Original Article
Liver tissue metabonomics in rat after acute paraquat poisoning gas chromatography-mass spectrometry

Peiwu Geng[1*], Jinzhang Cai[2*], Lijing Zhang[3], Mengzhi Xu[3], Zezheng Liu[3], Jing Zhang[3], Zhiyi Wang[2], Xianqin Wang[3], Congcong Wen[3], Jianshe Ma[4]

[1]The Laboratory of Clinical Pharmacy, The People's Hospital of Lishui, Lishui 323000, China; [2]The Second Affiliated Hospital & Yuying Children's Hospital of Wenzhou Medical University, Wenzhou 325000, China; [3]Laboratory Animal Centre, [4]Function Experiment Teaching Center, Wenzhou Medical University, Wenzhou 325035, China. *Equal contributors.

Abstract: In this study, we developed a liver tissue metabolomic method based on gas chromatography-mass spectrometry (GC-MS) to evaluate the effect of acute paraquat poisoning on rats. The acute paraquat poisoning group rats were given 36 mg/kg of paraquat by intragastric administration, control group were given saline by intragastric administration. Liver tissue samples were collected from the rats from the acute paraquat poisoning group and control group. Pattern recognition analysis, including both principal component analysis (PCA) and partial least squares-discriminate analysis (PLS-DA) revealed that acute paraquat poisoning induced liver tissue metabolic perturbations. Compared to the control group, the level of (Z,Z)-9,12-octadecadienoic acid, D-gluconic acid, oleic acid, 4,7,10,13,16,19-docosahexaenoic acid, 5,8,11,14,17-eicosapentaenoic acid, d-glucose, phthalic acid in liver tissue of acute paraquat poisoning group increased, while the level of d-mannose, heptadecanoic acid, D-glucuronic acid decreased. In conclusion, liver tissue metabolomic method based on GC-MS may be useful to elucidate acute paraquat poisoning through the exploration of biomarkers. According to the pathological changes of liver tissue at difference dosage, paraquat is hepatotoxic.

Keywords: Metabolomics, GC-MS, acute paraquat poisoning, rat, liver

Introduction

Paraquat (1,1'-dimethyl-4, 4'-bipyridinium) was introduced in 1962. It is an organic heterocyclic herbicide that is potent, highly toxic and widely used in agriculture, especially in Asian countries [1]. The prevalence of paraquat poisonings has increased dramatically in the past two decades, paraquat poisoning is a medical problem in many parts of Asia and the Pacific especially in China [2]. It came into disrepute because of accidental or intentional ingestion leading to a high mortality. The mortality rate has been reported to be greater than 90% [3].

Metabolomics is widely used in life sciences and other fields such as safety evaluation, drug development, toxicity screening markers, disease diagnosis, gene function currently [4, 5]. In recent years, metabolomics has been widely applied to uncover biomarkers [6] and meta-bolic fingerprint in drug discovery and clinical toxicology [7], especially to investigating systematic metabolic responses to toxins [8] and the associated mechanisms [9].

Paraquat is actively taken up against a concentration gradient into lung tissue through the highly developed polyamine uptake system, which can lead to pneumonitis and lung fibrosis. Although the lung is the primary organ affected, paraquat can exert toxic effects against other organs such as the liver [10, 11]. The liver, which is the primary site for xenobiotic transformation, has a high potential for generating ROS, and thus, it is at high risk for toxic damage [12]. In fact, the liver has been regarded as a key target of paraquat poisoning [13, 14]. The primary goal of this study is to study systematically the liver metabolic pathway changes induced by acute paraquat poisoning in rats.
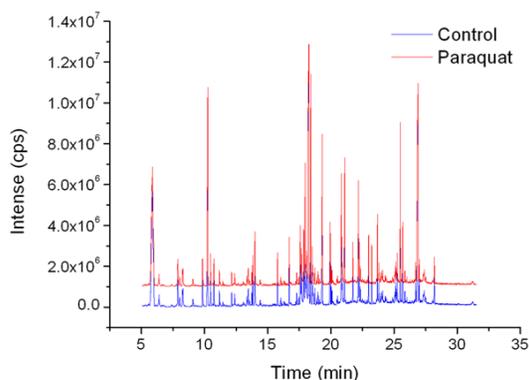
Figure 1. Typical GC-MS total ion chromatogram of in rat liver tissue after acute paraquat poisoning.

