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Determination of reference interval values for inorganic elements in whole blood samples of humans and laboratory animals by X-ray fluorescence spectrometry

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Abstract Inorganic elements are responsible for essential bodily functions, such as osmotic regulation, cardiac frequency and contractibility, blood clotting and neuromuscular excitability. The determination of inorganic elements in corporeal fluids such as blood, serum, plasma and urine is used as a monitor for a part or the whole organism; their values, then, are compared with reference interval values. In this study, the energy dispersive X-ray fluorescence spectrometry (EDXRF), applying the Fundamental Parameters method, for the determination of inorganic elements in whole blood samples from humans and laboratory animals, was used. Peripheral blood samples were collected and, before coagulation, 100 μL of sample were deposited onto Whatman No. 41 filter paper and dried, using infrared spotlight. The reference interval values for healthy Brazilian population of Na were found to be 1,788–1,826 μg g^{-1}, of Mg 63–75 μg g^{-1}, of P 602–676 μg g^{-1}, of S 1,519–1,718 μg g^{-1}, of Cl 2,743–2,867 μg g^{-1}, of K 1,508–1,630 μg g^{-1}, of Ca 214–228 μg g^{-1}, of Fe 170–184 μg g^{-1}, of Cu 4–6 μg g^{-1} and of Zn 1–3 μg g^{-1}. The reference interval values for golden hamster (*Mesocricetus auratus*) of Na were found to be 1,714–1,819 μg g^{-1}, Mg 51–79 μg g^{-1}, P 970–1,080 μg g^{-1}, S 1,231–1,739 μg g^{-1}, Cl 2,775–2,865 μg g^{-1}, of K 1,968–2,248 μg g^{-1}, of Ca 209–257 μg g^{-1}, of Fe 145–267 μg g^{-1}, of Cu 4–6 μg g^{-1} and of Zn 3–5 μg g^{-1}. A comparative study between EDXRF and instrumental neutron activation analysis data was carried out and the results for both techniques are statistically equal ($\alpha = 0.05$). The results contribute for the establishment of reference interval values for Na, Mg, P, S, Cl, K, Ca, Cu and Zn in the healthy Brazilian population and the referred laboratory animal species.

Keywords Reference interval values · Whole blood · Energy dispersive X-ray fluorescence spectrometry

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Introduction

Blood is a suspension of cells contained in complex liquid called plasma. The term "whole blood" refers to samples with both parts, solid (cells) and liquid (plasma). Inorganic elements, though being minor constituents, having concentration in the parts per million range, of blood, are responsible for essential bodily functions, such as osmotic regulation (Na^+, Cl^-, K^+), cardiac frequency and contractibility (K^+, Ca^{2+}, Mg^{2+}), blood clotting (Ca^{2+}, Mg^{2+}), hemoglobin and myoglobin synthesis (Fe^{2+}, Fe^{3+}), tissue growth (Zn^{2+}). Recent studies [1, 2] have indicated that several diseases can be diagnosed based on the change in the concentrations of inorganic elements, for example, Co in vitamin D.

Conventional techniques of analyses of inorganic elements in blood, namely atomic absorption spectrometry and colorimetry, are time consuming and demand large volume of samples. In this study, two nuclear analytical techniques, energy dispersive X-ray fluorescence (XRF) and neutron activation analysis (NAA), are presented as an alternative to the conventional ones. The determination of inorganic elements in human [3] and animals' whole blood

samples [4] have been performed at the Nuclear Structure Laboratory and the X-Ray Fluorescence Laboratory at IPEN, São Paulo, Brazil.

For clinical merit, any laboratory test result must be compared with established reference values on which a diagnosis can be based on. Moreover, the individuals should be selected under defined clinical criteria.

In Brazil, there is at present no available data on the reference intervals of inorganic elements regarding healthy individuals. Hence, laboratory test results cannot be compared to present international reference values, furthermore, because of different regional dietary habits, variation may exist.

Several medical studies are dependent on the use of animal model. The golden hamster (*Mesocricetus auratus*) is a species widely used in parasitological and biochemical studies [5, 6]. There is no information regarding the inorganic elemental content of this particular species.

In this study, the elements Na, Mg, P, S, Cl, K, Ca, Fe, Cu and Zn were determined quantitatively by EDXRF technique, using the Fundamental Parameters (FP) method in human and golden hamster whole blood samples. The results were compared with NAA analysis.

Experimental

Sample preparation

The human samples were obtained from blood banks from northeastern and southeastern regions of Brazil. Blood was collected following Brazilian Health Agency (ANVISA) norms for blood donors: individuals should be between 18 and 65 years old and weighing above 50 kg. Circa 2 mL of peripheral venous blood was collected in a plastic tube without any anticoagulant agent, since the anticoagulant contains high concentrations of Na^+ and K^+. Immediately after collection, 100 μL of whole blood was deposited on Whatman 41 filter paper (deposition area $\sim$500–700 mm^2) and dried for a few minutes with an infrared lamp; assuming a homogenous cellular distribution. Part of the collected blood was sent to conventional serology tests to validate the patients' health.

