**Introduction**

Chronic hepatitis B viral (HBV) infection continues to be a worldwide liver problem with approximately 240 million people carrying the virus (Schweitzer et al., 2015). Ten to twenty percent of patients with chronic hepatitis B (CHB) can develop liver cirrhosis in five years (Amantonico et al., 2010). Cirrhosis precedes most cases of hepatocellular carcinoma (HCC), with 70%–90% of HCC developing from the background of liver cirrhosis (Zhang et al., 2013c; Wanich et al., 2016). These data clearly indicate the critical importance of early diagnosis of liver cirrhosis. Although liver biopsy is currently recommended as the gold standard method of staging fibrosis in patients with CHB, it has several disadvantages such as invasive protocol, risks of complication, poor patient compliance and sampling error, which limits its usefulness for dynamic surveillance and follow-up. Therefore, a reliable, non-invasive diagnostic procedure to predict and assess treatment and prognosis of liver cirrhosis is needed. Metabolomic analysis has been shown to be a powerful tool for the diagnosis, treatment, and prevention of human diseases (Zhang et al., 2012; Zhang et al., 2013b).

Metabolomics, which is defined as the measurement of low molecular weight metabolites in an organism at a specified time under specific environmental conditions (Wu et al., 2009), has been shown to be an effective tool for disease diagnosis (Claudino et al., 2007; Wikoff et al., 2007), biomarker screening (Bogdanov et al., 2008; Xue et al., 2008b; Silva et al., 2011), and characterization of biological pathways (Nicholson et al., 2002). Recently, metabolomic profiling approaches have been increasingly used to elucidate significant changes in tumor metabolism and to explore candidate biomarkers from a huge number of endogenous metabolites. Blood and urine are the most common types of samples employed for exploring the systematic alteration in human metabolome. However, urine sample is more preferable as it enables non-invasive monitoring of metabolic changes.

Metabolomic studies generally employ techniques such as nuclear magnetic resonance (NMR), fourier transform infrared (FT/IR) spectroscopy, liquid chromatography/mass spectrometry (LC/MS), and gas chromatography/mass spectrometry (GC/MS).
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...GC/MS has been described as a sensitive and reproducible method, which has been proposed as an ideal tool for metabolomic profiling of urine samples (Zhang et al., 2007; Wu et al., 2009).

There are few studies concerning CHB. Soga et al., (2011) applied LC/MS to analyze the serum from CHB patients and proposed γ-glutamyl threonine as a feature to discriminate CHB from other liver diseases. Zhou et al., (2012) analyzed the metabolites in serum from CHB patients and a control group by LC/MS and discovered distinctive metabolites that were involved in fatty acids, amino acids, bile acids and energy metabolism pathways. Yang et al., (2016) further investigated the serum and urine metabolic alterations of CHB by GC/MS, and they found that glycine and fatty acid metabolism may be reprogrammed in CHB patients. To date, there are still few metabolomic studies about CHB so it is a research domain that needs to be expanded.

The aims of this study were to compare metabolite profiles of urine samples from CHB patients and healthy subjects using GC/MS and to establish a diagnostic model from these metabolic biomarkers to distinguish CHB from the normal subjects.

Materials and Methods

Subjects and sample collection

The research was approved by the Committee on Human Right Related to Research Involving Human Subjects of Ramathibodi Hospital (ID 02-58-22). The study was carried out according to the Helsinki Declaration (1964) as revised in 2013. Informed consent was obtained prior to subject enrollment. Twenty CHB patients were recruited to the study from the liver clinics of Ramathibodi Hospital, Mahidol University, Bangkok, along with 20 normal control subjects. Exclusion criteria were presence of cancer, significant concomitant diseases such as congestive heart failure, renal insufficiency, respiratory failure, or those who used antiviral or herbal medication. Normal controls were healthy subjects without medical disease. Clinical information was obtained from medical records and the hospital database. Blood samples were collected for biochemical testing. Urine samples were collected and centrifuged at 3,000 rpm for 10 min at 4°C for removal of solid debris. The samples were stored at -80°C until GC/MS analysis.

