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Quantification of proteins in whole blood, plasma and DBS, with element-labelled antibody detection by ICP-MS

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Keywords: ICP-MS, ELISA, whole blood, element-labelled antibodies

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Abstract

Over recent years, quantification of multiple proteins in body fluids has become increasingly prominent, which is beneficial to a number of scientific fields, not least biomedical. Several techniques have been developed based on conventional ELISA; one of these techniques is analysis of proteins labelled with element-tagged antibodies by ICP-MS in serum, allowing quantification of multiple targets within a single sample. This research aimed to quantify albumin and immunoglobulin G (IgG) levels in plasma, whole blood and dried blood spots using NANOGOLD® and Europium labelled antibodies analysed by ICP-MS. Before the proteins were quantified simultaneously, albumin and IgG concentrations were measured separately and compared to protein levels obtained by ELISA. It was found that protein concentrations for both albumin and IgG obtained with element-labelled antibody detection correspond to those determined by ELISA. Furthermore, albumin and IgG levels measured simultaneously by ICP-MS correspond to concentrations found when the proteins were analysed separately by ICP-MS. Finally, development of this method has provided a positive indication that it can be extended to quantification of additional proteins, which could be related to a disease or as a minimum provide additional information for a protein profile of an individual.

1.0 Introduction

Over the last few decades biological markers have become more prominent for diagnosis of diseases [1,2]. A biological marker or biomarker is a characteristic found in biological fluids or tissues that can be measured objectively and evaluated as a sign of normal or abnormal biological processes [2–4]. Biomarkers can be divided in two types: biomarkers of exposure and biomarkers of disease [1]. Biomarkers of exposure are used to predict the risk of developing a disease, especially when the disease is toxin or chemical based. The second type, biomarkers of disease, are often used in screening, diagnosis and monitoring of disease progression. Although protein quantification is more common in clinical diagnosis, it is used in other disciplines as well - for example forensic science. Proteins can provide information about anthropometrical measures of an individual or identify life style factors that influence ageing of an individual [5]. Furthermore, a post-mortem interval estimation can be given by proteins that are involved in cellular changes in blood after death [6]. Although a biomarker can be used to detect difference in biological fluids and therefore the human body, analyses of a single protein is often not sufficient due to limited specificity and sensitivity of biomarkers for clinical diagnosis [7].

One of the most common techniques used for protein quantification in biological fluids is Enzyme-Linked ImmunoSorbent Assay (ELISA) [8]. An ELISA quantifies proteins using antibodies and enzyme-substrate complexes that induce colour formation, which can be detected by a microplate reader [9]. Unfortunately, ELISA is restricted to quantification of a single protein per analysis and to quantify multiple biomarkers in one sample multiple ELISAs must be running simultaneously leading to an increase in cost and time. To enable simultaneous analyses of multiple proteins several multiplex ELISA techniques have been developed, such as dissociation-enhanced lanthanide fluorescence immunoassay (DELFIA™; PerkinElmer, Boston, USA), Quantibody: Multiplex ELISA Array (RayBiotech, Norcross, GA), Luminex™ (Luminex Corporation, Austin TX, USA) and Bio-plex® Multiplex Immunoassays (BioRAD, Hemel Hempstead, UK). Both Luminex™ and Bio-plex® systems employ colour coded beads where primary antibodies for one specific protein are attached to one colour. The secondary antibodies are labelled with a fluorescent dye and the beads are detected with flow cytometry. The Quantibody and DELFIA™ systems make use of both antibody detection and fluorescence, where Quantibody uses a streptavidin-conjugated fluorescent label, and DELFIA™ labels antibodies with fluorescent lanthanides. Other research has focused on using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) alone or in combination with Laser Ablation (LA) or Flow Cytometry (Mass Cytometry) for detection of multiple proteins with element-tagged antibodies, which opens a variety of possibilities for protein quantification in biological fluids [3,7,10–14]. ICP-MS analyses elements by nebulizing the sample into plasma, which ionizes the elements in the sample, after which they are separated by mass spectrometry based on their mass to charge ratio [15]. Differences between ICP-MS, LA-ICP-MS and Mass Cytometry can be found in sample introduction, such as a nebulizer for ICP-MS and a laser for LA-ICP-MS, and sample preparation, such as the production of single-cell suspensions for Mass Cytometry and liquid samples for ICP-MS samples [10,15]. Mass Cytometry is a variation of Flow Cytometry combined with ICP-MS and separates single cells by labelling multiple internal and external markers with element-tagged antibodies instead of labelling these markers with fluorophore dyes, which are used in Flow Cytometry [10]. With Mass Cytometry it is possible to simultaneously analyse up to 31 different markers, which shows the applicability of ICP-MS for

element-tagged antibody detection [11,16]. In comparison to other immunoassay techniques, ICP-MS has additional advantages, such as detection limits of around 1 part per trillion (ppt) for almost every element and a linear dynamic range greater than 7 orders of magnitude [3,11]. Furthermore it is possible to simultaneously analyse eight proteins with LA-ICP-MS by labelling the proteins with antibodies tagged with eight different lanthanides [11].

