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Trace ^{56}Fe, $^{63/65}$Cu and ^{64}Zn metal analysis in whole blood by SEC-HPLC-ICPMS

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Abstract

Trace metals have important biological roles to play within the human body. This work investigated the presence of ^{56}Fe, $^{63/65}$Cu and ^{64}Zn in blood and utilised dried blood spot sampling (DBS) technology. Blood was taken from one female subject and extraction conditions were optimised. Extracted samples were analysed by SEC-HPLC for protein separation and SEC-HPLC-ICP-MS for metal detection. Proteins from the blood samples separated via SEC-HPLC were found to be haemoglobin, myoglobin and transferrin; SEC-HPLC-ICPMS metal determination saw correlated peaks of ^{56}Fe, $^{63/65}$Cu. However, ^{64}Zn peaks were much weaker and could therefore only be tentatively identified.

Introduction

Blood diseases are very common amongst the world's population, with anaemia being one of the world's most common health disorders in itself and also as a result of another illness such as gastrointestinal cancer, chronic kidney disease, and others. Anaemia is associated with the deficiency of iron. In order to be able to diagnose blood diseases such as anaemia, blood samples must be taken and subsequently analysed. Conventionally this is done via venepuncture – the process of extracting blood from the veins of a patient via the use of a needle. This procedure is invasive and can be painful, especially for young children and the elderly and must be carried out by properly trained persons. Additionally, the process carries potential dangers to the healthcare professional conducting the procedure as patient's blood may be infectious and pose a risk to their own health (Bishop, 2009) [1].

Over the past decade, the use of dried blood spots has grown in clinical settings (Déglon et al., 2012) [2]. The sampling process involves drops of capillary blood collected onto filter paper or card via a simple finger or heel prick facilitating a minimally invasive alternative from venous sampling. This allows samples to be taken in non-clinical environments by the patient themselves at home or by less extensively trained technicians, this therefore would be a less expensive service for the NHS and other healthcare providers to use. The procedure requires only small sample volumes (40 µL was used in this investigation) compared to the volume required for venous blood samples which is approximately 5 mL although this larger volume facilitates the running of several clinical tests, not solely compound determination discussed in this research. DBS samples have increased stability, can be stored at room temperature, shipped more easily, do not require any anticoagulants and reduce risk of infected pathogens such as Hepatitis C and HIV/AIDS [3].

Materials and Methods

Sample Collection

The integrity of the blood sample can be adversely affected by the sampling procedure so care must be exercised through the sampling process. The use of alcohol wipes to sanitise the local site of sampling on the patient is one common practice utilised to obtain a high quality samples. The hands of a healthy, 38-year-old, female subject were sanitised before sample collection and Unistik 3 Extra lancets (Owen Mumford; Oxford, UK) were used to pierce the skin of the subject's finger and samples were collected in 40 µL Lithium Heparin capillary tubes (Cholestech LDX; Alere North America). The collected blood samples were then spotted onto Perkin Elmer 226 Spot Saver (Perkin Elmer, Greenville, South Carolina, USA) cards via capillary plungers. Capillary tubes were used to allow the acquisition of accurate and consistent sample volumes rather than applying drops of blood sample directly to the spot saver card. Spots were left to dry for 24 hours with a continuous ambient air flow on both the front and the back of the card as can be seen in *Figure 1*.

Experiments were all performed in accordance with the Bioscience Research Ethics review group at Sheffield Hallam University.

Figure 1. Blood spots drying

DBS Sample Preparation and Extraction

Blood spots were cut from the spot saver cards using metal scissors as no ceramic scissors were available at the time. It should be noted that commercial punches are available that facilitate automation of this process, not accessible during this project. Blood spot discs were added into universal tubes containing 2 mL of ultra-pure deionised water and placed into a 40°C water bath for 60 minutes. Optimisation of this process was undertaken to ensure that recoveries were optimum and that no denaturing occurred; several time and temperature combinations were trialled and this was found to be the most suitable (data not shown). After tubes were removed from the bath, samples were left to cool at room temperature before discs were removed using tweezers. Samples were then ready for analysis. At each step of this process, control samples were prepared using blank cards.

