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8 **Sample preparation of blood plasma enables baseline separation of iron metalloproteins by**

9 **SEC-GFAAS**

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Highlights

- The analysis of plasma for diagnostically relevant proteins is challenging
- This separation problem can be overcome if plasma is analyzed for metalloproteins
- A hyphenated chromatographic method was developed to detect Fe metalloproteins
- The chelating agent mediated mobilization of Fe from Tf allowed baseline separation
- The practical feasibility of the developed SEC-GFAAS approach is demonstrated

Abstract

The analysis of human plasma for biomarkers holds promise to revolutionize disease diagnosis, but is hampered by the inherent complexity of the plasma proteome. One way to overcome this problem is to analyze plasma for a sub-proteome, such as the metalloproteome. Previous studies employing size-exclusion chromatography (SEC) coupled on-line to an inductively coupled plasma-atomic emission spectrometer (ICP-AES) have revealed that plasma contains ~12 copper, iron and zinc metalloproteins. This included the iron metalloproteins transferrin (Tf) and a recently identified haptoglobin-hemoglobin (Hp-Hb) complex, which is formed in plasma when red blood cells rupture. Since this SEC-ICP-AES method required a sample volume of 500 μ L to generate diagnostically useful results, we sought to develop an alternative SEC-based hyphenated approach using a smaller SEC column (150 x 5 mm I.D.) and a graphite furnace atomic absorption spectrometer (GFAAS) as the iron-specific detector. A designed interface enabled the integration of the SEC system with the GFAAS. Baseline separation between the Hp-Hb complex and Tf was achieved by developing a sample preparation procedure which involved the chelating agent-based mobilization of Fe from Tf to a small molecular weight Fe complex. Spiking of human plasma (1.0 mL) with red blood cell lysate (1-2 μ L) increased only the intensity of the Fe peak corresponding to the Hp-Hb complex, but not that of Tf. Since the developed SEC-GFAAS method requires only 50 μ L for analysis, it can now be employed for the cost-effective quantification of the clinically relevant Hb-Hp complex in human plasma in <50 min.

1. Introduction

Human disease specific biomarkers can be quantified in a variety of bodily fluids, including urine, saliva, cerebrospinal fluid and blood [1]. Among these, blood plasma is one of the most information-rich biological fluids owing to the fact that it is in continuous contact with all organs and it is easily accessible [1]. Thus, the analysis of plasma or serum for protein biomarkers is a vibrant research area owing to its inherent diagnostic value [2, 3], but these biological fluids also represent one of the most challenging proteomes to characterize as they contain thousands of proteins, which span a dynamic concentration range of more than 10 orders of magnitude.[4] The inherent difficulty that is associated with the separation of plasma into individual plasma proteins, however, can be dramatically reduced if plasma is analyzed for the metalloproteome, which refers to all plasma proteins that contain a bound transition metal [5]. This task can be achieved by analyzing blood plasma with size exclusion chromatography (SEC) and employing element-specific detectors. To this end, SEC coupled with an inductively coupled plasma atomic emission spectrometer (ICP-AES) has been recently employed successfully to analyze plasma (0.5 mL) for ~12 copper (Cu), iron (Fe) and zinc (Zn)-containing metalloentities [5, 6]. The latter biomolecules were qualitatively identified either by immunoassays, enzymatic assays or spiking with pure metalloprotein standards [7]. Thus, the analysis of plasma by SEC-ICP-AES represents a feasible approach that is complementary to conventional mass spectrometry-based proteomics methods to identify potential biomarkers [2, 8]. While SEC-ICP-AES allows to obtain diagnostically useful results, this particular instrumental analytical approach is not only costly in terms of the equipment, but also cumbersome in terms of the analysis and the conversion of the analytical raw data into plasma metalloprotein concentrations. Owing to these disadvantages it is desirable to identify

alternative instrumental analytical approaches, which may be adopted by clinical laboratories that routinely analyze blood plasma/serum for diagnostically relevant plasma proteins [9].

