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Quantification of trace elements in human serum fractions by liquid chromatography and inductively coupled plasma mass spectrometry

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On-line coupling of LC and ICP-MS has been used for fractionation and detection of species of Cu, Fe, I, Se and Zn in human serum. It has been shown that anion exchange chromatography provided better separation capability (both intra- and inter-element) than size-exclusion chromatography. The mobile phases for ion exchange chromatography consisted of Tris–HNO_3 buffer and ammonium salt (nitrate, acetate or formate). Formate was found to be the best mobile phase counter ion, enabling good chromatographic separation, and is acceptable for mass spectrometry too. The quantitative evaluation of element concentrations adhering to individual fractions was performed by the peak area normalization method. The repeatability of results ranged from 3 to 15% (depending on the element concentration level) and represented the main part of the result uncertainty. The accuracy of Cu and Zn fraction determinations was confirmed by comparison with the isotope dilution technique.

KEYWORDS: serum; trace elements; metalloproteins; liquid chromatography; inductively coupled plasma mass spectrometry

INTRODUCTION

Trace element analysis of human body fluids can be utilized for diagnostic purposes. However, knowledge of the total concentrations of trace elements in body fluids and tissues without differentiation of individual forms is of lower signification for understanding their roles in the body. Trace elements (especially elements included into this project) are normally bound to various proteins, peptides and other ligands. The separation of metalloproteins is frequently accomplished by high-performance liquid chromatography (HPLC). This technique is convenient since it can be easily hyphenated with an element analyser, which consequently serves as an element-specific detector. Size-exclusion chromatography (SEC) is the separation method most frequently used. From among the recently published works applying SEC, the speciation of Cu, Fe, Se and Zn in serum,[1] Pb in erythrocytes,[2] Cd, Cr, Se, Th and U in serum,[3] Cu, Fe, Mn and Zn in plasma and erythrocytes[4] and a study of complexation of Pt and Rh metallodrugs with plasma proteins[5] can be mentioned. The mobile phases used in these works are very similar. Solutions containing 0.01–0.1 mol/l Tris (hydroxymethyl) aminomethane–HCl buffer of pH = 6.8–8.4 have been used most often. Another simple method is ion-exchange chromatography (IEC), used, for example, for speciation analyses of Al,[6,7] Cu, Fe and Zn,[8] Mg[9] or Sb[10] in serum or blood.

All separations were done using a strong anion exchanger while the mobile phase was mostly ammonium acetate solution. Reversed-phase chromatography can be used too, especially for speciation of elements covalently bound to proteins such as Se[11] or I.[12] These chromatographic methods can be combined to achieve better separation efficiency. Two-step chromatographic separation comprising SEC and IEC was used for the determination of Al species in serum of dialysed patients.[13] Affinity chromotography can be useful for selective removal of certain high molecular species from

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serum samples, e.g. an immobilized heparin column can be used for retention of selenoprotein P[14,15] and a Cybacron blue column can serve for albumin depletion.[15]

Detection and quantification of elements bound to fractions of human blood or serum can be conveniently accomplished by the use of inductively coupled plasma mass spectrometry (ICP-MS), which is very sensitive (detection limits are frequently less than 1 ng/ml) and allows easy multielemental analysis.[16] However, application of on-line hyphenation of liquid chromatography (LC) and ICP-MS is limited by mobile phase composition. The ionic strength of the mobile phase as well as the proportion of organic solvents should be kept low because a high concentration of salts causes signal depression and blockage of cone orifices, and high concentration of organic matter produces instability of the argon plasma. Compromising mobile phase composition enforced by the specific demands of ICP-MS can lead to somewhat worse chromatographic resolution of separated species. Therefore the selection of the proper stationary phase is of primary importance. Another difficulty of ICP-MS application consists of the spectral interferences caused by polyatomic ions. These interferences can be removed by various types of collision cell, e.g. for the determination of V (disturbed by ClO particles)[17] and Se (disturbed by HBr particles).[18]

Up to now, less effort has been devoted to the metrological point of view, i.e. precision, accuracy and uncertainty of results of element species fractionation studies. Although this study covers some aspects of optimization of chromatographic separation (the choice of mobile phase for IEC separation of Cu, Fe, I, Se and Zn species), it is focused on the optimization of ICP-MS determination of trace elements in the individual chromatographic fractions of human serum (choice of isotopes free of interferences and choice of internal standards) and validation of the quantification step. In this part of the study little stress is put on identification of species of analysed elements. However, some of them are well known and could be easily recognized (transferrin, ceruloplasmin, albumin, selenoprotein, GPX, etc.). As the method is intended for rapid screening of trace element distributions among individual species fractions, it utilizes a single separation step (SEC or IEC) and the simplest quantification procedure (normalization of peak areas). The uncertainties of quantification are estimated. In the case of Cu and Zn, the accuracy of the method has been proved by comparison with the isotope dilution method (ID).

