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Metabolic urinary profiling of alcohol hepatotoxicity and intervention effects of Yin Chen Hao Tang in rats using ultra-performance liquid chromatography/electrospray ionization quadruple time-of-flight mass spectrometry

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ABSTRACT

This paper was designed to study metabonomic characters of the hepatotoxicity induced by alcohol and the intervention effects of Yin Chen Hao Tang (YCHT), a classic traditional Chinese medicine formula for treatment of jaundice and liver disorders in China. Urinary samples from control, alcohol- and YCHT-treated rats were analyzed by ultra-performance liquid chromatography/electrospray ionization quadruple time-of-flight mass spectrometry (UPLC/ESI-QTOF-MS) in positive ionization mode. The total ion chromatograms obtained from the control, alcohol- and YCHT-treated rats were easily distinguishable using a multivariate statistical analysis method such as the principal components analysis (PCA). The greatest difference in metabolic profiling was observed from alcohol-treated rats compared with the control and YCHT-treated rats. The positive ions *m/z* 664.3126 (9.00 min) was elevated in urine of alcohol-treated rats, whereas, ions *m/z* 155.3547 (10.96 min) and 708.2932 (9.01 min) were at a lower concentration compared with that in urine of control rats, however, these ions did not indicate a statistical difference between control rats and YCHT-treated rats. The ion *m/z* 664.3126 was found to correspond to ceramide (d18:1/25:0), providing further support for an involvement of the sphingomyelin signaling pathway in alcohol hepatotoxicity and the intervention effects of YCHT.

1. Introduction

Low molecular mass metabolites, which are key components of cellular regulatory and metabolic processes in biological systems, have been shown to respond to environmental changes [1,2]. In order to study one or two metabolites in clinical chemistry, metabolomics is a multitargeted analysis of low molecular mass (1000 Da) endogenous and exogenous metabolites [3]. As one of the important systems biology, metabolomics is a promising tool for investigating toxicity and disease states in biological systems. A lot of studies have investigated the effects of toxicity in experimental animals, but to fully understand genetic differences and their effects on toxicological damage and disease development, the metabolomics combined with the other "omics" sciences needs to emerge [4,5]. The evolution of systems biology will allow metabolites to be correlated to their respective metabolic pathways and will establish a statistical relationship between metabolites, protein levels, and gene expression levels.

The metabolomics is highly analytical in nature. To obtain useful diagnostic information, as many low molecular mass metabolites as possible must be quantified and compared with normal samples. Nowadays, the metabonomic data are commonly analyzed by means of two key techniques: mass spectrometry (MS) coupled with gas chromatography (GC/MS) [6,7] or liquid chromatography (LC/MS) [8,9], and nuclear magnetic resonance (NMR) spectroscopy [4,10], which are all powerful analytical tools when combined with multivariate statistical analyses.

Alcohol liver disease (ALD) remains one of the most common pathogenic factor of chronic liver diseases in the world [11], ALD and chronic viral hepatitis are leading causes of cirrhosis and hepatocellular carcinoma in the world [12,13]. It has been reported that several mediator systems were involved with the development of ALD from fatty liver to advanced liver injury, such as inflammation and necrosis [14]. Alcohol fatty liver was much more suscepti-

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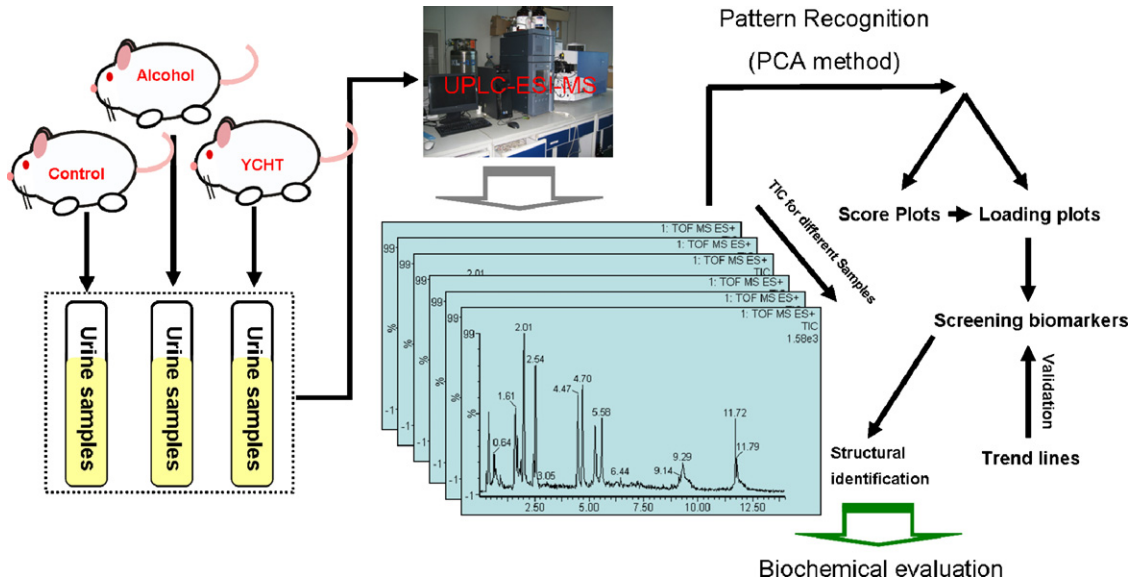

Fig. 1. Flowchart of metabonomic analysis in this study.

ble to many inflammatory stimuli [15], the bacterial endotoxins mainly participated in the inflammatory process. Moreover, oxidative stress or ischemic damage also seemed to aggravate the hepatic inflammation induced by alcohol [16].

