nutrición clínica y Dietética Hospitalaria

Colaboración corta

Nutr. clín. diet. hosp. 2018; 38(2):133-138
DOI: 10.12873/382escobedo

Comparison study between colorimetric method and flame atomic absorption spectrophotometry in serum zinc status

Estudio comparativo entre la espectrofotometría de absorción atómica de llama y el método colorimétrico en el estado del zinc sérico

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Recibido: 25/abril/2018. Aceptado: 4/julio/2018.

ABSTRACT

Introduction: Zinc is an essential trace element for human life and its deficiency affects human growth and development. Serum zinc concentration (SZnC) provides useful information in the clinical categorization of deficiency and toxicity states.

Objectives: This paper presents a comparison study of Flame Atomic Absorption Spectrometry (FASS) and the Colorimetric method in the analysis of SZnC and hypozincemia.

Methods: The serum concentrations of zinc of 93 patients (1 to 31 years old) with chronic diseases were used for analysis. Statistical analytical: for SZnC, Pearson correlation coefficient, simple linear regression analysis, Bland & Altman method (B&A), and concordance correlation coefficient (CCC) were valued; and for hypozincemia, the differences were studied and Cohen's Kappa index was used.

Results: The main results indicate the means of the SZnC by both methods presented neither significant difference (p=0.328) nor linear relation (R=0.18, p=0.077). Furthermore, the percentage of cases of hypozincemia by the Colorimetric method was almost double (13%) than by the FASS (8%). There was only one coincident case in both methods at <70 μg/dl.

Discussion: The Colorimetric method in hypozincemia ranges predicted lower values with the FASS. This concordance poor between both methods was corroborated with a concordance correlation coefficient (CCC) lowly of 0.17. Moreover, the Cohen's Kappa index (-0.013) shown a concordance poor between both methods, too. In other studies, the variability of SZnC by Colorimetric method is more than FASS.

Conclusion: In summary, despite that, the mean of serum concentrations of zinc by both methods is similar; the diagnosis of cass with hypozincemia is not. The degree of agreement between methods is poor, with a poor strength of concordance to diagnosis hypozincemia. Therefore, we recommended the use of FASS to evaluated zinc status and diagnosis of hypozincemia, instead of the Colorimetric method.

KEY WORDS

Serum zinc concentration, hypozincemia, Colorimetric method, flame atomic absorption spectrophotometry.

ABBREVIATIONS

SZnC: Serum zinc concentration.

FASS: Flame Atomic Absorption Spectrometry.

CCC: concordance correlation coefficient.

B&A: Bland & Altman.

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INTRODUCTION

Zinc is an essential micronutrient for all life forms. Cellular, tissue and whole-body zinc homeostasis are tightly controlled to sustain metabolic functions over a wide range of zinc intakes, making it difficult to assess zinc insufficiency or excess[1]. A sensitive, specific biomarker of zinc nutrition has not been identified for the individual diagnosis of zinc deficiency. Plasma o SZnC is the only biochemical indicator to assess the zinc status of populations recommended by WHO/UNICEF/IAEA/IZiNCG[2]. For this analysis, reference methods such as the FAAS, the most commonly used, are required[3]. If the Colorimetric method is an alternative method to FAAS, it is interesting to determine the concordance between the two methods. Therefore, the aim of this study is the comparison between the Colorimetric method and FAAS in the serum zinc state in a group of patients with chronic diseases.