Material and methods

Chemicals and animals

Trimethylchlorosilane (TMCS) and N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) were purchased from Sigma-Aldrich Corporation (Shanghai, China). Pyridine and methylhydroxyl-amine hydrochloride were purchased from Aladdin Industrial, Inc. (Shanghai, China). HPLC-grade n-heptane and acetonitrile were purchased from Tedia Reagent Company (Shanghai, China). Sprague-Dawley rats (male, 220± 20 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd.

Instrumentation and conditions

Agilent 6890N-5975B GC-MS equiped with HP-5MS (0.25 mm × 30 mm × 0.25 mm), were purchased from Agilent Company (Santa Clara, California, USA). The GC oven was initially set at 80°C and was kept at this temperature for 5 min. The temperature was then gradually increased to 260°C at a rate of 10°C/min, and then kept at 260°C for 10 min. Mass detection was conducted in EI mode with electron energy of 70 eV, in full-scan mode with m/z 50-550, by splitless mode injection [15, 16].

Sample preparation

The 250 µL of acetonitrile was added to 100 µL of liver tissue, the mixture was stored at -80°C for 20 min then grinded for 2 min by a SCIENTZ-48 Tissue Grinder, the grinding parameter was set at 64 Hz and 1800 r/s. The tubes were vortex mixed for 1.0 min, then kept in an ice-bath for 15 min, and then 10,000 g were centrifuged for 10 min at 4°C. The 200

µL of the supernatant was transferred to a GC vial and evaporated to dryness under a stream of nitrogen gas. Methoximation was carried out at 70°C for 1 h after 50 µL of methylhydroxylamine hydrochloride (15 mg/mL in pyridine) was added. The 50 µL MSTFA (with 1% TMCS as the catalyst) was added and kept at 70°C for another 1 h, and then vortexed after adding 150 µL n-heptane [16].

