Development and validation of a novel ICP-MS method to quantify different copper species in human plasma from patients with Wilson disease

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INTRODUCTION

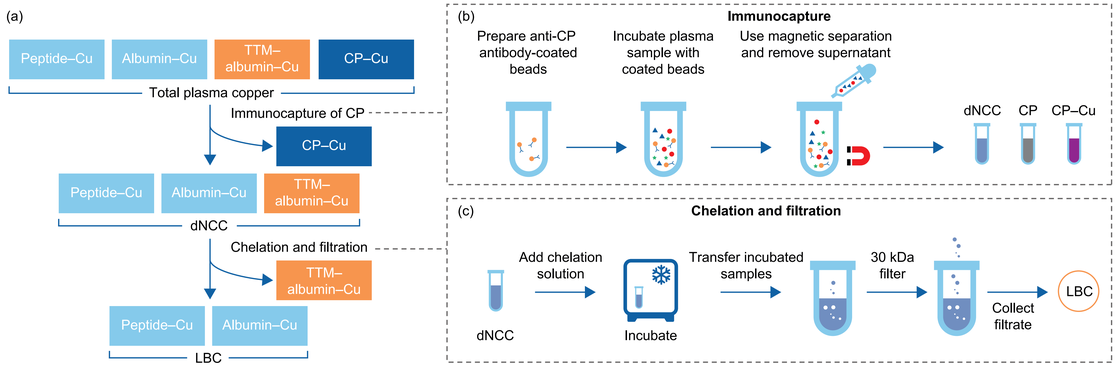
- Ceruloplasmin (CP) is a major copper-carrying protein in the blood; CP-bound copper (CP–Cu) is considered to be nontoxic because it is nonexchangeable at physiological pH, whereas non-CP-bound copper (NCC) is exchangeable and therefore potentially reactive.[1,2]
- In healthy individuals, approximately 90% of copper in plasma is bound to CP.[3]
- In patients with Wilson disease (WD) – a rare, autosomal recessive condition – defective ATP7B leads to inadequate loading of copper into CP, resulting in accumulation of copper in the tissues and an increase in exchangeable copper, or NCC, in the blood.[3,4]
  - Successful treatment of WD relies on agents that can remove excess copper from the body.[5]
  - Standard of care (SoC) involves removal of excess copper using metal chelators and limiting copper absorption using zinc; however, in many patients, symptoms persist or worsen.[6]
- Currently, assays recommended in guidelines from the American Association of Liver Disease and the European Association for the Study of the Liver indirectly estimate the level of NCC by subtracting the concentration of CP–Cu from the total serum copper concentration (calculated NCC [cNCC]).[5,7] This method assumes that six copper atoms are bound to each CP molecule; in reality, the ratio may vary.[8]
- In addition, the existing guideline-recommended method may not accurately capture NCC levels in patients being treated with ALXN1840.
  - ALXN1840 (bis-choline tetrathiomolybdate [TTM], formerly named WTX101) is an investigational copper-binding agent that mobilizes blood and tissue copper and has demonstrated a significant NCC-lowering effect in a phase 2 study.[9]
  - The mode of action of ALXN1840 is unique; it forms an inert TTM–albumin–copper tripartite complex (TPC) that does not contribute to the exchangeable copper pool.[9,10] To calculate NCC in ALXN1840-treated patients, the TPC would need to be measured and subtracted from the NCC fraction.[9] A direct assay is therefore needed.

AIM

- To develop and validate a novel assay that isolates multiple copper species (CP–Cu, directly measured NCC [dNCC] and labile bound copper [LBC]) from plasma.

METHOD

Figure 1. Summary of novel assay process[a]



Method scheme. (a) Overview of assay procedure (TTM, the active moiety of ALXN1840, which can bind to copper and albumin). (b) Immunocapture steps to obtain dNCC, CP and CP–Cu species. (c) Chelation and filtration steps to isolate LBC.
[a]Capture of the TTM–albumin–Cu tripartite complex is important for quantifying LBC in patients treated with ALXN1840, but it is not necessary for measuring dNCC nor relevant for LBC data from samples collected at baseline.
All samples described herein were collected at baseline.
CP, ceruloplasmin; Cu, copper; dNCC, directly measured non-ceruloplasmin-bound copper; LBC, labile bound copper; TTM, tetrathiomolybdate.

- The assay directly quantifies multiple copper species from human plasma using a combination of steps: immunocapture of CP using magnetic beads coated with a monoclonal antibody; chelation; filtration and inductively coupled plasma mass spectrometry (ICP-MS). These combined sequential processes enable bioanalysis of CP and various copper species, including CP–Cu, dNCC and LBC, from human plasma (**Figure 1**).
- Guidance from the US Food and Drug Administration for methodological validation was followed, incorporating measures for precision, accuracy, selectivity and stability.[11] The following validation parameters were examined: linear range, sensitivity, intra- and inter-run precision and accuracy, selectivity, carryover and sample stabilities (freeze/thaw and short- and long-term stabilities).
- Plasma samples from 207 patients enrolled in the phase 3 study of ALXN1840 in WD (NCT03403205) were assessed at baseline (before treatment) using the novel assay. For comparison, samples from 17 healthy volunteers enrolled in a phase 1 study (NCT04594252) were also evaluated.

