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Research Article

Method validation of an inductively coupled plasma mass spectrometry (ICP-MS) assay for the analysis of magnesium, copper and zinc in red blood cells

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ABSTRACT

Background: Laboratory measurements of trace elements such as magnesium (Mg), copper (Cu), and zinc (Zn) in red blood cells (RBCs) are essential for assessing nutritional status and diagnosing metal toxicity. The purpose of this study was to develop and validate an ICP-MS method for quantifying these elements in RBCs.
Methods: Packed RBCs were aliquoted and diluted in an alkaline diluent solution containing internal standards, 0.1 % Triton X-100, 0.1 % EDTA, and 1 % ammonium hydroxide. The resulting diluted specimen was analyzed using inductively coupled plasma mass spectrometry (ICP-MS) to quantitatively determine the levels of Mg, Cu, and Zn. The method underwent validation for accuracy, precision, method comparison, linearity, analytical sensitivity, and carryover. Additionally, retrospective data were analyzed, and non-parametric reference intervals were calculated.
Results: Accuracy and linearity fell within the expected range of $\leq \pm 15$ % for all analytes. Within-run, between-run, and total imprecision were ≤ 15 % coefficient of variation. All other validation experiments met the established acceptance criteria. Retrospective data analysis was conducted on patient samples using the method. The application of Tukey's HSD test for multiple comparisons revealed statistically significant mean differences ($p < 0.05$) in Mg, Cu, and Zn concentrations between all pairwise groups of age and sex, except for the mean Cu concentration in adult males versus females and the mean Mg concentrations in adult versus minor males.
Conclusions: The presented method was successfully validated and met the criteria for clinical use. Retrospective data analysis of patient results demonstrated the method's suitability for assessing nutritional deficiency and toxicity.

1. Introduction

Trace elements play a critical role in human growth and development, even in small quantities. These elements primarily act as catalysts in various enzymatic reactions within the body. It is essential to ensure an adequate supply of these essential trace elements to maintain a high quality of life [1]. Thus, clinical measurement of trace elements is helpful in nutritional assessment and may be necessary for determining potential deficiencies and/or toxicities for patient management. Laboratory assessments of microelements, such as magnesium (Mg) and trace elements like copper (Cu) and zinc (Zn) in red blood cells (RBCs) are utilized to evaluate patients' nutritional statuses [2]. Hemodialysis patients commonly face systemic Zn deficiency. However, solely monitoring plasma Zn levels may fail to consider intricate interactions

Abbreviations: NH4OH, ammonium hydroxide; AMR, analytical measurement range; CLRW, clinical lab reagent water; CV, coefficient of variation; Cu, copper; Ga, gallium; ICP-MS, inductively coupled plasma mass spectrometry; ITSDs, internal standards; KED, kinetic energy discrimination; LOD, limit of detection; LOQ, limit of quantitation; LOB, limit of the blank; LLOQ, lower limit of quantification; Mg, magnesium; QC, quality controls; RBCs, red blood cells; RIs, reference intervals; Sc, scandium; Zn, zinc.

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occurring within RBCs. Improper Zn supplementation may potentially result in Cu deficiency, underscoring the importance of maintaining balanced levels. To gain comprehensive nutritional insights, it is crucial to recognize systemic Zn deficiency and evaluate Zn and Cu dynamics within RBCs [3]. Therefore, measuring multiple elements in RBCs, such as Mg, Cu, and Zn, collectively provides insights into the intracellular stores and overall homeostasis levels of these essential minerals.

Mg is the fourth most abundant cation in the human body and is considered an essential mineral [4], serving as a cofactor for over 325 enzymatic reactions in cells [5]. It is found in various foods, including fruits, vegetables, nuts, meats, fish, and dairy products, as well as in drinking water. Mg plays a vital physiological role in calcium and potassium transport, cell signaling, and energy metabolism in organs, such as the heart, muscles, and brain. A deficiency in Mg can lead to conditions like heart disease, diabetes, bone disorders, and neurological impairment [6,7]. At the same time, excessive levels of Mg in the human body can also be harmful [8]. While serum is commonly used to measure Mg in healthy individuals, it may not accurately reflect the actual Mg status due to strict regulatory mechanisms in the body, potentially leading to misleading results [9–11]. The majority of the body's Mg is stored in bones, muscles, soft tissues, and RBCs. RBCs, in particular, carry a comparatively high Mg concentration, with studies indicating lower Mg levels in RBCs among individuals following prolonged Mg-depleted diets [12,13]. Additionally, Mg concentration in RBCs correlates with malignancy severity in cancer patients, although the underlying mechanism remains unclear [14,15]. Thus, RBCs are the preferred specimen type for measuring Mg concentration in body stores [16].

Cu is a crucial micronutrient in the biological system, which may exist in the +1 or +2 valence state. It acts as a cofactor for oxidation–reduction reactions of different metalloprotein-dependent enzymatic reactions [17]. Cu can be obtained from shellfish, meat, chocolate, and whole grain [18]. Cu participates in approximately 300 enzymatic reactions in the human body by cycling between oxidized and reduced forms. It is predominantly absorbed in the gastrointestinal tract ileum, followed by transport to the portal blood, often binding with albumin, transcuprein, and/or metallothionein. It exhibits higher levels in women, particularly during pregnancy, oral contraceptive use, or estrogen treatment in menopause [19,20]. Some essential biological functions of Cu include energy production [21], iron metabolism [22], neurotransmitter signaling in the central nervous system [23], catalyzation of melanin synthesis [24], prevention of oxidative damage [25] and promotion of angiogenesis [26]. Cu deficiency, resulting from malabsorption, Zn supplementation [27], or bariatric surgery, can lead to anemia, hematological abnormalities [28], cardiac damage [29], Menkes disease [30] and myeloneuropathy [31]. Excessive ionized, unbound Cu accumulation in the liver, kidneys, and brain can cause adverse effects through Cu-induced cellular toxicity [32]. Genetic disorders like Wilson disease and Cu-contaminated food or water can also induce toxicity [33] causing hepatic disease, skeletal abnormalities, neurodegenerative changes, and myocardial disease [34]. Laboratory measurements of Cu concentration in RBCs, serum and/or plasma and urine specimens are used to assess individuals at risk of toxicity and/or deficiency [35]. Cu is an essential element for erythropoiesis and competes with Zn for absorption, making RBC Cu concentrations reflective of intracellular levels, general homeostasis, and nutritional status.