Protein Standards

Gel marker filtration kit (mw 12,000 – 200,000 Da, MWGF200), ferritin from equine spleen (Type 1 in saline solution), myoglobin from equine heart, human haemoglobin (lyophilised powder) and transferrin bovine were all obtained from Sigma Aldrich, UK. Gel marker filtration kit and myoglobin stored at -20°C, all others stored at 4°C.

Chemicals and Reagents:- NaCl (laboratory reagent), Sodium diphosphate monobasic (NaH_2PO_4), Sodium diphosphate dibasic (Na_2HPO_4), Fe, Cu and Zn 1000 ppm standards (in nitric acid) were all obtained from Fisher Scientific, UK.

SEC-HPLC Separation

The SEC-HPLC analysis was carried out [4] using a Perkin Elmer HPLC system composed of a series 200 pump, series 200 vacuum degasser and series 200 UV/Vis detector with a 20 µL injection loop. The chromatographic column used was

the Tricorn™ Superdex 200 10/300 GL high performance column (GE Healthcare; bed dimensions; 10 x 300 -310 mm, column volume 24 mL; particle size 13 µm).

The column was first calibrated using gel marker filtration kit (mw 12,000 – 200,000 Da) which is a standard protocol for SEC separation. The column was further calibrated with 4 metal containing proteins: haemoglobin, myoglobin, transferrin and ferritin. SEC-HPLC analysis was performed under the following conditions. Flow rate 0.5 mL/min, mobile phase composed of 50 mM $Na_2HPO_4.2H_2O$ and 0.15 M NaCl adjusted to pH 7.2 with NaCl. Proteins were detected by UV/Vis detector at 280 nm. Column pressure was between 120-130 psi to allow for additional back pressure in the system (column max 217 psi). Ambient lab temperatures were recorded daily (as is common practice by students working in our laboratories), temperatures ranged from 21-29°C throughout the course of analysis, however it should be noted that SEC systems operate in such a way that temperature does not affect retention times. Figure 2 shows the 4 metal containing proteins used in this investigation.

