Multielemental Fractionation in Human Peripheral Blood Mononuclear Cells by Size Exclusion Liquid Chromatography Coupled to UV and ICP-MS Detection

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Abstract

An analytical methodology is presented in this work to determine metal-biomolecule complexes size distribution patterns of several elements, among different compounds present in human peripheral blood mononuclear cells (PBMC). A hyphenated technique based on size exclusion chromatography (SEC) coupled online to UV and inductively coupled plasma mass spectrometry (ICP-MS) detection is used. Two different SEC columns with separation ranges between 1,500–1,000,000 relative molecular mass (M_r) (Nanofilm SEC-250) and 5,000 and 100,000 relative molecular mass (M_r) (TSK-Gel G2000 SW) are used with 10 mmol/L tris-HCl at pH 7.3 as mobile phase. Retention behavior (retention time and peak-area ratios) remained unchanged for several successive separations. Metal-containing compounds are found to a wide range of M_r. Copper-zinc superoxide dismutase, copper and zinc metallothionein, and copper and zinc transferrin are identified in PBMC samples. A high M_r (147,000) metal-binding protein containing copper and zinc and a high M_r (107,000) manganese-binding protein were also found; however, these remained unknown.

Introduction

The biological function of essential elements depends mainly on their specific chemical property of building up multivalent metal-organic complexes, e.g., in different enzymes and protein structures (1–3). It is necessary to investigate both the nature of proteins and their bound elements to obtain detailed information about metabolic processes (4). However, most studies carried out are referred to total element determinations, which only provide incomplete information about trace element status in biological systems. The biological behavior (bioavailability, effects, and toxicity) of a given element strongly depends on the concentration and the chemical form in which this element occurs in the biological sample (5–8). This fact explains the need to carry out chemical speciation studies rather than total element determinations to get more comprehensive information about the distribution pattern of the essential elements in a tissue or a body fluid, which is vital in many areas of medical and biomedical sciences (9–11). To separate metal-containing proteins, a method that has little influence on the composition of the sample has to be employed. For the retention mechanism of size exclusion chromatography (SEC), there is theoretically no interaction among the sample parts and the separation phase. It is often used for metal-containing proteins chemical speciation investigations in biological samples (12–19). SEC is based on the molecular sieve effect and enables species to be separated according to their size and, to a lesser extent, their shape. The time average of a substance remains in the pores can usually be related directly to its molecular weight (13), which might be used to predict the type of association between the metal and the molecules present in the sample. Experimentally, some changes in metal-containing proteins can occur due to interactions with the column matrix (non-specific binding of metal ions or proteins) during their separation. To avoid it, Raab and Brätter (12) reported those separation parameters by SEC (buffer type, concentration, and pH) that should be determined and adapted to the sample matrix.

Several authors have recently described chemical speciation studies in serum and blood fractions, mainly plasma and erythrocytes, by coupling SEC, hydrophobic interaction chromatography (HIC), and anion exclusion chromatography (AEC) with element-specific detectors such as ICP–OES (14,20) or ICP–MS (15,21). Other papers have emphasized the metal quantification of proteins through X-ray, atomic absorption, and mass spectrometric techniques, among others (19). As well as the authors of this paper know, any speciation analysis using SEC coupled online to UV and ICP-MS detection has been undertaken on peripheral blood mononuclear cells (PBMC), despite the fact that these cells, unlike serum and plasma, are less affected by factors such as acute infection, inflammatory response, stress, myocardial infarction, physical exertion, and nutritional status (22–25). Additionally, because PBMC are immune cells, these have been used like markers under different pathological conditions (26,27). On the other hand, the PBMC might reflect whole body essential elements status more effectively, and it would be expected to provide more reliable measurements of intracellular trace and ultra-trace elements in cells metabolically active and short life-span.

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Thus, the aim of this work is to present metal-biomolecule complexes size distribution patterns in human PBMC using an analytical methodology based on SEC coupled online to UV and ICP–MS detection.

Experimental

Reagents

Sodium heparin 1000 IU/mL was purchased from Farmionni Scalpi (Bogotá, Colombia). Histopaque-1077, PBS (phosphate buffer saline 0.01 mol/L; NaCl 0.138 mol/L; KCl 0.01 mol/L, pH 7.4 to 25ºC), TRIZMA base (99.9%), and methanol were obtained from Sigma Aldrich (St. Louis, MO). Blue dextran, transferrin, albumin, β-lactoglobuline, superoxide dismutase from *E. coli*, superoxide dismutase from human erythrocytes, lysozyme, and metallothionein were purchased from Sigma. Stock single element standards of copper, zinc, and manganese (1000 μg/L each one) were purchased from Fluka (St. Louis, MO). Deionized water (18 MΩ·cm) from an Easy Pure UV compact ultrapure water system (Barnstead, Boston, Canada). Nitric acid (65%) and hydrogen peroxide (35%) were from Riedel de Häen (Seelze, Germany). Nitric acid was purified using a Berghof BSB-939-IR sub-boiling distillation system (Eningen, Germany). Hydrochloric acid (37%) and sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany). All the reagents used were of analytical reagent grade and found to be free from trace metals.

