Improved limit of detection and quantitation development and validation procedure for quantification of zinc in Insulin by atomic absorption spectrometry

Muhammad Abdul Qadir, Mahmood Ahmed, Iftikhar-ul-Haq and Saghir Ahmed
Institute of Chemistry, University of Punjab, Lahore, Pakistan

Abstract: A simple and expeditious analytical method for determination of zinc in human insulin isophane suspension by flame atomic absorption spectrophotometer (FAAS) was validated. The method was carried out on atomic absorption spectrometer with 0.4nm bandwidth, 1.0 filter factor on deuterium (D_2) background correction. The integration time was set at 3.0 second with 5.0mA lamp current. The parameters of method validation showed adequate linearity, efficiency and relative standard deviation values were between 0.64%-1.69% (n=7), 1.31%-1.58% (n=10) for repeatability and intermediate precision respectively. The limit of detection 0.0032µg/mL, 0.0173µg/mL, 0.0231µg/mL and limit of quantitation 0.0107µg/mL, 0.0578µg/mL, 0.0694µg/mL based on signal to noise (SN), calibration curve method (CCM) and fortification of blank (FB) were obtained respectively. The percentages of recovery for low, medium and high spiked concentration levels of zinc in human insulin were 99.38±0.04 to 100.3±0.03, 98.45±0.38 to 100.3±0.07 and 99.42±0.03 to 99.42±0.08 respectively. With the use of this method, five samples from each vial of human insulin isophane suspension were analyzed and the zinc content was determined. The zinc content were 22.1±0.025µg/mL and 24.3±0.028 µg/mL which compliance the British Pharmacopoeia standard.

Keywords: Insulin, zinc, AAS, validation.

INTRODUCTION

The pancreas which is a large gland lies near the stomach produced insulin hormone. The correct use of food by body especially sugar is controlled by insulin hormone. Humans became the victim of diabetes when enough insulin is not produced by pancreas to fulfill the body's need. Insulin helps to keep blood glucose at a nearly normal level (JDF 2003, Eli Lilly, USA). Zinc has a vital role through all stages of insulin metabolism, production then storage and utilization. The beta cell destruction is protected by zinc. Response of pancreatic beta cells to body for production and secretion of insulin is affected by lack of zinc. The glucose level in the body becomes higher when production and secretion of insulin by pancreas is inadequate; also it happens when insulin does not work properly. Insulin sensitivity lacked by zinc deficiency, not enough glucose is entering the cells because insulin receptors on the cell are being inhibited (Dilina NM *et al*., 2004). Zinc in insulin can be determined by various techniques like x-ray fluorescence, neutron activation, fluorimetry, polarography but atomic absorption spectrometry preferred because of simplicity, sensitivity, precision and cheaper analysis cost (MiKac DD, 1970; Sunderman FW Jr, 1973). To obtain consistent, reliable and accurate data by analytical measurement then validation of analytical method play a major role. The consistency of analytical results, quality and reliability of method is judged from method validation. Also analytical method's validation required by most quality and regulatory standards that has impact on laboratories. The validation measures the different effects in the whole analytical system which influences the result and ensures that there are no other effects which have not been considered (ICH, 1996; Paul DB and Helmut G, 2005).

The matrix effects, sample concentration, instrument sensitivity and purity of reagents used in analysis can be problematic with the detection and quantification of an analyte. The objective of present work was to consider the limit of detection (LOD) and limit of quantification (LOQ) as integral part of method validation for quantification of total zinc in human insulin isophane suspension. Three different methods were used for derivation of the LOD and LOQ. Signal-to-noise ratio called instrument detection limit (IDL), calibration curve method and fortification of blank with analyte standard called method detection limit (MDL).

MATERIALS AND METHODS

Reagents

Zinc 1000mg/L standard solution ((Fisher scientific, UK), Hydrochloric acid (Fisher scientific, UK), Triton X-100 (Baker Analyzed, Phillipsburg, NJ, USA). Deionized water with a specific resistivity of 18 MΩ cm obtained from a Milli-Q system (Millipore, USA).

Working standards and solutions preparation

0.02N HCl solutions used in this work was prepared from concentrated hydrochloric acid by appropriate dilution.