To identify an alternative instrumental analytical approach, we have investigated if the previously employed SEC-based separation of plasma proteins can be miniaturized and the ICP-AES replaced by a more cost-effective element-specific detector, namely a graphite furnace atomic absorption spectrometer (GFAAS). While GFAAS can only detect one element at a time, it requires less operator skills than an ICP-AES, requires small sample volumes for analysis (20-100 μ L) and perhaps most importantly operates in an off-line mode. It was therefore necessary to develop a suitable interface, which allowed for the introduction of SEC effluent fractions into the GFAAS in a controlled manner. Considering that a Superdex 200 HR SEC column (300 x 10 I.D. mm column) was previously used for the analysis of blood plasma for metalloproteins [7], we decided to use the same stationary phase, but smaller column dimensions (150 x 5 mm I.D. column) to analyze plasma for the two major iron metalloproteins. Transferrin (Tf, 79 kDa) is a classical Fe plasma protein (concentrations 1.78-3.72 g/L in adults) that plays an important role in the distribution of dietary Fe to organs, while a haptoglobin-hemoglobin (Hp-Hb) complex (~500 kDa) is formed in plasma after the rupture/lysis of red blood cells and the subsequent binding of the released Hb to the plasma protein Hp [10, 11]. The interaction between Hp and Hb is highly specific and has been exploited for the purification of Hp after the generation of a Hb-affinity column [12]. Therefore, the quantification of the Hp-Hb complex in human plasma is of inherent diagnostic significance as it allows to detect events that are directly linked to the lysis of red blood cells [13]. Our overall goal was to develop a robust and rapid SEC-GFAAS based method for the quantification of clinically relevant iron metalloproteins in human blood plasma.

2. Experimental

2.1 Chemicals

Phosphate buffered saline (PBS) powder, sodium hydroxide (>97%), human holo-Transferrin (Tf, >98%), deferiprone (DFP, 1,2-Dimethyl-3-hydroxy-4(1H)-pyridon, >98%) and Krebs-Ringer bicarbonate buffer powder were obtained from Sigma-Aldrich (St. Louis, MO, USA). Mixed type haptoglobin (2-1, >95%) was obtained from Athens Research and Technology (Athens, GA, USA). All aqueous solutions were prepared using DI water from a Simplicity water purification system (18.2 MΩ•cm, Millipore, Billerica, MA, USA) after filtration through 0.45-μm nylon-filter membranes (Mandel Scientific, Guelph, ON, Canada). PBS-buffered saline (10 mM phosphate, 2.7 mM KCL and 137 mM NaCl) was prepared by dissolving PBS powder in the appropriate volume of MQ water (1 packet per 1.0 L of MQ water). The pH of the mobile phase was adjusted to 7.4 with hydrochloric acid using a VWR Symphony SB20 pH meter (Thermo Electron Corporation, Beverly, MA, USA). A DFP stock solution was prepared by dissolving 20.25 mg in 1215 μL of PBS-buffer.

2.2 Blood plasma preparation

The collection of human blood from a healthy male volunteer (12 h fasting) was approved by the Calgary Conjoint Health Ethics Board (ethics approval REB15-1138-REN5). Blood was collected into trace metal grade heparinized vacutainer tubes (Becton Dickinson, Franklin Lake, New Jersey) and centrifuged at 3000 x g for 20 min (4 °C) to separate blood into plasma and erythrocytes. The supernatant plasma was pooled into a 150 mL Teflon container, gently mixed

and aliquots of plasma (1.0 mL or 2.0 mL) were stored in a liquid nitrogen Dewar. All experiments were conducted with this plasma stock.

2.3 Red blood cell (RBC) lysate preparation

Following the centrifugation of whole blood (3000 x g, 20 min, 4 °C) and the removal of plasma and the buffy coat, the pellet was washed twice with an equal volume of Krebs-Ringer buffer (9.5 g/L) and centrifuged (3000 x g, 10 min, 4 °C). The RBC's were lysed with an equal volume of deionized (DI) water (22 °C) followed by centrifugation at 20,000 x g for 60 min to remove cell debris. The clear lysate was pooled into a 150 mL Teflon container, gently mixed and aliquots of the lysate (1.0 mL or 2.0 mL) were flash frozen and stored in a liquid nitrogen Dewar.

2.4 Preparation of the Hp-Hb complex

The Hp-Hb complex was prepared by reconstituting 1.0 mg of haptoglobin (Hp) with 300 µL of DI water. After adding 4 µL of RBC lysate to 850 µL DI water, this mixture was added to 150 µL of the Hp solution. This solution was injected on a system comprised of size-exclusion chromatography (SEC, Superdex 200 Increase SEC column (300 x 10 mm I.D.) coupled on-line to an inductively coupled plasma atomic emission spectrometer (ICP-AES). Iron-specific detection allowed to detect the Hp-Hb complex and ~1800 µL fractions thereof were collected. The collected fractions were then concentrated 9-fold using Centricon tubes (30 kDa cut-off, Merck Millipore, Burlington, Massachusetts) using a 5804R centrifuge (Eppendorf, Germany) operated at 4000 rpm for 7 min.