Sample preparation

The urine samples were thawed at room temperature. A 10 μL 1 volume of internal standard (0.5 mg/ml diphenylamine in methanol) was added into 2.0 ml aliquot of urine in a 8 ml glass tube and vortex-mixed for 15 s. A 200 μL of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s. A 200 μL l of KOH (5M), 2.0 ml tert-butyl methyl ether (TBME), and 3 g anhydrous sodium sulfate were added into each tube. The tubes were then capped with Teflon-lined screw-top caps, shaken horizontally for 15 s.

Data processing and statistical analysis

After GC/MS analysis, each sample was represented by a GC/MS total ion chromatogram (TIC), and the peak areas of assigned compounds were integrated. The peak area ratio of each compound to the internal standard was calculated and used as the response. The GC/MS data were imported into the Mass Profiler Professional (MPP) software (Agilent Technologies, Santa Clara, CA, USA) for differential analysis. MPP was used for data filtering and statistical analysis, and compound identification was performed using the NIST library and Agilent MassHunter ID Browser. Independent-sample t-test statistics was used for comparison of the metabolite levels to determine the significant differences between the CHB group and the control group. Differentially expressed compounds with p-values of <0.05 were considered to be statistically significant.

Principal component analysis (PCA) was used to differentiate the samples, using the MPP software. All of the data from the differentially expressed compounds were used for constructing PCA models. The score plots of the first three principal components allowed visualization of the data and compared samples between the CHB and control groups. Group prediction employed the partial least square discriminant analysis (PLS-DA) model. PLS-DA was constructed using the significant metabolites of filtered data using four components including auto scaling, N-fold validation type, three numbers of fold, and with ten numbers of repeats. Sensitivity and specificity were also calculated from the constructed model. Forty Samples were randomly...
selected and validated through the constructed model. A portion (~75%) of the 40 subjects (15 CHB patients and 15 healthy individuals) was used as a training set to explore the specific biomarkers associated with chronic hepatitis B. The remaining ~25% samples (5 CHB patients and 5 healthy individuals) was used as a test set to validate the diagnostic capability of the two combined markers discovered from the training set.

Metabolic pathway and metabolite biofunction analysis was performed using the network database (KEGG PATHWAY Database, http://www.kegg.jp/kegg/pathway.html). The impact of CHB on metabolic pathways was evaluated through an online MetaboAnalyst 3.0 software suite for metabolomic data analysis (http://mirror.metaboanalyst.ca/MetaboAnalyst/faces/home.xhtml).

Results

**Metabolomic profiling of urine samples**

Demographic, clinical and laboratory data were collected and showed in Table 1. As expected, AST and ALT levels were significantly increased in CHB patients. Representative GC/MS total ion chromatograms (TICs) are shown in Figure 1, with large diverse sets of metabolites in the urine from a normal control group selected and validated through the constructed model. A portion (~75%) of the 40 subjects (15 CHB patients and 15 healthy individuals) was used as a training set to explore the specific biomarkers associated with chronic hepatitis B. The remaining ~25% samples (5 CHB patients and 5 healthy individuals) was used as a test set to validate the diagnostic capability of the two combined markers discovered from the training set.

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**Table 1. Clinical Characteristics and Liver Function of the Study Subjects**

