nebulized material is transferred to the ICP system. However, Black et al. (35) used ICP alone for serum and tissues, as did Barnes and Fodor (36) for urine samples alone. Three other methods are not so widespread: photometry is mentioned by Borner et al. (9) and Mehnerl (37), fluorometry by Rubito et al. (38), and spectrochemical emission is only used by Hambidge and Droegenmueller (39) for plasma and hair and by Yoakum et al. (40) for body tissues.

From a close examination of the research published to date, we find the method most commonly used in clinical chemistry for determining manganese is EAAS.

Operating Conditions in EAAS

Wavelength and slit width. Hollow-cathode lamps (single- or multi-element) are the most frequently used, generally set at the most sensitive wavelength, 279.5 nm. The smallest slit width should be chosen (generally 0.2, or 0.7 nm if the available energy is not sufficient at 0.2 nm), as determined by the optical characteristics of each apparatus.

Carrier gas. Although some authors use nitrogen (41-46), argon is preferred to avoid nitricure generation. Furthermore, Brodie (47) found that argon offers a greater sensitivity.

Background correction. Continuum source background correction is the most widespread system. Deuterium arc lamps are the most frequently used, and in recent times the Zeeman effect has been used for improved background correction (48-53). Grafflage et al. (54) and especially Favier et al. (55, 56) found that this correction is absolutely essential to free the determination from the effect caused bycovolatilized matrix (55). More importantly, background correction is almost always necessary to avoid errors near the detection limit.

Furnaces. Pyrolytically coated tubes are the most commonly used (45, 47, 53, 54, 57, 58). According to our experience, they offer a better sensitivity and higher thermal stability than that of standard tubes. The L'vov pyrolytic platform, generally associated with a matrix modifier, is used for complex media such as seawater (59, 60), but its use in analysis of biological materials is likely to increase (61, 62). Some manufacturers suggest the use of graphite "cup-type" cuvettes (48, 51).

Injected volumes. Injected volumes depend on the dimensions of the furnace. They range from 2 (52) to 50 μL (54, 56, 63, 64); the most common are between 20 and 50 μL. For such volumes, a wetting agent is added to the sample, to spread out the drop in the furnace. Injection of the sample into the furnace may be manual or automatic. Automatic injection of course offers the advantage of better within-run precision. It is recommended to cut the catheter tip regularly to avoid adsorption (56). For small volumes (5 μL), Manning and Slavin (65) used a special apparatus with a deposit of the sample on tungsten wire, prepared beforehand.

Graphite furnace settings. Drying depends on the volume and type of sample. Times range from 5 s for Wei et al. (57) to 2 min for Bek et al. (66), with various numbers of stages (47, 51, 67, 68).

Various charring temperatures are used (46, 54), the most suitable being 1100 °C (24, 41, 47, 54, 56, 64, 66, 69-72), with a ramp time of 10 s and a hold time of 30 s for a 20-μL sample deposit. No loss of Mn can be observed at this temperature.

For atomization, the most frequently mentioned temperatures are 2400 °C and 2600 °C (48, 50, 63, 64, 73-75). Fast heating (0 s ramp time) is recommended to increase the atomic concentration in the vapor phase. Narrower and sharper absorption peaks are observed if the furnace is heated very rapidly (76, 77), and the sensitivity and linearity are improved (55).

Specimen Collection

Manganese may be measured in serum, plasma, whole blood, hair, and urine. Its presence in hair is of particular interest as a good indicator of the current body burden of manganese (5, 32, 70). Assay of whole-blood specimens has been preferred to serum, owing to the strong concentration of Mn in the erythrocytes (3, 26, 78).

Because the concentrations of manganese ordinarily present in the biological materials analyzed are very low (<1 μmol/L or 1 μmol/g), certain conditions must be met when samples are collected, to avoid contamination.

Blood

Blood samples must be obtained with sampling devices free of manganese and this preferably in the morning after overnight fasting (9, 39, 79). Some studies mention much Mn contamination when disposable syringes and stainless-steel needles are used (69, 80). However, other authors have not noted any contamination from the use of stainless-steel needles (12). Versieck et al. (81) recommended, however, that when this procedure is used, the first few milliliters of blood collected should be discarded. Use of a nonmetallic catheter is clearly preferred (41, 54, 69). The use of evacuated containers does not seem to pose any major problems of contamination (48, 56, 82, 83). For collection and storage, plastic tubes are most frequently used (polypropylene, polyethylene, or polyethylene), previously decontaminated by washing in diluted hydrochloric or nitric acid (39, 54, 69, 80). Generally, the samples are stored at −20 °C (39, 42, 48, 80, 84). Glass tubes are not recommended for storage. Hulnik et al. (80) have observed noticeable contamination from this material, and Grafflage et al. (54) noted adsorption of manganese by the glass.