2.2 Instrumental System

The SEC-GFAAS system was comprised of a Agilent 1200 HPLC pump, a 50 μ L PEEK injection loop, an interface, a SuperdexTM 200 Increase High Resolution SEC column (150 mm x 0.5 cm I.D., GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA; particle size: 8 μ m; fractionation range ~600-10 kDa) and a MGA-1000 graphite furnace atomic absorption spectrometer (GFAAS, Lumex Instruments, Mission, BC, Canada), which offers a detection limit for Fe of 1 ppb (40 μ L sample volume). The mobile phase was a PBS-buffer (pH 7.4), which was delivered at a flow rate of 0.45 mL/min or 0.10 mL/min. Iron (Fe) was detected in the SEC effluent at 372.0 nm using a hollow cathode lamp operated at 8 mA. The photomultiplier tube was 500 V.

The interface that allowed to hyphenate the SEC system with the GFAAS was machined from Nylon Plastic 6/6 obtained from McMaster Carr (Elmhurst, IL, USA). The diameter of the interface was chosen to fit into the GFAAS autosampler tray. The round extension on the bottom of the nylon interface fit snugly into a hole on the autosampler bottom plate and ensured stability during the analysis. The actual dimensions of the interface are depicted in Figure 1. The SEC effluent was fed into the bottom of the well of the interface and the liquid was removed with a water aspirator (Figure 1). In this manner, the well volume was maintained at 61 μ L during the analysis and the GFAAS sampling arm was able to consecutively withdraw samples for analysis.

The autosampler arm of the GFAAS transferred every 120 s 40 μ L of the column effluent from the well of the interface into the graphite cuvette (Schunk Carbon Technology, Germany). Argon (99.999%) was used as a sheath gas (0.7 L/min) and the Ar flow rate was reduced to 0 during the atomization step. The injected aliquots were dried at 130°C for 36 s, atomized at 2100°C for 2 s, cleaned at 2500°C for 2 s and then cooled down for 60 s before the next sample could be analyzed.

All analyses (pure metalloproteins or hemoglobin-spiked and unspiked human plasma) were executed in triplicate. The atomic absorption units (AU) that were obtained for an individual metalloprotein injection were first baseline corrected by subtracting the average absorption that was obtained for the analysis of PBS-buffer (n=3), then added up and the standard deviation from three replicate analyses is reported. The baseline absorption varied with each graphite tube, ranging between 0.004-0.007 AU.

3. Results and Discussion

The quantification of biomarker proteins in biological fluids is useful for diagnostic/pathological purposes and for therapeutic monitoring [4, 14, 15]. The analysis of blood plasma for specific proteins is particularly relevant, but requires powerful analytical methods that offer the required selectivity, sensitivity, are cost efficient and require a small sample volume. To date, human blood plasma has been most frequently analyzed for metalloproteins by SEC-ICP-MS [16-20], 2-D PAGE-LA-ICP-MS [21, 22], as well as SEC-ICP-AES [5]. The latter technique has been recently applied for the detection of a Hp-Hb complex in human plasma [7]. SEC-ICP-AES offers the advantage that a PBS-buffer – which resembles the salinity of blood plasma – can be used as the mobile phase, which contributes to leave potentially labile plasma protein-metal bonds intact during the analysis [5]. While non-transferrin bound iron in human blood plasma has been quantified by GFAAS after its separation from iron metalloproteins using ultrafiltration [23], HPLC-GFAAS has only infrequently been used for the quantification of selenium [24] and arsenic species [25, 26] in environmental samples, such as mushrooms. We established a SEC-GFAAS system to demonstrate its feasibility for the direct analysis of human plasma (50 μ L) for the two major Fe metalloproteins, namely the Hp-Hb complex and Tf [7].

