Determination of Cu, Fe, Mn, and Zn in blood fractions by SEC-HPLC-ICP-AES coupling

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The binding of Cu, Fe, Mn, and Zn to proteins in blood and in blood fractions was investigated, since their interactions in free radical metabolism in humans is of great interest. An HPLC-ICP-AES technique was developed allowing adequate separation of metalloproteins and of inorganic and organic metal species. For the separation of metalloproteins in erythrocytes and blood plasma a Merck Superformance Fractogel EMD BioSEC 650 (S) column was used. Size exclusion chromatography (SEC)-HPLC was hyphenated to ICP-AES both on-line and off-line for the detection of trace elements in the fractions resulting from HPLC separations. HPLC parameters, pH, temperature, flow rate and salt concentration were optimized for the protein separation and the optimal conditions were applied for the hyphenation to the ICP-AES detector. The separation column was calibrated with five standard proteins. For the element determination by ICP-AES a line selection with respect to the sensitivity was performed. Three different methods were used for the determination of trace elements in blood: direct determinations, on-line and off-line SEC-HPLC-ICP-AES measurements. For the optimizing experiments blood samples of one female subject were used. The direct determination by ICP-AES of the elements was performed in blood and blood fractions of ten different subjects to obtain the average concentration ranges. From the results the identification of the protein Cu/Zn superoxide dismutase in erythrocytes was possible. The LOD were 0.03 μg mL⁻¹ for Cu, 0.026 μg mL⁻¹ for Fe, 0.8 ng mL⁻¹ for Mn, and 0.09 μg mL⁻¹ for Zn in a synthetic blood matrix.

Introduction

Under normal physiological conditions cells of aerobic organisms generate potentially deleterious, reactive oxygen metabolites. A permanent generation of reactive oxygen radicals is system immanent. An imbalance of pro-oxidants and antioxidants in blood and blood cells is very dangerous and may cause damage.1–3

The phenomenon of oxygen toxicity is inherent in the atomic structure of oxygen. Molecular oxygen is a biradical that upon single electron addition is transformed into the partially reduced molecules O_{2}⁻, H₂O₂, OH⁻, which may generate an array of additional reactive oxygen metabolites by further reactions, and cause extensive oxidative damage to biological macromolecules.4–6

Free radicals are formed in vivo as organic and inorganic superoxides, peroxides, hydroxyl radicals or lipid peroxides. Breakdown of these species is controlled by antioxidant reagents. Increases in concentrations of oxygen and pro-oxidants in blood cause an increased production of reactive oxygen radicals leading to the disturbance of the equilibrium of the oxygen metabolism and finally to oxidative stress. DNA strands can break, and cell membranes and lipids can be oxidized leading to complete cell destruction. Decreased concentrations or defects of the antioxidant reagents result in oxidative stress as well.7–11

Proteins working as antioxidative reagents are involved in chronic diseases like diabetes mellitus, arteriosclerosis or cancers. They can influence the resulting after-effects of radical reactions in the cells. In relation to the oxidative stress defense system the concentrations of trace elements vary due to the changes in human metabolism. This may be regarded as an indication for disorders.12–15

Antioxidant enzymes regulating the metabolism of oxygen free radicals, superoxide dismutases (SODs), and glutathion peroxidase (GPX), are known to contain the essential trace elements Cu, Zn, Mn, and Se. Element species of Fe and Cu or other trace metals like Cr may also affect the balance of pro-oxidants and antioxidants in blood.16,17

Since blood fractions (blood plasma, erythrocytes, lymphocytes) contain a large variety of substances ranging from high molecular mass proteins to small metallic ions, there is a need for effective separation methods. In erythrocytes, hemoglobin, which represents up to 94% of the total amount of proteins, complicates the identification of other proteins, such as albumin in blood plasma. A separation of the proteins according to size is useful. The size is approximately directly proportional to the mass of the particles.

Merck developed a solid phase tentacle system for the size exclusion chromatography (SEC) technique, which enables the preservation of the native form of the proteins in the separation systems. The column fill consists basically of a silica gel matrix carrying different polymeric chains with active ends.18–20

SEC enables a separation according to size and therefore to mass of the substances of interest thus giving the first important information as a result of the chromatographic procedure itself. Combination with other techniques enables an even more effective separation.21,22 Other techniques such as ion, affinity, and reversed-phase chromatography can lead to protein denaturation.

