Applied Radiation and Isotopes 110 (2016) 189–192

Contents lists available at ScienceDirect

Applied Radiation and Isotopes

journal homepage: www.elsevier.com/locate/apradiso

Comparative study of inorganic elements determined in whole blood from Dmd^{mdx}/J mice strain by EDXRF and NAA analytical techniques

M.M. Redígolo [a], I.M. Sato [a], S. Metairon [b,*], C.B. Zamboni [b]

[a] Instituto de Pesquisas Energéticas e Nucleares, IPEN – CNEN/SP, Centro de Química e Meio Ambiente, CQMA, Av. Professor Lineu Prestes 2242, 05508-000 São Paulo, SP, Brasil

[b] Instituto de Pesquisas Energéticas e Nucleares, IPEN – CNEN/SP, Centro do Reator de Pesquisa - CRPq, Av. Professor Lineu Prestes 2242, 05508-000 São Paulo, SP, Brasil

HIGHLIGHTS

- The EDXRF technique using the Fundamental Parameters method showed to be adequate and viable for the determination of Ca, Cl, Fe, K, Mg, Na and S in whole blood samples.
- EDXRF and NAA analysis showed agreement for all elements concentration investigated in mice (Dmd^{mdx}/J and C57BL/6J strains) offering a new contribution for the Duchenne Muscular Dystrophy (DMD).
- These analytic techniques are complementary and showed their applicability for biochemistry tests in blood, requiring small amount of samples, short time of analysis and simple sample preparation.

ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form
22 January 2016
Accepted 22 January 2016
Available online 23 January 2016

Keywords:
Whole blood
Energy Dispersive X-ray Fluorescence
Neutron Activation Analysis
Animal model
DMD

ABSTRACT

Several diseases can be diagnosed observing the variation of specific elements concentration in body fluids. In this study the concentration of inorganic elements in blood samples of dystrophic (Dmd^{mdx}/J) and C57BL/6J (control group) mice strain were determined. The results obtained from Energy Dispersive X-ray Fluorescence (EDXRF) were compared with Neutron Activation Analysis (NAA) technique. Both analytical techniques showed to be appropriate and complementary offering a new contribution for veterinary medicine as well as detailed knowledge of this pathology.

1. Introduction

Inorganic elements are of great importance in studies of clinical biochemistry, being responsible for several bodily functions. Any disturbance in their concentration can be related to diseases, such as calcemia, chloremia, natremia, etc.

This work shows in details an investigation of inorganic elements of clinical and nutritional relevance (Br, Ca, Cl, Fe, K, Mg, Na, and S) in whole blood of Dmd^{mdx}/J dystrophic mice caused by Duchenne Muscular Dystrophy (DMD), a progressive neuromuscular disease. This disorder is caused by a genetic mutation on the humans X chromosome. Unlike of most genes, which come in pairs in both sexes and stay active throughout life, in males there is only one X chromosome, for these reason this disorder usually affected much more boys than girls (1/ 3500 boys (Bushby, 2010; Matsuo, 1995; Emery, 2002)). The symptoms beginning at the age of 5. The life expectancy has increase up to 20 years old (Goyenvalle et al., 2011). There is no cure for DMD and the treatment is aimed at control of symptoms (muscle weakness, premature death, and instability of the membrane that involves the muscle fibers-causing functional/structural abnormalities (Goyenvalle et al., 2011; Regence Medical Police Manual, 2015; Muntoni and Wells, 2007). Its main characteristic is the degeneration of the membrane that involves the muscular cell (sarcolemma), which leads to muscular necrosis and that is caused by absence of the dystrophin protein in muscles. This gene has 79 exons (largest of the human genome) forming the genetic code for the dystrophin protein. Duchenne patients have mutations in their DMD gene. The most common mutation is when one exon or more are missing from this gene (deletion).