MATERIALS AND METHODS

Instrumentation

All ICP-MS measurements were carried out using an ICP mass spectrometer Elan 6000 (Perkin Elmer, Norwalk, CT, USA) equipped with a Meinhard nebulizer, cyclonic glass spray chamber and a Gilson 212 peristaltic pump for sample aspiration. pH values of buffer solutions were measured using a pH 03 instrument (Labio, Prague, Czech Republic). The HPLC apparatus consisted of an anion-exchanger column Mono-Q HR 5/5 (dimensions 5 × 50 mm, Amersham Pharmacia Biotech, Uppsala, Sweden), Varian Inert 9012 high pressure pump (Varian, Walnut Creek, CA, USA) and a Rheodyne 9125 injector equipped with a 100 µl PEEK sample loop. A Superdex 200 HR 10/30 column (dimensions 10 × 300 mm, Amersham Pharmacia Biotech; optimal fractionation range 10–600 kDa) was used for SEC analyses. PEEK or PTFE capillaries (internal diameter 0.25 mm) connected all parts of the apparatus.

Standards and reagents

Reagents serving for the preparation of the mobile phases were: tris (hydroxymethyl) aminomethane (Tris), ammonium acetate of Micro-Select and purissimum grade, respectively (both Fluka, Neu-Ulm, Germany), ammonium nitrate (analytical grade, Lachema, Brno, Czech Republic) and ammonium formate (Sigma, St Louis, MO, USA). Stock solutions ($c = 2$ mol/l) of all these compounds were purified before use by passing through a column packed with chelating resin Chelex 100 (Merck, Darmstadt, Germany) in NH_4^+ form. Nitric acid (65%) and ammonia solution (25%), used to adjust the pH solutions, was of Suprapur® grade (Merck). Copper, indium, iron, rhodium, selenium, tellurium, yttrium and zinc stock solutions ($\rho = 1\,000$ mg/l) were obtained from Merck too. Stock solution of iodate (equivalent iodine concentration $\rho = 1000$ mg/l) was prepared by dissolving potassium iodate (purissimum grade, Merck). These solutions served for the preparation of calibration solution or solutions of internal standards. Stock solution serving for preparation of serum matrix-matched calibration solution contained 8 g NaCl, 0.4 g KCl, 0.6 g $Ca(NO_3)_2 \bullet 4H_2O$, 6.6 g cysteine hydrochloride monohydrate, 0.07 g KBr and deionized water up to 100 ml. All reagents were of Suprapur grade and were obtained from Merck, except for cysteine hydrochloride monohydrate, which was supplied by Sigma and was of SigmaUltra grade. Triton X-100 (Aldrich, Milwaukee, WI, USA) was used for serum stabilization.

Oak Ridge National Laboratory (Oak Ridge, TN, USA) ^{65}Cu-enriched metal copper (0.30% ^{63}Cu and 99.70% ^{65}Cu) and ^{68}Zn-enriched zinc oxide (0.12% ^{64}Zn, 0.11% ^{66}Zn, 0.05% ^{67}Zn, 99.71% ^{68}Zn and 0.01% ^{70}Zn) were used for preparation of isotope-enriched solutions. Stock solutions containing approximately 100 µg/g (expanded uncertainty 0.2 µg/g) of the element were prepared by dissolution of 10.00 mg of ^{65}Cu-enriched metal copper and 12.36 mg of ^{68}Zn-enriched zinc oxide, respectively, in diluted nitric acid and making up to 100 g with deionized water. Precise weighing of isotope-enriched substances was performed in the Laboratory of Mass Measurements of the Czech Metrological Institute, Prague. A working isotope-enriched solution containing 150.0 ± 0.5 ng/ml Cu and 25.0 ± 0.1 ng/ml Zn was prepared daily by two-step dilution of both stock solutions. Distilled deionized water (Milli-Q, Millipore, Bedford, MA, USA) was used for preparation of all solutions.

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The samples

Seven healthy volunteers (three males and four females, age 23–54 years) donated blood for our study. They all gave their informed consent prior to entering the study. The blood was obtained by venepuncture using Vacutainer tubes (Becton Dickinson Vacutainer Systems, UK). After coagulation of blood and centrifugation (3000 rpm, 10 min, 4 °C) serum was separated and stored in polyethylene tubes decontaminated by washing with nitric acid solution and deionized water. The samples were held at 4 °C and analysed as soon as possible (within several hours after blood sampling).

Procedures

Chromatographic analysis

In the case of IEC separation mobile phase A was 0.05 mol/l Tris–HNO_3 buffer solution (pH = 7.4) while mobile phase B was a mixture containing 0.05 mol/l of Tris and 1 mol/l of ammonium salt (one of: nitrate, acetate or formate), the pH value of which was adjusted to 7.4 by addition of either nitric acid or ammonia solution. Before each chromatographic run the column was conditioned by 5 ml of mixture A + B solutions (1 : 1) and then washed with 5 ml of A solution. The total duration of one chromatographic run was 60 min; 100% A in the mobile phase was kept for 5 min after the sample injection (100 µl). Between 6 and 50 min the portion of mobile phase B was uniformly increased from 0 to 50%. The total mobile phase flow was 0.5 ml/min. In the case of SEC separation only mobile phase A was used; the flow rate was 0.5 ml/min as well. The temperature of the column was in both cases (IEC and SEC) was maintained at 20 ± 1 °C. The column effluent was joined together with a T piece with a flow of mixed solution of internal standards (In, Rh, Te and Y, all 50 ng/ml) and isotope-enriched standards ^{65}Cu (150 ng/ml) and ^{68}Zn (25 ng/ml), flow rate 0.5 ml/min. At 55 min, when all compounds had eluted, this solution was replaced by a solution of 200 ng/ml Cu and 100 ng/ml Zn, both of natural isotopic composition. The measurements performed during the last 5 min served for mass bias correction, which is necessary for isotope dilution quantification. The mixed flow was delivered by peristaltic pump to the nebulizer of ICP-MS. The following nuclides were measured: ^{57}Fe, ^{63}Cu, ^{65}Cu, ^{66}Zn, ^{68}Zn, ^{82}Se and ^{127}I (analytes) and ^{89}Y, ^{103}Rh, ^{115}In, and ^{130}Te (internal standards); details of ICP-MS measurement are summarized in Table 1. The choice of proper internal standard is discussed below. In order to evaluate the chromatographic behaviour of interferents, the ^{37}Cl and ^{79}Br nuclides as well as $^{32}S^{16}O$ particles were observed.