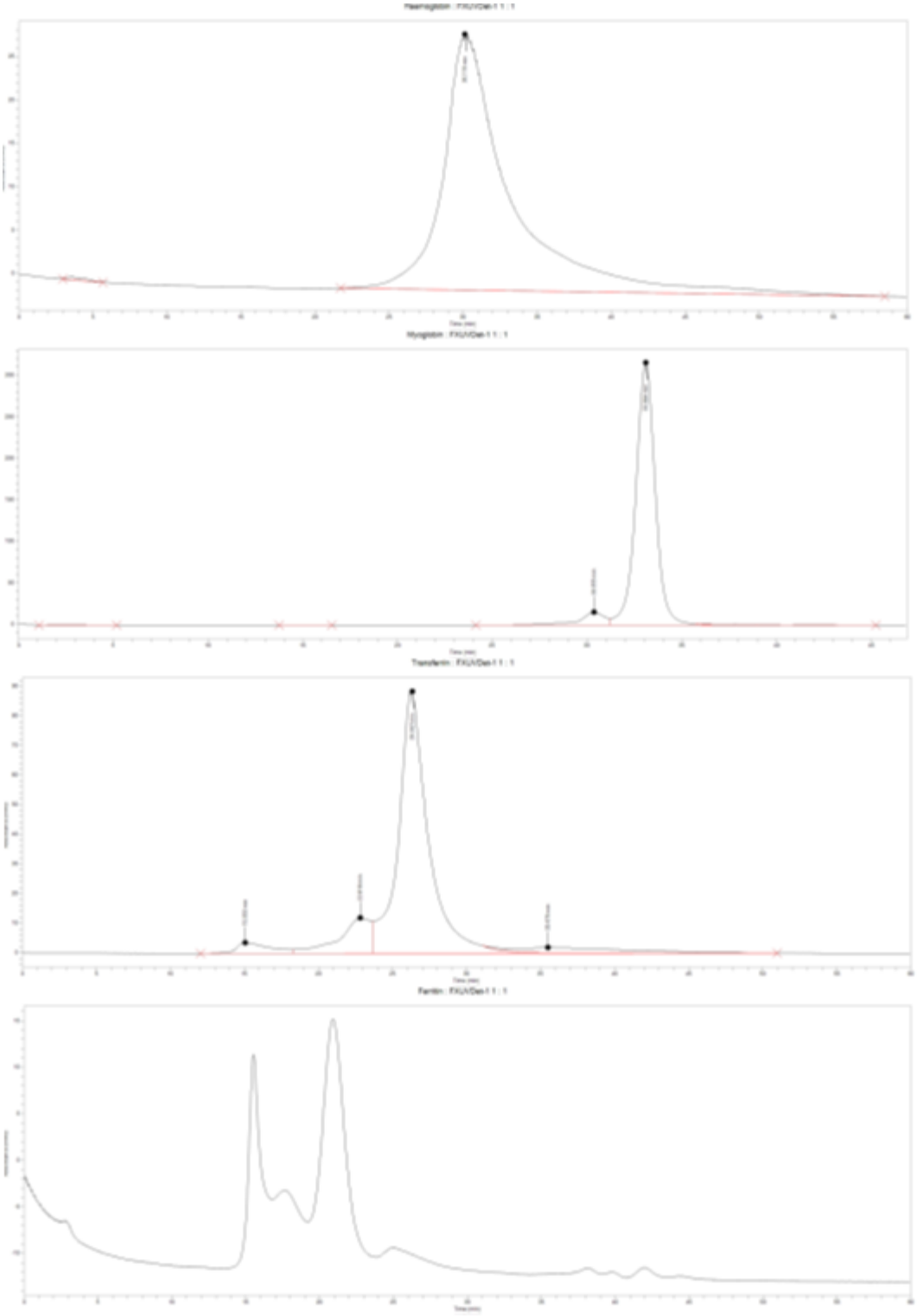

Figure 2. From bottom to top, chromatograms of Haemoglobin, Myoglobin, Transferrin and Ferritin.

ICP-MS analysis

Analysis of all samples and standards [5] was performed on a Perkin Elmer NexIon 350X ICP-MS, in KED mode, tuned using NexIon Setup Solution (Perkin Elmer, UK). Fe, Cu and Zn standards were produced and diluted in 1% HNO_3 to the following concentrations: 0, 0.05, 0.1, 0.5, 1 ppm.

SEC-HPLC-ICPMS analysis

SEC-HPLC was hyphenated to the ICP-MS instrument previously discussed using the same conditions, using Syngistix software. Protein standards, blood extracts and control samples were analysed.

Results & Discussion

This section is composed of results acquired during method development procedures as well as genuine investigative results.

SEC-HPLC Method Development

Gel Filtration Marker Kit Calibration

Conditions initially used for SEC-HPLC analysis were 50 mM phosphate buffer + 0.15M NaCl at pH 7.2 with a flow rate of 0.5 mL/min and a run time of 180 minutes, as the time for protein separation was unknown under these specific conditions. To test the separation capabilities of the column and gauge the separation time of the proteins, proteins from a gel marker filtration kit (mw 12,000 – 200,000 Da, MWGF200 Sigma) were separated on the column. Duplicate runs were performed for all protein separations in this analysis.

The smallest molecular weight protein in the kit, Cytochrome C, was analysed first to approximate how long run times would be. Following this the largest molecular weight protein was run followed by all other proteins to determine each individual retention time. In this case, the void volume was calculated using blue dextran. This was prepared to a concentration 1 mg/mL. A combined mixture of standards was also prepared and run.

Metal-containing Proteins Calibration

All protein standards were first run separately in duplicate to determine individual retention times. Set concentrations were used for proteins as a means to produce peaks of similar height. The concentrations of the myoglobin, transferrin and haemoglobin standards were 3 mg/mL, while the standard of ferritin, which has a

higher rate of absorbance, was prepared at a concentration of 0.3 mg/mL. Myoglobin and Cytochrome C are close in molecular weight and could not be separated under these conditions.

