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High Performance Liquid Chromatography−Mass Spectrometry for Metabonomics: Potential Biomarkers for Acute Deterioration of Liver Function in Chronic Hepatitis B

Jun Yang,[†] Xinjie Zhao,[†] Xiaoli Liu,[‡] Chang Wang,[†] Peng Gao,[†] Jiangshan Wang,[†] Lanjuan Li,[‡] Jianren Gu,[§] Shengli Yang,[†,§,∥] and Guowang Xu*[,†]

National Chromatographic R. & A. Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 116023 Dalian, China, The First Affiliated Hospital of Medical College, Zhejiang University, Hangzhou, China, National Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai Jiao Tong University, Shanghai 200032, China, and Shanghai Research Center of Biotechnology, Chinese Academy of Sciences, Shanghai, China

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Metabonomics methods have been successfully applied to the drug discovery, toxicology, phytochemistry, and clinical fields. Here, we report a self-developed metabonomics platform which is based on high performance liquid chromatography−mass spectrometry (HPLC−MS) technique and applied to the investigation of acute deterioration of liver function in chronic hepatitis B to find the potential biomarkers. Sera from 50 healthy persons and 37 patients with acute deterioration of liver function in chronic hepatitis B were analyzed by HPLC−MS after removal of proteins. After de-noise, peak detection and peak alignment, the data of metabolites were fed to partial least squares discriminant analysis (PLS-DA) to find the potential biomarkers. According to the corresponding tandem mass results, several potential biomarkers were identified: Lysophosphatidyl Choline (LPC) C18:0, LPC C16:0, LPC C18:1, LPC C18:2, and glycochenodeoxycholic acid (GCDCA) (or its isomer glycodeoxycholic acid (GDCA)). On the basis of the relevant literature and pathway databases, the biological significance of the present study is discussed.

Keywords: metabonomics • hepatitis • LC • MS • PLS-DA • serum • biomarker

Introduction

Metabonomics as a novel methodology arising from the post-genomics era has been successfully applied in many fields such as drug discovery,[1] toxicology,[2,3] phytochemistry,[4,5] and clinical research.[6−8] According to quantitative measure of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification,[9] the toxicological mechanisms and disease process could be investigated using induction way.[10] Due to the diversities of molecules in polarity, molecular weight, and concentrations, it is generally accepted that a single analytical technique could not provide sufficient visualization of the metabolome, and therefore, multiple technologies are needed for a comprehensive view. Among the analytical technologies, NMR,[7,11−27] the chromatographic techniques[8] and their hyphenated techniques[6,28−37] are the most popular methods used in the metabonomics researches. For the high sensitivity and resolution, LC−MS based platforms are preferred to analyze the bio-fluid samples: serum, plasma, and urine.

Different from the chromatographic techniques, the LC−MS and GC−MS data afford the three-dimensional information of the metabolites: retention characteristics, mass-charge-ratio (m/z), and peak intensities. The added m/z information brings higher resolution and throughput, which are ideal for a metabonomics platform. At the same time, more difficulties in alignment and deconvolution are encountered with the added m/z information. To be able to compare samples in large databases, data must fulfill following specific criteria: (i) each sample is characterized by the same number of variables, (ii) each of these variables is represented across all observations, and (iii) a variable in one sample has the same biological meaning or represents the same metabolite as in all other samples.[38] How to extract the useful information from the raw data has become an obstacle for GC−MS and LC−MS to metabonomics applications. Several strategies[38−41] have been developed to transfer the acquired GC−MS and LC−MS data to fulfill these criteria. In the original works,[40,41] the authors accomplished the peak alignment using different techniques to get the same number of variables. In the successive works,[38,39] they introduced resolution techniques, alternating regression (AR) and multivariate curve resolution (MCR) to

* To whom correspondence should be addressed. Fax: 0086-411-84379559. E-mail: xugw@dicp.ac.cn.
[†] Dalian Institute of Chemical Physics, Chinese Academy of Sciences.
[‡] The First Affiliated Hospital of Medical College, Zhejiang University.
[§] Shanghai Cancer Institute, Shanghai Jiao Tong University.
[∥] Shanghai Research Center of Biotechnology, Chinese Academy of Sciences.

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Table 1. Demographic Information of Control Groups and Hepatitis B Group[a]

	control group ($n = 50$)	hepatitis group ($n = 37$)
sex (male/female)	34/16	28/9
age (yr)	43.89 ± 13.74	45.88 ± 12.24
ALT (U/L)	22.16 ± 3.64	33.22 ± 4.68
TB (μmol/L)	11.76 ± 4.92	474.18 ± 165.68
PT	12.08 ± 0.79	32.80 ± 17.10

[a] Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; TB, total bilirubin; PT, prothrombin time.

deconvolution and data reduction. Although these approaches have resolved many problems, there is still a lot of work required before applying GC–MS and LC–MS to the metabonomics research successfully.