| Samples | Control (n = 20) | CHB (n = 20) | p value |
|---------|-----------------|-------------|---------|
| Age (years)a | 36.5 ± 10.2 | 47.9 ± 13.3 | 0.006 |
| Maleb | 12 (54.5) | 9 (42.8) | 0.251 |
| BMI (kg/m2)c | 22.3 ± 1.2 | 22.5 ± 2.3 | 0.278 |
| AST (U/L)d | 22.4 ± 4.2 | 63.1 ± 68.2 | <0.05 |
| ALT (U/L)d | 27.0 ± 15.5 | 108.8 ± 158.9 | <0.05 |
| ALB (g/L)d | 40.1 ± 2.2 | 39.7 ± 2.6 | 0.605 |
| Total bilirubin (mg/dL)d | 0.7 ± 0.2 | 0.8 ± 0.2 | 0.247 |
| Glucose (mg/dL)d | 91.3 ± 7.0 | 91.8 ± 15.6 | 0.943 |
| BUN (mg/dL)d | 10.8 ± 2.5 | 12.4 ± 3.1 | 0.110 |
| Creatinine (mg/dL)d | 0.87 ± 0.2 | 0.89 ± 0.2 | 0.853 |
| Triglyceride (mg/dL)d | 91.4 ± 25.9 | 115.3 ± 71.7 | 0.287 |
| Cholesterol (mg/dL)d | 196.4 ± 33.5 | 197.8 ± 35.2 | 0.911 |
| PLT (10^3/mm^3) | 249.0 ± 66.0 | 203.1 ± 39.7 | 0.084 |

CHB, Chronic hepatitis B; BM, Body mass index; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase; ALB, Albumin; TB, Total bilirubin; BUN, Blood urea nitrogen; PLT, Platelet; a mean ± SD; b n (%)

(TICs) are shown in Figure 1, with large diverse sets of metabolites in the urine from a normal control group.

![Figure 1. Comparison of Typical GC/MS Total Ion Current (TIC) Chromatograms of Urine from Chronic Hepatitis B (CHB) and Control Group (IS: Internal standard, diphenylamine)](image1)

![Figure 2. Distribution of Identified Urine Metabolites of Chronic Hepatitis B (CHB) and Control Groups According to Classes of Compounds](image2)
and a CHB patient. The difference between urinary GC/MS profiles of normal control subject and CHB patient were observed. In all, 377 metabolites were presumptively identified in urine samples of the normal control and CHB groups. These include a variety of chemical compounds that were identified to be involved in multiple biological functions. The 377 metabolites were subsequently classified into ketones, aldehydes, acids, alcohols, terpenoids, benzene derivatives, sulfur compounds, phenols, esters, naphthalene derivatives and miscellaneous Figure 2.

Pattern recognition and function analysis

Twelve compounds with high abundances were identified using MPP and all of them were found to be present at significantly higher levels in the CHB group. These are palmitic acid, stearic acid, oleic acid, benzoic acid, butanoic acid, cholesterol, glycine, 3-heptanone, 4-heptanone, hexanal, 1-tetradecanol and naphthalene Table 2. A PCA model was constructed using the marker metabolite intensities as variables. The PCA scores plot showed that the samples are scattered into two regions Figure 3. Hierarchical cluster analysis (HCA) was performed to produce a dendrogram for clustering of samples groups using normalized intensities of 12 significant metabolites. HCA of these 12 metabolites again divided the samples into CHB and control groups as shown in Figure 4.

Class prediction model and test

Using the 12 statistically significant metabolites, PLS-DA algorithm was used to classify the samples into discrete classes. A clear separation was observed between the CHB and control groups in the PLS-DA scores plot (Figure 5). Sensitivity and specificity were also calculated for the constructed model. The sensitivity and specificity were 95% and 85%, respectively, and the overall accuracy of the model was 90% (Supplemental Table 1). The model was used to validate an independent or blind-test set of 10 urinary samples (5 healthy controls and 5 CHB patients). PLS-DA classifier correctly predicted the presence of CHB in 5 out of 5 patients, and healthy control in 4 out of 5 samples, resulting in 100% sensitivity and 80% specificity, respectively.