Recently, the herbal drugs played an important role in the therapy of liver disorders [17,18]. Yin Chen Hao Tang (YCHT) is a classical traditional Chinese medicine (TCM) formula, which is an aqueous extract from the mixture of three herbal drugs: flos of *Artemisia capillaries* Thunb (Compositae), fruit of *Gardenia jasminoides* Ellis (Rubiaceae), and root of *Rheum officinale* Baill (Polygonaceae), used for treating hepatitis C [19], primary biliary cirrhosis [20], and cholestatic liver diseases [21,22] in clinical.

The purpose of this study is to investigate the metabolic profiling of hepatotoxicity induced by alcohol and the positive intervention effects of YCHT in rats using a metabonomic method. The overall flowchart has been shown in Fig. 1.

2. Experimental

2.1. Chemicals and reagents

Yin Chen Hao Tang recorded in "*Shanghanlun*" [23] consists of accurate 18.0 g of the flos of *A. capillaries* Thunb (Compositae) (YCH), 9.0 g of the fruit of *G. jasminoides* Ellis (Rubiaceae) (ZZ), and 6.0 g of the root of *R. officinale* Baill (Polygonaceae) (DH). The flos of *A. capillaries* Thunb (Compositae) was purchased from China Company of Xinneitian Pharmaceutical Company of Japan (Tianjin, China); the fruit of *G. jasminoides* Ellis (Rubiaceae) and the root of *R. officinale* Baill (Polygonaceae) were purchased from Harbin Tongrentang Drug Store (Harbin, China). All these crude drugs were authenticated by Professor Xijun Wang of the Department of Pharmacognosy, Heilongjiang University of Chinese Medicine. The experimental samples in the form of freeze-dried powders were produced according to the method recorded in "*Shanghanlun*". First, 18 g of YCH was boiled in 1400 mL of H_2O until the solution volume was reduced to 700 mL, then 9.0 g of ZZ and 6.0 g of DH were added and the solution was kept boiling for 10 min. The mixture was filtered through five-layer bandage; finally, the filtrate was prepared as freeze-dried powders for experimental usage.

Acetonitrile (HPLC grade) was purchased from Sigma–Aldrich (MO, UK); formic acid (analytical grade) was purchased from Beijing Reagent Company (Beijing, China); the deionized water was purified by Milli-Q system (Millipore, Bedford, MA, USA); leucine enkephalin was purchased from Sigma–Aldrich (MO, UK); alcohol was purchased from Beijing Reagent Company (Beijing, China); the assay kits of alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyltranspeptidase (γ-GT), triglyceride (TG), and alcohol dehydrogenase (ADH) were purchased from Zhongsheng Beikong Biological Technology Limited Corporation (Beijing, China); the assay kits of malondialdehyde (MDA), and glutathion (GSH) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.2. Animal handling and sampling

30 male Wistar rats (age, 6 weeks; body weight, 200 ± 20 g) were purchased from the Shanghai Slac Laboratory Animal Limited Corporation (Shanghai, China). All animals were kept in animal room with temperature: 25 ± 2 °C, humidity: 60 ± 5%, and 12 h dark to light cycle for 1 week before the experiment. All rats were randomly divided into control group (CG), ethanol group (EG), high dosage group of YCHT (HG), middle dosage group of YCHT (MG), and low dosage group of YCHT (LG). All animals were allowed to acclimatize in metabolism cages for 3 days prior to treatment. The CG rats were orally administrated by water between 11:00 and 12:00 a.m. from day 1 to day 7, the EG rats was orally administrated by water between 11:00 and 12:00 a.m. from day 1 to day 2, and was orally administrated by 50% alcohol at a dose of 5 g/(kg/day) between 11:00 and 12:00 a.m. from day 3 to day 7. The HG, MG and LG rats were orally administrated by freeze-dried powders of YCHT at a dose of 4.8, 2.8, and 0.6 g/(kg/day), respectively, between 8:00 and 9:00 from day 1 to day 7, simultaneously, all rats of HG, MG and LG were orally administrated by 50% alcohol at a dose of 5 g/(kg/day) between 11:00 and 12:00 a.m. from day 3 to day 7. The control morning urine was collected from CG rats from 18:00 p.m. on day 7 to 6:00 a.m. on day 8. The individually dosed morning urine was collected respectively from EG, HG, MG and LG rats from 18:00 p.m. on day 7 and 6:00 a.m. on day 8. Food (standard diet) and water (uniform labeled water) were available between 12:00 and 6:00 throughout the study. Samples were stored at −20 °C prior to analysis; for UPLC/MS samples were prepared by centrifugation at 13,000 rpm (5 °C).