* Corresponding author.
E-mail addresses: marcelo.redigolo@usp.br (M.M. Redígolo), imsato@ipen.br (I.M. Sato), metairon@live.com (S. Metairon), czamboni@ipen.br (C.B. Zamboni).

http://dx.doi.org/10.1016/j.apradiso.2016.01.022
0969-8043/

In this work, inorganic elements were determined quantitatively by EDXRF technique, using the Fundamental Parameters (FP) method in whole blood samples of Dmd^{mdx}/J (dystrophic) and C57BL/6J (control group) mice strain and, the results were compared with recent data of NAA analysis (Metairon et al., 2013).

2. Experimental

2.1. Sample preparation

The animal models used for the control group were C57BL/6J male (10) and female (7) and for dystrophic group Dmd^{mdx}/J male (9) and female (9). The adult mice strain (4 months-old) were obtained from Jackson Laboratory (Maine, USA) and later bred at IPEN – CNEN/SP (São Paulo, Brazil). The 100 μL blood collection was performed without the use of any anticoagulant agent. The blood was then deposited onto Whatman 41 filter paper (deposition area ~500 to 700 mm^2). The samples were dried for a few minutes with an infrared lamp. All samples were prepared in duplicate and stored, separately, in plastic bags at room temperature.

2.2. Equipment

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

The neutron activation analysis was carried out at the nuclear reactor IEA-R1 (3.5–4 MW, pool type) at IPEN using the same samples (non-destructive procedure). Each sample was irradiated in the IEA-R1 nuclear reactor for few minutes in a thermal flux of 4.5×10^{12} N cm^{-2} s^{-1}. Standard solutions obtained from high purity metals and salts were prepared following the same procedure. For ^{38}Cl ($T_{1/2}$=37 min, E_γ=1642 keV) and ^{24}Na ($T_{1/2}$=15 h, E_γ=1368 keV) determination an irradiation time of 2 min followed by 5 min of counting time was used. For ^{80}Br ($T_{1/2}$~16 min, E_γ=616 keV), ^{49}Ca ($T_{1/2}$~9 min, E_γ=3084 keV), ^{27}Mg ($T_{1/2}$~9 min, E_γ=1012 keV), ^{42}K ($T_{1/2}$~12 h, E_γ=1525 keV) and ^{37}S ($T_{1/2}$=5 min, E_γ=3104 keV) sample and standard were irradiated for 5 min and, after a decay time of 60 s, they were counted by 20 min for Br, Ca, Mg, and S determination and followed by 4 h of counting for K. For ^{59}Fe ($T_{1/2}$~44.5d, E_γ=1099 keV) determination each sample was irradiated for 8 h in a neutron flux of 7.1×10^{12} cm^{-2} s^{-1} and, after a decay of a week at least, was counted for 6 h. More details about experimental conditions, nuclear instrumentation, methods of analysis and the results of quality control using NAA technique are presented in a previous study (Metairon et al., 2013). The present study shown the activation measurements performed for the female control group samples for determination of Br, Ca, Cl, Fe, K, Mg Na and S as well as a comparison with EDXRF measurements

Table 1
Measurement conditions from the EDXRF spectrometer.

Parameter	Condition
X ray tube	Rh target
Voltage	15 kV
Current	Adjustable (40–100 μA)
Atmosphere	Vacuum
Detector	Si(Li)
Collimator	5 mm
Fixed time count	100 s
Emission line	Kα for all elements

2.3. Methodology

The whole blood mice strain samples were analyzed by EDXRF spectrometry using the Fundamental Parameters (FP) method. The FP method (Beckhoff et al., 2006) is an algorithm applied for correction of matrix effects (inter-elemental and physics) which uses the instrumental sensitivity curve calculated from spectrometer nuclear data library. To analyze biological samples, an experimental sensitivity curve was obtained using a biological reference material, NIST SRM 1577c-Bovine liver. The reference material IAEA-A-13 Animal Blood, from the International Atomic Energy Agency, was used to validate the method. Approximately, 100 mg of the IAEA A-13 was deposited in a sample carrier and analyzed directly, without additional pre-treatment. The Ca, Fe, K, Mg, Na and S elements were determined using the experimental sensitivity curve. From a set of data of 10 measurements, the measurement uncertainty (u) and the relative standard deviation (RSD%) were calculated to evaluate the precision. The Z-score test was calculated to evaluate accuracy, according to ISO 17025 and EURACHEM/CITAC norms. The same methodology was applied for the determination of inorganic elements in whole blood samples in previous studies (Redígolo et al., 2013); in this reference the homogeneity of blood samples was also discussed.