In this study, the determination of reference values for inorganic elements in human blood was performed analyzing more than 250 samples by NAA technique and circa 50 samples by EDXRF technique. For animal blood samples, a reduced number of samples, 17, were analyzed.

The golden hamster samples were obtained from two renowned Brazilian public health research laboratories, Instituto Butantan and Centro de Pesquisas Aggeu Magalhães. The same procedure described above was followed in the preparation of these samples.

All samples were prepared in duplicate and stored, separately, in plastic bags at room temperature.

Equipment

The X-ray fluorescence analysis was carried out at the SHIMADZU Co. EDXRF spectrometer, model Rayny 720, which was coupled to the FP method software. The instrumental measurement conditions are shown in Table 1 (Rh target, vacuum atmosphere, collimator, detector, fixed time count). Two run groups analyses were established, one from Na to Ca and another from Fe to Zn. The Kα emission line was used for all elements. The irradiation area available in the spectrometer ranges from 1.5 to 30 mm^2, in this study the latest area was used (30 mm^2).

Methodology

The elemental content determination was carried out by analyzing triplicate measurements of 50 whole blood samples from humans and 17 from golden hamsters, using the FP method. The FP method [7] is an algorithm used to correct matrix effects (inter-elemental and physics) that 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 analyze 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 Rigaku spectrometer's sample carrier and analyzed directly, without additional pre-treatment. The elements Na, Mg, P, S, Cl, K, Ca, Fe, Cu and Zn were determined using the experimental sensitivity curve. From a

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

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 Z score test (Eq. 3) was calculated to evaluate accuracy, according to ISO 17025 [8] and EURACHEM/CITAC [9] norms.

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

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

$$\text{RSD}\% = \frac{s_{\text{det}}}{x_{\text{det}}} \cdot 100 \tag{2}$$

where x_{det} = determined value, s_{det} = standard deviation of the determined value

$$Z = \frac{x_{\text{det}} - x_{cert}}{\sqrt{s_{\text{det}}^2 + s_{cert}^2}} \tag{3}$$

where x_{cert} = certificated value, s_{cert} = deviation of the certificated value.

The sensitivity of the method was evaluated by the limit of quantification (LQ), according to Rousseau [10] statement, considering the confidence level and the distribution of data as influenced by factors such as sample preparation, counting statistics and instrument (Eq. 4).

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

where $C_m = m$th concentration value, $\bar{C}$ = mean concentration value, n = number of measurements

The duplicate samples were analyzed by EDXRF and NAA and a comparison of results was carried out by the Student's t test ($\hat{t}_v$) for statistically unequal variances (Eq. 5).

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

where in this study $\bar{x}_1$ = sample mean of results obtained by EDXRF technique, $\bar{x}_2$ = sample mean of results obtained by NAA technique, s_1^2 = sample variance obtained by EDXRF technique, s_2^2 = sample variance obtained by NAA technique, n_1 = number of samples analyzed by EDXRF technique, n_2 = number of samples analyzed by NAA technique, $\nu = n_1 + n_2 - 2$, 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.

The precision of the method is considered satisfactory when values of relative standard deviation are below 10 % [8]. RSD% values for Na, P, S, K, Fe, Cu and Zn elements are below 6.2 %, showing a good repeatability of the FP 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 [8]. According to Table 2, Z-score values for all elements are below 1; hence, the accuracy is satisfactory for the determination of all elements.

The LQ is significant when it is 100–1,000 times smaller than the determined concentration [9]. 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).

Table 2 CRM IAEA-A-13 data

Element	Confidence interval (μg g^{-1})	$x$det ± $u$det (μg g^{-1})	RSD%	Z-score values	LQ (μg g^{-1})
Na	11,600–13,500	12,523 ± 249	3.1	−0.1	113 (<110×)*
Mg	81–139	100 ± 7	9.2	−0.1	1 (<106×)
P	690–1,120	954 ± 34	5.0	0.2	8 (<120×)
S	6,000–7,000	6,052 ± 270	6.2	−0.7	42 (<145×)
K	2,100–2,700	2,264 ± 79	4.9	−0.4	15 (<150×)
Ca	226–332	258 ± 19	10.0	−0.2	3 (<90×)
Fe	2,200–2,500	2,179 ± 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×)

* (<110×) means 110 times lower than determined values

Table 3 Human whole blood analyses, μg g^{-1}

Element	$x \pm u$ EDXRF	Median	Minimum value	Maximum value
Na	1,807 ± 19	1,816	1,606	1,897
Mg	69 ± 6	72	57	90
P	639 ± 37	647	530	836
S	1,618 ± 99	1,516	1,424	2,015
Cl	2,805 ± 62	2,825	2,525	3,097
K	1,569 ± 61	1,614	1,429	1,855
Ca	221 ± 7	220	175	260
Fe	177 ± 7	177	125	210
Cu	4.9 ± 1.0	4.9	4.1	5.5
Zn	2.3 ± 1.0	2.3	1.6	2.9

Human whole blood samples

The results can be statistically described as a normal distribution; values of mean and median are close for all elements and the maximum and minimum values are not extreme (Table 3).