Table 1
UPLC gradient elution program

Time (min)	A/% (0.1% formic H_2O)	B/% (0.1% formic ACN)	Curve
Initial	98	2	Initial
2	98	2	6
3	80	20	6
7	5	95	6
9	5	95	6
11	98	2	6
15	98	2	6

2.3. UPLC-ESI-MS conditions

The UPLC/MS analysis was carried out using a Waters ACQUITY UPLC system (Waters Corporation, Milford, USA) coupled with a with a Waters Micromass Q-tof micro™ Mass Spectrometer (Manchester, UK). The data were obtained using MassLynx 4.1 (Waters Corporation, Milford, USA), and the MarkerLynx Application Manager (Waters Corporation, Milford, USA) was used for the peak detection and for principal component analysis.

For the reversed-phase UPLC analysis, the ACQUITY UPLC™ BEH C_{18} column (50 mm × 2.1 mm i.d., 1.7 μm, Waters Corporation, Milford, USA) was used. The column temperature was maintained at 4 °C. The flow rate of the mobile phase was 400 μL/min. The injection volume was fixed at 5 μL. Mobile phase A consisted of 0.1% formic acid in water, while mobile phase B consisted of 0.1% formic acid in acetonitrile. The column was eluted with a linear gradient (Table 1).

An ESI interface was used, and the profile data for positive ions from *m*/*z* 50–900 were recorded. The source temperature was set at 110 °C with a cone gas flow of 100 L/h, a desolvation gas temperature of 300 °C and a desolvation gas flow of 600 L/h were employed. The capillary voltage was set at 3.2 kV in positive ion mode and 2.6 kV in negative ion mode and the cone voltage was set 35 V. A scan time of 0.4 s with an inter-scan delay of 0.1 s was used throughout, with collision energy of 4 eV. A lock-mass of leucine enkephalin at a concentration of 0.2 ng/μL, in acetonitrile (0.1% formic acid): H_2O (0.1% formic acid) (50:50, v/v) for positive ion mode ($[M+H]^+ = 556.2771$) were employed via a lock spray interface. Data were collected in centroid mode, the lock spray frequency was set at 5 s and the lock mass data was averaged over 10 scans for correction. A "purge–wash–purge" cycle was employed on the auto-sampler, with 90% aqueous formic acid used for the wash solvent and 0.1% aqueous formic acid used as the purge solvent, this ensured that the carry-over between injections was minimized. The mass spectrometric data was collected in full scan mode from *m*/*z* 50 to 900 from 0 to 10 min in positive ion mode.

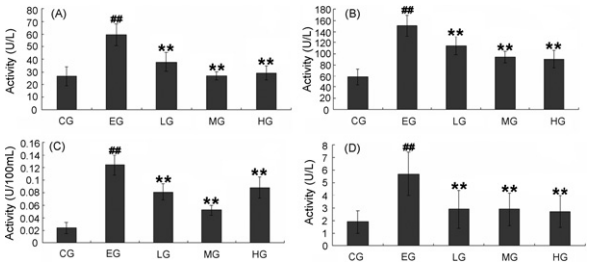

Fig. 2. Effect of alcohol on activities of AST, ALT, γ-GT and ADH in rat serum. The data were expressed as Mean ± S.D. ##Significant difference compared with CG groups ($p < 0.01$); **Significant difference compared with EG groups ($p < 0.01$). A, ALT; B, AST; C, γ-GT; D, ADH.

2.4. Clinical chemistry and histopathology

On day 8, all rats were sacrificed. The collected samples of rat serum and liver tissue of every group were characterized on an Automated Synchron LX20 Beckman-Coulter analyzer by following clinical chemical parameters according to the corresponding method: ALT, AST, γ-GT, TG, ADH, MDA, and GSH. The liver histological sections of control, alcohol-, and YCHT-treated rats were examined by light microscopy after being stained with hematoxylin and eosin (H and E).

3. Results

3.1. Clinical chemistry and histopathology

The activities of the plasma enzymes: ALT, AST, γ-GT, and ADH were markedly increased after exposing to alcohol for 5 days compared with CG, and all these increases were obviously intervened by YCHT in different groups, the intervention effect in HG was most obvious (Fig. 2). The contents of TG and MDA were significantly increased, the activities of hepatocytes enzymes of GSH were significantly decreased after exposing to alcohol for 5 days,

