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Analytical methodology

Investigating the influence of standard staining procedures on the copper distribution and concentration in Wilson's disease liver samples by laser ablation-inductively coupled plasma-mass spectrometry

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ABSTRACT

The influence of rhodanine and haematoxylin and eosin (HE) staining on the copper distribution and concentration in liver needle biopsy samples originating from patients with Wilson's disease (WD), a rare autosomal recessive inherited disorder of the copper metabolism, is investigated. In contemporary diagnostic of WD, rhodanine staining is used for histopathology, since rhodanine and copper are forming a red to orange-red complex, which can be recognized in the liver tissue using a microscope. In this paper, a laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) method is applied for the analysis of eight different WD liver samples. Apart from a spatially resolved elemental detection as qualitative information, this LA-ICP-MS method offers also quantitative information by external calibration with matrix-matched gelatine standards. The sample set of this work included an unstained and a rhodanine stained section of each WD liver sample. While unstained sections of WD liver samples showed very distinct structures of the copper distribution with high copper concentrations, rhodanine stained sections revealed a blurred copper distribution with significant decreased concentrations in a range from 20 to more than 90%. This implies a copper removal from the liver tissue by complexation during the rhodanine staining. In contrast to this, a further HE stained sample of one WD liver sample did not show a significant decrease in the copper concentration and influence on the copper distribution in comparison to the unstained section. Therefore, HE staining can be combined with the analysis by means of LA-ICP-MS in two successive steps from one thin section of a biopsy specimen. This allows further information to be gained on the elemental distribution by LA-ICP-MS additional to results obtained by histological staining.

1. Introduction

Wilson's disease (WD) is a rare genetic disorder of the copper metabolism, leading to various hepatic, neurological, and psychiatric symptoms due to a copper accumulation in the liver and the central nervous system of the affected patients [1]. Because of manifold and unspecific symptoms, the diagnosis of WD is complicated and therefore, different guidelines with scoring systems and algorithms for the diagnosis are available [2–4]. These guidelines include measurements of biomarkers in urine and serum, examinations of liver biopsies concerning the histopathology and hepatic copper concentration, as well as genetic studies [2–4]. Due to the fact that there are various mutations of gene *ATP7B*, which is responsible for WD, genetic testing does not provide a universal diagnosis and thus, liver biopsies are still performed in many cases [3].

For histopathology of these liver biopsies, rhodanine, orcein, and Timms sulfur staining are applicable for the detection of copper in hepatocytes [3,5]. However, it is well known that the hepatic copper distribution exhibits large fluctuations in different stages of WD and, in addition to this, the lack of copper, identifiable by histological staining, does not exclude WD [3,6]. Moreover, staining methods have the disadvantages of a low sensitivity and the fact that they are poorly

Abbreviations: LA-ICP-MS, laser ablation-inductively coupled plasma-mass spectrometry; WD, Wilson's disease; HE, haematoxylin and eosin

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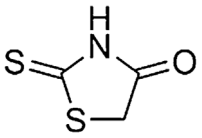

Fig. 1. Structural formula of the rhodanine molecule.

quantifiable [3]. Although, a combination of different staining methods for copper is recommended in the literature, staining with rhodanine (Fig. 1) using the protocol according to Lindquist is considered as method of choice for the histochemical detection of copper and copper associated proteins as red to orange-red complex in the liver tissue [7,8].

However, it cannot be excluded that sample preparation, such as staining, possibly shows an effect on the analyte distribution and concentration within the tissue. Nevertheless, an investigation of the influence of rhodanine staining on the copper distribution and concentration has not been described in the literature. For this purpose, laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) can be performed for the spatial resolved detection of copper in unstained and stained sections of liver samples. LA-ICP-MS for the analysis of biological samples was first applied by Wang et al. in 1994 and offers a high spatial resolution in a micrometer range down to 1 µm and a high sensitivity with limits of detection in the lower $\mu g \cdot kg^{-1}$ range [9–11]. Besides qualitative information, LA-ICP-MS also provides quantitative data using internal or external calibration [12]. Using these advantages, different applications of LA-ICP-MS for the analysis of liver samples are described in the literature [13–17].

In this paper, the influence of rhodanine and haematoxylin and eosin (HE) staining on the copper distribution and concentration is investigated by means of LA-ICP-MS. Therefore, a quantitative LA-ICP-MS method for the analysis of copper and iron in paraffin-embedded liver needle biopsy specimen is applied, which was developed by our group in previous work [13]. Copper and iron are quantified by external calibration with matrix-matched gelatine standards. Besides copper, iron is monitored since it is also elevated in WD [18,19]. The utilized LA-ICP-MS method offers a small spotsize of 10 µm, suitable for the typical small specimen size of a liver biopsy, and features limits of detection for copper and iron, appropriate for the analysis of human liver tissue. In total, the sample set of this study includes eight liver samples, which were collected from WD patients during a liver needle biopsy. Two parallel sections of each WD liver sample were analyzed, while one section was analyzed by LA-ICP-MS after deparaffinization and the other section after rhodanine staining. In addition to this, a further parallel section of one WD liver sample was analyzed after HE staining in order to evaluate the influence of this staining method on the copper distribution and concentration within the liver tissue.