The ICP-MS record of chromatogram consisted of 1000 steps each of 3.6 s. After smoothing of data using a Golay–Savitzky digital filter the obtained peaks of analytes were evaluated by two methods. The first method comprised the normalization of peak areas and comparison of the relative areas of individual peaks with the total amount of element in the injected portion of the sample. The increase in the ammonium salt concentration in the mobile phase during analysis caused strong time-dependent non-spectral interference. To compensate for this effect, the ratio of signals of analyte and internal standard was evaluated instead of the analyte signal only. In the case of Cu and Zn determination, the signals of ^{63}Cu and ^{66}Zn were used. The next quantification (Cu and Zn only) consisted of the ID technique. Isotope ratios ^{63}Cu : ^{65}Cu and ^{66}Zn : ^{68}Zn were used for result evaluations. Calculation of the total mass of analyte bound in a particular chromatographic fractions was based on summation of individual increments m_{inc} (ng),

Table 1. The main operating conditions of the ICP-MS Elan 6000

Parameter	Total content	SEC/ICP-MS[a]
RF power	1100 W	
Ion lens voltage	AutoLens mode optimized for maximum signal of Be, Co, In and Pb	
Sample Ar flow	0.7–0.8 l/min (optimized daily)	
Measurement mode	Peak hopping	
Measured isotopes	^{57}Fe, ^{65}Cu, ^{66}Zn, ^{77}Se, ^{103}Rh, ^{127}I	^{57}Fe, ^{63}Cu, ^{65}Cu, ^{66}Zn, ^{68}Zn, ^{82}Se, ^{103}Rh, ^{127}I, ^{130}Te (^{129}Xe, ^{137}Ba—isobaric overlaps correction)
Dwell time	50 ms	27 ms for Rh 30 ms for others
Sweeps/replicate	10	10
Acquisition time per one replicate	3.2 s	3.6 s
No. of replicates	10	1 000
Total acquisition time	32 s	3 600 s
Solution uptake	1.0 l/min	0.5 ml/min of column effluent +0.5 ml/min of internal standards and isotope-enriched standard solution

[a] Optimized procedure comprising selected isotopes only.

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representing mass of analyte per step of a chromatogram:

$$m_{inc} = \frac{m_{iso} \cdot A_{1S} - f \cdot m_{iso} \cdot A_{2S}}{f \cdot A_{2N} - A_{1N}} \cdot \frac{M_N}{M_S}$$
$$m_{iso} = \rho_{iso} \cdot F_{iso} \cdot t \quad (1)$$

where m_{iso} represents the mass of isotope-enriched element (ng) per chromatogram point, f is the measured isotope ratio of selected isotopes corrected for mass discrimination, M_N is the molar mass of the natural element, M_S is the molar mass of the isotope-enriched element, A_1 are abundances of the first element isotope in serum (index N) and in the isotope-enriched solution (index S), and A_2 are abundances of the second (reference) element isotope in serum (index N) and in the isotope-enriched solution (index S). ρ_{iso} represents concentration of analyte in the working isotope-enriched solution (ng/ml), F_{iso} is the flow rate of the working isotope-enriched solution (approximately 0.5 ml/min) and t is the step duration (0.06 min). The value of F_{iso} somewhat depends on the status of the peristaltic pump tubing and was re-determined before each analysis. For more details of the procedure and for uncertainty estimation see Mestek *et al*.[19]

Determination of total concentration of elements

Analyses were performed using the procedure, which was similar to a previously described one.[20,21] The paper[20] also describes the uncertainty estimation. The method comprises the 10-fold dilution of a sample, acidification by HNO_3 (final concentration 0.15 mol/l), an addition of Triton X 100 (final content 0.1%) and the internal standard (Rh final concentration 50 ng/ml), and ICP-MS analysis. The calibration standard (Cu and Zn, 200 ng/ml; Fe, 400 ng/ml; Se and I, 40 ng/ml; Rh, 50 ng/ml) and reagent blank were spiked (1 + 99) with a solution simulating a matrix of human serum. ^{57}Fe, ^{65}Cu, ^{66}Zn, ^{77}Se and ^{127}I (analytes) and ^{103}Rh (internal standard) nuclides were measured; for details see Table 1. Verification of the method was carried out using Seronorm Trace Elements reference materials Whole blood and Serum. The ^{77}Se nuclide was preferred to the ^{82}Se one since the former showed a higher signal–background ratio.[21] Moreover the concentration of chloride ion in human plasma (98–107 mmol/l ≈ 3.5–3.8 g/l)[22] and subsequently its effect on ^{77}Se measurement is more stable than that of bromide (2–5 mg/l)[23] ion, affecting ^{82}Se measurement.

