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^{31}P High Resolution NMR Spectroscopy in Analysis of Phosphate-containing Compounds of Bile

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^{31}P high resolution nuclear magnetic resonance (NMR) spectroscopy was used to examine phospholipid metabolism and to analyze the phosphate-containing compounds in the bile in the transplanted liver recipients, the cholelithiasis patients' and the living donors' groups. Three signals of NMR spectrum of raw bile were determined: inorganic phosphate (P_i), lysophosphatidylcholine (LPtdC), and phosphatidylcholine (PtdC) in all investigated groups. Pi concentration was significantly higher in the recipients' group than in the living donors' group (Mann-Whitney test, $p < 0.05$). LPtdC and PtdC concentrations were significantly higher (Mann-Whitney test, $p < 0.05$) in the cholelithiasis patients' group in comparison to the recipients' group. Between the cholelithiasis patients' group and the living donors' group no significant differences in the three analysed compounds were found. The chemometric analysis for the ^{31}P NMR spectral data set provided good classifications between the living donors' and recipients' groups and the poor one among all groups. Results of our study suggest that ^{31}P NMR spectroscopy in vitro may be used for assessment of graft function, for the early signs of rejection and for the predisposition to gallstone formation.

K e y w o r d s: phospholipids, orthothopic liver transplantation, metabolomics

Abbreviations:
^{31}P NMR – Nuclear Magnetic Resonance of phosphorus nuclei
HPLC – High Performance Liquid Chromatography

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TLC – Thin Layer Chromatography
VIP – Variable Importance Plot
PLS – Partial Least Square
PLS-DA – Partial Least Square – Discriminant Analysis
MS – Mass Spectroscopy
UV – Ultraviolet
P_i – inorganic phosphate
LPtdC – lysophosphatidylcholine
PtdC – phosphatidylcholine;
OLTx – orthothopic liver transplantation
OD – outer diameter

1. Introduction

Phospholipids are widespread in nature. Well-known phospholipids are phosphatidylcholine and phosphatidylethanolamine. There are other phospholipids together with their degradation products, about 20 different phospholipids are known. The analysis of these compounds is difficult. The molecules have hydrophobic and hydrophilic parts. The resulting surface activity causes problems in the chromatography of phospholipids. Additionally, there are problems with UV detection. Some HPLC methods have been developed, but chromatographic resolution and dynamics of detection are not satisfactory. For each source of phospholipids special standards are needed due to the different distribution of the fatty acids. These standards are expensive and in some cases they are not available. Another problem occurs when analyzing phospholipids in complex matrices. In many cases their separation is not possible or is very difficult due to the surface activity, which is a positive property for many other applications but not for the analysis of these compounds. It is desirable to have a method which is selective in the detection of phospholipids in order to avoid the need to separate them from the matrix. ^{31}P NMR spectroscopy meets this requirement [1].

^{31}P spectroscopy differentiates between the various phospholipids and is able to quantify very small amount of phospholipids contained in the other more abundant components. Only phosphorus containing substances are detected. The analysis is not disturbed by non-phosphorus compounds [1].

The phosphorus resonance frequency depends on the chemical environment within the molecule. Phospholipids with different structure are therefore recorded at distinct frequencies. Each phospholipid is represented by a single signal. Separation of the various phospholipids is not necessary [1]. For qualitative analysis no specific standards are needed. Only one external or internal standard with a known concentration is used for all compounds analyzed by NMR.

The purpose of this study was to examine the function of liver using ^{31}P nuclear magnetic resonance (NMR) spectroscopy of bile specimens. The application of the high

resolution ^{31}P NMR spectroscopy for estimation of composition of the raw bile samples allowed to determine of their phosphate-containing compounds in the bile excreted after implantation of liver into recipients and in the bile samples from patients with biliary obstruction. This advanced technique gives a unique opportunity to measure the components of the bile in very small quantities and is a potential new tool in clinical practice for assessment of the graft in early and late postoperative period.

Bile is an organic fluid, secreted by the liver into the intestinal tract where it plays a key role for lipid digestion and absorption. Water is the main constituent of bile, representing more than 95% of its total mass. Bile can also be described as an excretory route for both endogenous and exogenous compounds. It is composed of phosphatidylcholine (PtdC), which constitutes more than 95% of biliary lipids, of phosphatidylethanolamine and of the traces of sphingomyelin [2]. PtdC is also referred to as lecithin. Phospholipids are important for the proper function of the liver and for and removal of cholesterol and prevention together with bile acids against gallstone formation [3]. The secretion of lipids into the bile is critical for the maintenance of cholesterol homeostasis, as well as for an efficient intestinal absorption of dietary lipids [4, 5]. When relatively low amounts of phospholipids are present, cholesterol crystal formation occurs in the supersaturated bile, which is the beginning of gallstone formation [6].