In this study, we developed a homemade metabonomics platform, which consists of LC–MS method, peak detection and peak alignment scripts and biomarker identification strategy. After this, the acute deterioration of liver function in chronic hepatitis B was selected as an example. Several potential biomarkers: LPCs and GCDCA (or its isomer GDCA) were found and identified. On the basis of the relevant literature and pathway databases, the biological significance is discussed.

Experimental Section

Study Population and Sample Collection. Thirty-seven chronic hepatitis B patients hospitalized for acute deterioration of liver function were enrolled in this study. They all had severe impairment of liver function. The control group consisted of blood samples from 50 healthy individuals who came to The First Affiliated Hospital of Medical College, Zhejiang University for physical check-up. They all had normal liver biochemistry tests and had no evidence of diseases. The demographic information of the samples is given in Table 1.

With the consent, the fasting blood samples were collected before breakfast at the time of admission. Blood samples were drawn into tubes and stored at room temperature for 1 to 2 h.

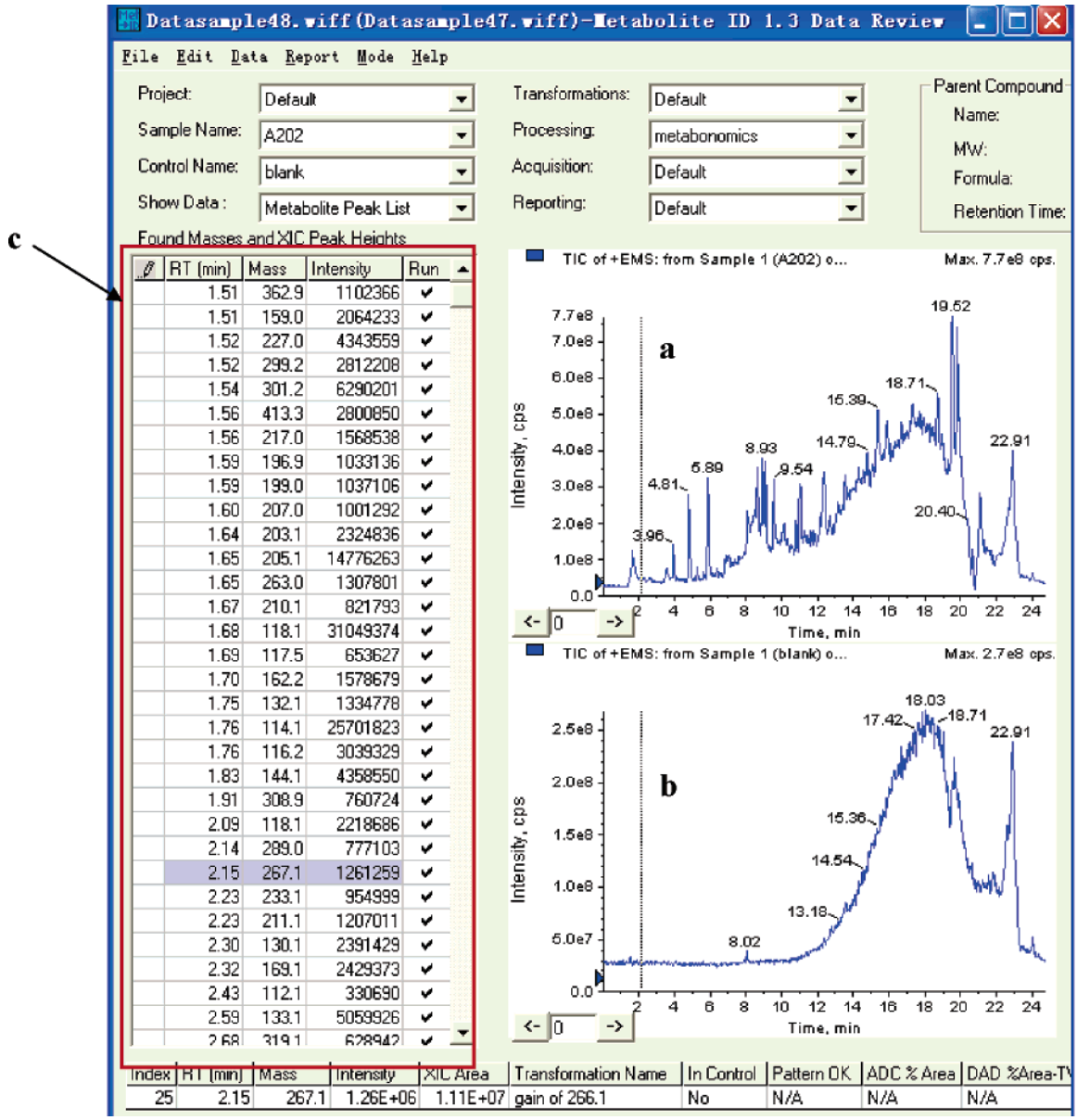

Figure 1. Typical chromatograms shown in Metabolite ID software. (a) Sample chromatogram; (b) Blank chromatogram; (c) Peak list.

