Spectroscopy 21 (2007) 161–167
IOS Press

Hepatic iron quantification by atomic absorption spectrophotometry: Full validation of an analytical method using a fast sample preparation

A.C. Wortmann [a,*], P.E. Froehlich [b], R.B. Pinto [a], R.B. Magalhães [a], M.R. Álvares-da-Silva [a], J.J. Ferreira [b] and T.R. Silveira [a]

[a] *Centro de Pesquisas – Hospital de Clínicas de Porto Alegre, Programa de Pós-Graduaçao em Ciências Aplicadas à Gastroenterologia – Faculdade de Medicina da Universidade Federal do Rio Grande do Sul, Porto Alegre-RS, Brazil*

[b] *Laboratório de Produção de Padrões Secundários, Programa de Pós-Graduação em Ciências Farmacêuticas, Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul, Porto Alegre-RS, Brazil*

Abstract. Atomic absorption spectrophotometry is considered the method of choice for hepatic iron quantification. The objective of the present study was to perform full validation assays of hepatic iron quantification by atomic absorption spectrophotometry, using a fast sample preparation procedure, following the guidelines from the International Conference on Harmonization. The following parameters were evaluated: specificity, linearity/range, precision, accuracy, limit of detection and limit of quantification. A good linear correlation was found (0.9948) in the concentration range evaluated (20–120 ppb). The relative standard deviations were below 15% for accuracy, and below 10% for both day-to-day reproducibility and within-days precision, and the repeatability of injections was 0.65%. Limit of detection was 2 ppb, and limit of quantification was 6 ppb. Fresh bovine liver tissue was used to evaluate the procedure of collecting samples by liver biopsies. These findings indicate that hepatic iron quantification by atomic absorption spectrophotometry can be reliably performed at the established conditions, and suggest the method is suitable for further use in clinical practice. Hepatic iron quantification by AAS is validated by the experiments performed in the present study.

Keywords: Iron, liver, quantitative methods, atomic absorption spectrophotometry, validation

1. Introduction

The determination of the hepatic iron concentration (HIC) is important for the diagnosis and research of hemochromatosis and secondary iron overload states [1,2]. Hereditary hemochromatosis is one of the most prevalent inherited diseases in Caucasian populations, and secondary iron overload states may be associated with a variety of causes of chronic liver disease, like viral hepatitis, alcoholic liver disease, post-transfusional states and nonalcoholic fatty liver disease. Independent of the etiology, the extent of

*Corresponding author: Andre Castagna Wortmann, Rua Jose Mario Monaco 349/601, Bento Goncalves-RS, Brazil, CEP 95700-000. Tel.: +55 54 452 0071; Fax: +55 54 451 1605; E-mail: wortmannbento@terra.com.br

liver iron overload is associated with hepatocelular damage, fibrogenesis and progression to cirrhosis. Despite some recent advances in diagnostic strategies of these disorders, liver biopsy is still important for histological grading of liver disease and biochemical analysis to quantify the HIC [3–7].

The measurement of the HIC can be performed by biochemical or colorimetric methods, both described as technically reliable. Atomic absorption spectrophotometry (AAS) is considered the method of choice, and allows iron quantification using very small-sized samples [8]. It can either be done with flame burners or using a graphite furnace [9].

There are not many studies addressing the issue of validation procedures for iron measurement in liver tissue [9,10]. The first step to develop and incorporate the method to the routine clinical practice is to perform its validation, evaluating parameters such as those reported by the International Conference on Harmonization (ICH) and the Food and Drug Administration (FDA) [11,12]. These guidelines specify the parameters to be addressed, such as precision, accuracy, linearity and concentration range.

The present study is aimed to develop and fully validate a method for hepatic iron quantification by AAS, according to current guidelines.

2. Material and methods

2.1. Instrumentation

All iron analysis were performed on an automated Perkin-Elmer Analyst-300 atomic absorption spectrophotometer with deuterium-arc background correction, equipped with a Perkin-Elmer HGA-800 graphite furnace, an AS-72 auto sampler (Welleslay, MA, USA). AAWinlab software was used for data acquisition. The emission source was a single element (Fe) hollow cathode lamp, and pyrolitic graphite-coated tubes with L'vov platforms, all form Perkin-Elmer. The purge was argon. A sonicator and an oven were used for sample digestion. Fresh bovine liver samples were lyophilized (Micro Moduli 97, BOC Edwards, Wilmington, MA, USA) to constant weight before digestion.