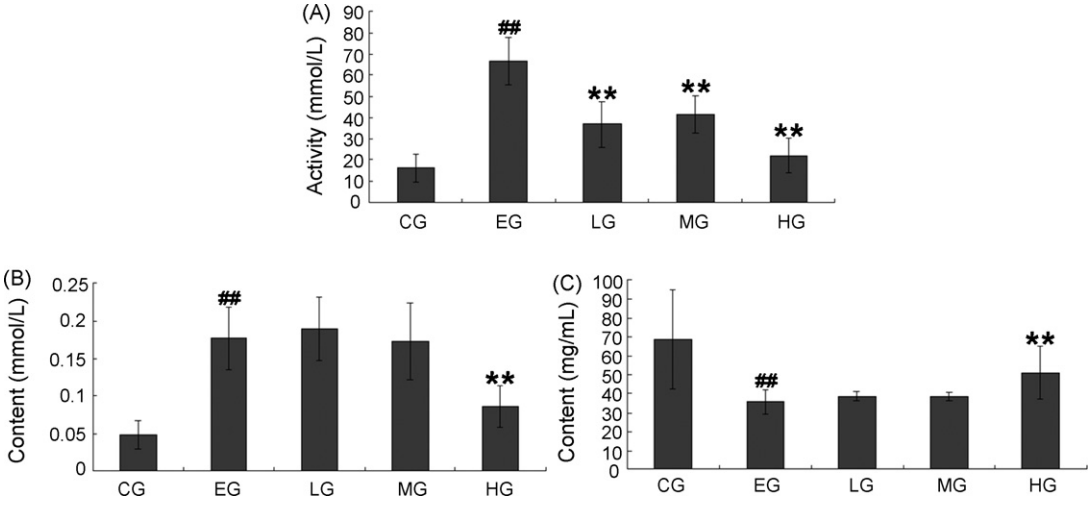

Fig. 3. Effect of alcohol on content of TG and MDA, and activity of GSH in rat hepatocytes. The data were expressed as Mean ± S.D. ##Significant difference compared with CG groups ($p < 0.01$); **Significant difference compared with EG groups ($p < 0.01$). A, TG; B, MDA; C, GSH.

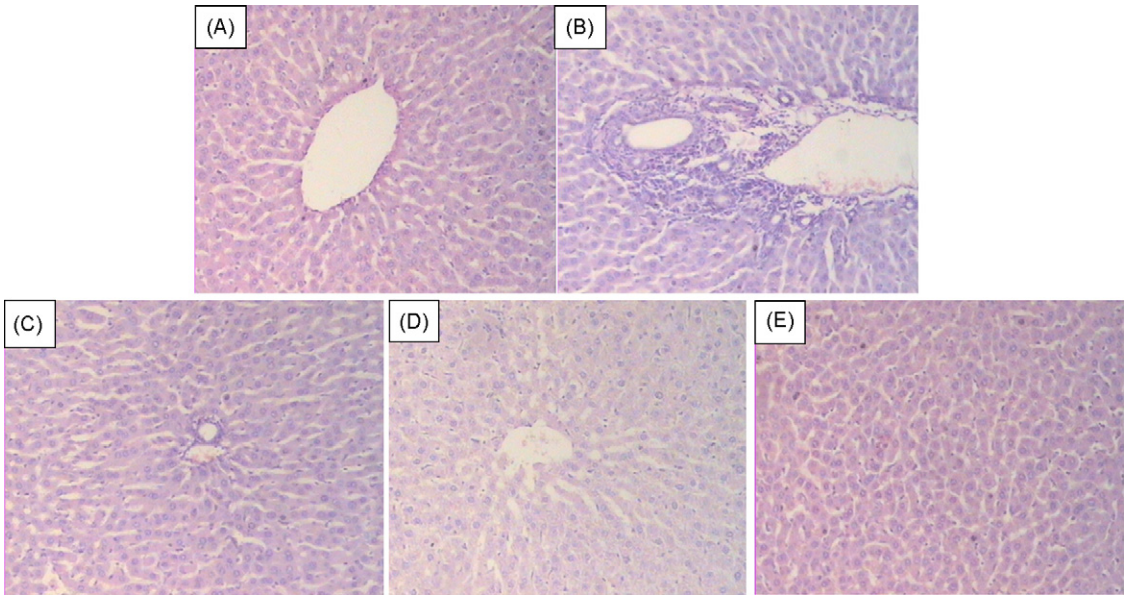

Fig. 4. Hepatohistological observation. A, control rat liver; B, alcohol-treated rat liver; C, alcohol + LG-treated rat liver; D, alcohol + MG-treated rat liver; E, alcohol + HG-treated rat liver.

but these changes were significantly improved by YCHT only in HG (Fig. 3).

Histopathological changes of rats' liver were shown in Fig. 4, the liver of alcohol treated rats showed markedly feathery degeneration, micro- and macrocellular fatty changes, periportal fibrosis and vascular congestion compared with CG. In the YCHT-treated group, the pathological abnormalities were improved obviously, and the effect of HG was most noticeable, which almost showed normal histology with mild congestion of central vein. It was concluded that the animal model of alcohol hepatotoxicity in this study was successfully reproduced and YCHT was demonstrated to intervene its effect positively, and action of HG was mostly strong, so metabolic profiling in following experiments only paid close attention to the urine samples of high dose group rats.