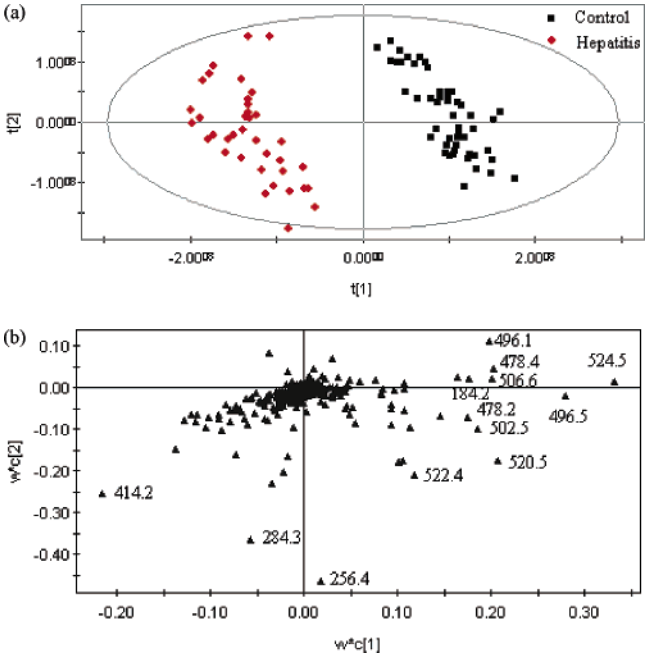

Figure 2. (a) PLS-DA score plot for the first two components showing the separation between the hepatitis B group and healthy control. (♦) Hepatitis patients and (■) Healthy control. (b) PLS-DA variable loadings plot for the two first components (w^*c_2/w^*c_1) explaining the above separation between hepatitis B and healthy groups. The triangles were labeled by *m*/*z* values.

Table 2. Potential Biomarkers and Their Identification Results

variable no.	VIP[a]	retention time (min)	*m*/*z*	identification result
VAR_5229	41.23	17.22	524.5	LPC[b]C18:0
VAR_4177	33.07	15.33	496.5	LPC C16:0
VAR_4167	20.35	15.25	478.2	fragment of LPC C16:0
VAR_5226	18.78	17.21	506.6	fragment of LPC C18:0
VAR_3850	18.04	14.75	520.5	LPC C18:2
VAR_686	17.28	7.78	235.2	UN[c]
VAR_644	16.33	7.55	235.2	UN[c]
VAR_4422	16.16	15.85	522.4	LPC C18:1
VAR_3849	12.55	14.75	502.5	fragment of LPC C18:2
VAR_2266	12.20	12.34	414.2	fragment of GCDCA or GDCA
VAR_6169	11.45	19.81	282.4	UN[c]
VAR_4417	10.06	15.84	504.4	fragment of LPC C18:1
VAR_4022	9.759	15.05	478.4	fragment of LPC C16:0
VAR_4024	9.582	15.05	496.1	LPC C16:0
VAR_4021	8.195	15.05	184.2	phosphatidylcholine moiety of LPC C16:0
VAR_5104	7.956	16.89	524.4	LPC C18:0
VAR_4178	7.585	15.34	479.3	isotope compound of 478.4
VAR_741	5.628	7.99	235.3	UN[c]

[a] VIP: Variable Importance in the Projection, which reflects the influence on *Y* of every term (x_K) in the model, is the sum over all model dimensions of the contributions of variable influence.[42] [b] LPC: Lysophosphatidyl Choline. [c] UN: un-identified.

After centrifugation at 3000 × *g* for 10 min at 4 °C, serum was stored −70 °C until analysis.

Sample Preparation. The sera were thawed before analysis. 150 μL acetonitrile was added to150 μL of the serum and shaken vigorously (30 s), and then the mixture was allowed to stand for 5 min and centrifuged at 12 000 rpm for 3 min. The supernatant was lyophilized.

HPLC−MS Analysis. Serum material obtained following acetonitrile precipitation was dissolved in 150 μL 0%, 20%, 50%, 80%, 100% acetonitrile solutions, respectively. The results indicated that more components could be extracted using 80% acetonitrile. So, 80% acetonitrile solution was selected as the reconstructed sample solution for all the samples.

Chromatography was performed on an Agilent 1100 liquid chromatography series (Agilent Corporation, USA), coupled to a QTrap tandem mass spectrometer (Applied Biosystems Instrument Corporation, USA) equipped with an electrospray source after split with 1:5 split ratio.