Nitrogen (99%) and liquid argon were purchased from AGA (Maracay, Venezuela). An argon vaporizer in the tank provided argon gas.

Instrumentation

The chromatographic system consisted of a Varian Inert 9012 solvent delivery system (Varian, Palo Alto, CA) and a Rheodyne 9010 PEEK (Rheodyne, Cotati, CA) injection valve fitted with a 20-μL loop. A Hewlett-Packard Series 1100, (Agilent Technologies, Santa Clara, CA) UV-vis absorption detector was employed for molecular detection, and the data obtained was processed in an EZChrom version 6.8 (Scientific Software, Lincolnwood, IL) Chromatography Data System.

The chromatographic separations were carried out on two SEC columns. A Sepax Nanofilm SEC-250 column (300 mm × 4.6 mm i.d.) with calibration range between 1,500–1,000,000 relative molecular mass (M_r) (Sepax Technologies, Inc., Newark, DE) was protected with a Phenomenex Security Guard Cartridge System One for all (Phenomenex, Torrance, CA). The other column was a TSK-Gel G2000SW (300 × 7.5 mm id) with a calibration range between 5,000–100,000 relative molecular mass (M_r) (Tosoh Bioscience, Tokyo, Japan). This column was protected with a TSK-Gel SW guard column.

The ICP-MS detector was a Perkin Elmer SCIEX model Elan 6000 (Ontario, Canada) operating with an ICP RF power of 1100 W. A concentric micronebulizer MicroMist 100 and a cyclone spray chamber both from Glass Expansion (Pocasset, MA) were used as nebulization device for SEC. The aerosol carrier gas flow rate was 1.1 L/min, and the sample liquid uptakes were 100 and 500 μL/min by total determinations and SEC analysis, respectively. The monitored isotopes were ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn, and ^{55}Mn.

The SEC–UV-ICP-MS coupling was performed connecting the outlet of the SEC column to the UV detector and the outlet of this connected to the liquid sample inlet of the ICP–MS nebulizer using Teflon tubing (30 cm × 0.1 mm i.d.).

A 5810R refrigerated ultracentrifuge Eppendorf (Hamburg, Germany) with swinging bucket rotor was used to separate the human PBMC from the whole blood samples.

The extraction procedure was carried out under an inert atmosphere in a glove bag (Atmos Bag, Sigma Aldrich). To break the membrane cells, a homogenization pistill Sonic–dismembrator 100 (Fisher Scientific, Hampton, MA) was used. For cell counting, a hemacytometer (Electron Microscopy Sciences, Hatfield, PA) and a digital microscope (Leica Microsystems, Weltzlar, Germany) were used.

Procedures

Blood samples collection and PBMC isolation

The samples were obtained from volunteers. For the analysis, 20 mL blood samples were drawn by venipuncture in sterile Becton Dickinson syringes (to avoid contamination), taking care to avoid hemolysis. Aliquots were collected into sterile polypropylene centrifuge tubes containing sodium heparin to prevent coagulation. All materials used, were first soaked in 10% (v/v) nitric acid for approximately 48 h, followed by six rinses with deionized water. Finally, the last water fraction was analyzed by ICP-MS and found to be free from trace metals. The closed blood tubes were kept in a room temperature until their use. PBMC isolation from whole blood was performed for no more of 3 h after the blood was drawn.

PBMC were separated from the whole blood by centrifugation [400 × g, 30 min and room temperature (RT)] on Histopaque-1077. After centrifugation, the PBMC layer was collected carefully by aspiration with a micropipette and transferred into a clean centrifuge tube. The harvested cell suspension was washed twice with PBS solution and centrifuged at 250 × g for 10 min at RT, then was resuspended into 5 mL of PBS solution, and a 20-μL aliquot was used for cell counting. Finally, PBMC suspensions were further centrifuged at 250 × g, 10 min at RT, and the isolated cells were divided into two portions. One was used the same day of fractionation by SEC, and the other one was stored at –30°C until their total element determinations.