METHODS

In a prospective study of SZnC, 93 patients from 1 to 31 years old (11 ± 6 years), with chronic diseases of different aetiologies, were consecutively referred for nutritional assessment to the Paediatrics service –University Clinical Hospital in Valladolid–, during 18 months. Two samples of blood were taken in fasting. The first sample was transported refrigerated in polypropylene tubes to the Laboratory of Instrumental Techniques of the Chemistry Department of the Valladolid University. The second sample was sent to the laboratory of the University Clinical Hospital, where Zn In Vitro - Colorimetric Method for the Quantitative Determination of Zinc, marketed by Wako Chemicals GmbH148 (Normal value 73-127 μg/dl) was used[4]. The accuracy and precision of the FASS were valued previously. A CONTOX® Trace Metal Serum Control A level II (CAT0146, TM14647495R02, commercialized for KAULSON Laboratories) kit was used as a certified reference material (CRM)[5].Then, the normal distribution of SZnC in both samples was verified, the data were expressed as the mean ± SD or median (min-max) where appropriate. The Pearson correlation coefficient, the simple linear regression analysis, the B&A method –the difference between methods and the difference between the methods expressed as a percentage [(FAAS - Colorimetric method)/percentage of the mean]–, and CCC were computed to study relations between methods. Finally, the comparison of hypozincemia between methods was based upon the percentages of cases, using the following cut-off points: SZNC <70 μg/dl in children under 10 years of both sexes and in women aged 10 or older years, and <74 μg/dl in men aged 10 years or more[2]. Different cut-off points greater than <70 μg/dl were compared to visualize the coincidence of hypozincemia cases obtained by both methods. The degree of agreement between the methods with the Cohen's Kappa index was valued. A statistical analytical procedure was performed using the SPSS/PC software.

RESULTS

Accuracy and precision of the FASS

In the beginning, zinc levels were obtained in μg/dl of the certified serum. The results obtained from the certified serum used were compared with the certified values, in order to estimate the accuracy, expressed as the percentage of recovery (%R); and precision, expressed as the relative standard deviation (RSD) (Table 1). The percentage R **[%R = ($X_{calculated}$/ $X_{certified}$) x 100]** was calculated from the quotient between the average of the replicas of the CRM ($X_{calculated}$) and the certified values soluble in royal water ($X_{certified}$), multiplied by 100. Besides, the RSD **[RSD (%) = (SD/X) x 100]** was used to evaluate the precision of the method, where SD is the standard deviation and X is the arithmetic mean obtained after analysing the different replies[4]. As the percentage of %R was 96% (range 80-120%), and the RSD was 15% (≤20%), we conclude that both values are within the accepted range and adequate for obtaining the SZnC by this method.

Table 1. Evaluation of the accuracy and precision of the Flame Atomic Absorption Spectrophotometry in analysis of zinc certified (μg/dl).

	Zinc obtained (μ/dl)	References values
Value certified en μg/dl	73 ± 10	
$X_{calculated}$ ± DE	69.75 ± 10.55	
Recovery percentage	**95.55**	80 – 120 %
Relative standard deviation	**15.12**	≤ 20 %

Serum zinc concentration

This study reveals that the SZnC of both samples developed a pattern of normal distribution (Kolmogorov Smirnov's test of 0.89, p=0.402 for the FASS and 0.81, p=0.533 for the Colorimetric method). Although the media SZnC by the Colorimetric method (88.78 μg/dl) was slightly higher than by FASS (86.66 μg/dl), this difference was not significant (Student t-test, p=0.328). Despite the fact that the stockings of the SZnC for the Colorimetric method were major in the different studied conditions –age group, sex, state of malnutrition and of sexual development–, these differences were not significant either. The Pearson correlation coefficient was 0.1843 (p=0.077), and in the simple regression analysis, R Square indicates that only 3.4% of the variations of the FASS can be explained by the Colorimetric method. First, to verify the assumption of normality of differences B&A method used a graphical approach. First, the distribution of the values of the difference between the methods was also normal (K-S = 0.56, p=0.909). After, we obtained the average of the differ-

ence of the means (ADM), which was -2.10 µg/dl. This mean of the difference (MD) is not zero, and this means that on average the Colorimetric method measures 2.10 µg/dl more than the FASS. Now, we can use SD (20.95) to define the limits of agreement. In this way, 95% of the difference would be **MD -1.96SD = –2.10 - (1.96x20.95) = –43.16** and **MD +1.96SD = –2.10 + (1.96x20.95) = 38.96 (**Table 2). Therefore, the results measured by the FASS can be 39 units above or 43 below the Colorimetric method[7]. Second, the difference between the two pairs of measurements is plotted against the average of the two measures (Fig. 1). The graph includes the mean of the difference between the methods or bias of -2.10 µg/dl (red arrow tip), which is represented between the x-axis, and the parallel line for the x-axis -2.10 units. Likewise, the concordance limits are represented, from –1.96 (–43.06, IC95% –50.58 to –35.54) to +1.96DE (38.96, IC95% 31.44 to 46.48), with their corresponding confidence intervals (zones between arrows). The B&A chart also allows differences to be expressed as a percentage of the values on the x-axis –proportionality of the measured quantities [(FAAS - Colorimetric method)/percentage of the mean], versus the mean of the two methods (Fig. 2). The areas indicated between arrows present the limits of the confidence intervals for the mean and the limits of agreement. The slope (difference of means) was -9.96%, with limits of agreement, are -109.99% at 90.07%. Finally, the Lin CCC follows the next equation: **CCC = [A2 + B2 – C2] / [A2 + B2 + D2].** Where: A^2 is the variance of the FASS, B^2 is the variance of