The sensitivity of the method was evaluated by the limit of quantification (LQ), according to Rousseau statement (Rousseau, 2001), considering the confidence level and the distribution of data as influenced by factors such as sample preparation, counting statistics and instrumentation.

3. Results and discussion

The method was evaluated using the reference material IAEA-A-13 Animal Blood. The values determined for this reference material ($\mathbf{x}_{det} \pm \mathbf{u}_{det}$) as well as the relative standard deviation (RSD %) and Z-score are presented in Table 2. The precision of the method is considered satisfactory when values of relative standard deviation (RSD%) are below 10%. According to Table 2 the RSD% values for Fe, K, Na and S elements are below of 5%, while Ca and Mg are a bit higher (7.4% and 8.7%, respectively) but also showing a good repetition of the method. It could be related to Ca and Mg 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 follows: values of $|Z| < 2$ are satisfactory; values of $2 < |Z| < 3$ are questionable; values of $|Z| > 3$ are unsatisfactory. 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 quantification limit (LQ) is significant when it is 100–1000 times smaller than the determined concentration. The experimental QL showed values between 104 and 151 times smaller than determined values; except for Ca (86 times), thus the method presents adequate sensitivity (Table 2).

Table 2
Reference Material IAEA-A-13 Animal Blood data.

Elements	$\mathbf{x}_{RM} \pm \mathbf{u}_{RM}$	Present study $\mathbf{x}_{det} \pm \mathbf{u}_{det}$	RSD%	$\lvert Z\text{-score}\rvert$ values	QL	$[\mathbf{x}_{det}/QL]^*$
Ca, mg kg^{-1}	286 ± 0.054	258 ± 19	7.4	0.5	3	< 86
Fe, g kg^{-1}	2.40 ± 1.44	2.28 ± 0.93	4.1	0.8	19	< 120
K, g kg^{-1}	2.50 ± 0.35	2.26 ± 0.79	3.5	0.7	15	< 151
Mg, mg kg^{-1}	99 ± 29	104 ± 9	8.7	0.2	1	< 104
Na, g kg^{-1}	12.60 ± 1.01	12.52 ± 0.25	2.0	0.1	113	< 113
S, g kg^{-1}	6.50 ± 0.52	6.05 ± 0.27	4.5	0.9	42	< 144

* Means number of times lower than determined values.

Table 3
Whole blood mice strains concentrations by XRF and NAA techniques.

Elements	C57BL/6J (control group)		Dmd^{mdx}/J (dystrophic group)	
	MV ± 1SD XRF	MV ± 1SD NAA	MV ± 1SD XRF	MV ± 1SD NAA [a]
Br, mg kg^{-1}				
M	< *QL*	5.6 ± 0.6[a]	< *QL*	3.7 ± 0.2
F		4.9 ± 0.6		3.8 ± 0.3
Ca, mg kg^{-1}				
M	242 ± 32	289 ± 66[a]	203 ± 36	244 ± 85
F	261 ± 28	270 ± 29	181 ± 21	156 ± 34
Cl, g kg^{-1}				
M	2.95 ± 0.44	3.13 ± 0.26[a]	3.10 ± 0.30	2.71 ± 0.18
F	2.90 ± 0.37	2.91 ± 0.30	nd	2.99 ± 0.28
Fe, mg kg^{-1}				
M	147 ± 19	163 ± 0.13	139 ± 46	nd*
F	150 ± 17	nd*	107 ± 12	
K, g kg^{-1}				
M	2.15 ± 0.52	2.45 ± 0.48[a]	nd	2.39 ± 0.32
F	2.00 ± 0.31	2.12 ± 0.34		2.14 ± 0.43
Mg, mg kg^{-1}				
M	29 ± 7	27 ± 9[a]	17 ± 2	17 ± 2
F	31 ± 9	26 ± 11	13 ± 4	10 ± 3
Na, g kg^{-1}				
M	2.21 ± 0.16	2.37 ± 0.11[a]	nd	2.38 ± 0.20
F	2.33 ± 0.13	2.41 ± 0.11		2.46 ± 0.32
S, g kg^{-1}				
M	1.23 ± 0.20	1.17 ± 0.22[a]	0.99 ± 0.13	1.02 ± 0.17
F	1.20 ± 0.19	1.08 ± 0.24	0.98 ± 0.08	0.92 ± 0.16