Metabolomics study

Rats were housed under a natural light-dark cycle conditions with controlled temperature (22°C). All forty rats were housed at Laboratory Animal Research Center of Wenzhou Medical University. All experimental procedures were approved ethically by the Administration Committee of Experimental Animals of Wenzhou Medical University.
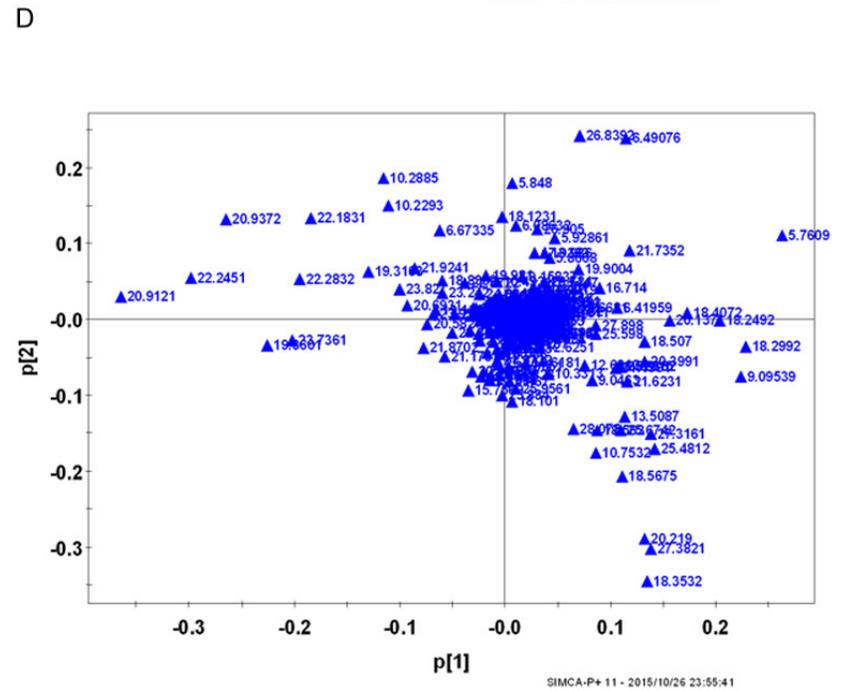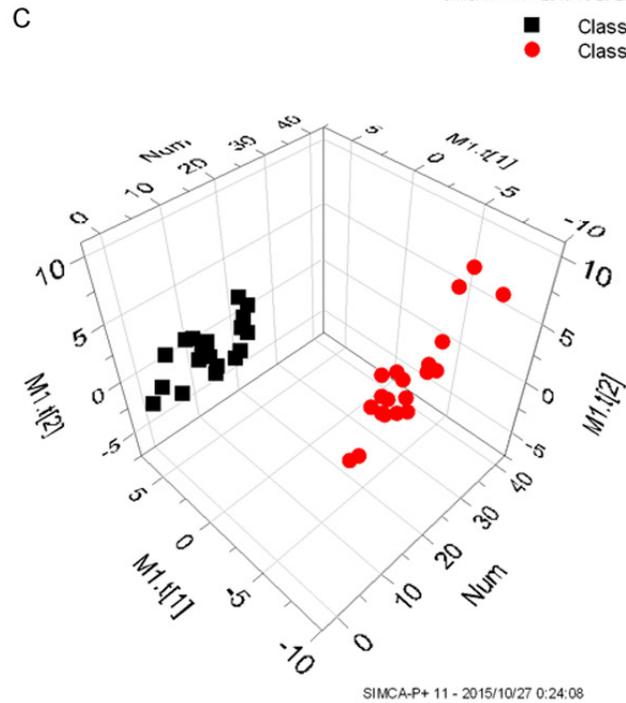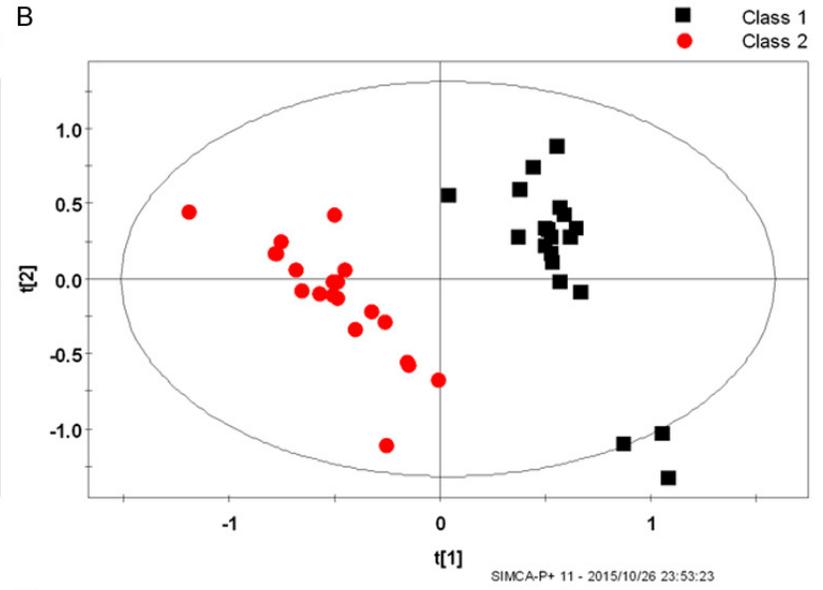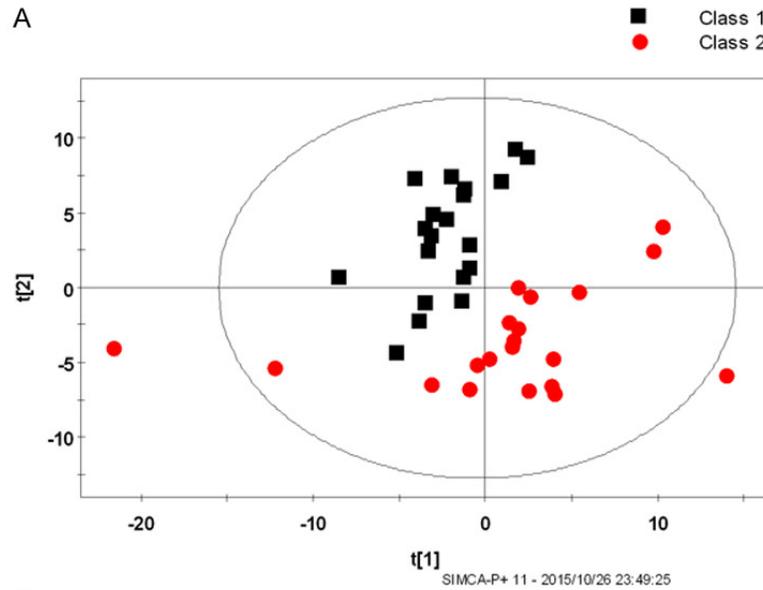
Forty rats (220±20 g) were randomly divided to acute paraquat poisoning group and control group, twenty rats in each group. Acute paraquat poisoning group were given paraquat (36 mg/kg) by intragastric administration, control group were given saline by intragastric administration. Liver tissue samples were collected from the rats from the control group and acute paraquat poisoning group at 8:00 am after 21 days, respectively. The liver tissues were stored at -80°C until analysis.