Sample sources used in publications about analysis of multiple proteins are human serum and cell lysates of several cell lines. However, for biological analysis in clinical diagnosis or therapeutic drug monitoring human serum and plasma obtained by venepuncture are often used [17,18]. Over recent years more interest has developed in the collection of blood by the finger prick method, after which blood drops are collected on a filter paper and dried, to produce so-called 'dried blood spots' (DBS). This method is based on the heel prick method used for newborn screening in which the DBS are analysed for more than 50 treatable inborn disorders [19,20]. The collection of blood with a finger prick instead of a venepuncture is less invasive and the collection of DBS provides other advantages, such as lower costs for storage, analyte stability and deactivation of pathogenic agents by the filter paper [17]. Another field that benefits from the growing development in the analysis of DBS is forensic sciences; for example the detection of drugs of abuse [21]. Furthermore, it is possible to collect basic anthropometrical measures for an individual from DBS [5].

Although multiple protein quantification methods are developed for Mass Cytometry and LA-ICP-MS, there is still much to gain in method development for multiple protein quantification with ICP-MS. Several publications can be found on element-tagged antibody detection analysed with ICP-MS, which confirm the possibility of quantifying proteins with this mass spectrometry technique [3,13,14,22]. Here we present in a single manuscript a novel proof of concept study to develop a method for simultaneous quantification of proteins in human plasma, whole blood and dried blood spots (DBS) using element-tagged antibodies analysed with ICP-MS. In this research the possibility of analysing the two most abundant blood proteins, as a simple model, albumin and immunoglobulin G (IgG), with element-tagged antibodies analysed by ICP-MS was shown. In addition, it was found that both proteins cannot only be analysed in human plasma, but also in whole blood samples and dried blood spots with ELISA and ICP-MS. Furthermore, it was shown that albumin and IgG can be analysed simultaneously with element-tagged antibodies analysed by ICP-MS. This work also further highlights how the DBS specimen could be exploited more widely than is currently used in a clinical setting.

2.0 Materials and methods

2.1 Blood sampling and preparation

Finger prick blood samples of small volumes (up to 40 µl) were collected from a healthy female volunteer. Prior to sampling the working area and hands of the volunteer were sanitized using antiseptic swabs. Fingers were pricked with Unistik 3 Neonatal & Laboratory lancets (Gauge: 1.2mm; Depth: 1.8 mm; Owen Mumford, Woodstock, UK) and 40 µl whole blood was collected with lithium heparin-coated capilliary tubes (Cholestech LDX, Alere, San Diego, USA). Samples consisting of 40 µl whole blood were either used in fluid state immediately or spotted onto Spot Saver cards (PerkinElmer, Greenville, USA) before being left to dry for 24 hours at room temperature. Bloods spots were cut from Spot Saver cards and blood was extracted from the discs by immersing in tubes containing 4 ml of ultra-pure deionised water. The tubes were placed in a 40°C water bath for 60 minutes. After 60 minutes the tubes were removed from the water bath and cooled at room temperature before removing the disks. The samples were used immediately or stored at 4°C prior to analysis. Human plasma was separated from 80 µl of whole blood by centrifuging at 14000 rpm for 10 minutes and used immediately or stored at -20°C. For analysis whole blood samples and dried blood spots were diluted 1:5000 and plasma samples were diluted 1:10000.

Samples for a set of experiments were taken at the same timepoint. Biological replicates were three for plasma samples and two each for DBS and whole blood. For technical replicates (where standard deviation is shown in Results) - IgG is 4 or 5 samples and albumin is 3 or 4 samples.

2.2 ELISA

(A review of ELISA methodology can be found in [9].) Human albumin was analysed with a competitive Albumin Human ELISA kit (Abcam, Cambridge, UK) according to manufacturer's protocol. 0.05% Tween 20 (Sigma-Aldrich, Gillingham, UK) in Dulbecco's Phosphate Buffered Saline (D-PBS; Gibco®, ThermoFisher Scientific, Paisley, UK) was used as a wash buffer. The microplates were analysed with a Wallac Victor^2 1420 multilabel counter microplate reader (PerkinElmer) at a wavelength of 450 nm.