Dried Blood Spot Extraction Method Development

Blood spots were extracted under varying conditions of time and temperature as well as in different solutions to determine an optimal procedure. However, all early trial methods gave satisfactory extraction results and therefore, all subsequent extractions were done in 2 mL of water at 40°C for 60 minutes. All control samples were subject to the same conditions.

DBS SEC-HPLC

The SEC-HPLC conditions described in the standard analysis in Section 3.1 were utilised for this analysis of the DBS samples. Duplicate runs were done for all separations in this stage.

All dried blood spot samples extracted were separated via SEC-HPLC to determine the metalloproteins present and also to determine if the varying extraction conditions had an effect on separation results. From the retention times of the proteins separated in the samples, the proteins within the blood samples were identified as transferrin and haemoglobin. It was observed that the different extraction conditions did not affect the results of separation in the DBS samples. All samples show the same two proteins at similar retention times. *Figure 3* illustrates these chromatograms. The two peaks present in the ferritin chromatogram can be attributed to heavy and light forms of ferritin being simultaneously present in the sample. Light and heavy ferritin have molecular weights of 19 kDa and 21 kDa respectively.

Figure 3. from left to right shows the SEC-HPLC chromatograms for Myoglobin, Haemoglobin, Transferrin, Ferritin and an extracted blood sample. Retention times of characteristic peaks were 33.09, 30.19, 26.34, 14.93 and 19.61, 29.93 minutes respectively.

DBS SEC-HPLC-ICP-MS

Using samples from the extraction method (including a blank for Fe, Cu and Zn) SEC-HPLC-ICPMS was performed (*Figure 3)*. The software on the instrument allowed detection of all 3 metals simultaneously. Peaks containing Fe and Cu were detected. However, Zn analysis yielded lower than expected results.

Due to residue build-up on the ICP-MS cones throughout the investigation, the concentration of buffer was dropped from 0.5 mM to 0.2 mM for a trial period. However, as this made minimal difference, the original 0.5 mM buffer was used for the remainder of the investigation and a proportion of the flow was diverted at the start. In future, different types of buffer could be trialled in order to minimise wear and tear on the instrument.

It is not clear why Fe-containing protein peaks (e.g.; myoglobin) also illustrated presence of Cu (*Figure 4)*. Many proteins can bind copper non-specifically through histidine residues, so there may have been contamination during the analyses allowing myoglobin to pick copper up, potentially from the buffer.

Figure 4. SEC-HPLC-ICP-MS chromatograms for Myoglobin and Haemoglobin including Fe, Cu and Zn. Additionally, separate profiles are included for Fe, Cu and Zn traces within an extracted blood sample

Conclusion

The DBS extraction procedure in this investigation proved to work well, with all blood being *visually* extracted from spot card. The volume of blood used (40 µL) proved to be enough to gain promising results in both the SEC-HPLC and SEC-HPLC-ICP-MS analysis. The conditions used for SEC-HPLC analysis generally provided good protein separation across all samples and identification of all proteins separated in this analysis were made.

Overall, this investigation using SEC-HPLC in conjunction with ICP-MS proved to be a useful and promising technique for detecting the metals Fe and Zn in dried blood spot samples as well as for the identification of the metalloproteins the metals are associated with, by retention time.

Future work to better this investigation could start with trialling different buffers for SEC-HPLC analysis.

In order to assess the scope of this method in comparison to whole blood samples, the investigation should be repeated using whole blood samples to provide comparison against the DBS samples.

With regards to SEC-HPLC-ICP-MS, the analysis could be trialled with an increased injection volume, such as 1mL to increase the response. Additionally, analysis of more Zn and Cu binding/containing proteins would be useful to expand further possibilities for investigation.

A simple and ethically approved study could be carried out over a longer period of time on individuals suffering from disorders related to metalloproteins such as anaemias [6]. This would allow investigation into protein changes in response to treatments such as medication, chelation therapy or diet alterations.

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