Viability test

The viability of the PBMC was assessed by trypan blue exclusion test. The number of unstained cells (alive) was counted on a hemacytometer employing a microscope to obtain the number of PBMC per milliliter suspension.

Total element determinations in PBMC

To find the best digestion procedure by total element determinations in PBMC samples, an experimental design was applied using three different digestion media (HNO_3, H_2O_2, and

HNO_3/H_2O_2). The procedure was carried out as follows: different volumes of HNO_3 and H_2O_2 were added to the samples. The mixture was homogenized by a vortex-mixer during 5 min and exposed to both warming (90ºC at two different times: 15 and 30 min) and not warming (RT, during 30 min) patterns. Later, the samples were diluted to 3 mL with deionized water, and the solution was filtered through a 0.2-μm PTFE membrane syringe filter, and the concentrations of Cu, Mn, and Zn were determined by ICP-MS.

To consecutive determinations of the concentration of elements in PBMC, the samples were treated by adding of a mixture HNO_3–H_2O_2 (0.20–0.020 mL, respectively). Then, they were vortex-mixed for 5 min and maintained to RT during 30 min to complete the digestion process. The next steps are the same to those described previously.

Extraction of metal-containing compounds

The isolated PBMC were thawed on ice and homogenized. The cells were suspended into 1 mL of 10 mmol/L Tris-HCl buffer (pH 7.3). These cell suspensions were sonicated with a titanium pistill for 10 min at 40 W and kept on ice during the process. Each step was carried out under N_2 atmosphere in a glove bag to prevent contamination and/or degradation of the species in the sample. The homogenates were centrifuged (250 × g, 5 min, 4ºC), and the supernatant was filtered through a 0.2-μm PTFE membrane filter and used as extract. Due to the instability of some metal-containing compounds, PBMC extractions and SEC analysis were performed not more than 24 h after the isolation of PBMC was done.

Analysis of metal-biomolecule complexes by SEC–UV-ICP-MS

With the purpose of knowing the profiles of metal-biomolecule complexes, a preliminary analysis of a PBMC extract was carried out on a Nanofilm SEC-250 analytical column with a wide separation range of molecular weight, coupled online to UV and ICP-MS detection. The SEC parameters were previously optimized (28).

To obtain more detailed molecular mass information about metal-binding compounds, a TSK-Gel G2000SW analytical column with narrow separation range of molecular weight (in comparison with a Nanofilm SEC-250 column) was employed, and the SEC parameters were optimized. Concentrations among 5 mmol/L and 50 mmol/L of tris-HCl buffer solution (pH 7.3) were tested. The flow rates of mobile phase were varied between 0.5 mL/min (the lowest value of the flow rate that can be used on the chromatographic column, according to the manufacture specifications) and 1 mL/min.

Finally, the fractionation of PBMC extracts was also performed on the two columns previously mentioned that were connected in series and coupled to UV and ICP–MS detectors.

Calibration and quality control of PBMC samples

The optimization of instrumental conditions and the calibration of the ICP-MS were achieved by using multielemental standard solutions containing 10 μg/L of Cu, Mn, and Zn in 2% HNO_3. Multielemental solutions were daily prepared from the stock single-element standards (1000 μg/L) by dilution with deionized water.

Due to lack of Certified Material Standards (CMS) from PBMC or from a similar matrix, the total element determination was validated by a recovery test. Six quality control samples were prepared by spiking control PBMC samples with multi-elemental standards of Cu, Mn, and Zn at two concentration levels (30.0, 60.0 μg/L) on the day of the PBMC analysis. The six spiking samples together with the three samples unspiking were treated (following the same procedure described in the total element determinations in PBMC section) and analyzed at each concentration level in each sample sequence.

Several considerations are taken into account in order to overcome the problems related to losses and stability of the species during analysis and identification difficulties.

To evaluate the non-specific adsorption between SEC matrices and species in PBMC extracts, the columns were effectively cleaned after each sample injection. To do this, 400 mmol/L NaCl (pH 2.3) (0.5 mL/min) was passed through the column for 1 h. During this time, the eluate was monitored with UV and ICP–MS detection. Following a mixture MeOH–H_2O (0.4:1) at the same flow rate (0.5 mL/min) was passed through the column for 1 h to remove hydrophobic proteins and lipoproteins. The columns were rinsed with 3–5 column volumes of deionized water after each solution. These mobile phases were monitored with UV detection. This procedure was repeated several times among consecutive injections of sample. After cleaning, the columns were equilibrated with the mobile phase (10 mmol/L Tris-HCl at pH 7.3) until the UV and ICP–MS baseline stabilized before injecting the next sample.

