Journal of Pharmaceutical and Biomedical Analysis 53 (2010) 559–566

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Metabonomic study of biochemical changes in the urine of Morning Glory Seed treated rat

Chao Ma, Kaishun Bi, Ming Zhang, Dan Su, Xinxin Fan, Wei Ji, Chao Wang, Xiaohui Chen*

School of Pharmacy, Shenyang Pharmaceutical University, Wenhua Road 103, Shenyang 110016, PR China

ARTICLE INFO

Article history:
Received 30 January 2010
Received in revised form 24 March 2010
Accepted 25 March 2010
Available online 31 March 2010

Keywords:
Morning Glory Seed
Nephrotoxicity
Metabonomics
UPLC/MS

ABSTRACT

This paper was designed to study metabonomic characters of the nephrotoxicity induced by Morning Glory Seed (MGS), a well-known traditional Chinese medicine which was used for the treatment of edema, simple obesity and lung fever. Urinary samples from control and MGS treated rats were analyzed by ultra-performance liquid chromatography/mass spectrometry (UPLC–MS) in positive ionization mode. Blood biochemistry and histopathology were examined to identify specific changes of renal damage. The results affirmatively suggested that ethanol extract of Morning Glory Seed (EMGS), instead of water extract of Morning Glory Seed (WMGS), should be responsible for the nephrotoxicity caused by this herbal medicine. The UPLC–MS analysis revealed that the levels of 8 endogenous metabolites as biomarkers were significantly changed in urine from EMGS treated rats. The underlying regulations of EMGS-perturbed metabolic pathways were discussed according to the identified metabolites. The present study proves the potential of UPLC–MS based metabonomics in mapping metabolic response for toxicology.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Morning Glory Seed, also called QianNiuZi (Chinese name), Semen Pharbitidis (Latin name) and Pharbitis seed (English name), is prepared from dried mature seeds of *Pharbitis nil* (L.) Choisy or *Pharbitis purpurea* (L.) Voigt. It is well known as a valuable traditional Chinese medicine because of the therapeutic effect on relieving constipation by purgation, dispersing phlegm and washing excessive fluid. Morning Glory Seed is widely used for the treatment of edema, ascites, simple obesity and lung fever [1,2]. As an obstetrical medicine, Morning Glory Seed could induce labor effectively [3]. It is also reported that Morning Glory Seed diets could decrease the serum total cholesterol, triglyceride, LDL-cholesterol and γ-GTP levels that were increased by chronic ethanol administration [4]. This medicine has been officially listed in the Chinese Pharmacopoeia for a long time because of its definite therapeutic effect.

However, previous clinical and animal study indicated that excessive or long-term use of this herbal medicine will cause serious renal and nervous system damage, the clinical manifestations varied from lumbago, oliguria to hematuria [5–8]. Nephrotoxicity is of critical concern when selecting new drug candidates during the early stage of drug development. Because of its unique metabolism, the kidney is an important target of drug toxicity. Although the mechanism responsible for the nephrotoxicity induced by Morning Glory Seed was not yet completely understood, there's no doubt that excessive or long-term use of this herbal medicine will finally result in renal damage. To our knowledge, the studies on the diagnosis of nephrotoxicity mainly focused on routine test of some traditional biochemical markers, which could not fully expounded this disease and predict it in advance.

Metabonomics is a new methodology arising from the post-genomics era which is defined as the quantitative approach to study "the dynamic metabolic response of living systems to pathophysiological stimuli or genetic modification" [9,10]. Metabonomics has the ability to rapidly assess potential toxicity and monitor toxicity during chronic drug administration without the scientific bias associated with predefined clinical measurements. The ability to measure global alterations in metabolism in tissues and biofluids that precede conventional biochemical and pathological changes has contributed to the emergence of metabonomics as a promising scientific platform for safety assessment.

Analytical methods based on MS and NMR have been more and more used in metabonomics. Compared with NMR, MS is a more sensitive technique. When MS is coupled to liquid chromatography, higher resolution and sensitivity can be achieved for the low abundance metabolite. To obtain a higher throughput and more comprehensive profiles, new LC systems and columns with sub-2 μm particle stationary phases have arisen and shown their advantages in the metabolic profiling studies. UPLC is one of these

* Corresponding author. Tel.: +86 24 23986296; fax: +86 24 23986259.
E-mail address: cxh_syphu@yahoo.com.cn (X. Chen).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.03.034

techniques and has been widely adopted in many metabonomics investigations.

Based on the reasons above, in the present study, ultra-performance liquid chromatography coupled to mass spectrometry (UPLC/MS) system was used for the metabolic profiling of rat urine. With principal component analysis (PCA), we investigated the difference of metabolic profiling among the urine samples of rats in model groups treated with water extract and ethanol extract of Morning Glory Seed, the positive control group treated with extract of Caulis Aristolochiae Manshuriensis and the healthy control group. Furthermore, global metabolite profiling was used to study whether changes in the metabolite profile were associated with changes in physiopathology. The results of this investigation will provide useful information for the further study in toxicity assessment of Morning Glory Seed.

2. Experimental

2.1. Chemicals, regents and herbal material process

Acetonitrile and formic acid (HPLC grade) were purchased from Fisher Corporation (USA), water was purified by redistillation and was filtered through 0.22 μm membrane filter before use. The standards of methionine, tryptophan, phenylalanine, creatinine, citric acid, homocysteine and cholic acid were purchased form Sigma–Aldrich (MO, USA). Morning Glory Seed and the positive control drug Caulis Aristolochiae Manshuriensis (CAM) were provided by Liaoning Chinese Herbal Medicine Factory (Shenyang, China) and authenticated by Professor Qi-shi, Sun in Traditional Chinese Medicine College, Shenyang Pharmaceutical University.