RESULTS AND DISCUSSION

Selection of isotopes for mass spectrometric measurement

When the ICP-MS instrument equipped with a common quadrupole ion filter without collision cell is to be used, the selection of proper isotopes is of great importance. During ICP-MS analysis serum matrix elements (S, P, Cl, Br, etc.) together with oxygen, hydrogen and/or plasma argon can form several polyatomic ions of the same mass as some analysed elements. Chosen isotopes of zinc (^{66}Zn and ^{68}Zn) were selected with respect to potential spectral overlap with various SO_2^+ ions. The abundance sensitivities of zinc isotopes (i.e. the ratio of the isotopic abundance of the interfering sulfur dioxide ion and the abundance of the analysed zinc nuclide) are 1.9 for ^{64}Zn, 0.16 for ^{66}Zn and ≪0.01 for ^{68}Zn. This means that ^{66}Zn is disturbed 12-fold less than ^{64}Zn. Sulfur is a very important interferent since its concentration in human plasma can reach the level of 1–2 g/l,[22,24] and it disturbs all relevant zinc nuclides as well as ^{65}Cu nuclides. During IEC chromatography with ammonium formate mobile phase (see Fig. 1), the sulfur compounds eluted in three fractions corresponding to retention times 2–3 min (void volume), 16–20 min (the first main zinc fraction) and 21–26 min (the right tail of the second zinc peak), respectively. Possible interferences caused by sulfur were examined by nebulization of two cysteine solutions (equivalent sulfur concentration 100 and 1000 mg/l) and ICP-MS measurement of $^{48}(SO^+)$ particles and relevant species of SO_2^+ under optimized instrument conditions. The signal of $^{66}(SO_2^+)$ is 70 times lower than that of $^{48}(SO^+)$, the signal of $^{68}(SO_2^+)$ is 210 times lower and the signal of $^{63}(SO_2^+)$ is 800 times lower than that of $^{48}(SO^+)$. Considering the relative magnitude and positions of sulfur, zinc and copper signals (peaks) observed in real serum (see Fig. 1), this means that the sulfur could not significantly disturb both measured zinc isotopes and ^{63}Cu nuclide as well. Interference of SO_2^+ ions on the ^{65}Cu signal, which is necessary to measure for ID quantification was almost negligible as the signal of $^{65}(SO_2^+)$ was found to be 35 times lower than that of $^{48}(SO^+)$. Because of the relatively high concentration of ^{65}Cu in the isotope enriched solution, the ID measurement was not markedly affected. The influence of phosphorus-containing particles on ICP-MS detection of copper (namely $^{31}P^{16}O_2^+$ on ^{63}Cu and $^{31}P^{16}O^{18}O_2^+$ and $^{31}P^{17}O_2^+$ on ^{65}Cu) is negligible because the phosphorus content in serum (up to 150 mg/l)[25] is considerably lower than that of sulfur and a preliminary measurement of ammonium phosphate solution (10 mg/l P) proved that the $^{63}(PO_2)^+$ signal is 300 times lower than that of $^{47}(PO)^+$. Another interference caused by doubly charged barium ions ($^{132}Ba^{2+}$ and $^{136}Ba^{2+}$ on ^{66}Zn and ^{68}Zn respectively) can also be neglected owing to the low natural occurrence of barium in human plasma (up to 100 μg/l)[26] and the low abundance of the respective barium nuclides.

In the case of Se species detection, the ^{82}Se isotope is preferred to ^{77}Se since the main interfering element of the former, bromine (owing to $^1H^{81}Br^+$ ion formation),[27] is eluted in one narrow peak, whereas chlorine, the main element disturbing ^{77}Se detection (owing to $^{40}Ar^{37}Cl^+$ ion formation), is eluted in a wide-tailed peak, see Fig. 2. Hence, the Se peak eluting at $t_R = 41$ min during SEC analysis and the Se peak eluting at $t_R = 15$ min during IEC analysis, representing false positive signals of HBr^+ ions. In both cases (SEC and IEC) the ratio of heights of proper bromine ($m/z = 79$) and false positive selenium ($m/z = 82$) peaks fit well with a value of 330

