Quotient Sciences

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Towards a biomarker assay: bridging the gap from total copper to non-ceruloplasmin bound copper using an LC-ICPMS speciation approach

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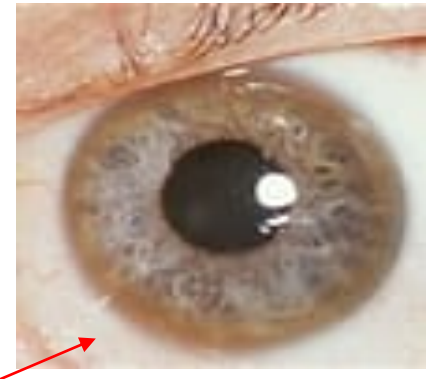
Contents

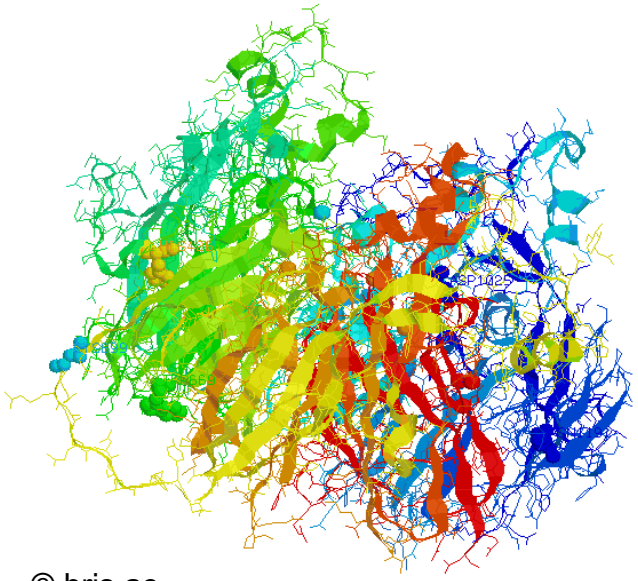
Wilson's Disease
Causes and symptoms

- We ingest copper in our diet. Shellfish, whole grains, beans, nuts, potatoes, and organ meats are good sources of copper. Dark leafy greens, dried fruits and yeast are also sources of copper.
- Wilson disease (WD) is an autosomal recessive disorder of copper (Cu) transport caused by mutations of the ATP7B gene
- It leads to an accumulation of copper in the brain, kidneys and cornea, where a key feature of the disease can be the development of Kayser-Fleischer rings, which form gold-yellow rings around the pupils.
- Untreated, the disease will progress and can lead to severe symptoms such as hepatic disease, movement disorders and even death.
- Worldwide it affects around 1 in 30,000 people



Treatments and NCC

- **Ceruloplasmin (Cp) is a globulin type serum copper transport protein**
- In Wilson's disease, the incorporation of copper into apoceruloplasmin (copper-absent Cp) to produce holoceruloplasmin (copper-containing Cp) is impaired (ratio 6 Cu:1Cp)
- Although copper levels are lower in WD patients, the proportion of unbound 'free' (NCC) is increased and it is this which drives the clinical manifestations of Wilson's Disease.
- Current treatments are chelating agents to remove circulating copper eg penicillamine, trientine and agents to prevent absorption e.g Zinc Acetate
- CoU: Client need to measure NCC in clinical trial subjects to assess therapeutic effect and efficacy relative to standard treatment; biomarker



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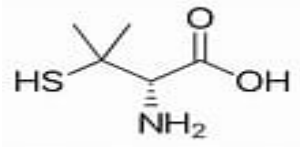


