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Determination of iron content in whole blood in different mouse strains using a portable XRFS spectrometer

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Abstract Iron has an important role in blood as an indicator of a great number of anomalies. Anemia due to iron-deficiency in the world is a public health problem in all ages and socioeconomic levels. Nowadays, Brazil's pharmaceutical companies are testing iron compounds to reduce the costs of those new drugs. In this study, Energy Dispersive X-Ray Fluorescence Technique was applied to determine Fe concentrations in blood samples of different mice strains using a Portable XRF Spectrometer. These data may help researchers choose the convenient mice strain that best meets its medical investigation, reducing costs and optimizing their researches.

Keywords Iron · Blood · Mouse strains · PXRFS

Introduction

Iron has an important role in blood as part of the hemoglobin molecule for oxygen transportation to all the body. A reduced daily intake of iron or excessive loss by hemorrhage or parasitism can be an indicator of a great number of anomalies [1]. Approximately 50 % of cases of anemia in the world are considered to be due to iron deficiency [2]. According to the last global estimative, anemia due to iron-deficiency is a public health problem in the world: it affects mainly children (~43 %), pregnant women (~38 %) and women of reproductive age (~29 %) [3]. If it occurs in early childhood and infancy, it impairs cognitive performance in language skills, motor ability and coordination [4]. In the last decade, according to National Health Surveillance Agency (ANVISA) several strategies have been adopted for preventing iron deficiency in the Brazilian population, such as: dietary improvement or modification, fortification and use of supplements [5, 6]. This action reduced the anemia rate however, it is still a public health problem in Brazil: the prevalence among children under five years and pregnant is in a range of 20.0–39.9 % [3]. Recently, attention is given to investigation of new drugs. Although several drugs (Fe-containing) are available in the Brazilian market (such as, ferrous sulfate, iron amino chelate and iron polymaltose) there is no scientific research proving superiority in terms of effectiveness for normalization of hemoglobin levels. Moreover, the price variations are up to 1500 % [7]. These data show the need to investigate new drugs for iron deficiency combining high efficiency and low cost.

Nowadays, Brazil's pharmaceutical companies are testing iron compounds to attend populations of low income. Drug development is complex and extensive (usually take years to be approved and marketed). It is a very expensive endeavor undertaken by scientists and pharmaceutical companies. Investment in new drugs requires several steps and it is mandatory to carry out various screening tests before starting the studies in human being. These tests are called pre-clinical and comprises the following fields of study: biopharmaceutical (formulation), in vitro pharmacological studies and in animals in vivo tests for the evaluation of potential clinical efficacy. While in vitro tests are used to identify the pharmacological properties (pharmacodynamics effects) of new drug, in vivo tests are

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performed in order to evaluate the safety of new drugs prior to any clinical trial with humans [8–10].

Considering the low cost, easy handling and simplicity related to the legal implications, the pre-clinical tests are usually performed in mice [9]. There are several mice strains developed for the research of new drugs. The most used strains are C57BL/6J, A/J and BALB/c. Some of them are inbreed for high and low antibody-producer (HIII and LIII), some for maximum and minimum inflammatory response AIR_{MAX} and AIR_{MIN}, some for autoimmune diseases (B10.RIII, NZW and NZB), others are mutants for muscular dystrophy (SJL/J and Dmd^{mdx}/J) [11, 12]. This study aims to determine the Fe concentration in whole blood of these distinct mice strains. The results will be correlated with human whole blood estimation for checking the similarities, a fundamental requirement to start the pre-clinical tests of news drugs [8–10]. The Energy Dispersive X-Ray Fluorescence Technique (EDXRF) was applied to determine Fe concentrations in whole blood samples of twelve mice strains. These measurements were performed using a portable X-Ray spectrometer (PXRFS). This procedure has been chosen because it requires a small amount of blood for Fe analyses [13–15] (ten times less comparatively to the conventional tests performed in serum [16]) an important condition when the biological material is scarce (the weight mice is about 20 mg and total body blood is $\sim$1.2 mL).

Materials and methods

The biological mice samples came from IPEN, Instituto Butantan and the Bioscience Institute, all research centers located at São Paulo city (SP, Brazil) where the mice were bred. The whole blood samples were collected from 90 male adult mice (4 month old, mass range from 20 to 25 g). Blood (at least 300 µL) was collected by the retro-orbital venous plexus and aliquots of 100 ± 0.5 % µL (in triplicate, when possible) were transferred to the filter paper (Whatman, no. 41). For the human whole blood collection, a description in a preview research [17] was made and its preparation followed the same procedure as the mice blood samples preparation (100 µL dropped in paper, in duplicate). The MINI-X spectrometer (Amptek XR-100SDD model) consisting of an Ag X-ray tube was used to perform the EDXRF measurements. The characteristic of the fluorescence intensity of Fe K_α (6.4 keV) line was measured with a Si Drift detector (25 mm^2 × 500 µm) with Be window (12.5 µm). Each whole blood sample (both, mice and human) was irradiated for 300 s using 30 kV and 5 µA excitation conditions and the analysis of the spectra was performed using the WinQxas Software. Standards (certified iron solution was prepared following the same procedure of the blood samples) were irradiated using the same excitation conditions. For each sample, three repetitions were made.