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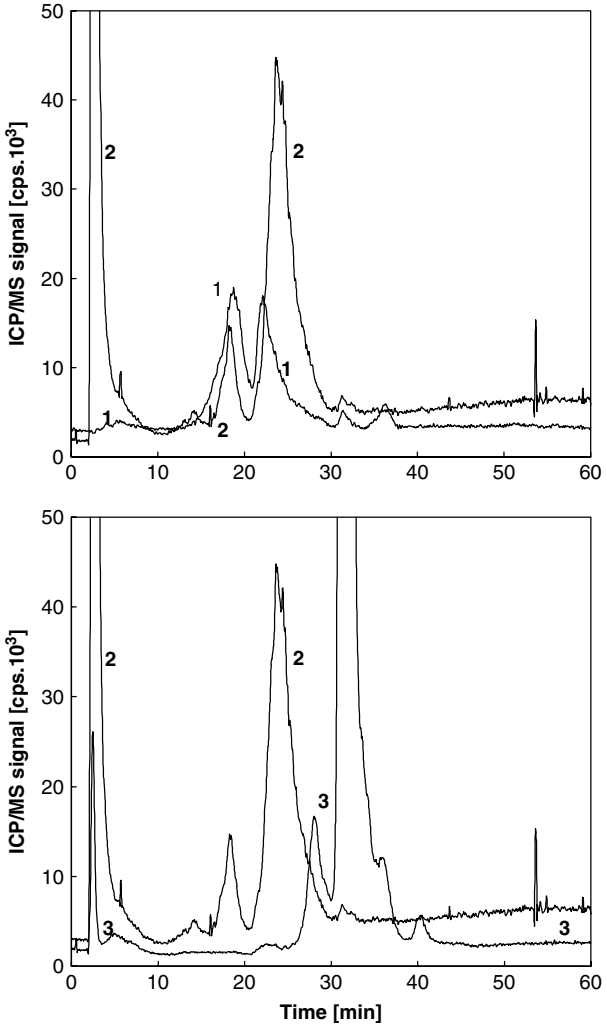

Figure 1. Course of chromatographic separation of zinc and sulfur species on IEC column: **1**, ^{66}Zn signal; **2**, $^{32}S^{16}O$ signal; **3**, ^{63}Cu signal, all curves exhibit real background.

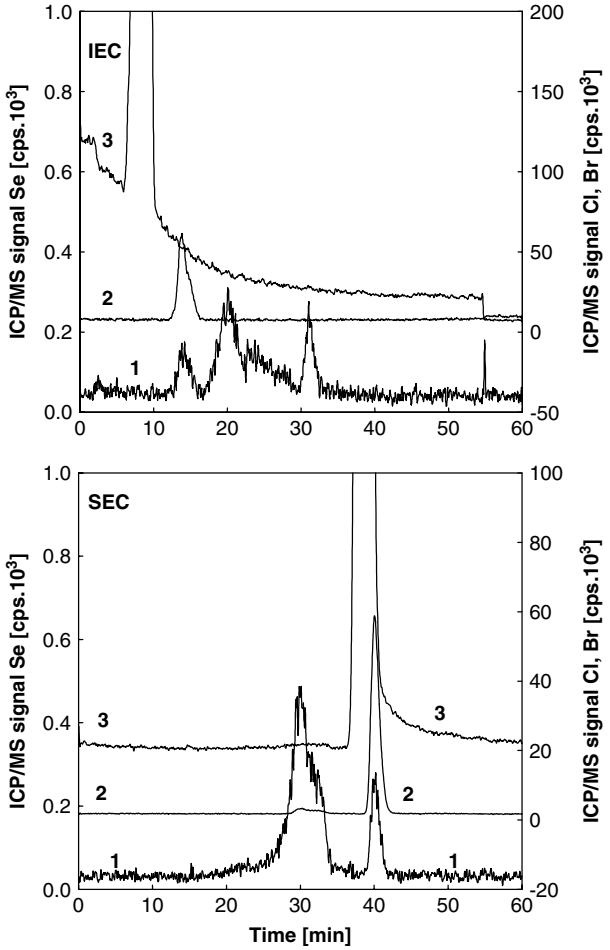

Figure 2. Course of chromatographic separation of selenium, bromine and chlorine species: **1**, ^{82}Se signal; **2**, ^{79}Br signal; **3**, ^{37}Cl signal; all curves exhibit real background.

corresponding to $^{79}Br^{+}/^{1}H^{81}Br^{+}$ determined by preliminary measurement of KBr solution containing 10 mg/l Br.

Composition of mobile phase and selection of internal standards

The choice of a suitable mobile phase represents a substantial part of LC/ICP-MS coupling experiments. It should satisfy two antagonistic demands: to ensure good chromatographic separation of element species and to be acceptable for ICP-MS. In order to prevent the ICP-MS sampling system blockage only such compounds, capable of being thermally decomposed to gaseous products, were tested. They included Tris, used for preparation of the basic buffer solution, and three ammonium salts (acetate, formate and nitrate), applied individually as a counter ion component.

The first step of optimization of the IEC mobile phase composition was focused on finding the counter ion concentration necessary for quantitative elution of all element species from the column. During these tests serum samples of previously determined total content of trace elements were analysed using a somewhat modified chromatographic process. Five minutes after sample injection the concentration of counter ion in the mobile phase was promptly changed to the target value. After elution of all species the calibration solution was injected using the second injector equipped with a 200 µl sample loop. This injector was inserted between the column and the T piece. After smoothing, the recorded signals of individual elements were integrated and the respective calibration equations were established. The elements quantities eluted from the serum sample were then ascertained and compared with the total element content in the serum. It was found that a 0.5 mol/l concentration of all tested salts was sufficient for total elution of analysed trace elements.