To obtain an accurate measurement of the M_r of biomolecules in the PBMC extracts, the two SEC columns were calibrated with protein standards of M_r: transferrin (80,000 M_r), albumin (66,500 M_r), β-lactoglobulin (36,500 M_r), lysozyme (14,300 M_r), metallothionein (7,000 M_r), and the linearity of the curve (log M_r versus retention time) was evaluated. The void retention time was determined using blue dextran (2,000,000 M_r).

Results and Discussion

Determination of total element concentrations in PBMC

The results obtained in the digestion process indicated that the unwarmed HNO_3–H_2O_2 mixture was the most efficient. Therefore, it was used for the digestion of the PBMC samples. The validation of this method was performed using a recovery test. The results of the recoveries test were 87% and 102% for Cu, 90% and 98% for Zn, and 101% for Mn at each concentration level in the samples. The results of the recovery percentage indicate that these elements can be quantitatively recovered from PBMC in all the steps of the analysis procedure.

The total Cu, Zn, and Mn concentrations in PBMC were obtained by a sample analyzed in triplicate: (0.00787 ± 0.00003) μg/10^6 cells, (0.0180 ± 0.0008) μg/10^6 cells, and (0.00073 ± 0.00001) μg/10^6 cells, respectively. The detection limits, defined as three standard deviations (SD) of the blanks, were 0.000004 μg/10^6 cells (0.12 μg/L) for Cu, 0.00005 μg/10^6 cells (0.33 μg/L) for Zn, and 0.000003 μg/10^6 cells (0.06 μg/L) for Mn. The quantification limits, given as 10 SD of the blanks, were 0.000014
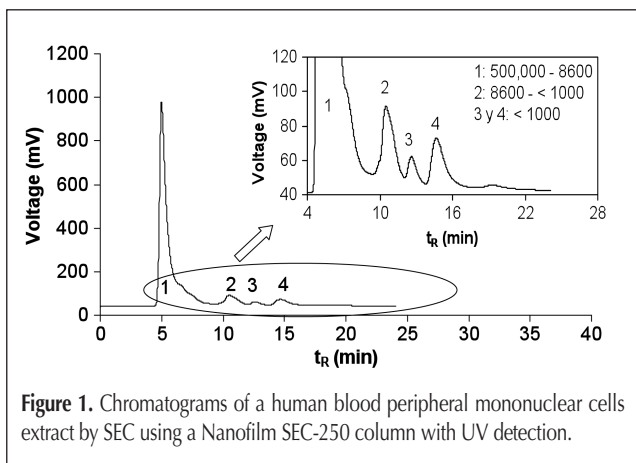
Figure 1. Chromatograms of a human blood peripheral mononuclear cells extract by SEC using a Nanofilm SEC-250 column with UV detection.
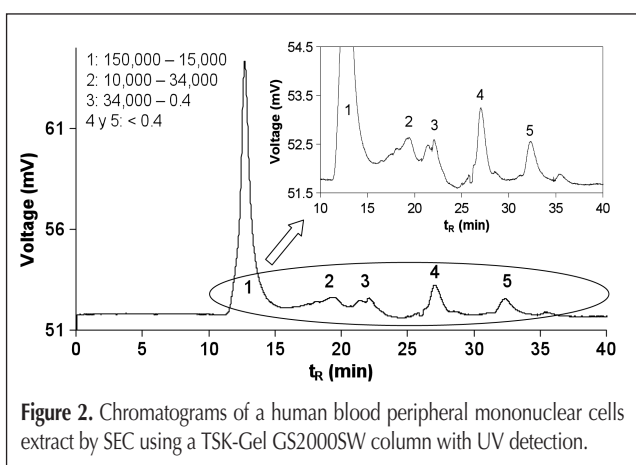