The dried and pulverized plant material samples of Morning Glory Seed were processed as follows: 1000 g Morning Glory Seed was divided into two parts equally, they were macerated with 2000 ml water and ethanol respectively for 12 h .The maceration was performed 3 times and the solvents were removed under reduced pressure. Then the ethanol and water extract of Morning Glory Seed were redissolved with water and diluted to a volume equivalent to 1.5 g MGS plant material per milliliter (1.5 g MGS/ml). 500 g CAM was refluxed with 1000 ml water for 60 min twice, and the solvents were evaporated in vacuum. The CAM solution for experiment use was diluted to 1.5 g CAM/ml.

2.2. Animals and treatment

28 male Wistar rats of 6–8 week-old weighing 200–220 g from Shanghai Slack Laboratory Animal Co. Ltd., were used in the study. Animal care was in accordance with the Guidelines for Animal Experimentation of Shenyang Pharmaceutical University (Shenyang, China) and the protocol was approved by the Animal Ethics Committee of the institution. The animals were fed with a certified standard diet and tap water *ad libitum*, and were allowed to acclimate for a period of 7 days before they were placed into metabolic cages for 14 days. During the study, room temperatures remained within 18–21 °C, daily relative humidity remained within 45–65%. To reduce contamination, the rats were placed in clean cages each day. After one week of acclimatization, the rats were randomly divided into 4 groups ($n = 7$/group): WMGS group (WMGSG), oral gavage with water extract of Morning Glory Seed (WMGS) at a dose of 15 g MGS/kg/day; EMGS group (EMGSG), oral gavage with ethanol extract of Morning Glory Seed (EMGS) at a dose of 15 g MGS/kg/day; CAM Group (CAMG), oral gavage with water extract of CAM at a dose of 15 g CAM/kg/day; Healthy Control Group (HCG), oral gavage with the approximately same volume water as the other groups.

2.3. Sample collection and preparation

The urine samples were collected at various time-points (24 h pre-dose, and 48 h, 96 h, 144 h, 192 h, 240 h, 336 h post-dose), during which the rats were deprived of food to avoid solid debris pollution, but were allowed tap water *ad libitum*. Then the urine samples were kept at −20 °C. Prior to the analysis, urine samples were thawed and centrifuged at 13,000 rpm for 3 min to aid settling of coarse material. Supernatant was removed, diluted at a ratio of 1:1 with Milli-Q water and vortex mixed for LC–MS analysis.

2.4. Serum biochemistry test

One milliliter of blood sample for biochemistry measurements was collected from the retro-orbital venous plexus in 48 h pre-dose and 360 h post-dose. Serum samples were then removed from the coagulated blood after centrifugation (13,000 rpm, 3 min) and biochemical examination was performed. Serum creatinine and BUN were used in the evaluation of nephrotoxicity disorders.

2.5. Histopathology

The sample part of kidney was fixed in 10% formalin for at least 12 h, processed to 3 μm wax sections. Tissue sections were subsequently stained with haematoxylin and eosin, and were examined under a light microscope.

2.6. Chromatography

Chromatographic separation was performed on a 5 cm × 2.1 mm ACQUITY 1.7 μm C_{18} column (Waters, USA) using an ACQUITY^{TM} Ultra-Performance Liquid Chromatography system (Waters, USA). The column was maintained at 35 °C, a 10 μl aliquot of the sample was introduced to the column. The UPLC mobile phase consisted of 0.1% formic acid in acetonitrile (solution A) and 0.1% formic acid in water (solution B). The gradient duration was 17 min at a flow rate of 0.3 ml/min. The linear gradient increased from 5% to 20% A in 8 min, and increased to 50% A in another 3 min. Then the solution A was continuously increased from 50% to 90% in 4 min. Finally, the solution A was linearly decreased back to the initial condition of 5%.

2.7. Mass spectrometry

The mass spectrometric data were collected on a Micromass Quattro micro^{TM} API mass spectrometer (Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface and triple quadruple mass analyser. Mass spectra were obtained on a full-scan operation in positive ion mode. The capillary voltage was set at 3.0 kV, and the cone voltage was optimized at 30 V. The source temperature of 120 °C, a desolvation gas temperature of 350 °C, and a nebulization gas flow of 9.0 l/min were used. Data profiling of positive ions from m/z 100 to 900 was recorded at a speed of 1 s/scan with 0.1 s as the inter-scan delay during analysis. In the MS/MS experiments, argon was employed as the collision gas and collision energy was set from 10 eV to 30 eV. The data were collected in centroid mode, and the mass was corrected with NaCsI before the study.

2.8. Data analysis

The mass data acquired were imported to Markerlynx (Waters, UK) within Masslynx software (version 4.0) for peak detection and alignment. The retention time and m/z data for each peak were determined by the software. All data were normalized to the summed total ion intensity per chromatogram. The parameters of

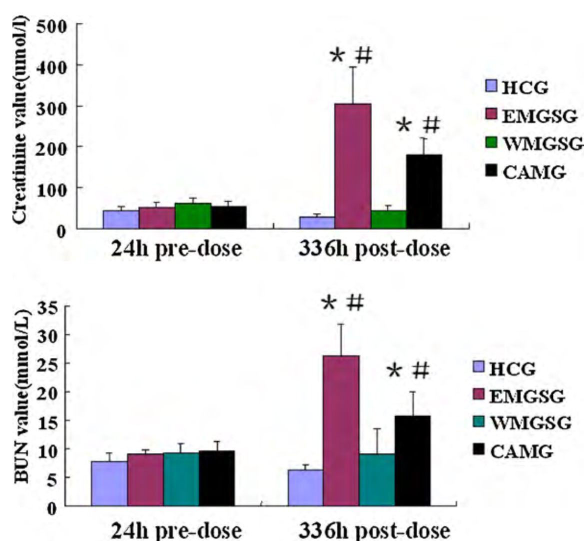

Fig. 1. *Compared with HCG in the time point of 336 h post-dose. #Compared with EMGSG and CAMG themselves in the time point of 24 h pre-dose ($p < 0.05$).