**Corresponding author:* e-mail: mabdulqadir@gmail.com

The working standards (0.20-1.00 µg/mL of Zn) were prepared immediately before use from a 1000 mg/L zinc stock solution by serial dilutions in 0.02N HCl. 0.1% Triton X-100 is neutral dispersant added to samples and standards to overcome any residual viscosity that affected the nebulization efficiency. The continuous aspiration with 0.5N HCl for rinsing was used to avoid the fouling of the burner slots.

Sample preparation

Shake the preparation gently and dilute 1ml sample containing 100 IU of human insulin to 50.0 ml with 0.02N hydrochloric acid and 0.1% Triton X-100 in equal volumes .

Instrumental conditions

A PG-990 atomic absorption spectrometer equipped with fully integrated atomizers that is a burner system for flame atomization equipped with a deuterium background corrector. The system was operated from an interfaced computer running AA Win Lab software. Zinc hollow cathode lamps were used as line sources for analyte. The optimal operating conditions for flame atomization of zinc are presented in table 1.

Table 1: Optimal operating conditions for flame atomization of zinc by AAS

Flame	Air - Acetylene
Calibration mode	Absorption, Peak Height
Measurement time	3.0 s
Lamp current	5 mA
Slit width	0.4 nm
Burner height	6.0 mm
Wavelength	213.9 nm
Background correction	Deuterium background

METHODS

Method validation

Selectivity and specificity of method

Method selectivity is accurate measure of analyte while the specificity is ability to assess the analyte in sample with present of unexpected matrix which interfere the results of analyte in sample. The results for standard deviation of absorption values (n=6) for zinc working standards and zinc in insulin isophane suspension was within range of acceptance criteria (%RSD ≤2). The results presented in table 2 and spectra of zinc standards are shown in fig. 1.

Linearity and range used for analytes

The absorption is directly related and proportional to concentration of standard solution in their lower and upper limits. The interval between the upper and lower levels of analyte is the range of an analytical method that has been demonstrated to be quantified. The linearity and range for analytes were determined by analyzing five point association standard calibration curves (n=6). The five different standard solutions used to calculate determination of coefficients from linear regression equation. The calibration curve is shown in fig. 2 and regression equation presented in table 6.

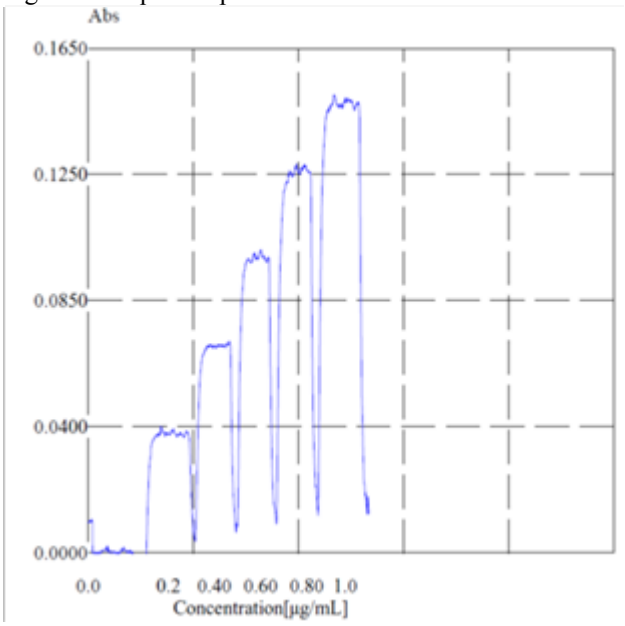

Fig. 1: Spectra of zinc standard solutions

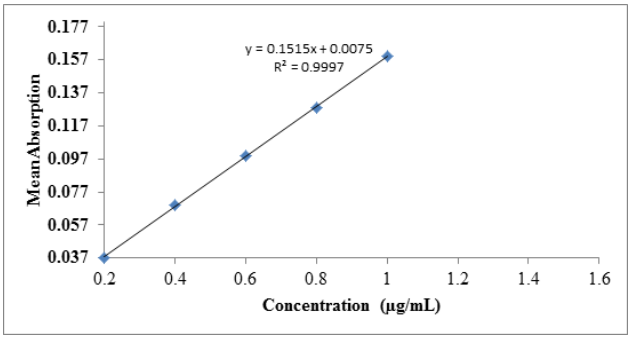

Fig. 2: Calibration curve for zinc standard solutions absorption expressed as mean (n=6)

Accuracy and precision of method

Three different levels of concentration in triplicates (low, medium and high) for evaluating the recoveries study which interpret accuracy of the method analyzing. Precision reported as percentage relative standard deviation (%RSD) by intra-day (repeatability) and inter-day (intermediate precision) assays.