Metabolomic studies employing NMR, HPLC and MS methods have been used for a number of years to measure the extent of organ injury caused by storage and the rate of the post-transplantation organ recovery and to identify prognostic and diagnostic markers of the organ rejection and dysfunction. The small number of organ-specific markers for liver function suggests that metabolomics may have much to offer for the liver transplant community [7]. In our study for analyzing of NMR spectra of phosphate-containing compounds in bile metabolomics was used.

2. Material and Methods

2.1. NMR Spectroscopy

High resolution proton-decoupled ^{31}P NMR spectra were measured with a Varian Inova 400 spectrometer at ^{31}P frequency of 161.9 MHz. The NMR spectra were collected from raw bile and lipid extracts of each bile sample. The acquisition parameters were: 16k data points, relaxation delay 5s, spectral width 4k Hz, and 2k scans. The line broadening of 5 Hz was applied and the data were zero-filled prior to the Fourier transformation. The spectra were phase and baseline corrected for further analysis.

85% H_3PO_4 in H_2O solution was used as the ^{31}P external reference of the chemical shift (0 ppm) and for the quantitative analysis. Due to the severe reference signal overlapping for each sample two spectra were measured, one with and one without the external standard. In the former one the magnitude of the P_i signal was calibrated

against the external standard, while in the latter one the PtdC and LPtdC signals were calibrated against the magnitude of the P_i one. Concentrations of the compounds were expressed as arbitrary units/ml of the raw bile.

2.2. Bile Samples

The studied patients' group consisted of the cholelithiasis patients' group (n = 7), living donors' group (n = 3) and recipients' group (n = 13) – patients undergoing orthotopic liver transplantation. The mean age of the patients in the cholelithiasis patients' group was 46±10 (range 18–57), 1 male and 6 females, in the living donors' group it was 30±7 years (range 24–40 years), 1 male and 2 females, and in the recipients' group was 45±9 years (range 24–60 years), 7 males and 6 females. The study was approved by the institutional ethics committee. Written consent was obtained from each participant. The bile samples in the recipients' group were obtained from the bile duct at the end of the orthotopic liver transplantation just before completion of homeostasis and surgical closure of the wound. The bile samples from the living donors' group (n = 3) were obtained with the fine, flexible catheter introduced into biliary tree and before cholangiography, and from the cholelithiasis patients' group, contained the patients having biliary tract surgery, the samples were obtained during the revision of the bile ducts. The bile samples (1.5 ml) were kept frozen at –70ºC until analyzed.

Before the NMR analysis, the bile samples were centrifuged and supernatants were placed in 5mm OD NMR tubes. Extraction of lipids from the hepatic bile was performed according to Bligh and Dyer method [8].

2.3. Statistical Analysis

Three NMR signals of the raw bile were analyzed: P_i, LPtdC, PtdC [9, 10]. The statistical analysis was done for the NMR signal magnitudes. Nonparametric tests Kruskal-Wallis and Mann-Whitney U test were used for analysis. A p-value of less than 0.05 was considered to be indicative of statistical significance. The statistical analyses were performed using the Statistica software (StatSoft, Inc., 2005. STATISTICA version 7.1).

As the multivariate statistical analysis, PLS-DA analysis was performed. PLS-DA, partial least squares discriminant analysis, is derived from the partial least squares (PLS) method which is a general multiple regression method dealing with multiple collinear predictor and response variables. In this study, we have investigated the use of PLS-DA to identify the biochemical data which could contribute in the group separation. The goodness of fit was reported as the cumulative score across all the components' Q2cum. This was used to determine whether the model had any predictive power and predicted class membership better than chance. The theoretical maximum was 1 for a perfect prediction. Mean-centering was performed column-wise to remove the offsets. The most important compounds for classification were

identified using the variable importance plot (VIP) [11]. The model validation was carried out via permutation. Q2cum is a measurement of the predictive ability of the model, and R2cum is related to the goodness of fit of the model. The VIP criterion used for PLS-DA is more robust and discriminative [12].

3. Results

Concentrations of the phosphate-containing compounds in the human biliary secretions were quantified using the ^{31}P NMR spectroscopy. The spectra were collected from the raw bile and from the chloroform lipid extracts. Figure 1 A and B details the representative ^{31}P NMR spectra of raw bile and its chloroform lipid extract for a living donor's subject. Resonances of particular interest were P_i (2.55 ppm), LPtdC (–0.3 ppm) and PtdC (–0.87 ppm). These assignments were confirmed by measuring the standards obtained from SIGMA-ALDRICH (St. Louis, MO, USA). The signal assignments were against the external reference standard. In the ^{31}P NMR spectrum of the chloroform lipid extracts of bile only phospholipids signals were observed. Signals in this spectrum correlated very well with the phospholipids signals of the raw bile spectrum (Fig. 1).