3.2. Validation of UPLC conditions

The flow rate into the electrospray ion source recommended was limited within 0.4 mL/min in order to reach the best ionization efficiency and avoid the ion suppression which will seriously influence the analytical sensitivity. Considering sensitivity and resolution meanwhile, the ultimate flow rate was optimized at 0.4 mL/min in this study. Furthermore, the effects of acid modifier (e.g., formic acid) with different concentrations (1, 0.1, and 0.01%) were characterized, the optimal peak resolution and shape was obtained by 0.1% formic acid. The liquid chromatography column temperature was set at 25 °C to reduce the column pressure resulting from a higher flow rate. The gradient elution was adopted to obtain better separation. Six batches of urinary samples from the same rat were injected on the UPLC column for evaluating the reproducibility of the chromatography. The retention time of visible peaks varied with the R.S.D. values within the range of 0.05–0.8% during the analysis, suggesting that the retention time reproducibility of the system was good for large-scale sample analysis. For testing the ability of the UPLC system to differentiate, the control, alcohol- and YCHT-treated rat urine were analyzed. Typical chromatograms of these samples were displayed in Fig. 5. Visual examination of these chromatograms showed distinct among different samples.

3.3. Optimization of mass conditions

The desolvation gas flow rate was set at 600 L/h, and desolvation temperature at 300 °C, respectively to remove redundant solvent resulting from a higher flow rate of 0.4 mL/min for mass spectrometer. Cone voltage was optimized to the corresponding values ranging from 15 to 90 V for the broad ranges of low molecular weights metabolites (50–900 Da). The results indicated that a cone voltage of 35 V was sufficient to produce product ions in the ES+ mode for metabolites. As LC peaks become narrower for UPLC system, the mass spectrometer was required to acquire data faster. The faster duty cycle of the TOF instrumentation should allow rapid data collection and good quality MS/MS analysis. In this study, the LC eluent was analyzed in positive ion electrospray without splitting eluent. The data were collected along with alternating collision energy, at 5 eV for parent ion information and range of 15–25 eV for fragment ion information. The data acquisition rate of 100 ms per channel with a 20 ms interchannel delay was used. The resolution was greater than 5000 in both high and low collision energy modes. The Q-TOF instrument was switching with a frequency of 100 ms and we could see that the frequency was sufficiently rapid to keep the chromatographic information while still obtaining good MS/MS data.

3.4. Analysis of metabolic profiling

Fig. 6 showed the representative BPI chromatograms for the reversed-phase UPLC-ESI-TOF-MS analysis of urine samples collected from the control rats, alcohol-treated rats, and YCHT-treated rats at day 9. Although some differences could be visually noted among the three sets of detail lustrated in Fig. 6, more subtle changes could be visualized using a pattern recognition approach, such as principal components analysis (PCA). Fig. 6 presented the PCA results from samples of the 9th day. It was clear that a distinct clustering of control rat, alcohol-treated rat, and YCHT-treated rat urine samples has been achieved from the PCA scores plot. The corresponding loadings plot indicated that the ions and their chromatographic retention times were attributable to the clustering observed in the scores plot. A large number of peaks could be

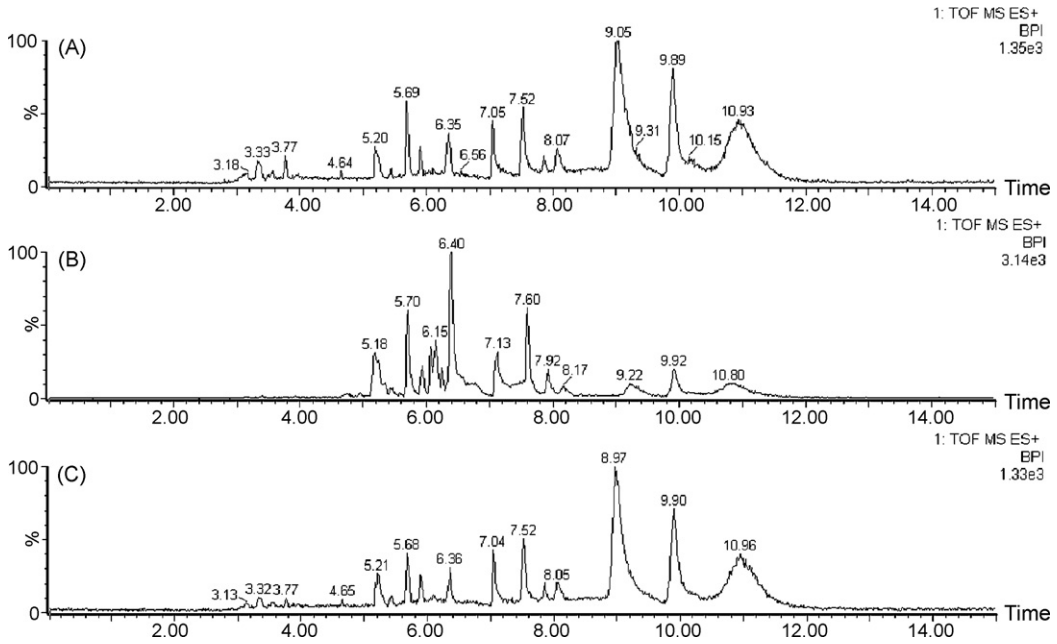

Fig. 5. BPI chromatograms of (A) control rat; (B) alcohol-treated rat; (C) YCHT-treated rat urine samples at day 7 using reversed-phase UPLC-ESI-TOF-MS.