Repeatability

Over a short period of time within a day (Intra-day) within a laboratory, the analytical procedures done with same equipment by the same analyst demonstrate the repeatability. The intra-day precision studies of zinc standard solutions are presented in table 3.

Table 2: Selectivity and Specificity of Method

Zinc concentration (µg/mL)	Mean Absorbance (n=6)	SD	%RSD
0.20	0.037	0.0006	1.53
0.40	0.069	0.0010	1.45
0.60	0.099	0.0010	1.02
0.80	0.128	0.0015	1.20
1.00	0.159	0.0010	0.65
Zinc in vial 1	0.073	0.0006	0.76
Zinc in vial 2	0.079	0.0007	0.86

Table 3: Intra-day precision study of zinc

Zinc standard concentration (µg/mL)	Zinc measured concentration mean (µg/mL) (n=7)	SD	%RSD
0.20	0.1987	0.0013	0.64
0.60	0.5985	0.0011	0.18
1.00	1.0130	0.0171	1.69

Table 4: Inter-day precision study of zinc

Zinc standard concentration (µg/mL)	Zinc measured concentration (µg/mL) (n=10)			Mean (µg/mL)	SD	%RSD
	Day 1	Day 2	Day 3			
0.20	0.198	0.203	0.204	0.202	0.0032	1.58
0.60	0.592	0.605	0.591	0.596	0.0078	1.31
1.00	1.021	0.998	0.996	1.005	0.0139	1.38

Table 5: Signal to noise based detection limits

Mean absorbance (n=10)	SD	Limit of detection µg/mL (3xSD)	Limit of quantitation µg/mL (10xSD)
0.0014	0.00107	0.0032	0.0107

Table 6: Linear regression equation of the calibration curves (n=6)

Component	Linear range (µg/mL)	Regression equation	Intercept (mean ± SD)	Slop (mean ± SD)	R^2 (mean ± SD)
Zinc	0.20-1.00	y = 0.1515x + 0.0075	0.0075±0.00021	0.1515±0.00052	0.9997±0.00012

Table 7: Calibration curve method based detection limits

sB	Limit of detection µg/mL	Limit of quantitation µg/mL
0.00088	0.0173	0.0578

Table 8: Fortification of blank based detection limits

Mean absorbance (n=7)	SD	Limit of detection µg/mL	Limit of quantitation µg/mL
0.022	0.00069	0.0231	0.0694

Intermediate precision

It is the method of variation determination in analysis on different days (Inter-day) within a laboratory. Intermediate precision was evaluated after repeating the experiment using freshly prepared samples on three separate days. The inter-day precision studies of zinc standard solutions are presented in table 4.

Limit of detection and limit of quantitation

Detection limits based on signal to noise ratios

The detection limits based on the signal to noise ratio was determined after calculating the standard deviation (SD) of 10 signal responses of blank reagent which is generally known as instrumental detection limits. Signal to noise based detection limits presented in table 5.

Detection limits based on the calibration curve method

The limit of detection and the limit of quantitation were determined by calibration curve method using the following equations:

$LOD = y_B + 3s_B$

$LOQ = y_B + 10s_B$

Where y_B is intercepts of regression line and s_B is standard deviation of intercepts of regression line (James

Table 9: Assay results for zinc in human insulin

Insulin	Content	Mean±SD (n=5) µg/mL	%RSD
Vial 01	Zinc	22.1±0.025	0.97
Vial 02	Zinc	24.3±0.028	0.84

Table 10: % Recovery study of zinc

Spiked level of Concentration	%Recovery ± SD		Day
	Zinc - Vial 01	Zinc - Vial 02	
Low	100.3±0.03	99.87±0.03	1
Medium	98.72±0.24	100.3±0.07	
High	99.90±0.04	99.50±0.04	
Low	99.38±0.04	98.63±0.02	2
Medium	98.45±0.38	99.90±0.03	
High	99.79±0.05	99.42±0.03	
Low	100.10±0.09	99.50±0.07	3
Medium	99.28±0.16	99.20±0.04	
High	99.79±0.10	99.83±0.02	

NM and Jane CM, 2010). The linear regression equation of the calibration and detection limits based on calibration curve presented in table 6 and 7 respectively.

