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An Assay for Determination of Hepatic Zinc by AAS – Comparison of Fresh and Deparaffinized Tissue

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1. Introduction

Atomic absorption spectroscopy (AAS) is a reliable method to determine metal concentrations. Zinc is a fundamental trace element because of its role in several essential biochemical functions. It is a component or co-factor of several enzymes, such as alcohol dehydrogenase and superoxide dismutase. It is of fundamental importance in cell division, genetic expression, and physiological processes, such as growth and development, immunity and wound healing; also, it plays a structural role in stabilizing biomembranes (Hambidge, 2000; Kruse-Jarres, 2001).

The importance of the determination of the hepatic concentration of certain metals is clearly established in the investigation of hereditary hemochromatosis and Wilson's disease (Pietrangelo, 2003, Roberts et al; 2003). As well, some interesting studies were published on the importance of zinc related to hepatic diseases. Decreases in plasma (Halifeoglu et al., 2004; Schneider et al., 2009; Pereira et al., 2011) or serum (Hamed et al., 2008, Matsuoka et al., 2009) zinc concentrations have been described in patients with chronic liver diseases. Authors that measured zinc in the liver parenchyma of adults and children with liver cirrhosis found low zinc levels (Milman et al., 1986; Göksu & Özsoylu, 1986; Sharda & Bhandari, 1986; Kollmeier et al., 1992; Adams et al., 1994). In patients with alcoholic cirrhosis, studies found abnormal zinc concentrations not only in the liver parenchyma (Rodriguez-Moreno et al., 1997), but also in subcellular fractions of the liver (Bode et al., 1988). In other diseases, such as biliary atresia (Bayliss et al., 1995; Sato et al., 2005), Indian childhood cirrhosis (Bhardwaj et al., 1980; Sharda & Bhandari, 1986), and chronic hepatitis B (Gür et al., 1998), also were found low liver zinc concentrations.

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Paraffin-embedded liver tissue usually stored in Pathology Laboratories may be used for analysis when fresh tissue is not available. Some hepatic diseases present a severe imbalance in the metabolism of metals, for example the excess of copper in Wilson's disease and iron in hemochromatosis. Recently, Wortmann and colleagues have validated an analytical method similar to ours for hepatic iron quantification, following the guidelines recommended by the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use (Wortmann et al., 2007). Due to the fact that zinc is a protective metal to human health, the assessment of zinc status has a great importance in clinical investigation. Since there is only a few data available in the literature about methods to determine zinc concentration in hepatic tissue, the assay proposed can be helpful to this analysis. Therefore, the purpose of this study was to compare zinc concentrations in fresh and deparaffinized tissues and to determine whether the concentration of this metal in liver specimens is influenced by tissue processing in paraffin blocks.

2. Material and methods

This study was conducted after the validation of the graphite furnace AAS method to determine zinc concentration in bovine liver tissue (Fröehlich et al., 2006), in accordance with the guidelines established by ICH, FDA and ANVISA (ICH, 2005; FDA, 2001; ANVISA, 2003). We used a standard zinc solution (1 mg/ml) and standard bovine liver material from the National Institute of Standards and Technology (NIST, SRM 1577b) with known zinc concentrations (127 ± 16µg/g dry weight).

A steel scalpel was used to obtain 29 wedge biopsies from the same bovine liver obtained from a local market. Each specimen measured about 4 x 2 cm, and 2 were excluded from each group due to contamination and loss of material. Each of the 27 remaining specimens was divided in half, and two groups of samples were formed.

This study was approved by the Ethics in Research Committee of the Research and Graduate Studies, Hospital de Clínicas de Porto Alegre, Porto Alegre, Brazil.

2.1 Group 1

The samples in group 1 were placed in eppendorf tubes previously decontaminated with 10% nitric acid (HNO_3). Samples were lyophilized, using a lyophilizer (Micro moduli 97, Edwards®) for 72 hours. After lyophilization, 500 µL of concentrated HNO_3 (twice distilled, Zn concentration < 0.5 µg/g) was added to each sample, and sonication was applied for 1 hour. The samples were then placed in an incubator (303, Biomatic®) at 60º C for 1 hour to complete digestion of organic matter. From each of the 27 solutions prepared, 50 µl were poured into automatic sampler vials, and 950 µl of pure water (Milli-Q Plus, Millipore®) was added. Concentrations were then determined with a graphite furnace (HGA 800, Perkin-Elmer®) AAS (AAnalyst-3000, Perkin-Elmer®) and an automatic sampler (AS-72).