198 Considering that GFAAS requires a minimum total analysis time of 120 s for a 40 μ L sample
199 volume (this involves a drying, an atomization and a cleaning step), it was of the utmost importance
200 to carefully tailor the SEC flow rate to the GFAAS sampling rate to avoid missing the detection of
201 an entire iron metalloprotein peak. We used pure Fe metalloproteins to develop a suitable SEC
202 ‘flow rate regime’, which was a prerequisite to achieve a baseline separation of the Hp-Hb complex
203 and Tf. To this end, we employed the flow rate that was recommended by the manufacturer to
204 separate the pure Fe metalloproteins and thereafter the flow rate was decreased to a value that
205 allowed to detect each metalloprotein by more than one GFAAS signal in the SEC effluent. The
206 optimized overall ‘flow rate regime’ involved the injection of a sample using a flow rate of 0.45
207 mL/min for 165 s, followed by a decrease to 0.10 mL/min. After a 15 s delay, GFAAS analysis
208 was initiated and 15 consecutive measurements were made to obtain the Fe-specific
209 chromatograms. All results were carried out with the developed SEC-GFAAS system and the
210 optimized ‘flow rate regime’ and all obtained cumulative Fe signal intensities are depicted in Table
211 1.

212 To develop the desired metalloprotein separation, we first injected pure Tf and observed a Fe signal
213 cluster with a peak maximum at 15 min (Figure 2A, blue line, GFAAS signal cluster between 11-
214 21 min). To try to rationalize these results, we injected red blood cell lysate, which predominantly
215 contains the Fe metalloprotein hemoglobin (Hb) and detected a Fe signal cluster with a peak
216 maximum at 15 min (Figure 2A, red line, GFAAS cluster between 13-19 min). These retention
217 times confirm that reliable results were obtained, as Tf and Hb have rather similar sizes of 79 kDa
218 and 66 kDa, respectively. We then injected a pure Hp-Hb complex (see Experimental) and detected
219 a Fe signal cluster with a peak maximum at 9 min (Figure 2A, black line, GFAAS signal cluster
220 between 7-13 min). The comparatively shorter retention time of this Fe-peak demonstrates that

221 the Hp-Hb complex has a larger hydrodynamic radius than Tf and Hb. The superimposition of the
222 Fe-specific chromatograms that were obtained for the Hp-Hb complex and Tf peak (Fig. 2A, blue
223 and black line), however, clearly indicates that no baseline separation between these
224 metalloproteins is achievable under these conditions.

225 To tackle this separation problem, one may take advantage of the fact that Fe^{3+} is reversibly bound
226 to Tf [27] and that chelating agents, such as deferiprone (DFP), have previously demonstrated to
227 mobilize this metal from its binding pockets [28]. Thus, the addition of DFP to Tf should result in
228 the formation of a significantly smaller Fe species [i.e. a $(\text{DFP})_3\text{Fe}$ complex], which should elute
229 with a longer retention time on a SEC column compared to Tf and thus result in the desired baseline
230 separation between the Hp-Hb complex and 'Tf'. A prerequisite to achieve this 'peak shift',
231 however, is to quantitatively transform the Tf-bound Fe^{3+} into a $(\text{DFP})_3\text{Fe}$ complex. We therefore
232 added DFP (200 μg) to Tf (600 μg)(see Experimental) and investigated if a shift in the elution of
233 an iron peak can be achieved by ultrasonication for 10, 20 and 30 min. This attempt resulted in a
234 rapid increase of the water bath temperature to $>60^\circ\text{C}$, which is undesirable as the conditions to
235 achieve this 'peak shift' cannot be adequately controlled. Since the incubation of the DFP/Tf
236 mixture for up to 60 min at room temperature did not shift the Fe-peak to longer retention times,
237 we incubated the DFP/Tf reaction mixture in a water bath to 50°C and analyzed the mixture at
238 different time points (Fig. 3A-E). These results revealed that a 15 min incubation time was
239 sufficient to shift the retention time of Tf to longer retention times, which indicated that a $(\text{DFP})_3\text{Fe}$
240 complex had formed [28].

241 The developed sample preparation protocol was tested with pure Tf and revealed a Fe signal cluster
242 with a peak maximum at 17 min (Figure 2B, blue line, GFAAS cluster between 15-25 min), which
243 clearly demonstrates an essentially quantitative mobilization of Fe from Tf to a $(\text{DFP})_3\text{Fe}$ complex.