For analysis of human IgG, a Nunclon™ Delta Surface 96 wells plate (ThermoFisher Scientific) was coated with 0.8 µg/ml Goat-anti-human IgG (Sigma-Aldrich) in D-PBS for 2 hours at room temperature or overnight at 4°C. Samples and standards were diluted in D-PBS with 0.05% Tween 20 and 1% bovine serum albumin (BSA; Sigma-Aldrich) and incubated for 90 minutes at room temperature. Pentaglobin® (Biotest, Dreieich, Germany) was used as an IgG standard. After washing with 0.05% Tween 20 in D-PBS, 100 ng/ml Mouse-anti-human IgG Europium (Eu)-labelled antibody (DELFIA®; PerkinElmer) was diluted in D-PBS with 0.05% Tween 20 and 1% BSA and incubated for 1 hour. After washing 100 µl of DELFIA® Enhancement solution (PerkinElmer) was added per well and incubated for 15 minutes at room temperature on a shaker to release Europium, which can then form a bright enhancing chelate enabling analysis with the Europium programme of the Wallac Victor^2 1420 multilabel counter microplate reader.

For the ELISAs the samples were measured in duplicate and the ELISA performed at least 3 times. On the final occasion n=5.

2.3 Instrumental parameters

Elemental compositions were determined using a NexIon 350X ICP-MS instrument (PerkinElmer). Typical operating parameters are listed in Table 1. Samples were introduced into the instrument with a S10 Autosampler for ICP-MS (PerkinElmer).

To eliminate carryover of gold, wash solutions containing 5% *aqua regia* and 10 mM L-cysteine (Sigma-Aldrich) were used. Before gold analysis the instrument was rinsed for 60 minutes with both wash solutions (this method development is included in the Supplementary Materials).

Operating parameters	
Plasma power	1600 W
Plasma gas (Ar) flow rate	18 L/m
Auxiliary gas (Ar) flow rate	1.2 L/m
Nebuliser gas (Ar) flow rate	0.89 L/m
Sample flush time	55 s
Sample flush speed	24 rpm
Read delay	35 s
Delay/Analysis speed	20 rpm
Sweeps/Reading	200
Readings/Replicate	1
Replicates	3
Wash delay	60 s
Wash speed	24 rpm
Applied liquid flow rate	1 mL/m
Dwell time per isotope	50 ms
Isotopes monitored	^{197}Au, ^{193}Ir; $^{151/153}$Eu, ^{115}In

Table 1. *Typical operating parameters of the NexIon 350X ICP-MS.*

2.4 Element based single protein detection assays

Human albumin was analysed with NANOGOLD® (Universal Biologicals, Cambridge, UK) labelling using a competitive Albumin Human ELISA kit. The assay protocol was based on the protocol of the Albumin Human ELISA kit and the method developed by Thompson *et al.* [3] for analysis of VEGF with NANOGOLD® labelling. After the samples and the biotinylated albumin were incubated for one hour at room temperature the wells were washed with 0.05% Tween 20 in D-PBS. Subsequently 50 µl of NANOGOLD®-streptavidin (1:500 diluted in 0.1% FCS in PBS) was added to the wells and incubated for another hour at room temperature. The wells were washed again, 200 µl of 2% HNO_3 (Sigma-aldrich) was added and the plate was shaken for 30 minutes at ~180 rpm. The samples were diluted further in 3 or 6 ml of 2% HNO_3 spiked with Iridium (PerkinElmer) as an internal standard for analysis with the ICP-MS instrument.

Human IgG was analysed with a Mouse-anti-IgG Eu-labelled antibody using the IgG ELISA protocol. After incubation of the Eu-labelled antibody the wells were washed and 200 µl of 2% HNO_3 was added after which the plate was shaken for 30 minutes at ~180 rpm. The samples

were diluted further in 3 or 6 ml of 2% HNO_3 spiked with Indium (BDH spectrosol; VWR international, Radnor, USA) as an internal standard for analysis with the ICP-MS instrument.

ICPMS was repeated at least 3 times and on the final occasion n=5. Calibration curves were measured in duplicate pre- and post-run and the samples themselves just once (but the instrument taking 3 measurements and giving an average).

