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Microanalytical Determination of Trace Elements from Liver Biopsy Materials of Patients with Chronic Diffuse Liver Diseases with Different Ultrasound Attenuation

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1. Introduction

Liver biopsy remained the gold standard in the diagnostics of chronic diffuse liver diseases despite the effectivity of some recent noninvasive diagnostic tools for the detection of fatty liver (Amacher, 2011; Copel, 2003; Germani et al, 2011; Karamashi, 2008; Lewindon, 2011; Sporea, et al., 2008; Strauss, 2010; Talwalkar, 2002).This means, that liver biopsy is applied in the diagnostic procedure of almost every patient with suggested chronic diffuse liver disease. Beside establishment of the diagnosis, liver biopsy specimens can be used also for research projects, aiming to obtain knowledge from the pathophysiological background of these conditions.

Trace element contamination is growing with the great progress of industry. Consequently, the trace element load of living organisms is also increasing. Some elements can play a role in the formation of malignant tumors. (Boffetta, 1993; Hayes, 1997; Navarro Silvera, 2007; Sky-Peck, 1986; Wingren & Axelson, 1993). It is well known that the liver is involved in the metabolism of compounds containing also certain trace elements. Thus, determination of these elements in biopsy samples and searching for correlations between element content and some liver diseases seems to be promising.

Ultrasonography is widely used in the diagnostic procedure of patients with liver diseases. Chronic diffuse liver diseases produce the characteristic ultrasound image of bright liver (Lonardo et al., 1997; Joseph et al., 1979). On the basis of in vivo measurements of liver ultrasound attenuation (α), two major appearances of bright liver were differentiated, the low (DI type) and the high (DII type) attenuation types. It was proved previously that low (DI type) bright liver shows increase of connective tissue content, while high (DII) attenuation type bright liver is associated with fatty liver, correlating with subcutaneous fat thickness (SCF) and body mass index (BMI). (Szebeni et al., 1999; Szebeni et al., 2006).

2. Objectives

The main objective was to determine the trace element content of the liver from biopsy samples. It was also studied that the trace element content is similar or differing from each other in patients with normal ultrasound liver pattern as well as in patients with chronic diffuse liver diseases showing low attenuation (DI) type or high attenuation (DII) type bright liver. Another objective of the present study was the determination of possible contamination of biopsy samples by steel-metals during sample collection using a porcine liver model. Additional objective was the determination and comparison of intra-individual variability of element concentrations in a porcine liver as well as in a human liver obtained from cadaveric donor with liver steatosis. The choice of analytical methods was a key question. An appropriate method should be capable of measuring very low concentrations simultaneously and sample consumption should be restricted to the possible minimum. The suitability of histochemical staining methods and two microanalytical methods were studied. Inductively coupled plasma mass spectrometry (ICP-MS) has favourable detection limits and was selected for simultaneous determination of micro and trace elements (Labat, et al., 2003; Millos, et al., 2008). Total reflection X-ray fluorescence spectrometry (TXRF) is also a suitable and powerful technique for analysis of small-mass biopsy samples, because it requires small amount of substance (Marco, et al., 2004).

3. Material and methods

3.1 Material
183 patients (110 males, age 26-80; and 73 females, age 23-70) were examined because of the suggestion of chronic diffuse liver disease. After clinical (history, physical examination, BMI, abdominal circumference, waist/hip ratio), laboratory (liver function tests, total se cholesterol, low density and high density cholesterol, trigliceride, INR, platelet count, etc…) and ultrasound examinations, liver biopsy was performed for establishing the correct diagnosis. Semiquantitative histopathological analysis was also done in these patients. Biopsy materials were used retrospectively for research purposes, namely for analysis of the concentration of the following trace elements: Cr, Mn, Fe, Ni, Cu, Zn, Rb, Mo and Pb.

3.2 Methods
3.2.1 Ultrasound examinations
Ultrasound scanning of the liver – as part of a general abdominal ultrasound examination - and subcutaneous fat thickness determinations were made by a B-K Medical Hawk 2102 EXL scanner. For liver scanning 5 MHz curved transducer, for subcutaneous fat thickness measurements 12 MHz linear transcducer was used. Attenuation of the liver was measured with the aid of a homogeneous tissue equivalent reference phantom with known attenuation. After scanning of the phantom and the patient's liver with the same equipment setting, a special software was applied, capable to digitizing the image, as well as obtain and compare their brightness diagrams and evaluate attenuation of the patient's liver (Szebeni et al., 2006).

3.2.2 Percutaneous liver biopsy
Before the intervention, detailed information was given to the patient about the procedure and importance of percutaneous liver biopsy. Thereafter a statement of permission was subscribed by the patient. 30 minutes before the biopsy slight sedation was applied (0,07 mg/kg midazolam was given intramuscularly). 15 minutes before the intervention

0,5 mg atropin was injected subcutaneously. The biopsy was made in the left decubitus position using generally applied disposable Braun Hepafix needle. The site of the biopsy was determined by percussion between the anterior and median axillary line according to the hepatic dullness. 1% Lidocain injection was used for local anaesthesia. The puncture was made in deep outbreath in most cases blindly, but sometimes under ultrasonic guidance. The obtained biopsy specimen was fixed in 4% neutral formalin solution for histological examination. For trace element determination a small part of the material was deep-freezed and stored on -20° C till the analytical process.

3.2.3 Semiquantitative histopathologic evaluation

Histopathological studies have been performed in formalin-fixed, paraffin-embedded biopsy materials. In addition to the routine hematoxylin-eosin (H&E), periodic acid-Schiff (PAS) stains, the specimens were also stained by picrosirius red (Szendrői et al., 1984), a 1% alcoholic solution of rubeanic acid (dithiooxamide) counterstained with Nuclear Fast Red (Vacca, 1985). The amount and distribution of connective tissue was visualized by the picrosirius red stain. Fibrosis was diagnosed when the retained lobular architecture was surrounded by the collagen fibers, and it was semiquantitatively graded as mild, intermediate and severe. In cirrhotic livers the lobular architecture has been distorted. Rubeanic acid method is principally used for identification of copper in histological preparates, but other metals are also identifiable: while the copper granules are characteristically greenish-black, the nickel is bluish-violet, and the cobalt is yellowish-brown (Quicke,1979, Vacca, 1985).

The severity of the fatty change was semiquantitatively scored in H&E stained samples. Mild form was diagnosed when the lipid droplets occupied up to 25% of the liver, intermediate between 25-65%, and severe when they exceeded 65%. The necroinflammatory reaction was evaluated in at least 20 portal of intralobular areas, and graded as mild (1-5 portal tracts involved), intermediate (6-10 portal tracts) or severe (over 10 portal tracts affected).

3.2.4 Trace element analysis

3.2.4.1 Sample collection and preparation

For reference measurement twelve porcine liver portions (different size, in the range from 7 mg to 545 mg wet weight) were cut by a quartz blade, immediately weighed on a microbalance and freeze-dried. Time relationship of element release from biopsy needles was investigated applying 0.1, 1 and 24^h contact time. Disposable Braun-Hepafix liver biopsy needles were used for sampling porcine and human cadaver liver and the same sample preparation was applied. The sample preparation was performed in a clean bench and the porcine liver was stored at 4°C during the exposure intervals. Suprapure nitric acid (Merck, Darmstadt, Germany) and high-purity water from a Milli-Q system were used throughout the work. Polypropylene microvials (used for sample storage) were cleaned with 0.5 mol/l nitric acid for 1^h then rinsed with high-purity water and dried in a clean bench. The samples were digested in a laboratory microwave system according to the method described in our previous work (Varga et al., 2005). Distribution of the elements within the liver was investigated taking biopsy samples from different localizations of a cadaveric liver with steatosis with the same technique described before.

3.2.4.2 Instrumentation and technique

Total reflection X-ray fluorescence (TXRF) analysis was performed using an Atomika EXTRA IIA spectrometer equipped with line focused X-ray tubes and an energy dispersive Si(Li) detector. Mo Kα 17.4 keV, W continuum (Bremsstrahlung) 35 keV excitation and 1000 s data acquisition live time were applied. K lines were used for Cr, Mn, Fe, Ni, Cu, Zn, Rb and Mo, L line was used for Pb determination. 100 µl of sample solution was used to prepare specimens for the TXRF analysis in the following manner. 25 µl of sample solution was pipetted onto a previously siliconized quartz glass carrier and allowed to dry in a clean bench at 40°C. This procedure was repeated until 100 µl total volume of sample solution was reached. Finally, 10 µl yttrium chloride solution containing 0.1 mg/dm^3 yttrium was added as an internal standard for the quantification of TXRF measurements. Biopsy samples were analyzed by TXRF spectrometry after nitric acid digestion. Small volume microwave digestion was developed especially for biopsy samples and proved to be applicable for liver biopsies having sample size as small as only 1 mg or less. An efficient XRF method was developed for autopsy samples having sample mass of about 500 mg without digestion. Samples were analyzed by a benchtop XRF spectrometer -PANalytical MiniPal2 (Almelo, the Netherlands)- equipped with low power, Rh anode X-ray tube and a Si-PIN semiconductor detector (Fig. 1).

Fig. 1. Atomika Extra IIA TXRF Spectometer and PANalytical MiniPal2 benchtop XRF Spectrometer

TXRF analytical method was validated applying NIST 1577a Bovine Liver Certified Reference Material (Table 1.)

	Certified values (µg/g)		Measured values (µg/g)	SD	Recovery (%)
P	11100	± 400	11780	880	106.1
S	7800	± 100	7290	550	93.5
K	9260	± 70	11300	830	113.5
Mn	9.9	± 0.8	11.8	0.9	119.2
Fe	194	± 20	193	14	99.5
Pb	0.135	± 0.015	n.d.	-	-
Cu	158	± 7	156	11	98.5
Se	0.71	± 0.07	0.56	0.21	78.9
Zn	123	± 8	127	9	103.3
Rb	12.5	± 0.1	11.9	0.8	94.8
Sr	0.138	± 0.003	n.d.	-	-
Mo	3.5	± 0.5	3.7	0.4	105.7

(4 independent replicate, concentrations in µg/g corresponding to dry weight)
n.d. signifies concentration under the limit of detection (LOD)

Table 1. TXRF analysis results of NIST 1577a Bovine Liver CRM.