The following criteria of mobile phases selection were pursued during the subsequent tests: separation of various species of one element, mutual separation of various species

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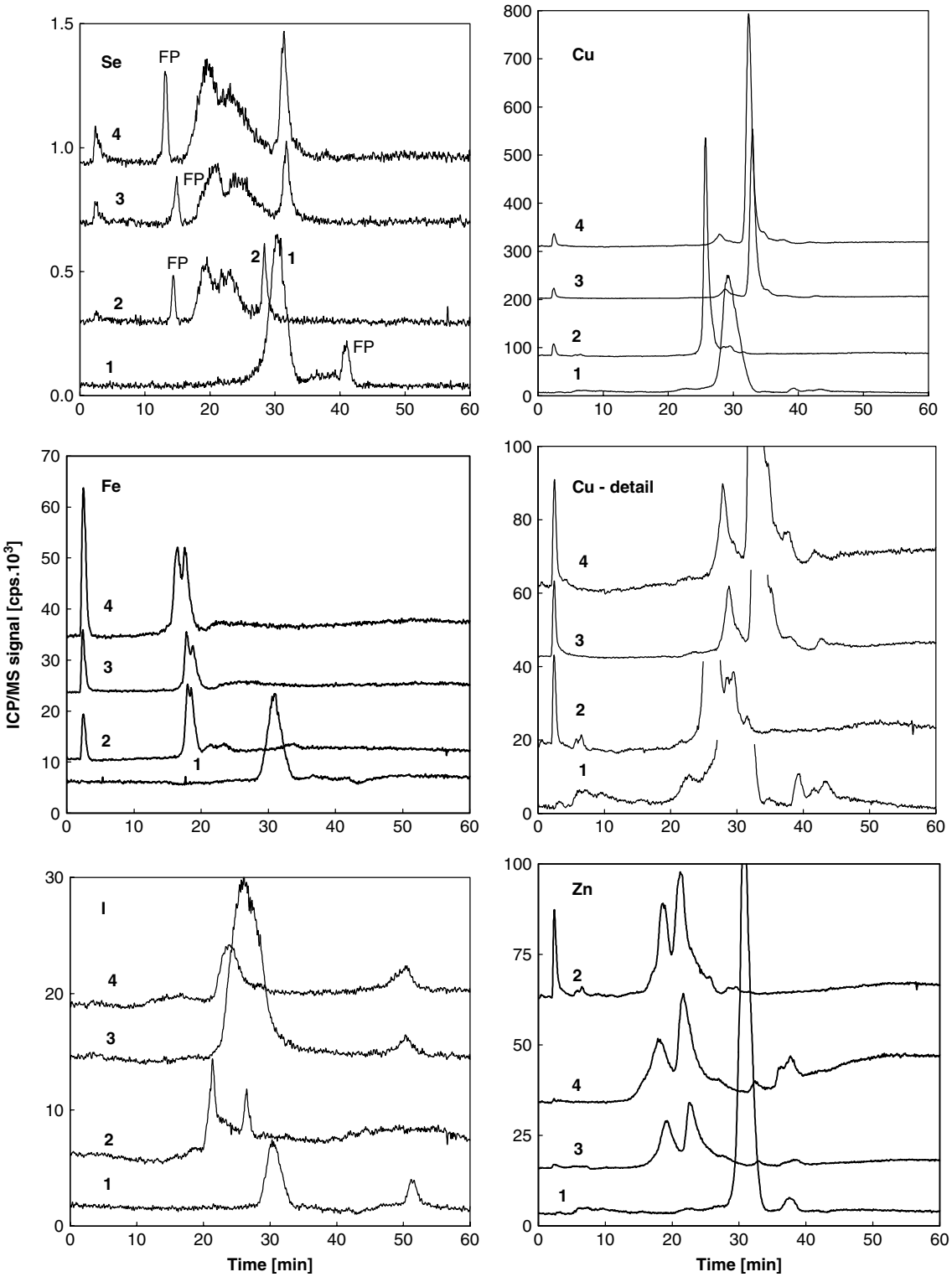

Figure 3. Course of chromatographic separation of element species: **1**, SEC; **2**–**4**, IEC; **2**, nitrate mobile phase; **3**, formate mobile phase; **4**, acetate mobile phase; FP, false positive; only curve 1 exhibits real background.

of various elements, ICP-MS sampling system blockage and the influence of mobile phase composition on the sensitivity of ICP-MS detection. A comparison of the separation capability of tested chromatographic arrangements is shown in Fig. 3, containing chromatograms of the same sample. During these tests IEC and SEC were also compared; however IEC and

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SEC separate the individual species fractions according to different principles and there is no connection between values of retention times of both chromatographic techniques. Generally all IEC analyses give much better separation of element species fractions than SEC. In the case of selenium, SEC gives two peaks: a main peak at retention time 30 min and a small broad zone eluted between 33 and 43 min. IEC separates selenium compounds into four fractions. Similarly, iron compounds are in the case of SEC eluted in one fraction, whereas in the case of IEC they are separated into three or four fractions. The separation efficiency of the copper species is similar for both chromatographic techniques; however IEC concentrates the main compound of copper (ceruloplasmin) into a much narrower peak and allows better resolution from compounds of other elements. The IEC separation of zinc species is also better than the SEC one: whereas only a major zinc fraction and a minor zinc one are found in SEC chromatograms, the IEC analyses resolve two major and two minor zinc peaks. The separation of iodine species is poor on both types of column: they are distributed between two fractions corresponding to iodide ($t_R = 51$ min) and the sum of the other iodine species, respectively. Mutual separation of individual iodine compounds can probably be performed only after their release from the protein carrier.[12] The use of SEC is also inadequate for mutual inter-element species separation as the majority of iron, copper and zinc (and selenium) eluted together in one fraction at $t_R = 30$ min.