Because the metabolites in human serum were very complicated, it is impossible to analyze all of them with single method. The mass spectrometer was operated in positive ion mode to analyze as many metabolites as possible in a single injection. The source temperature was set at 250 °C with a curtain gas pressure of 25 psi, a nebulization gas pressure of 30 psi, an auxiliary heat gas pressure of 40 psi. The ion sprayer voltage was set at 5 kV for positive ion mode with a declustering potential of 45 V. The Enhanced MS mode was employed to scan from 100 to 800 *m*/*z* with Q_0 trapping on and 50 ms LIT fill time. The collision energy was set at 10 V and collision gas was set at high.

A 10 μL aliquot of the reconstructed solution was injected onto a 4.6 × 150 mm Zorbax Eclipse XDB−C8 5 μm column. The column was eluted with a linear gradient of 2−98% of 0.1% formic acid in acetonitrile (B) over 0.1−18 min; the composition was held at 98% of B for 2 min then returned to 2% of B at 22 min; at last, the composition was held at 2% of B for another 3 min at an eluent flow rate of 1 mL min^{-1}. To avoid the cross contamination, the blank run was inserted between the consecutive samples.

HPLC−MS/MS Experiment. To get identification information of the metabolites, information dependent acquisition (IDA) mode was employed to get the tandem mass spectra of the metabolites. Using IDA mode of QTrap system, the survey scans such as EMS and dependent scans-EPI (Enhanced Product Ion) are sequentially performed, and repeated in the entire duration of the HPLC analysis, thus offering the maximum information from a single injection. The HPLC and EMS parameters were identical to the ones in the HPLC/MS experiment except that the scan rate was changed to 4000 amu/s to get a higher duty cycle. In the EPI scan mode, the resolution of Q1 was set at LOW (~2 amu) to get higher sensitivity and Collision Energy (CE) was set at three different values: 20, 30, and 60 eV to get abundant structure information under the various fragment degrees.

Peak Detection and Alignment. After data acquisition using HPLC−MS, the de-noising, baseline correction and peak detection were followed. For the high complexity of serum, it is impossible to let all compounds be completely eluted from the column. Therefore, even with the blank runs, several strong retentates' peaks (for example, the peak with 22.91 min in Figure 1) could be found at the end of the chromatogram of each run. To exclude these peaks in the following analysis, Metabolite ID Software (Applied Biosystems Instrument Co., USA) was employed to accomplish the de-noising and peak detection. The steps included: subtract average blank spectra from sample for time slices (0.2 min); combine masses into a unique mass list, calculate extracted ion chromatograms (XIC), find peaks above the MS threshold (200 000 cps, the noise ion level of the front baseline), remove isotope peaks, remove peaks

