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Original Article

Development of In-house Standard Reference Material for Estimation of Metals in Whole Blood

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Abstract

Objective: To develop the standard in order to assure that a test run is valid and results are reliable and the shelf life of the reference material will be at least six months.
Study type, settings and duration: Quantitative analysis of metals in clinical samples PCSIR Laboratories, Karachi during six months.
Materials and Methods: For this purpose 500ml human blood sample, obtained from blood bank was used to make a reference solution. This solution was prepared by acid digestion. After diluting to an appropriate volume, working solutions were freshly made and calibrated for each metal using the method of standard addition after a certain interval of time. E. Merck stock standards of 1000mg/l, were used for each metal to make the standard addition solutions. Percentage recoveries of standard addition solutions were calculated using the standard formula.
Results: The analysis showed that the recoveries of most of the metals for both flame and graphite furnace were found to be within 4% of the actual values. The estimation of elements after an interval of about six weeks (four times) did not showed any difference from the intial concentration. This indicates the reproducibility of the results of metals was satisfactory. Similarly the internal spiking also showed the accuracy and comparability of the results.
Conclusion: The prepared reference blood solution can be stored at ambient temperature and used as a reference standard at least for 6 months without change in the concentration of metals. It may be used as reference material if certified reference material is not available.
Key words: Whole blood, open wet digestion, recovery, metals, atomic absorption spectrophotometer.

Introduction

Imbalance in trace metal metabolism may lead to metal interactions that may be of patho-phsiological importance. There are many pathological conditions which can be attributed to abnormal (low or high) levels of metal concentration in blood[1-4]. Accurate estimation of trace metals is needed to asses the data of control and diseased patients[5]. Blood contains organic matrix which often compromises the accuracy of results of quantitative analysis[6]. Sample preparation must follow a protocol which ensures minimum error. The development of such protocols depends on the analytical performance characteristics of the technique selected as well on the nature of the samples. Different methods for elemental determinations in biological samples are available[7-9]. For this study, we have chosen Atomic Absorption Spectroscopy including both flame as well as electro thermal atomization techniques. Liquids may be amenable to direct analysis but matrix interferences in many cases usually necessitate some form of sample pretreatment[6]. The choice of flame atomization atomic absorption spectrophotometer (FAAS) or electro thermal atomization (ETAAS) depends on the concentration of the element sought and analytical sensitivity. For the analysis by flame atomization (FAAS) it is good to decompose organic matter and convert metals into a relatively simple inorganic or ionic form in solution. Appropriate sample preparation is required in which contaminants and interfering substances are removed to get accurate results[1-6,10,11]. Wet digestion and dry ashing are the two main techniques employed in tissue destruction[1-6,10,12.] Wet digestion method includes decomposition by acids either alone or in mixture, carried out in open vessels in tube on a hot plate or an aluminum heating block. The other method is microwave digestion which is an attractive method especially for small samples. Nitric acid is used commonly since it produces the fewest polyatomic interference in contrast to $HClO_4$ and H_2SO_4.

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In our study, emphasis is placed on atomic absorption spectrophotometer and prerequisite sample preparation because of the practicality of the technique and its present widespread use[6]. In the present work the method of sample preparation and calibration of laboratory made standard is described. This procedure can be applied when certified reference material (CRM)/ standard reference material (SRM) are not available. This study may be helpful to the researchers and pathologists to get the accurate and precise data regarding metals in body fluids.

Materials and Methods

Distilled water taken from distillation unit "Stuart" was used throughout the study. The water samples were tested for zero response by Atomic Absorption Spectrophotometer for each element under estimation.

Blood was collected and stored in freezer. Before digestion it was first brought to room temperature according to the requirement of the method of analysis[13]. Blood collection tubes, nozzles of micro digital pipettes, disposable syringes, plastic tubes for graphite furnace and all glassware were soaked at least for 24 hrs in 20% nitric acid. This procedure ensures metal free surface[10]. They were then washed well with distilled water. Plastic wares were dried at 70-80°C while glass ware at 105°C. Concentrated nitric acid of analytical grade (Merck) was used for wet digestion of blood. Blood was processed in accordance with reference[14]. It was digested in 1:1 ratio with concentrated nitric acid under control temperature of $120C^0$ until a clear yellow solution was obtained. It was filtered and the final concentration of acid in solution was kept 1.0 % in order to avoid corrosion of metal sampler[15].