If an anticoagulant is used, it must be rigorously controlled. The studies of Delvee et al. (82) have pointed out the high concentrations of manganese in some heparins. However, heparin is the anticoagulant most frequently used in manganese determination (24, 39, 48, 83), although Muzzarelli and Rocchetti used oxalate (43), Nath (84) recommended citrate, and Case et al. used dipotassium EDTA (75).

Urine

Urine collected in normal urination or, more often, in diuresis over 24 h is acidified and collected in plastic vials previously washed in acid (14, 69, 85). The specimens are stored at −20 °C (15).

Hair

Hair specimens are taken in sufficient quantities (about 20 mg is needed for good precision) and washed by various methods (each author has a preference) before determination of manganese. The washing should eliminate adsorbed elements without, however, desorbing the manganese actually in the hair itself (25, 86). Many different procedures have been used for washing the hair (87). This difficult
problem has long been a point of contention, as emphasized by Chittleborough (88). Organic solvents (28, 39, 70, 71), detergents (53, 74, 89), and ultrasonic treatment with water (25) have been shown to remove surface contamination from hair effectively without extracting the Mn contained in the shaft, but it is difficult to conclude which wash is the best (53). We recommend sequential washings with non-ionic detergents (such as Triton X-100), water (several times), 95% ethanol solution, and again with water. After drying at 60 °C the specimens can be stored at 20 °C until determination. The use of a chelating agent should be avoided.

Tissue

Tissue biopsies are carefully soaked several times in a solution of wetting agent, rinsed with de-ionized water, and placed in a flask previously washed with a diluted acid (80, 90). The samples may be stored at −20 °C after lyophilization, or not (80, 91).

Preparation and Treatment of the Sample

The analysis for manganese in biological media may be carried out in two different ways: directly, with or without dilution and perhaps with the addition of a matrix modifier, or indirectly, after mineralization with or without chelation/extraction.

Direct methods. In EAAS, the introduction of pure biological media limits the risks of contamination but requires lengthy temperature ramps. Interferences, matrix effects, and carbon residues may be important in this procedure. Very small quantities of liquid media may be injected into the graphite furnace in a pure state without any treatment or dilution (46, 50, 54, 63, 66, 72, 92). Tissues may be placed directly in the graphite furnace after lyophilization (52). For hair, Alder et al. (71, 93) decomposed the sample directly in the graphite tube. In direct methods with dilution, de-ionized water (43, 46, 54, 68, 69, 73, 94, 95), Triton X-100 (24, 47, 48, 92) or other wetting agents (42, 96), acids (75, 96), ammonium compounds (57), or quaternary amines (51, 97, 98) are commonly used.

Indirect methods. Complex biological substances may be simplified by acid digestion followed by simple dilution (41, 45, 54, 99), or by acid digestion followed by chelation and extraction with a solvent (14, 15, 46, 54, 100–102). Guided by our experience we recommend, for urine and blood serum, direct analysis with dilution, which allows good practicability and minimizes contamination.

For tissues, three procedures of mineralization and solubilization have been proposed: solubilization with quaternary amines (97, 98, 103, 104); acid digestion without extraction (45, 49, 67, 72, 90, 105); and acid digestion with chelation/extraction (44, 101).

Hair is dissolved, after washing (as described above), by acid digestion and Mn is determined after simple dilution (53, 89). Saner et al. (70) dissolved the residue in HCl after dry ashing.

Standards

The standard solution of manganese is prepared either from the pure metal dissolved in sulfuric acid (92) or from a commercial standard solution made from different compounds of manganese, such as MnCl₂ (15, 52, 106) or MnO₂ (98), in water (14), nitric acid (44), or 0.5 mol/L sulfuric acid (96).