2.2. Reagents and other materials

Analytical-grade reagents were used throughout the study. Nitric acid was reagent's grade (Merck, Germany) and distilled twice in a glass distiller. Standard iron solutions were prepared from a 1 g/l stock solution (Titrisol® – Merck, Germany). Ultra-purified water (Milli-Q Plus® – Millipore, USA) and Triton X-100 (Merck, Germany) were used. Glassware and plastic sample vials and pipette tips were decontaminated with 10% nitric acid solution (immersed for 4 h), rinsed thoroughly with water and let over a plastic tray in an oven at 60°C to dry. Surgical materials were also washed with nitric acid and water prior to sterilization, and further protected in decontaminated plastic bags until they were used. Bovine liver tissue used was a standard reference material (SRM) 1577b, from National Institute of Standards and Technology (NIST; Gaithesburg, MD, USA). Fresh bovine liver was acquired at a local store, and kept under cool temperatures until the performance of liver biopsies.

2.3. Methods

Validation procedures were performed as outlined in a recent paper from our group on zinc quantification [13], following current guidelines [11,12,14]. The following parameters were addressed: specificity,

linearity/range, precision (repeatability of injections, intra and inter-day), accuracy, limit of detection (LOD) and limit of quantification (LOQ).

First, assays were performed using a commercially available iron standard solution, with different concentrations in order to evaluate the linear concentration range of the method. For the construction of each calibration curve (concentration versus peak area of standard), five different iron concentrations were analysed in triplicate. Linearity of three calibration curves was tested by ANOVA and linear regression analysis.

The next procedures were carried out using the NIST bovine liver standard, to evaluate the precision, accuracy, concentration range and linearity. Samples were acid digested in closed eppendorf tubes with 1 ml of nitric acid in a sonicator for 30 min, then transferred to an oven at 60°C for 1 h and later submitted to dilution to yield the desired concentrations. Intra and inter-day precision were determined from quality control samples in three different concentrations. Accuracy of the method was evaluated comparing the results of the quality control samples obtained in the first day ($n = 6$) with the result stated in the NIST Certificate of Analysis, taking into account the sample sizes and the dilutions made. Precision and accuracy are expressed in terms of relative standard deviations (RSD).

Following validation with NIST standard, we analyzed samples of fresh bovine liver tissue, obtained percutaneously by tru-cut needle. These procedures intended to simulate those used for human sampling. The sample includes 29 specimens, since 1 fragment was lost after the biopsies were performed. Before digestion, the samples were lyophilized to constant weight.

2.4. Instrumental conditions

Analytical settings for iron quantification are:

- Iron hollow cathode lamp (Perkin Elmer)
- Wavelength (nm): 248.3
- Slit width (nm): 0.2
- Lamp current (mA): 15
- Background correction (deuterium lamp): on
- Volume injected (μl): 10.

3. Results

3.1. Linearity/range

Figure 1 shows a calibration curve of one day of the experiments. This allows to evaluate the method's linearity. Calibration curves were linear in the 20 to 120 ppb range.

Average coefficient of determination (R^2) of nine experiments (showed in Table 1) was 0.9948 (SD $\pm$ 0.0028).

3.2. Limit of detection/limit of quantification

The lower detection limit was 2 ppb, and the lower quantification limit was 6 ppb, both calculated based on the signal to noise ratio, according to the previously mentioned literature [11,12,14].

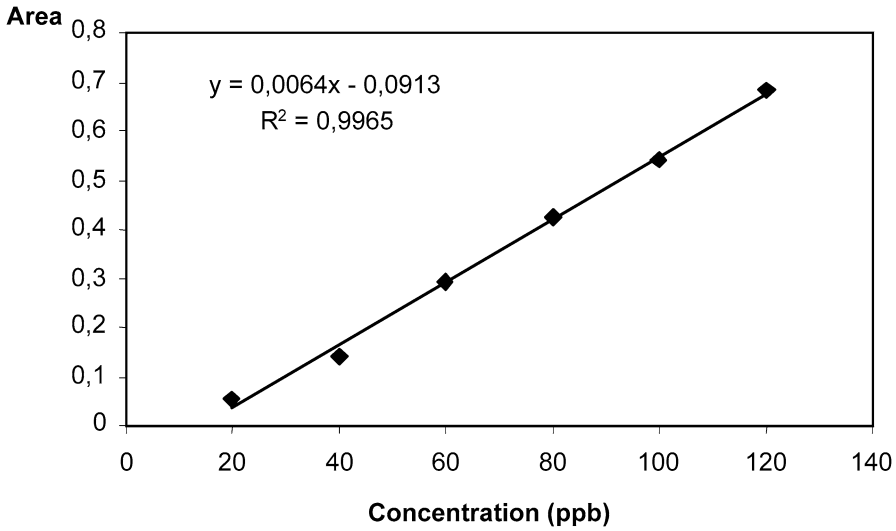

Fig. 1. Example of a calibration curve using a standard iron solution by Atomic Absorption Spectrophotometry (from day 1).