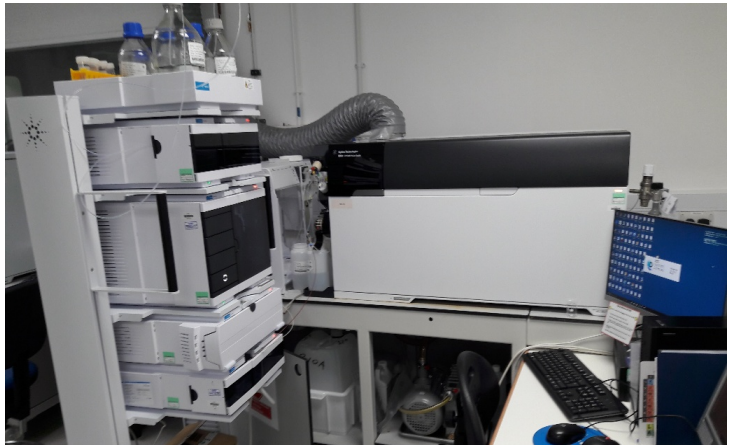
penicillamine

Measuring NCC
Various methods: pros and cons, rationale

- **Measure total serum copper and ceruloplasmin & subtract, correcting for Cu in Cp**
  - Cp measured using a kit. These kits are not validated/regulated for diagnostic use
- **Chelate NCC with EDTA, filter and measure the filtrate**
  - Proved to be inaccurate; affected by high molecular weight artefact binding copper

  from albumin retained above the 30KDa filter resulted **in an underestimation** of true NCC; possible imprecise titration in WD patients
- **Alternative: use ICPMS combined with LC-ICPMS**

to measure copper species, total copper and thereby provide a measure of NCC



- This can be validated, is more accurate and was the technique selected for NCC monitoring during the pivotal study

Analytical Methods
Total copper in serum

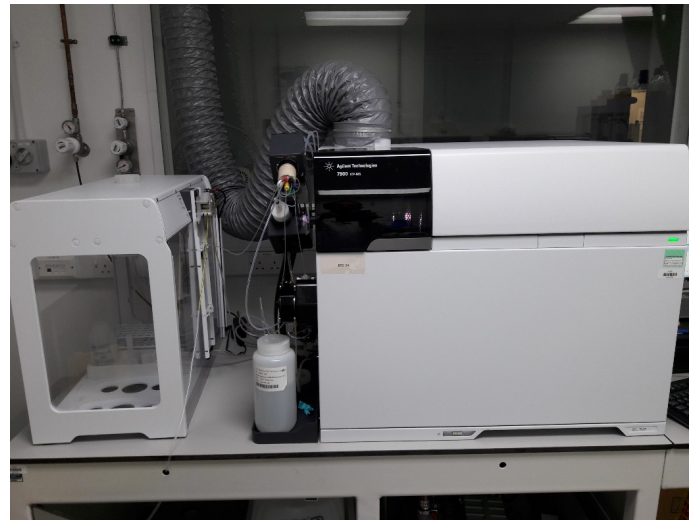
- Takes 50uL human serum and digest with TMAH/chelating agent solution with addition of matched (Rhodium) internal standard
- Agilent 7900x single quad ICP-MS with ASX-500 autosampler
- Running in He cell gas mode to remove isobaric interferences &

monitoring Cu isotope 63 and Rh isotope 105

- Analytical range 20- 2000 ng/mL; endogenous assay using a

spiked surrogate calibration curve and matrix matched serum QC samples

- High throughput, fully validated assay



7900x with plasma on

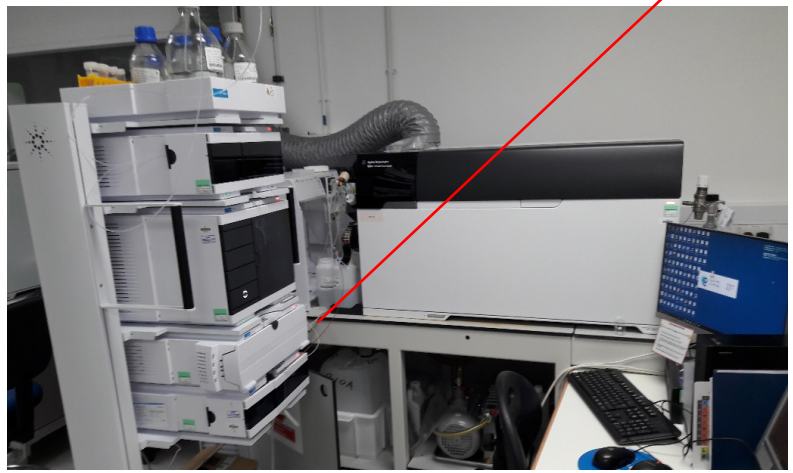
Samples infused from tubes into nebuliser via peristaltic pump