Statistical analysis was carried out only for the raw bile and initially for all groups. Kruskal-Wallis test shows significant differences ($p < 0.05$) between the groups for P_i and LPtdC. Concentrations of the P_i in bile were significantly higher (2.95-fold) in the cholelithiasis patients' group and 5.8-fold in the recipients' group (Kruskal-Wallis test, $p < 0.05$) than in the living donors' group. The LPtdC concentrations were lower, 1.2-fold for the cholelithiasis patients' group and 2.8-fold for the recipient group than in the living donors' group. To find the differences between each group Mann-Whitney test was carried out. The LPtdC and PtdC concentration

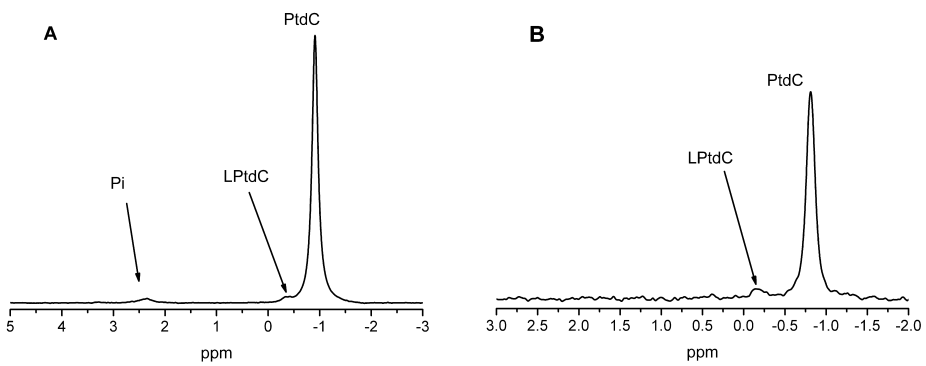

Fig. 1. ^{31}P NMR spectrum of raw bile obtained from living donors group specimen. A) Raw bile spectrum. Three signals were assigned as follows: PtdC at –0.87 ppm, LPtdC at –0.3 ppm and P_i at 2.55 ppm. All signals were assigned against the external standard (H_3PO_4) at 0 ppm. B) ^{31}P NMR spectrum of bile chloroform lipid extract from the same bile sample

were significant, 2.4-fold and 3-fold higher (Mann-Whitney test, $p < 0.05$) in the cholelithiasis patients' group in comparison with the recipients' group, respectively. The P_i concentration was significantly 5.8-fold higher for the recipients' group than in the living donors' group (Mann-Whitney test, $p < 0.05$) (Fig. 2).

Multivariate analysis PLS-DA for two and three groups was performed. At first two groups were analyzed: the living donors' group and the recipients' group. The best model in the PLS-DA analysis has two components (Fig. 3). The Q2cum value for this model was 0.677, and R2cum was 0.822. This model was valid for both groups. That means that the model good fitted the data and predicted them very good.

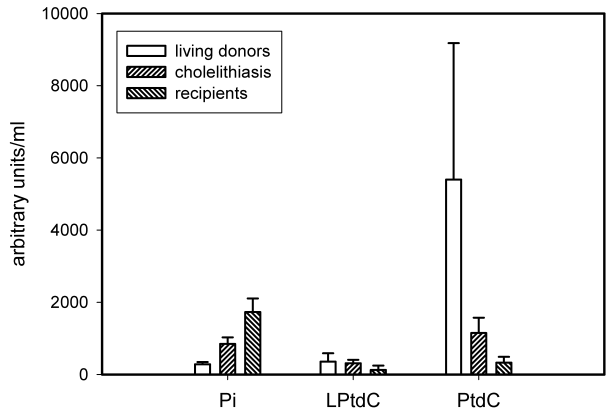

Fig. 2. Comparison of concentration of P_i, LPtdC, PtdC level compared to the external reference signal (H_3PO_4 in D_2O) is illustrated in the figure. The results are expressed as mean ± SEM values in arbitrary units/ml of bile sample

