

Metabonomics delineates allergic reactions induced by *Shuang-huang-lian* injection in rats using ultra performance liquid chromatography-mass spectrometry

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[ABSTRACT] *Shuang-huang-lian* Injection (SHLI) is the first successfully developed drug from traditional Chinese medicine (TCM) powder for injection, since its use for the treatment of acute respiratory tract infection, pneumonia, influenza, etc. At the same time, its allergic reactions have also emerged, which limits clinical applications. However, few scholars pay attention to the mechanism of allergic reactions. In this present study, metabonomics technology was used to explore the changes in endogenous metabolites in urine of the rat model of SHLI induced allergic reaction; we analyzed the metabolites, metabolic pathway, and the mechanism which were closely related to the allergic reactions. The levels of serum histamine and tryptase were examined and changes in histomorphology were also observed. Based on the UPLC-Q-TOF/MS metabonomics, we carried out the pattern recognition analysis, selected potential biomarkers associated with allergic reactions, and explored the pathological mechanism for SHLI induced allergic reaction, which laid the foundation for the safety research of SHLI. Our results showed that SHLI increased the levels of serum histamine and tryptase in rats with allergic reaction; we determined 15 biomarkers in rat allergic reaction model induced by SHLI and found multiple metabolic pathways involved, such as metabolism of linolenic acid, phenylalanine, amino acid, 2-oxo acid, and purine and other metabolic pathways.

[KEY WORDS] *Shuang-huang-lian* injection; Biomarker; Metabonomics; Allergic mechanism; Metabolites

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Introduction

Allergic reaction, including sensitization and stimulation, is a type of tissue injury with physiological disorder caused by the same antigenic substance, which is considered as an abnormal and pathological immune response. *Shuang-huang-lian* Injection (SHLI) is one of the earliest injection formulations

developed from traditional Chinese medicine (TCM) powder, which is composed of three herbs, *Lonicera japonica*, *Baical skullcap* root, and *Forsythia suspensa*, streamlining from the *Yinqiaosan* ("Warm Febrile Diseases"). SHLI is used for the treatment of viral and bacterial acute upper respiratory tract infection, acute bronchitis, tonsillitis, pharyngitis, and urinary tract infections. However, with a wide range of clinical applications, adverse reactions reports have also increased year by year, especially the allergic reactions, and the reasons are not clear. Moreover, few researchers have focused on the mechanism of allergic reactions. For thousands of years, TCM has made a great contribution to healing the wounded and rescuing the dying. Nowadays, TCM is widely concerned by the whole world because of its good therapeutic effect and low toxicity. Therefore, it is necessary to study the mechanism of SHLI induced allergic reactions and to lay the foundation for the safe application of TCM injections.

Metabonomics is a newly developed technology in post-genome era, with high separation efficiency, high sensitivity and low detection limit, which can be used to study the metabolic

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pathways of biological systems by investigating the changes of their metabolites or their changes over time after stimulating or disturbing the biological system (*e.g.*, a particular gene variation or environmental changes) [1–4]. In 2005, Wrona *et al.* introduced a new mass spectrometry technique for metabolic studies [5]. Recently, ultra performance liquid chromatography (UPLC) technique is suggested to be suitable for metabonomics, because of its high sensitivity in detecting metabolites and high efficiency in separation, especially for large-scale untargeted metabonomics [6]. The mass spectrometry^{Elevated Energy} technique has been proven to be a powerful tool for confirming their presence and for identification of trace components in complex mixtures. As the emergence of more effective UPLC-MS^E technologies, metabolomics investigations have been greatly expanded [7–11]. Nowadays, UPLC-MS^E is one of the most versatile techniques, offering a significant potential for discovery of novel biomarkers from disease samples [12–17]. It has a wide dynamic range, high sensitivity and high resolution that are required for comprehensive analysis of metabolites; at the same time, with improvements in quantitation accuracy, robustness, and throughput, UPLC-MS can analyze volatile, non-volatile, and polar compounds, over a wide mass range and does not require sample derivatization.