Analytical Methods

LC-ICPMS Copper speciation method

- **Protein Anion exchange: negatively charged proteins**
- **QE Mono Q5/50 GL column** $-CH_2-N^+(CH3)_3$
- **Mobile Phase A: 50 mM Tris-HCl (pH 7.4)**
- **Mobile Phase B: 50 mM Tris-HCl, 500 mM ammonium acetate (pH 7.4)**
- **Agilent 8900x ICPMS monitoring isotope 63>63 in He cell gas mode**

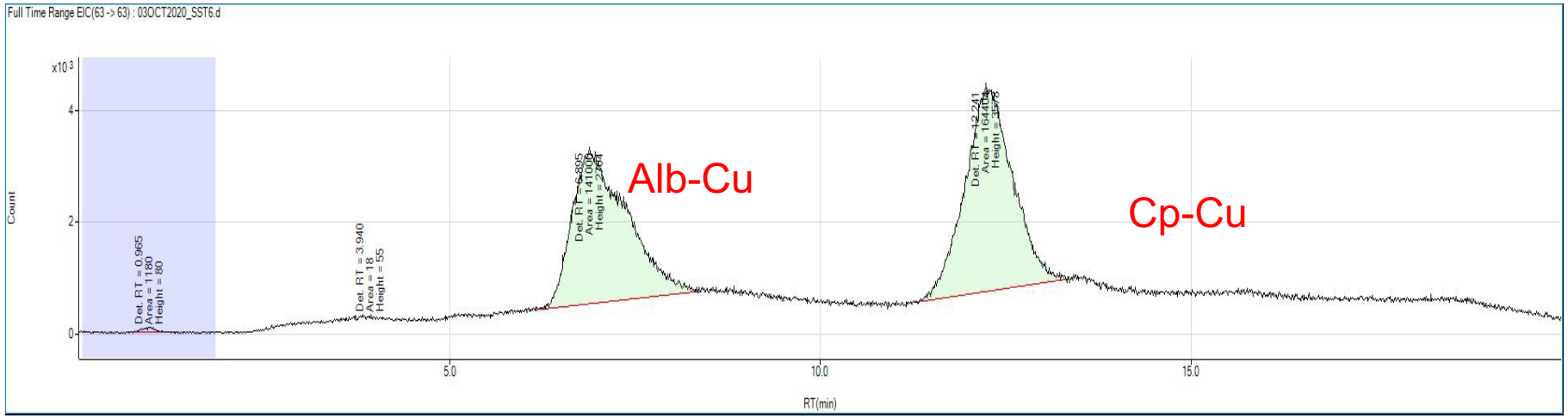
Time [min]	A [%]	B [%]
0.00	100.0	0.0
1.00	90.0	10.0
4.00	55.0	45.0
14.00	0.0	100.0
17.00	0.0	100.0
20.00	100.0	0.0



- **Flow rate 1mL/minute**
- **Analogous to Solovyev et. al**

Mixed Reference Standard

Separation of ceruloplasmin and albumin



- The Ceruloplasmin standard (~135 KDa protein supplied as a lyophilised powder) was unsuitable for use as an external standard for direct ceruloplasmin-bound copper quantification – low purity (27%), possible instability.
- It was proposed and agreed that Cp-Cu concentration could be derived from the percent relative abundance of Cp-Cu by LC-ICP-MS speciation, together with the total serum copper as determined quantitatively by ICP-MS.
  LC-ICP-MS determines Cp-Cu indirectly by the speciation of Cu-containing proteins

