Comparative study of inorganic elements determination in whole blood from Crioula breed horse by EDXRF and NAA analytical techniques

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Abstract The World Health Organization states that envenomation is responsible for a high number of deaths per year, especially in equatorial areas. The only effective specific treatment is the use of hyperimmune serum (antivenom). In Brazil, Crioula breed horses are used for antivenom production, with great importance in the maintenance of public health programs. A strict biochemical and metabolic control is required to attain specificity in antiserum. Inorganic elements represent only a small fraction of whole blood. Nonetheless, they play important roles in mammalian metabolism, being responsible for controlling enzymatic reactions, respiratory and cardiac functions and ageing. In this work, whole blood samples from Crioula breed horses were analyzed by EDXRF technique. The reference interval values were determined for the elements Na (1955–2013 $\mu g\ g^{-1}$), Mg (51–75 $\mu g\ g^{-1}$), P (523–555 $\mu g\ g^{-1}$), S (1628–1730 $\mu g\ g^{-1}$), Cl (2388–2574 $\mu g\ g^{-1}$), K (1649–1852 $\mu g\ g^{-1}$), Ca (202–213 $\mu g\ g^{-1}$), Cu (4.1–4.5 $\mu g\ g^{-1}$) and Zn (2.4–2.8 $\mu g\ g^{-1}$) and a comparative study with NAA results was outlined. The samples were obtained from Instituto Butantan. Both techniques showed to be appropriate for whole blood sample analyses and offer a new perspective in Veterinary Medicine.

Keywords EDXRF · Whole blood · Inorganic elements · Crioula breed horse

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Introduction

According to the World Health Organization (WHO) estimate, 5 million people are bitten by snakes around the world each year, with up to 50% of the cases leading to envenomation. The annual mortality is, at least, 100,000 people and around three times as many amputations and other permanent sequelae. Unlike other health conditions, a very effective treatment for envenomation exists: the hyperimmune serum (snake antivenom immunoglobulin), which is the only specific treatment. Deaths and amputations of envenomed patients are, therefore, preventable and, for this reason, antivenom is included in the *WHO List of Essential Medicines* [1]. The production of antiserum involves the fractionation of plasma obtained from large domestic animals, like horses, immunized against venoms. The antiserum, when injected in a patient, is capable of neutralizing the venom used in its production. Antiserum production is a laborious process and demands a strict biochemical and metabolic control in order to obtain high quality and efficient product. The health of horses employed in the process has to be monitored by parameters such as hemoglobin, hematocrit, erythrocyte and leukocyte counts plus inorganic elements concentration in blood [2]. In order to consider a subject healthy or unhealthy, one

should compare such parameters with reference values. Reference values or intervals are established by testing individuals selected according to defined criteria. The results are statistically described as a normal distribution with significance level ($\alpha = 0.05$) [3].

Blood is a complex matrix composed of mainly two parts, cells and plasma; the term *whole blood* refers to samples containing both parts. Inorganic elements represent only a small fraction of whole blood; nonetheless, they play important roles in mammalian metabolism, since they are responsible for controlling enzymatic reactions (Mg^{2+}, Ca^{2+}), respiratory and cardiac functions (K^+, Mg^{2+}, Ca^{2+}), osmotic regulation (Na^+, Cl^-, K^+), oxidation processes (Fe) and hemoglobin synthesis (Mn) [4]. Recently, a large number of publications indicate the possibility of diagnosing different diseases by observing variations in the concentration of inorganic elements in human blood [5, 6].
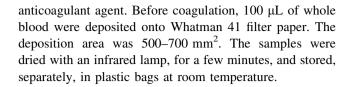
The conventional clinical analyses determine different elements using diverse analytical techniques, namely ion specific electrode, colorimetry and titrimetry, requiring a large volume of samples for a multi-element determination. Lately, analytical techniques such as neutron activation analysis (NAA) and X-ray fluorescence (XRF) spectrometry have shown great progress in the analytical field, owing to their characteristics of non destructive multi-elemental analysis, short time of analysis and a small amount of sample requirement. Custódio et al. [7] and Khuder et al. [8] have determined several inorganic elements in lyophilized human blood samples by EDXRF. The Nuclear Structure Laboratory and the X-Ray Fluorescence Laboratory at IPEN, São Paulo, Brazil, have performed the determination of inorganic elements in human [9] and animals' whole blood samples [10]. Inorganic elements' reference interval values in laboratory animal blood by EDXRF are not present on literature.