In agreement with the holistic concept of TCM, metabolomics has shown potential in pharmacological and toxicological evaluation of TCM [18–19]. The UPLC-MS^E-based metabolomics platform may become a powerful tool for pharmacological and toxicological evaluation of TCM. Based on UPLC-MS^E metabolomics technique, Zhang *et al.* have reported pharmacological activity and biochemical action mechanism of rhubarb on chronic kidney disease [20]. Their results have demonstrated that rhubarb can restore upregulation expression of α smooth muscle actin, collagen I, fibronectin and plasminogen activator inhibitor-1 proteins against tubulointerstitial fibrosis and show a beneficial nephroprotective effect [21]. The aristolochic acid-induced nephrotoxicity has been systematically evaluated by UPLC-MS^E metabolomic approach using serum, urine and tissue samples [22–24]. Metabolomics results have demonstrated that the dysregulation of lipid abnormalities is closely associated with oxidative stress, inflammation, fibrosis, and Nrf2 dysfunction in aristolochic acid-induced nephropathy [25].

Metabonomics is an important part of biology system that combines overall, dynamic, and comprehensive analysis using various methodologies, which is consistent with the overall concept in medicine [26]. In the present study, we used the bovine serum albumin and SHLI induced acute allergic reaction model to investigate the changes in endogenous metabolites, determine the regulation of SHLI on metabolism and identify the biomarkers associated with SHLI induced allergic reaction [27], further elucidating the mechanism of allergic reaction. It was hoped that our results will help expand the application of metabonomics in studying drug aller-

gic reaction [28–29], by demonstrating [30–33].

Materials and Methods

Chemicals and reagents

SHLI (lyophilized, 600 mg each) was obtained from the Second Chinese Medicine Factory of Harbin Pharm. Group Co., Ltd. (Product batch No. 1409405; Harbin, China). HPLC-grade acetonitrile was acquired from Fisher Corporation (Waltham, Massachusetts, USA). Watsons Water was purchased from Guangzhou Watsons Food Co., Ltd. (Guangzhou, China). Formic acid (HPLC grade) was obtained from Tianjin Kemiu Chemical Reagent Co., Ltd. (Tianjin, China). Leucine enkephalin (LENK) was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA). Rat serum histamine detection kit was purchased from Shanghai Blue-based Biotechnol. Ltd. (Shanghai, China). Trypsin was purchased from Beijing Soledad Bao Technology Co., Ltd. (Beijing, China). Sodium chloride injection (0.9%, *W/V*), as a negative control drug and solvent, was purchased from Zhejiang Doubang Pharmaceutical Co., Ltd. (Quzhou, China). Bovine serum albumin (BSA) formulated in 0.9% sodium chloride injection was purchased from Sigma Chemical Co., Ltd. (St. Louis, MO, USA).

Apparatus

KDC-160HR high speed freezing centrifuge was purchased from HKUST Innovation Co., Ltd. Jia Branch (China); microplate reader was purchased from Shanghai Thermo Fisher Instrument Co., Ltd. (Shanghai, China); ultra-low temperature freezer was purchased from Shanghai Thermo Fisher Instrument Co., Ltd. (Shanghai, China); KQ5200E ultrasonic cleaner was purchased from Kunshan Ultrasonic Instrument Co., Ltd. (Kunshan, China); JA2003 Precision electronic balance was purchased from Shanghai Ryohei Instrument Factory Co., Ltd. (Shanghai, China).