Zn is an essential trace element that serves as a co-factor for more than 200 metalloenzymes, playing a vital role in biological pathways. It is distributed throughout the body's tissues, but is mostly found in muscle and bone [36]. Notably, Zn concentration in RBCs is 6–10 times higher than in plasma [37]. Deficiency in Zn, stemming from genetic diseases or poor dietary intake, can lead to various conditions, such as immune suppression, susceptibility to infections, hair loss, diarrhea, psychiatric or mental health impairments, and weight loss [38]. Conversely, elevated Zn levels due to supplementation can interfere with Cu absorption and result in excess concentrations of Zn, leading to conditions like acute respiratory distress syndrome, gastrointestinal distress, metal fume fever, anemia, and secondary copper deficiency [39]. Laboratory assessments for Zn deficiency or overdose use RBCs, serum, plasma, and urine specimens. The preference for RBC samples stems from their ability to reflect zinc's intracellular levels, providing insights into general homeostasis and nutritional status.

Accurately measuring Mg, Cu, and Zn within RBCs is crucial for assessing nutritional status and overall health. In the past, methods for measuring Cu and Zn in RBCs required outsourcing, leading to inefficiencies. Outdated technology hindered historical Mg measurements in RBCs. To address these challenges, this study introduces a newly validated inductively coupled plasma mass spectrometry (ICP-MS) method for simultaneous quantification of Mg, Cu, and Zn in RBCs, effectively overcoming past limitations. Laboratory methods play significant roles in assessing nutritional status and diagnosing metal toxicity, and ICP-MS is considered the gold standard for precise elemental determinations based on specific mass-to-charge ratios. The primary objective of this study was the development and validation of an ICP-MS method for quantifying Mg, Cu, and Zn in RBCs. Method development and validation experiments assessed accuracy, linearity, sensitivity, imprecision, and carryover. In addition, retrospective analysis was conducted on a dataset spanning one and a half years following assay validation, using post-validation patient data.

2. Materials and methods

2.1. IRB protocol

The University of Utah Institutional Review Board (IRB Protocol #00082990) approved this study's retrospective analysis of clinical samples from human subjects.

2.2. Source of specimens

This validation study utilized pooled human whole blood and/or RBCs enriched with Mg, Cu and Zn, along with previously analyzed de-identified patient specimens. Whole blood samples were collected and then subjected to centrifugation to separate the RBCs from plasma. The isolated RBCs, measuring 2 mL, were transferred to ARUP trace-element-free collection tubes for testing. To minimize exposure to environmental contaminants, the ICP-MS tests for trace and toxic elements were conducted in a controlled clean room environment. Calibrators and quality control samples were prepared by fortifying Mg, Cu, Zn into lysed RBCs. The reference ranges for Mg, Cu, and Zn in RBCs were adopted from a reference laboratory to which we previously outsourced samples for analysis.

2.3. Reagents

All Reagents used in this method were reagent grade. Nitric acid and Ammonium Hydroxide (NH_4OH) and EDTA were purchased from VWR Scientific (Radnor, PA). Triton X-100 was purchased from Sigma-Aldrich, Inc (St. Louis, Mo). Clinical Lab Reagent Water (CLRW) was obtained from Milli-Q Water Purification System. Mg, Cu, Zn, Scandium (Sc), and Gallium (Ga) stock were purchased from Thermo Scientific, LLC (Waltham, MA). Four levels of quality controls (QC) were prepared by the ARUP Reagent Lab. Every batch of patient samples received matrix-matched QC specimens during the analysis process.

2.4. Instrumentation

The multi-element RBC panel method employed an Agilent 7700 ICP-MS instrument coupled with a workstation and syringe injection CETAC MVX 7100 autosampler system. Helium gas was utilized to mitigate potential polyatomic interferences in this method.

2.5. Instrument settings

Instrument settings are outlined in Table 1. Prior to each run, performance tune checks were conducted to verify the gas parameters for Kinetic Energy Dissociation. The manufacturer's recommended sensitivity was set at >1000 CPS for mass 59 in the tuning solution. To improve the signal-to-noise ratio in the method settings and enhance *m/z* 59 tune counts, acceptance criteria were established as follows: oxidized % of *m/z* 156/140 to be ≤1.5, and double charge % of *m/z* 70/140 to be ≤3.0.

2.6. Sample preparation

A volume of 20 μL was taken from calibrators, QC, and patient samples, which were then combined with 750 μL of Alkaline Diluent w/ 0.1 % EDTA, 0.1 % Triton X-100 and 1 % NH_4OH, with ^{45}Sc and ^{71}Ga) added as internal standards (ITSDs). This mixture was then placed into a 96-well microplate and thoroughly mixed. For the RBC panel assay, calibration curves were established using each batch of specimens. The calibration concentrations ranged from 2 to 12 mg/dL for Mg, 40 to 180 μg/dL for Zn, and 400 to 2000 μg/dL for Zn.