The identification of the metal–protein complexes of interest is not possible according to size only, because blood fractions contain many proteins similar in size. Metals bound to proteins can be determined by element specific analytical methods and thereby the proteins can be identified.

Atomic spectroscopic methods are frequently used for the specific determination of the mostly very low element concentrations in biological samples. In most of the matrices concerned inductively coupled plasma-atomic emission spectrometry (ICP-AES) and electrothermal atomic absorption spectrometry
(ETAAS) are methods which enable a precise element specific determination of the elements in question after a high performance liquid chromatographic separation (HPLC) of the proteins.\textsuperscript{23–32}

In this work ICP-AES was used for determination of the metals of interest. Three different methods were used: direct ICP-AES determination of the metals in whole blood and blood fractions after wet digestion; off-line coupling of SEC-HPLC to ICP-AES for the evaluation of the metal concentrations after the protein separation; and finally on-line hyphenation of SEC-HPLC and ICP-AES.

The aim of this work was to optimize the hyphenation of ICP-AES and HPLC for isolation and identification of Cu, Fe, Mn, and Zn particularly as metal–protein complexes in blood plasma and erythrocytes. The optimization of the coupled system was performed using the iron–hemoglobin complex.

**Experimental**

**Chemicals**

Ficoll-Paque was provided by Pharmacia Biotech (Uppsala, Sweden); Heparin Immuno (5,000 I.E. mL\textsuperscript{-1}) by Immuno (Vienna, Austria), nitric acid suprapur, hydrochloric acid suprapur, hydrogen peroxide (30\% H\textsubscript{2}O\textsubscript{2}) pro Analysi (p.A.), NaH\textsubscript{2}PO\textsubscript{4} p.A., NaCl p.A. and single element standards p.A. (Cu, Fe, Mn, and Zn) by Merck (Darmstadt, Germany).

**Sample preparation and separation of proteins**

All experiments for the optimization of the methods were performed using blood samples of one female subject (29 years old, 170 cm tall, weight 55 kg). In whole blood, erythrocytes, blood plasma, and in the collected fractions after SEC-HPLC separation the Fe, Cu, Zn, and Mn concentrations were determined by ICP-AES. In addition these elements were determined in whole blood of ten different subjects for comparison.

Blood samples were collected by Venflon to avoid metal contamination. The first 3 mL were discarded. Plasma and erythrocytes were separated by centrifugation (1200 \(g\) for ten minutes). After washing the cells with a physiological sodium chloride solution they were stored at \(-20^\circ\text{C}\) for further use.

For HPLC analysis the erythrocytes were thawed, homogenized by a vortex mixer, centrifuged at 1200 \(g\) for ten minutes and filtered by a Micro Prep-Disp Filter [Bio-Rad (Richmond, CA, USA)] PTFE membrane, pore size 5 \(\mu\text{m}\) prior to injection. The plasma was thawed, homogenized and centrifuged as described for erythrocytes.

Afterwards the proteins of blood plasma and erythrocytes were separated by SEC-HPLC. The SEC-HPLC procedure was carried out using an isocratic system from Hewlett-Packard (Avondale, PA, USA) (HP 1090 Liquid Chromatograph, PC HP 9000, Chem Station HP 9153C, printer HP Think Jet). A 250 \(\mu\text{L}\) injection loop was used (Hamilton, Reno, NV, USA, 250 \(\mu\text{L}\) Microliter Syringe). The chromatographic column used was Merck Superperformance. Fractogel EMD BioSEC 650 (S), 600 \(\times\) 16 mm id, particle size 20–40 \(\mu\text{m}\), produced by Merck, Darmstadt, Germany. The column was thermostatted by a water cooling system.

For the separation of the proteins an optimization of the parameters \(\text{pH}(6.5–7.2),\) temperature (25, 30 and 35 \(^\circ\text{C}\)), flow rate (0.8–1.2 mL min\textsuperscript{-1}), and ion strength in the mobile phase (0.1–0.5 M NaCl) was performed. The mobile phase eluent solution (0.02 M NaH\textsubscript{2}PO\textsubscript{4} + 0.3 M NaCl, at \(\text{pH} = 6.8\) and \(r = 30^\circ\text{C}\) was used, injection volumes varied between 100 and 250 \(\mu\text{L}\). The proteins were detected by a UV/VIS diode array detector (DAD). The pressure on column was 38–42 bar (1 bar \(=10^\text{5}\) Pa). The optimized column conditions were used both for the off-line and the on-line coupling with ICP-AES.