An inductively coupled plasma mass spectrometer (ICP-MS) is capable for analysis of 70-80 elements in multielemental mode, from 1-5 cm³ volume of a sample, moreover the detection limits of elements are in µg/kg-ng/kg (ppb-ppt) concentration range. Nowadays there is very important to analyze growingly smaller concentrations of elements. An ICP-MS has different physical and chemical interfering effects analyzing various samples. The smaller the concentration of an analyte and the larger the concentration of the matrix the larger the interfering effects (Kovács et al., 2006). From the spectroscopic analytical instruments generally the inductively coupled plasma mass spectrometer is capable of analyzing the smallest concentration of elements.

Fig. 2. The applied inductively coupled plasma mass spectrometer

As the human origin samples (e.g. human liver) contain small enough concentration of elements so an inductively coupled plasma mass spectrometer (Fig. 2.) was applied to analyze the various elements.

An X7 type (Thermo Elemental, Winsford, UK) inductively coupled plasma quadrupole mass spectrometer was used to detect the elements.

The instrument was operated with a Peltier cooled impact bead spray chamber, single piece quartz torch (1.5 mm i.d. injector) and a conventional glass concentric nebulizer.

The following isotopes were measured during the research work: ^{25}Mg, ^{52}Cr, ^{53}Cr, ^{55}Mn, ^{54}Fe, ^{56}Fe, ^{58}Ni, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{64}Zn, ^{66}Zn, ^{75}As, ^{78}Se, ^{80}Se, ^{82}Se, ^{111}Cd, ^{114}Cd and ^{208}Pb. Table 2. shows the applied operating instrumental parameters.

ICP-MS system:	X7 type (Thermo Elemental, Winsford, UK)
RF power:	1400 W
Nebulizer gas flow:	0.80 L/min
Auxiliary gas flow:	0.95 L/min
Cool gas flow:	15.0 L/min
Sample uptake rate:	0.88 mL/min
Interface:	Xi interface cones (Ni)
Data acquisition:	
Dwell time per isotope:	20 ms
Number of sweeps:	21
Number of replicates:	3
Sample uptake and wash time:	35 s
Calibration mode:	Peak jump
Number of integration:	3/sample 5/blank

Table 2. ICP-MS operating and data acquisition parameters

3.2.4.3 Intra-individual variability of element concentrations in human liver

A small liver biopsy specimen does not represent the liver as a whole. In order to be able to draw conclusions regarding differences of trace element contents of liver samples, we have to assume uniform trace element distributions throughout the whole organ. This assumption should be checked by comparison of samples taken from different lobes of the same liver. The latter study can be performed only on autopsy samples.

Intra-individual variability of elemental concentrations was investigated by the analysis of multiple human liver biopsy samples obtained from cadaveric donor with liver steatosis. The purpose of these investigations was the high hepatic Ni concentration observed in the human liver and its uneven distribution. Similar sampling was made on porcine liver for comparison. Concentrations of Fe, Ni, Cu, Zn, Rb, and Mo determined by total reflection X-ray fluorescence spectrometry are listed in Table 3. Cr, Mn, Co and Pb were measured only by inductively coupled plasma-atomic emission spectrometry. Element concentrations determined by both techniques were in good agreement and not presented in Table 3. as a repetition. The variability of element concentrations was between 8.7 and 17.6 % RSD. Exceptions were Pb, Ni and Cr having variability of 27.8, 73.0 and 68.6 % RSD, respectively. In case of porcine liver the intra-individual variability was less than 13.5 % RSD for each element. It can be also emphasized, that nickel distribution was quite even in porcine liver and average Ni concentration (0.16 µg/g dry weight) was two orders of magnitude lower compared to the value measured in the investigated human liver.

	Cr[b]	Mn[b]	Fe[a]	Co[b]	Ni[a]	Cu[a]	Zn[a]	Rb[a]	Mo[a]	Pb[b]
1	1.80	6.2	789	0.20	45.2	21.0	402	9.7	3.50	2.53
	±0.09	±0.4	±106	±0.03	±1.7	±1.5	±44	±0.7	±0.12	±0.14
2	1.15	7.3	910	0.17	26.3	15.3	401	9.3	3.66	1.54
	±0.16	±0.5	±81	±0.02	±1.4	±1.6	±32	±1.1	±0.17	±0.11
3	0.82	6.5	843	0.13	12.9	14.5	395	6.8	6.16	1.23
	±0.08	±0.5	±77	±0.02	±1.7	±1.2	±40	±1.0	±0.18	±0.12
4	2.28	6.8	865	0.23	20.8	17.2	397	9.4	2.98	1.81
	±0.15	±0.4	±99	±0.02	±1.1	±1.4	±36	±0.9	±0.21	±0.09
5	1.42	6.8	790	0.20	37.9	19.5	349	7.2	3.18	2.52
	±0.15	±0.6	±92	±0.01	±2.1	±1.4	±38	±0.6	±0.13	±0.10
6	3.66	7.7	980	0.15	34.7	19.5	425	8.9	3.23	1.51
	±0.18	±0.7	±68	±0.03	±1.5	±1.8	±28	±0.6	±0.14	±0.11
7	0.65	7.0	1205	0.13	15.5	18.0	523	10.9	3.00	1.22
	±0.10	±0.5	±88	±0.02	±0.9	±1.4	±44	±0.7	±0.18	±0.10
8	4.08	7.4	944	0.19	30.8	17.6	480	9.2	3.15	1.89
	±0.22	±0.5	±76	±0.02	±1.5	±1.5	±39	±0.8	±0.11	±0.12
9	0.76	5.6	842	0.19	99.1	15.0	384	9.9	2.73	2.27
	±0.11	±0.5	±53	±0.03	±2.4	±1.2	±30	±0.7	±0.16	±0.10
mean	1.85	6.8	906	0.18	35.6	17.5	417	9.0	3.18	1.84
RSD%	68.6	9.5	14.4	17.6	73.0	12.9	12.7	14.3	8.7	27.8

Table 3. Intra-individual variation of element concentrations in a human liver obtained from cadaveric donor. (concentrations in µg/g dry weight, a: measured by TXRF, b: measured by ICP-MS)

4. Results

4.1 Investigation of possible contamination from biopsy needles

Percutaneous human liver biopsies taken from living patients could not be repeated frequently; therefore considerable contamination was indirectly disproved. In the present study, the possible contamination of biopsy samples during sample collection was determined using a porcine liver model. Availability in large amount was the purpose of using porcine liver for the method development. Twelve portions from a porcine liver were freeze-dried. Portions of porcine liver were cut by a quartz blade and treated as same as the steel needle biopsy samples. Concentrations determined in samples taken by a quartz device represented the non-contaminated values and were used to determine reproducibility of measurement and intra-individual variations. The precision of the drying process, the sample preparation and the analytical measurements was tested by the analysis of porcine liver. Calculated mean dry/wet mass ratio was 0.347 with an acceptable low relative standard deviation (RSD) of 2.2 %. Freeze-dried samples were subjected to microwave digestion in concentrated nitric acid (Suprapure, Merck). The precision of the concentration determination was found to be better than 14.2 % RSD by TXRF and 13.9 %RSD by ICP-MS for Mn, Fe, Cu, Zn, Rb and Mo (Cr, Co and Ni in the porcine liver was below the detection limit of TXRF in the given conditions: LODs of TXRF measurement were 0.92, 0.89 and 0.85 µg /g d.w. for Cr, Co and Ni).

All materials, solutions and tools used during the sample collection and preparation were tested for possible contamination: Analysis of repeated procedural blank samples showed that no measurable contamination of steel-metals originates from labware or reagents. Biopsy needles and scalpels were digested and analyzed previously. The material of biopsy needles was found to be a standard stainless steel with a main composition of Cr:Fe:Ni / 18:72:9 and Mn content of 0.1 % w/w. These four metals would be expected as possible contaminants. To investigate the possible release of elements from the steel needle biopsies the samples were allowed to contact with the needle for different time in a refrigerator at 4°C. According to the present data no observable contamination could be determined after 6 minutes contact of the porcine liver tissue and the biopsy needle. After 1 hour contact the tissue concentration of Cr and Ni was significantly higher than those measured in the samples taken by a quartz blade (0.41 and 0.29 µg/g instead of 0.06 and 0.15 µg/g d.w., respectively). The change of Mn and Fe concentration was in the range of measurement uncertainty.
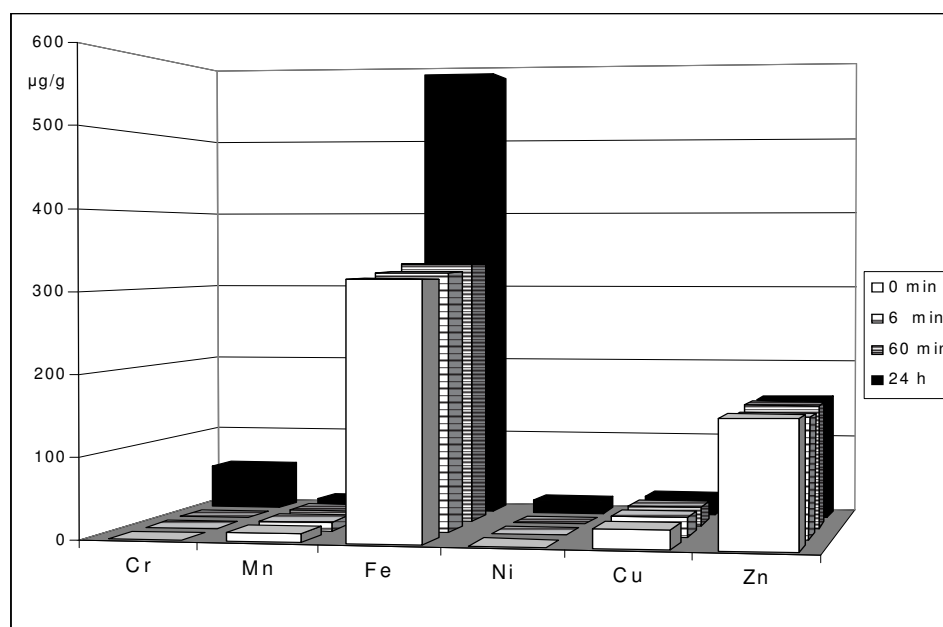
Fig. 3. Concentration of different elements in porcine liver samples after increasing contact time with biopsy needle.