Table 1
Validation results from hepatic iron quantification by Atomic Absorption Spectrophotometry

Linearity (average of 9 calibration curves)		0,9948	
Detection limit		2 ppb	
Quantification limit		6 ppb	
Concentration range		20–120 ppb	
Precision (CV %)	50 ppb	75 ppb	110 ppb
Within day (3 curves plus 3 controls (1 from each concentration))	5.2	3.3	2.8
Day to day (two different days)	2.1	8.2	5.1
Repeatability of injections		0.65%	
Accuracy	85.7–107%	87.5–113.8%	91.1–114.4%

3.3. Precision

Reproducibility was investigated by measuring the iron content of the standard several times. Precision of iron measurements according to repeatability of injections, day-to-day precision and within-day precision were accessed respectively in 10, 6 and 2 consecutive sets of assays. Table 1 summarizes the results of the validation carried out using NIST standard. The relative standard deviations (RSD) obtained were within the acceptable reference values.

3.4. Accuracy

Accuracy values ranged within the 15% acceptable limit of variation (see Table 1).

Table 2
Characteristics of 29 consecutive liver biopsy specimens from bovine liver: weight (mg), absorbance (in UA) e HIC (ppm or μg/g)

Sample	Dry weight (mg)	CFH (ppm)*
1	3.88	37.58
2	2.89	43.32
3	3.45	45.39
4	4.04	44.48
5	3.74	44.17
6	5.39	41.30
7	4.23	49.60
8	4.52	49.89
9	4.36	53.37
10	4.25	55.04
11	4.92	43.96
13	4.18	53.73
14	4.77	44.42
15	4.81	43.04
16	4.41	45.01
17	4.34	49.12
18	3.77	52.49
19	5.09	48.23
20	4.50	52.64
21	4.33	49.56
22	4.17	50.53
23	4.81	41.83
24	4.99	42.53
25	4.02	45.80
26	3.79	45.30
27	3.73	46.73
28	4.68	46.97
29	4.99	44.89
30	4.39	47.52
Average	4.32	46.84
SD	0.53	4.21
RSD (%)	12.27	8.98

*μg/g dry weight.

3.5. Fresh bovine liver tissue

The results of the determination of HIC in bovine liver biopsy specimens are shown in Table 2. The average weight was 4.33 mg (SD 0.54; RSD 12.50) and the average HIC was 46.84 μg/g (dry weight) (SD 4.21; RSD 8.98).

4. Discussion

The present study followed the current international guidelines to develop and fully validate an analytical assay for iron quantification in hepatic tissue.

The steps involving the preparation of the sample for further iron measurement are of great importance for the purpose of iron quantification, since it concerns the degree of specificity of the method [11]. Potential sources of contamination were controlled in the experiments, as described in Section 2. Regarding the method under study, the AAS is a very specific method [15].

The present study employed a freeze-drying method to obtain dried hepatic tissue [16], as opposed by most of the studies that have performed oven-driven or similar processes [8,17–19]. Freeze-drying is described as much more efficient than the other available methods, therefore justifying the preference of the authors [16].

Most studies employing AAS for hepatic iron measurement did not have fully validated the method according to the current guidelines available [9,10], and another study have mentioned validation assays, though using different sample preparation [17]. Therefore, the relevance of performing such a study is highlighted by the fact it was designed to follow both strict validation parameters and the current requirements on description of analytical methodology studies [11,12,14].

Concerning bioethical issues, it is of great importance that this step of developing a new technique at our setting have been investigated first in a laboratory setting, using standard solutions, and bovine standard reference material and liver tissue. This allows future application of the validated method for human liver biopsy samples and further clinical perspectives.

In relation to the results of the validation of iron measurement by AAS in the present study, our findings are adequate compared to those reported in the literature [9,10]. We have observed good correlation on the method's linearity for the concentration range of 20 to 120 ppb. The relative standard deviations (RSD) were below 15% for accuracy, and below 10% for both day-to-day reproducibility and within-days precision; the repeatability of injections was 0.65%. The results observed from the determination of the HIC in the liver biopsy specimens show that this procedure is reproducible. These data confirm that hepatic iron quantification by AAS can be reliably performed at our setting.

In conclusion, AAS determination of liver iron content is fully validated by the present study.

Nonstandard abbreviations

Atomic absorption spectrophotometry, AAS; National Institute of Standards and Technology, NIST; Standard reference material, SRM; relative standard deviations, RSD; parts per billion, PPB; parts per million, PPM; SD, standard deviation.

Acknowledgements

The authors thank Dr. Marcelo Vasconcellos Vieira, who devoted his time to obtain the liver biopsy samples, Dr. Carlos Roberto Galia and Ms. Tielle Muller de Mello, from Banco de Tecidos do Hospital de Clínicas de Porto Alegre, for their support and technical assistance in the process of lyophilization of the samples, and also the students Marcela Heck and Adriana Steffens, from Faculdade de Farmácia da Universidade Federal do Rio Grande do Sul, who helped in the preliminary assays of iron quantification by AAS. Research support from CAPES/Ministério da Educação and Fundo de Incentivo à Pesquisa (FIPE)/Hospital de Clínicas de Porto Alegre (HCPA).

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