Detection limits based on fortification of blank

Zinc standard solution with concentration of 0.1µg/mL was aspirated into the flame under the optimal conditions of atomization and the detection limit was calculated by 0.0044 x C/A, where C is concentration of zinc standard and A is absorption (n=7) also called method detection limit (MDL) which determine the sensitivity (characteristics concentration) of zinc to produce a signal of 0.0044 absorption units. The limit of quantitation is three times of limit of detection. Fortification of blank based detection limits presented in table 8.

Zinc content in human insulin

The proposed method was performed for quantification of zinc in human insulin isophane suspension. To validate the usefulness of method proposed for determinations of zinc in human insulin isophane suspension, five samples were taken from each different vial of same batch number (label as vial 1 & 2) of insulin. The results of assay presented in table 9.

Ruggedness of data (% Recovery ± SD)

The lack of influence of environmental and operational variables on results of the analytical method is ruggedness of data. The recovery of the analytes spiked at three different concentrations: low (80% of analyte), medium (100% of analyte) and high (120% of analyte) provide the information that how method is rugged. The recovery data obtained for each concentration level on three days performed in five replicates presented in table 10.

STATISTICAL ANALYSIS

The detection limits were statistically analyzed at level of significance set α=0.05 by applying one-way analysis of variance (ANOVA). P-value > 0.05 would indicate no significant differences in detection limits by different methods and vice versa using SPSS 15.0 for Windows®.

RESULTS

The best spectroscopic conditions for proposed flame atomic absorption spectrophotometric method were adequately selected for the quantification of zinc in human insulin isophane suspension. The detection limit was the main objective of present work along with validation studies, all the results are presented in table 2-9.

DISCUSSION

The method was specific and selective with %RSD 0.65 to 1.53 in the presence of matrix in human insulin. The calibration curves for zinc were constructed by plotting the absorbance versus concentration. Linearity was observed in a concentration range from 0.20 to 1.00 µg/mL of zinc and the value of the coefficient of determination (R^2 = 0.9997) showed excellent linearity of the calibration curve for the method. Repeatability (n=7) was studied by calculating the relative standard deviation (%RSD) for low (0.20 µg/mL), medium (0.60µg/mL) and high (1.00µg/mL) standard concentration of zinc was 0.64, 0.18 and 1.69 respectively moreover for the intermediate precision (n=10) %RSD was 1.58, 1.31 and 1.38 obtained for low, medium and high standard concentration of zinc under the same experimental conditions respectively. The detection limits were calculated on base of three different methods, LOD, 0.0032µg/mL and LOQ, 0.0107µg/mL based on the signal to noise was significantly different ($p<0.05$) from calibration curve method and fortification of blank and also improved as reported by I.G. Tănase *et al* (0.01µg/mL). There is no significant difference ($p>0.05$) between the detection limits calculated based on

calibration curve method and fortification of blank. The ruggedness and accuracy of data was evaluated by recovery studies from five replicates determinations of human insulin isophane suspension sample spiked with three different levels of zinc standard solutions as low (80% of analyte), medium (100% of analyte) and high (120% of analyte). The percentage recoveries (%Recovery ± SD) were obtained between the 99.20±0.04 and 100.3±0.03 indicating the method is rugged and accurate.

CONCLUSION

The data validation indicates that AAS method is accurate, rugged and possesses precision characteristics and excellent linearity. Atomic absorption spectrophotometric technique has become the method of choice because of relatively simple, sensitive, and precise. The direct dilution method presented here successfully used for the quantification of zinc in human insulin isophane suspension only required few seconds per sample. This method is cheap and can be used in each laboratory.

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