The analysis of biopsy samples having 24ʰ contact with the needle showed considerable increase of chromium, manganese, iron and nickel concentration: the increments were 54.9 for Cr, 269 for Fe 15.6 for Ni and 2.1 for Mn in µg/g d.w. The results are demonstrated in Fig. 3. In contrast of steel metals, concentration of essential elements, such as copper and zinc remained constant as expected. Although the steel needles in the present study could not be substituted by polypropylene or Teflon utensils, it was demonstrated that the application of needle biopsy sampling in the reported analysis does not involve measurable contamination if contact time is kept to several minutes as usual in the clinical practice.

4.2 Intra-individual variability of element concentrations in human liver
A small specimen from liver biopsy does not represent the liver as a whole. In order to able to draw conclusions regarding differences of trace element contents of liver samples, we have to assume uniform trace element distributions throughout the whole organ. This

assumption should be checked by comparison of samples taken from different lobes of the same liver. The latter study can be performed only on autopsy samples.

Intra-individual variability of elemental concentrations was investigated by the analysis of multiple human liver biopsy samples obtained from cadaveric donor with liver steatosis. The purpose of these investigations was the high hepatic Ni concentration observed in the human liver and its uneven distribution. Similar sampling was made on porcine liver for comparison. Concentrations of Fe, Ni, Cu, Zn, Rb, and Mo determined by total reflection X-ray fluorescence spectrometry are listed in Table 3. Cr, Mn, Co and Pb were measured only by inductively coupled plasma-atomic emission spectrometry. Element concentrations determined by both techniques were in good agreement and not presented in Table 3. as a repetition. The variability of element concentrations was between 8.7 and 17.6 % relative standard deviation (RSD). Exceptions were Pb, Ni and Cr having variability of 27.8, 73.0 and 68.6 % RSD, respectively. In case of porcine liver the intra-individual variability was less than 13.5 % RSD for each element. It can be also emphasized, that nickel distribution was quite even in porcine liver and average Ni concentration (0.16 µg/g d.w.) was two orders of magnitude lower compared to the value measured in the investigated human liver.

4.3 Correlation between ultrasound data and trace element concentration

From the 183 examined patients 54 normal liver pattern was found (Fig. 4.), 53 showed low attenuation type (DI) (Fig. 5.) and 76 had high attenuation type (DII) bright liver (Fig. 6.). Average attenuation in the normal group was 0,64 ± 0,07 dB/cm/MHz, in the DI group 0,75 ± 0,12 dB/cm/MHz and in the DII group 1,34 ± 0,28 dB/cm/MHz. SCF values proved to be 6,8 ± 3,8 mm in the normal group, 8,4 ± 3,9 mm in the DI type, and 14,5 ± 5,5 mm in DII type bright liver.
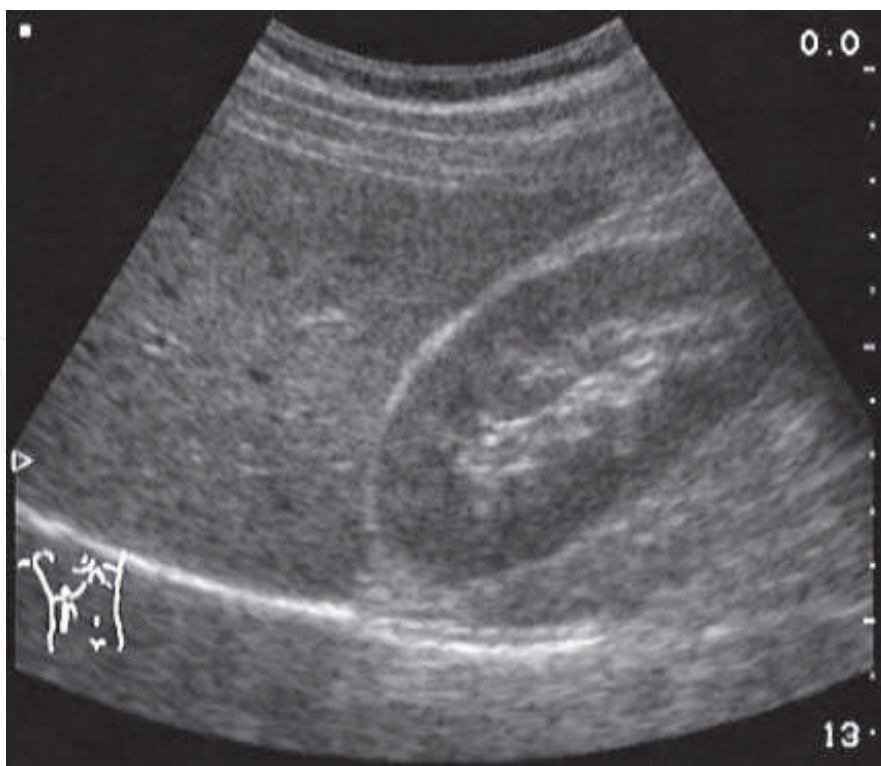

Fig. 4. Normal ultrasound liver pattern. Echogenicity and echodensity of the liver and the kidney are similar.
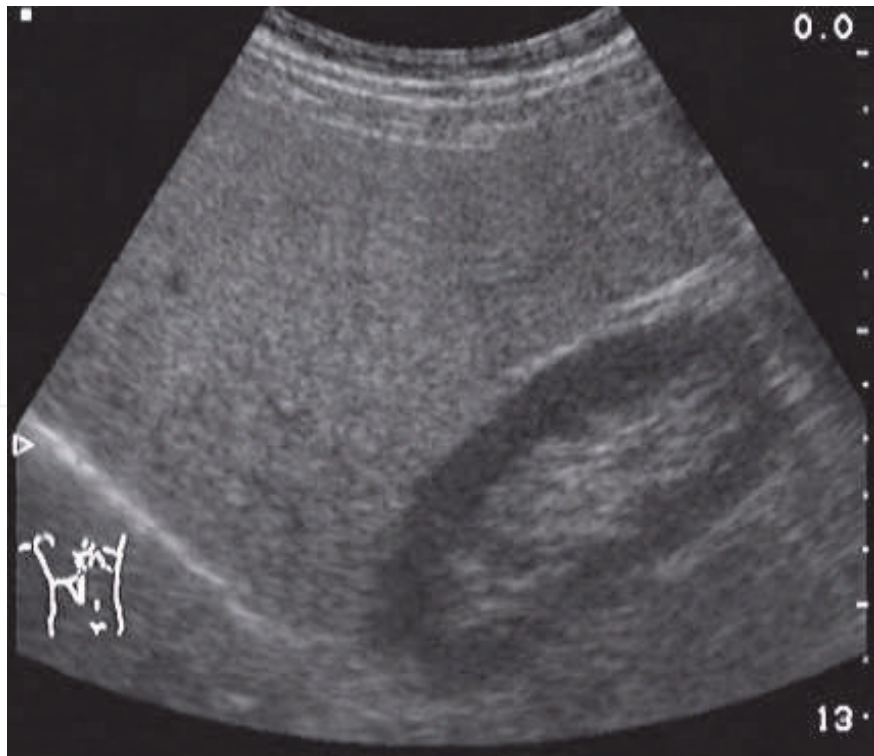
Fig. 5. Low attenuation (DI) type bright liver pattern. Echogenicity and echodensity of the liver and the kidney are different. The bright liver pattern is seen throughout the whole depth of the liver.
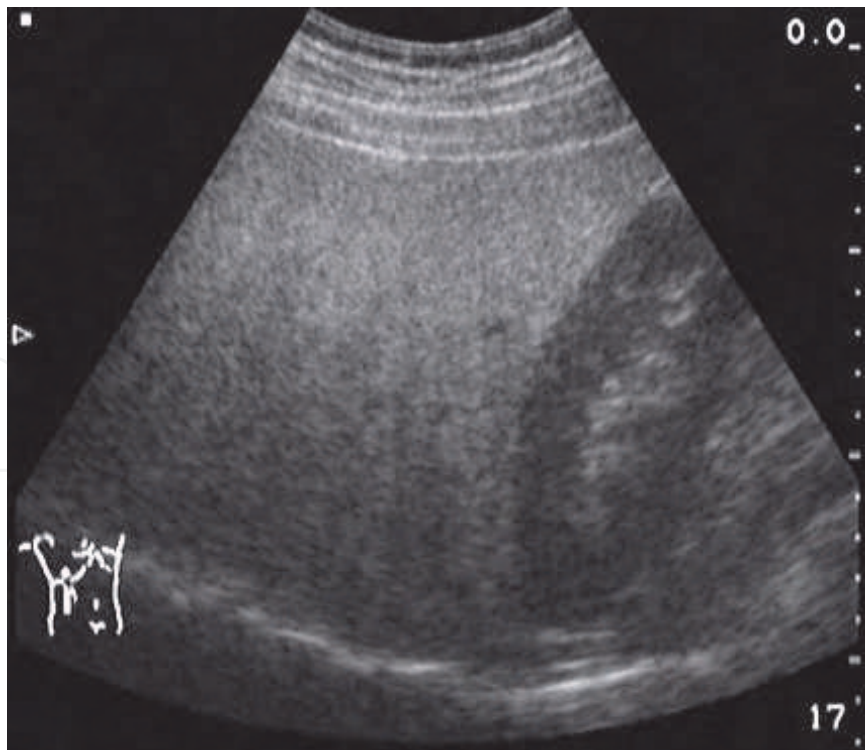

Fig. 6. High attenuation (DII) type bright liver pattern. Echogenicity and echodensity of the liver and the kidney are different. The bright liver pattern is seen mainly superficially, toward the depth of the liver gradually decreasing.