The calibration of the SEC column was performed with five standard proteins under optimized conditions (30 \(^\circ\text{C}\); \(\text{pH} = 6.8\); 0.3 M NaCl, flow rate 1 mL min\textsuperscript{-1}). Myoglobin (18 kDa), ovalbumin (45 kDa), BSA (67 kDa), conalbumin (78 kDa), and \(\gamma\)-globulin (150 kDa) were injected separately (0.5 mg mL\textsuperscript{-1} of each protein in 100 \(\mu\text{L}\)) and as a mixture (0.1 mg mL\textsuperscript{-1} of each protein in 100 \(\mu\text{L}\)).

For the identification of the Cu/Zn–SOD, GPX, and hemoglobin, single standards and mixtures were injected (250 \(\mu\text{L}\)). The samples used are listed in Table 1.

After separation by HPLC the amount of Cu, Fe, Mn, and Zn was determined by ICP-AES.

**ICP-AES determination**

For the element detection and determination an ARL 3520 ICP spectrometer (ARL, Ecublens, Switzerland) was used. All instrument parameters are listed in Table 2.

(a) **Line selection and determination of the limits of detection (LOD).** Prior to analysis line selections were performed. Two spectral lines for Cu (324.694 and 327.396 nm), three spectral lines for Fe (259.940, 271.440 and 258.588 nm), two spectral lines for Mn (257.61 nm), and one spectral line for Zn (231.856 nm) were investigated.

For the direct determination of the elements in whole blood, erythrocytes and blood plasma samples, scans using a synthetic blood matrix (containing 122.7 \(\mu\text{g}\) mL\textsuperscript{-1} of Mg, 371.6 \(\mu\text{g}\) mL\textsuperscript{-1} of P, 489.9 \(\mu\text{g}\) mL\textsuperscript{-1} of Na, 406.5 \(\mu\text{g}\) mL\textsuperscript{-1} of Ca, 3503.7 \(\mu\text{g}\) mL\textsuperscript{-1} of K, 547.4 \(\mu\text{g}\) mL\textsuperscript{-1} of Fe, and 2404.7 \(\mu\text{g}\) mL\textsuperscript{-1} of S = ‘matrix’) and single element standards (10 \(\mu\text{g}\) mL\textsuperscript{-1} each) were performed. For the line selection the following solutions were scanned: (a) matrix; (b) matrix + 10 \(\mu\text{g}\) element standard mL\textsuperscript{-1}; (c) 5 \(\mu\text{g}\) element standard mL\textsuperscript{-1} in 1.4 M HNO\textsubscript{3}; and (d) 1.4 M HNO\textsubscript{3}.

For the line selection for the determination of the elements after the SEC-HPLC separation blood plasma and erythrocytes were diluted with the HPLC eluent solution. The resulting solutions were scanned. Scans using (a) eluent; (b) 1 mL blood plasma in eluent (1 + 24); (c) 1 mL erythrocytes hemolyzate (1 + 9 in H\textsubscript{2}O) in eluent (1 + 24); (d) the solution (b) + 5 \(\mu\text{g}\) mL\textsuperscript{-1} of each element standard.

**Table 1** Identification of the proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>Single proteins</th>
<th>Mixture 1</th>
<th>Mixture 2</th>
<th>Mixture 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc./ mg mL\textsuperscript{-1}</td>
<td>Volume/ (\mu\text{L})</td>
<td>Conc./ mg mL\textsuperscript{-1}</td>
<td>Volume/ (\mu\text{L})</td>
</tr>
<tr>
<td>Erythrocytes</td>
<td>—*</td>
<td>200</td>
<td>—*</td>
<td>200</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>5</td>
<td>200</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>SOD</td>
<td>5</td>
<td>200</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>GPX</td>
<td>1</td>
<td>100</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Erythrocyte hemolyzate diluted 1 + 9 with doubly distilled water.
of Cu, Mn, and Zn; (e) the solution (c) + 5 μg mL⁻¹ of Cu, Mn, and Zn; (f) 5 μg element standard mL⁻¹ in 1.4 M HNO₃; and (g) 1.4 M HNO₃ were produced.