Histopathology

After metabolomics experiment, rats were deeply anesthetized with 10% chloral hydrate (i.p., 20 mg/kg). The liver tissues were rapidly isolated and immersed in freshly prepared 4% w/v formaldehyde (0.1 M phosphate buffers, pH 7.2) for 48 h and embedded in paraffin. Then 4-µm-thick histologic sections were prepared and stained with hematoxylin and eosin (HE) by routine HE method. The morphological changes were observed under light microscope.

Data analysis

The GC-MS data was exported into Microsoft Excel, with the peaks normalized to the total sum of spectrum prior to multivariate analyses. The resulting data was processed through principal component analysis (PCA) and partial least squares discriminate analysis (PLS-DA) using SIMCA-P 11.5 software (Umetrics, Umea, Sweden).

A

■ Class 1
● Class 2

SIMCA-P+ 11 - 2015/10/26 23:49:25

B

■ Class 1
● Class 2

SIMCA-P+ 11 - 2015/10/26 23:53:23

C

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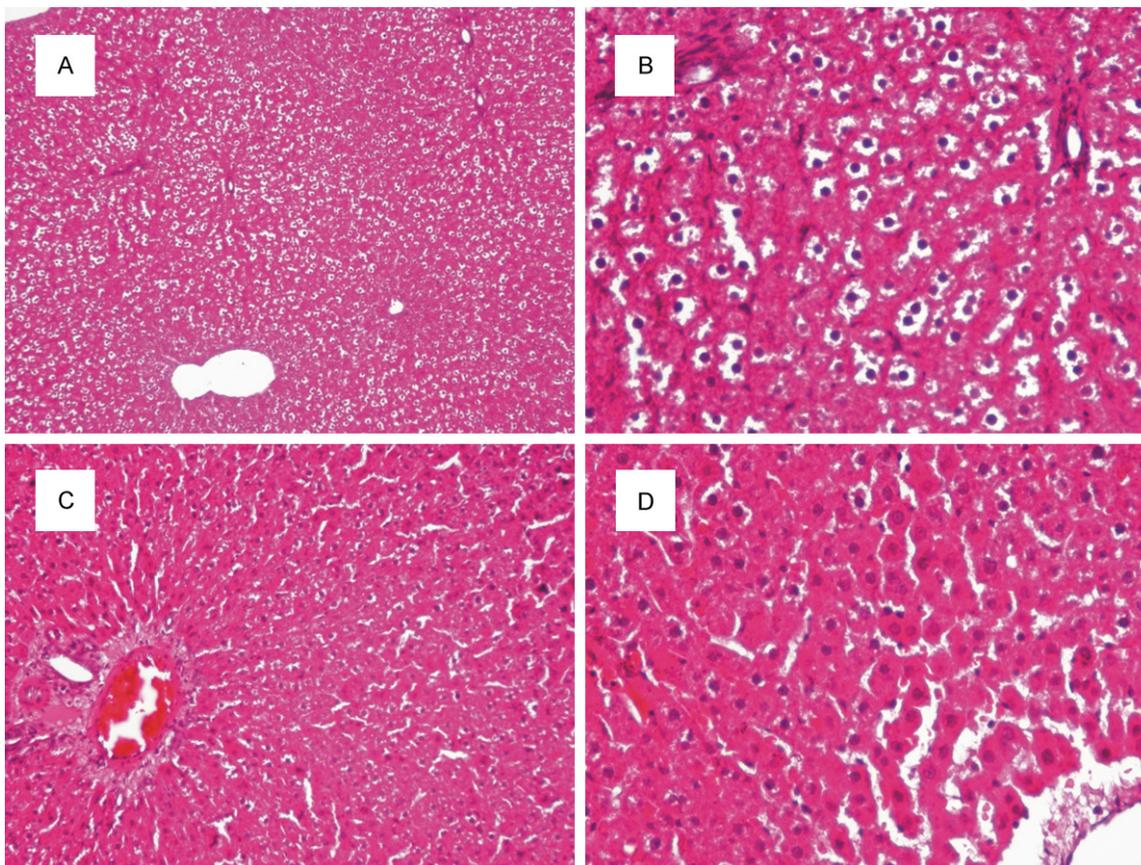SIMCA-P+ 11 - 2015/10/27 0:24:08