Mild fatty degeneration did not alter the ultrasonic reflectivity and echodensity, but the DII type bright liver pattern was always accompanied by severe fatty change (Fig. 7.). In hepatic samples from patients with normal ultrasound pattern, the amount of connective tissue was either normal, or just a slight fibrosis was seen (5/54 cases). In DI type bright liver pattern the typical histopathological alteration was the moderate or severe accumulation of connective tissue (Fig. 8.), accompanied by necroinflammation (Fig. 9.), but in DII type bright liver there typical morphological finding was just a slight portal fibrosis (37 samples).
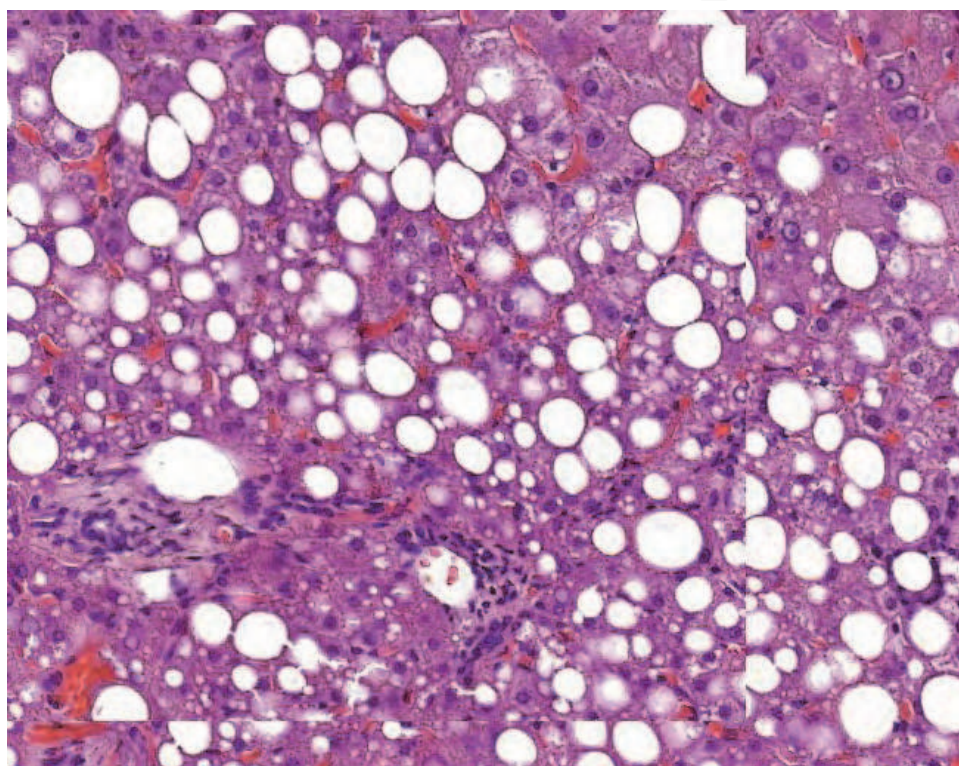
Fig. 7. In the liver multiple, large lipid vacuoles are seen representing a severe fatty degeneration (HE 400x).

Copper was demonstrated in 12, while nickel in 2 cases. None of the copper-positive patients suffered from Wilson disease. The amount of the metal granules was mild, and showed uneven distribution (Fig. 10.). In all but one specimens there were evidence of fatty degeneration, accumulation of connective tissue (septal fibrosis or cirrhosis), and inflammatory reaction. Presence of copper was observed in 1 case with normal liver. Histologically visible nickel was demonstrated just in 2 cases. One sample had a fatty change accompanied by infiltration of chronic inflammatory cells, the other specimen was taken from an alcoholic patients displaying fatty change and incomplete cirrhosis.
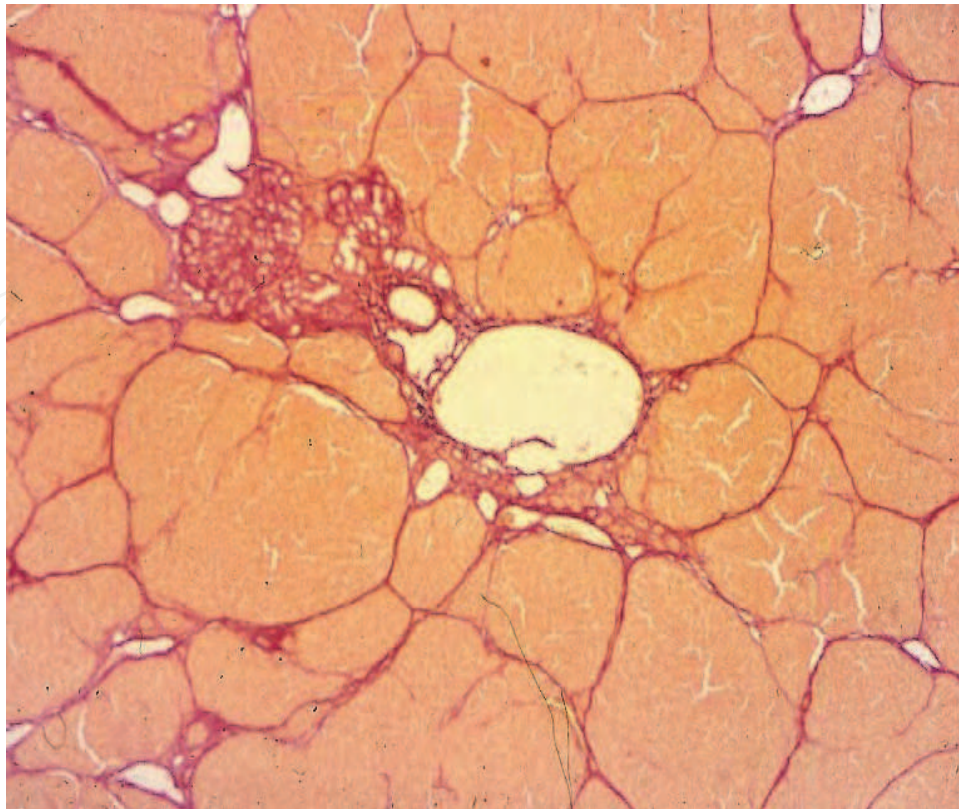
Fig. 8. The picrosirius red stain reveals a micronodular cirrhosis (200x).
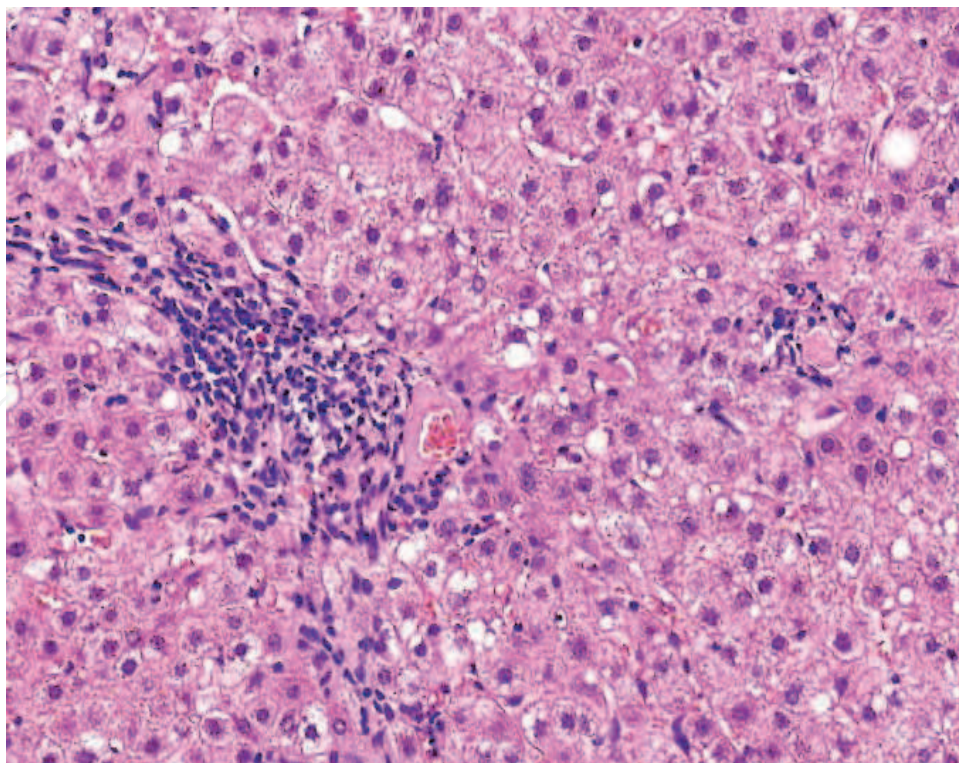