The limits of detection (LOD) were calculated according to Boumans³⁰ using 3σ. LOD were determined in pure element standards and in the synthetic matrix.

(b) Direct determination by ICP-AES. For the direct determination of the elements in whole blood, erythrocytes, and blood plasma the samples were digested in glass vessels with a mixture of nitric acid and hydrogen peroxide. For each series of digestions a reagent blank was prepared.

The system was calibrated using mixed aqueous standards. The concentration ranges for the elements were: 0.5 to 5 μg mL⁻¹ of Cu, Mn and Zn for all samples, and 0.5 to 10 μg mL⁻¹ of Fe for blood plasma samples, and 10 to 100 μg mL⁻¹ for 1 + 9 dilutions of erythrocytes and whole blood samples, respectively.

(c) Off-line coupling of SEC-HPLC-ICP-AES. After the SEC-HPLC separation of proteins, fractions of 2 mL, volume were collected and analyzed by ICP-AES for Cu, Fe, Mn, and Zn. The fractions were collected by the fraction collector L 5200 Merck Hitachi. Calibration for ICP-AES was performed using adequate concentrations of the elements as described in (b) dissolved in the eluent solution.

(d) On-line coupling of SEC-HPLC-ICP-AES. The determination of the Fe–hemoglobin complex in erythrocytes was performed using SEC-HPLC-ICP-AES coupling on-line. As a separation system a Fractogel EMD BioSEC 650 (S) column, a Merck Hitachi L-6200A Intelligent Pump, and a PTFE tube (1.5 id, 1 m long) were applied. For these experiments an erythrocyte hemolyzate diluted with doubly distilled water (1 + 9) was used.

Results and discussion

In order to understand the mechanisms and processes by which trace elements are absorbed, transported and incorporated into proteins it is important to monitor most of the essential trace elements because of their various interactions.

The development of an analytical method for studying the protein binding and speciation of metals needs several prerequisites to be fulfilled: contamination has to be avoided, the separation system has to be optimized, interferences during spectroscopic detection have to be eliminated and the sensitivity for the metals of interest has to be optimized in the matrix given.

For reducing the risk of contamination several precautions were applied. Glass-ware was purified by steaming with acid. The tendency for adsorption of metal species on glass was tested using standard solutions, e.g., 0.5 to 5 ng mL⁻¹ for manganese, since the lowest concentrations were expected for Mn. The recovery was 98 to 101%.

The contamination caused by the reagents was corrected by a blank for iron, since the iron contamination of the prepared blank solutions was in the range of 0.5 μg mL⁻¹. For copper, zinc, and manganese the contamination was under the LOD, so a blank correction was not applicable. These data refer to measurements of pure aqueous standards. (see Table 2)

The efficiency of the SEC-HPLC protein separation is influenced by the parameters pH, temperature, salt concentration, and flow rate. For both protein separation and coupling with ICP-AES the HPLC parameters had to be optimized with respect to the physical properties of the native proteins. For the separation of erythrocyte and blood plasma proteins the conditions described in the experimental section were selected.

A pH of 6.8 was used, presenting a value in-between the known isoelectric values of the proteins of interest (hemoglobin 6.8, albumin 4.9, SOD 4.7–4.9, γ-globulin 5.8–7.3, co-eruloplasmin 4.4, catalase 5.8–6.5, cytochrome-c oxidase 10.6). Physiological processes take place under neutral conditions. Large amounts of erythrocyte proteins cannot be separated by the SEC column because of the similar size of the proteins and because of the overload of the column caused by hemoglobin. For blood plasma proteins the SEC method was not satisfactory either, but the albumin fraction could be separated from the other moieties. As a compromise a pH of 6.8 was chosen for all experiments.

No significant changes in the resolution could be observed for temperatures between 25 °C and 35 °C. The results are shown in Fig. 1. Two replicates of each temperature were compared. All following experiments were performed at 30 °C, because of the best reproducibility of the retention time at this temperature.