2.7. ICP-MS analysis

The prepared 96-well plate containing the RBC samples was loaded into the CETAC MVX-7100 workstation autosampler, which facilitated the aspiration of diluted specimens into the ICP-MS system. The ICP-MS procedure involved introducing the aspirated samples into a plasma torch within an argon atmosphere, leading to aerosol ionization. The plasma torch, maintained at around 6000 degrees Kelvin with up to 2.5 kW of radio frequency power, enabled efficient ionization. To address potential interferences, helium gas was introduced at the collision cell entrance using the kinetic energy discrimination (KED) method. This helped eliminate polyatomic interferences that arise when combined atoms match the natural isotope's mass of the analyte. Other interferences, such as those from heavier elements with double charge states, were also evaluated (Table 2). These interferences could result in elevated element concentrations, necessitating correction during measurement. Targeted ions and interfering molecules underwent selective filtration through an energy filter to remove interfering ions from the desired analytes. These ions were then filtered by the main quadrupole, transduced into electrons, and amplified by the ion multiplier for quantification. The calibration curve used for quantifying the three elements was established by assessing ratios between varying amounts of calibrator components and a constant ISTD quantity.

Table 1
Instrument settings.

CETAC MVX 7100 autosampler settings parameters:	
Instrument	**Setting Parameters**
Sample volume/loop volume	360 μL
Carrier Dispense rate	575 μL/min
Carrier Dispense volume	665 μL
Quick Push	Enabled with a volume of 750 μL
Extra Rinse	Enabled
Sample Mixing	Disabled
Agilent 7700 ICP-MS instrument settings parameters:	
Instrument	Setting Parameters
Helium gas flow/rate	4.5 mL/min
Peak Pattern	1 Point
Replicates	3
Sweeps/Replicate	30
Integration Time/Mass	0.3 s for Mg, Cu, and Zn
Energy Discrimination	5.0 V
Mode of analysis	Gas Mode
Octopole (OctP) bias	18.0 V
Radio frequency (RF) Power	1550 W
Sample Depth	8.0 mm
Option Gas and makeup/dilution gas	0
S/C Temperature	2 °C
Extract 1	0 V
Cell Entrance	38 V
Cell Exit	−55 V
Calibration mode	Standard Addition
Calibration weighting	None for Mg, Cu, and Zn

2.8. Method validation

2.8.1. Accuracy

To assess accuracy, we compared patient and matrix-matched spiked specimen test results with the method-generated results from those specimens. For the accuracy study, a minimum of eight samples per day were analyzed over five days for each analyte.

2.8.2. Method comparison

The analytical accuracy of the multi-element panel was evaluated through a rigorous testing process. For Mg, we conducted assessments using patient RBC samples that had previously undergone testing on the production Mg RBC assay (n = 48). To comprehensively evaluate the accuracy of our method for Cu and Zn, we took a comparative approach. Patient RBC samples were split and sent to another accredited laboratory for Cu and Zn testing. The results from the other laboratory were then compared to our calculated values for Cu (n = 39) and Zn (n = 42) in patient RBC spiked specimens. Deming and linear regression analyses were employed for result comparison and to determine the slope, intercept, and correlation coefficient of each element. The acceptance criteria for the slope was set to be between 0.9 and 1.10, while the y-intercept was required to be less than the lower limit of quantification (LLOQ) for each element. Furthermore, a correlation coefficient value ≥0.95 was deemed acceptable for Mg, Cu, and Zn.

2.8.3. Linearity

In the linearity study, five standards were prepared by combining different ratios of elements to achieve the target concentrations (Table 3). The calibration standards were analyzed through at least five separate runs, conducted on a minimum of two different days, at each mix level. The concentration range targeted for analysis encompassed values below, within, and above the desired Analytical Measurement Range (AMR).

Linear regression analysis was utilized to compare the results and calculate correlation coefficients, imprecision, and accuracy for each element. For acceptance criteria, the following thresholds were defined: a correlation coefficient value of ≥0.99, an imprecision coefficient of variation (CV) of ≤15 %, and an accuracy of ≤±15 %.

2.8.4. Imprecision

For this assay, within-run, between-run, and total imprecision were

Table 2
Possible source of interferences from polyatomic and doubly-charged species.

Elements	Polyatomic species
^{25}Mg	$^{12}C_2^1H^+$, $^{12}C^{13}C^+$, $^{50}Ti^{++}$, $^{50}V^{++}$, $^{50}Cr^{++}$, $^{24}Mg^1H^+$, $^{7}Li^{18}O^+$, $^{9}Be^{16}O^+$
^{45}Sc	$^{12}C^{16}O_2^1H^+$, $^{28}Si^{16}O^1H^+$, $^{29}Si^{16}O^+$, $^{14}N_2^{16}O^1H^+$, $^{13}C^{16}O_2^+$, $^{90}Zr^{++}$, $^{9}Be^{36}Ar^+$, $^{10}B^{35}Cl^+$, $^{44}Ca^1H^+$, $^{27}Al^{18}O^+$
^{63}Cu	$^{31}P^{16}O_2^+$, $^{40}Ar^{23}Na^+$, $^{47}Ti^{16}O^+$, $^{23}Na^{40}Ca^+$, $^{46}Ca^{16}O^1H^+$, $^{36}Ar^{12}C^{14}N^1H+$, $^{14}N^{12}C^{37}Cl^+$, $^{16}O^{12}C^{35}Cl^+$, $^{126}Te^{++}$, $^{27}Al^{36}Ar^+$, $^{26}Mg^{37}Cl^+$, $^{28}Si^{35}Cl^+$, $^{62}Ni^1H^+$, $^{45}Sc^{18}O^+$
^{66}Zn	$^{50}Ti^{16}O^+$, $^{34}S^{16}O_2^+$, $^{33}S^{16}O_2^1H^+$, $^{32}S^{16}O^{18}O^+$, $^{32}S^{17}O_2^+$, $^{33}S^{16}O^{17}O^+$, $^{32}S^{34}S^+$, $^{33}S_2^+$, $^{132}Xe^{++}$, $^{26}Mg^{40}Ar^+$, $^{29}Si^{37}Cl^+$, $^{132}Ba^{++}$, $^{31}P^{35}Cl^+$, $^{65}Cu^1H^+$, $^{50}Cr^{16}O^+$
^{71}Ga	$^{35}Cl^{18}O2^+$, $^{37}Cl^{16}O^{18}O^+$, $^{37}Cl^{17}O_2^+$, $^{36}Ar^{35}Cl^+$, $^{36}S^{17}O^{18}O^+$, $^{38}Ar^{33}S^+$, $^{142}Ce^{++}$, $^{142}Nd^{++}$, $^{31}P^{40}Ar^+$, $^{34}S^{37}Cl^+$, $^{36}S^{35}Cl^+$, $^{70}Zn^1H^+$, $^{70}Ge^1H^+$, $^{55}Mn^{16}O^+$