M: male.
F: female.
nd: not determined due to a sample damage (long irradiation time in NAA).
nd*: not determined (below of the detection limit for NAA).
QL: Quantification Limit.
[a] From Ref. Metairon et al. (2013).

In Table 3 the whole blood element concentrations of dystrophic mice (Dmd^{mdx}/J) and control group (C57BL/6J) by XRF and NAA are presented as the mean value (MV) and standard deviation (± 1SD) from duplicate analyses.

In Table 4 the ratios (in percentage) between dystrophic and control groups are presented as well as the ratios between dystrophic mice by gender (last column). The concentrations ratios for Ca, Mg and S were obtained using the mean values from both techniques.

The Ca, Cl, Fe, K, Mg, Na and S concentrations (Mean Value and Standard Deviation) determined by EDXRF are in agreement with those determined by NAA technique for a confidence interval of 95% (Table 3). The Student's *t*-test for unequal variances was applied for results comparison between gender (control and dystrophic). The results from both techniques for Fe, Cl, Mg and S are considered statistically equal ($p > 0.05$) and for Ca ($p < 0.001$). In addition, for the control group there is also an agreement between genders ($p < 0.05$). In Fig. 1 the visualization concentrations proportion between control and dystrophic groups (by gender) are presented.

Table 4
Comparison concentration ratios between dystrophic (C_{DMD} male and C_{DMD} female) and control group (C_{CG}) mice and by gender between dystrophic (C_{DMD}^{F}/C_{DMD}^{M}) mice.

Elements	C_{DMD}/C_{CG} male %[a]	C_{DMD}/C_{CG} female %[a]	C_{DMD}^{F}/C_{DMD}^{M} %[b]
Br	< 34	< 22	~1
Ca	< 16	< 36	< 25
Cl	< 14	~1	~1
Fe	< 5	< 29	< 23
K	< 2	~1	< 10
Mg	< 39	< 41	< 29
Na	~1	~1	~1
S	< 16	< 17	~1

M: male.
F: female.
C_{CG}: mean of blood concentration for the Control Group.
C_{DMD}^{male}: mean of blood concentration for the dystrophic Male Group.
C_{DMD}^{female}: mean of blood concentration for the dystrophic Female Group.
< decrease.
> increase.
[a] C_{CG}/C_{CG}: CG concentration ratio assumed 100%.
[b] C_{DMD}^{M}/C_{DMD}^{M}: DMD (male) concentration ratio assumed 100%.

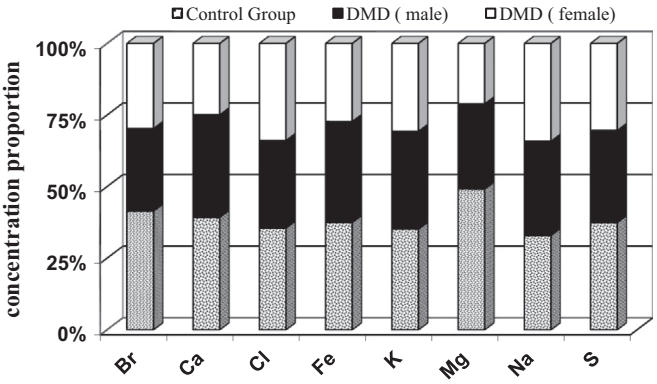

Fig. 1. Concentrations proportion between control and dystrophic mice groups.