The concentration of NaCl in the eluent buffer solution was varied between 0.1 M and 0.5 M. The optimum concentration

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Table 2 Operating conditions for ICP-AES and line selection

<table>
<thead>
<tr>
<th>Element</th>
<th>Wavelength/nm</th>
<th>LOD in matrix/μg mL⁻¹</th>
<th>LOD in standards/μg mL⁻¹</th>
<th>Background correction/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu/1</td>
<td>324.694</td>
<td>0.03</td>
<td>0.01</td>
<td>±0.04</td>
</tr>
<tr>
<td>Cu/2</td>
<td>327.396</td>
<td>0.05</td>
<td>0.03</td>
<td>±0.04</td>
</tr>
<tr>
<td>Fe/1</td>
<td>259.940</td>
<td>0.026</td>
<td>0.024</td>
<td>±0.04</td>
</tr>
<tr>
<td>Fe/2</td>
<td>271.440</td>
<td>0.384</td>
<td>0.358</td>
<td>±0.04</td>
</tr>
<tr>
<td>Fe/3</td>
<td>258.588</td>
<td>0.072</td>
<td>0.068</td>
<td>±0.05</td>
</tr>
<tr>
<td>Mn/1</td>
<td>257.610</td>
<td>0.0008</td>
<td>0.0003</td>
<td>±0.05</td>
</tr>
<tr>
<td>Zn/1</td>
<td>213.856</td>
<td>0.086</td>
<td>0.044</td>
<td>±0.04</td>
</tr>
</tbody>
</table>

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Fig. 1 Optimization of the temperature, two replicates at each temperature: ..., 25 °C; — 30 °C; —— 35 °C.
was found to be 0.3 M NaCl leading to a satisfactory peak resolution for the proteins in blood plasma and in erythrocytes.

Despite the long separation time a flow rate of 1 mL min\(^{-1}\) was chosen to enable hyphenation of the separation column to the ICP-AES. This flow rate is recommended by Merck as optimal for the separation column. This is in good agreement with the experiments performed using 0.8 mL min\(^{-1}\) up to 1.2 mL min\(^{-1}\) in 0.1 mL min\(^{-1}\) steps. For 1.0 mL min\(^{-1}\) the highest signal was obtained.

After optimization of the HPLC system for the separation of the proteins, the column was calibrated with five proteins of different size. Fig. 2 shows the chromatograms of the single standards and of their mixture. The retention times of the single protein standards are in very good agreement with the peaks obtained by the separation of their mixture. The resulting calibration curve corresponds with the figure published in ref. 33 (selectivity curve).

For the detection of the proteins only one wavelength is required, because for the given problem there is a need for the detection of the native proteins only. Two dimensional plots of erythrocytes and blood plasma chromatograms at 230 nm and 405 nm are presented in Fig. 3. The chromatograms of erythrocytes show a heme peak at 405 nm. In blood plasma only the signals of the amino acids could be observed (see dotted line at 230 nm). These chromatograms were compared to the profiles obtained by ICP-AES measurements (see below).

The atomic spectrometric methods had to be optimized for the metal detection in the given matrix, as described below.

ICP-AES determination

(a) Line selection and determination of the limits of detection (LOD). Line selection was performed to choose the most sensitive analytical wavelength for the elements of interest. Spectral lines for analysis were selected with respect to two important criteria. The most sensitive line without spectral interferences in the sample matrix was used for analysis. For the given matrix three interference free lines for Fe, two for Cu, one for Zn, and one for Mn, respectively, were tested for their LOD in the matrix and in the eluent solution. In blood plasma and erythrocyte hemolyzate both diluted with eluent [solutions (b) and (c)] and the Fe concentration was high enough to be registered in the scans. For the other elements Cu and Zn and in particular for Mn single element standards (5 μg mL\(^{-1}\) final concentration) were added [solutions (d) and (e)], because otherwise the signals obtained were too small for the line selection routine.

For the determination of Cu the line at 324.694 nm, for Fe the line at 259.940 nm, for Mn the line at 257.610 nm, and for Zn the line at 213.856 nm were selected, because of their lowest LOD in the matrix given. (Table 2)

(b) Direct determination by ICP-AES. The concentration of the elements in question were determined by direct measurement in whole blood, erythrocytes, and blood plasma samples of ten subjects (\(n = 10\)). The results are listed in Table 3.

The second column of Table 3 gives the range of the measured concentration in digested samples (whole blood, erythrocytes, blood plasma) of 10 different individuals and the RSD range for three replicates. The RSD range from 0.1 to 9.8%. The highest RSD is caused by measurement of Cu in erythrocytes since the Cu content is very low in this case (approximately 0.3 μg mL\(^{-1}\) in a complex matrix).