Table 2. Measures used for the analysis of differences by the B&A method.

	Unit	Standard Error	Confidence	95% Confidence Interval	
				Lower	Upper
Mean of the difference (**MD**)	-2.10	2.17		-6.41	2.22
MD +1.96 SD	38,96	3,78	7,52	**31,44**	**46,48**
MD -1.96 SD	-43,06	3,78	7,52	**-50,58**	**-35,54**
Average of the difference of the means (**ADM**)	-9.96	10.37	20.60	-30.56	10.64
ADM +1.96DE	90,07	18,06	35.94	**54.13**	**126.01**
ADM -1.96DE	-109,99	18,06	35.94	**-145.63**	**-74.05**

Figure 1. Bland and Altman plot of the differences against mean between both methods.

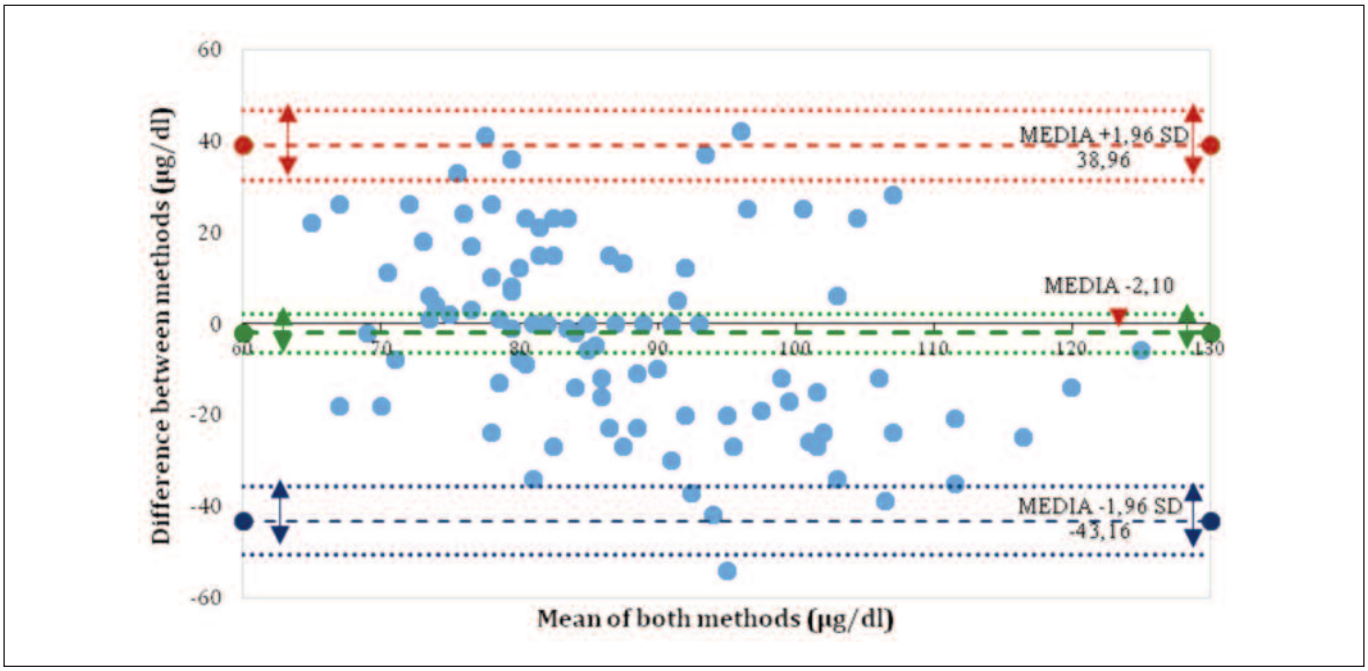
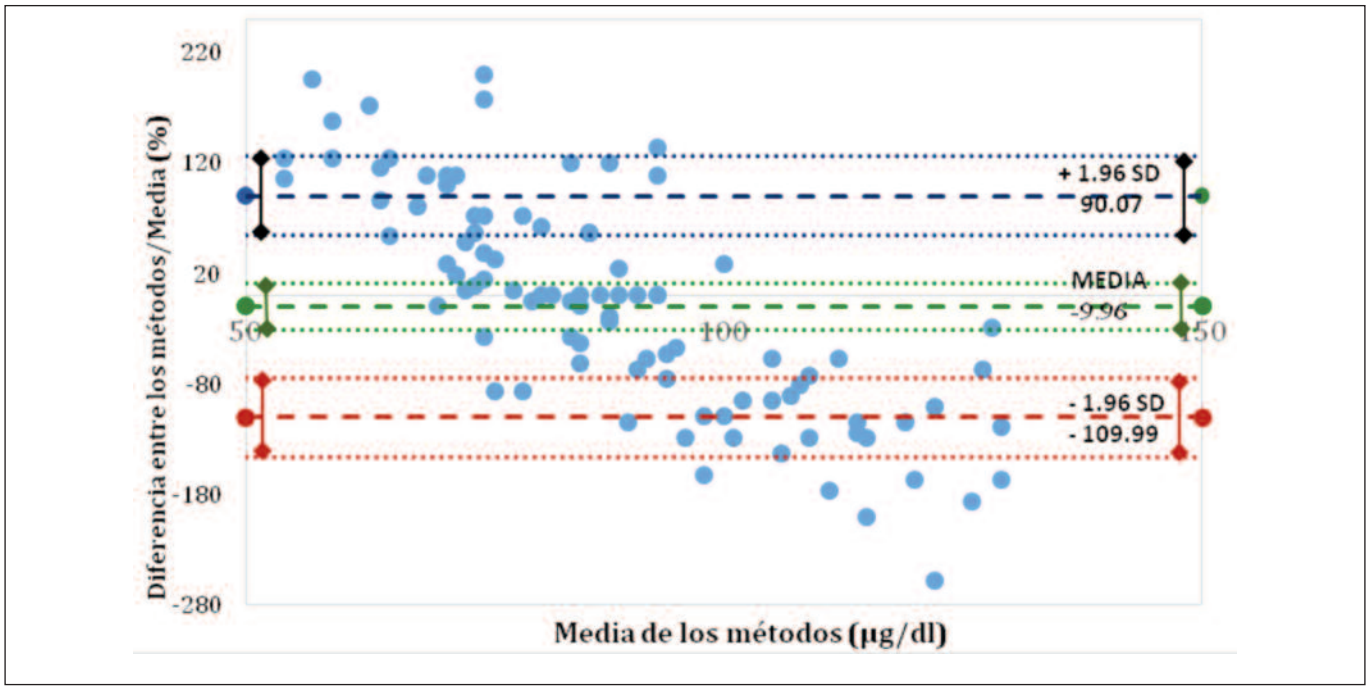


Figure 2. Differences between the methods expressed as a percentage of the values [(method EAA-Colorimetric method)/percentage of the mean], versus the mean of the two methods.



the Colorimetric method, C^2 is the variance of the difference between the methods and D^2 is the average difference of the methods[8]. The CCC is equal to 0.17[9].