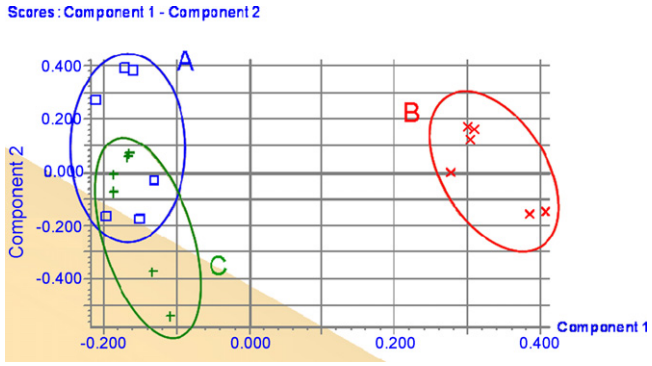

Fig. 6. Resulting scores plot from PCA of reversed-phase UPLC-ESI-TOF-MS data obtained from (A) control rat; (B) alcohol-treated rat; (C) YCHT-treated rat urine samples collected from day 7. Symbols, control rat (□); alcohol-treated rat (×) and YCHT-treated rat (+).

detected using the positive ion mode compared with using the negative ion mode. Therefore, the positive ion mode was selected in this study. Among them, three dominant ions *m*/*z* 664.3126 (9.00 min), 155.3547 (10.96 min) and 708.2932 (9.01 min), respectively (Fig. 7), and the amount of three ions from alcohol-treated group was statistically different from that of the control and YCHT-treated group. The trend lines for three ions were shown in Fig. 8. These three compounds were considered to be intrinsic compounds and were detected from both the control and YCHT-treated groups. The existence ratio of *m*/*z* 155.3547 in the control and YCHT-treated rats was approximately 3-fold higher compared with that in the alcohol-treated rats at day 9. The existence ratio of *m*/*z* 664.3126 in the alcohol treated rats was approximately fivefold higher compared with that in the control rats and YCHT-treated group at day 9, and the existence ratio of *m*/*z* 708.2932 in the alcohol-treated rats was approximately accordant with that in the control and YCHT-treated rats at day 9. The results suggested that these three compounds

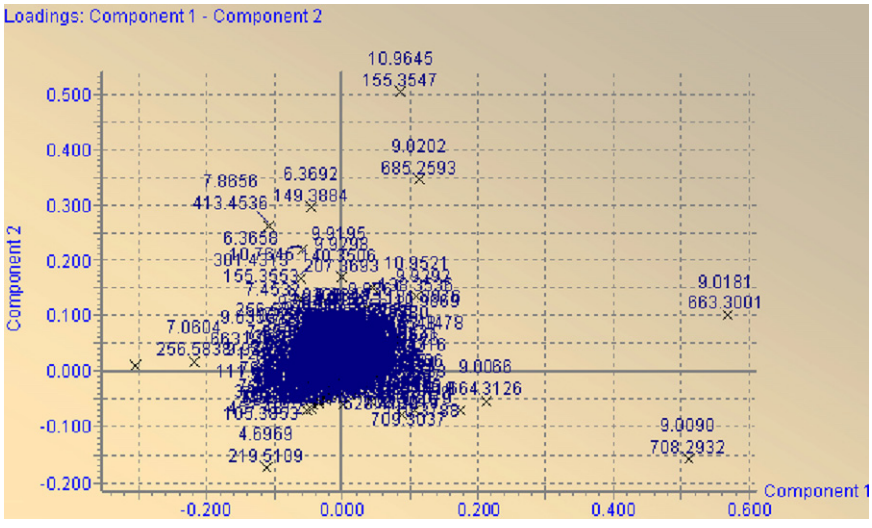

Fig. 7. Resulting loadings plot from PCA of reversed-phase UPLC-ESI-TOF-MS data obtained from control rat, alcohol-treated rat and YCHT-treated rat urine samples collected from day 7.

