A METROLOGICAL APPROACH TOWARDS ABSOLUTE QUANTIFICATION OF PROTEIN BIOMARKERS OF METAL METABOLISM DISORDERS

Heidi Goenaga Infante, Estela del Castillo Busto, Christian Deitrich

LGC NML, Queens Road, Middlesex, TW11 0LY, United Kingdom

Metal metabolism disorders (MMD) are those with genetic origin and, for which the functions and levels of physiologically relevant metals in the blood are controlled by specific proteins. Inherited metabolic disorders can result in protein malfunction and therefore, deficiency or toxic accumulation of metals in the body. There are several examples of MMD, of which Wilson's disease (toxic copper levels accumulate in the liver, brain, and other organs) and hemochromatosis (the intestines absorb excessive iron, which builds up in the liver, pancreas, joints, and heart) are very important with regards to metal accumulation/toxicity [1,2]. Diagnosis usually involves gene mutation testing, clinical observations and bio-chemical testing [e.g. non Ceruloplasmin (CER)-bound Cu or exchangeable Cu for Wilson's disease and total blood Fe, plasma Ferritin (light chain) for hemochromatosis].

In Wilson's disease, exchangeable Cu (CuEXC) is currently measured by nephelometry as the amount of total Cu minus that of CER-bound Cu. The main limitation of this test lies in the inaccuracy of measuring CER by immunological methods not able to distinguish between the apo-CER and the active holo-CER, thus leading to biased results. In Fe disorders, Ferritin is the main storage protein for iron in tissues and is engaged in its uptake, accumulation and release in cells. Circulating Ferritin is normally predominantly in the light subunit form. The level of plasma Ferritin directly reflects the level of stored iron and is normally quantified using an antibody test that detects the Ferritin protein, to diagnose iron-related disorders like hemochromatosis. For Ferritin determination, the WHO, which revised its global guidelines for the use of Ferritin thresholds in patient groups with iron deficiency and those at risk of iron overload, recognises that there is no specific recommendation on variability among analytical methods and commutability [3]. From the foregoing account, there is an urgent need for SI traceable methods for the quantification and identification of biochemical markers used for the early diagnosis and treatment monitoring of MMD.

In this short article, we describe a strategy towards development of traceable speciation methods to underpin quantification of biomarkers of MMDs such as non Ceruloplasmin (CER)-bound Cu or exchangeable Cu and plasma Ferritin light chain.

Determination of non Ceruloplasmin (CER)-bound Cu in human plasma

A standardless protein quantification approach was developed and characterised, in terms of accuracy and uncertainty for the determination of plasma Cu-protein content relevant to WD monitoring of CuEXC [4]. It makes use of anion-exchange HPLC hyphenated with ICP-MS to determine the concentration of Cu associated to the protein fraction on the basis of relative peak area distribution and the total Cu concentration in the sample. Typical chromatograms obtained for a mixture of human albumin standards and human plasma using a strong anion-exchange FPLC MonoQ™ column and an ammonium acetate gradient (0-250 mM, pH 7.4) in 50 mM Tris buffer (pH 7.4) at a flow rate of 1.0 mL min^{-1} for 30 min is presented in Figure 1. ^{63}Cu and ^{65}Cu isotopes were detected using triple quadrupole ICP-MS (Agilent Technologies, Palo Alto, CA, USA) in MS/MS mode using O_2 as reaction gas.

Due to the lack of speciated Cu-reference materials, protein recovery was assessed by comparison with that of species-specific (SS) isotope dilution (ID). For this, a double SS HPLC-ICP-IDMS method for Cu-Albumin was developed and assessed for accuracy and uncertainty by analyis of three human sera (two frozen: LGC8211 and ERM®-DA250a, and one lyophilised: Seronorm™ Human). Cu-protein recovery measurements by the peak area distribution approach determined against

analysis of the same serum materials using SS HPLC-ICP-IDMS on Cu-ALB led to recovery values ranging from (102 ± 8)% to (145 ± 12)% for Cu-ALB serum concentrations between 455 and 112 µg kg^{-1} Cu.