Workflow

Sample Preparation, system suitability & ‘elementogram’

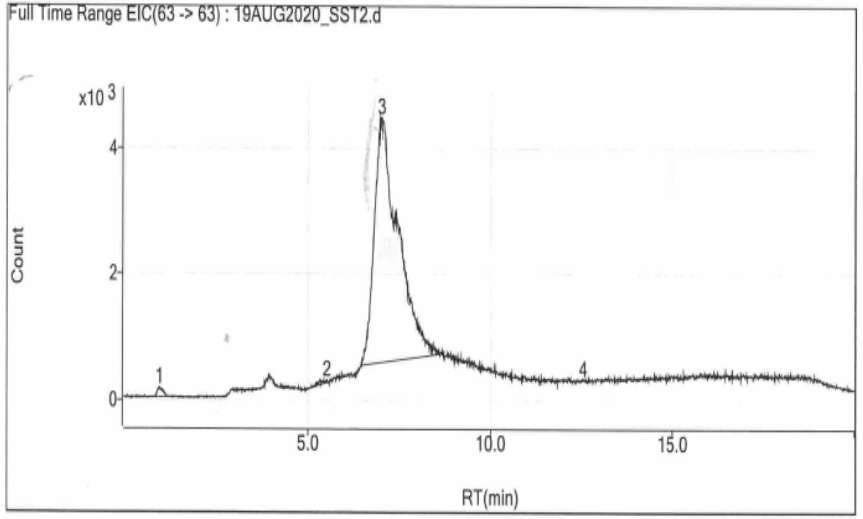
1) Sample Preparation:

80.0 µL of 20 mM Tris-HCL (pH 7.4) buffer solution is combined with 20 µL of human serum in a low-bind Eppendorf tube prior to injection.

Injection volume: 50uL

2) Suitability test

3) Sample Analysis



Suitability test

QC0

↓

Cp-1

↓

QC0

↓

→ Alb-1

↓

QC0

↓

Combined-1

Calculation of NCC
and diagnostic values

- Measure total serum Cu for each sample (healthy serum total copper concentrations are in the range **600 to 2000 ng/mL**)
- Run an aliquot of the same sample by LC-ICPMS & integrate peaks to obtain proportion of Cp-Cu relative to the total
- Multiply total copper concentration by the proportion of Cp-Cu to obtain concentration of Cp-Cu in ng/mL.
- Subtract **[Cp-Cu]** from **[total Cu]** to obtain **NCC**

Adequately controlled Wilsons means serum non-ceruloplasmin bound copper (NCC) level between

≥ 25 and ≤ 150 ng/mL* Based on lab values

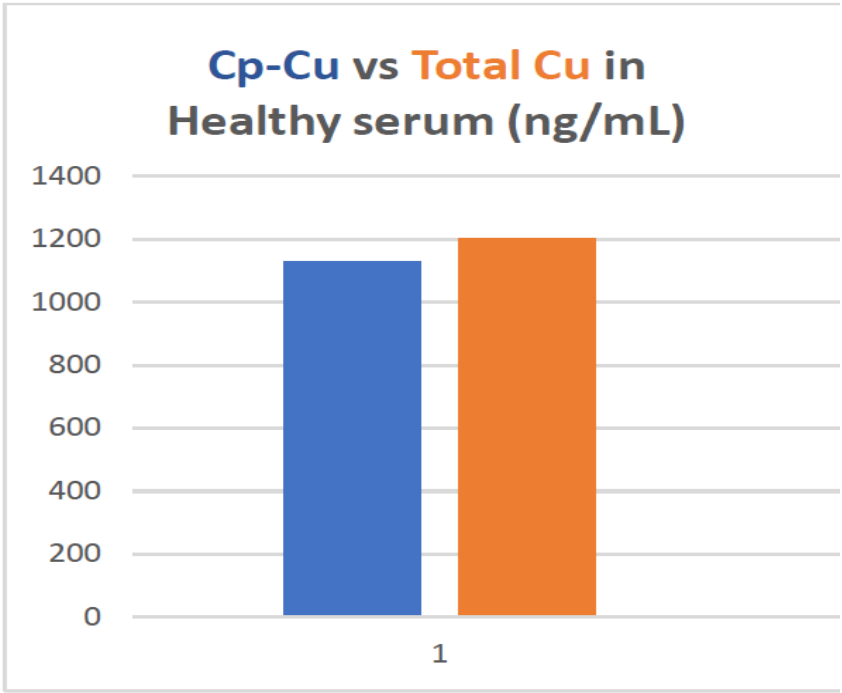
- Other measures e.g. urinary copper excretion, clinical findings also taken into account during the study