2.5 Element based multiple protein detection assay

For simultaneous quantification of IgG and Albumin a Nunclon™ Delta Surface 96 wells plate was coated with 0.8 µg/ml Goat-anti-human IgG and 1:60.000 Rabbit-anti-human Albumin (Sigma-Aldrich) and incubated for 2 hours at room temperature or overnight at 4°C. Standards and samples were diluted in PBS with 1% BSA and 0.05% Tween 20 and incubated for 90 minutes. 75 µl sample or standard was added to each well with 25 µl of 2x Biotinylated Albumin (Albumin Human ELISA Kit; Abcam). Standards employed were Pentaglobin® (Biotest) and Albumin standard of the Albumin Human ELISA Kit (Abcam). After washing with 0.05% Tween 20 in PBS 100 ng/ml Mouse-anti-human IgG Eu-labelled antibody (DELFIA®; PerkinElmer) and 1:500 NANOGOLD®-streptavidin was incubated for 1 hour. After incubation of the Eu-labelled antibody and NANOGOLD®-streptavidin the wells were washed and 200 µl of 2% HNO_3 was added after which the plate was shaken for 30 minutes at ~180 rpm. The samples were diluted further in 3 or 6 ml of 2% HNO_3 spiked with indium and iridium as internal standards for analysis with the ICP-MS instrument. Experiments were repeated twice for DBS and fresh blood, and three times for plasma. Figure 1 shows a schematic representation of the method.

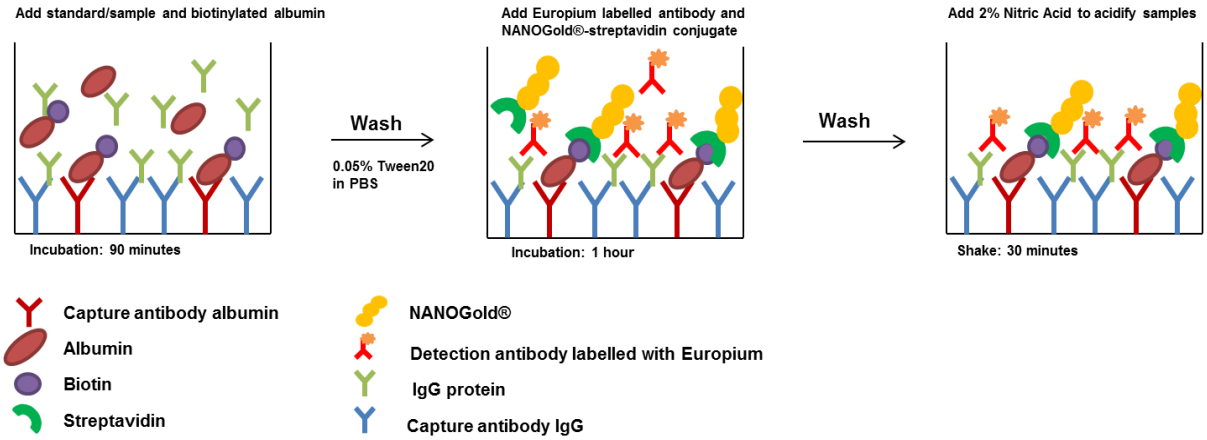

Figure 1. *Schematic representation of the element based detection of albumin and IgG.*

3.0 Results

3.1 Quantification of albumin levels with ELISA and ICP-MS

Albumin is one of the most abundant proteins present in whole blood and was therefore chosen to be quantified by ICP-MS following labelling with NANOGold®-tagged antibodies. Iridium was used as an internal standard to normalize the data obtained by ICP-MS analysis. Figure 2C shows that gold counts normalized using the internal standard iridium provided a superior calibration curve compared to a gold calibration curve without the use of an internal standard (Figure 2A). For both calibration curves containing gold, the gold values of standard 1 were higher in comparison to standard 2. To confirm that there was no carry-over from iridium a calibration was done leading to a R^2 of 0.9999, which can be seen in Figure 2B.

To prove the feasibility of protein quantification with ICP-MS measured albumin levels were compared to albumin levels measured with an ELISA. It was found that there was no difference between albumin levels measured with ELISA or ICP-MS (Figure 3A). Furthermore, it was found that there was no significant difference between whole blood and plasma albumin levels in both ELISA and ICP-MS measurements. However, albumin levels in dried blood spots were significantly lower in comparison to whole blood and plasma albumin levels measured with both ELISA and ICP-MS. Although albumin levels measured with ICP-MS had a larger standard deviation in comparison to albumin levels quantified with ELISA, there was a strong correlation between albumin levels measured by both techniques (Figure 3B). The correlation analysis showed a R^2 of 0.9025 and a p value of 0.0004, which indicates that the correlation is not due to random sampling. Figure 3C and D show albumin calibration curves measured with ELISA and ICP-MS from which albumin concentrations were determined. (A negative curve is observed as expected for competitive ELISA in both cases.) For ICP-MS lower albumin concentrations (0, 1.5, 3, 6 and 12 µg/ml) were observed, indicating a higher sensitivity of protein quantification with ICP-MS.