Fig. 9. The portal tract is loaded with moderate amount of chronic inflammatory cells. Moreover, focal, unicellular, intralobular necrosis is shown, surrounded by inflammatory cells (HE 300x).
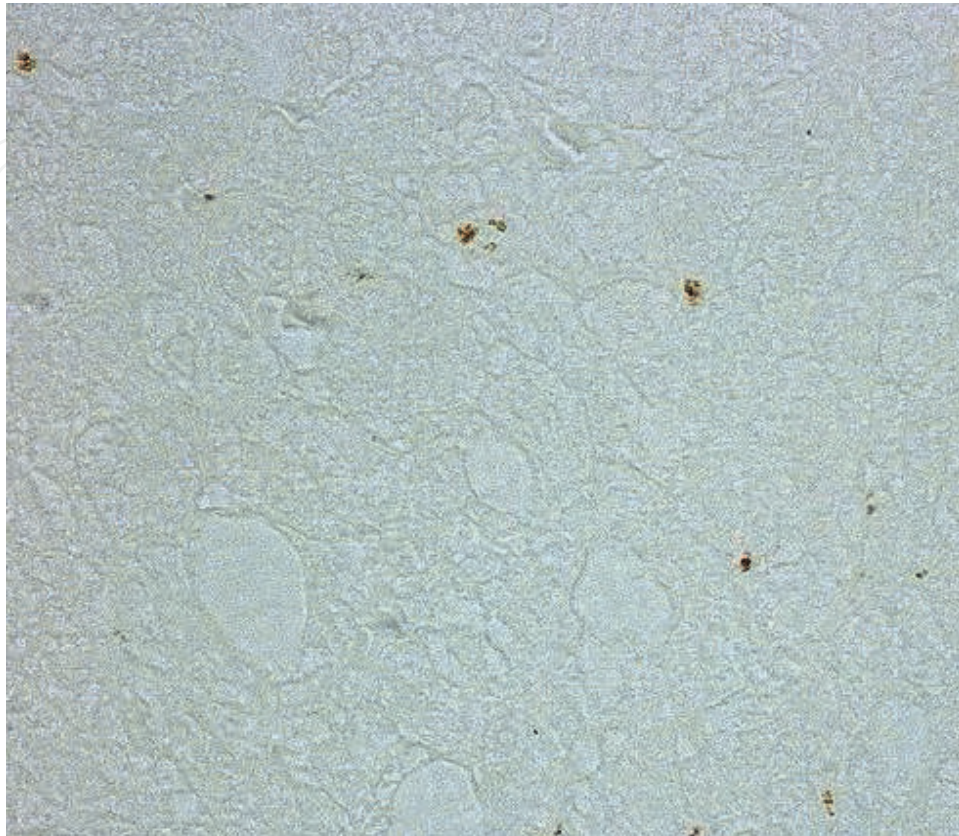
Fig. 10. Greenish-black copper-granules in the liver with fatty degeneration of intermediate degree. Rubeanic acid, 400x

Significant correlation was found between trace element determinations and the ultrasound and histopathological data in the case of Ni (Varga et al., 2005). This finding was confirmed in the present study with a larger sample size (Fig. 11.). In the normal and low attenuation (DI) type bright liver groups very low Ni concentrations were found, close to the detectability limit (Fig. 11.). On the contrary, in the high attenuation (DII) type bright liver group the Ni concentrations were substantially higher in all cases and some of them were extremely high, up to 700 µg/g (Fig. 11.). Decreased Fe concentration was also observed in the group of patients with steatosis. Distribution of elements within the liver was investigated taking biopsy samples from different localizations of a cadaveric liver. It is seen on table 3. in 3.2.4.2 , that the essential elements are uniformly distributed in the liver except the Cr. Ni showed surprisingly uneven distribution. The possible contamination with Ni can be excluded on the basis of our studies. Therefore the high Ni content of the liver can be taken as concomittant sign of fatty degeneration.
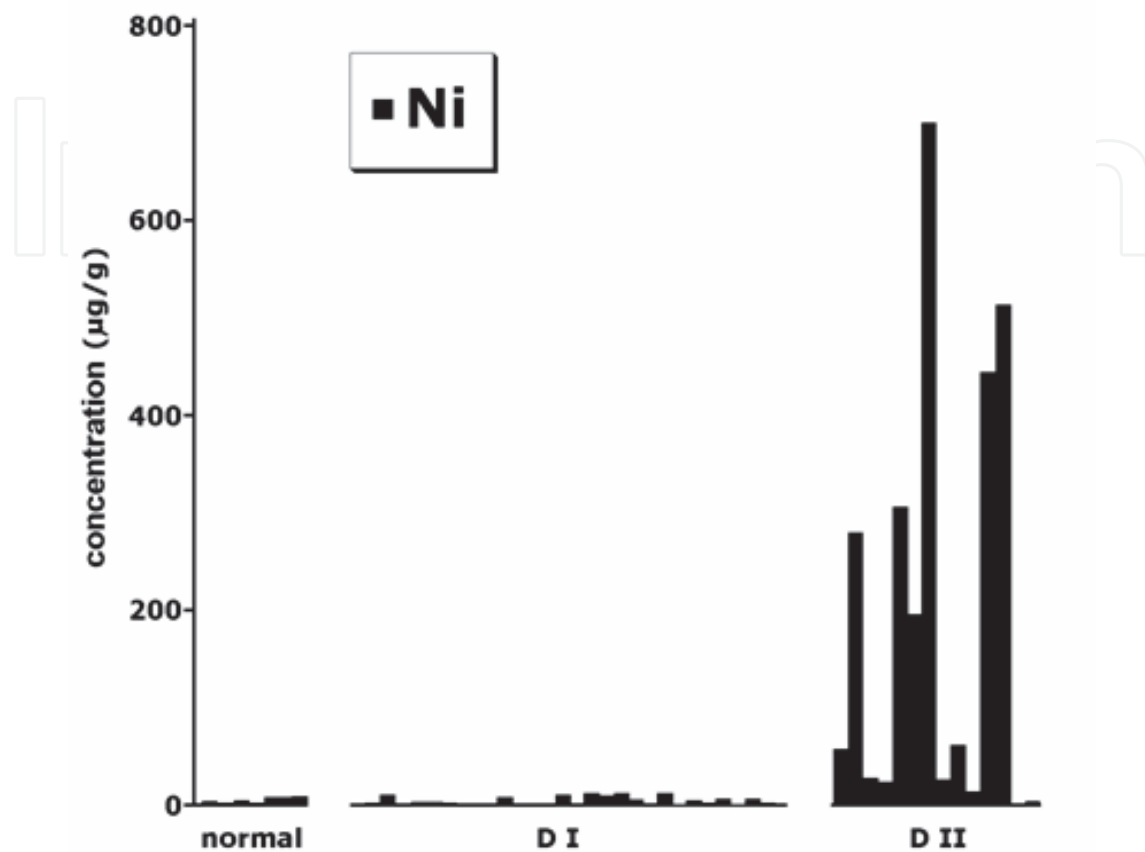
Fig. 11. Concentration of Ni in the three ultrasound based groups of patients. It is well seen, that the Ni content is much higher in the DII. type bright liver group, representing fatty liver, than in the normal, or DI type bright liver groups.