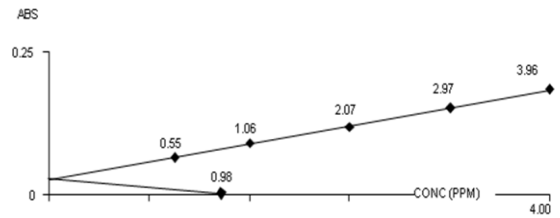
The digested residue was dissolved in water and diluted with nitric acid. Working standard for each element used for standard addition technique was diluted from respective stock solution of 500 or 1000 ppm (Merck, Darmstadt, Germany).The stock sample solution was analyzed for the determination of test metals after a time interval of about 5-6 weeks. At one time a set of solutions of different concentrations were run in triplicate for each trace metal. Whole study lasted for about six to seven months. The stock reference solution had been kept at room temperature.

Hitachi Model Z-8000 Atomic Absorption Spectrophotometer was used for the estimation of metals. It was equipped with Zeeman background corrector and a data processor. All the parameters were set according to the instrument manufacturer's instructions. Percentage recoveries of standard addition solutions were calculated using the standard formula i.e. R% = apparent concentration x 100/ actual concentration.

Results

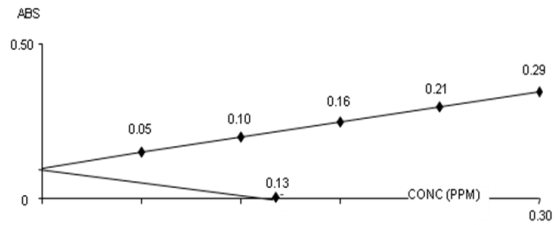
To standardize the blood stock solution, metals were determined using standard addition technique. For this purpose five standard working solutions of different concentrations were made as shown in Table-1. A spectroscopically pure solution of each analyte in 1% nitric acid was treated as sample on each standard addition plot. E. Merck standard stock solutions were used. These are the conventional standards used in atomic absorption spectroscopy. These solutions were run in ascending order regarding their concentration to get the calibration curve. In a graphic representation the absorbance values are plotted against the added concentration values of each metal and extrapolation indicates the concentration of metal which was estimated to be present in the sample (Figures-(a-f)). For chromium, lead and nickel, graphite furnace atomization was used while for remaining metals in this study flame atomization was used. Percentage recoveries of metals were found quite satisfactory and it proves the good reproducibility of the results.

Fig a: Calibration curve of calcium in blood.



Standard Addition Method

Fig b: Calibration curve of magnesium in blood.



Standard Addition Method

Each metal studied was estimated four times by preparing fresh working standards after a time interval of at least 5-6 weeks. At one time solution was analyzed three times for each trace metal. The mean of three was considered single run. As it can be seen in Table-2, there is very narrow range of concentration of each metal which proves that the stock blood solution, prepared in this manner may remain stable and there is no significant change of concentration with the passage of time.

Table 1: Standard additions and their recoveries in blood.

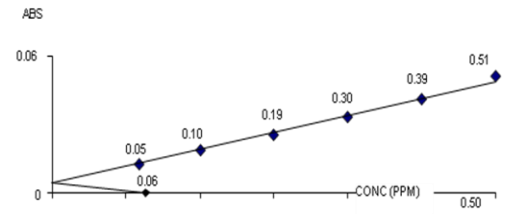
Elements	*Technique*	*Std. Addition*	*Mean Recoveries (%)*	*Mean of Means*	*Standard Deviation*	*C.V (%)*
Calcium µg/ml	F.A	0.50, 1.0, 2.0, 3.0, 4.0	102.1, 104.2, 100.9, 101.0	102.1%	1.53	1.50
Magnesium µg/ml	F.A	0.05, 0.10, 0.15, 0.20, 0.30	104.2, 102.7, 102.4, 101.7	102.8%	1.05	1.03
Zinc µg/ml	F.A	0.10, 0.20 0.30, 0.40, 0.50	101.3, 100.8, 100.8, 100.0	100.7%	0.54	0.53
Copper µg/ml	F.A	0.10, 0.20, 0.30, 0.40, 0.50	101.6, 99.64, 100.8, 101.3	100.8%	0.86	0.86
Chromium ng/ml	G.A	5, 10, 15, 20, 25	101.2, 105.2, 98.53, 95.37, 101.1, 103.2, 99.65	100.6%	3.19	3.17
Lead ng/ml	G.A	10, 20, 40, 60, 80	95.79, 91.20, 109.1, 94.54	97.66%	7.87	8.06

F.A: Flame atomization G.A: Electro-thermal atomization

Table 2: Analytical data for standard blood solution.