These results show that it is possible to quantify albumin levels with element-tagged antibodies quantified by ICP-MS. In addition, it was shown that the competitive Albumin Human ELISA kit cannot only be used to quantify albumin levels in plasma, but can quantify albumin levels in whole blood and dried blood spots as well.

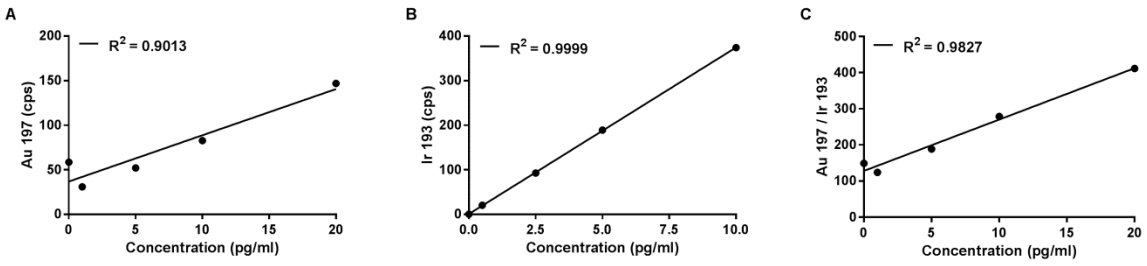

Figure 2. *A) Calibration curve of gold. B) Calibration curve of iridium. C) Calibration curve of gold with iridium as internal standard.*