Hypozincemia

Regarding the zinc deficiency, the percentage of cases with hypozincemia by the Colorimetric method was almost double (13.9%) compared with the FASS (8.6%), without statistical significance (Fischer's exact test, p=285). There is only one case with hypozincemia, determined by both methods when we used the cut-off point at <70g/dl. Likewise, the number of cases that coincide with hypozincemia between the Colorimetric method and the FAAS remain few (1-2 cases) when we establish the cut-off point between <72 y <74 µg/dl. Only when the cut-off point is greater than <76 µg/dl, the coincidences of cases between both methods increase from 9 to 14 cases. The estimation by the Kappa index follows equation: Kappa = P0 – Pe / 1 – Pe. Where P0 is the observed concordance ratio, Pe is the expected concordance ratio by chance and 1 - Pe, represents the maximum possible agreement or agreement not due to chance. In our study, P0=79.6% coincidences were observed. Whether both methods will score independently, the distribution expects Pe=79.8%, random coincidences. That is to say -0.013 (CI95% 0.183 to -0.208 and CI99% 0.245 to -0.27)[10]. Landis and Koch proposed a qualitative interpretation of the Kappa index classically used in which the concordance force is qualified as poor o weak (0.40), moderate (0.41 – 0.61), good (0.61-0.80) and very good (>1.13)[11]. Therefore, the concordance between the methods according to the Cohen Kappa index appears only at the cut-off point <76 with good concordance force (0.86), compared to the previous cut-off points.

DISCUSSION

In terms of the assessment of the usefulness of the Colorimetric method as an alternative test to the FASS, what matters is to determine if both measurements are similar in magnitude, not if they are associated. In fact, they must be associated, since they are two measurements of the same characteristics in the same individuals or samples[12]. Prior to the comparison of the methods, it was demonstrated that the FASS presented a recovery percentage of zinc (96%) and a relative standard deviation (15%) within the accepted and suitable range for obtaining the SZnC by this method. Both samples exhibited a normal distribution pattern. The Pearson's low correlation coefficient (0.1843, p=0.077) indicates that there is no significant linear relationship between the two methods. Moreover, the simple regression analysis (R2=0.034, p=0.077) informed us that the Colorimetric method could only explain 3.4% of the variations of the FAAS and that both methods were not linearly related. The regression equation would be FASS = 75.41 + 0.13 Colorimetric. That is when a patient presenting an SZnC of for example 50 µg/dl (hypozincemia) by the method Colorimetric we would obtain an SZnC of 81.91 µg/dl (normal) per FASS. This would mean a difference very high that is not acceptable for clinical purposes since one of the ob-

jectives of the assessment of the SZnC is the identification of patients with hypozincemia. Consequently, we value the concordance between the methods.

B&A shows the agreement or concordance between two quantitative measures. Therefore, if the FAAS is considered a reference method, the difference between the methods, whose values followed a normal distribution (K-S 0.56, p=0.909), could be compared with the mean of the two paired values[7]. A negative trend of values seems to be obvious in the graph (Fig. 1), and that measurements do not match, as suggested by the Pearson's correlation coefficient. In like manner, the Colorimetric method provides higher serum zinc values than the FAAS, with a mean difference of -2.10 µg/dl; and the limits of concordance indicate that the values of the method are between 38.96 µg/dl above the Colorimetric method and 43.06 µg/dl for below. Besides, it is possible to say that the bias is significant because the line of equality is within the confidence interval of the mean of the difference. Otherwise, the intervals are wide, reflecting the size of the sample and the great variation of the differences. Furthermore, if the expected differences fall within the MD±2SD, they would not be clinically important, and we could use the two analytical methods interchangeably. However, the SZnC by the FAAS would be 39 µg/dl above or 43 µg/dl below the Colorimetric method, which would not be acceptable for clinical purposes[7].

With regard to the graph of differences as a percentage by the method of B&A, a useful option when there is an increase in the variability of the differences when the magnitude of the measurements of the magnitudes increases[7]. A slope of -9.96% was observed, almost constant for all measured concentrations. As in the method of the differences of B&A, this slope is significant, since the equality line is included in the confidence interval. Moreover, as the limits of the agreement range from -109.99% to 90.07%, these differences are also not acceptable to consider the two methods equivalent. Therefore, whether the problem of interest is to determine the similarity of measurements of the same variable continues in the same samples or individuals, made with different methods, equipment or technicians, it is appropriate to use a measure of agreement, particularly the coefficient of correlation of agreement[12]. Lin (1989) developed a proposal to evaluate the concordance between continuous variables through the CCC[8]. This coefficient ($\mathbf{r_c}$) can vary between -1 and 1 and its absolute value cannot be greater than the Pearson's correlation coefficient (**R**) so that the following relationship can be established: $\mathbf{-1 \leq - |R| \leq r_c \leq |R| \leq 1}$. The correlation coefficient of Lin's agreement can only be zero if the Pearson's correlation coefficient is also zero agreement[12]. In our study, we obtained a CCC or Lin coefficient of 0.17, lower than the Pearson's correlation coefficient of 0.18. Therefore, the degree of agreement is poor, which is congruent with the differences described above.