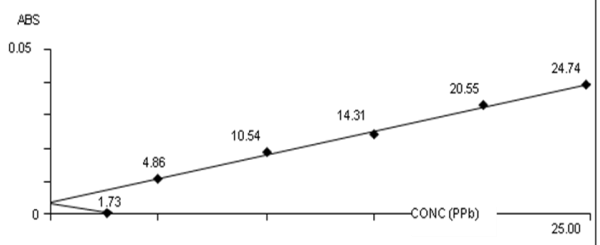
Elements	*No. of Runs*	*Conc.found*	*Mean Conc.*	*Range*
Calcium µg/ml	04	203,205,205,203	204	202-205
Magnesium µg/ml	04	36.0,35.5,38.0,35.5	36.25	36.25-38.0
Zinc µg/ml	04	6.52,6.00,7.50,6.50	6.63	6.00-7.50
Copper µg/ml	04	0.52,0.62,0.60,0.52	0.56	0.50-0.62
Chromium ng/ml	07	29.60,35.35,29.60, 36.40,33.02,35.55, 36.10	33.66	29.60-36.40
Lead ng/ml	04	132.5,135,132.55,134.35	133.6	132.5-135

Fig c: Calibration curve of zinc in blood.



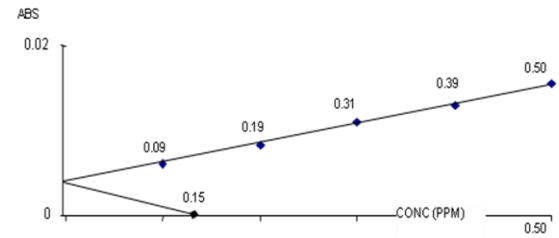
Standard Addition Method

Fig e: Calibration curve of chromium in blood.



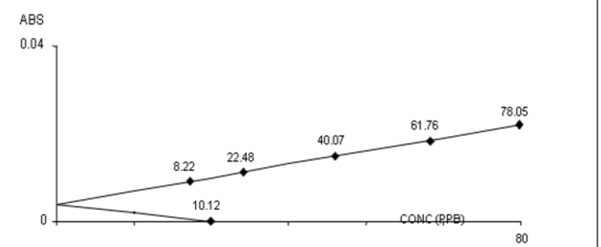
Standard Addition Method

Fig d: Calibration curve of copper in blood.



Standard Addition Method

Fig f: Calibration curve of lead in blood.



Standard Addition Method

To make sure the accuracy of the results, internal spiking was also done (Table-3).The esimated value of the sum of the concentration (observerd conc before spiking and after spiking) was found to be satisfactory.

Discussion

In order to minimize matrix differences, standard solution of blood was made. The result of this study clearly show that matrix interferences can be minimized by standard addition technique. This solution may be used as a reference material for the estimation of metals. Low acid concentration might cause the

Table 3: Accuracy of the method by internal spiking.

Element	*Conc. Before Spiking*	*Internal Spiking*	*Conc. After Spiking (Observed Value)*	*Actual value*	*% age recovery*
Calcium µg/ml	204	50	255.01	254	100.40
Magnesium µg/ml	36.25	15	50.85	51.25	99.22
Zinc µg/ml	6.63	10	16.30	16.63	98.02
Copper µg/ml	0.56	02	02.49	2.56	97.27
Chromium ng/ml	33.66	20	53.67	53.66	100.02
Lead ng/ml	133.6	50	185.20	183.60	100.87

formation of black carbon residue and low recovery of some elements like copper[16]. Nitric acid was chosen for decomposition by wet digestion. The purpose was that it produces least polyatomic interferences in contrast with $HClO_4$ and H_2SO_4[5]. Later two acids introduce polyatomic ions such as ClO+ and SO+. Long neck open vessels were used to reduce the loss of analyte. Aluminum block was used to control and kept uniform temperature[5] Elements under study were estimated in their solution by using standard addition method. Calibration with a standard reference solution minimizes the effect of differences of matrix, providing that the solutions of blood under test should made up in the same way as their respective standard stock solutions. As shown in our previous study[17], Concentration of elements changes with acid strength so the concentration of acid was kept identical in all working solutions which were tested for metals estimation.

As no reference material is available for metal estimation in whole blood, the accuracy of the method for the estimation of metals in blood was counter checked by internal spiking as shown in Table-3. Results obtained by the two methods (internal spikng and standard addition method) agreed with each other. Data from selected investigation is compared with previous study conducted in Pakistan[18], Japan[19], Spain[20], Banaladesh[21], Italy[22] and China[23]. In our study concentration of copper is found low as compare to other countries. But the Zinc level is found almost same as in Canada and Italy[22]. As far as Magnesium is concerned, its concentration is compatible with previous study done in Pakistan and also with Canada[22] and China[23]. Wherever the ranges are reported, Variation in ranges among different countries is observed. The variation in metal level is reasonable because there are many contributing factors on which metals level depend like methodology, analytical technique used, environmental conditions, diet gender etc.

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