Pathway analysis

The biological pathways involved were analyzed using the online MetaboAnalyst 3.0 software Figure 6. All matched pathways are shown according to p-values from the pathway enrichment analysis (y-axis) and pathway impact values from pathway topology analysis (x-axis), with the most impacted pathways colored in red (Xia and Wishart, 2011). Consequently, eight pathways were considered closely related to CHB as shown in Figure 6. These include fatty acid biosynthesis, fatty acid elongation in mitochondria, the biosynthesis of primary bile acid and steroid hormone and the metabolism of cyanoamino acid, thiamine, glutathione and phenylalanine. The altered metabolic pathways were generated using the reference map by searching KEGG database, as shown in supplemental materials

| Metabolites          | FC ([CHB] vs [Control]) | Regulation | p value       |
|----------------------|-------------------------|------------|---------------|
| 3-Heptanone          | 1.3996902               | up         | 2.05 x 10^-6 |
| Stearic acid         | 1.7362183               | up         | 4.69 x 10^-7 |
| Palmitic acid        | 5.1711727               | up         | 1.04 x 10^-3 |
| 1-Tetradecanol       | 1.2326117               | up         | 8.04 x 10^-4 |
| Naphthalene          | 1.7234368               | up         | 1.16 x 10^-4 |
| 4-Heptanone          | 2.2869997               | up         | 1.76 x 10^-4 |
| Glycine              | 1.9506816               | up         | 6.89 x 10^-5 |
| Hexanal              | 3.8837343               | up         | 1.96 x 10^-7 |
| Oleic acid           | 1.4874096               | up         | 2.55 x 10^-2 |
| Butanoic acid        | 1.2648753               | up         | 7.09 x 10^-4 |
| Benzoic acid         | 2.2584724               | up         | 1.52 x 10^-5 |
| Cholesterol          | 1.3778079               | up         | 3.95 x 10^-3 |

CHB: Chronic hepatitis B; FC: Fold change

Table 2. Twelve High Abundance Compounds Identified Using the Mass Profiler Professional (MPP) Software and Filtered Using t-test

Figure 3. Sample Pattern Recognition Using Principal Component Analysis (PCA). PCA Analysis of the Twelve Significantly Abundant Compounds Results in two Distinctive Groups of Samples. The Blue and Red Circles Indicate Healthy Volunteers (n=20) and Chronic Hepatitis B (CHB) Patients (n=20), Respectively
Supplemental Figure 1-8.

Discussions

GC/MS-based urine metabolomics coupled with multivariate statistical analysis clearly differentiated CHB patients from normal subjects with high sensitivity (95%) and specificity (85%) and able to identified the metabolite biomarkers.

In this study, we have carried out the analysis of the clinical and biochemical indicator. There was no significant difference in mean age, sex, BMI, ALB,
TB, BUN, creatinine, glucose, triglyceride, cholesterol and PLT among these two groups. On the contrary, a significant increase in the activities of AST and ALT was observed in patients with CHB, indicating considerable hepatocellular injury (Ganem and Prince, 2004). According to the GC/MS TIC chromatograms displayed in Figure 1, the majority of the peaks in the chromatograms were identified by NIST mass spectra library, including ketones, aldehydes, fatty acids, amino acids, organic acids, and so on. These metabolites are known to be involved in multiple biochemical processes, especially in energy and lipid metabolism (Postic et al., 2004; Wu et al., 2009). In this study, we constructed a PCA, HCA, and PLS-DA model to obtain metabolomic profiling. A clear separation was observed between the CHB and control groups. These results indicate that the models had a good ability of explaining and predicting.

Discriminatory metabolites were significantly identified as palmitic acid, stearic acid, oleic acid, benzoic acid, butanoic acid, cholesterol, glycine, 3-heptanone, 4-heptanone, hexanal, 1-tetradecanol and naphthalene Table 2. The present study adds evidence to support the previous results of metabolomic study that the levels of some fatty acids and amino acids are altered in CHB patients (Zhang et al., 2013a; Yang et al., 2016). Previous reports have shown significant increases in levels of glycine, benzoic acid and butanoic acid in CHB patients compared with the control groups (Zhao et al., 2013). Lu et al., (2015) have also reported increased levels of glycine, stearic acid, oleic acid and cholesterol, in a study performed by LC/MS and GC/MS, when comparing the urinary metabolomic profiles of CHB patients and control groups. Moreover, a recent GC/MS-base metabolomics study of CHB patients identified the metabolites glycine, cholesterol, palmitic acid, stearic acid and oleic acid as discriminatory metabolites which corroborate the finding in this study (Yang et al., 2016). In our study, we observed increased levels of saturated fatty acids, such as palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and cholesterol in CHB patients, which suggests that chronic HBV infection may interfere with fatty acid metabolism. In addition, Lu et al., (2015) have reported increased levels of fatty acids in hepatitis B related HCC that might be the results of energy requirement and cell membrane synthesis due to aggressive cell proliferation.