The separation capabilities of all IEC versions (using ammonium nitrate, formate and acetate) are very similar; minor differences in individual chromatograms can be explained by random variability and/or by the age of the sample. Only in the case of copper has different behaviour been observed: the use of nitrate does not allow mutual separation of the main copper and zinc peaks, whereas in acetate or formate mobile phase the copper peak is shifted towards a longer retention time. In further experiments the acceptability of the mobile phase for the mass spectrometer was tested. The mobile phase for SEC contained only low amount of salts and therefore did not cause any difficulty either with the sample injection system of mass spectrometer or with the ICP-MS signal. The mobile phase containing ammonium nitrate, used for IEC, was also very well tolerated by the mass spectrometer. On the other hand, both organic salts cause deposit formation in the sample injector tube of the plasma torch. The use of ammonium acetate mobile phase, in particular, leads to formation of a sticky film and causes considerable contraction of the injector's inner diameter. The effect of the ammonium formate mobile phase is not as strong.

All salts used as the counter-ion components in the mobile phase for IEC somewhat affect the ICP-MS signal as well. The most important interference was observed in the case of zinc when acetate containing mobile phase was used (see Fig. 3: the zinc background follows the gradient of ammonium acetate in the mobile phase). The effect of other salts was less significant. The results of all the tests are summarized in Table 2. Considering all criteria the best choice is a formate containing mobile phase, which ensures sufficient chromatographic separation of species and does not trouble the operation of ICP-MS.

The effect of the ammonium formate containing mobile phase on the sensitivity of ICP-MS determination and the feasibility of a proper internal standard to compensate for these interferences was investigated in detail in the subsequent tests. During these tests a chromatographic session was performed without sample injection. The column effluent was formed by mobile phase only and it was coupled by a T piece together with a flow of mixed solution of tested internal standards (In, Rh, Te and Y, all 50 ng/ml) and analysed elements (Cu, Zn, 200 ng/ml; Fe, 500 ng/ml; I, Se, 40 ng/ml). In order to evaluate the efficiency of individual internal standard corrections, the difference between the ICP-MS signal at the beginning of analysis (zero concentration of formate) and that at $t_R = 50$ min (max. concentration of formate) was ascertained. When no internal standard correction was applied, the signals of Cu, Fe and I increased by approximately 10%, the signal of Zn increased by approximately 15% and the signal of Se increased by as much as 35%. This signal drift can be corrected by particular internal standards; the use of Rh for determination of Cu, Fe, I and Zn and the use of Te for determination of Se were found to be most capable. Nevertheless, some of the interference (approximately 2%) remained uncorrected. The signal of Zn was somewhat increased; on the other hand, the signal for Se was overcorrected and therefore decreased. This signal fluctuation was incorporated into the uncertainty of the results.

Quantification of individual element species: precision and uncertainty

ID represents a sophisticated quantification technique exhibiting the highest level of accuracy and precision. It was

Table 2. Comparison of types of liquid chromatography separation procedures

Chromatography type	Species separation	Interelement separation	Sample injector tolerance	ICP-MS sensitivity
SEC	Poor	Poor	Good	Good
IEC—nitrate	Good	Sufficient	Good	Sufficient
IEC—acetate	Good	Good	Poor	Poor
IEC—formate	Good	Good	Sufficient	Sufficient

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recently also successfully applied for quantification of species of copper, iron and zinc in human serum.[8] However, owing to poor availability of some isotopic enriched standards and problems with spectral interferences, the general use of ID is questionable. Therefore in this study the much simpler and generally applicable method of peak area normalization (PAN) was tested. This method consists of simple distribution of total element concentration (ρ_{tot}) among individual fraction (ρ_i) according to the ratio of their peak areas (A_i) and the sum of all peak areas A_{tot}:

$$\rho_i = \frac{A_i}{A_{tot}} \cdot \rho_{tot} \quad (2)$$

In order to examine the variability of quantitative results, the serum samples of seven volunteers were analysed in two replicates. Both replicates were analysed immediately one after another. In order to characterize the variability of results, two chromatographic fractions with high and low concentrationd of each elements were selected for quantification and relative standard deviations of repeatability were calculated. The selected fractions of copper were: the main fraction eluted at $t_R = 32$ min (Cu I) and the minor fraction eluted at $t_R = 29$ min (Cu II). Two adjacent iron fractions, $t_R = 18$ (Fe I) and 19 min (Fe II), selenium fractions eluted at $t_R = 20$ min (Se I) and 32 min (Se II) and zinc fractions eluted at $t_R = 23$ min (Zn I) and 37 min (Zn II) were chosen as well. Owing to poor separation, quantification of iodine species was not performed. One could assume similar statistical behaviour of peaks of similar areas. However, this presumption was not quite proved. Table 3 shows that the s_r values of most fractions range from 0.03 to 0.07, depending on the relative abundance of proper fraction. There is one exception: the s_r value of the Cu II fraction is 0.16. This fact can be explained in terms of significant negative correlation. The average value of correlation coefficient between Cu concentration in Cu I and Cu II fractions calculated for individual replicates is −0.71. As the relative abundance of Cu I fraction is very high (≥90%) small changes of Cu concentration in the Cu I value evoke substantial alterations of Cu concentration in the Cu II fraction.