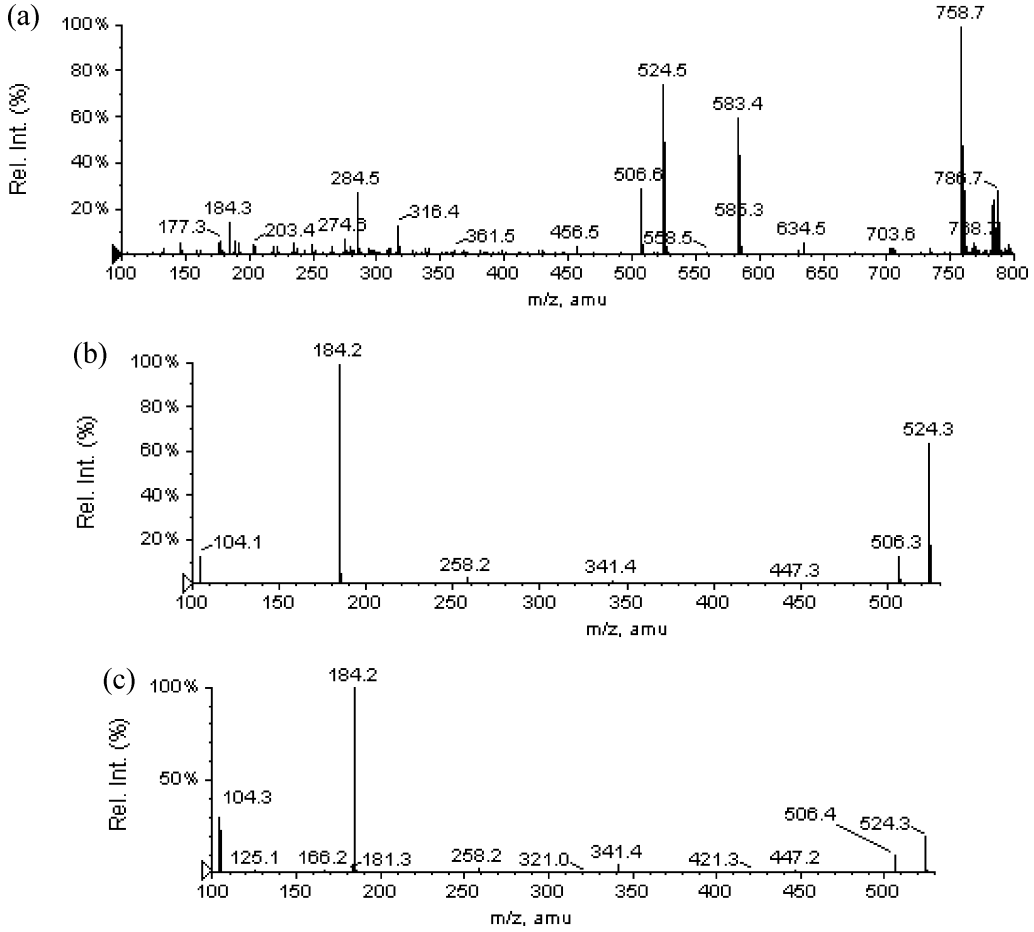

Figure 3. (a) Mass spectrum of the Var_5226: *m*/*z* 506.6; (b) Tandem mass spectrum of the quasi-molecular ion: 524.3; (c) Spectrum of the commercial standard Lysophosphatidylcholine C18:0.

found in the blank based on XIC peak height ratio (2:1). And the peak list was exported to a CSV file, which includes the information of the *m*/*z*, retention time, height, and area of the XIC peaks. A typical view of the software was given in Figure 1. Using the software, we could avoid the influence from the change of the column and the noise more efficiently. On the other hand, the retention time (22.91 min in Figure 1) of the strong retentate was found to be very reproducible, it could be used to monitor the reproducibility of the injections.

The next step was that all of the samples' peak lists were aligned using in-house program written in MATLAB software (Mathworks, Natick). At first, the peak lists with the corresponding retention time and *m*/*z* were merged together to a total peak list. Then the total peak list was sorted according to the retention time and *m*/*z*. After the windows of the *m*/*z* and retention time were set, the adjacent peaks located in the same retention time and *m*/*z* window were considered as the identical peak. A reference peak list was constructed and each sample was assigned with the identical number of peaks according to the reference peak list, which was represented as a *K* (number of the reference peaks) × 4 (*m*/*z*, retention time, height, area) matrix. All of the samples have the same number of variables with the same biological meaning. To use height or area information, the corresponding vectors of all samples were extracted to create an *N* (number of the samples, 87 in this paper) × *K* matrix.

Multivariate Data Analysis. Partial least-squares discriminant analysis (PLS-DA) was used as the classification method for modeling the discrimination between the hepatitis B patients and healthy controls based on serum metabolites. Multivariate analysis was performed using the SIMCA-P 10 demo version (Umetrics AB, Umeå, Sweden).

Results and Discussion

The 7347 peaks were found in the final reference peak list after merged. The area and height data were fed to the SIMCA-P, respectively. When the area data were selected, the model could describe 52.0% of the variation in *X* ($R^2X = 52.0\%$), 97.0% of the variation in the response *Y* (class) ($R^2Y = 97.0\%$), and predict 94.8% of the variation in the response *Y* ($Q^2Y = 94.8\%$). There were many variations in *X* that were not related to *Y*, or in other words, just a part of the raw information (*X*) was related to the class information (*Y*), disease or not. Because of this the other part perhaps was describing the variation of the sex, dietary, and other stimuli. However, the model presented satisfied classification ability between the hepatitis patients and the healthy controls (the high R^2Y and Q^2Y). The peak height data gave the similar result.

The score and loading plot of the PLS-DA are given in Figure 2. From the score plot, it can be found that the hepatitis group was clearly separated from the healthy control group clearly. To find the potential biomarkers, a parameter VIP (Variable