Three typical X-ray fluorescence spectra illustrate simultaneous analysis and distibution of maior, minor and trace elements obtained from liver biopsy samples. The only significant difference between the three ultrasonically determined groups was in the concentration of Ni and Fe as demonstrated on figures 12., 13. and 14. It is seen in figure 12., (normal ultrasonic pattern), that the Ni concentration is low, near to the quantification limit of the applied analytical method. On the 13. figure, originated from a liver biopsy sample of a patient with DI type bright liver the Ni concentration is in the normal range. On the contrary, on figure 14. the spectrum of a liver biopsy sample of a patient with fatty liver (ultrasonically DII type bright liver), high Ni concentration can be observed. Intense peak appearing at 2.7 keV energy is due to scattered radiation of the X-ray tube (Rh K-lines). As concerning the iron content, in the patient with liver steatosis (Fig. 14.), lower Fe concentration was found, than in the patients from the other groups (Figs. 12.,13.).
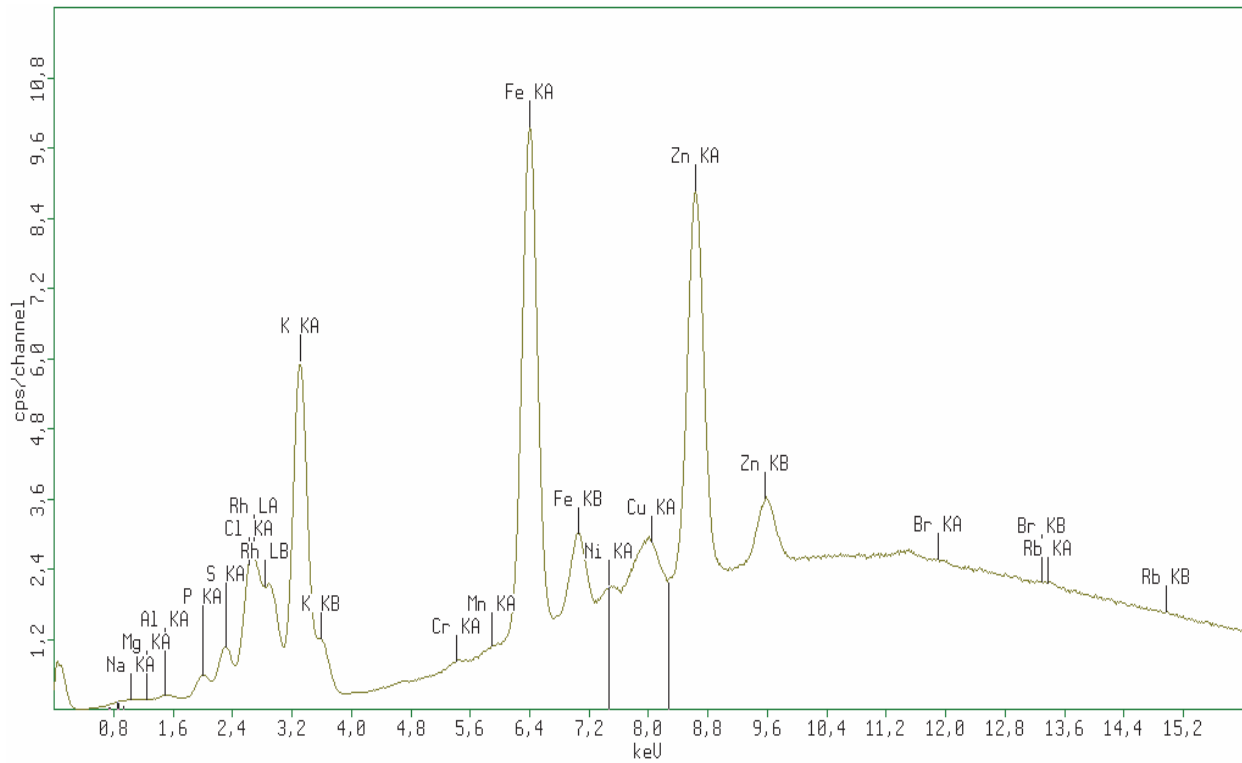
Fig. 12. TXRF spectrum of a liver biopsy sample of a patient from the normal group.
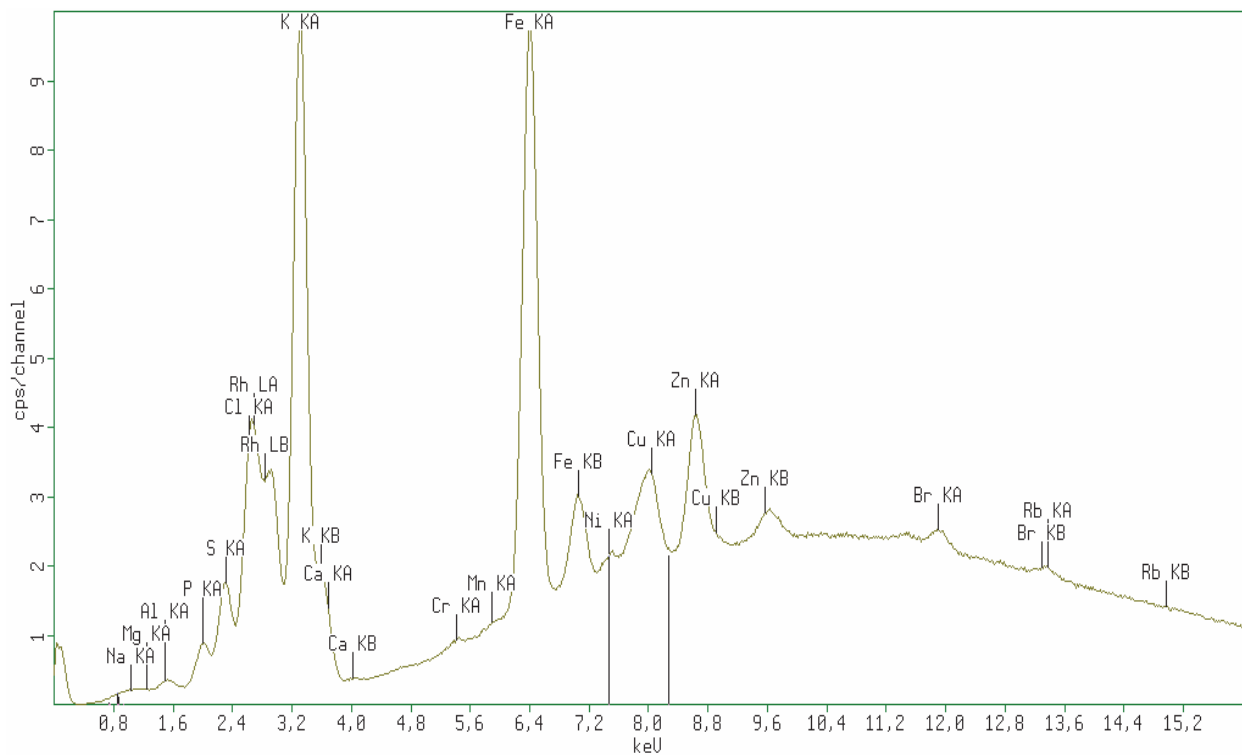

Fig. 13. TXRF spectrum of a liver biopsy sample of a patient from the DI type bright liver group.
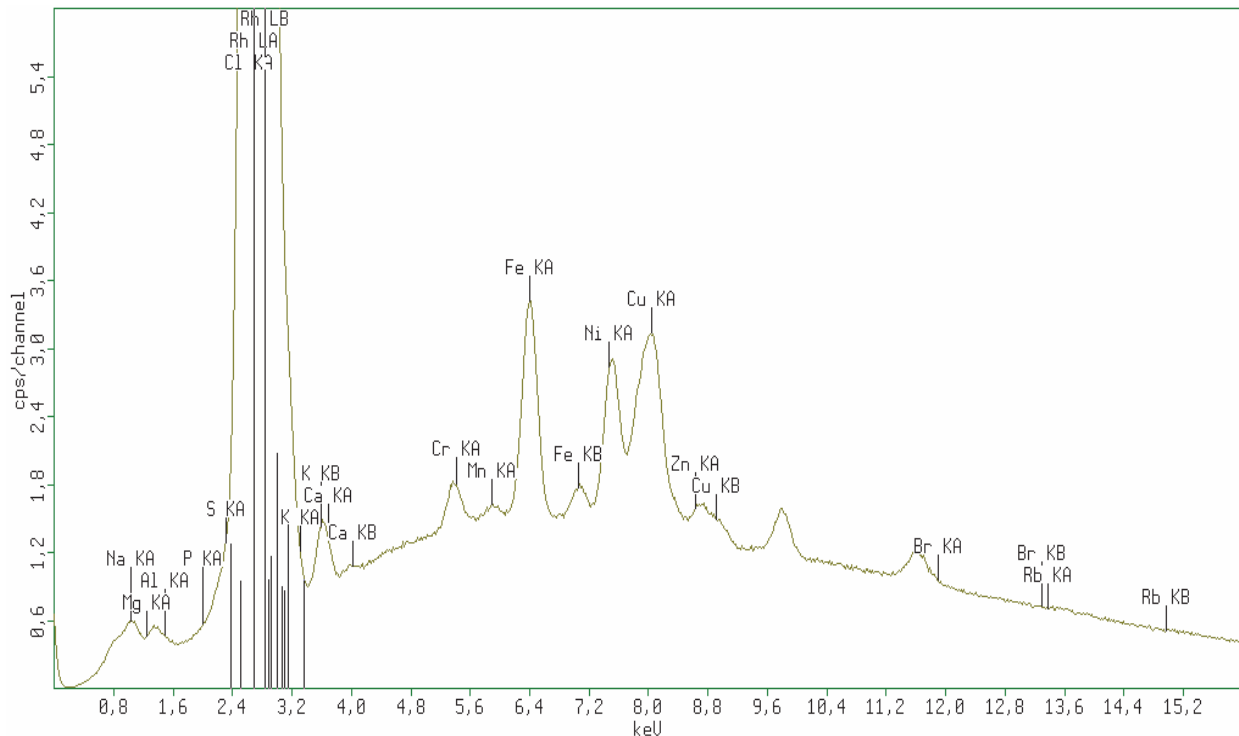
Fig. 14. TXRF spectrum of a liver biopsy sample of a patient from the DII type bright liver group (high Ni content).

5. Discussion

For the investigation of the pathological background of the different types of bright liver, quantitative measurement of the liver attenuation is necessary; a simple and reliable method was elaborated together with J.D. Satrapa (Satrapa, & Szebeni, 1996; Szebeni, & Satrapa, 1996). Evaluation of trace element analysis in subjects with normal ultrasound liver pattern and in patients with chronic diffuse liver diseases showing bright liver demonstrated, that the two types of ultrasound attenuation had different pathological background and also was not uniform from the point of view of trace element content. On the basis of attenuation measurements and semiquantitative histopathological analysis it was proved that low attenuation (DI) type bright livers show increase of connective tissue content while high attenuation (DII) type bright livers are associated with lipid deposition dominance (Szebeni, A. et al., 2006). It was also proved that some parameters, e.g. body mass index (BMI), subcutaneous fat thickness (SCF), could well be associated with the groups of liver ultrasound attenuation (Szebeni et al., 1999).

The different attenuation patterns reflect various histopathological alterations: fibrosis, fatty degeneration, chronic inflammation, alone, or in combination. DI pattern was mainly due to chronic, fibrotizing inflammation, while the severe fatty change was the major finding in DII cases.

Although trace metals are relatively frequently stored in the liver, the routine histological techniques rarely display their presence, therefore, if these metals do not reach the toxic levels, they may remain unidentified. Some technical notes also seem important, because it was found that reliable histological assessment for copper is only possible in formalin fixed

liver tissue (Hoffmann et al., 2009), and not after alcohol fixation. The rubeanic acid stain is a very useful method for demonstration of copper in Wilson disease and in various liver diseases with cholestasis, but in our positive cases both possibilities could have been excluded. It has long been known that accumulation of intrahepatic copper is most frequently demonstrable in alcoholic cirrhosis, but it is not related to the concomitant cholestasis or to the activity of the process (Berresford et al., 1980). The cirrhotic process by itself, however, was not a decisive feature in our material, because in 10, histologically proven incomplete or complete cirrhosis no rubeanic acid positivity was observed. It is interesting that in most of the copper-containing liver specimens a fatty degeneration was seen. The relationship between the two condition is not clear, but it was published that in cultured rat hepatocytes Cu induced lipid peroxidation, while similar effect was not seen after Ni administration (Furono et al., 1996).

Nickel was histochemically identified just in 2 cases. In both cases fatty degeneration was seen. In the literature very few data are available about the non-toxicological appearance of this metal. Rezuke et al. have determined nickel concentrations in various human tissues from 10 postmortem specimens by using electrothermal atomic absorption spectrophotometry with Zeemann background correction (Rezuke et al., 1987). In their reference work they have found a relatively higher Ni-concentration in thyroid and adrenal gland as compared with other organs, and it was suggested that biliary excretion may be a significant route for the elimination of nickel in humans. It was found that in vitro Ni uptake by rat hepatocytes was partly through the Ca channel transport processes (Funakoshi, 1997). Exposure of HepG2 human hepatoma cells to nickel(2+) ions resulted in a stimulation of Ser/Thr Akt and this activation is most likely independent of oxidative processes, since no oxidation of cellular glutathion was detected (Eckers et al., 2009). The mitochondrium also seems to be the place of its effects, because there are data that Ni is a potent competitive inhibitor of calcium transport in mitochondria (Bragadin et al., 1997, Ligeti et al., 1981). So the hepatic fatty change and the presence of copper and nickel in these samples are not necessary independent findings.