External standards. The aqueous standard solutions are prepared with hydrochloric acid (68, 91, 106) or nitric acid (44, 49, 69), or with the same diluents as for sample dilution (42, 56). For Favier and Ruffieux (42), Muzzarelli and Rocchetti (43), and Wei et al. (57), the results obtained with an aqueous solution and standard additions are comparable. On the other hand, Smeyers-Verbeke et al. (64) do not recommend the use of aqueous solutions because the chemical evolution of manganese depends heavily on the composition of the medium.

Determination by standard additions. This is the method preferred by most researchers (14, 43, 46, 47, 58, 63, 64, 67, 75, 90, 92, 96, 107).

Addition calibration. Pleban and Pearson (48), like Boiteau et al. (51), set up a range of standards by additions of standard to the same medium (serum pool or whole blood with very low manganese concentrations). For each sample, the concentration of manganese is taken directly from a graph after deducting for each sample the value of a blank reagent. The two last procedures of standardization are the most suitable, taking into account the matrix effects.

Precision

Within-Run Precision

For whole blood, serum, or plasma: Better within-run results are obtained when the concentration in manganese is high: reported CVs range from 1.7% (1019.5 mmol/L) to 12.5% (114.6 mmol/L) (48, 51). Favier et al. (56) demonstrate the importance of prior dilution of the sample: precision was poor for a non-diluted sample (CV 27%), owing to high background, and for one diluted 20-fold (CV 33%), probably owing to low concentration after dilution. The best results (CV 4%) are obtained with a fivefold dilution of the sample in 10-fold-diluted ethylene glycol (56).

For urine: The within-run CV is generally <10%. Halls and Fell (69) found a within-run CV of 11.9% for undiluted urine and 7.4% for a twofold-dilution of the same urine. The twofold dilution decreases the background and gives a much smaller range for analytical recoveries (69). Lekahal and Hanoq (14) compared two techniques for treating urine: extraction with and without prior acid mineralization with two different methods of determination (AAS and EAAS); within-run results were comparable, and CVs ranged between 0.4% and 3%.

For hair: Guillard et al. (53) and Friel and Nguyen (74) observed, for non-exposed subjects, within-run CVs of 3.9% and 8%, respectively.

For tissue: The CV ranged between 2% and 5.6% (49, 72).

Between-Run Precision

CVs range from 2.3% (46) to 16% (54) for serum. The results improve as the concentrations in manganese increase: Meissner et al. (73) observed CVs of 5.6, 3.8, and 2.4% for concentrations of 1.8, 18.2, and 182 mmol/L, respectively. For determination in tissue, the between-run CV was between 6.3 and 18.5% (46, 49, 52, 72).

Accuracy/Specificity

Recovery. Analytical recovery ranges between 90 and 110% (14, 44, 46, 49, 51–53, 58, 68, 74, 78, 101).

Interferences. Matrix effects are significant (69), and they increase in proportion to the amount of the deposit. The use of background correction (deuterium lamp or Zeeman effect) eliminates nonspecific interferences. Smeyers-Verbeke et al. (64) specify that slow thermal pretreatment results in a more effective reduction of interference by matrix. In most
cases, positive interferences can be decreased when the concentration is calculated on the basis of the peak areas (64).

The significance of the interferences seems to be related to the temperature chosen for the mineralization. Bek et al. (66) observed a high background noise at a charring temperature of 750 °C, and d'Amico and Klawans (63) say this temperature must be 800 °C. Alt and Massmann (107) find 1000 °C to be apparently insufficient to avoid interference by absorption of molecular sodium chloride (107).

Sodium chloride in concentrations greater than 10 to 20 mmol/L causes a reduction in the signal (44, 73). As a result, the following are required: dilution of the sample (73), extraction (44), addition of ammonium nitrate, or, as mentioned above, an increase in the charring temperature from 850 to 1100 °C. Favier et al. (56), like Sekiya et al. (99), observed no significant interferences by sodium. For Smeyers-Verbeke et al. (64) only sodium in sulfate compounds increased the signal (by 25%). Belling and Jones (44) noted a negative interference from potassium, not observed by Sekiya et al. (99) or Favier et al. (56). Interferences from chlorides should be compensated by the background correction (108). There appear to be no interferences from iron, copper, or zinc (56). Several authors point out negative interference from calcium and magnesium ions (44, 56, 109), which can be eliminated by the addition of EDTA (56, 109) or by extraction (44). Smeyers-Verbeke et al. (64) studied the influence of very high concentrations (18.2 mmol/L) of calcium and magnesium in different salts (chlorides, nitrates, phosphates, and sulfates). Calcium and magnesium salts, according to their different natures, affected the signal in different ways (from −80% to +50%). With calcium and magnesium chlorides, the signal reduction can be explained by a masking of the manganese by these salts. Smeyers-Verbeke et al. (109) observed an increase in the atomization speed in the presence of calcium or magnesium nitrate. Favier et al. (56), however, did not observe any major interferences in the case of sulfates and phosphates.