Figure 2. Chromatograms of a human blood peripheral mononuclear cells extract by SEC using a TSK-Gel GS2000SW column with UV detection.
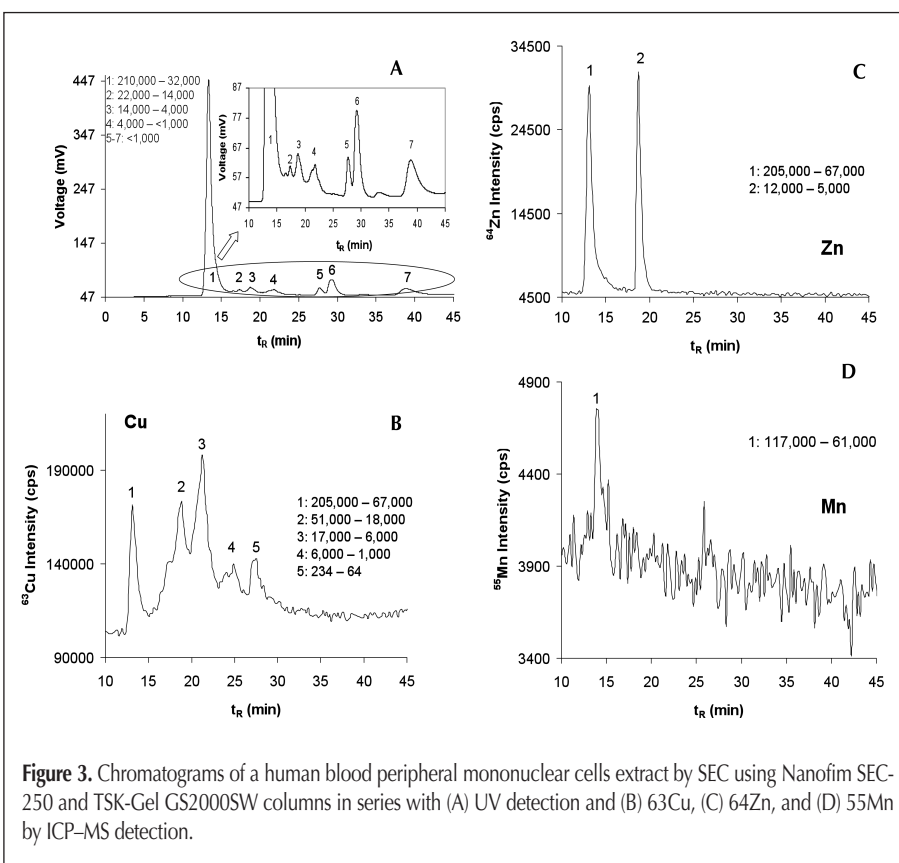

Figure 3. Chromatograms of a human blood peripheral mononuclear cells extract by SEC using Nanofim SEC-250 and TSK-Gel GS2000SW columns in series with (A) UV detection and (B) 63Cu, (C) 64Zn, and (D) 55Mn by ICP–MS detection.

μg/10^6 cells (0.41 μg/L) for Cu, 0.00016 μg/10^6 cells (1.09 μg/L) for Zn, and 0.000010 μg/10^6 cells (0.18 μg/L) for Mn. The equations of straight lines were: ^{63}Cu Intensity (cps) = 10481[Cu] (μg/L) – 330.1, r^2 = 0.9999, ^{64}Zn Intensity (cps) = 6767.5 [Zn] (μg/L) + 1443.1, r^2 = 0.9996, and ^{55}Mn Intensity (cps) = 16772 [Mn] (μg/L) + 217.1, r^2 = 0.99998.

Analysis of metal-biomolecule complexes by SEC–UV-ICP-MS
Analysis of PBMC extracts with a Nanofilm SEC-250 analytical column.
    The equation of calibration obtained for the column was: log (M_r) = –0.5952 t_R(min) + 8.426, r^2 = 0.9939.
    The information about the distribution of compounds in different ranges of M_r was obtained from the UV absorbance chromatogram at 280 nm (Figure 1). The results show that the main compounds were found in the high range of M_r.
    ICP-MS detection revealed the distribution of the elements bound to biomolecular compounds. ^{63}Cu and ^{64}Zn compounds are found in a wide range of M_r. Significant amounts of ^{63}Cu and ^{64}Zn were found in the middle range of M_r (between 56,000 and < 1,000 for ^{63}Cu, and between 43,000 and < 1,000 for ^{64}Zn). Finally, only one peak is obtained for ^{55}Mn that is related to high M_r compounds. The reproducibility obtained (n = 4) by the retention times in SEC-UV and SEC–ICP-MS chromatograms was < 0.6%.