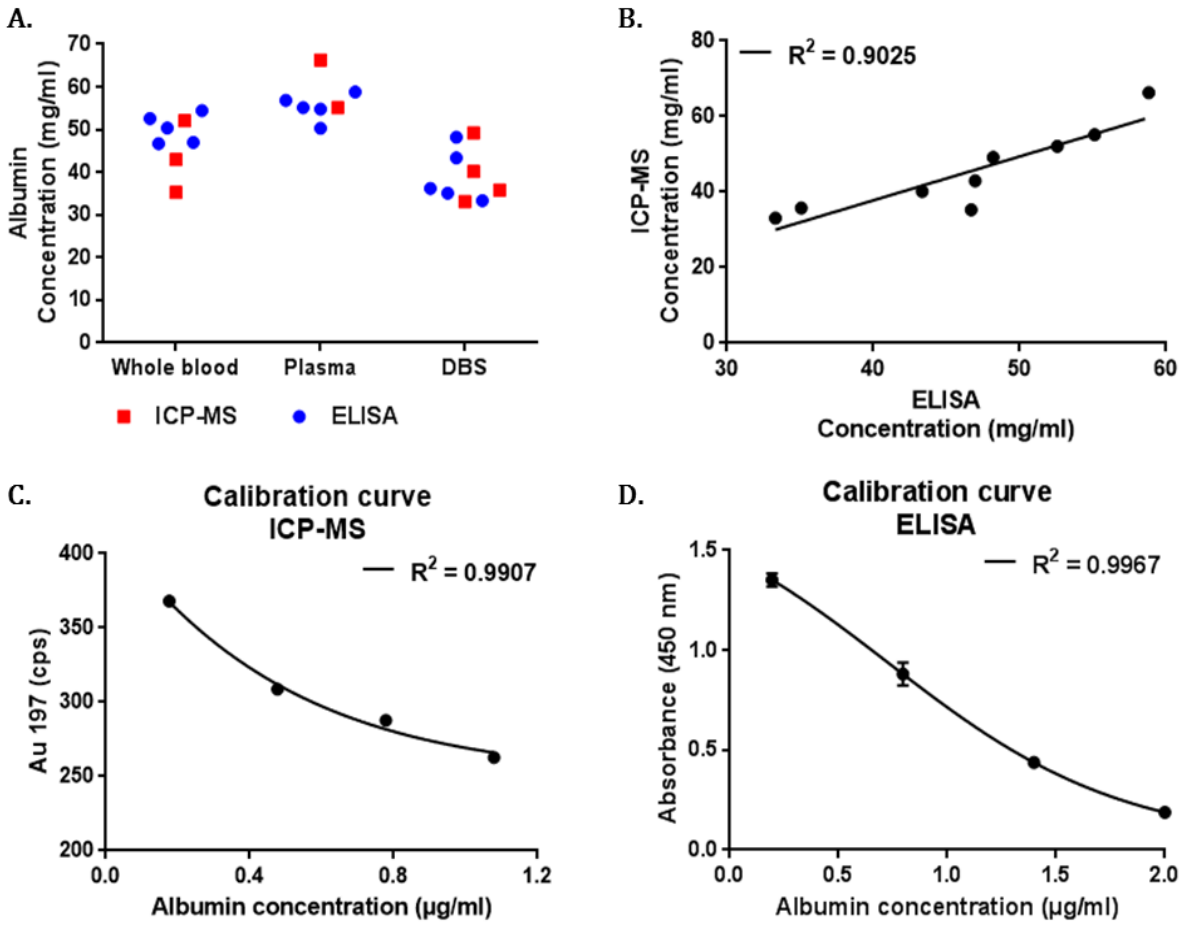

Figure 3. *A) Albumin concentrations in whole blood, plasma and dried blood spots analysed by ICP-MS and ELISA. B) Correlation analysis of albumin levels measured with ELISA and ICP-MS with a p value of 0.0004. C) Albumin calibration curve measured with ICP-MS. D) Albumin calibration curve measured with ELISA. [DBS: Dried blood spots]. NB. Where error bars are smaller than the spots, they are not shown.*

3.2 Quantification of IgG levels with ELISA and ICP-MS

Immunoglobulin G was chosen to be quantified as the second protein in this study. IgG was labelled with a europium-tagged antibody for analysis with ICP-MS. In this instance, indium was used as an internal standard. In Figure 4 calibration curves of both europium isotopes (151 and 153) and indium are shown. All three calibration curves gave a perfect fit with R squared values between 0.9998 and 1.000, suggesting there is no carry-over effect between standards.

IgG levels were measured in whole blood, plasma and DBS with ICP-MS and ELISA and no significant difference in IgG concentrations obtained by both techniques was found (Figure 5A). In addition, there was no significant difference in IgG concentrations found between plasma and whole blood samples. However, IgG levels in DBS were significantly higher in comparison to IgG levels found in plasma and whole blood samples. A correlation analysis of IgG levels measured with ELISA and ICP-MS confirm that there was no difference between IgG concentrations in plasma, whole blood and DBS measured with both techniques (Figure 5B). Figure 5C and D show the calibration curves measured with ICP-MS and ELISA that were used for determination of IgG levels in the samples.

These results show that it is possible to quantify albumin levels with element-tagged antibody detection using ICP-MS.

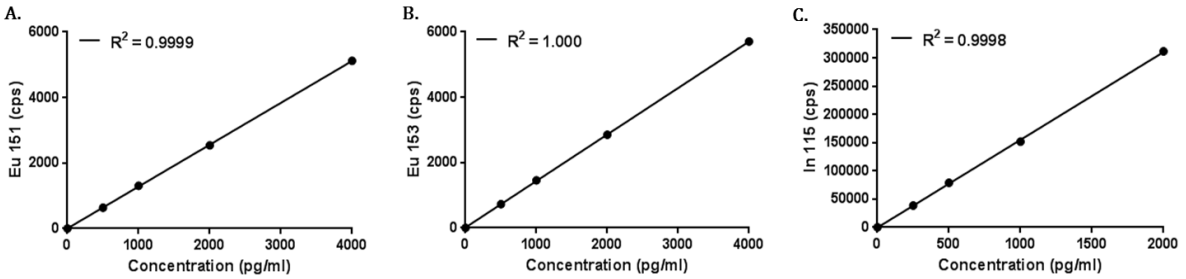

Figure 4. *A) Calibration curve of europium 151. B) Calibration curve of europium 153. C) Calibration curve of indium 115.*