Considering the availability of biological material for the human, we also performed the blood investigation using INAA. For sample preparation, aliquots of 500 µL of human blood were transferred to a cylinder plastic bag [17]. The blood samples (prepared in duplicate) and reference material (IAEA-A13) were irradiated for 4hs in the IEA-R1 nuclear reactor (IPEN) and gamma counted for eight hs using HPGe detector (FWHM = 1.92 keV). Fe concentrations was determined using in-house software [18]. The Student *t* test was applied to compare results from both techniques (INAA and EDXRF).

Results

For the PXRFS calibration, standard solutions containing varying concentrations of Fe were prepared in the range of 10−500 µg mL^{-1}. The calibration curve (Fig. 1) was performed using the fluorescence intensity of Fe K_α line.

Elemental sensitivity (*S*) value for Fe and its detection limit (DL) were obtained as described in reference [19]. It was observed that the *S* value was 25.41 cps mg L^{-1} and the DL of the method for Fe in whole blood was 0.31 mg L^{-1}. The precision of the method was checked by calculating the standard deviation (for Fe concentration) of the ten samples. It was below of 4.4 % in triplicate analysis.

The X-ray spectrum of whole blood sample (C57BL/6J of Table 1) showed a characteristic peak of Fe as well as S, Cl, K, Ca peaks (Fig. 2). The Ar peak was due to its presence in air.

The results for Fe concentration in whole blood mice strains samples using EDXRF technique are shown in Table 1 and, they were expressed by mean value, standard

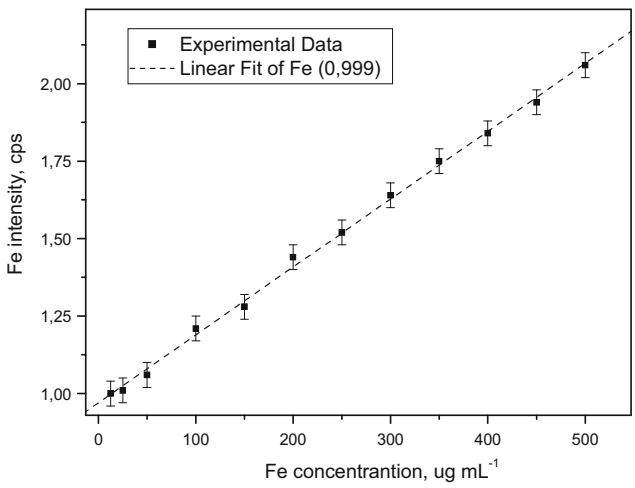

Fig. 1 Calibration curve for Fe using PXRFS

Table 1 Whole blood iron concentrations

Species (*n*)	Fe, mg L^{-1}			
	Mean value	±2 SD	Minimum	Maximum
C57BL/6J (12)	147	38	123	174
Dmd^{mdx}/J (8)	154	76	109	240
NZW (6)	244	16	234	253
L_{III} (6)	256	46	218	280
AIR_{MIN} (6)	262	66	222	303
B10.RIII (6)	274	40	247	295
H_{III} (10)	277	98	207	358
A/J (6)	329	10	323	335
BALB/c (6)	343	26	328	360
NZB (6)	357	64	325	388
AIR_{MAX} (6)	362	88	313	420
SJL/J (12)	375	78	322	416
Human (32) [range][a]				
XFR	320 [188–452]	66	224	419
NAA	347 [239–455]	54	238	430

n Number of samples

[a] Confidence interval of 95 % usually adopted for clinical practices

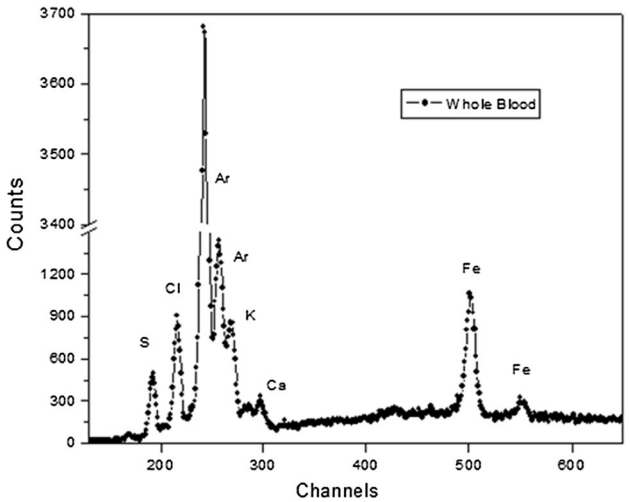

Fig. 2 Spectrum of whole blood sample (C57BL/6J) after 300 s of the excitation time

deviation (±2 SD), minimum and maximum values. In this table, the human whole blood Fe data, for both techniques (EDXRF and INAA), as well as the range for confidence interval of 95 % (usually adopted for clinical practices) were also included. To visualize, in Fig. 3 are shown the results of Fe concentrations in whole blood by EDXRF analysis of these species; the human being estimation and the normal range (±1 and ±2 SD) were also included for comparison.