Nowadays a lot of analytical laboratory use an inductively coupled plasma optical emission spectrometer (ICP-OES) or an inductively coupled plasma mass spectrometer (ICP-MS) for multielemental analysis of various samples. Generally analyses of various essential elements and potential toxic elements with relatively low concentration are required, for which an ICP-MS is needed due to much smaller detection limits of the elements.

To elaborate analytical methods for analysis of the examined elements with an inductively coupled plasma mass spectrometer the most important interfering effects (problems) were evaluated before analysis: 1) isobaric elemental, 2) isobaric molecular and 3) physical interferences (Montaser, 1998; *www.epa.gov)*. EPA 6020A describes the interferences in ICP-MS techniques (www.epa.gov):

- Isobaric elemental interferences:
  These types of interferences in ICP-MS are caused by isotopes of different elements forming atomic ions with the same nominal mass-to-charge ratio (m/z). An evaluation software must be used to correct for these interferences, however these types of interferences are not easily corrected.
- Isobaric molecular and doubly-charged ion interferences:
  These interferences are caused by ions consisting of more than one atom or charge, respectively. Most isobaric interferences that could affect ICP-MS determinations have been identified in different literature.

- Physical interferences:
  These are associated with the sample nebulization and transport processes as well as with ion-transmission efficiencies. Nebulization and transport processes can be affected if a matrix component causes a change in surface tension or viscosity. Changes in matrix composition can cause significant signal suppression or enhancement (Beauchemin et al., 1987). Dissolved solids can deposit on the nebulizer tip of a pneumatic nebulizer and on the interface skimmers (reducing the orifice size and the instrument performance). One or more suitable internal standards can be used to correct for physical interferences, if it is carefully matched to the analyte so that the two elements are similarly affected by matrix changes.
- Memory interferences:
  Sample deposition on the sampler and skimmer cones, spray chamber design, and the type of nebulizer affect the extent of observed memory interferences. The rinse period between samples must be long enough to eliminate significant memory interference.

The isobaric elemental, isobaric molecular and doubly-charged ion interferences caused by various ions consisting of one or more than one atom or charge. These types of possibly emerging interferences are listed in Table 4., which are described in the Plasmalab software (2007) made by Thermo Fisher for analysis of different samples and build an appropriate analytical method. Before analysis these interferences were evaluated and were taken into consideration for decrease or eliminate these interfering effects.
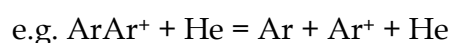
To eliminate the doubly-charged ion interferences, before analysis the tuning parameters were optimized considering the appropriate plasma temperature which ensures the minimum level of doubly-charged ions.

The interferences (Plasmalab, 2007) caused by various dimer, trimer or tetramer ions consisting of two, three or four elements were decreased by gas mixture using 7% hydrogen in helium (7% H_2 + 93% He) as collision cell technology (CCT) gas.
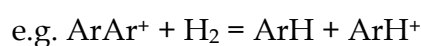
Collision/reaction cell technology has proved to be effective methods for decrease/eliminate the interferences caused by various dimer, trimer or tetramer ions and thus allow the determination of the major isotopes (^{52}Cr, ^{56}Fe and ^{80}Se). This method needs a collision/reaction cell, which is composed of a multipole (quadrupole, hexapole or octopole) before the quadrupole analyzer. A collision/reaction gas is introduced into the cell where, by a number of different ion-molecule collision and reaction mechanisms, most of poly-atomic interfering ions were converted to harmless non-interfering species. The analyte ions continue the way into the quadrupole analyzer for normal mass separation and detection. Owing to the collision/reaction cell technology the most abundant and thus most sensitive isotope can be used for the analysis of a given element. Different collision and reaction gases or gas mixtures have been selected to apply in an ICP-MS. Generally a mixture of H_2 and He gases (7-8% H_2 in He), NH_3 (1% NH_3 in He) and CH_4 gases are widely applied in ICP-MS. Moreover O_2, N_2O or other gases or mixtures can be used as collision/reaction gases also.

In the case of the analysis of the most abundant selenium isotope (^{80}Se) by ICP-MS the next possible mechanisms can be for reduction/elimination of polyatomic interferences.
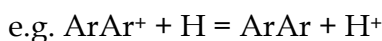
- collisional dissociation:

$$e.g.\ ArAr^+ + He = Ar + Ar^+ + He$$

- chemical reaction:

$$e.g.\ ArAr^+ + H_2 = ArH + ArH^+$$

- charge transfer:

$$e.g.\ ArAr^+ + H = ArAr + H^+$$

- collisional retardation/energy filtering:

$$e.g.\ ArAr^{+*} + He = ArAr^+ + He^*$$

Symbol	Abundance	Interferences
^{25}Mg	10.11	^{40}Ar+^{15}N, ^{16}O+^{39}K, ^{14}N+^{41}K, ^{1}H+^{54}Fe, ^{1}H+^{54}Cr, ^{15}N+^{40}Ca, ^{36}Ar+^{19}F, ^{12}C+^{43}Ca, ^{109}Ag^{++}, ^{110}Pd^{++}, ^{110}Cd^{++}
^{52}Cr	83.76	^{40}Ar+^{12}C, ^{36}Ar+^{16}O, ^{1}H+^{51}V, ^{12}C+^{40}Ca, ^{17}OH+^{35}Cl, ^{13}C+^{39}K, ^{16}O+^{36}S, ^{103}Rh^{++}, ^{104}Ru^{++}, ^{104}Pd^{++}
^{53}Cr	9.55	^{40}Ar+^{13}C, ^{17}OH+^{36}Ar, ^{14}N+^{39}K, ^{1}H+^{52}Cr, ^{16}O+^{37}Cl, ^{12}C+^{41}K, ^{13}C+^{40}Ca, ^{18}O+^{35}Cl, ^{17}OH+^{36}S, ^{106}Pd^{++}, ^{105}Pd^{++}, ^{106}Cd^{++}
^{55}Mn	100	^{40}Ar+^{15}N, ^{16}O+^{39}K, ^{14}N+^{41}K, ^{1}H+^{54}Fe, ^{1}H+^{54}Cr, ^{15}N+^{40}Ca, ^{36}Ar+^{19}F, ^{12}C+^{43}Ca, ^{109}Ag^{++}, ^{110}Pd^{++}, ^{110}Cd^{++}
^{54}Fe	5.9	^{54}Cr, ^{40}Ar+^{14}N, ^{14}N+^{40}Ca, ^{17}OH+^{37}Cl, ^{1}H+^{53}Cr, ^{12}C+^{42}Ca, ^{15}N+^{39}K, ^{19}OH+^{35}Cl, ^{107}Ag^{++}, ^{108}Pd^{++}, ^{108}Cd^{++}
^{56}Fe	91.52	^{40}Ar+^{16}O, ^{1}H+^{55}Mn, ^{16}O+^{40}Ca, ^{17}OH+^{39}K, ^{12}C+^{44}Ca, ^{14}N+^{42}Ca, ^{36}Ar+^{20}Ne, ^{112}Cd^{++}, ^{111}Cd^{++}, ^{112}Sn^{++}
^{58}Ni	67.76	^{58}Fe, ^{40}Ar+^{18}O, ^{12}C+^{46}Ti, ^{17}OH+^{41}K, ^{1}H+^{57}Fe, ^{14}N+^{44}Ca, ^{13}C+^{45}Sc, ^{16}O+^{42}Ca, ^{18}O+^{40}Ca, ^{19}OH+^{39}K, ^{115}In^{++}, ^{116}Sn^{++}, ^{116}Cd^{++}, ^{115}Sn^{++}
^{59}Co	100	^{19}OH+^{40}Ar, ^{14}N+^{45}Sc, ^{40}Ar+^{19}F, ^{1}H+^{58}Ni, ^{12}C+^{47}Ti, ^{17}OH+^{42}Ca, ^{36}Ar+^{23}Na, ^{1}H+^{58}Fe, ^{19}OH+^{40}Ca, ^{16}O+^{43}Ca, ^{118}Sn^{++}, ^{117}Sn^{++}
^{60}Ni	26.16	^{1}H+^{59}Co, ^{40}Ar+^{20}Ne, ^{12}C+^{48}Ti, ^{14}N+^{46}Ti, ^{16}O+^{44}Ca, ^{15}N+^{45}Sc, ^{36}Ar+^{24}Mg, ^{12}C+^{48}Ca, ^{17}OH+^{43}Ca, ^{120}Sn^{++}, ^{119}Sn^{++}
^{65}Cu	30.91	^{14}N+^{51}V, ^{17}OH+^{48}Ti, ^{1}H+^{64}Zn, ^{40}Ar+^{25}Mg, ^{12}C+^{53}Cr, ^{16}O+^{49}Ti, ^{1}H+^{64}Ni, ^{13}C+^{52}Cr, ^{17}OH+^{48}Ca, ^{130}Te^{++}, ^{129}Xe^{++}, ^{130}Xe^{++}, ^{130}Ba^{++}
^{64}Zn	48.89	^{64}Ni, ^{12}C+^{52}Cr, ^{40}Ar+^{24}Mg, ^{16}O+^{48}Ti, ^{1}H+^{63}Cu, ^{17}OH+^{47}Ti, ^{14}N+^{50}Ti, ^{14}N+^{50}Cr, ^{13}C+^{51}V, ^{36}Ar+^{28}Si, ^{14}N+^{50}V, ^{19}OH+^{45}Sc, ^{16}O+^{48}Ca, ^{127}I^{++}, ^{128}Te^{++}, ^{128}Xe^{++}
^{66}Zn	27.81	^{14}N+^{52}Cr, ^{1}H+^{65}Cu, ^{40}Ar+^{26}Mg, ^{12}C+^{54}Fe, ^{17}OH+^{49}Ti, ^{16}O+^{50}Ti, ^{16}O+^{50}Cr, ^{12}C+^{54}Cr, ^{15}N+^{51}V, ^{16}O+^{50}V, ^{18}O+^{48}Ti, ^{13}C+^{53}Cr, ^{132}Xe^{++}, ^{131}Xe^{++}
^{75}As	100	^{40}Ar+^{35}Cl, ^{16}O+^{59}Co, ^{12}C+^{63}Cu, ^{17}OH+^{58}Ni, ^{1}H+^{74}Ge, ^{14}N+^{61}Ni, ^{1}H+^{74}Se, ^{17}OH+^{58}Fe, ^{36}Ar+^{39}K, ^{19}OH+^{56}Fe, ^{149}Sm^{++}, ^{150}Sm^{++}, ^{150}Nd^{++}
^{78}Se	23.61	^{78}Kr, ^{14}N+^{64}Zn, ^{12}C+^{66}Zn, ^{1}H+^{77}Se, ^{16}O+^{62}Ni, ^{17}OH+^{61}Ni, ^{14}N+^{64}Ni, ^{13}C+^{65}Cu, ^{15}N+^{63}Cu, ^{19}OH+^{59}Co, ^{156}Gd^{++}, ^{155}Gd^{++}
^{80}Se	49.96	^{80}Kr, ^{40}Ar+^{40}Ar, ^{40}Ar+^{40}Ca, ^{17}OH+^{63}Cu, ^{1}H+^{79}Br, ^{16}O+^{64}Zn, ^{14}N+^{66}Zn, ^{12}C+^{68}Zn, ^{16}O+^{64}Ni, ^{15}N+^{65}Cu, ^{159}Tb^{++}, ^{160}Gd^{++}, ^{160}Dy^{++}
^{82}Se	8.84	^{82}Kr, ^{1}H+^{81}Br, ^{17}OH+^{65}Cu, ^{16}O+^{66}Zn, ^{12}C+^{70}Ge, ^{14}N+^{68}Zn, ^{13}C+^{69}Ga, ^{40}Ar+^{42}Ca, ^{12}C+^{70}Zn, ^{19}OH+^{63}Cu, ^{164}Dy^{++}, ^{163}Dy^{++}, ^{164}Er^{++}
^{111}Cd	12.86	^{12}C+^{99}Tc, ^{40}Ar+^{71}Ga, ^{17}OH+^{94}Zr, ^{16}O+^{95}Mo, ^{1}H+^{110}Pd, ^{12}C+^{99}Ru, ^{1}H+^{110}Cd, ^{14}N+^{97}Mo, ^{17}OH+^{94}Mo, ^{36}Ar+^{75}As, ^{13}C+^{98}Mo, ^{18}O+^{93}Nb
^{114}Cd	28.81	^{114}Sn, ^{40}Ar+^{74}Ge, ^{12}C+^{102}Ru, ^{16}O+^{98}Mo, ^{14}N+^{100}Ru, ^{1}H+^{113}Cd, ^{14}N+^{100}Mo, ^{17}OH+^{97}Mo, ^{1}H+^{113}In, ^{16}O+^{98}Ru, ^{40}Ar+^{74}Se, ^{12}C+^{102}Pd, ^{15}N+^{99}Tc, ^{13}C+^{101}Ru
^{208}Pb	52.38	^{16}O+^{192}Os, ^{17}OH+^{191}Ir, ^{14}N+^{194}Pt, ^{40}Ar+^{168}Er, ^{12}C+^{196}Pt, ^{1}H+^{207}Pb, ^{16}O+^{192}Pt, ^{13}C+^{195}Pt, ^{15}N+^{193}Ir, ^{12}C+^{196}Hg, ^{40}Ar+^{168}Yb