2. Materials and methods

2.1. Chemicals

All chemicals were used in the highest quality available. Nitric acid (65%, Suprapur), multielemental standard IV (1000 $mg \cdot L^{-1}$), copper (II) sulfate pentahydrate, ethanol, and xylene were obtained from Merck KGaA (Darmstadt, Germany). Iron (III) sulfate was purchased from Sigma Aldrich (Steinheim, Germany). Rhodium (1000 $mg \cdot L^{-1}$) and gallium (1000 $mg \cdot L^{-1}$) ICP standard solutions were purchased from SCP Science (Baie D'Urfé, Canada). Gelatine was obtained from Grüssing GmbH (Filsum, Germany). All standard solutions were prepared with doubly distilled water generated by an Aquatron Water Still purification system model A4000D (Barloworld Scientific, Nemours Cedex, France).

2.2. Liver sample preparation and histological staining

WD liver samples were provided by the Institute of Pathology of the University Medical Center Freiburg and by the Institute of Pathology of the Technical University of Munich. The sample set consisted of eight WD liver samples, which were collected by a needle biopsy within medical investigations from different patients. Information concerning chelation therapy of the patients on this study is not available. All patients gave their written consent for usage of these samples in clinical studies.

For histopathology and analysis by means of LA-ICP-MS, all specimens had been fixed in neutral formalin, embedded in paraffin, and stained by the common routine methods. The histochemical rhodanine staining was performed with a commercially available kit (Morphisto 12315, Frankfurt a. M., Germany) according to the manufacturer's instructions. Briefly, tissue sections were deparaffinized and incubated 18 h in the detection solution at room temperature. After washing in demineralized water a staining with Hematoxylin (Roth T8653, Karlsruhe, Germany) for 4 min proceeded. For HE staining, Hematoxylin was incubated for 1 min followed by the differentiation afterwards in floating tap water for 5 min and pursued with Eosin (Sigma HT110232, Sigma-Aldrich, Taufkirchen, Germany) staining for 1 min.

In order to remove the cover slides of the stained samples for the analysis by LA-ICP-MS, the samples were incubated with xylene for 24 h. A further paraffin embedded and unstained parallel section of each WD liver sample was deparaffinized by washing with xylene, ethanol, and water and subsequently used for the analysis by LA-ICP-MS. Prior to the ablation procedure, microscopic images of all tissue sections were captured with a BZ-9000 inverted fluorescence/bright field microscope (Keyence, Osaka, Japan).

2.3. Preparation of matrix-matched calibration standards

Copper and iron within the WD liver samples were quantified by an external calibration with matrix-matched standards based on 10% gelatine in aqueous standard solution as described by our group in a previous work [13]. Thus, gelatine calibration standards for copper and iron were prepared in a concentration range from 10 to 5000 $\mu g \cdot g^{-1}$ and from 1 to 500 $\mu g \cdot g^{-1}$, respectively. According to the thickness of the sample, gelatine calibration standards were sectioned at 3 µm and 4 µm for unstained and stained liver samples, respectively, and ablated under the same conditions as the WD liver samples. For validation processes, the copper and iron concentrations of the gelatine standards were determined additionally by bulk analysis [13].

2.4. Instrumentation and experimental parameters

Laser ablation experiments were carried out using a commercial laser ablation system LSX-213 $G2^+$ (Teledyne CETAC Technologies, Omaha, USA) with a wavelength of 213 nm, which was equipped with a HeIEx Active 2-Volume Cell (Teledyne CETAC Technologies). For MS detection, a quadrupole-based ICP-MS iCAP Qc (Thermo Fisher Scientific, Bremen, Germany) was applied. Based on experience gained during previous work of our group, a spotsize of 10 µm at a scan rate of 20 $\mu m \cdot s^{-1}$ was used for a suitable spatial resolution due to the small specimen size of a liver biopsy. The further experimental parameters of the laser ablation and MS system were applied as described earlier [13]. For the analysis of the HE stained parallel section of WD liver sample 1 the following isotopes were monitored with a dwell time of 0.1 s each: ^{27}Al, ^{56}Fe, ^{63}Cu, ^{69}Ga, and ^{79}Br.

2.5. Data analysis for elemental bioimaging

Copper and iron concentrations within the WD liver samples were determined via linear regression using the external calibration with