Smeyers-Verbeke et al. (64) demonstrated the influence of acids on the signal. Phosphoric acid, 100-fold and 20-fold diluted (by vol), increases absorption by 15%. On the other hand, hydrochloric acid in dilutions lower than or equal to 10-fold and sulfuric or nitric acids, 100-fold diluted, have no effect. The sulfuric or nitric acids in 10-fold or 20-fold dilutions (by vol) reduce the signal by 20% to 60%.

The volatility of manganese varies according to the concentration and the nature of the salts, so it is necessary to test an extended series of concentrations and different temperature programs in accordance with different matrices. The use of aqueous standards does therefore not seem to be suited for the determination of manganese in biological materials.

Detection Limits

The detection limits observed are reported in Table 1.

Linearity

Very few authors mention the upper limit of linearity in their methodologies. Favier et al. (56) found it to be 910 nmol/L for serum, and Pleban and Pearson (46, 49) found it to be 364 nmol/L for serum, 728 nmol/L for whole blood, and 820 nmol/L for solubilized tissues.

Reference Values

The data presented in the following tables were obtained from the available published mean values, which vary greatly from one author to another, especially for serum and urine (Tables 2 and 3). This discrepancy is due in part to the sample-collection conditions, possible sources of contamination, as well as individual variations in addition to the different methodologies (9, 14, 15, 30, 46, 54, 57, 107, 110). For serum, neutron activation leads to low values, while flame atomic absorption and photometry give high values. For urine, the methodology seems to play a less-important role. Values for plasma (Table 2) vary greatly, probably owing to the type of anticoagulant used. Reported reference values for manganese in whole blood and erythrocytes (Table 2) are more uniform. A 30-fold greater concentration of manganese in erythrocytes than in serum may explain this uniformity of results.

Reference values for manganese in hair are also widely disparate (Table 3). The effect of hair pigmentation (see below and 111) and the method used for washing are the predominant factors here.

Physiological Variations

Manganese in the serum may be subject to a nyctohemeral cycle (42), with a peak at 0800, although Borner et al. (9) did not observe such a cycle. Manganesemia does not vary according to sex (2, 8, 12, 42, 54). Masiak et al. (30), however, observed slightly higher concentrations in women.

There also does not appear to be any variation in manganesemia in relation to age in adolescents or adults (42, 50, 54). During gestation, manganesemia increases early, then stabilizes, reaching normal values for adults at the time of delivery (42).

The use of birth-control pills leads to an increase in manganese in urine (112).

In hair, the concentration varies in relation to the pigmentation. A study by Guillard et al. (111) demonstrates that dark hair is richer in manganese than grey hair. The concentration of manganese in hair is low at birth (0.19 ± 0.11 μg/g dry wt), increases rapidly during the first few weeks after birth (0.965 ± 0.39 μg/g dry wt), and then decreases between the sixth week and the third year (0.398 ± 0.21 μg/g dry wt) (89).
Liver tissue showed no variation in concentrations related to age or sex (72).

Conclusion

The determination of manganese in biological materials by EAAS still leaves us with many problems to be solved, as is illustrated by the discrepancy in usual values and in methods used for treating the sample before it is injected into the graphite furnace. Certain analytical parameters have now been well established and these include wavelength (279.5 nm), slit width (generally 0.2 or 0.7 nm), the necessity of using a background correction (D2 lamp or Zeeman effect), and the use of pyrolytic tubes. A wetting agent (such as Triton X-100) also seems useful. Charring and atomization temperatures do vary from author to author, but it seems that 1100 °C and 2400 to 2600 °C are the most suitable. The importance of interferences makes us favor internal standard additions or calibration by standard additions as opposed to external standards. Precision and accuracy are generally acceptable, while the limits of detection, values for which vary from author to author, often approach the normal reference values, especially for biological materials such as serum, plasma, urine, and spinal fluid.