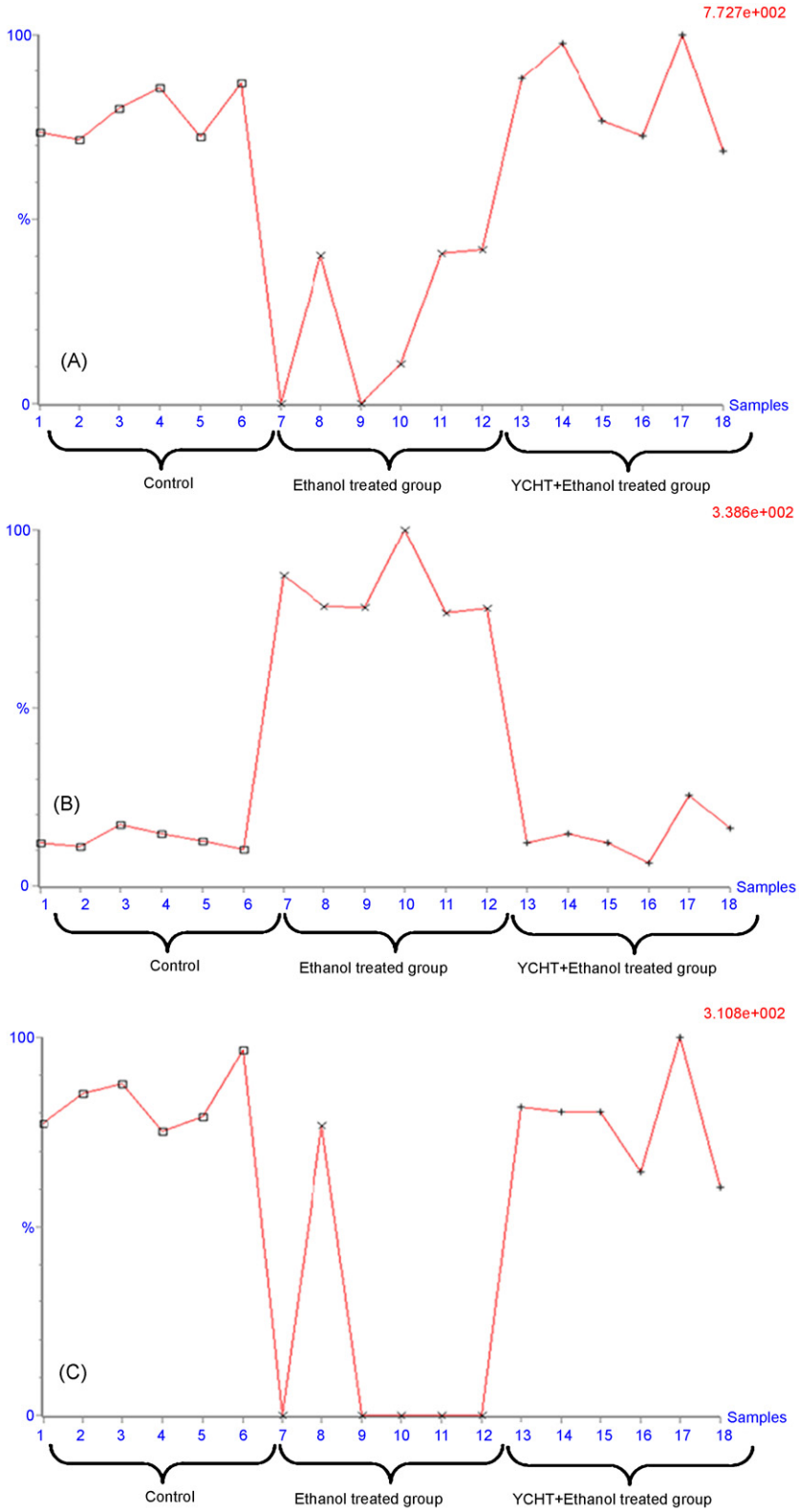

Fig. 8. Trend lines for the *m*/*z* 155.3547, 664.3126, and 708.2932 ions among control, alcohol-treated rat and YCHT-treated rat's urinary samples at day 7. Symbols, control rat (□); alcohol-treated rat (×) and YCHT-treated rat (+).

might be used as biomarkers for diagnosis of alcohol hepatotoxicity. The structure of *m*/*z* 664.3126 was preliminarily identified as ceramide (d18:1/25:0). We have been detecting the structures of *m*/*z* 155.3547 and *m*/*z* 708.2932.

4. Discussion

Based on the evaluations of clinical chemistry and histopathology, a rapid and validated UPLC/ESI-QTOF-MS method has been

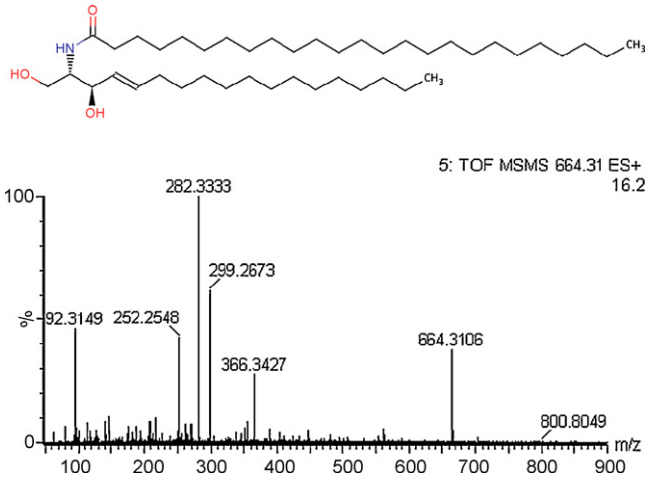

Fig. 9. Chemical structure and mass fragments information of ceramide (d18:1/25:0).

developed to investigate the metabolic profiling of alcohol hepatotoxicity and intervene of YCHT in rats. It was clear that a distinct clustering of urine samples from alcohol-treated rats has been demonstrated in scores plot compared with control rats. However, a weakly different clustering of urine samples was found between control and YCHT-treated rats, and these results suggested that YCHT have interfered partially the alcohol hepatotoxicity in preliminary. The corresponding loadings plot indicated that the ions and their chromatographic retention times were attributable to the clustering observed in the scores plot. Among these ions, three dominant ions *m*/*z* 664.3126 (9.00 min), 155.3547 (10.96 min) and 708.2932 (9.01 min) were observed. These results obviously demonstrated that three compounds were marker compounds, which might be related to developing of alcohol hepatotoxicity. The structure of *m*/*z* 664.3126 was preliminarily identified as ceramide (d18:1/25:0) (d18:1/25:0) (Fig. 9). The structures of *m*/*z* 155.3547 and *m*/*z* 708.2932 have been in progress.