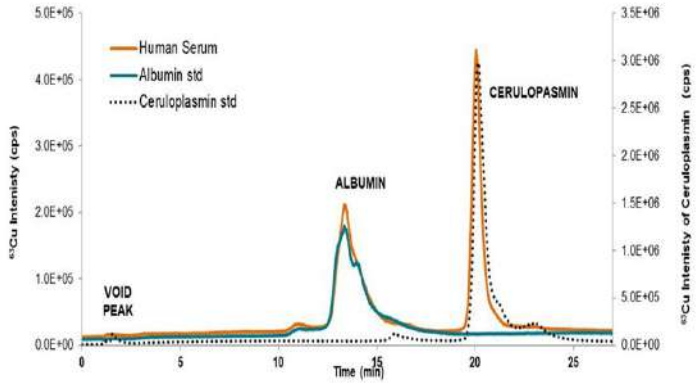


Figure 1

Figure 2 shows the workflow of the SI-traceable quantification of Cu bound to Albumin (Cu-Albumin) in human serum by double SS HPLC-ICP-IDMS

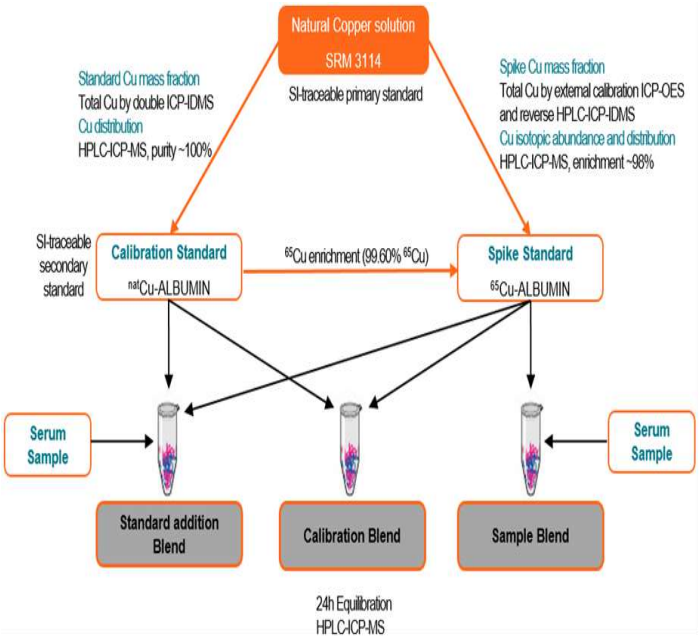


Figure 2

The validated relative approach, with relative expanded uncertainties (k=2) between 5.7 and 10.1% for Cu-Albumin concentrations ranging from 112 to 455 µg kg^{-1} Cu, was found able to discriminate between healthy and WD populations in terms of Cu-Albumin content. Also, using such methodology, underestimation of CuEXC by the classical EDTA/ultrafiltration method was demonstrated [4]. Finally, the high throughput of this method made it attractive for Cu-protein screening in a clinical trial where a large number of samples have had to be analysed within their stability window.

<u>Towards absolute quantification of Ferritin light chain in plasma using SS HPLC- ICP-IDMS *via* Sulphur</u>

First efforts to achieve chromatographic selectivity for ferritin light chain in plasma and to prepare a natural ferritin standard as well as ^{34}S-enriched ferritin species (spike) are discussed here.

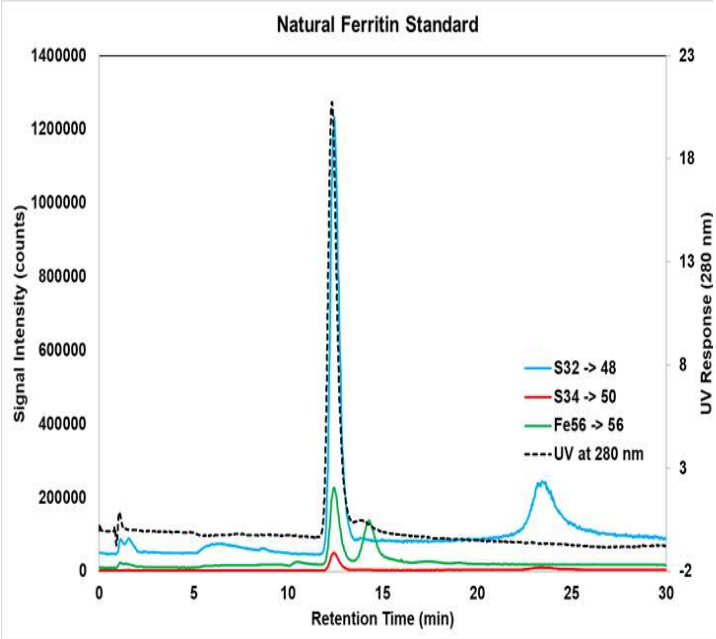
Expression and purification of human Ferritin light chain protein in HEK-293 mammalian cells was undertaken in a media, dosed with either natural methionine or ^{34}S-enriched methionine. Analysis of cultures by SDS-PAGE was undertaken to establish optimum yielding conditions.

To obtain information about the S speciation in the standard and spike of ferritin, methodology to achieve the selective separation of light chain from heavy chain ferritin and from other plasma proteins such as transferrin and albumin was developed. For this purpose the separation methodology used for Cu proteins on strong anion-exchange FPLC MonoQ™ column was revisited by slower increase of the concentration of mobile phase B within the first five minutes.