Table 3
Calibrator concentrations and linearity results.

Elements	LLOQ	Standard 1	Standard 2	Standard 3	Standard 4	Approved dilution factor	Linear equation	Correlation coefficient
Mg (mg/dL)	2.0	3.7	5.8	9.9	13.9	5x	$y = 0.9922x + 0.125$	$R^2 = 0.9998$
CV (%)	2.60 %	1.70 %	1.40 %	1.80 %	1.00 %			
Cu (μg/dL)	40.1	82.6	106.4	163	221.8	2x	$y = 0.9971x + 2.3925$	$R^2 = 0.9986$
CV (%)	2.4 %	1.6 %	1.3 %	1.8 %	1.00 %			
Zn (μg/dL)	400	849.9	1168.7	1877.5	2600.1	2x	$y = 1.0159x + 3.8092$	$R^2 = 0.9988$
CV (%)	2.4 %	1.6 %	1.4 %	1.5 %	1.1 %			

evaluated. To assess within-run imprecision, four specimens covering the reporting range were each run 20 times in a single day and in a single run for each element. The concentrations of these specimens can be found in Table 4. Regarding between-run imprecision, four samples spanning the reporting range were run five times a day for four consecutive days for each component. To calculate total imprecision, data from the between-run imprecision study were consolidated. The % CV for within-run, between-run, and total imprecision was calculated for each element, with an acceptability threshold set at ≤15 %.

2.8.5. Sensitivity

Analytical sensitivity was assessed through the determination of the limit of the blank (LOB), the functional limit of detection (LOD), and the limit of quantitation (LOQ). The LOB was calculated by averaging the results of diluent-only blank samples over five measurements per day for four consecutive days and adding three times the standard deviation. The LOB criterion was set to be below the LOQ for each element.

The LOD and LOQ were established by analyzing a pool designed to represent half of the lower end of the reporting range. This pool was tested five times a day over a period of four days. The LOD CV was utilized for informational purposes, while the LOQ CV target was set to be less than 20 %. The requirement for LOQ accuracy was ≤±20 %.

2.8.6. Carryover

We assessed carryover interference using a red blood cell pool spiked at two different levels. High concentrations were set at three times above the calibration range (36 mg/dL, 540 μg/dL, and 6000 μg/dL for Mg, Cu, and Zn, respectively), while low concentrations were positioned towards the lower end of the calibration range (3 mg/dL, 55 μg/dL, and 700 μg/dL for Mg, Cu, and Zn, respectively). These samples were analyzed in the following sequence on three separate days: L1, L2, L3, H1, H2, L4, H3, H4, L5, L6, L7, L8, H5, H6, L9, H7, H8, L10, H9, H10, L11.

The higher concentrations of the low samples were immediately followed by the low samples (L4, L5, L9, L10, L11), and the results were compared to the low samples following the low samples (L2, L3, L6, L7, L8). This sequencing was designed to indicate potential carryover. Carryover was calculated by subtracting the average of the high-low samples from the average of the low-low samples. The value was divided by the average of the high samples and multiplied by 100 to express it as a percentage.

This experiment was conducted on three different days, and we compared the percent carryover from these three days to an acceptability criterion of 1 %. This criterion represented calculated values less than or equal to three times the standard deviation of the low-concentration samples.

Table 4
Total Imprecision (%CV).

Elements	Conc. 1	% CV	Conc. 2	% CV	Conc. 3	% CV	Conc. 4	% CV
Mg (mg/dL)	2.5	2.1	4.9	2.9	8.7	1.8	10.6	2.6
Cu (mg/dL)	43.9	2.1	74.9	3.1	98.9	2.8	147.4	3.2
Zn (mg/dL)	481.4	2.5	872.7	3.3	1235.5	2	1823.5	3.3

2.8.7. Dilution

To evaluate the dilution strategy, we utilized the RBC pool spiked at a concentration 1.5 times higher than the upper limit of the AMR for each element. The validation process involved a 2-fold dilution for all elements except for Mg, which was validated up to a 5-fold dilution. This dilution was achieved by mixing with clinical laboratory reagent water.

The diluted and undiluted specimens were analyzed five times within a single batch over a day. For each element, the target concentration was calculated to determine the percentage deviation, which was then compared to the mean of the detected concentration.

Our acceptance criteria were set within a ±15 % range of the target concentration, with an expected CV of ≤15 % established as our performance benchmark.