In this work, the elements Na, Mg, P, S, Cl, K, Ca, Fe, Cu and Zn were determined quantitatively by EDXRFS, using the Fundamental Parameters (FP) method in Crioula breed horse whole blood samples; and results were compared with NAA analysis.

Experimental

Sample preparation

The samples were obtained from a renowned Brazilian public health research laboratory, Instituto Butantan, which is one of the largest producers of antivenom in the country. For this work, Crioula breed horses, 12–36 months old, both female and male, from São Joaquim farm (São Paulo State) were used. 2 mL of whole blood were collected by jugular puncture in a vacuum plastic tube, without any anticoagulant agent. Before coagulation, 100 μL of whole blood were deposited onto Whatman 41 filter paper. The deposition area was 500–700 mm^2. The samples were dried with an infrared lamp, for a few minutes, and stored, separately, in plastic bags at room temperature.

Equipment

The X-ray fluorescence analysis was carried out at an EDXRF spectrometer, from SHIMADZU Co., model Rayny 720, which was coupled to the Fundamental Parameters method software. The instrumental measurement conditions are shown in Table 1.

Methodology

The inorganic elements determination was carried out by analyzing triplicate measurements of 10 whole blood samples from Crioula breed horses, using the Fundamental Parameters method.

The Fundamental Parameters method [11, 12] is an algorithm used to correct matrix effects, inter-elemental and physics, which are predominant in XRF analyses. The FP method uses the instrumental sensitivity curve calculated from nuclear data library in the spectrometer. Commercial spectrometers offer sensitivity libraries based on fluorescent intensities measured from pure oxides and metallic samples. To perform analyses on biological samples, an experimental sensitivity curve was obtained using a biological reference material, NIST SRM 1577c-Bovine liver.

The certified reference material IAEA A-13 Animal Blood, from the International Atomic Energy Agency, was used for the methodology evaluation. Approximately, 50 mg of the CRM IAEA A-13 were deposited in a sample carrier and analyzed directly, without additional pre-treatment. The elements Na, Mg, P, S, Cl, K, Ca, Fe, Cu and Zn

Table 1 Measurement conditions from the EDXRF spectrometer

Parameter	Condition
X ray tube	Rh target
Run group analysis	Na, Mg, P, S, Cl, K and Ca (15 kV)
	Fe, Cu and Zn (50 kV)
Current	Adjustable (40 μA maximum)
Atmosphere	Vacuum
Detector	Si(Li)
Collimator	5 mm
Fixed time count	100 s for each group
Emission line	Kα for all elements
Irradiated area	30 mm^2

were determined using the experimental sensitivity curve. From a set of data of 10 measurements, the measurement uncertainty (u, Eq. 1) and the relative standard deviation (RSD%, Eq. 2) were calculated to evaluate the precision. The accuracy of the method was evaluated by the Z score test (Eq. 3), according to ISO 17025 [13] and EURACHEM/CITAC [14] norms.