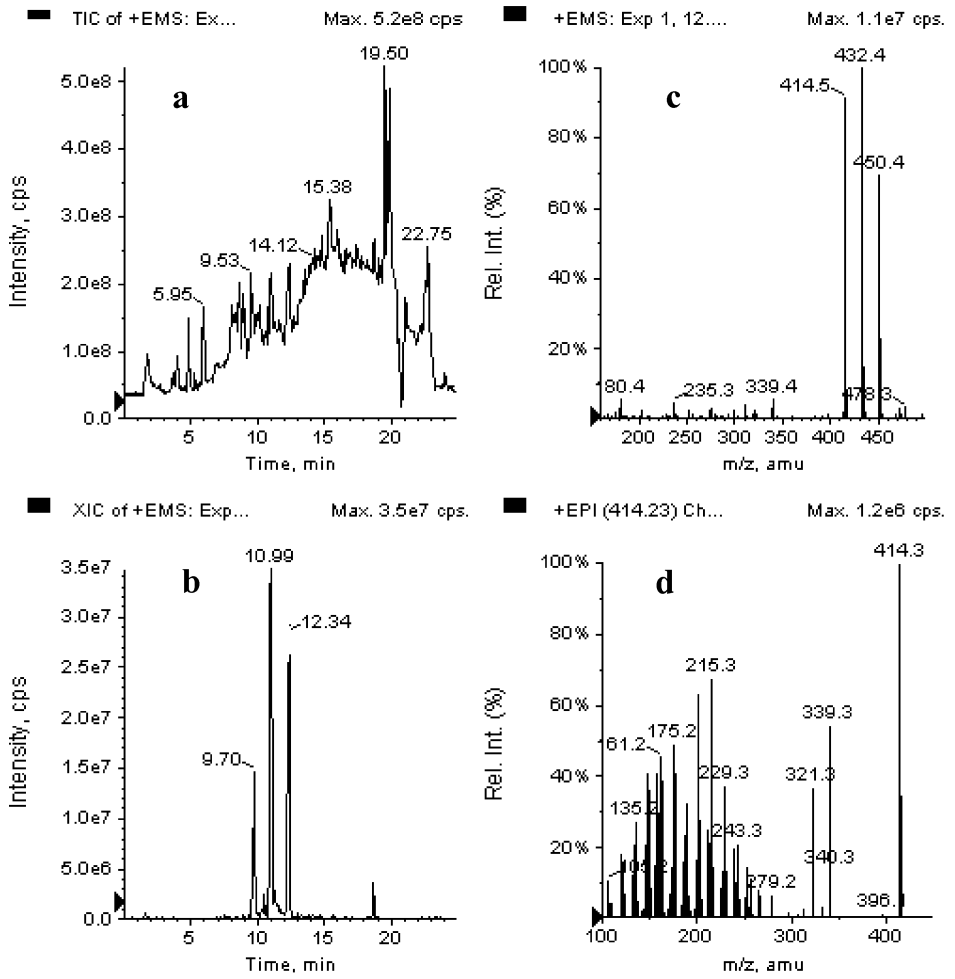

Figure 4. IDA view of Var_2266. (a) Total ion chromatogram; (b) XIC of the ion (*m*/*z* 414.2); (c) Spectrum in the retention time (t_R = 12.34 min); (d) Tandem mass spectrum of the ion (*m*/*z* 414.2, t_R = 12.34 min).

Importance in the Projection) was employed to reflect the variable importance. It could be calculated according to the formula (1)

$$\mathrm{VIP}_{AK} = \sqrt{\left(\sum_{a=1}^{A} (w_{ak}{}^2 * (\mathrm{SSY}_{a-1} - \mathrm{SSY}_a)) * \frac{K}{\mathrm{SSY}_0 - \mathrm{SSY}_A}\right)} \quad (1)$$

where A is the number of components of the model; K is the number of the variables; w_{ak} is the PLS weight; SSY is the explained SS of the a^{th} PLS dimension; and SS is the residual sum of squares of the previous component.[42]

According to VIP value, the most important 20 variables (potential biomarkers) were first selected as the candidate of potential biomarkers. Then t-test was employed to these 20 variables, significant differences ($P < 0.05$) were found except for Var_6014 and Var_6461.So, 18 variables were considered as the potential biomarkers (Table 2).

Several markers with different *m*/*z* in one spectrum perhaps came from an identical compound: the quasi-molecular ions, the ion adducts and the fragments produced in the ion source. So, when the potential biomarkers were identified, the quasi-molecular ion should be found at first. As an example, when identifying the Var_5226 (*m*/*z* 506.6), we found a higher mass (524.2) than it had in the spectrum (Figure 3). It could be deduced that the Var_5226 was the corresponding ion losing water from the 524.2, which is a very common phenomena in mass spectrum.

The tandem mass spectrum was searched for the further identification from the IDA data of the LC−MS/MS experiment. The characteristic fragment ion of lyso-phosphatidyl choline-*m*/*z* 184 (choline moiety)[43, 44] was found in several tandem spectra. According to the fragment mechanism of the LPCs and the spectra of the standards purchased from Sigma, their identification results were given in Table 2.

For other potential biomarkers, we searched candidates from the ligand database of the KEGG (www.kegg.com) and PubChem (pubchem.ncbi.nlm.nih.gov) using the molecular weight at first. Taking VAR_2266 as an example, the corresponding quasi-molecular ion was 450.4 (Figure 4c) and 14 items were found from the KEGG with respect to *m*/*z* 449 (the molecular weight). For the cues of loss of water (*m*/*z* 450.4 → 432.4, 432.4 → 414.5) in Figure 4c, there must be two hydroxyl groups in the structure. According to this fact, 5 items were removed from the candidate list. Another 4 items were removed for another hydroxyl group (414.5 → 396.1) cues in Figure 4d. Now, only 5 items survived and their mass fragment information was searched from the previous researches about these metabolites. When compared with the fragment ions of glycocholic acid (GCA), a similar fragmentation pattern was found, with the exception of the 2 amu shift.[45] The possible fragment mecha-