Markerlynx method were set as follows: mass tolerance 0.1 Da; noise elimination level 100; the high and low mass was set as 100 and 900 respectively; the initial and final retention time was set as 0 min and 16 min.

2.9. Identification of the endogenous metabolites

All collected urine samples were analyzed and low molecular weight metabolites were represented as the chromatographic peaks in the total ion current (TIC) chromatograms. Mass spectra were interpreted with available biochemical databases, such as KEGG (http://www.genome.jp/kegg/), METLIN (http://metlin.scripps.edu/), and SciFinder (https://scifinder.cas.org/). The collision induced dissociation (CID) experiment was implemented to get fragmentation patterns of these potential urine biomarkers. Furthermore, identification of metabolites was performed using the commercial available standards by comparing their MS spectra and retention time.

3. Result and discussion

3.1. Clinical chemistry results

In the serum biochemistry result, as Fig. 1 reported, the average values of selected clinical chemistry parameters for the nephrotoxicity study in EMGSG and positive control group were significantly different ($p < 0.05$) from their time-matched control group. The creatinine and BUN, which are markers of renal damage were elevated both in EMGSG and CAMG comparing to HCG, presenting a marked reduction of renal function. CAM was a well-known nephrotoxic Chinese herbal [11–13] and was chosen as positive control drug to test the effectiveness of toxicology experimental system. Compared with the CAMG, the EMGSG rats exhibited slightly higher creatinine and BUN levels. In contrast, the creatinine and BUN of WMGSG did not show significant difference in comparison with HCG.

3.2. Histopathology

Histopathological findings after the administration of Morning Glory Seed are summarized in Fig. 2. As it was shown in Fig. 2a and b, the kidney section of the HCG rat showed apparently normal structure in renal cortex and medulla. In contrast, the kidney section from EMGSG rat showed significant glomerulus atrophy and bowman space expansion (Fig. 2e), at the same time, obvious vacuolization and dilation in kidney tubules were observed in renal medulla (Fig. 2f). The kidney section isolated from rat treated with CAM showed similar pathological state (Fig. 2g and h) with EMGSG rat, but as reported [11], the glomerulus is normal. The kidney of WMGSG animals revealed a nearly regular morphology of epithelial tubular cells (Fig. 2c) and glomerulus (Fig. 2d).

3.3. UPLC–MS analysis

Urine contains thousands of endogenous metabolites, including amino acids, fatty acids, sugars, lipids, hormones, and sulfate conjuncts, they are different greatly in physicochemical properties such as polarity, size and molecular weight, so there are no universal analytical techniques to analyze these compounds simultaneously. In this study, the LC and mass conditions were optimized to detect as many peaks as possible.

Fig. 3 presented the typical positive base peak intensity (BPI) chromatograms of representative urine sample from HCG, WMGSG, EMSEG and CAMG rat. Acetonitrile was chosen as the organic phase

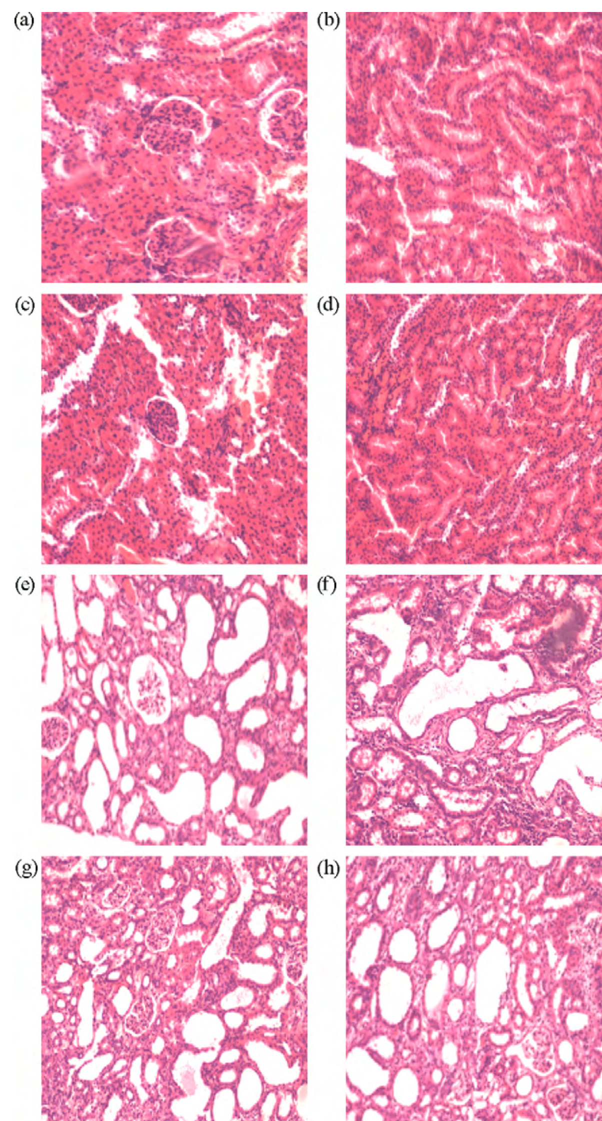

Fig. 2. Histopathological photomicrographs of rat kidney sections in the time-point of 360 h post-dose. (a) HCG renal cortex, (b) HCG renal medulla, (c) WMGSG renal cortex, (d) WMGSG renal medulla, (e) EMGSG renal cortex, (f) EMGSG renal medulla, (g) CAMG renal cortex, and (h) CAMG renal medulla.