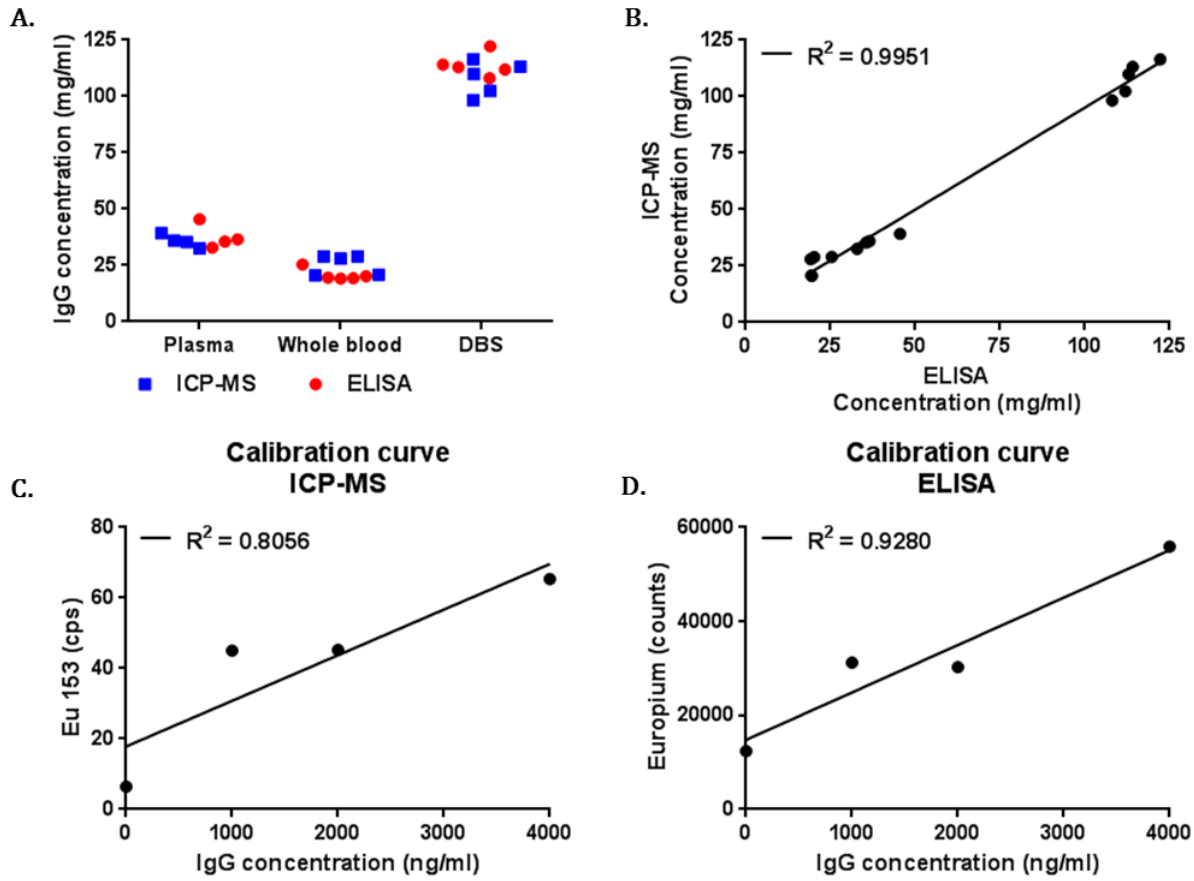

Figure 5. *A) IgG levels in plasma, whole blood and dried blood spots analysed by ICP-MS and ELISA. B) Correlation analysis of IgG levels measured with ELISA and ICP-MS with a p value of 0.0001. C) IgG calibration curve measured with ICP-MS. D) IgG calibration curve measured with ELISA. [DBS: Dried blood spots].*

3.3 Albumin and IgG simultaneous quantification

After it was shown that blood proteins albumin and IgG can be analysed using NANOGold® and europium-labelled antibody detection coupled with ICP-MS, concentrations of both proteins were measured simultaneously. Albumin and IgG levels were measured separately and

simultaneously in plasma, whole blood and DBS to prove the feasibility of simultaneous protein quantification. Albumin levels measured in all three sample sources showed no significant difference between separate analysis of albumin and analysis of albumin in combination with IgG (Figure 6A). In addition, there was no significant difference in albumin levels between plasma, whole blood and DBS. In Figure 6B it is shown that there was no significant difference in IgG concentrations determined with separate analysis and analysis of IgG combined with albumin. However, there was a significant difference between IgG levels measured in plasma, whole blood and DBS.

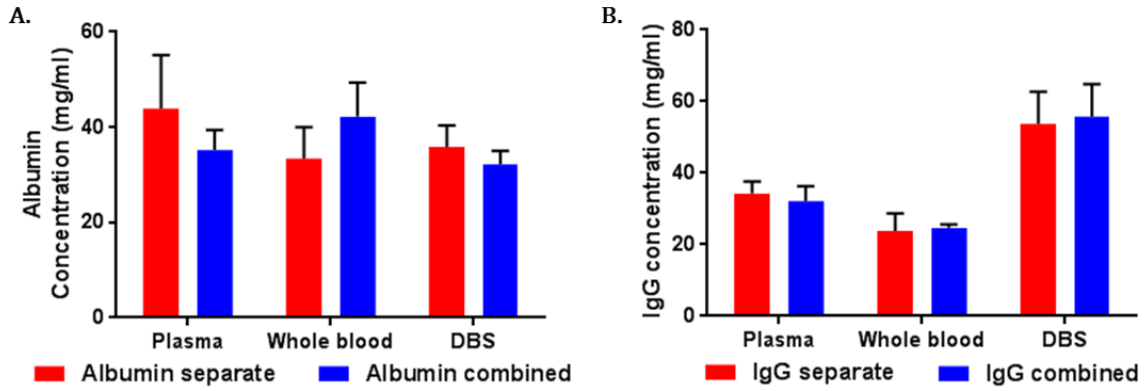

Figure 6. *A) Albumin concentrations analysed in whole blood, plasma and DBS in a separate assay and an assay combined with IgG. B) IgG concentrations analysed in plasma, whole blood and DBS in a separate assay and an assay combined with albumin. n = 5 for all [DBS: dried blood spots].*