2.8.8. Retrospective patient data analysis

Data was collected for retrospective data analysis from the internal laboratory information system at ARUP laboratories (Salt Lake City, UT). Retrospective data analysis results from the new analytical method were used to calculate the reference range and compare it with the adopted reference range for each element. Multiple tests run for the same patient were retained due to the deidentification of the patient data. The medical history and dietary nutrition of the patients were unknown for this study's patients.

2.8.9. Statistical analysis

Retrospective data analysis was conducted using R data analysis software. Two-way ANOVAs and Tukey's HSD test were employed to examine statistical differences between sex and age groups. The test results followed a normal distribution; therefore, a standard normal deviation test was utilized to determine if reference intervals (RIs) should be stratified by sex and age group (adult and pediatric) as recommended by the CLSI guidelines. Outliers were eliminated through Tukey outlier deletion before determining RIs.

RIs were determined using a non-parametric approach, with RIs defined at the 2.5th and 97.5th percentiles. Mean, standard deviation, % CV, slope, y-intercept, and correlation coefficient were calculated using Microsoft Excel (Microsoft, WA, USA).

3. Results

3.1. Accuracy and method comparison

Linear regression analysis was performed, with predefined criteria for slopes between 0.90 and 1.10 and intercepts below the LOQ for each element. Additionally, a correlation coefficient equal to or greater than 0.95 was mandated. All elements in the assay satisfied the specified accuracy criteria. Refer to Fig. 1 for the accuracy linear regression and bias plots of each element.

3.2. Linearity

The calibration curve was constructed from the lowest calibrator concentration to the highest calibrator concentration using the average

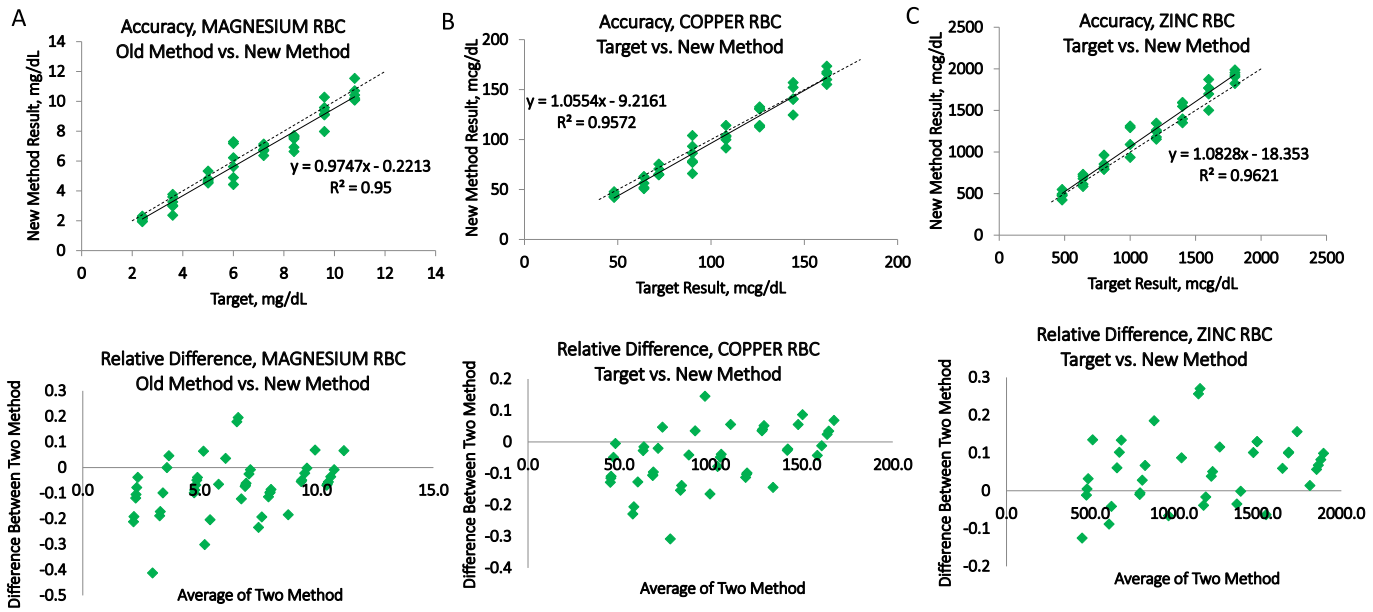

Figure 1: Accuracy study – Linear regression and Bias plots, magnesium (A), Copper (B) and Zinc (C)

Private Information

Fig. 1. Accuracy study – Linear regression and Bias plots, magnesium (A), Copper (B) and Zinc (C).

of each standard for each element. Linearity data are presented in Table 3. All three elements met the linearity criteria.

3.3. Imprecision

The imprecision of the multi-element panel was evaluated by examining four different concentrations for each element. While both within-run and between-run imprecision were calculated, the focus was on presenting data for total imprecision, as it is the most relevant aspect of this study. Importantly, all imprecision criteria were satisfied by the three elements analyzed. Table 4 provides the data for the lowest and highest concentrations in the imprecision study, including their respective percent % CV.

3.4. Sensitivity

The LOB was 0.0 mg/dL for Mg, 1.2 µg/dL for Zn, and 0.4 µg/dL for Cu. The LOQ was established at 2 mg/dL for Mg, 400 µg/dL for Zn, and 40 µg/dL for Cu.

3.5. Carryover

The calculated carryover for Mg, Cu, and Zn ranged from −0.1 % to 0.5 %. All three elements met the carryover criteria. The contribution of carryover from the high-concentration specimen was minimal and did not lead to a falsely elevated result for a low patient sample.