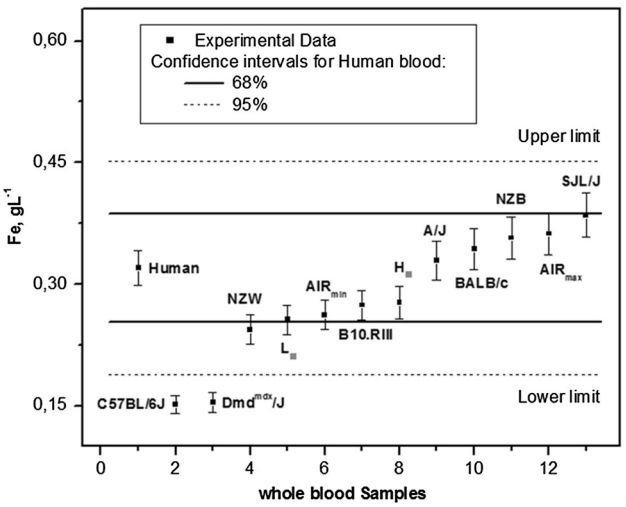

Fig. 3 Fe concentrations in whole blood samples of mice strains by EDXRF analysis. The human being estimation and their confidence intervals (±1 and ±2 SD) were included

The comparative study involving humans and mice strains (Table 1) indicate the necessity to establish a specific measurement in the mice strains to allow the correct choice for pre-clinical tests. In this table, according to the student *t* test, ten of the species investigated H_{III}, L_{III}, AIR_{MAX}, AIR_{MIN}, B10.RIII, BALB/c, NZW, A/J and NZB, as well as the mutant for muscular dystrophy, SJL/J strain, have results compatible with the iron in human blood estimation ($p > 0.05$). However, the Fe concentration result (147 ± 38 mg L^{-1}) for C57BL/6J mice strain (usually used for pre-clinical drug tests) shows that it is not in agreement with human Fe levels estimation, even considering the lower limit (188 mg L^{-1}) for a confidence interval of 95 %. The comparison of C57BL/6J mice strain

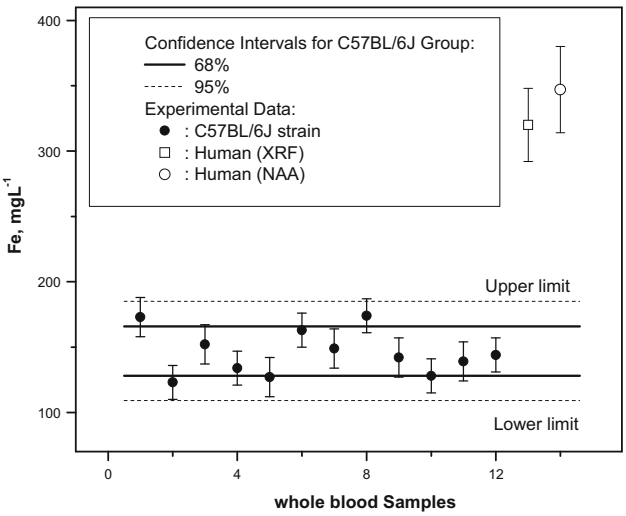

Fig. 4 Fe concentrations in whole blood samples of C57BL/6J mice strains by EDXRF analysis. The human being estimations were included for comparison

with human being estimation can be seen in Fig. 4. The results suggest that A/J (329 ± 10 mg L^{-1}) and BALB/c (343 ± 26 mg L^{-1}) strains are more suitable to be adopted. This recommendation is also extended for the dystrophic species (Dmd^{mdx}/J), specifically to perform drug tests that are used for treating muscle disorders [20]. The present results emphasize the choice of SJL/J (375 ± 78 mg L^{-1}) that may be more appropriate than Dmd^{mdx}/J strain (154 ± 76 mg L^{-1}).

Conclusions

The EDXRF analyses using PXRFS provides a fast and efficient way for the determination of Fe in blood, which emphases that this methodology is very promising for the clinical practice, especially when the availability of biological material is scarce. Related to the Fe concentrations in whole blood for mice strains investigated, the most used (C57BL/6 J) is not suited for performing pre-clinical tests when the similarity with human being is an essential condition to be adopted. The present results suggest that A/J and BALB/c (343 ± 26 mg L^{-1}) strains are more suitable. In addition, the possibility to select a similar strain as a reference, facilitates and reduces the cost in pre-clinical tests of new drugs.

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