The fourth column gives the mean concentration found for all persons tested (10) and the biological standard deviation (the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Measured conc./μg mL(^{-1})</th>
<th>RSD (%)</th>
<th>Conc. ± SD/μg mL(^{-1})</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0.73–1.02</td>
<td>2.0–5.1</td>
<td>0.82 ± 0.12</td>
<td>14.6</td>
</tr>
<tr>
<td>Fe</td>
<td>183.49–267.01</td>
<td>0.1–1.2</td>
<td>223.18 ± 24.14</td>
<td>10.8</td>
</tr>
<tr>
<td>Mn</td>
<td>0.0039–0.0095</td>
<td>0.9–5.9</td>
<td>0.0063 ± 0.0016</td>
<td>25.4</td>
</tr>
<tr>
<td>Zn</td>
<td>4.28–6.44</td>
<td>0.4–3.1</td>
<td>5.34 ± 0.7</td>
<td>12.7</td>
</tr>
<tr>
<td><strong>Erythrocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0.34–0.62</td>
<td>1.2–9.8</td>
<td>0.45 ± 0.13</td>
<td>28.9</td>
</tr>
<tr>
<td>Fe</td>
<td>431.81–527.07</td>
<td>0.9–1.4</td>
<td>484.04 ± 34.08</td>
<td>7.0</td>
</tr>
<tr>
<td>Mn</td>
<td>0.0086–0.0177</td>
<td>1.9–5.8</td>
<td>0.0134 ± 0.0029</td>
<td>21.3</td>
</tr>
<tr>
<td>Zn</td>
<td>9.42–12.12</td>
<td>1.2–3.7</td>
<td>10.5 ± 1.1</td>
<td>10.3</td>
</tr>
<tr>
<td><strong>Blood plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0.84–1.45</td>
<td>5.7–8.1</td>
<td>1.07 ± 0.21</td>
<td>19.6</td>
</tr>
<tr>
<td>Fe</td>
<td>2.47–13.10</td>
<td>0.1–1.3</td>
<td>5.64 ± 3.56</td>
<td>63.0</td>
</tr>
<tr>
<td>Mn</td>
<td>0.0004–0.0027</td>
<td>0.7–5.0</td>
<td>0.0012 ± 0.0006</td>
<td>54.8</td>
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<tr>
<td>Zn</td>
<td>0.46–1.67</td>
<td>0.1–4.3</td>
<td>0.96 ± 0.36</td>
<td>37.5</td>
</tr>
</tbody>
</table>

* RSD gives the variation of the concentration according to biological differences, \(n = 10\).
fifth column RSD%). It is evident that the biological range is rather high for the element investigated.

It has to be stressed that the erythrocytes represent approximately 45% of the whole blood (hematocrit). In Table 3 the concentrations of elements in erythrocytes are not calculated according to their number per mL of whole blood, but are given for 1 mL of concentrate obtained after centrifugation.

The element concentrations in whole blood, erythrocytes, and in blood plasma of the female subject used for the optimizing experiments were determined direct by ICP-AES as well. The measured concentration of Cu was 2.0 µg mL⁻¹ in whole blood, 1.0 µg mL⁻¹ in erythrocytes and 3.3 µg mL⁻¹ in blood plasma. The measured concentration of Fe was 213.7 µg mL⁻¹ in whole blood, 459.9 µg mL⁻¹ in erythrocytes and 6.2 µg mL⁻¹ in blood plasma. The measured concentration of Mn was 8.8 ng mL⁻¹ in whole blood, 17.0 ng mL⁻¹ in erythrocytes and 1.0 ng mL⁻¹ in blood plasma. The measured concentration of Zn was 6.1 µg mL⁻¹ in whole blood, 9.5 µg mL⁻¹ in erythrocytes and 1.3 µg mL⁻¹ in blood plasma.

(c) Off-line coupling of SEC-HPLC-ICP-AES. The collected 2 mL fractions after the SEC-HPLC separation of the proteins were analyzed for Cu, Fe, Mn, and Zn by ICP-AES.

Fig. 4a shows the determination of Fe in erythrocytes and in blood plasma. The determination of Fe in erythrocytes was easily performed in the collected fractions, because of the high Fe concentration (upper diagram). The main signal is caused by the Fe–hemoglobin complex. For the determination of Fe in blood plasma it was necessary to collect the fractions of three column separations to register sufficient intensities.

