Validation of a method for quantification of Lead, Chromium, Magnesium, Zinc & Copper in human blood and serum using Atomic Absorption Spectrometry

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Abstract

The quality of analytical data coming from the analysis of biological samples like blood, serum and human hair is of fundamental importance for judging the limit of contaminants. Since this analysis is exposed to errors due to sampling, sample preparation and instrument, it is important to validate the analytical methods used for such measurements. A method for quantification of lead, chromium, copper, magnesium and zinc in blood and in serum using electrothermal and flame atomic absorption spectrometer was established. Full validation was done to ensure the suitability of the developed method for the purpose of analysis. The main parameters evaluated in the validation study were the limit of detection (LOD), limit of quantification (LOQ), linearity range, accuracy, precision and expanded uncertainty of the measurement results of in case of each element. The results obtained revealed that the analytical method is valid for the analysis of biological samples.

Key words: Blood samples, trace elements, method validation, uncertainty, traceability

1. Introduction

Validation of an analytical method is a necessary step in controlling the quality of quantitative analysis. Validation can be defined as the process by which it is established by laboratory studies that the analytical parameters of the method meet the requirements for the intended analytical applications [1]. Thus, with the background knowledge of linearity, detection and quantification limits, precision, accuracy, specificity and robustness of the analytical method, it is relatively easy to derive the confidence and reliability of the analytical data obtained with it [1]. Also biomonitoring of trace elements in human blood has become an important tool for occupational and environmental health [2]. Among several elements, chromium, zinc, copper, lead, nickel, magnesium and manganese are of great importance because they are largely used in metal industry including welders and alloy smelter works. Lead is a harmful element since it is particularly taken in through the respiratory tract and the gastrointestinal tract. Its toxicity has been known from ancient times and many studies have explored the mechanisms and symptoms of this toxicity through the years [16]. Copper and zinc are known to be necessary for the activity of some enzymes [3]. Copper is an essential micronutrient. However it can be toxic when present in excess, the most noticeable chronic effect being liver damage [17]. Magnesium is an essential nutrient mainly found in foods like cereals, nuts, and vegetable [1]. Many techniques have been used to measure trace elements in blood such as inductively coupled plasma mass spectrometry (ICP-MS), inductively coupled plasma optical emission spectrometry (ICP-OES) and atomic absorption spectrometry (AAS). The analytical results must be reliable which reflect unambiguous, true and clear values of sample composition [4]. The aim of this work is to develop and validate an analytical method used to determine chromium, Zinc and Magnesium in blood serum using atomic absorption spectrometry. Also full validation was done for a published method used to determine lead and copper in whole blood and serum [2,5].

2. Materials and methods

Hydrogen peroxide 30%, ammonium dihydrogen phosphate 99.999 %, magnesium nitrate hydrate 99.999% and Triton X-100 were obtained from Alpha Aesar, Germany. Nitric acid, 65%, was obtained from Analar, England and potassium chloride 99%, was obtained from Merck, Germany. Standard calibration solutions of Pb, Cr, Mg, Cu & Zn were prepared using NIS certified reference
Blood samples were collected and were stored at 4°C and analyzed within short times. For validation of the analytical method of lead, blood samples were collected in heparin containers. 100 µL of sample was diluted with 20 µL 0.1% (V/V) Triton X-100 and completed to 1 mL with 0.5% (V/V) ammonium hydroxide solution in auto sampler caps [2]. A solution of 1% of ammonium dihydrogen phosphate was prepared as a modifier for lead. For validation of the analytical method of copper, blood serum was obtained by centrifuging the blood samples for 5 minutes at a radial centrifugal acceleration of 3000 rps. One mL serum was mixed with 9 mL of 0.1% potassium chloride solution in 15 mL plastic cup and then was directly injected [5]. Blank samples were also prepared. The average signal of blank solutions was subtracted from analytical signals of unknown samples.

Development of a method for analysis of Chromium, Magnesium & Zinc
Blood sample of 1 mL was mixed with 4.5 mL nitric acid and 2.5 mL H₂O₂ in teflon container and the mixture was placed in microwave digestion system. After cooling, the mixture was transferred to 25 mL measuring flask and was diluted to the final volume with deionized water. 0.1 % of magnesium nitrate was prepared to be used as a modifier for chromium.