D

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Figure 3. Morphological changes of liver in acute paraquat poisoning group (A, B) and control-group (C, D) (hematoxylin-eosin, AC × 100, BD × 400).

Statistical analysis

Statistical analysis was carried out using SPSS software (Version 18.0, SPSS). Independent samples T-test was applied in order to detect significant differences in all metabolites between two groups. A *P* value of < 0.05 was considered statistically significant.

Results and discussion

Metabolomics study

Metabolomics is a newly emerging omics approach to the investigation of metabolic phenotype changes induced by environmental or endogenous factors [17-21]. It has shown promising results in healthcare fields, especially in disease diagnosis and drug-toxicity assessment, as reviewed recently [22, 23]. In drug-

toxicity assessment, metabolomics is often concerned with finding toxicity-related biomarkers by investigating the changes in metabolic signatures induced by drug exposure [15, 24].

Figure 1 provides the typical metabolic profiles of liver acquired through GC-MS technique. Metabolic profile data pretreatment resulted in a final dataset consisting of sixty-four metabolic features from GC-MS analyses. The endogenous metabolites in the liver were identified using the NIST 2005 mass spectrometry database.

In order to explore the metabolic profile changes of acute paraquat poisoning in rats, we compared the GC-MS spectrum of PLS-DA of the acute paraquat poisoning group with the rats in the control group (**Figure 2**). **Figure 2A** PCA and

Table 1. Summary of the changes in relative levels of metabolites in rat liver tissue after acute paraquat poisoning

NO.	Renten time/min	Metabolite	VIP	Paraquat
1	20.9121	(Z,Z)-9,12-Octadecadienoic acid	5.31519	↑,**
2	5.76095	Butanoic acid	4.32686	-
3	22.2451	Arachidonic acid	4.31601	-
4	19.3601	D-Gluconic acid	4.15781	↑,**
5	20.9372	Oleic acid	3.57703	↑,**
6	23.7361	4,7,10,13,16,19-Docosahexaenoic acid	3.52773	↑,**
7	18.2492	d-Mannose	3.46751	↓,**
9	22.2832	5,8,11,14,17-Eicosapentaenoic acid	3.02946	↑,**
10	18.4072	d-Glucose	2.86254	↑,**
12	20.1371	Heptadecanoic acid	2.60582	↓,**
14	18.507	Phthalic acid	2.32054	↑,**
15	21.7352	D-Glucuronic acid	2.05531	↓,**

Note: Variable importance in the projection (VIP) was acquired from the PLS-DA model with a threshold of 2.0. Marks indicate the direction of the change, i.e. ↓for decrease, ↑for increase, -for no change. Compared acute paraquat poisoning group with control group, **P < 0.01, as indicated by the statistical analysis T-test.

Figure 2B PLS-DA score chart showed that the first principal components of the rats in the acute paraquat poisoning group were distinguished from the rats in the control group. The PLS-DA result was better than that of PCA. PLS-DA 3D (**Figure 2C**) score chart showed that the rats in acute paraquat poisoning group were distinguished from the rats in the control group.

