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

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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-ICP-MS 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 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 Carolina, USA) were used to 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.

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 phosphate monobasic (NaH₂PO₄), Sodium diphosphate dibasic (Na₂HPO₄), 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 Na₂HPO₄ •2H₂O and 0.15 M NaCl at pH 7.2 with a run time of 180 minutes, as 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.

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 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.

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.

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

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 Cu in dried blood spot samples as well as for the identification of the metalloproteins the metals are associated with, by retention time.

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.

References/ Bibliography


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