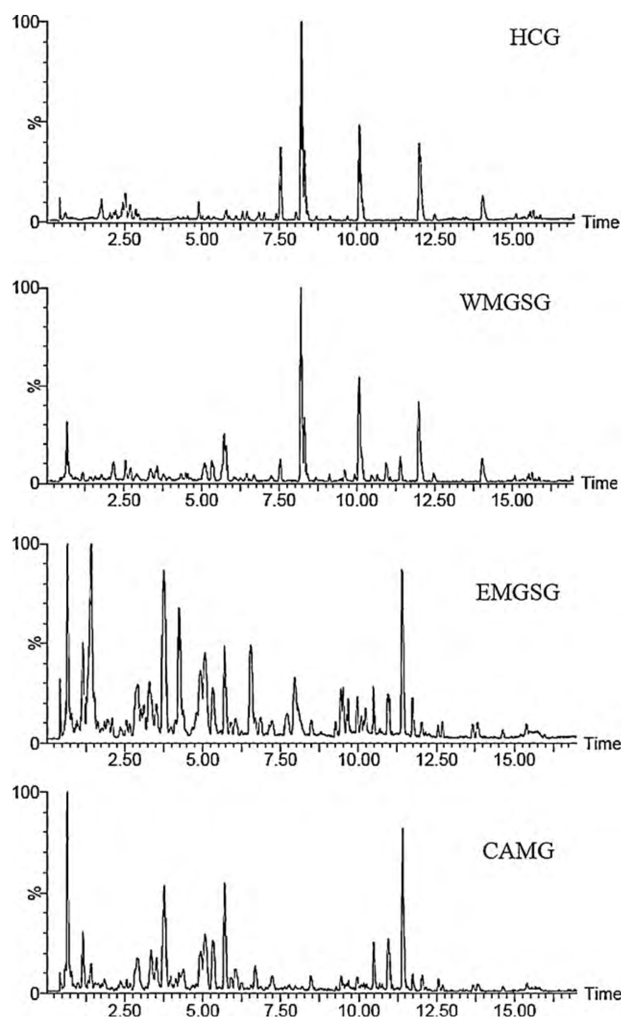

Fig. 3. The positive base peak intensity (BPI) chromatograms of representative urine sample in the time-point of 336 h post-dose.

in the study. The elution time was reduced to 17 min, due to the fact that few compounds with reasonable peak intensities were obtained after a 17 min retention time. Considering the sensitivity on the single mode, full-scan detection was set as positive ion mode. Cone and capillary voltage were also optimized, so that molecular ions $[M+H]^+$ accounted for the majority of the mass spectrum. In the MS–MS analysis, the collision energy was elevated gradually to determine the suitable value for representing the fragment information.

During this study, more than 50 urine samples were analyzed, the applied method was validated prior to the analysis of the experimental samples, including the precision of injection, the within-day stability and the repeatability of sample preparation. Extracted ion chromatographic peaks of seven ions (with the retention time and mass pairs of 1.4_297, 11.4_355, 7.9_441, 4.5_591, 3.8_618, 7.9_745, and 11.4_840, in positive ion mode) were selected for method validation. The relative standard deviations (R.S.Ds) of peak intensities and retention time for the selected ion in pooled urine samples were calculated. Precision of injection was carried out by the continuous detection of six injections of the same sample. Within-day stability was investigated by six injections in 24 h with an interval of 4 h from the same urine sample. Then six parallel samples were prepared for the repeatability of sample preparation study. The results were shown in Table 1. The good precision, stability, and repeatability indicated that the method could be utilized to the analysis of urine samples.

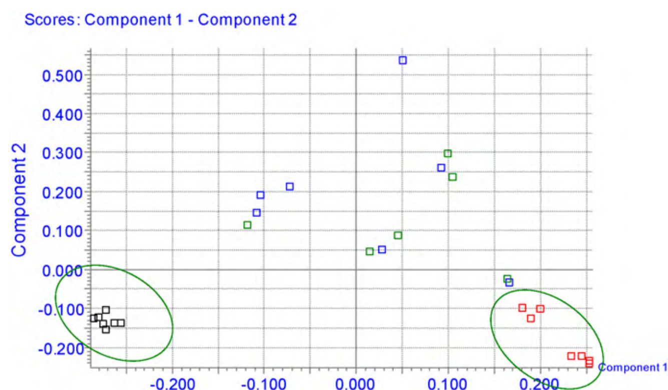

Fig. 4. The score plot for PCA at the time-point of 336 h post-dose (a) for HCG, WMGSG, EMGSG and CAMG.
HCG WMGSG EMGSG □ CAMG.

3.4. Data analysis

As it was clearly shown in Fig. 3, some of the metabolic changes in the urine sample of EMGSG (Fig. 3c) could be found directly in the TIC chromatograms compared with HCG (Fig. 3a), but the TIC chromatogram of WMGSG (Fig. 3b) did not show obvious difference in comparison with its time-matched control group. In order to gain a comprehensive view of the metabonome, PCA was used in the subsequent data analysis.

The obtained PCA score plots from the processing of data were shown in Figs. 4 and 5. As it was shown in Fig. 4, at the time point of 336 h post-dose, the PCA score plot could be readily divided into three clusters: EMGSG, CAMG, and the region which WMGSG mixed with HCG. The samples of EMGSG tend to cluster in the right part, while the CAMG located in the left part, they were clearly separated from other groups. However, the border between the WMGSG and HCG was ambiguous, large part of them was overlapped. Additionally, we can find that EMGSG and CAMG concentrated into a small area respectively, but WMGSG and HCG dispersed to a relatively large region. Based on the phenomenon showed by the PCA score plot coupled with the clinical chemical and histopathology results, some conclusions could be made as follows: the presence of substantial kidney damage after administration of EMGS was confirmed. As the positive control drug, CAM also showed its clear nephrotoxicity as reported. The separation between the EMGSG and CAMG indicated that EMGS could cause renal damage based on the different mechanisms compared to CAM. WMGSG did not show obvious nephrotoxicity according to the clinical chemical result and its mixed distribution with HCG in the PCA score plot. In contrast with the scattering of WMGSG and HCG caused by individual difference, the concentration trend of EMGSG showed the similar pathological state of rats caused by EMGS.