Morphological changes of liver tissue

There was significant morphological change in the two groups, acute paraquat poisoning group and control-group, according to HE staining method (**Figure 3**). In control group, the liver lobules were intact, hepatic lobe, liver rope, and disciple district could be obviously observed. At high magnification, the hepatocytic plates were separated by sinusoids, and liver cells were arranged tightly along with central veins.

While, there was diffusive lesions in acute paraquat poisoning group, which could be observed at low magnification. It was hard to recognize hepatic lobe and liver rope clearly. And at high magnification, the regular arrangements of hepatocyte along with central veins were disappeared, the cell nucleus condensed, and the cytoplasm was swollen. It indicated that acute paraquat poisoning has caused irreversible damage for liver.

Changes in metabolite

Metabolomics comprises the measurement of endogenous metabolites, including amino acids, nucleic acid precursors, lipids, and degradation products of chemical intermediates in catabolism and biosynthesis. The advantage of metabolomics is that it provides the most functional measure of cellular status and can help to describe an organism's phenotype [25].

In this study, the changes of metabolites in liver tissues between acute paraquat poisoning groups and their control group were shown in **Table 1**. Compared to the control group, the level of (Z,Z)-9,12-octadecadienoic acid, D-gluconic acid, oleic acid, 4,7,10,13,16,19-docosahexaenoic acid, 5,8,11,14,17-eicosapentaenoic acid, d-glucose, phthalic acid in liver tissue of the acute paraquat poisoning group increased, while the level of d-mannose, heptadecanoic acid, D-glucuronic acid decreased.

These finding may be useful for new evidences in acute paraquat poisoning study. Additional prospective studies will be required to better understand these observations.

Conclusion

These biomarkers ((Z,Z)-9,12-octadecadienoic acid, D-gluconic acid, oleic acid, 4,7,10,13,16,19-docosahexaenoic acid, 5,8,11,14,17-eicosapentaenoic acid, d-glucose, phthalic acid, d-mannose, heptadecanoic acid, D-glucuronic acid) were the additional evidence. According to the pathological changes of liver at difference dosage, paraquat is hepatotoxic. We demonstrated that metabolomic methods based on GC-MS could provide a useful tool for exploring biomarkers to elucidate acute paraquat poisoning.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Congcong Wen, Laboratory Animal Centre, Wenzhou Medical University, Wenzhou 325035, China. E-mail: bluce494949@163.com; Dr. Jianshe Ma, Function Experiment Teaching Center, Wenzhou Medical University, Wenzhou 325035, China. E-mail: jianshema@gmail.com

References

[1]   Xu L, Xu J and Wang Z. Molecular mechanisms of paraquat-induced acute lung injury: a current review. Drug Chem Toxicol 2014; 37: 130-134.

[2]   Wunnapuk K, Mohammed F, Gawarammana I, Liu X, Verbeeck RK, Buckley NA, Roberts MS and Musuamba FT. Prediction of paraquat exposure and toxicity in clinically ill poisoned patients: a model based approach. Br J Clin Pharmacol 2014; 78: 855-866.

[3]   Qin CZ, Ren X, Tan ZR, Chen Y, Yin JY, Yu J, Qu J, Zhou HH and Liu ZQ. A high-throughput inhibition screening of major human cytochrome P450 enzymes using an in vitro cocktail and liquid chromatography-tandem mass spectrometry. Biomed Chromatogr 2014; 28: 197-203.

[4]   Griffin JL and Bollard ME. Metabonomics: its potential as a tool in toxicology for safety assessment and data integration. Cur Drug Metab 2004; 5: 389-398.

[5]   Nicholson JK and Lindon JC. Systems biology: Metabonomics. Nature 2008; 455: 1054-1056.

[6]   Boudonck KJ, Mitchell MW, Nemet L, Keresztes L, Nyska A, Shinar D and Rosenstock M. Discovery of metabolomics biomarkers for early detection of nephrotoxicity. Toxicol Pathol 2009; 37: 280-292.

[7]   Beger RD, Sun J and Schnackenberg LK. Metabolomics approaches for discovering biomarkers of drug-induced hepatotoxicity and nephrotoxicity. Toxicol Appl Pharmacol 2010; 243: 154-166.