$$u = t_{n-1\left(\frac{\alpha}{2}\right)} \cdot \frac{s_{\det}}{\sqrt{n}} \qquad (1)$$

where $t_{n-1(\alpha/2)}$ is t-Student value, at 95% confidence level; $s_{\det}$ is the standard deviation of the determined value; n is the number of measurements; α is significance level, 0.05

$$\mathrm{RSD\%} = \frac{s_{\det}}{x_{\det}} \cdot 100 \qquad (2)$$

where $x_{\det}$ is determined value; $s_{\det}$ is the standard deviation of the determined value

$$Z = \frac{x_{\det} - x_{\mathrm{cert}}}{\sqrt{s_{\det}^2 + s_{\mathrm{cert}}^2}} \qquad (3)$$

where x_{cert} is certificated value; s_{cert} is the deviation of the certificated value

The sensitivity of the method was evaluated by the limit of quantification (LQ), according to Rousseau [15] statement, considering the confidence level and the distribution of data as influenced by factors such as sample preparation, counting statistics and instrument (Eq. 4).

$$LQ = 2 \cdot \sqrt{\frac{\sum_{m=1}^{n} (C_m - \bar{C})^2}{n-1}} \qquad (4)$$

where C_m is mth concentration value; $\bar{C}$ is the mean concentration value; n is number of measurements

One of the objectives of this work was to provide more data for inorganic elements reference intervals values, in whole blood, of Crioula breed horses. The samples analyzed by XRF were previously analyzed by NAA and a comparison of results was carried out by the Student's t test ($\hat{t}_v$) for statistically unequal variances (Eq. 5).

$$\hat{t}_v = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}} \qquad (5)$$

where, in this work $\bar{x}_1$ is the mean of results obtained by EDXRF technique; $\bar{x}_2$ is mean of results obtained by NAA technique; s_1^2 is variance obtained by EDXRF technique; s_2^2 is variance obtained by NAA technique; n_1 is number of samples analyzed by EDXRF technique; n_2 is number of samples analyzed by NAA technique; v is $n_1 + n_2 - 2$, degrees of freedom

Results and discussion

The adequacy of the method was evaluated for Na, Mg, P, S, K, Ca, Fe, Cu and Zn determination using the CRM IAEA-A-13, Animal blood. The certified and determined values, RSD% and Z score values are given in Table 2.

Relative standard deviation evaluates the precision of the method and is considered satisfactory when their values are below 10% [13]. RSD% values for Na, P, S, K, Fe, Cu and Zn elements are below 6.2%, showing a good repeatability of the method. The elements Mg and Ca presented higher RSD% values (9.2 and 10.0%, respectively). The lower repeatability could be related to their low concentration, in relation to others elements, associated to EDXRF low efficiency for light elements determination.

The evaluation of the Z score test is as it follows: values of $|Z| < 2$ are satisfactory; values of $2 < |Z| < 3$ are questionable; values of $|Z| > 3$ are unsatisfactory [13]. According to Table 2, Z score values for all elements are below 1; hence, the accuracy is satisfactory for the determination of all elements.

Table 2 CRM IAEA-A-13 data

Element	Confidence interval ($\mu g\ g^{-1}$)	$x_{\det} \pm u_{\det}$ ($\mu g\ g^{-1}$)	RSD%	Z score values	LQ ($\mu g\ g^{-1}$)
Na	11600–13500	12523 ± 249	3.1	−0.1	113 (<110×)[a]
Mg	81–139	100 ± 7	9.2	−0.1	1 (<106×)
P	690–1120	954 ± 34	5.0	0.2	8 (<120×)
S	6000–7000	6052 ± 270	6.2	−0.7	42 (<145×)
K	2100–2700	2264 ± 79	4.9	−0.4	15 (<150×)
Ca	226–332	258 ± 19	10.0	−0.2	3 (<90×)
Fe	2200–2500	2179 ± 95	6.1	−0.9	17 (<130×)
Cu	3.7–4.8	4.6 ± 0.2	4.1	0.5	0.03 (<150×)
Zn	12–14	13 ± 0.6	4.2	0.2	0.06 (<215×)