On the basis of the analysis above, it is confirmed that EMGS should be responsible for the nephrotoxicity of Morning Glory Seed. So a further study on the progression of renal damage induced by EMGS was performed in another PCA score plot (Fig. 5a). According to the time after administration of EMGS, PCA score plot could be divided into seven zones basically, suggesting that EMGS-induced kidney morbidity seemed to progress in a stepwise fashion. Furthermore, with the passage of time, the points of post-dose were gradually moving far away from the points of pre-dose, providing a time-dependent tendency and indicating the progression of renal damage.

Though being a preliminary work for the nephrotoxicology study, the obtained PCA data did show the difference between the control and dosed rats when the animal experiment and urine sample analysis by LC–MS were conducted in the same way, suggesting

Table 1
Precision of injection, within-day stability, repeatability of sample preparation of the urine analytical method ($n = 6$).

m/z (t_R)	Precision of injection		Within-day stability		Repeatability of sample preparation	
	t_R (RSD%)	Intensity (RSD%)	t_R (RSD%)	Intensity (RSD%)	t_R (RSD%)	Intensity (RSD%)
297 (1.4)	1.41	4.00	1.20	6.79	0.37	4.35
355 (11.4)	0.18	6.02	0.07	11.10	0.25	6.85
441 (7.9)	0.07	8.03	0.12	11.55	0.05	9.59
591 (4.5)	0.10	12.68	0.22	4.89	0.17	7.45
618 (3.8)	0.55	8.73	0.29	2.57	0.11	6.54
745 (7.9)	0.15	7.88	0.36	10.71	0.50	9.66
840 (11.4)	0.07	5.53	0.10	3.78	0.10	3.63

that the metabonomic study could be used to indicate whether the animal had been dosed with Morning Glory Seed or not.

3.5. Biomarker elucidation

The PCA loadings plot was shown in Fig. 5b. The distance of an ion from the origin represents the influence of that ion on the PCA components. A number of ions which were found predominantly in the loading plot, were chosen as biomarkers, as it was listed in Table 2. Furthermore, to investigate the change tendency of the biomarkers, we compared the intensity of biomarkers in urine of EMGSG rats in different time-points (Fig. 6). In our study, biomarkers were tentatively identified based on mass measurements via MS, MS/MS experiments, and comparison to authentic standards. Here, the identifications of methionine, tryptophan, phenylalanine, creatinine, citric acid, homocysteine and cholic acid were using the commercial available standards by comparing their MS/MS spectra and retention time.

Due to lack of reference standard, it was challenging to identify the metabolite eluted at 2.9 min, which showed peak at m/z 313. With different collision energy, the corresponding MS/MS information was showed in the Fig. 7 and was compared with product ion spectra presented in the literature [14–16]. At the end, it was tentatively identified as the characteristic fragment of 5-methyltetrahydrofolate (5-CH_3 THF).

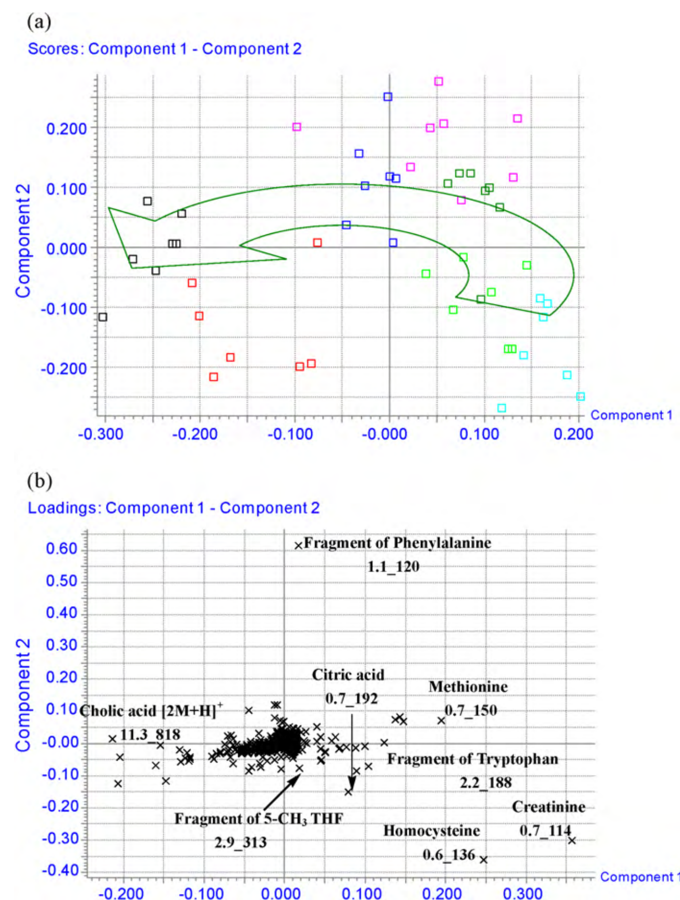

Fig. 5. The score plot (a) reflecting the tendency of the urine metabolic fluctuations according to time after oral EMGS and the loading plot (b) for biomarker recognition. □ 24 h pre-dose □ 48 h post-dose □ 96 h post-dose □ 144 h post-dose □ 192 h post-dose □ 240 h post-dose □ 336 h post-dose.

3.6. Biochemical interpretation

For healthy mammalian, all of the glucose, and amino acids, >90% of the uric acid and 60% of inorganic salts are reabsorbed by active transport. However, concentrations of many amino acids in urine were found increased after the EMGS exposure in this study. So we deduced that renal function was decayed by intoxication of EMGS.