Assay Verification

Scientific Validation of Cp-Cu in human serum

- Six different individual lots (batches) of human serum analysed (n=6) on three separate occasions to generate precision data.
- Accompanying satellite verification tests: freeze thaw, carryover, matrix stability etc
- Column mass balance study – are we losing anything?

Assay Variability: Ceruloplasmin-bound copper	Batch	Mean Calculated Cp-Cu Conc (ng/mL)	Total Cu Conc (ng/mL)	Within-run Precision (RSD%)
	HMN398561	1234	1320	0.915
	HMN398563	1151	1220	0.794
	HMN398564	828	890	0.791
	HMN398560	1138	1210	0.729
	HMN398559	1151	1220	0.795
	HMN398558	1269	1350	0.723



Assay Verification
Column mass balance & healthy volunteers

- The copper mass balance in six separate human serum samples was assessed by following the collection of sample eluent before and after passing through the ion exchange column.
- Each eluate was collected, freeze-dried, reconstituted with pure water and analysed for total copper content using the serum total Cu method
- Mean recovery for 6 human samples, post column vs. pre-column was **97.7%**

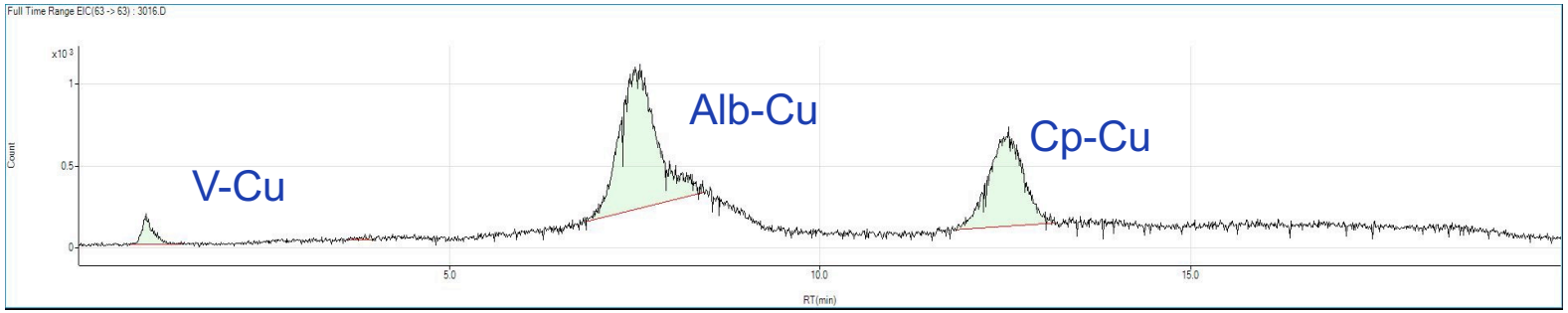
To establish baseline levels for healthy volunteers, serum samples from 50 subjects were analysed for Cp-Cu in triplicate

- Relatively high levels of total copper in serum, most (80-95%) of which is in the form of Cp-Cu