3.6. Retrospective data analysis

In our retrospective patient data analysis, we examined information obtained through the new multi-element method. A total of 25,147 unique samples were collected for Mg, 1,823 for Cu, and 6,567 for Zn, covering various age groups from 0 to 105 years. The details of age and sex groupings are presented in Table 5(A) and 5(B) for reference. Two-way ANOVAs were performed to evaluate the impact of age and sex on analyte concentrations. The results of the two-way ANOVAs indicated statistically significant effects of both age group and sex on the concentrations of Mg, Cu, and Zn ($p = 0.003$, $p = 0.0003$, $p < 0.0001$, respectively).

Further analysis revealed that age group significantly influenced Cu and Zn concentrations ($p < 0.0001$), while sex had a significant effect on Mg and Zn concentrations ($p < 0.0001$). Additionally, Tukey's HSD test was used for multiple comparisons, uncovering statistically significant mean concentration differences among nearly all pairwise groups of age and sex ($p < 0.05$), with a few exceptions. Specifically, differences in mean Cu concentration between adult males (≥18 to 105 years) and adult females (≥18 to 105 years), as well as mean Mg concentrations between adult males and young males (0 to < 18 years), were not statistically significant.

RIs were determined by age and sex based on standard normal deviation test results. Table 5(A) and 5(B) present details including the total number of patients, age distribution, RI per element, and the percentage of the population within or outside of the adopted and calculated RI.

Table 5(A)
Retrospective patient data analysis.

Elements (RBC)	Age Range (years)	Original Reference interval (RBC)	Total subjects (N)	% of patient within the reference interval	% of patient above the reference interval	% of patient below the reference interval
Magnesium (Mg)	0–105	3.6–7.5 mg/dL	24,858	100.0 % (N = 24858)	0.00 % (N = 0)	0.00 % (N = 0)
Copper (Cu)	0–105	59–91 µg/dL	1774	92.0 % (N = 1632)	3.78 % (N = 67)	4.23 % (N = 75)
Zinc (Zn)	0–105	794–1470 µg/dL	6423	85.4 % (N = 5485)	14.51 % (N = 932)	0.09 % (N = 6)

Table 5(B)
Retrospective patient data analysis.

Elements (RBC)	Age Range (years)	Gender	Calculated Reference interval (RBC)	Original Reference interval (RBC)	Total subjects (N)	% of patient within the reference interval	% of patient above the reference interval	% of patient below the reference interval
Magnesium	0–17	F		3.6–7.5 mg/dL		100.0 % (N = 579)	0 % (N = 0)	0 % (N = 0)
			4.2–6.4 mg/dL		579	96.5 % (N = 559)	1.38 % (N = 8)	2.07 % (N = 12)
	18–105			3.6–7.5 mg/dL		100.0 % (N = 16688)	0 % (N = 0)	0 % (N = 0)
			4.2–6.7 mg/dL		16,688	95.5 % (N = 15929)	2.06 % (N = 343)	2.49 % (N = 416)
	0–17	M		3.6–7.5 mg/dL		100.0 % (N = 498)	0 % (N = 0)	0 % (N = 0)
			4.3–6.7 mg/dL		498	95.6 % (N = 476)	2.41 % (N = 12)	2.01 % (N = 10)
	18–105			3.6–7.5 mg/dL		100.0 % (N = 7083)	0 % (N = 0)	0 % (N = 0)
			4.2–6.8 mg/dL		7083	96.1 % (N = 6810)	1.79 % (N = 127)	2.06 % (N = 146)
Copper	0–105	F		59–91 μg/dL		93.0 % (N = 1088)	3.59 % (N = 42)	3.42 % (N = 40)
			57.7–92.7 μg/dL		1170	95.0 % (N = 1112)	2.48 % (N = 29)	2.48 % (N = 29)
	0–17	M		59–91 μg/dL		82.9 % (N = 102)	16.26 % (N = 20)	0.81 % (N = 1)
			62.5–105.8 μg/dL		123	95.1 % (N = 117)	2.44 % (N = 3)	2.44 % (N = 3)
	18–105			59–91 μg/dL		92.1 % (N = 442)	1.25 % (N = 6)	6.67 % (N = 32)
			55.8–89.7 μg/dL		480	95.0 % (N = 456)	2.5 % (N = 12)	2.5 % (N = 12)
Zinc	0–105	F/M		794–1470 μg/dL		83.5 % (N = 5485)	15.55 % (N = 1021)	0.93 % (N = 61)
			871.8–1718.3 μg/dL		6567	95.0 % (N = 6240)	2.48 % (N = 163)	2.5 % (N = 164)

4. Discussion

Analysis of RBC specimens proves vital in assessing the nutritional status and potential toxicity of elements like Mg, Zn, and Cu. RBCs contain a variety of circulating elements, making them an ideal specimen type for evaluating element concentrations. Our laboratory employs this method to measure Mg, Zn, and Cu levels within RBC specimens. Prior to validating this multi-element method, measurements of Cu and Zn in RBCs were outsourced, and the reference ranges were adopted from the external reference laboratory. Furthermore, historical Mg measurements within RBCs were conducted using the PerkinElmer DRC ICP-MS instrument, which was eventually retired due to outdated technology, inadequate performance, and costly service contracts. This outdated instrumentation for Mg measurements presented limitations such as analytical inaccuracies, extended run times, and increased specimen volume requirements.

The new method was developed using an Agilent 7700 ICP-MS instrument coupled with a workstation and a syringe injection CETAC MVX 7100 autosampler system. Calibrators and quality control samples were employed to assess the acceptability of the calibration process, instrument drift, and test results. Our Reagent Lab prepares these QC and calibrators with varying levels of Mg, Zn, and Cu spiked into lysed red blood cells. This matrix-matched QC and these calibrators have significantly improved the analytical accuracy of this multi-element method.