Analysis of PBMC extracts with a TSK-Gel G2000SW analytical column.
    The equation of calibration obtained for the column was log (M_r) = –0.501 t_R (min) + 12.015, r^2 = 0.9966.
    In the previous section, it was indicated that in PBMC extracts, the main compounds of ^{63}Cu and ^{64}Zn was found in the middle range of M_r. Therefore, a TSK-Gel G2000SW column was used to obtain more detailed molecular mass information about the species bound to copper, zinc, and manganese in this range.
    The ionic strength of the mobile phase buffer plays an important role in maximizing the molecular sieving mechanism and minimizing secondary effects, such as rearrangements or non-specific adsorption by ionic and hydrophobic interaction between the sample and the column packing material during separation by SEC (12,16). In this way, separation conditions related to the concentration of mobile phase solution were optimized using UV detection and found that increasing the ionic strength of the mobile phase (Tris-HCl buffer solution) between 20–50 mmol/L does not improve the chromatographic separation of the metal-containing compounds found in PBMC extracts. Therefore, according to the results obtained for several successive separations, the best separation was

obtained when a 10 mmol/L Tris-HCl buffer solution was used. Additionally, the buffer pH was maintained near the physiological pH (7.3) to keep those metals that are bound to proteins intact during the chromatographic separation.

The effect of the mobile phase flow rate was evaluated from 0.5 to 1 mL/min to enable subsequent hyphenation of the SEC column to the ICP-MS instrument. A 0.5 mL/min flow rate of 10 mmol/L Tris-HCl buffer solution was chosen.

Once optimized the SEC parameters, the SEC–UV-ICP-MS coupling was performed. Chromatogram SEC–UV obtained for a PBMC extract is shown in Figure 2. Chromatogram SEC–UV shows a species distribution over a wide M_r range, with a major peak at high M_r, near to void volume. Most species of ^{63}Cu, ^{55}Mn, and ^{64}Zn were presented in the high and middle range of M_r.

Relative standard deviations ($n = 4$) for the retention times measured in SEC–-UV and SEC-ICP-MS chromatograms were < 1.1%.

Analysis of PBMC extracts with a Nanofilm SEC-250 and a TSK-Gel G2000SW columns in series.

According to the results presented previously, the individual columns separated compounds at different ranges of molecular mass. The Nanofilm SEC-250 column mainly separated compounds of high and middle M_r, while the TSK-Gel G2000SW column separated compounds of middle and low M_r that had not been separated for the first column. Therefore, using both chromatographic columns at the same time might make it possible to separate all these species in one single chromatographic run, and thus from the knowledge of the molecular mass, it can obtain more detailed information about of metal-binding biomolecules in PBMC extracts without using lyophilizing methods, which uses more time and might affect the integrity of the metal-biomolecule complexes found in PBMC samples (29).

Hence, to perform the subsequent chromatographic separations, the two SEC columns (Nanofilm SEC-250 and TSK-Gel G2000SW) were connected in series. The equation of calibration obtained for this configuration of the columns was: $\log (M_r) = -0.209\, t_R$ (min) $+ 7.933$, $r^2 = 0.9981$. Chromatograms SEC–UV and SEC–ICP-MS from a PBMC extract are shown in Figure 3. Both chromatograms SEC–UV (Figure 3A) and SEC–ICP-MS (especially for ^{63}Cu) (Figure 3B) show a major number of peaks in comparison with chromatograms SEC–UV and SEC–ICP-MS (for ^{63}Cu) gotten when the individual columns were used. SEC–ICP-MS chromatogram of ^{63}Cu shows three main peaks corresponding to high and middle M_r (210,000–6,000 M_r) compounds, and two minor peaks related to middle and low M_r compounds. SEC–ICP-MS chromatogram of ^{64}Zn (Figure 3C) shows two well-defined main peaks in a high and middle range of M_r, analogous to those obtained with individual columns. Meanwhile SEC–ICP-MS chromatogram of ^{55}Mn (Figure 3D) showed one single peak in a high range of M_r.

According to these results, the configuration of two columns connected in series provides comprehensive information about distribution patterns of molecular mass of metal-binding compounds in PBMC extracts without lengthening the total analysis time because the total time to complete a chromatographic run with ICP-MS detection is < 30 min.

Relative standard deviations ($n = 4$) for the retention times measured in SEC–UV and SEC–ICP-MS chromatograms were < 0.4%.

Non-specific adsorption between material columns and PBMC compounds was investigated. Once the PBMC extract was injected, several mobile phases with different ionic strength were passed through the columns in order to elute any compound retained on the column. The mobile phase signal at the exit of the columns was monitored. During this time, any peaks were observed either in UV or ICP-MS detectors. This means that there is not a non-specific retention on the material columns that would affect the integrity of the metal-biomolecule complexes in PBMC extracts. The procedure was also valid in the case of the individual columns, and it offers the advantage of not requiring exhaustive cleaning process among consecutive sample injections. Hence, it is only necessary to rinse the columns with the working mobile phase (10 mmol/L Tris-HCl buffer, pH 7.3) at the working flow rate before injecting the next sample.