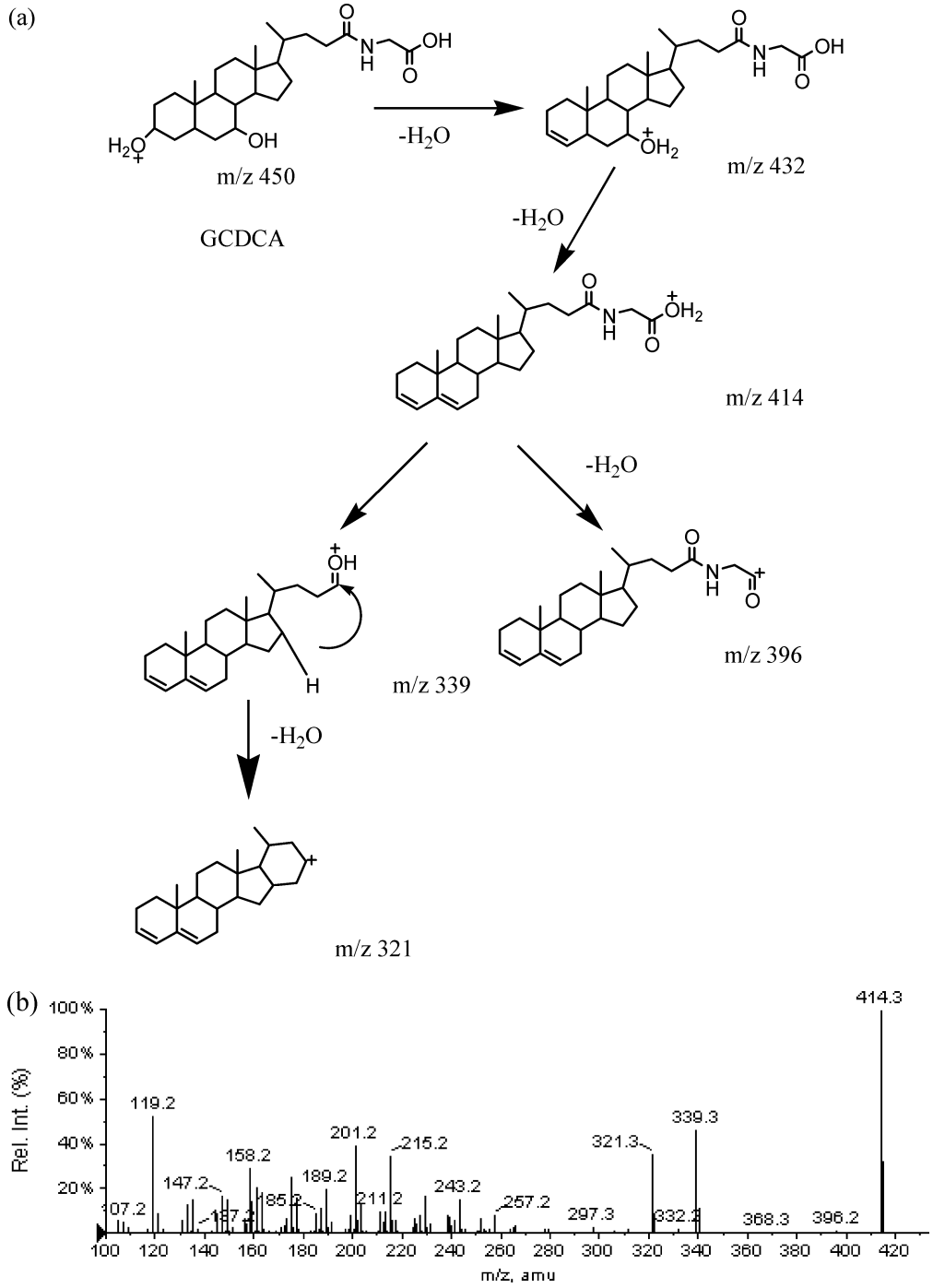

Figure 5. Possible fragment mechanism of GCDCA (a) and mass spectrum of the commercial standard-sodium glycochenodeoxycholate (GCDCA+Na^+) (b).

nism was given in Figure 5a. So, this potential biomarker (Var_2266) was likely to be glycochenodeoxycholic acid (GCDCA) or its isomer glycodeoxycholic acid (GDCA). It was confirmed by the spectrum of GCDCA standard purchased from the Sigma (Figure 5b) at last. It should be pointed out that because of unavailable standard sample of GDCA, we still do not know whether the GCDCA has been separated from the GDCA.