2.2 Group 2

Samples in the second group were fixed in formalin, embedded in paraffin, and later deparaffinized. The material was kept in paraffin blocks for about 7 days. The samples were

deparaffinized by placing them in an incubator at 60º C for about 30 minutes to dissolve the paraffin block, and then in an average of two xylene baths (about 30 minutes each) alternating with 4 baths of alcohol (99%) and distilled water until the paraffin was totally removed. The samples were lyophilized for 72 h and concentrations were determined using AAS, following the same procedure described for samples in group 1. Mean dry weight of the 27 samples after lyophilization was 39.9 mg, and, after deparaffinization, 38 mg. Reagents were analyzed to assess contamination by zinc, which was negligible.

2.3 Statistical analysis

The software Microsoft Excel for Windows® and the Statistical Package for Social Sciences® 12.0 (SPSS) were used to create a database and to conduct statistical analysis. Measures of central tendency and dispersion were used to describe data, with means and standard deviations for quantitative variables. The Student t test for paired samples was used for comparisons between groups. The level of significance was established at $p<0.05$.

3. Results

3.1 Group 1 – Analysis of fresh liver tissue

Zinc concentration in group 1 was 173.6 ± 37.9 μg/g dry tissue (mean ± standard deviation). Table 1 shows the results for the 27 fresh bovine liver samples analyzed after lyophilization.

3.2 Group 2 – Liver tissue analyzed after deparaffinization

Zinc concentration in group 2 was 220.2 ± 127.0 μg/g dry tissue (mean ± standard deviation). Table 1 shows the results of 27 bovine liver samples that were embedded in paraffin, deparaffinized, lyophilized and then analyzed.

3.3 Comparison between analysis of fresh and deparaffinized samples

Liver zinc concentrations in fresh and deparaffinized samples were compared by Student t test for paired samples, and there were no statistically significant differences between these two groups ($p = 0.057$).

Samples	*Concentration fresh liver (μg/g dry tissue)*	*Concentration deparaffinized liver (μg/g dry tissue)*
1	213.3	246.6
2	178.5	148.0
3	169.5	163.3
4	241.2	153.8
5	154.3	201.8
6	154.0	201.4
7	179.4	171.6
8	169.2	193.7
9	170.8	185.1

Samples	*Concentration fresh liver (μg/g dry tissue)*	*Concentration deparaffinized liver (μg/g dry tissue)*
10	168.0	200.2
11	188.2	215.4
12	221.6	212.9
13	136.6	216.4
14	299.0	744.7
15	170.0	523.7
16	179.8	166.5
17	160.5	260.8
18	151.1	153.1
19	155.9	220.2
20	163.8	175.0
21	124.1	141.9
22	145.6	161.4
23	140.0	164.4
24	190.3	179.9
25	174.2	149.0
26	103.7	169.3
27	184.3	225.2
Mean	173.6	220.2
SD[a]	37.9	127.0
RSD[b]	21.9	57.5

[a] SD = standard deviation,[b] RSD = relative standard deviation (%).

Table 1. Liver zinc concentration in samples of fresh and deparaffinized bovine liver tissue.

4. Discussion

Zinc is an essential trace element to human health. It plays an important role in membrane stabilization and in cell protection against oxidative stress because it is part of structure of superoxide dismutase, the main enzyme in endogenous control of some types of free oxygen radicals. It also inhibits transition metals, such as copper and iron, from producing reactive types of oxygen (Powell, 2000). This metal is also essential for DNA and RNA polymerase, which has an important effect in hepatic regeneration (Sato et al., 2005).

The liver is one of the main organs in the metabolism of zinc. Disorders in zinc metabolism have been described in patients with chronic liver disease, and several studies found a decrease in plasma, serum or liver zinc concentrations (Loguercio et al., 2001; Halifeoglu et al., Schneider et al., 2009; Matsuoka et al., 2009, Milman et al., 1986; Göksu & Özsoylu, 1986; Sharda & Bhandari, 1986; Bode et al., 1988; Kollmeier et al., 1992). The decrease of zinc in liver disease seems to be associated with decreased intake, poor absorption associated with portal hypertension, and greater urinary excretion (Loguercio et al., 2001). Collagenase is a zinc-metalloenzyme and zinc is the most effective inhibitor for prolylhydroxylase, an

enzyme which plays a key role in collagen synthesis. These two assumptions could explain the role of zinc in collagen deposition and reabsorption in liver disease, the role played by zinc in liver fibrosis and in the evolution of chronic hepatitis toward cirrhosis (Faa et al., 2008).