Now let us study what happens with the state of zinc. The percentage of cases with hypozincemia by the Colorimetric method is almost double (13.9%) compared to the FAAS (8.6%), without significant difference (p=285). That is, there is only one case with hypozincemia in the Colorimetric method that coincided with the FAAS when the cut-off point was established at <70 µg/dl; only when the cut-off point was greater than <76 µg/dl, the coincidences of the cases with hypozincemia increased. This observed difference could be due to the weak correlation and poor concordance in the SZnC between both samples. At the same time, we evaluated Cohen's Kappa index, which is a measure of the agreement between dichotomous evaluations, such as the determination of the state of hypozincemia in our study. An observed quantity (P_0) higher than expected (P_e) would indicate concordance, and a lower quantity, discrepancy[11]. As in our study, P_0 (79.6%) ≤ Pe (79.8%), we did not find more concordance than what is expected by chance. In addition, the value of Cohen's Kappa index was -0.013 (IC95% from 0.183 to –0.208). This result, together with the qualitative interpretation of the Cohen's Kappa index, indicates a discrepancy in the methods with a poor strength of concordance. Otherwise, the concordance between the methods according to this index appears only at the cut-off point <76 µg/dl with a good concordance force (0.86), compared to the previous cut-off points; the concordance force decreased with <78 µg/dl.

Coronado et al. (2014) reported on a comparative study of level serum zinc in 120 adult women, concentrations were different showing a weak correlation between Colorimetric method of Zincon (104.45±19.34 µg/dl) and those obtained by FAAS (p <0.05) with an R2 = 0.1644, indicating that the method adapted Zincon is not comparable to that of FAAS[13]. Nevertheless, the manufacturers have reported a good correlation between results given by the Wako kit and FAAS. Wako Pure Chemical Industries Ltd uses the chromogen 5-Br-PAPS, together with citrate, dimethylglyoxime and salicylaldoxime to mask reaction with other minerals (iron, copper and nickel). However, there is a positive bias associated with the colorimetric assay, indicating a failure to compensate for all of the interference, which could explain the different results between methods found in our study[14]. In contrast, from January 1994 to September 1995 the laboratory performance scores from the Guildford Trace Elements External Quality Assessment Scheme (serum and urine zincs), which includes more than 100 participants from about 20 countries; were significantly better for FAAS compared with colorimetric methods; with mean (SD) scores of 22.0 (7.1) and 19.6 (6.0), respectively. The better performance is indicated in this scheme by a numerically higher score[14].

The observation of our result is congruous with the finding of Arnaud et al. in a European and North American study about quality specifications based on biological intra- and interindividual variability were calculated and compared to

those currently used by various trace element external quality assessment schemes (TE-EQASs) for plasma or serum copper, zinc, and selenium concentrations. For Zn, 11% of unacceptable results [Abs (Z score) >2] were found when FAAS, ICP-MS, or ICP-AES/OES were used. However, this percentage increased to 34% with Colorimetric methods[15]. Serum concentrations of zinc provide useful information in the clinical categorization of deficiency and toxicity states. Even though colorimetric assays are available, they are liable to interferences, which cannot always be easily eliminated and are variable between samples[14]. Even though our results favoured the use of FAAS over the Colorimetric method, its use requires further investigation for confirmation.

CONCLUSIONS

In summary, despite that, the mean of SZnC by both methods is similar; the diagnosis of cases with hypozincemia is not. The Colorimetric method provides higher serum zinc values than the FAAS. The degree of agreement between methods is poor, with a poor strength of concordance to diagnosis hypozincemia. Therefore, it is encouraging to see that the findings of this study have provided some support to the assumption that the use of FAAS to evaluated zinc status and diagnosis of hypozincemia is better than Colorimetric method.

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