Fig. 4b shows the signal intensities of Cu, Mn and Zn in erythrocytes after collection of the fractions of three column separations to register sufficient intensities. The zinc peak is very small and is situated approximately at the same retention time as Cu. We conclude that the Zn signal belongs to Cu/Zn–SOD, which is also proved by the results of the protein separation and UV detection of the SOD standard on the same column (see also Fig. 6).

Fig. 4c deals with Cu and Mn in blood plasma. As mentioned for Fig. 4b due to the low amounts of the elements present in blood plasma a ten-fold collection of the fractions was necessary to get measurable intensities. Cu in blood plasma seems to correspond to Cu bound to albumin, (the same retention time as the main signal in the chromatogram of blood plasma) since it is the main protein in this blood fraction. The second Cu peak like the Mn peaks could not be correlated to proteins investigated in plasma. We speculate that these element signals are caused by free metal ions present (see also Fig. 6).

The profiles obtained by ICP-AES measurements (Fig. 4a–c) were compared to the two dimensional plots recorded at 230 nm (Fig. 3). (The comparison is shown in Fig. 6.)

(d) On-line coupling of SEC-HPLC-ICP-AES. In order to identify and to quantify the metal–protein complexes, the HPLC separation system was coupled directly to the ICP-AES sample introduction device. The concentrations of Cu, Mn, and Zn protein complexes in blood are too low for the optimization of the hyphenated HPLC-ICP-AES method described. Therefore the Fe–heme complex of hemoglobin in erythrocytes was chosen to prove the reliability of the method. Hemoglobin represents up to 94% of the total protein in erythrocytes. In blood plasma the Fe present is primarily bound to transferrin and albumin.

By coupling of the SEC-HPLC separation column and the ICP-AES detector the following chromatogram was obtained: Fe in erythrocytes at pH 6.8 in Fig. 5. The Fe intensity signal/V was plotted vs. separation time/min. Because of the relatively high separation volume of the SEC column (119 mL) it took 120 min to separate the proteins. Optimal protein separation was obtained using a sample flow rate of 1 mL min⁻¹, which is also a resonable flow rate for ICP-AES detection. The greatest challenge was to quantify the Fe intensity changing during the separation (peak) using a transient measuring mode. The Fe intensity was registered at time intervals of 0.5 s by the PC program ‘Nextview’ (BMC Puchheim-München, Germany). The statistical evaluation of the results was performed using the program ‘origin’ (MicroCal Software, Inc.).

To summarize (c) and (d), Fig. 6 describes the applicability of the method developed. In part a the results of the protein separations and in part b the results of the off-line and on-line
element detection are shown. The symbols mark (a) the retention times of the peak height maximum and (b) the fractions with the highest intensity.

Conclusion

The ICP-AES detection of Cu, Fe, Mn, and Zn in blood and blood fractions in combination with the separation of metalloproteins is a promising and suitable tool for the identification of proteins carrying these metals.

The hyphenation of SEC-HPLC and ICP-AES was successfully applied off-line for the identification of the enzyme Cu/Zn–SOD in the erythrocytes. On-line only the determination of iron bound to hemoglobin in erythrocytes was performed in order to test the coupling system. For SOD-proteins containing copper and zinc, or manganese, the system has to be modified. The most important step will be the preconcentration of the protein complexes by column switching using different separation systems (hydrophobic-interaction chromatography or ionexchange chromatography). A limitation of the method described above is the multifold dilution of the sample during HPLC elution, therefore the dimensions of the separation system should be optimized as well. The method worked out was used for identification of the metal–protein complexes, but not for quantification. Especially for the on-line hyphenated system the calibration has to be performed in another study. The transient signals obtained have to be analyzed statistically to allow calibration.

The calibration of the off-line coupling method could be performed easily. Iron could be detected in the 2 mL fractions after one separation run. The measured intensities were too low for manganese and not satisfying for copper and zinc after one separation run, since the proteins of interest are present in a very small concentration in blood and only 250 μL of the samples could be injected. A suitable separation system has to be found to overcome the collection of fractions of several separation runs.

For the ICP-AES sample introduction system different nebulizers should be tested because of the high salt concentrations of the eluent used and other performances of different types of nebulizers. The flow rate has to be optimized according to the separation system used and to the requirements of the argon-plasma as discharge unit.

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