Clinical interest in Mn determination is not yet well established in cases of liver disease (cirrhosis), psychological and neurological disorders (epilepsy, Parkinson-like syndrome), and in certain cases of chronic ulcers (prolactase deficiency). On the other hand, Mn determination is of special clinical interest in the detection of overloading as a result of workplace exposure, and among hemodialysis patients when the content of manganese in the dialysis liquid is high. In this case, it may be as important as aluminum.

References


Table 2. Reference Values for Manganese In Blood

<table>
<thead>
<tr>
<th>Method</th>
<th>n</th>
<th>Reference values, nmol/L</th>
<th>Ref. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>25</td>
<td>10.7 ± 2.2</td>
<td>79</td>
</tr>
<tr>
<td>NAA</td>
<td>2</td>
<td>10 ± 2.5</td>
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<tr>
<td>NAA</td>
<td>95</td>
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<tr>
<td>AAS</td>
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<td>EAAS</td>
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<td>EAAS</td>
<td>145 ± 318</td>
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<td>EAAS</td>
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<td>43</td>
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<tr>
<td>EAAS</td>
<td>18 ± 3.4</td>
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<td>EAAS</td>
<td>91 ± 364</td>
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<tr>
<td>EAAS</td>
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<td>EAAS</td>
<td>40 ± 11</td>
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<td>EAAS</td>
<td>37</td>
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<td>6.5 ± 17.4</td>
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<td>EAAS</td>
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<td>Photometry</td>
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<td>Plasma</td>
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<td>10.5 ± 3.3</td>
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<tr>
<td>EAAS</td>
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<tr>
<td>EAAS</td>
<td>10</td>
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<td>Whole blood</td>
<td>18</td>
<td>153 ± 50</td>
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<td>EAAS</td>
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<td>Erythrocytes</td>
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<td>EAAS</td>
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<tr>
<td>Erythrocytes</td>
<td>303</td>
<td>94 ± 9</td>
<td>115</td>
</tr>
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*Mean ± SD, or range. 6Men. Women. 7Children up to 14 years. 8Boys and girls, 2 months to 15 years old. 9Adults.

Table 3. Reference Values for Manganese In Urine, Cerebrospinal Fluid, Hair, and Liver Tissue

<table>
<thead>
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<th>Method</th>
<th>n</th>
<th>Reference values, nmol/L</th>
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<tr>
<td>Urine</td>
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<td>NAA</td>
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<tr>
<td>AAS</td>
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<td>AAS</td>
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<tr>
<td>AAS</td>
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<tr>
<td>AAS</td>
<td>339 ± 26</td>
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</tr>
<tr>
<td>EAAS</td>
<td>220 ± 16</td>
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<tr>
<td>EAAS</td>
<td>181 ± 327</td>
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<tr>
<td>EAAS</td>
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<tr>
<td>EAAS</td>
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<td>69</td>
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<tr>
<td>Cerebrospinal fluid</td>
<td>3.4 ± 2.4</td>
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<tr>
<td>Hair</td>
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<tr>
<td>EAAS</td>
<td>12 ± 2</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td>NAA</td>
<td>213 ± 64</td>
<td>114</td>
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</tr>
<tr>
<td>NAA</td>
<td>231 ± 7</td>
<td>25</td>
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<tr>
<td>AAS</td>
<td>5 ± 39</td>
<td>116</td>
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</tr>
<tr>
<td>AAS</td>
<td>3.3 ± 2.1</td>
<td>89</td>
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<tr>
<td>AAS</td>
<td>5 ± 0.9e</td>
<td>53</td>
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</tr>
<tr>
<td>AAS</td>
<td>4.3 ± 0.54</td>
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<tr>
<td>AAS</td>
<td>43.6 ± 21.8</td>
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<tr>
<td>AAS</td>
<td>13.47 ± 1.1</td>
<td>74</td>
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<tr>
<td>Liver tissue</td>
<td>30</td>
<td>21.8 ± 6.5</td>
<td>101</td>
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<tr>
<td>EAAS</td>
<td>35 ± 24</td>
<td>72</td>
<td></td>
</tr>
</tbody>
</table>

*Mean ± SD, or range. 6Birth. 7Brown, unexposed. 8White. 9Brown.