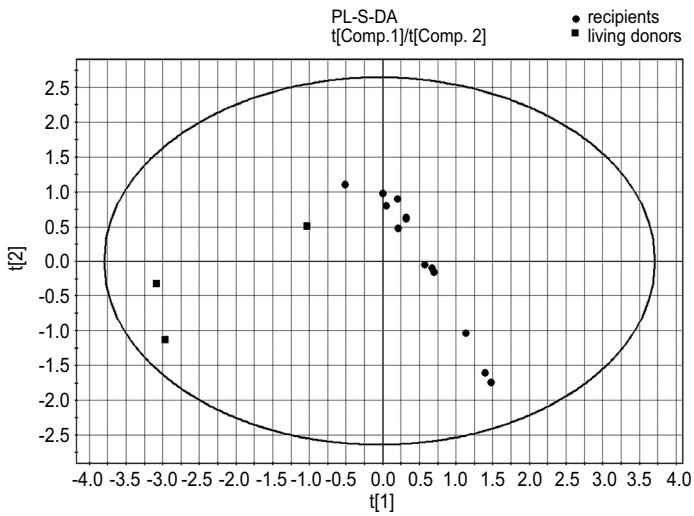

Fig. 3. A plot of principal component scores (t[1] versus t[2]) for a set of bile samples. Two groups were analysed: living donors and recipients

All patients from the recipients' group were classified correctly but only 66.67% (2 from 3) were classified correctly to the living donors' group. The most important term in this group the separation model was the PtdC concentration.

For all three groups the model of analysis resulted not as good as for two groups. The best model had only a one component. Q2cum was only 0.25 and R2cum was 0.36. The model was not valid for the cholelithiasis patients' group but was valid for the living donors' group and the recipients' group. All patients from the living donors' group and the recipients' group were classified correctly, and no one was classified correctly in the cholelithiasis patients' group – four were classified to the recipients' group and one to the living donors' group.

4. Discussion

In vitro ^{31}P NMR spectroscopy of bile allowed us to identify and quantify the phosphate-containing constituents of human bile and have the significant advantages over the conventional techniques as requiring almost no sample preparation, no prior knowledge of the constituent compounds, and no chemical pretreatment step to be able to identify them. Our result indicated that the spectra of raw bile and chloroform lipid extracts both showed phospholipids, the used method of analysis obviated the need to extract the lipids from the samples. Concentrations of the phosphate-containing compounds in human biliary secretions were quantified using the ^{31}P NMR spectroscopy of raw bile [13].

Since the majority of the samples in the living donors' group were very small, the significance of these findings still remains to be determined by the more extensive studies on more numerous groups.

Earlier studies showed that the disturbances in phospholipids concentration had led to gallstone formation [6, 14–19]. Our studies confirmed these results and showed a novel method that could be used for analysis of phospholipids in bile and therefore predict the predisposition to gallstone formation in the patients after liver transplantation or other patients after endoscopic retrograde cholangiopancreatography examination.

When the transplanted liver begins excretion, the bile contains a small amount of PtdC and LPtdC, and a great amount of P_i compared to the bile of the living donors. The bile of the cholelithiasis patients' group also contained a small amount of PtdC and LPtdC compared to the living donors' group, but the concentration level of those components were significantly higher than in the recipients' group. The level of P_i was also higher than in the living donors' group and higher than in the recipients' group as well.

Our results could indicate that biliary obstruction leads to impairment of liver anabolic functions and that transplanted liver had impaired hepatocytes secretion function compared to the living donor's liver. Most probably, it reflects the extent of the ischemic damage of the graft. The obtained information may have clinical implica-

tions in bile investigations using ^{31}P NMR spectroscopy to assess the graft function, the early signs of the rejection and predisposition to gallstone formation [20–22].

The conventional statistical analysis showed significant differences between the groups, but compounds responsible for the group separation were different for each pair of groups. Other compounds differentiated the patients' groups and other ones each patients' group and the living donors' group.

The chemometric analysis for the ^{31}P NMR spectral data set provided good classifications between the living donors' group and the recipients' group and poor ones between all the groups. This study showed the power of the combination of the NMR technique and the pattern recognition method for the analysis of biochemical phosphate-compounds of bile.

5. Conclusions

Multivariate statistical analysis demonstrated that the most significant compound involved in separation of the analyzed groups was PtdC, which is the most important phospholipid in bile. Bile phospholipids play an important role in proliferation of membrane-bound organelles, lipoprotein synthesis, and signal transduction affecting the processes of cell proliferation, and apoptosis [3]. The secretion of lipids into the bile is necessary for the maintenance of cholesterol homeostasis [4, 5].

In liver transplantation, metabolite measurements were performed to monitor two key aspects of the organ's physiology: the organ reperfusion injury and the organ function (or dysfunction) [23]. Our study confirmed that the ^{31}P NMR spectroscopy of the phosphate-containing bile allowed to measure concentrations of those compounds and correlate those results with patients' condition. The significant advantage of the new NMR approach includes the obviation of the loss of phospholipids because there is no extraction procedure involving organic solvents. The ^{31}P NMR spectroscopy is a reliable tool to analyze the phospholipids being non-invasive, rapid, simple, and one-step procedure. It is significantly more accurate than conventional analyses of phospholipids, that requires complicated and time-consuming extractions [13].

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