Additionally, neither ICP-MS determination of total elements nor on-line ICP-MS detection in chromatographic fractions are not distorted either by non-spectral or spectral interferences.
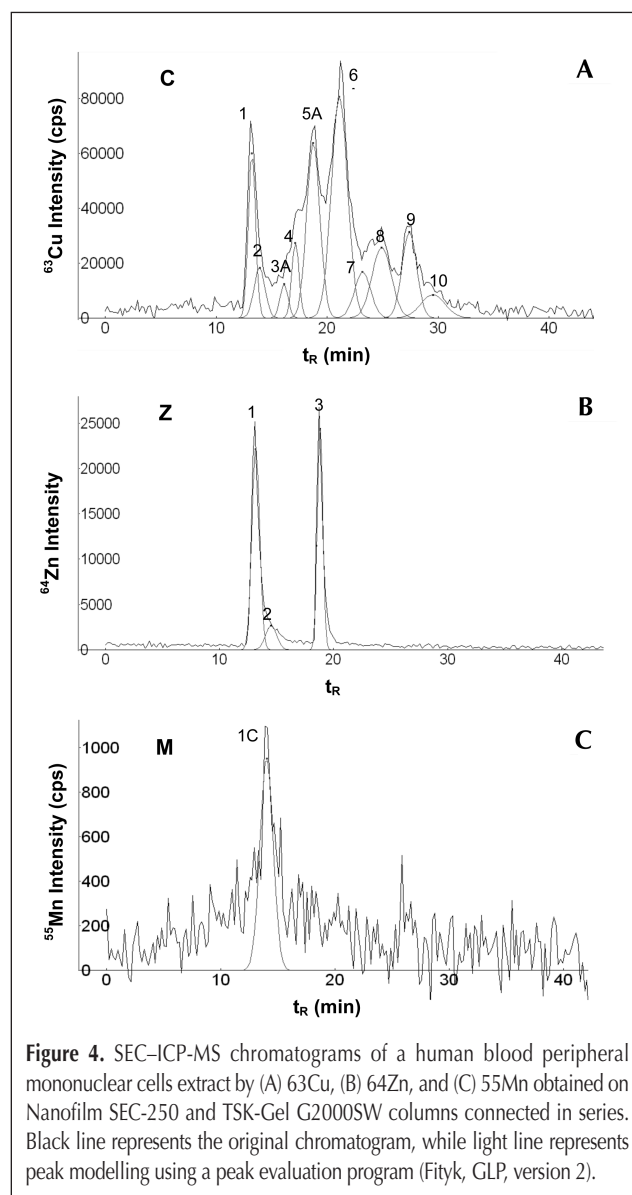

Figure 4. SEC–ICP-MS chromatograms of a human blood peripheral mononuclear cells extract by (A) 63Cu, (B) 64Zn, and (C) 55Mn obtained on Nanofilm SEC-250 and TSK-Gel G2000SW columns connected in series. Black line represents the original chromatogram, while light line represents peak modelling using a peak evaluation program (Fityk, GLP, version 2).

On the other hand, it was observed that the behavioral retention (retention time and peak-area ratios) of the molecular mass distribution patterns of metal-binding compounds found in PBMC samples remained unchanged for several successive separations every 2 h. However, the height of chromatographic peaks decreased at the end of an 8–12 h work session. After 24 h, even though molecular mass distribution patterns remained (in terms of retention times), strong degradations (approximately of 80%) were observed in those peaks corresponding to high M_r metal-binging species monitored by ICP-MS (unpublished data). Other authors (18) have reported a similar behavior in fractionation of Mn species from liver extracts.

The instability observed in metal-biomolecule complexes probably is due to dissociation of high M_r metalloproteins, which are formed by several sub-units (26–30). For this reason, the fractionation of PBMC extracts was performed during the first 6 h after PBMC separation from whole blood and extraction of metal-binding compounds from PBMC.

Molecular mass distribution of metal-biomolecule complexes in PBMC extracts

SEC-ICP-MS chromatograms obtained with the chromatographic columns in series were processed with a peak evaluation program (*Fityk*, General Public License, version 2) for mathematical resolution of peaks unresolved (Figure 4) (18). Both, in the chromatogram of ^{63}Cu (Figure 4A) and in the chromatogram of ^{64}Zn (Figure 4B) is observed a peak to high range of M_r. The compounds related to it have a M_r calculated of approximately 147,000 each one, and according to the chromatograms of ^{63}Cu, and ^{64}Zn showed in this work, it seems to be the same metalloprotein binding to both Cu and Zn.