Tryptophan and phenylalanine are essential amino acids which cannot be synthesized by the body. They are either incorporated into proteins or broken down for energy and metabolic intermediates. Changes of the two amino acids in EMGS treated rats may indicate an alteration of the balance between nutrition intake and consumption [17].

It is noteworthy that amino acid is widespread in Chinese herbal including MGS. So we must make it clear whether the increased amino acids level in urine was caused by renal damage or just by the elimination of MGS components. The amino acid biomarkers were found based on renal damage caused by ethanol extract of Morning Glory Seed (EMGS). We preliminarily investigated the profile of EMGS with same UPLC–MS condition as the urine samples analysis, and did not detected the amino acid biomarkers in corresponding retention time. This result may be due to the fact that solubility of some polar amino acid in cold ethanol is very small.

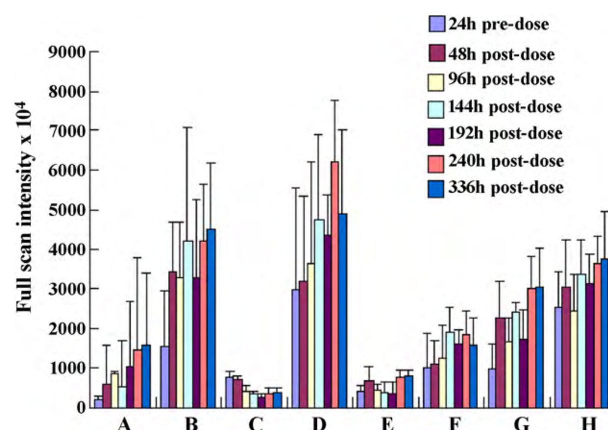

Fig. 6. The trends of biomarker intensity for EMGSG.
(A) Cholic acid, (B) creatinine, (C) citric acid, (D) phenylalanine, (E) methionine, (F) tryptophan, (G) Homocystein, and (H) 5-CH_3THF.

Table 2
Potential biomarkers of nephrotoxicity induced by EMGS.

Retention time (min)	m/z (Da)	scan mode	quasi-molecular ion	Metabolites	Levels in abnormal urine
0.7	150 148	+ –	$[M+H]^+$ $[M-H]^-$	Methionine	↑
2.2	188 203	+ –	$[M+H-H_2O]^+$ $[M^-H]^-$	Tryptophan	↑
0.6	114 112	+ –	$[M+H]^+$ $[M-H]^-$	Creatinine	↑
1.1	166 164 120	+ – +	$[M+H]^+$ $[M-H]^-$ $[M+H-HCOOH]^+$	Phenylalanine	↑
0.7	192 190	+ –	$[M+H]^+$ $[M-H]^-$	Citric acid	↓
0.6	136 134	+ –	$[M+H]^+$ $[M^-H]^-$	Homocysteine	↑
2.9	313	+	Characteristic Fragment of 5-CH_3 THF	5-CH_3 THF	↑
11.3	818 816	+ –	$[2M+H]^+$ $[2M-H]^-$	Cholic acid	↑

Therefore, we deduced that increased amino acids level in urine was caused by renal dysfunction instead of elimination of EMGS components.

The concentration of citric acid in urine was found to be reduced after EMGS exposure in our experiment. Since citric acid is one of the key compounds in the TCA cycle, we infer that the decrease of citric acid is due to dysfunction of TCA. In addition, a dramatic augment of creatinine was observed. According to assessed metabolic pathway, creatinine is a waste product formed by the slow spontaneous degradation of creatine-phosphate [18]. While creatine is charged with energy by the enzyme creatine kinase which transfers the high-energy phosphate bond of ATP to make creatine-phosphate in vivo. Creatine and creatine-phosphate exist in a reversible equilibrium and creatine-phosphate functions as a “battery” that stores the energy of excess ATP. When EMGS caused energy decline (according to the dysfunction of TCA cycle), ATP insufficiency resulted in shifting the equilibrium to left and thus increased the total amount of free creatine which subsequently degraded to creatinine. Then, urinary levels of creatinine were augmented.

Homocysteine (Hcy) is a well-established biomarker of renal function. As its concentration increases in kidney tissues, renal function worsens [19]. Hcy is formed by the demethylation of methionine, and plays an important role in the activated methyl and folate cycles. The clearance of Hcy includes two metabolic pathways: in the transsulfuration pathway, Hcy condenses with serine to cystathionine, which is further metabolized to cysteine and sulfate; alternately, the remethylation of Hcy to methionine occurs through a methyl donation from 5-methyltetrahydrofolate (5-CH_3-THF), by means of methionine synthase. Herein, the elevated Hcy levels may be interpreted by another phenomenon observed in this experiment: 5-CH_3-THF presented increased level in urine due to the decreased reabsorbtion. The great loss of 5-CH_3-THF in urine will decrease the level of this metabolite in vivo. Due to lack of methyl donation, the transformation from Hcy to methionine will be decreased. Then, the Hcy level will be elevated. It is also reported