3. Instrumentation
The measurements were made using Zeenit 700 atomic absorption spectrometer (Analytik Jena, Jena, Germany). Lead, chromium, copper, magnesium and zinc hallow cathode lamps were operated according to the manufacturer instructions.

Recovery study
Spike sample was prepared by adding known amounts of the analyzed elements to the unknown sample. The spike was treated and analyzed by the same procedure used for the analysis of the unknown sample.

Linearity study
The calibration of flame and electrothermal atomic absorption spectrometer was performed by introducing different concentrations of every element standard solution.

4. Results and Discussion
The performance characteristics of the analytical method of each of the five elements in blood sample were evaluated. A blank sample was injected into atomic absorption ten times. The limit of detection (LOD) was calculated as LOD = 3 S and the limit of quantification (LOQ) was calculated as LOQ = 10 S, where S is the standard deviation. The results obtained for both parameters are shown in Table 1.

| Parameter | Pb   | Cr   | Cu  | Zn   | Mg  |
|-----------|------|------|-----|------|-----|
| LOD       | 0.16 µg/L | 0.06 µg/L | 0.029 mg/L | 0.0164 mg/L | 0.015 mg/L |
| LOQ       | 0.539 µg/L | 0.539 µg/L | 0.097 mg/L | 0.0547 mg/L | 0.050 mg/L |

4.1. Calibration curve and range of linearity
AAS was calibrated using external standard calibration method. The response factor was used to check the linear range of calibration of the atomic absorption. [6], the correlation coefficient r² was calculated as well. It was found that the correlation coefficient r² was 0.9999, 0.9973, 0.9998, 0.9989 and 0.9989 for Pb, Cr, Zn, Cu and Mg respectively in certain ranges. The traceability of measurement results to the SI units was established by using the NIS certified reference materials of the measured elements for calibration of flame and electro thermal atomic absorption spectrometer.

4.2. Accuracy
The analysis of spiked samples evaluates the accuracy of the procedure of the sample preparation. Also it reveals any errors made due to the sample preparation or matrix effect. The recovery study was used to evaluate the accuracy of the method. The % recovery was calculated from the following equation:

\[ \text{Recovery} = \frac{X_{\text{measured}}}{X_{\text{reference}}} \times 100 \]  

Where \( X_{\text{measured}} \) is the concentration found
\( X_{\text{reference}} \) is the spiked concentration
It was found that, the (%) recovery was between 95-102% for the analyzed elements which is in agreement with the criteria in the field of trace analysis [12].

4.3. Precision (repeatability & reproducibility)
Both repeatability and reproducibility were used to calculate the whole precision of the method. The repeatability was evaluated for Zn, Cu & Mg from five repeated measurements and for Pb & Cr from three repeated measurements. The reproducibility was evaluated from repeated measurements in five different days. The calculations were done using analysis of variance (ANOVA) method. Precision due to the repeatability conditions was found between 1% and 3.5% for all elements and was 5% in case of zinc. Precision due to the reproducibility was found 5% for lead up to 10% for the other elements measured by flame technique.

4.4. Uncertainty budget
Several approaches have been used for the evaluation of uncertainty of measurement results. Bottom-up approach of the Guide to the Expression of Uncertainty (GUM) [9] is based on the identification and estimation of all uncertainty sources in the analytical process. This approach quantifies the uncertainty associated with each individual effect that causes random errors of the measurements. In the present case of elemental analysis, the concentration (C) of each element was calculated from the following equation:

\[ C = c \times \text{d.f} / V \]

where c is the analyte concentration measured in the prepared solution
d.f is the dilution coefficient
V is the initial volume taken from the unknown sample

All uncertainty contributions must be quantified then combined to calculate the combined standard uncertainty, \( u \), as:

\[ u^2 = u_{\text{calibration}}^2 + u_{\text{cr}}^2 + u_{\text{repeatability}}^2 + u_{\text{sample preparation}}^2 \]

Each component is calculated as follows:

4.4.1. Calibration uncertainty
The calibration uncertainty is an important component in the uncertainty budget. It has been calculated using the individual relative uncertainties of the certified reference materials, the volumetric pipettes and the measuring flask used to prepare the calibration standards as well as the uncertainty due to the regression line. The uncertainty of the volumetric flask and pipette was calculated from the equation:

\[ u_{\text{volume}} = \sqrt{u_{\text{calibration}}^2 + u_{\text{cr}}^2 + u_{\text{repeatability}}^2} \]

The uncertainty of the regression line was calculated from the equation [5]:

\[ u(c) = \frac{S}{B} \sqrt{\frac{1}{n} + \frac{1}{n} + \left(\frac{c - c_0}{S_{xx}}\right)^2} \]

The combined standard uncertainty of the calibration for each element was calculated from the following equation:

\[ u_{\text{calibration}} = \sqrt{u_{\text{cr}}^2 + u_{\text{repeatability}}^2 + u_{\text{sample preparation}}^2 + u_{\text{calibration}}^2} \]

where C is the concentration of CRM, V is the volume of the flask and pipette, c is the analyte concentration.

4.4.2. Uncertainty of recovery
The uncertainty of recovery experiment was calculated from the following equation:

\[ u_{\text{recovery}} = \sqrt{u_{\text{repeatability}}^2 + u_{\text{cr}}^2 + u_{\text{calibration}}^2} \]

The standard uncertainty due to the repeatability was calculated from:

\[ u_{\text{repeatability}} = \frac{SD}{\sqrt{n}} \]

where SD is the standard deviation of the repeated measurements and n is the number of measurements.

4.4.3. Uncertainty of precision
It is calculated from the standard deviation of repeatability conditions and reproducibility conditions using equation No. (8).
The expanded uncertainty calculations

The expanded uncertainty ($U_{\text{exp}}$) was calculated by multiplying the combined standard uncertainty by the coverage factor $k$, which usually chosen to be $k = 2$ to provide a level of confidence of approximately 95%. Table 2 summarizes the uncertainty components, the combined standard uncertainty and the expanded uncertainty of each analyzed element.

Table 2: The uncertainty budget of the measured elements

| Element | $u_{\text{calibration}}$ | $u_{\text{recovery}}$ | $u_{\text{precision}}$ | $u_{\text{sample pr.}}$ | $u_{\text{combined}}$ | $U_{\text{exp}}$ (relative) |
|---------|--------------------------|------------------------|-------------------------|--------------------------|------------------------|-----------------------------|
| Pb      | 0.0120                   | 0.031                  | 0.009                   | 0.030                    | 0.04567                | 0.0913                      |
| Cr      | 0.0260                   | 0.051                  | 0.012                   | 0.024                    | 0.0632                 | 0.1264                      |
| Cu      | 0.0185                   | 0.025                  | 0.021                   | 0.009                    | 0.0386                 | 0.0772                      |
| Mg      | 0.022                    | 0.016                  | 0.025                   | 0.023                    | 0.0435                 | 0.0870                      |
| Zn      | 0.0162                   | 0.056                  | 0.030                   | 0.020                    | 0.0685                 | 0.1371                      |

5. Conclusion

Full validation was made for analytical methods used to analyze five elements (Pb, Cr, Mg, Cu and Zn) in blood and serum samples. It was proved that, the described methods are reliable for such measurements. Also the validated methods presented in this paper meet the requirements and the criteria set out in the international regulations of the European Union for the method validation. In comparison with previous publications, P. Olmedo et al [13] reported that, limit of detections were found 0.19 and 0.8µg/L for chromium and lead and the results of recovery of spiked samples varied from 96.3 to 107.8%. Anu Viitak et al [14] found that, limit of detection and limit of quantification for lead in biological samples were 1.2 µg/L & 2.8 µg/L respectively and the recovery of spiked samples was in the range 91-109%. Fábio Kummrow et al [15] reported that, limit of detection for lead in whole blood samples was 0.65 µg/L and repeatability ranged from 1.2 to 1.7% for Pb. The present method gave comparable results in terms of accuracy and recovery and better results for limit of detection and limit of quantification. Atomic absorption spectrometry was used because it permits the analysis of elements in biological samples without any separation from their matrix.

6. References

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