Finally, five potential biomarkers have been identified. Four of them were LPC. LPC regulates a variety of biological process including cell proliferation, tumor cell invasiveness, and inflammation. Produced by the action of Phospholipase A_2 (PLA_2) on phosphatidylcholine, it promotes inflammatory effects, including increased expression of endothelial cell adhesion molecules and growth factors, monocyte chemotaxis, and macrophage activation. It has recently been identified as ligands for the lymphocyte G protein-coupled orphan receptor G2A.[46] In another study, LPC was considered as potent superregulators of T cell activation inflammation at sites of tissue damage and in the early stages of atherosclerosis.[47] LPC has been related to many diseases such as Parkinson's disease (PD),[48] glomerular inflammation,[49] bronchospastic syndrome,[50] Alzheimer's disease,[51] ischemic canine heart.[52] PC[50] and LPC/PC[51] have been selected as marker of these diseases. Another

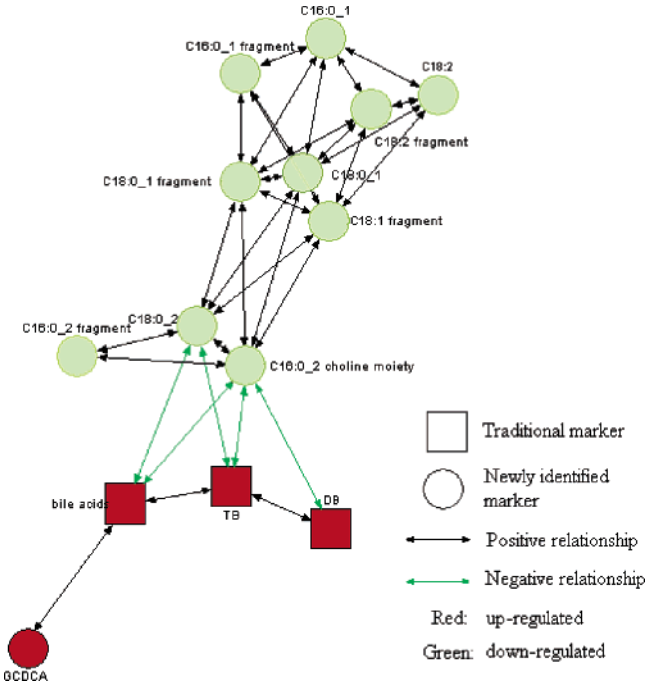

Figure 6. Correlation Network of biomarkers with $|C_{ij}| > 0.8$. The high relationship between the parent ions such as C16:0 and the corresponding fragment ions further indicated the reliability of the identification.

potential biomarker, GCDCA or GDCA, is one of the coagulated bile acids, which were helpful for the digestion of the lipids. It was related to a part of the traditionally diagnostic marker: CA (cholic acid)/CDCA (chenodeoxycholic acid). Compared with the case report of the samples, the same trend was found between the bile acid (traditional marker) and GCDCA. Compared to the healthy control, LPC levels in hepatitis patients were down-regulated and GCDCA (or GDCA) level was up-regulated.

Combined the marker list with the traditional marker data, the correlation analysis was performed. After the associations $|C_{ij}| < 0.8$ were removed, a network (Figure 6) was obtained. This method is similar to relevance networks introduced by Butte et al.[53] and van der Greef.[54] Images were generated using the Graph Package by Tom Sawyer Software (www.tomsawyer.com/git/). From Figure 6, it is found that GCDCA has very good relationship with bile acids, total bilirubin (TB) and direct bilirubin (DB), their concentrations were up-regulated. In reverse, the concentrations of LPCs in chronic hepatitis B patients hospitalized for acute deterioration of liver function were down-regulated. The fragments and their corresponding parent ions were also highly correlated, which is helpful for the structure identification.

It should be pointed out that the causal relationship of the identified biomarkers and HBV specific liver dysfunction is not firmly established. This could be best addressed by additional analyses of samples from patients with other liver diseases, e.g., intra- or extrahepatic cholestasis, or samples from chronically infected HBV patients without relevant liver dysfunction. In the absence of such data, the importance of LPC and GCDCA as pathophysiologically relevant marker for the diagnosis of HBV-mediated liver dysfunction needs be further confirmed in future studies.

Conclusions

Here, we described a method to accomplish the LC−MS based metabonomics research on acute deterioration of liver function in chronic hepatitis B to find the potential biomarkers. 5 of them were identified: LPC C18:0, LPC C18:1, LPC C18:2, LPC C16:0, GCDCA (or its isomer GDCA). GCDCA (or GDCA) was a classical biomarker, the other four was newly found. According to the relevance networks of the markers including the traditional markers and the newly identified ones, it is found that GCDCA has very good relationship with bile acids, total bilirubin (TB) and direct bilirubin (DB). The results showed that the technique was mature and the metabonomics method was a powerful way of potential biomarker discovery. To confirm the biomarkers found in this study, other liver diseases need to be analyzed in the future.

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