244 We also applied this sample preparation protocol to dilute red blood cell lysate (i.e. Hb), which
245 yielded the Fe-specific chromatogram depicted in Figure 2B (red line). The Fe signal cluster
246 (GFAAS cluster between 13-23 min) had a cumulative signal intensity of 0.300 ± 0.046 AU and
247 clearly indicated the presence of two Fe entities, with the first one corresponding to Hb (Figure
248 2A, red line), which had another Fe signal cluster on its long retention end. We suspected that this
249 latter Fe peak corresponds to a $(\text{DFP})_3\text{Fe}$ complex that was formed by DFP sequestering trace Fe
250 from the stationary phase. To test this hypothesis we injected DFP (200 μg) in 50 μL of PBS-
251 buffer and obtained the results depicted in the inset of Figure 2B. The obtained Fe signal cluster
252 had a peak maximum at 19 min (GFAAS cluster range 17-25 min) and a cumulative Fe signal
253 intensity of 0.064 ± 0.009 AU. These results demonstrate that the increased cumulative signal
254 intensity that was observed for the Hb-DFP mixture (0.300 ± 0.046 AU) can be rationalized by the
255 Hb peak (0.231 ± 0.003 AU) and the mobilization of Fe in from of a $(\text{DFP})_3\text{Fe}$ complex from the
256 stationary phase (0.064 ± 0.009 AU)(Table 1). We rationalize the higher than expected cumulative
257 Fe signal intensity for the Tf/DFP mixture (0.686 ± 0.074 AU) was due to a column cleaning
258 procedure that was performed prior to these measurements.

259 The developed sample treatment protocol was also applied to the Hp-Hb complex to confirm that
260 DFP does not adversely affect its integrity. After exposing a DFP/HP-Hb complex mixture for 15
261 min to 50°C , its analysis revealed two Fe signal clusters with peak maxima at 9 min and 17 min
262 (Figure 2B, black line, GFAAS clusters between 7-13 and 17-23 min). Since the cumulative Fe
263 signal intensity of 0.173 ± 0.015 AU can be rationalized by the addition of the corresponding value
264 for the Hp-Hb complex (0.113 ± 0.022 AU) and that of the Fe sequestered from the column
265 (0.064 ± 0.009 AU), the first Fe signal cluster corresponds to the Hp-Hb complex.

Since the aforementioned results imply that it should be possible to achieve a baseline separation of these Fe metalloproteins, we wanted to investigate if the developed method is amenable to the analysis of human plasma. We first injected human plasma (1.0 mL) that was spiked with 1.0 μ L of DI water (control), followed by human plasma (1.0 mL) that was spiked with 1.0 μ L and 2.0 μ L of red blood cell lysate (i.e. Hb), respectively. The spiking of human plasma with red blood cell lysate may be regarded as the ‘simulation of a hemolytic event’. Representative Fe-specific chromatograms of these experiments are depicted in Figure 4 and the cumulative Fe signal intensities in Table 2. The analysis of unspiked human plasma revealed the presence of the Hp-Hb complex and Tf (Fig. 4, black signals), which was expected based on previous results [7]. Spiking of plasma (1.0 mL) with 1.0 or 2.0 μ L of red blood cell lysate resulted in an increase of the Fe peak intensity corresponding to the Hp-Hb complex in a dose dependent manner ($R^2=0.9899$), while the Fe peak intensity corresponding to Tf did not change (Table 2). Using the Hb reference concentration in red blood cells of ~ 5.0 mM, the intensity of the Fe peak corresponding to the Hp-Hb complex in unspiked plasma corresponds to a Hb concentration of 0.40 μ M. The larger standard deviation of the Tf peak (Table 2) is attributed to the fact that this plasma protein co-eluted with human serum albumin (HSA) and that the employed GFAAS temperature program could not completely remove this protein (which constitutes the matrix) prior to the atomization step. While ferritin is another iron metalloprotein that can be present in human blood plasma, its concentration is too low to be detectable in 50 μ L with our developed SEC-GFAAS method [7, 29].

Human blood plasma is routinely analyzed by clinical biochemistry laboratories for a large number of plasma proteins as abnormal concentrations can provide important clues into the health status of a patient [9]. A variety of analytical methods that are based on spectrophotometry, immunoassay-based methods, and gel electrophoresis can be employed to quantify free Hp in