The method is operated in helium mode with KED to minimize background noise and reduce polyatomic interferences. Additionally, this assay was performed in a clean room with a detailed standard operating procedure to minimize external environmental contamination. Reagent-grade reagents and clinical laboratory reagent water were used to reduce possible background noise.

Before running each batch, instrument auto-tune was performed to adjust mass spectrometry parameters and other predefined criteria for the assay. Moreover, an alkaline and nitric acid rinse was completed both before the run, to prime the system, and after the run, to clean the residual sample matrix and prevent nebulizer blockage. Before running a calibration curve, controls, and patient samples for each batch, seven matrix-matched samples and one diluent-only sample were run on the instrument to reduce instrument drift and achieve steady performance state (also known as conditioning). Furthermore, analytical accuracy was improved by adding four matrix-matched standards and QC samples with every run.

The instrument was maintained daily, weekly, and monthly to improve the accuracy and reproducibility of test results, maintain turnaround time by reducing unnecessary and unscheduled downtime, and prolong instrument and equipment life.

Daily maintenance involved a visual inspection of the torch, connector tube, spray chamber, nebulizer, and skimmer cone for residue, cracks, and melted injector, followed by cleaning with hydrogen peroxide and de-ionized water. Damaged parts were promptly replaced with new ones.

Weekly maintenance included cleaning the torch, connector tube, spray chamber, end cap, and nebulizer with 20 % bleach, followed by thorough rinsing in de-ionized water.

Monthly maintenance involved cleaning staining from the ion lens stack with a non-matrix rinse. Equipment cleaning and maintenance aimed to minimize potential contamination and method carryover. Records of instrument maintenance were logged daily, weekly, and monthly in the laboratory information management system.

We analyzed retrospective patient data obtained through the new RBC multi-element method to compare patient values against the adopted RIs for each analyte. The current RIs for RBC Mg, Cu, and Zn are 3.6–7.5 mg/dL, 59–91 μg/dL, and 794–1470 μg/dL, respectively. An analysis revealed that the majority of patient results (ranging from 83.5 % to 99.9 %) fell within these existing intervals for all three elements.

In contrast, the newly calculated RIs for RBC Mg, Cu, and Zn are 4.2–6.7 mg/dL, 56.9–93.4 μg/dL, and 871.8–1718.3 μg/dL, respectively. Notably, the calculated intervals suggest a narrower range for RBC Mg compared to the current intervals, encompassing approximately 95 % of patient data. This narrower range may potentially lead to the over-classification of Mg deficiency or toxicity.

Conversely, the calculated intervals for Cu and Zn appear broader than the current ranges. Roughly 95 % of patient results align with the newly calculated intervals, while only 79–90 % fall within the current intervals. These differences may result from age and gender-based variations in the calculated intervals for Cu and Zn. For Cu, wider calculated intervals were observed in young males (0 to <18 years) compared to adult males (≥18 to 105 years), potentially influenced by age-related differences in Cu concentration

In contrast, the calculated Zn interval showed a different pattern, possibly due to the age-related inverse association of the Cu/Zn ratio, which tends to be higher in children than in adults. Thus, the lower calculated Zn interval for young populations compared to adult males and females may be linked to these age-related dynamics [40]. The calculated reference range supported the use of the adopted reference, which were used for patient result reporting.

We have presented data comparing combined and segmented age and sex groups, evaluating the currently adopted and calculated RIs for RBC Mg, Cu, and Zn. Our retrospective data analysis highlights the importance of incorporating age and gender-specific RIs for these elements in RBCs. It is essential to recognize the limitations of this retrospective analysis, including the lack of patient medical history, clinical

conditions, inpatient/outpatient status, supplement intake, specific diagnoses, and reasons for laboratory evaluations. Furthermore, the dataset for Cu was relatively smaller than that for Mg and Zn, possibly reflecting lower test orders for Cu compared to the other elements. In summary, the ICP-MS method satisfactorily met the expected validation criteria for monitoring Mg, Cu, and Zn elements in RBC specimens.

Ethics statement

The University of Utah Institutional Review Board (IRB Protocol #00082990) approved this retrospective analysis of clinical samples from human subjects.