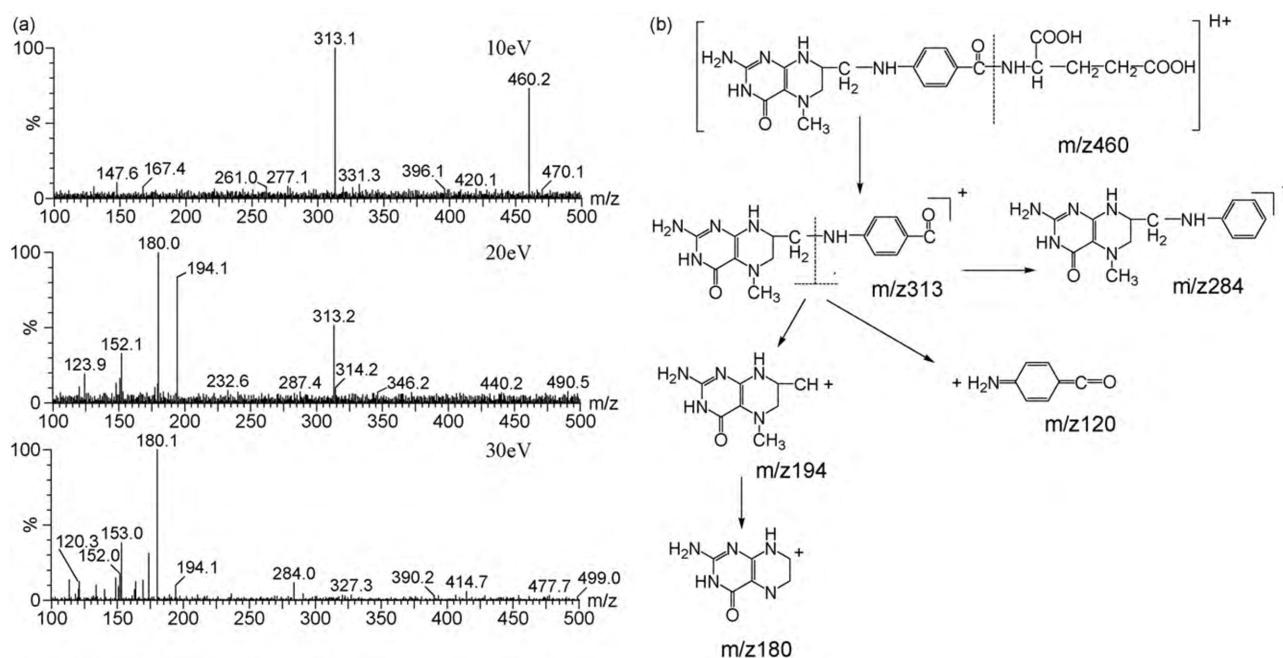

Fig. 7. (a) Product ion spectrum of m/z 460 (parent ion of biomarker m/z 313) in positive mode. (b) Possible MS fragmentation mechanism.

[20] that normal kidney tissue richly contains the methylase which plays an important role in the methylation of Hcy. When renal damage occurred, the loss of methylase will contribute to the elevated Hcy levels caused by deceleration of methylation.

Significant changes of cholic acid in EMGS treated rats were observed in our study. Bile acids are the major products of cholesterol catabolism. In health, only small quantities of bile acids are found in peripheral circulation and urine. However, in hepatobiliary and intestinal disease, disturbances of synthesis, metabolism, and clearance by the liver and absorption by the intestine will affect the concentration and profile of bile acids in various pool compartments (serum, liver, gallbladder, urine and feces). In our study, increased cholic acid level was observed in EMGS treated rats, the result may indicated that liver damage has also occurred simultaneously [21]. Bile acids could combine with taurine to form bile salts. In obstructive liver disease bile salts are known to accumulate in and damage specific kidney cells.

The kidney damage is mainly to proximal tubular cells including focal tubular necrosis and vacuolization of the tubular cells [22]. The ability to act as detergents are thought to be the mechanism by which bile salts damage kidney cells [23]. At concentrations near their critical micellar concentration, bile salts are capable of completely solubilizing cell membrane components, causing altered cell function or cell death [24]. In this experiment, the increased cholic acid may further aggravate renal damage

3.7. Preliminary understanding of the nephrotoxicity caused by ethanol extract of Morning Glory Seed (EMGS)

The extraction solvent of Chinese medicine is mostly water. However, Morning Glory Seed, as an exception, was commonly used as pill, powder and other solid dosage form. In the application of modern proprietary Chinese medicines, under most situations, the extraction solvent of Morning Glory Seed is ethanol. Ethanol extraction is effective method to extract the active compound responsible for the purgation activity of Morning Glory Seed: Pharbitin. Pharbitin, a kind of resin glycosides, was considered to be one of the possible chemical materials which were associated with this herbal medicine's nephrotoxicity. However, the mechanism was not yet completely understood and need further research.

3.8. Species specificity in the metabonomic study

Under certain situations, the differences of some metabolic pathways between human and animals make it difficult to predict toxicology effect on human being with metabonomics study results using animal model. Therefore, it is tremendously important for metabonomics investigation to choose suitable experimental animals which are close to human in genotype, phenotype and metabolic type. In our study, the metabolic pathways involved in the nephrotoxicity induced by EMGS are mainly amino acids excretion, folate–methionine cycle and tricarboxylic acid cycle. These metabolic pathways, which are widespread in human and other mammalian including rodents, have been successfully applied in many medical studies to demonstrate drug's toxicological or pharmacological action. However, for the future metabonomics study on certain complicated physiological and pathological mechanisms, experimental animals having more closer genetic relationship with humans such as primates and humanized animals should be chosen.

3.9. Viewpoint on the identification of isomers biomarkers

The separation of regio- and stereo-isomers is an important issue for biomarker identification. Because of the same molecular mass and similar chemical structure, it is difficult to distinguish isomers by common mass spectrometry alone. For the separation of single isomer, derivatization and mobile phase additives are effective methods. However, for the complex and mixed analyte of metabonomics research, fast and more sensitive analytical methods should be applied. Recent years, ion mobility mass spectrometry (IM-MS) is developing into an important analytical tool. Based on the difference of molecule size and conformation, the separation and identification of isomers could be achieved by IM-MS. Combined with other MS and chromatography technique, IM-MS will be a powerful tool for the isomer biomarker identification.

4. Conclusion

Morning Glory Seed-induced renal damage is still a challenging field for the clinic and experimental nephrotoxicology studies. In this work, blood biochemistry, histopathology and metabonomics method were combined to identify the fact that the ethanol extract of Morning Glory Seed (EMGS) should be responsible for the herb's nephrotoxicity. PCA based information-extracting method was provided to characterize the EMGS-induced metabolic disorders. Furthermore, significant differences in the urine levels of amino acids, citric acid, creatinine, cholic acid and 5-CH_3-THF were observed in EMGSG rats. Underlying relationship between pathological fluctuations and metabolic disequilibria caused by toxic stimulation was tentatively elucidated according to the identified biomarkers. This study demonstrates that the metabonomics based on UPLC–MS could reflect the balance of homeostasis and metabolism, and is a promising tool in clinical practice in the future.