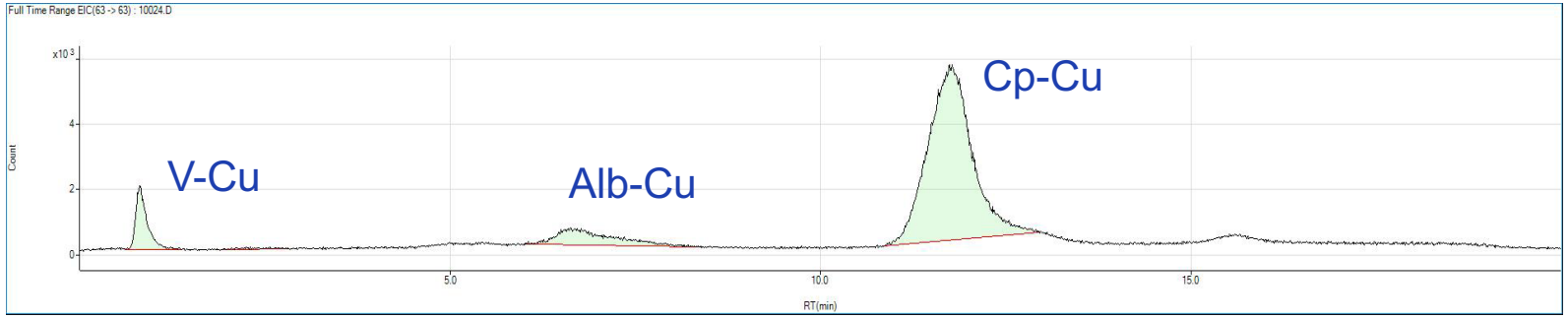
Comparative elementograms from study

Pre-dose vs. treated Wilson's

63>63 intensity vs. time



Pre-dose

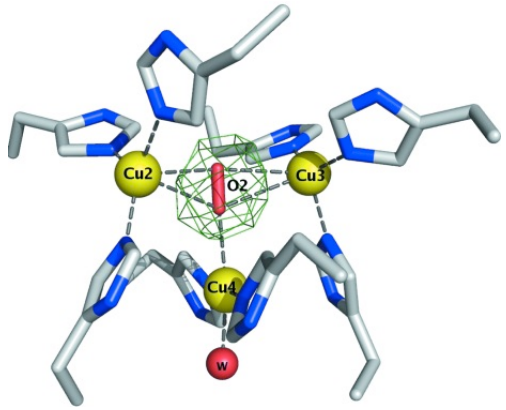


Treated

Absolute values are higher in the controlled (treated) patient, with a higher proportion of Cp-Cu (>80%)
In WD pre-dose total Cu lower, more variable (50-600ng/mL) with NCC approaching Cp-Cu

Summary

- A reliable and reproducible monitoring tool was necessary for the measurement of serum NCC, CoU to identify WD patients who are or are not within the established limits for well-controlled patients
- A diagnostic assay for NCC has been developed and validated using LC-ICPMS to assess the proportion of NCC and a total serum copper assay for quantitation
- NCC values in healthy volunteers have been established
- WD patients have lower, variable total Cu and higher NCC
- The assay is in routine production and monitoring NCC in trial samples
- **Total and LC-ICPMS provides a more accurate and reliable measure of NCC for diagnostic monitoring**



References

- The role of calculated non-caeruloplasmin-bound copper in Wilson's disease. *Duncan A, et al. Ann Clin Biochem 2017*

- Novel Copper Protein Speciation method for calculating Serum Non Ceruloplasmin Copper: A Comparative Analysis. Poster presented at The International Liver *Congress,* 56th Annual meeting of the European Association for the Study of the Liver (EASL) 23 – 26 June, 2021

- Defining the range of healthy volunteer non-ceruloplasmin copper using a new copper protein speciation assay. S Mc.Dougall and T Morley. Poster number 32, 14th European Bioanalysis Forum, November 2021

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