4.0 Discussion

Albumin was chosen to be the first protein to be analysed with element-labelled antibody detection, since it is the most abundant protein in blood [23]. Furthermore, normal albumin concentrations in blood are between 30 – 50 mg/ml and changes in albumin levels can indicate kidney or liver failure. In this study it was shown that albumin levels of the blood donor fall in this concentration range. In addition, it was shown that albumin concentrations obtained with a NANOGOLD® label correspond to levels obtained by a competitive ELISA for human albumin, indicating feasibility of this method. Although NANOGOLD® has advantages over other metal-based labels, such as lower detection limits, carry over effect of gold particles might be an issue for the use of this label for clinical diagnosis or forensic protein profiling [3]. Without the use of an efficient wash method, such as the one developed for this method, the gold counts observed cannot be deemed reliable and therefore it is not possible to accurately determine protein levels for an individual. With addition of an efficient wash method the protein concentrations obtained from the calibration curves are reliable, since the concentrations match protein levels found by ELISA. However, to improve the feasibility of this method and to reduce the time needed for the analysis it might be more efficient to use a lanthanide label, such as the Europium label used for quantification of IgG in this study.

For quantification of the second protein, antibody IgG, an ELISA was developed based on a DELFIA system. It was shown that protein levels obtained with europium-labelled antibody detection analysed by ICP-MS correspond to concentrations observed for IgG by ELISA. Europium is common in element-labelled antibody detection, since it is a rare earth element and it has fluorescent properties [11,13]. Should this method be developed further, the use of more lanthanides would be considered due to their high sensitivity in biological samples and the ease of lanthanides to bind to biomolecules [13].

In dried blood spots IgG levels were increased in comparison to IgG concentrations in plasma and whole blood, which might be caused by the extraction method of DBS. The extraction method used for this study is a common method and based on previous findings of the research group [24]. To extract DBS from the filter paper, spots were placed in 4 mL of ultra-pure deionized water and heated for an hour at 40°C. Normal body temperature is on average 37°C and a 3°C increase in temperature might induce degradation of proteins, which creates more IgG binding sites and therefore increasing IgG levels. Another possibility for a change in protein levels in DBS are storage conditions. Adam *et al.* [19] showed that low and high humidity can cause large changes in the degree and rapidity of degradation. In this study storage conditions could not be strictly regulated and fluctuations in humidity were possible.

5.0 Conclusion

Quantification of proteins plays an important role in a number of scientific fields and it is often necessary to analyse multiple proteins in one sample. In this research a method was developed to analyse two proteins, albumin and IgG, simultaneously in plasma, whole blood and dried blood spots with element-labelled antibody detection using ICP-MS. For both proteins it was shown that protein concentrations obtained by element-labelled antibody detection using ICP-MS correspond to those determined by ELISA. Furthermore, the possibility of simultaneous quantification of albumin and IgG with element-labelled antibody detection using ICP-MS was proven. The choice to use these two abundant proteins has enabled proof of concept and a simple model to be established that illustrates the effectiveness of ICPMS analysis via the DBS sample approach and serves as a reminder that this specimen can be as useful as liquid specimens but with major advantages

Although the methodology in this study is developed for quantification of human proteins in biological fluids, this method can be used for anabolic purposes as well. For example, the identification of anabolic-androgenic steroids (AASs) in biological fluids in doping controls [25]. AASs are used by athletes to improve their performance or physical appearance and the structures of AASs are based on ordinary human hormones, so the steroids can bind to receptors in the human body [26]. Since AASs are very similar to ordinary human hormones difficulties in detection are encountered. With use of element-labelled antibody detection and the production of antibodies with specific binding sites for AASs trace amounts of these steroids can be detected by ICP-MS.

For future research this method can be extended by incorporating the quantification of additional proteins through tagging with multiple elements. Any further extension to the methodology must take into consideration cross reactivity between antibodies. Previously it was mentioned that multiple protein quantification can be beneficial for two fields, medical and forensic. Proteins that will be selected for furthering this research should either be related to a disease, so a new diagnostic method can be developed, or give relevant information that could help form a forensic profile, such as proteins that are correlated with anthropometrical measurements [5]. Furthermore, the feasibility of this method for use in clinical diagnosis or forensic cases should be proven by re-diagnosis of patient samples or linking unknown blood samples to a suspect based on the protein profile of the sample.

Conflicts of Interest

None.

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Notes

Ethics approval was confirmed at Sheffield Hallam University through application number SHUREC2B HWB-BIO-04.

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