The estimation of uncertainty (according to definition, a parameter associated with the result of a measurement that characterizes the dispersion of the value that could reasonably be attributed to the measurand; for more details and methods of estimation see ISO[28]) of results obtained by the PAN method was made on the basis of the repeatability of results. In order that other sources of uncertainty not covered by repeatability could be involved, formal equation of measurement was set:

$$\rho = \rho_{found} \cdot f_{nonspect} \cdot f_{tot} \quad (3)$$

where ρ_{found} is the concentration of the element in a given chromatographic fraction calculated according to equation (2). Both included correction factors have a unitary value. $f_{nonspect}$ represents the correction for the remaining non-spectral interferences not compensated for by the internal standard correction, and its standard uncertainty was estimated to be 0.01. The second correction comprises the uncertainty of total element concentration: $u(f_{tot}) = u(\rho_{tot})/\rho_{tot}$. Hence the combined uncertainty of concentration of element bound in an individual fraction is given by:

$$u(\rho) = \rho \cdot \sqrt{s_r^2(\rho)/n + u(f_{nonspect})^2 + u(f_{tot})^2} \quad (4)$$

where n is the number of replicates.

Table 3. Element contents in serum samples and selected chromatographic fractions of human serum

Parameter	Copper	Iron	Selenium	Zinc
Total concentration				
mean (μg/l)	1130	1680	95	1050
range (μg/l)	740–2200	1100–2800	65–130	700–1600
relative standard uncertainty[a] u_r	0.020	0.020	0.030	0.020
Fraction I (higher concentration)				
t_R (min)	32	18	20	23
mean (μg/l)	1060	555	46	565
range (μg/l)	620–2 030	230–1 000	25–60	410–820
relative repeatability s_r	0.031	0.042	0.044	0.035
Fraction II (lower concentration)				
t_R (min)	29	19	32	37
mean (μg/l)	54	350	13	23
range (μg/l)	40–80	170–480	10–16	15–39
relative repeatability s_r	0.155	0.062	0.055	0.060

[a] Estimated previously in Mestek *et al.*[21]

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Table 4. Comparison of results of different quantification techniques

	Cu concentration (μg/l)				Zn concentration (μg/l)			
	Cu I fraction		Cu II fraction		Zn I fraction		Zn II fraction	
Sample	PAN[a]	ID[b]	PAN	ID	PAN	ID	PA	ID
1	622 ± 37	634 ± 32	40 ± 9	42 ± 5	525 ± 35	497 ± 25	17 ± 2	16 ± 2
2	1 000 ± 60	950 ± 48	44 ± 10	47 ± 6	818 ± 55	790 ± 40	39 ± 4	34 ± 3
3	825 ± 50	873 ± 44	64 ± 14	71 ± 9	408 ± 27	425 ± 21	18 ± 2	14 ± 2
4	2 030 ± 120	1 960 ± 98	81 ± 18	80 ± 10	461 ± 31	458 ± 23	18 ± 2	19 ± 2
5	1 130 ± 68	1 130 ± 57	59 ± 13	59 ± 7	532 ± 36	481 ± 24	23 ± 2	21 ± 2
6	955 ± 57	984 ± 49	48 ± 11	50 ± 6	560 ± 38	544 ± 27	15 ± 2	14 ± 2
7	880 ± 53	932 ± 47	40 ± 9	42 ± 5	653 ± 44	618 ± 31	29 ± 3	25 ± 3

Mean ± expanded uncertainty ($k = 2$).
[a] Uncertainty calculated according to eqn (4).
[b] For uncertainty estimation see Mestek *et al*.[20]

Quantification of individual element species: accuracy

The accuracy of the simple normalization method applied for the speciation of copper and zinc was proved by comparison of the results with those of the ID method. Table 4 summarizes the results of all analyses together with their expanded uncertainties. Mutual comparison of both techniques was performed by the Wilcoxon *T* pair test. Results of both copper fractions were in good accordance with ID data, but in the case of zinc the test proved the accuracy of fraction I results only. The second comparison consisted of testing the overlap of the PAN results uncertainty interval and the ID results uncertainty interval. The test gave excellent results for copper determination: all ID results fall within the limits given by the expanded uncertainty of PAN results. The results of the tests of zinc determination are worse: four results did not pass (fraction I of sample 7 and fraction II of samples 2, 3 and 7). However, the level of zinc concentration in the Zn II fraction was very low (the fraction represents approximately 5% of all serum zinc) and some level of bias is probably unavoidable.

CONCLUSIONS

Speciation analysis is an important discipline that allows us to study and understand better the pathway of elements in a human body. The proposed method, involving one-step chromatographic separation, can be easily used for the rapid screening of changes of distribution of trace elements among individual species fractions caused by various diseases. Quantification is done by the peak normalization method, which makes the procedure very simple. It was proved that, even under conditions of time-dependent concentration of counter-ion in the mobile phase, proper internal standards can ensure the almost constant sensitivity of the ICP-MS detector. The accuracy of determination of some elements was verified by the primary measurement method based on isotope dilution, and the uncertainties of results also met the intended purpose.

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