CRediT authorship contribution statement

Nazmin Bithi: Writing – original draft. **Daniel Ricks:** Writing – review & editing, Validation, Methodology, Formal analysis. **Brandon S. Walker:** Writing – review & editing, Software, Formal analysis. **Christian Law:** Writing – review & editing, Project administration. **Kamisha L. Johnson-Davis:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] A. Mehri, Trace elements in human nutrition (II)–an update, Int. J. Prev. Med. 11 (2020).
[2] A.S. Prasad (Ed.), Essential and Toxic Element: Trace Elements in Human Health and Disease, Elsevier, 2013.
[3] A. Takahashi, Role of zinc and copper in erythropoiesis in patients on hemodialysis, J. Ren. Nutr. (2022).
[4] A.A. Ismail, N.A. Ismail, Magnesium: a mineral essential for health yet generally underestimated or even ignored, J. Nutr. Food Sci. 6 (2) (2016) 1–8.
[5] M.-K. Choi, Y.J. Bae, Association of magnesium intake with high blood pressure in Korean adults: Korea national health and nutrition examination survey 2007–2009, PLoS One 10 (6) (2015) e0130405.
[6] Helmut Geiger, Christoph Wanner, Magnesium in disease, Clin. Kidney J. 5 (Suppl_1) (2012) i25–i38.
[7] Jeroen H.F. De Baaij, Joost G.J. Hoenderop, René J.M. Bindels, Regulation of magnesium balance: lessons learned from human genetic disease, Clin. Kidney J. 5 (Suppl_1) (2012) i15–i24.
[8] Y. Sakaguchi, et al., Hypomagnesemia is a significant predictor of cardiovascular and non-cardiovascular mortality in patients undergoing hemodialysis, Kidney Int. 85 (1) (2014) 174–181.
[9] W. Jahnen-Dechent, M. Ketteler, Magnesium basics, Clin. Kidney J. 5 (Suppl_1) (2012) i3–i14.
[10] D.e. Baaij, H.F. Jeroen, J.GJ. Hoenderop, R.JM. Bindels, Magnesium in man: implications for health and disease, Physiol. Rev. (2015).
[11] J. Fairley, et al., Magnesium status and magnesium therapy in critically ill patients: a systematic review, J. Crit. Care 30 (6) (2015) 1349–1358.
[12] M.J. Arnaud, Update on the assessment of magnesium status, Br. J. Nutr. 99 (S3) (2008) S24–S36.
[13] L.E. Basso, J.B. Ubbink, R. Delport, Erythrocyte magnesium concentration as an index of magnesium status: a perspective from a magnesium supplementation study, Clin. Chim. Acta 291 (1) (2000) 1–8.
[14] S. Sartori, et al., Serum and erythrocyte magnesium concentrations in solid tumours: relationship with stage of malignancy, Magn. Res. 5 (3) (1992) 189–192.
[15] W. Xiong, et al., Erythrocyte intracellular Mg2+ concentration as an index of recognition and memory, Sci. Rep. 6 (1) (2016) 1–12.
[16] M. Witkowski, J. Hubert, A. Mazur, Methods of assessment of magnesium status in humans: a systematic review, Magn. Res. 24 (4) (2011) 163–180.
[17] M. Wołonciej, E. Milewska, W. Roszkowska-Jakimiec, Trace elements as an activator of antioxidant enzymes, Postepy Hig. Med. Dosw. (Online) 70 (2016) 1483–1498.
[18] C. Kies, Food sources of dietary copper, Copper Bioavailability Metab. (1989) 1–20.
[19] N.A. Clark, et al., Trace element levels in adults from the west coast of Canada and associations with age, gender, diet, activities, and levels of other trace elements, Chemosphere 70 (1) (2007) 155–164.
[20] D.B. Milne, P.E. Johnson, Assessment of copper status: effect of age and gender on reference ranges in healthy adults, Clin. Chem. 39 (5) (1993) 883–887.
[21] R.B. Rucker, et al., Copper, lysyl oxidase, and extracellular matrix protein cross-linking, Am. J. Clin. Nutr. 67 (5) (1998) 996S–1002S.
[22] M. Arredondo, M.T. Núñez, Iron and copper metabolism, Mol. Aspects Med. 26 (4-5) (2005) 313–327.
[23] S. Lutsenko, A. Bhattacharjee, A.L. Hubbard, Copper handling machinery of the brain, Metallomics 2 (9) (2010) 596–608.
[24] V.J. Hearing, M. Jiménez, Mammalian tyrosinase—the critical regulatory control point in melanocyte pigmentation, Int. J. Biochem. 19 (12) (1987) 1141–1147.
[25] M. Valko, et al., Free radicals, metals and antioxidants in oxidative stress-induced cancer, Chem. Biol. Interact. 160 (1) (2006) 1–40.
[26] K. Ahmad, Copper moves across membranes during angiogenesis, Lancet Oncol. 8 (4) (2007) 289.
[27] M.S. Willis, et al., Zinc-induced copper deficiency: a report of three cases initially recognized on bone marrow examination, Am. J. Clin. Pathol. 123 (1) (2005) 125–131.
[28] Z.W. Myint, et al., Copper deficiency anemia, Ann. Hematol. 97 (9) (2018) 1527–1534.
[29] D.M. Danks, Copper deficiency in humans, Annu. Rev. Nutr. 8 (1) (1988) 235–257.
[30] M. Altarelli, et al., Copper deficiency: causes, manifestations, and treatment, Nutr. Clin. Pract. 34 (4) (2019) 504–513.
[31] B.P. Goodman, et al., Copper deficiency myeloneuropathy due to occult celiac disease, Neurologist 15 (6) (2009) 355–356.
[32] L.A. Videla, et al., Oxidative stress-mediated hepatotoxicity of iron and copper: role of Kupffer cells, Biometals 16 (1) (2003) 103–111.
[33] A. Ala, et al., Wilson's disease, Lancet 369 (9559) (2007) 397–408.
[34] V.W. Vanek, et al., ASPEN position paper: recommendations for changes in commercially available parenteral multivitamin and multi–trace element products, Nutr. Clin. Pract. 27 (4) (2012) 440–491.
[35] R. Squitti, et al., Measurements of serum non-ceruloplasmin copper by a direct fluorescent method specific to Cu (II), Clin. Chem. Lab. Med. 55 (9) (2017) 1360–1367.
[36] H. Tapiero, K.D. Tew, Trace elements in human physiology and pathology: zinc and metallothioneins, Biomed. Pharmacother. 57 (9) (2003) 399–411.
[37] K.L. Johnson-Davis, et al., Method validation for a multi-element panel in serum by inductively coupled plasma mass spectrometry (ICP-MS), Clin. Biochem. 82 (2020) 90–98.
[38] M.J. Tuerk, N. Fazel, Zinc deficiency, Curr. Opin. Gastroenterol. 25 (2) (2009) 136–143.
[39] Ulrika M. Agnew, Todd L. Slesinger, Zinc toxicity, 2020.
[40] M.F. Escobedo-Monge, et al., Copper and copper/Zn ratio in a series of children with chronic diseases: a cross-sectional study, Nutrients 13 (10) (2021) 3578.