[8]   Zhang L, Ye Y, An Y, Tian Y, Wang Y and Tang H. Systems responses of rats to aflatoxin B1 exposure revealed with metabonomic changes in multiple biological matrices. J Proteome Res 2011; 10: 614-623.

[9]   Huang Q, Tan Y, Yin P, Ye G, Gao P, Lu X, Wang H and Xu G. Metabolic characterization of hepatocellular carcinoma using nontargeted tissue metabolomics. Cancer Res 2013; 73: 4992-5002.

[10] Costa MD, de Freitas ML, Dalmolin L, Oliveira LP, Fleck MA, Pagliarini P, Acker C, Roman SS and Brandao R. Diphenyl diselenide prevents hepatic alterations induced by paraquat in rats. Environ Toxicol Pharmacol 2013; 36: 750-758.

[11] Chau KY, Korlipara LV, Cooper JM and Schapira AH. Protection against paraquat and A53T alpha-synuclein toxicity by cabergoline is partially mediated by dopamine receptors. J Neurol Sci 2009; 278: 44-53.

[12] Lattuca ME, Malanga G, Aguilar Hurtado C, Perez AF, Calvo J and Puntarulo S. Main features of the oxidative metabolism in gills and liver of Odontesthes nigricans Richardson (Pisces, Atherinopsidae). Comp Biochem Physiol B Biochem Mol Biol 2009; 154: 406-411.

[13] Han J, Zhang Z, Yang S, Wang J, Yang X and Tan D. Betanin attenuates paraquat-induced liver toxicity through a mitochondrial pathway. Food Chem Toxicol 2014; 70: 100-106.

[14] Malekinejad H, Rahmani F and Hassanpour F. Influences of sub-acute exposure to paraquat on cytochrome P450 3A2 expression in rat liver and lungs. Pestic Biochem Phys 2010; 96: 149-154.

[15] Wang X, Zhang M, Ma J, Zhang Y, Hong G, Sun F, Lin G and Hu L. Metabolic changes in paraquat poisoned patients and support vector machine model of discrimination. Biol Pharm Bull 2015; 38: 470-475.

[16] Zhang M, Deng M, Ma J and Wang X. An evaluation of acute hydrogen sulfide poisoning in rats through serum metabolomics based on gas chromatography-mass spectrometry. Chem Pharm Bull (Tokyo) 2014; 62: 505-507.

[17] Patti GJ, Yanes O and Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol 2012; 13: 263-269.

[18] Deng M, Zhang M, Huang X, Ma J, Hu L, Lin G and Wang X. A gas chromatography-mass spectrometry based study on serum metabolomics in rats chronically poisoned with hydrogen sulfide. J Forensic Leg Med 2015; 32: 59-63.

[19] Deng M, Zhang M, Sun F, Ma J, Hu L, Yang X, Lin G and Wang X. A gas chromatography-mass spectrometry based study on urine me-

tabolomics in rats chronically poisoned with hydrogen sulfide. Biomed Res Int 2015; 2015: 295241.

[20] Wen C, Zhang M, Ma J, Hu L, Wang X and Lin G. Urine metabolomics in rats after administration of ketamine. Drug Des Devel Ther 2015; 9: 717-722.

[21] Wen C, Zhang M, Zhang Y, Sun F, Ma J, Hu L, Lin G and Wang X. Brain metabolomics in rats after administration of ketamine. Biomed Chromatogr 2016; 30: 81-84.

[22] Monte AA, Heard KJ and Vasiliou V. Prediction of drug response and safety in clinical practice. J Med Toxicol 2012; 8: 43-51.

[23] Mamas M, Dunn WB, Neyses L and Goodacre R. The role of metabolites and metabolomics in clinically applicable biomarkers of disease. Arch Toxicol 2011; 85: 5-17.

[24] Kwon H, Park J, An Y, Sim J and Park S. A smartphone metabolomics platform and its application to the assessment of cisplatin-induced kidney toxicity. Anal Chim Acta 2014; 845: 15-22.

[25] Hong JH, Lee WC, Hsu YM, Liang HJ, Wan CH, Chien CL and Lin CY. Characterization of the biochemical effects of naphthalene on the mouse respiratory system using NMR-based metabolomics. J Appl Toxicol 2014; 34: 1379-1388.