Some reports found an increase in liver zinc concentrations in chronic liver disease. An increase in copper and zinc liver concentrations was found in Canadian children with chronic cholestasis (Phillips et al., 1996). Another case report described the increase in zinc concentration in hepatic tissue of a child with hepatosplenomegaly and symptoms of zinc deficiency, and the authors speculated about the existence of a zinc metabolism disorder (Sampson et al., 1997). A study that investigated the concentration of metals in liver tissue of adults with hereditary hemochromatosis found an increase in zinc in the liver parenchyma. The authors suggested that the concurrent increase in iron and zinc might be explained by the greater intestinal absorption of these metals (Adams et al., 1991).

The test usually conducted to determine body zinc is the measurement of plasma zinc concentration. However, plasma zinc concentrations do not seem to reflect the concentration found in the liver parenchyma (Göksu & Özsoylu, 1986; Sato et al., 2005). This may be explained by the fact that there are very efficient homeostatic mechanisms to correct plasma or serum zinc deficiencies, which makes it difficult to diagnose marginal deficiency by using this method. Therefore, the investigation of zinc concentration in liver tissue is important.

Studies report a great variation in liver zinc concentrations, maybe due to the different techniques used (Table 2). Kollmeier et al. (1992) studied the distribution of zinc in adult liver parenchyma from necropsy material, and found a small variation in intraorgan metal concentrations. They reported that zinc concentrations in the liver do not seem to be associated with sex or age (Kollmeier et al., 1992). Another study, conducted with children by Coni et al. (1996), confirmed these findings. They measured the concentration of metal in necropsy material from infants that died of sudden infant death syndrome and from pediatric control subjects, and found that a small liver sample is representative of liver concentration in the whole liver.

Author and year	Subjects and technique	Zinc concentration in liver tissues (μg/g dry tissue)
Adams et al. (1991)	Healthy controls (n=21) Flame AAS[a]	326.3 ± 65.4
Kollmeier et al. (1992)	Unselected necropsies (n=58) Flameless AAS[a]	280.0 ± 178.0
Bush et al. (1995)	Unselected necropsies (n=30) ICP-ES[b] AAS[a]	191.0 ± 56.3
Treble et al. (1998)	Unselected necropsies (n=73) Graphite furnace AAS[a]	118.3 ± 44.4
Hatano et al. (2000)	Healthy controls (n=21) Particle-induced X-ray emission	281.0 ± 25.5

[a] AAS = atomic absorption spectrophotometry.
[b] ICP-ES = inductively coupled plasma emission spectroscopy.

Table 2. Zinc concentration in hepatic tissue according to the literature.

Fresh tissue is not always available for chemical analysis, but formalin-fixed tissue often is. In our study, zinc concentration measurements in fresh liver tissue and deparaffinized tissue have been shown to be concordant. We have found in fresh e deparaffinized tissues respectively, 173,6 ± 37,9 μg/g dry weight and 220,2 ± 127 μg/g dry weight. Two deparaffinized tissue samples showed higher values of zinc than the others samples, causing a higher standard deviation. There was no evidence of mineral contamination during the embedding process to account for these divergent values.

We found only one study in the literature, conducted at the Mayo Clinic by Bush et al. (1995) that compared zinc concentration in fresh and deparaffinized liver tissue. Their study investigated the concentration of metals in several organs using material obtained from autopsy of 30 presumably healthy individuals. Zinc concentration found in fresh liver tissue was 191 ± 56.3 μg/g dry weight, and, in formalin-fixed tissue, 204 ± 63.2 μg/g dry weight. They concluded that formalin fixation long-term storage has little effect on zinc concentrations in tissue and that zinc was homogeneously distributed in liver.

Due to the clinical importance of zinc in liver diseases, the use of paraffin-embedded specimens for analysis is extremely useful when fresh tissue is not available. Stored material for analysis may be available even years after the biopsy or autopsy sample was obtained.

4.1 Conclusion

More than the results themselves, the proposed protocol for paraffinization/deparaffinization as well as for sample preparation for zinc determination by atomic spectroscopy in paraffinized samples were adequately established. According to the results of this study, paraffin embedding and deparaffinization do not significantly affect the determination of zinc concentrations in liver tissue, and, therefore, stored material can be used for analysis.

5. Glossary

AAS – Atomic Absorption Spectrophotometry.
ANVISA – Agência Nacional de Vigilância Sanitária (Sanitary Surveillance National Agency).
FDA – Food and Drug Administration.
ICH – International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

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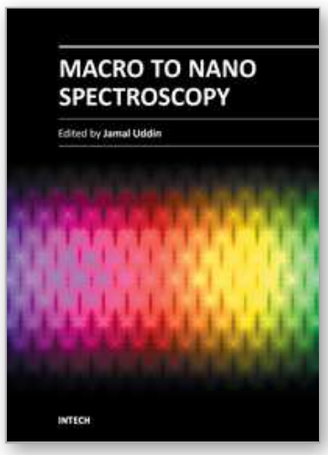

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