The ceramide (d18:1/25:0) is generated by de novo synthesis, sphingomyelin hydrolysis, and recycling of sphingolipids. Ceramide (d18:1/25:0) has been known to be involved in the regulation of several cellular processes, including differentiation, growth suppression, cell senescence, and apoptosis [24]. Importantly, cell apoptosis induced by ceramide (d18:1/25:0) has been attracted much attention in recent years. Ceramide (d18:1/25:0) has been also extensively studied for its role in the cellular response to stress and inflammation. Some key inducers and mediators of host in acute phase response (APR), such as bacterial lipopolysaccharide (LPS), interleukin-1b (IL-1b), and tumor necrosis factor-α (TNF-α), induced early generation of ceramide (d18:1/25:0) in the liver [25–27]. It has been reported that TNF-α was involved in liver injury during alcohol hepatitis [28]. The observation found that astrocytes prepared from rats prenatally exposed to alcohol were much more susceptible to the cytotoxic effect of TNF-α, suggesting that alcohol exposure could increase the sensitivity of astrocytes to the cytotoxic effect of TNF-α, which might be attributed to the altered metabolism of sphingolipids, resulting in the increased production of ceramide (d18:1/25:0) [29], finally further excreting into urine and elevating the level of urine ceramide.

Above results suggested that alcohol strongly affected the sphingomyelin signaling pathway in the liver by activation of TNF-α (Fig. 10). Then TNF-α activated ceramide (d18:1/25:0) in alcohol-treated group compared with the control and YCHT-treated groups. These factors could ultimately lead to hepatocyte necrosis. So the urine ceramide (d18:1/25:0) might be used as a biomarker for diagnosis of alcohol hepatotoxicity.

5. Conclusion

In this study, the UPLC-ESI-MS combined with PCA technology has been used for detecting urine metabolic profiling and data interpretation based on validated assessments of clinical chemistry and histopathology. The unsupervised PCA has been applied to sample classification. This method was applied to collect urine samples from control, alcohol- and YCHT-treated rats. From the PCA scores plot, a distinct clustering of the three groups based on their urine samples has been clearly demonstrated. Consequently, we have succeeded in detecting three potential biomarker associated

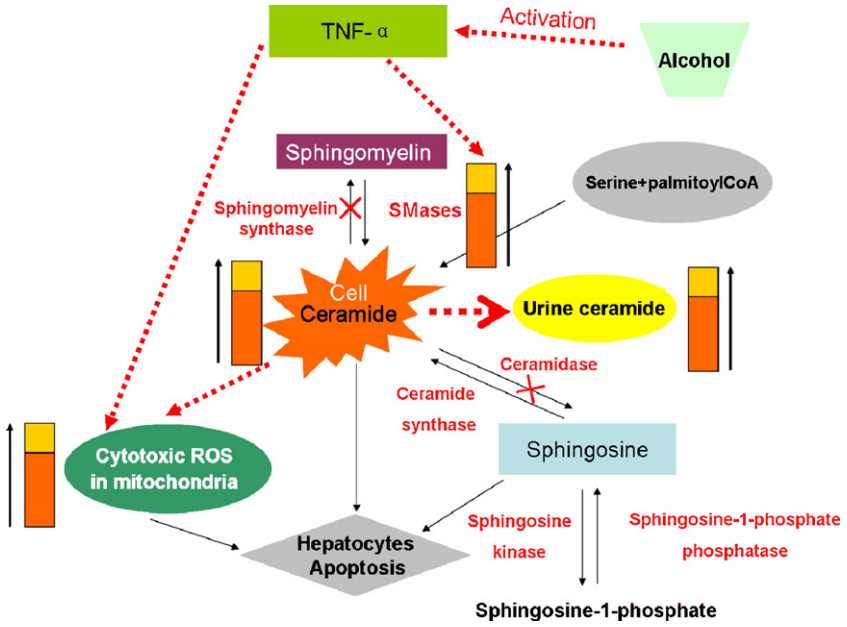

Fig. 10. The proposed metabolic pathway for explanation relationship between alcohol hepatotoxicity and the increased contents of ceramide (d18:1/25:0) in urine.

with alcohol hepatotoxicity. The trends lines revealed the contents of those biomarkers were obviously different in alcohol-treated rats from control and YCHT-treated rats. The results also demonstrated that alcohol has perturbed normal metabolic process of rats. YCHT indicated some intervention on alcohol hepatotoxicity by improving disturbed metabolic process. This simple study demonstrated that metabonomics, one of the most important systems biology platforms, showed a potential for identifying and characterizing biochemical responses of rat to chemicals (e.g., alcohol). Meanwhile, this strategy offered a practical method for performing intervened assessments of traditional Chinese medicines (TCM) in the future.

Acknowledgments

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