All of the SEC-ICP-MS analysis results obtained with the two columns in series, including retention time, calculated M_r and peak height (in counts per seconds), the percentage of metal associated with each metal-biomolecule (calculated from percentage of total peak area in SEC-ICP-MS chromatograms), and metal-biomolecule complexes identified in PBMC extracts, are summarized in Table I. All proteins showed in this table were identified with individual standard proteins.

Both 8% of Cu and a significant amount of Zn (46%) found in PBMC extracts were associated to the metalloprotein previously mentioned (147,000 M_r). However, in the bibliographic revision, authors did not find information about this metalloprotein. Six percent of Cu was binding to a compound of approximately 93,000 M_r. A peak with a similar M_r was found for ^{64}Zn (16.5%). This compound was identified as transferrin (Tf), a single-chain glycoprotein containing ca. 700 amino acids, whose M_r is approximately 80,000. This one has been identified in mammals and has potential capacity for binding to transition-metal ions (apart of Fe^{3+}) that enter the body, such as Cu^{2+} and Zn^{2+} (30). It has been suggested as carriers of zinc in plasma (31). In Figure 4, it was also observed that a minor peak of ^{63}Cu (4%) related

to a compound of approximately 40,000 M_r. This species was identified as CuZn superoxide dismutase (CuZnSOD) that in mammals has a M_r of approximately 32,000. It has been previously identified in lymphocytes (32). However, the mathematical model did not provide a peak of ^{64}Zn in this M_r probably because the ^{64}Zn sensibility is minor in comparison to the ^{63}Cu one.

Significant amounts of ^{63}Cu (19%) and ^{64}Zn (38%) were also found in middle M_r range with an important peak related to species of approximately 10,000 M_r. This species was identified as a metallothionein, which has a M_r between 6,000–7,000. Metallothioneins are an intracellular family of cysteine-rich metal-binding proteins that exist in several isoforms. They play an important role in the homeostasis of the trace elements copper and zinc, act as antioxidants, and are crucial for the detoxification of metals like cadmium and mercury (33). ^{63}Cu is also mainly associated to some smaller peptides among 3,000 M_r (26%) and 1,000 M_r (8%) and to compounds of low M_r that might be of an organic or inorganic nature. The Cu compound of 60 M_r that corresponds to 7% of the Cu in the PBMC extracts might be Cu inorganic, which has a molecular mass of 63. However, these compounds were not identified. On the other hand, the only manganese-binding protein found in PBMC extracts (Figure 4C) was not identified but showed a M_r of approximately 107,000 that, according to the literature (34), might be arginase.

Conclusions

Detailed information about molecular mass distribution patterns of metal-containing compounds was obtained when two SEC analytical columns with different separation range of molecular size were connected in series. The fractionation of human PBMC extracts by SEC–ICP-MS gave chromatographic profiles from metal-biomolecule complexes. The species identified in the PBMC samples were some metalloproteins as

Table I. Peak Parameters and Metal-Binding Compounds Found in Human Peripheral Blood Mononuclear Cells Extracts

Metal	Peak number	t_R (min)	M_r calculated	Peak height (cps)	% Metal bind to protein	t_R (min) protein standards	Metal-binding compound identified
Cu	1A	13.22	147,000	60372.8	8.14		*
	2A	14.18	93,000	17252.9	6.11	14.58	Cu-transferrin
	3A	15.91	40,000	13913.5	3.69	16.37	CuSOD
	4A	17.26	21,000	27051.0	5.10		*
	5A	18.83	10,000	64597.9	19.24	18.06	Cu-MT
	6A	21.08	3,000	82723.3	26.20		*
	7A	23.07	1,000	19809.7	7.78		*
	8A	24.84	550	26448.3	9.27		
	9A	27.38	160	28234.9	7.40		
	10A	29.33	60	11533.8	7.07		*
Zn	1B	13.23	147,000	23270.9	45.96		*
	2B	14.45	82,000	2971.3	16.45	14.58	Zn-transferrin
	3B	18.83	10,000	24171.6	37.60	18.06	Zn-MT
Mn	1C	13.88	107,000	1253.56	100		*

* Metal-binding compound not identified.

CuZnSOD, MT, and Tf. A protein of high molecular mass was also found in the PBMC samples, which seems to be bound to Cu and Zn; however, this species remained unknown. On the other hand, the molecular mass distribution patterns of Mn compounds showed only one peak that was not identified either. Due to SEC not permitting the separation of all species present in human PBMC extracts as individual chemical species, further orthogonal separation techniques like SEC and CZE coupled to ICP–MS detection might help to separate other metal-binding compounds that might not have been separated with the method used in this work.

Acknowledgments

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