RESULTS

Method validation

- Full validations were successfully performed for each copper fraction, as well as for CP, and demonstrated a linear range of 5 to 1000 ng/mL (0.08 to 15.75 µM) for CP–Cu, dNCC and LBC, and of 5000 to 800 000 ng/mL (0.04 to 5.97 µM) for CP.
- The precision and accuracy of intra- and inter-run comparisons met the predefined acceptance criteria for quality control (**Table 1**).
- Injection carryover for the analyte and the internal standard was within acceptable thresholds for validation (data not shown).
- In the selectivity evaluation, all individual plasma lots were within the accepted criteria for validation (100% ± 20% of the nominal concentration for each analyte).
- All stability measures were also acceptable for validation (**Table 1**).

Table 1. Intra- and inter-run validation parameters

	dNCC and CP–Cu	LBC	CP
Volume of human lithium plasma sample, µL	20	20	20
Platform	ICP-MS	ICP-MS	LC-MS/MS
Quantitation range, ng/mL (µM)	5 to 1000 (0.08 to 15.75)	5 to 1000 (0.08 to 15.75)	5000 to 800 000 (0.04 to 5.97)
QC intra-run precision, %CV[a]	1.1 to 16.8	1.1 to 14.4	2.9 to 13.3
QC intra-run accuracy, %bias[a]	–15.5 to 8.7	–19.7 to 18.6	–16.0 to 16.0
QC inter-run precision, %CV[a]	1.9 to 10.3	1.9 to 9.6	7.2 to 9.8
QC inter-run accuracy, %bias[a]	–10.3 to 5.6	–4.4 to 12.0	–14.0 to 7.4
Sample bench-top stability at room temperature, hours	19	17.5	19
Sample short-term stability at 4°C ± 4°C, hours	19	17.5	19
Freeze/thaw stability, number of cycles	4	4	4

[a]Results include combined %CV or %bias from all QC levels, including LLOQ, low, matrix low, mid, matrix mid, high and matrix high QCs. Acceptance criteria for QC samples were: %bias within 15.0% (within 20.0% for LLOQ) and %CV ≤ 15.0% (≤ 20.0% for LLOQ); the predefined acceptance criteria in human plasma were: %bias within 20.0% (within 25.0% for LLOQ) and %CV ≤ 20.0% (≤ 25.0% for LLOQ).
%CV, percentage coefficient of variation; CP, ceruloplasmin; CP–Cu, ceruloplasmin-bound copper; dNCC, directly measured non-ceruloplasmin-bound copper; ICP-MS, inductively coupled plasma mass spectrometry; LBC, labile bound copper; LC-MS, liquid chromatography–mass spectrometry; LLOQ, lower limit of quantitation; MS, mass spectrometry; QC, quality control.

Clinical sample testing

- The CP–Cu:CP molar ratio observed differed from the predicted ratio of six; the mean (standard deviation [SD]) was 4.68 (0.631) in healthy volunteers and 3.42 (3.089) in patients with WD (**Table 2**).
- Mean concentrations of CP–Cu and of plasma total copper were lower in patients with WD than in healthy volunteers (**Table 3**).
- In patients with WD who were enrolled in the phase 3 study of ALXN1840, 22% had negative cNCC values at baseline. For patients with positive values (n = 162), the mean (SD) baseline cNCC concentration was 2.06 (1.65) µmol/L.

Table 2. Molar ratio of CP–Cu:CP in patients with WD and in healthy volunteers

CP–Cu:CP ratio	Patients with WD (n = 207)	Healthy volunteers (n = 17)
Mean (SD)	3.42 (3.089)	4.68 (0.631)
Median (Q1, Q3)	2.93 (2.36, 3.49)	4.68 (4.31, 5.05)

CP, ceruloplasmin; CP–Cu, ceruloplasmin-bound copper; Q1, quartile 1; Q3, quartile 3; SD, standard deviation; WD, Wilson disease.

Table 3. Concentrations of CP–Cu, dNCC and LBC in patients with WD and in healthy volunteers

	Patients with WD (n = 207)	Healthy volunteers (n = 17)
CP–Cu concentration, µM, mean (SD)	3.03 (3.33)	11.30 (1.72)
dNCC concentration, µM, mean (SD)	1.03 (1.00)	0.51 (0.10)
LBC concentration, µM, mean (SD)	1.04 (0.87)	0.50 (0.10)
Plasma total copper concentration, µM, mean (SD)	4.86 (4.13)	13.75 (1.72)

CP–Cu, ceruloplasmin-bound copper; dNCC, directly measured non-ceruloplasmin-bound copper; LBC, labile bound copper; SD, standard deviation; WD, Wilson disease.

CONCLUSIONS

- Validation experiments confirmed that this novel assay directly measuring CP–Cu, NCC, LBC and CP conforms to the accepted criteria for precision, accuracy, selectivity and stability.
- Data also support existing evidence suggesting that the ratio of CP–Cu:CP can be lower than the theoretical value of six in healthy people and in those with WD, and that calculation of NCC using an assumption of six atoms of copper per CP molecule is not accurate.[8]
- This assay could potentially be used for efficacy assessment of ALXN1840 in clinical trials and may have broader utility in diagnosis and treatment monitoring in patients with WD.

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CONFLICTS OF INTEREST

At the time the research was carried out, the authors were all either employed by Alexion Pharmaceuticals, Inc., who validated the method, or involved in Alexion-sponsored projects at Frontage Laboratories, Inc., during which normal reference determination was performed.

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