Figure 3 shows S, Fe and UV-Vis (280 nm) profiles of the natural and spike ferritin materials under optimal separation conditions and detection by triple quadrupole ICP-MS in MS/MS mode using oxygen as reaction gas. The ^{32}S and ^{34}S signals observed for both natural stndard and spike at the retention time of approximately 24 mins match those of the procedural blank. Further work is ongoing for the characterisation of the standard and spike materials to be used for isotope dilution quantification of human plasma ferritin.

Using the same optimal separation and detection conditions, ^{32}S profiles were obtained for a protein mixture containing albumin (ALB), transferrin (TRF), immunoglobulin (IgG) and Ferritin light chain (FTL) and for a procedural blank. The obtained chromatogram is represented in Figure 4. As shown, a fair separation of the protein standards was achieved. Further work is investigating whether the chromatographic selectivity obtained is enough for direct application of the developed methodology to ferritin light chain detection in

high abundant protein plasma with and without combination of major protein removal (e.g. by chemical and heat treatment) without affecting the ferritin integrity. Further work will also involve the development of isotope dilution methodology for absolute quantification of Ferritin light chain in plasma.



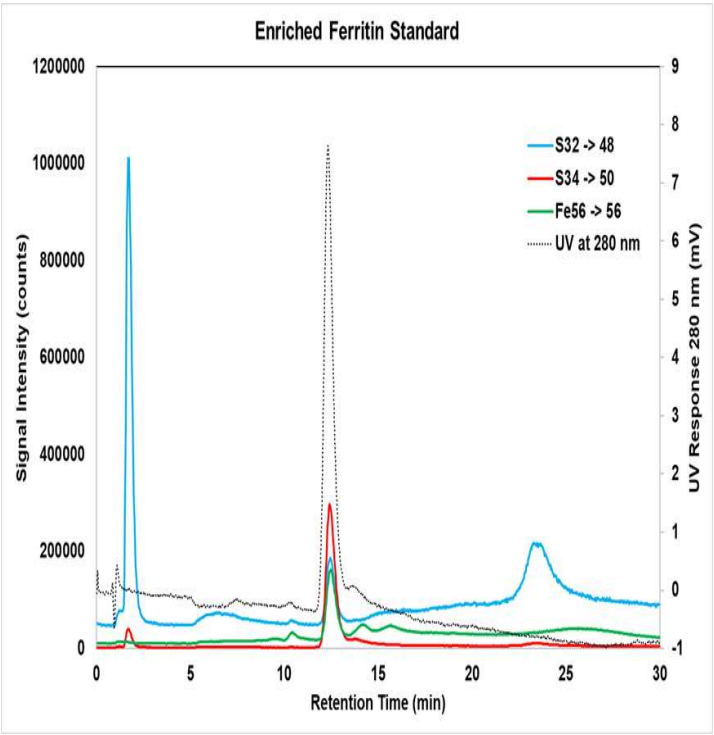


Figure3

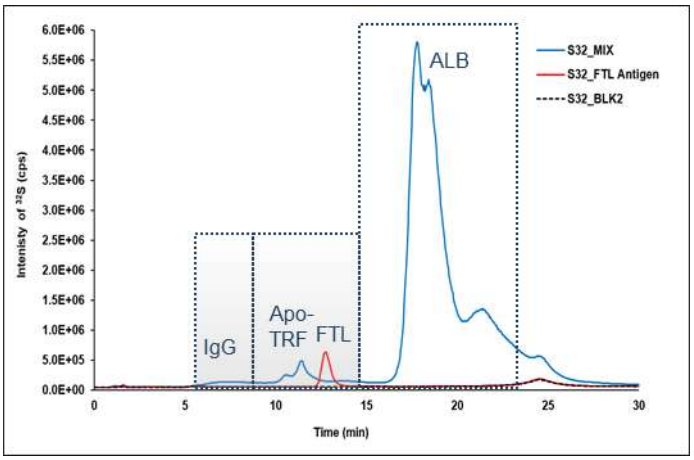


Figure 4

References

[1] M. Umair, M. Alfadhel, Cells, 2019, 8, 1598.

[2] C. R. Ferreira, W. A. Gahl, Trans. Sci. Rare Diseases, 2017, 2, 101.

[3] WHO guideline on use of Ferritin concentrations to assess iron status in individuals and populations, 2021.

[4] E. Del Castillo Busto, S. Cuello-Nunez, C. Ward-Deitrich, T. Morley and H. Goenaga Infante, Anal. Bioanal. Chem., 2021; 414, 561.