Moreover, the pathway analysis of our study confirmed the alteration of cholesterol, bile acid and bile salt metabolism, as shown in Supplemental Figure 3. Bile acids are steroidal amphipathic molecules, derived from the catabolism of cholesterol. Bile acids are involved in signal transduction pathways that regulate apoptosis (St-Pierre et al., 2001). Previous clinical observations showed increased conjugated bile acid as well in other HBV-induced liver diseases such as liver failure (Nie et al., 2014) and cirrhosis (Wang et al., 2016). Furthermore, a recent report revealed that the alterations of bile acid metabolism could characterize the different stages of CHB progression (Huang et al., 2016).

The urine level of glycine, a non-essential amino acid, was significantly increased in CHB patients compare with the control group, which suggested the abnormality of amino acid metabolism (Zhao et al., 2013). From a previous study, an increased urinary glycine level was also observed in hepatitis B related HCC, which may be attributed to the increased glycolysis in tumor (Yang et al., 2007; Wu et al., 2009). Glycine is involved in glutathione (GSH) metabolism and bile synthesis (Lu, 1999). Additionally, glycine exerts anti-inflammatory, cytoprotective, and immunomodulatory properties and rescues liver injury through attenuation of oxidative stress, and apoptosis (Chen et al., 2013).

Interestingly, the changes of benzoic acid and butanoic acid are associated with gut microflora. Benzoic acid is the metabolite from phenylalanine metabolism in bacteria. It is mainly produced when gut bacteria obtains polyphenols from food and aromatic amino acids from food protein decomposition (Rechner et al., 2004). Butanoic acid is a short-chain fatty acids mainly generated from dietary cellulose, starch and other undigested substances under microbial fermentation in the cecum or colon (Mao et al., 2012).

In addition, the levels of hexanal, heptanone and naphthalene were significantly increased in CHB patients comparing with control subjects. Hexanal is a breakdown product of lipid peroxidation that is formed as a result of oxygen free radical activity. The specificity of this marker for different cancers is under investigation (Xue et al., 2008a). The origin of 3-heptanone and 4-heptanone is still unknown but is derived probably from an exogenous source (Mills and Walker, 2001). It has been reported that 4-heptanone is produced from the in vivo metabolism of plasticizers in man (Walker and Mills, 2001). Moreover, the source of the metabolites identified to derive from naphthalene is not yet known; they may be the degradation products of steroids (Turfft, 1948; Annweiler et al., 2000).

There are some limitations of our study that need to be addressed, i.e. it was a cross-sectional study with a small sample size, and conducted in a single center. Further studies with larger population and multi-analytical techniques are required to confirm the findings.

In summary, GCMS-based urine metabolomics profiling coupled with multivariate statistical analysis is able to identify metabolite biomarkers which can significantly differentiate CHB patients from normal subjects with high sensitivity and specificity. The metabolic signature of CHB patients comprised metabolite changes associated with the metabolism of fatty acid, bile acid, amino acid and gut microbiomes. CHB-related alteration included palmitic acid, stearic acid, oleic acid, benzoic acid, butanoic acid, cholesterol, glycine, 3-heptanone, 4-heptanone, hexanal, 1-tetradecanol and naphthalene metabolites. Our study indicates that GC/MS-based metabolomics is a promising tool that could provide insights into the metabolomics study in CHB. Further study of these metabolites may facilitate the development of non-invasive biomarkers and more efficient therapeutic strategies for CHB patients.

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