Acknowledgement

This work was financially supported by Natural Science Foundation of China (No. 20875064).

References

[1] J.C. Tsai, S.L. Tsai, W.C. Chang, Comparison of two Chinese medical herbs, Huangbai and Qianniuzi, on influence of short circuit current across the rat intestinal epithelia, J. Ethnopharmacol. 93 (2004) 21–25.
[2] T.H. Lee, J.J. Choi, D.H. Kim, K.R. Lee, M. Son, M. Jin, Gastroprokinetic effects of DA-9701, a new prokinetic agent formulated with Pharbitis Semen and Corydalis Tuber, Phytomedicine 15 (2008) 836–843.
[3] P.M. Albert, The obstetrical use in ancient and early modern times of Convolvulus scammonia or Scammony: another non-fungal source of ergot alkaloids, J. Ethnopharmacol. 1 (1979) 193–195.
[4] S.H. Oh, Y.S. Cha, Effect of diets supplemented with Pharbitis seed powder on serum and hepatic lipid levels, and enzyme activities of rats administered with ethanol chronically, J. Biochem. Mol. Biol. 34 (2001) 166–171.
[5] G.M. Dugan, M.R. Gumbmann, Toxicological evaluation of morning glory seed: Subchronic 90-day feeding study, Food Chem. Toxicol. 28 (1990) 553–559.
[6] W.B. Rice, K. Genest, Acute toxicity of extracts of Morning-glory Seeds in mice, Nature 207 (1965) 302–303.
[7] L. Solyom, E. Kingstone, An obsessive neurosis following morning glory seed ingestion treated by aversion relief, J. Behav. Ther. Exp. Psychiatr. 4 (1973) 293–295.
[8] L.Q. Tian, Z.L. Zhang, B.S. Zhang, Research on the pharmacology, toxicity and clinical application of Morning glory seed, Chin. J. Nat. Med. 3 (2004) 146–147.
[9] J.K. Nicholson, J.C. Lindon, E. Holmes, "Metabonomics": understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data, Xenobiotica 29 (1999) 1181.
[10] J.K. Nicholson, Metabonomics and global systems biology approaches to molecular diagnostics, Drug Metab. Rev. 37 (2005) 10.
[11] X. Xue, Y. Xiao, L. Gong, S. Guan, Y. Liu, H. Lu, X. Qi, Y. Zhang, Y. Li, X. Wu, J. Ren, Comparative 28-day repeated oral toxicity of Longdan Xieganwan, *Akebia trifoliate* (Thunb.) Koidz., *Akebia quinata* (Thunb.) Decne. and *Caulis aristolochiae manshuriensis* in mice, J. Ethnopharmacol. 119 (2008) 87–93.
[12] Z.Z. Zhao, Z.T. Liang, Z.H. Jiang, Comparative study on the aristolochic acid I content of Herba Asari for safe use, Phytomedicine 15 (2008) 741–748.
[13] J.L. Tang, B.Y. Liu, K.W. Ma, Traditional Chinese medicine, Lancet 372 (2008) 1938–1940.
[14] B.C. Nelson, J.J. Dalluge, Preliminary application of liquid chromatography-electrospay ionization mass spectrometry to the detection of 5-methyltetrahydrofolic acid monoglutamate in human plasma, J. Chromatogr. B 765 (2001) 141–150.

[15] R.J. Pawlosky, V.P. Flanagan, Determination of 5-methyltetrahydrofolic acid in human serum by stable-isotope dilution high-performance liquid chromatography–mass spectrometry, Anal. Biochem. 298 (2001) 299–305.
[16] R.M. Kok, D.E.C. Smith, J.R. Dainty, 5-Methyltetrahydrofolic acid and folic acid measured in plasma with liquid chromatography tandem mass spectrometry: applications to folate absorption and metabolism, Anal. Biochem. 326 (2004) 129–138.
[17] M.R. Jones, J.D. Kopple, M.E. Swendseid, Phenylalanine metabolism in uremic and normal man, Kidney Int. 14 (1978) 169.
[18] M. Wyss, Creatine and creatine metabolism, Physiol. Rev. 80 (2000) 1107–1213.
[19] Y.E. Taes, J.R. Delanghe, N.H. Lameire, Creatine supplementation decreases homocysteine in an animal model of uremia, Kidney Int. 64 (2003) 1331–1337.
[20] A.G. Bostom, D. Shemin, J.T. Brosnan, B. Hall, M.R. Nadeau, J. Selhub, Net uptake of plasma homocystein by the rat kidney in vivo, Atherosclerosis 116 (1995) 59–62.
[21] H. Li, Y. Ni, M. Su, Y. Qiu, M. Zhou, M. Qiu, A. Zhao, L. Zhao, W. Jia, Pharmacometabonomic phenotyping reveals different responses to xenobiotic intervention in rats, J. Proteome. Res. 6 (2007) 1364–1370.
[22] J. Dawson, G. Stirling, Protective effect of mannitol on anoxic jaundiced kidneys, Arch. Pathol. 78 (1964) 254–259.
[23] B. Kaler, W. Morgan, P. Bach, T. Karram, I. Yousef, A. Bomzon, Are bile acids involved in the renal dysfunction of obstructive jaundice? An experimental study in bile duct ligated rats, Renal Failure 26 (2004) 507–516.
[24] W. Morgan, Y. Ding, P. Bach, The relationship between sodium chloride concentration and bile acid cytotoxicity in cultured kidney cells, Renal Failure 20 (1998) 441–450.