The reference intervals for Na, Mg, Cl, K and Ca determined by EDXRF are in agreement with those determined by NAA technique (Table 4). The reference interval values for Fe are not in agreement. For some transition metals, liquid sample deposition on thin layer may cause migration to the borders, depending on pH, called chromatographic effect. The lower values of Fe indicate the radial chromatographic effect for this element [7]. In this study, the sample irradiation area (30 mm^2) was smaller than deposited area (500–700 mm^2), which could have intensified the chromatographic effect. 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 smaller than those analyzed by NAA, the Student's t test for unequal variances was applied for comparison of results (Table 4). The $t_{calculated}$ values for Na, Mg, Cl, K and Ca are lower than t_{critic} ($t_{calc} < t_{crit}$); therefore, the results from both techniques are considered statistically equal. However, the Fe value $t_{calc} > t_{crit}$ (24.23 > 1.99) 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.

Golden hamster whole blood samples

Values of mean, median, maximum and minimum for the determination of all elements are shown in Table 5. The results can be described as a normal distribution.

The reference intervals for Na, Mg, S, Cl, K and Ca determined by EDXRF are in agreement with those determined by NAA technique. The Student's t test for unequal variances was applied for comparison of results and the values of $t_{calculated}$ values are lower than t_{critic} ($t_{calc} < t_{crit}$) for Na, Mg, S, Cl, K and Ca; therefore, the results are considered statistically equivalent (Table 6).

Table 5 Golden hamster whole blood analyses, μg g^{-1}

Element	$x \pm u$ EDXRF	Median	Minimum value	Maximum value
Na	1,767 ± 52	1,768	1,636	1,868
Mg	65 ± 14	63	44	87
P	1,025 ± 55	973	772	1,250
S	1,484 ± 254	1,440	1,161	1,748
Cl	2,820 ± 45	2,822	2,628	2,980
K	2,108 ± 140	2,108	1,954	2,377
Ca	233 ± 24	235	207	258
Fe	206 ± 61	194	153	248
Cu	5 ± 1	5	4	6
Zn	4 ± 1	4	2	5

Table 4 Reference intervals determined for humans, μg g^{-1}

Element	$x \pm u$ EDXRF	$x \pm s$ NAA	Reference intervals by EDXRF	Reference intervals by NAA	t^{a}_{calc}
Na	1,807 ± 19	1,770 ± 290	1,788–1,826	1,480–2,060	1.02
Mg	69 ± 6	57 ± 17	63–75	40–74	1.81
P	639 ± 37	–	602–676	–	–
S	1,618 ± 99	–	1,519–1,718	–	–
Cl	2,805 ± 62	3,020 ± 480	2,743–2,867	2,540–3,500	1.80
K	1,569 ± 61	1,610 ± 280	1,508–1,630	1,330–1,890	0.68
Ca	221 ± 7	233 ± 83	214–228	150–316	1.09
Fe	177 ± 7	395 ± 59	170–184	336–454	24.23
Cu	5 ± 1	–	4–6	–	–
Zn	2 ± 1	–	1–3	–	–

[a] ($\alpha = 0.05$, $t_{crit} = 1.99$)

Table 6 Reference intervals determined for golden hamsters (*Mesocricetus auratus*), μg g^{-1}

Element	$x \pm u$ EDXRF	$x \pm s$ NAA	Reference intervals by EDXRF	Reference intervals by NAA	t_{calc}
Na	1,767 ± 52	1,770 ± 370	1,714–1,819	1,400–2,140	0.01
Mg	65 ± 14	58 ± 16	51–79	42–74	0.60
P	1,025 ± 55	–	970–1,080	–	–
S	1,485 ± 254	1,490 ± 260	1,231–1,739	1,230–1,750	0.03
Cl	2,820 ± 45	2,930 ± 410	2,775–2,865	2,520–3,340	1.09
K	2,108 ± 140	2,070 ± 360	1,968–2,248	1,710–2,430	0.44
Ca	233 ± 24	230 ± 60	209–257	170–290	0.26
Fe	206 ± 61	–	145–267	–	–
Cu	5 ± 1	–	4–6	–	–
Zn	4 ± 1	–	3–5	–	–

($\alpha = 0.05$, $t_{crit} = 2.03$)

Conclusion

The EDXRF technique using the FP method showed to be adequate and viable for Na, Mg, P, S, Cl, K, Ca, Cu and Zn determination in whole blood samples from humans and animals used in experiments. The EDXRF and NAA analysis showed agreement for Na, Mg, Cl, K and Ca reference interval values for all samples. 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.

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