According to Table 4 the dystrophic mice (males and females) showed a decrease in Br, Ca, Fe, Mg and S levels in comparison with control group. Alterations in Ca and Mg levels can be related to the sarcolemma (muscle cell membrane) rupture, due the reduction or absence of dystrophin protein in body muscles (Han and Campbell, 2007; Berchtold et al., 2000), while Br and S variation can be related to administration of different rations. Although these animals have a controlled diet, the DMD mice have a more restrictive diet (low in fat and high in protein). The decrease of Fe levels (mainly in female dystrophic mice) can be due to the replacement of healthy cells by adipose cells reducing the number of red blood cells (Berchtold et al., 2000; Worton et al., 2001). Yet, for male dystrophic mice occurs a decrease in Cl level in comparison with the control group. Only Na and K show no changes.

Related to the gender (Table 4), in female dystrophic mice Ca, Fe and Mg present a significant decrease compared to male (25%, 23% and 29%, respectively) as well as a decrease in K level (10%). Although this muscle disease affects mainly males, the variation of the concentration of specific elements (mainly Ca, Fe, K and Mg) is accentuated in dystrophic female compared to the estimates obtained from males.

4. Conclusion

The EDXRF technique using the Fundamental Parameters method showed to be adequate and viable for the determination of Ca, Cl, Fe, K, Mg, Na and S in whole blood samples. Moreover, the XRF and NAA analysis showed agreement for all elements concentration investigated in mice (Dmd^{mdx}/J and C57BL/6J strains) offering a new contribution for veterinary medicine as well a detailed knowledge of Muscular Dystrophy (DMD), suggesting a different behavior for Ca, Fe, K and Mg based on gender.

These analytic techniques are complementary and showed their applicability for biochemistry tests in blood, requiring a small amount of samples, multi-elemental analysis, short time of analysis and simple sample preparation. Particularly for Fe determination, the EDXRF technique offers an efficient and fast evaluation for Fe blood analyses when the biological material is scarce.

References

Bushby, K., 2010. ⟨http://www.treat-nmd.eu/home.php⟩. (accessed 10.08.10).

Beckhoff, B., Kanngießer, B., Langhoof, N., Wedell, R., Wolff, H., 2006. Handbook of Practical X-ray Fluorescence Analysis. Springer, Berlin.

Berchtold, M.W., Brinkmeier, H., Muntener, M., 2000. Calcium ion in skeletal muscle: its crucial role for muscle function, plasticity, and disease. Physiol. Rev. 80 (3), 1215–1265.

Emery, A.E., 2002. The muscular dystrophies. The Lancet 359 (9307), 687–695.

Goyenvalle, A., Seto, J.T., Davies, K.E., Chamberlain, J., 2011. Therapeutic approaches to muscular dystrophy. J. Hum. Mol. Genet. 20 (1), R69–R78.

Han, R., Campbell, K.P., 2007. Dysferlin and muscle membrane repair. Curr. Opin. Cell Biol. 19, 409–416.

Matsuo, M., 1995. Duchenne muscular dystrophy. Southeast Asian J. Trop. Med. Public Health 26, 166–171.

Muntoni, F., Wells, D., 2007. Genetic treatments in muscular dystrophies. Curr. Opin. Neurol. 20, 590–594.

Metairon, S., Zamboni, C.B., Suzuki, Bueno, M.F., C.R., Sant'Anna, A.O., 2013. Elemental analysis of biological tissues of Dmd^{mdx}/J and C57BL/6J mice strains investigated by NAA. J. Radional. Nucl. Chem. 296 (1), 579–584.

Regence Medical Police Manual, 2015. ⟨http://blue.regence.com/trgmedpol/geneticTesting/gt69.pdf⟩. (accessed 26.05.15).

Redígolo, M.M., Aguiar, R.O., Zamboni, C.B., Sato, I.M., 2013. Determination of reference interval values for inorganic elements in whole blood samples of humans and laboratory animals by X-ray fluorescence spectrometry. J. Radional. Nucl. Chem. 297, 463–467.

Rousseau, R.M., 2001. Detection limit and estimate of uncertainty of analytical XRF results. Rigaku J. 18 (2), 33–47.

Worton, R.G., Molnar, M.J., Brais, B., Karpati, G., 2001. The muscular dystrophies, 8 ed. McGraw-Hill, New York, US, pp. 5493–5524.