Table 4. The most important isobaric interference ions (Plasmalab, 2007)

Those inductively coupled plasma mass spectrometers which use CCT or reaction gases, have the best detection limits, for example the detection limit of selenium approximately 0.001 µg/l for [80]Se isotope. These best detection limits origin from using the collision and/or reaction gases to reduce the interfering effects on various element peak. CCT can eliminate approximately 99.99% of the [40]Ar[40]Ar^{+} and [39]Ar[39]Ar^{+} interferences on [80]Se and [78]Se, respectively.

On the basis of the above we can conclude that a quadrupole inductively coupled plasma mass spectrometer which instrument applying a collision/reaction cell technique is the most appropriate to analyze ultra trace elements in routine analysis of human origin samples, which can decrease effectively the interference of polyadducts.

Sample contamination during autopsy was nominated the major source of uncertainty of trace element analysis of biological samples. Biopsies taken by the use of stainless steel needles were recommended to analyze only for non-steel metals (Versieck et al, 1973; Versieck et al, 1982). For urine and blood recommendations were outlined to eliminate possible contamination during sample collection (Cornelis et al., 1992). Burguera et al. (Burguera et al, 2005) reported that Mn contamination during Atomic Absorption Spectrometry (AAS) analysis of urine samples was avoided by using new plastic and glassware containers soaked for at least 4[h] in 2 mol/l nitric acid. Christensen (Christensen, 1995) gave a detailed overview on contamination control in trace element analysis. The author concluded: Although it is impossible in most instances, the whole analytical procedure recommended to be carried out in clean room facilities. Blood samples were analyzed by Zeeman-ETAAS taken in a clean room. After puncture steel needle was withdrawn, venous blood was collected using a teflon catheter in 6 vials. Vial No. 5 was used for analysis in order to avoid contamination by steel-metals (Kristiansen et al., 1997). Oral mucosa biopsies were taken by CO_2-laser bistoury technique for minimizing the contamination during sample collection and 36 elements were determined by radiochemical neutron activation analysis (Foglio Bonda et al., 2001).

Multielement analysis of porcine liver and human cadaveric liver biopsy samples applying total reflection X-ray fluorescence spectrometry and inductively coupled plasma mass spectrometry are described with a special respect to Ni content. Porcine liver can be successfully applied to estimate the reproducibility of biopsy sampling and to investigate possible contamination by steel metals originating from the biopsy needles. Evidence of tissue contamination by steel-metals during hours of contact with the steel biopsy needle was shown. However, contamination was not measurable applying less than 6 min. contact as usual in the clinical practice.

The intra-individual variability of element concentrations was determined by the analysis of biopsies taken from a cadaveric human liver. Variability of Mn, Fe, Cu, Zn and Rb concentration was in the range of 9-18 %RSD. Pb, Ni and Cr found to be inhomogeneously distributed in the liver having variability of 28, 73 and 69 %RSD, respectively. It was beyond the scope of the present study to explore the causes of inhomogeneity. The result presented in this paper demonstrated that despite of its uneven distribution, Ni concentration in human liver biopsy samples seemed to be suitable for classification of patients.

Analizing trace elements by microanalitical methods in human liver biopsy samples, collected from 52 individuals in a previous work (Varga et al., 2005), a probable correlation between high nickel concentration and hepatic steatosis was found: accumulation of nickel was exclusively observed in cases of steatosis. Decreased Fe concentration was also observed in the group of patients with-steatosis. In the present study this finding could be

confirmed on a larger sample size, at 183 patients (Fig. 11.). Investigations of cadaver liver showed that Ni was the only element distributed unevenly within the cadaver liver. Nevertheless, absolute values of Ni concentrations were so much higher in every segment of fatty livers, than the very low levels in normal one and in other diffuse liver diseases, that despite of the inhomogeneous distribution this sign is considered characteristic to fatty liver. The cause of Ni enrichment is not known so far.

6. Conclusions

Histological staining methods were not enough sensitive for the demonstration of nickel in cases with chronic dissuse liver diseases.
TXRF and ICP-MS methods were successfully applied for element analysis of liver biopsy samples. The two methods gave essentially same results; yet, for certain elements ICP-MS was more sensitive.
It was proved that no contamination occurred at the sampling and preparation procedures.
It was demonstrated, that distribution of the examined trace elements – except Ni - was homogeneous, thus microquantities of liver material is informative.
In fatty degeneration of the liver, established by ultrasound and histology, the enrichment of Ni was found by TXRF and ICPMS methods, in some cases to extremely great extent. The Ni concentration was much higher than normal in every fatty liver cases, therefore, despite of the inhomogeneous distribution, this phenomenon can be interpreted as characteristic to fatty liver.

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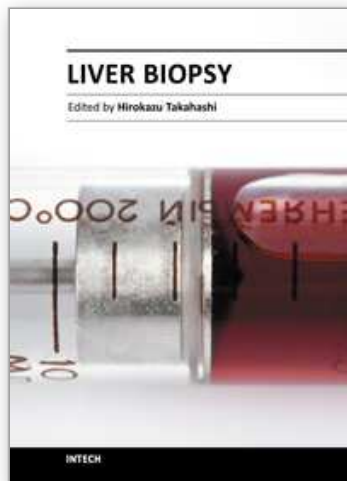
Liver Biopsy

Edited by Dr Hirokazu Takahashi

Liver biopsy is recommended as the gold standard method to determine diagnosis, fibrosis staging, prognosis and therapeutic indications in patients with chronic liver disease. However, liver biopsy is an invasive procedure with a risk of complications which can be serious. This book provides the management of the complications in liver biopsy. Additionally, this book provides also the references for the new technology of liver biopsy including the non-invasive elastography, imaging methods and blood panels which could be the alternatives to liver biopsy. The non-invasive methods, especially the elastography, which is the new procedure in hot topics, which were frequently reported in these years. In this book, the professionals of elastography show the mechanism, availability and how to use this technology in a clinical field of elastography. The comprehension of elastography could be a great help for better dealing and for understanding of liver biopsy.

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