human plasma [30] where it is present at concentrations between 0.27-2.46 g/L in males and 0.45-2.37 g/L in females (age 21-50)[9]. An abnormally low Hp plasma concentration can indicate the presence of a variety of disease states in patients, such as severe liver disease, red blood cell defects, genetic anhaptooglobinemia [9] or that a hemolytic event has occurred [13] since the rupture of red blood cells will release Hb, which will rapidly react with free Hp to form a Hp-Hb complex in plasma [30] that is then sequestered by macrophages [31]. The development of an affordable instrumental analytical method that can quantify the Hp-Hb complex in plasma is therefore of direct clinical relevance as the plasma concentration of this biomarker allows to infer that specifically a hemolytic event (e.g. stroke or paroxysmal nocturnal haemoglobinuria [13]) has occurred. The developed SEC-GFAAS based detection of a Hp-Hb complex in human plasma (Fig. 4) represents the first application of this hyphenated approach to quantify a clinically relevant iron metalloprotein in a biological fluid. In contrast to SEC-ICP-AES, the analysis of human plasma by SEC-GFAAS is less cumbersome and the baseline corrected Fe signals that correspond to the Hp-Hb complex need to be added and converted into the corresponding plasma metalloprotein concentration using a calibration curve (Table 2).

4. Conclusion

The complexity that is inherently associated with the analysis of the plasma proteome can be overcome by analyzing plasma for metalloproteins. Since the previously reported SEC-ICP-AES method required a sample volume of 500 μ L, we set out to develop an alternative analytical method that required less sample volume. Based on using a shorter SEC column (150 x 5 mm I.D.) to accomplish the separation of the desired metalloproteins, we employed a GFAAS as the Fe-specific detector and designed an appropriate interface to establish an integrated SEC-GFAAS

system. While the latter system required only 50 μ L of plasma for analysis, the baseline separation of the Hp-Hb complex and Tf required the quantitative conversion of Tf-Fe into a smaller (DFP)₃Fe complex which was baseline separated from the Hp-Hb complex. The developed sample treatment protocol was applied to analyze human plasma. The results revealed that unspiked human plasma contained a Hp-Hb complex and Tf, whereas the addition of red blood cell lysate to human plasma resulted in a dose dependent increase of the Fe peak that corresponded to the Hp-Hb complex, while the intensity of the Tf-peak remained unchanged. Thus, the developed integrated SEC-GFAAS system can now be used to quantify the Hp-Hb complex in plasma, which has important diagnostic ramifications since a variety of human diseases can result in the lysis of red blood cells [11, 13]. The developed methodology is of potential interest to clinical laboratories since it has been previously demonstrated that HPLC-GFAAS systems can be computer controlled to obtain fully automated analysis systems [24, 32]. Considering that the exposure of red blood cells to Hg²⁺ and Cd²⁺ has been demonstrated to contribute to their lysis [33, 34], the analysis of human plasma for the Hp-Hb complex may provide insight into the exposure of human populations to toxic metals [35], which is projected to affect more people in the future [36, 37].

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Figure Captions:

Figure 1 Conceptual drawing of the interface/flow through cell (well volume: 61 μ L) that was machined from Nylon Plastic 6/6 and enabled the integration of the SEC system with the GFAAS. All dimensions are provided in mm.

Figure 2 Representative superimposed SEC-GFAAS derived Fe-specific chromatograms (baseline corrected) obtained for the analysis of pure metalloproteins (A) and pure metalloproteins having undergone the optimized DFP sample preparation procedure (B). Aqueous solutions of pure hTf, dilute red blood lysate (i.e. Hb) and a purified Hp-Hb complex were analyzed individually. Column: Superdex™ 200 Increase HR SEC column (150 mm x 5 mm I.D.), Flow rate: 0.45→0.10 ml/min, Injection volume: 50 μ L, Detector: GFAAS at 372 nm, GFAAS sample volume: 40 μ L.

Figure 3 Representative SEC-GFAAS derived Fe-specific chromatograms (baseline corrected) obtained for the analysis of a Tf/DFP mixture that had been incubated at 50°C for 0 min (A), 5 min (B), 10 min (C), 15 min (D) and 20 min (E). Column: Superdex™ 200 Increase HR SEC column (150 mm x 5 mm I.D.), Flow rate: 0.45→0.10 ml/min, Injection volume: 50 μ L, Detector: at 372 nm, GFAAS sample volume: 40 μ L.

Figure 4 Representative SEC-GFAAS derived Fe-specific chromatograms (baseline corrected) obtained for the analysis of human plasma (A), human plasma (1.0 mL) spiked with 1 μ L of red blood cell lysate (B) and human plasma (1.0 mL) spiked with 2 μ L of red blood cell lysate (C). Column: Superdex™ 200 Increase HR SEC column (150 mm x 5 mm I.D.), Flow rate: 0.45→0.10 ml/min, Injection volume: 50 μ L, Detector: GFAAS at 372 nm, GFAAS sample volume: 40 μ L.

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