[a] 110 times lower than determined values

Table 3 Reference intervals determined for Crioula breed horses, µg g^{-1}

Element	$x \pm u$	Reference intervals by EDXRF	Reference intervals by NAA [17]	t_{calc}[a]
Na	1984 ± 29	1955–2013	1360–2800	−1.143
Mg	63 ± 12	51–75	30–74	1.096
P	539 ± 16	523–555	220–1400	−2.041
S	1679 ± 51	1628–1730	990–2790	−1.942
Cl	2481 ± 93	2388–2574	2020–3180	−0.494
K	1751 ± 38	1649–1852	1140–2740	−2.006
Ca	208 ± 5	202–213	89–369	−1.326
Fe	174 ± 8	166–182	381–689	−19.562
Cu	4.3 ± 0.2	4.1–4.5	–	–
Zn	2.6 ± 0.2	2.4–2.8	1.2–4.8	−1.995

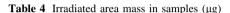[a] ($\alpha = 0.05$, $t_{\text{crit}} = 2.048$)

The limit of quantification (LQ) is significant when it is 100–1000 times smaller than the determined concentration [14]. The experimental LQ showed values between 106 and 215 times smaller than determined values; except for Ca (90 times), thus the method presents adequate sensitivity (Table 2).

The reference intervals for Na, Mg, P, S, Cl, K, Ca and Zn determined by EDXRF are in agreement with those determined by NAA technique (Table 3). The reference interval values for Fe, determined by EDXRF, are not in agreement with the NAA values. Liquid sample deposition on thin layer may cause for some transition metals migration to the borders, depending on pH, called chromatographic effect [16]. The lower values of Fe indicate the radial chromatographic effect for this element [16]. In this study, the sample irradiation area (30 mm^2) was smaller than deposited area (500–700 mm^2). This effect could be minimized by the reduction of the deposited area and the irradiated area enlargement.

As the number of samples analyzed by EDXRF was 10 and by NAA was 20, the Student's t-test for unequal variances was applied for comparison of results (Table 3). The $t_{\text{calculated}}$ values for Na, Mg, P, S, Cl, K, Ca and Zn are lower than t_{critic} ($t_{\text{calc}} < t_{\text{crit}}$); therefore, the results from both techniques are considered statistically equivalent. However, the Fe value $t_{\text{calc}} > t_{\text{crit}}$ (19.662 > 2.048) shows that the reference interval values determined by EDXRF are statistically different from values obtained by NAA analysis; this fact supports the Fe radial chromatographic effect.

The mass quantity on the irradiated area was calculated. The actual mass of the elements present on the filter paper samples is very low; for NAA analysis, the actual mass varied from 0.03 to 27.56 µg and for EDXRF technique from 0.003 to 2.20 µg, showing the good sensitivity of the latest technique (Table 4).

Table 4 Irradiated area mass in samples (µg)

Element	Mean concentration determined by NAA (µg g^{-1}) [17]	Total mass analyzed by NAA	Total mass analyzed by EDXRF
Na	2080	22.05	1.76
Mg	52	0.55	0.04
P	810	8.59	0.69
S	1890	20.03	1.60
Cl	2600	27.56	2.20
K	1940	20.56	1.65
Ca	229	2.43	0.19
Fe	535	5.67	0.45
Zn	3	0.03	0.003

Conclusion

The EDXRF technique using the Fundamental Parameters method showed to be adequate and viable for Na, Mg, P, S, Cl, K, Ca, Cu and Zn determination in whole blood samples from animals used in experiments.

The EDXRF and NAA analysis showed agreement for Na, Mg, P, S, Cl, K, Ca, Cu and Zn reference interval values. Both techniques showed their applicability, requiring a small amount of samples, multi-elemental analysis, short time of analysis and simple sample preparation. Moreover, the blood samples may be stored for a long period for future examination and their disposal may, also, be done as regular biohazard or by incineration processes.

Lastly, the blood collection from large animals is expensive, laborious and stressful to the animal. Hence, this work, also, offers an alternative analytical method for elemental blood analysis from large animals used in experiments.

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