UPLC-Q-TOF/MS

Ultra-performance liquid chromatography was performed with the Waters AcquityTM system (Milford, Massachusetts, USA equipped with quaternary pump, -line vacuum degasser, autosampler, Diode array detector, and column oven). Quadrupole time-of-flight mass spectrometry was accomplished with the Micromass Q-TOF microTM, system, (Milford, Massachusetts, USA) with electrospray ionization-positive and negative scanning method. The column used in the present study was an ACQUITY UPLCTM BEH C₁₈ column (1.7 μ m, 2.1 mm $\times$ 50 mm, Waters Corp, Milford USA). The column temperature was kept at 40 °C, the sample compartment temperature was at 4 °C, and the flow rate was set at 0.3 mL·min⁻¹. The injection volume was 3 μ L. Acetonitrile was used as mobile phase A and 0.1% aqueous formic acid (*V/V*) was used as mobile phase B. The linear gradient elution of A was performed as follows: 2%–40% A at 0–8 min, 40%–98% A at 8–10 min, 98%–100% A at 10–13 min.

The eluent was transferred to the mass spectrometer directly rather than a split; Electro-spray ionization (ESI) interface was used to scan in both positive and negative ion modes.

The desolvation temperature was set at 350 °C and with the flow rate was set at 750 L·h⁻¹ for both positive ion and negative ions. The flow rate of cone was set at 20 L·h⁻¹, the capillary voltage was set at 1.3 KV in positive ion and 1.5 KV in negative ion, and the sample cone voltage and extraction voltages were 60 and 5V, respectively. The source of temperature was set at 110 °C, collision energy was 100 V, and the gas of collision was Argon. For an accurate mass acquisition, a lock-mass of leucine enkephalin was used in positive ion mode and negative ion mode ($[M + H]^+$ m/z 556.277, $[M + H]^-$ m/z 554.2615). MS data were acquired in full-scan mode from m/z 100 to 1500 Da, MassLynx Station (V4.1) (Waters Corporation, Milford, USA) and MarkerLynx software (Waters Corporation, Milford, USA) were applied to the peak detection principal component analysis [34–35].

Animal groups and preparation of samples

Healthy SD male rats (SPF grade), weighting between 200 ± 20 g, obtained from GLP of Heilongjiang University of Chinese Medicine (China), License number: SYXK (HEI) 2013-012, were given standard laboratory feed and the temperature, kept the temperature at (22 ± 1) °C with 40%–50% humidity in controlled rooms and 12 h light to dark cycle, with free access to diet and water, the animals were kept in individual metabolic cages for 7 days, before being used in the study. All the animal experiments were in line with national law for the use of experimental animal requirements (China) [36].

Twenty-two SD rats were randomly divided into control group (K), SHL high dose group (H), SHLI medium dose group (M) and SHL low dose group (L) with 8 rats each group. The sensitized rats were administrated with H (720 mg·kg⁻¹), M (360 mg·kg⁻¹) and L (180 mg·kg⁻¹) by intraperitoneal injection of SHL once every other day, continuous injection of three times. In the first 14th and 21st days after the initial sensitization, the rats were injected by tail vein with the double dose. The control group were given the same volume of 0.9% sodium chloride injection, then observed the physical signs immediately to 30 min. Urine was collected in the next day (between 7: 00–8: 00 am) from metabolism cages within 12 hours of each rat and centrifuged at 13 000 r·min⁻¹ at 4 °C for 15 min, then the supernatants were stored frozen at –80 °C until metabonomics analysis, blood was collected from the eye venous plexus in the 14th and 21st days after the excitation in 30 min. After centrifuging the samples at 3000 r·min⁻¹ for 15 min, The supernatants were frozen at –80 °C until analysis [37–41]. After collecting the urine in the last time, the rats were anesthetized, fixed parts of the lung and trachea tissue placed in 4% poly formaldehyde, The pathological examination were carried as routine [42].

Biochemical index detection

We operated as the instructions of ELISA Histamine test kit, and detected the content of histamine in rat serum. The content of tryptase was detected by using a method of specific substrate [43].

Statistical analysis

The urine metabolic profile data of each group were in-

troduced into the Markerlynx software (Waters Corporation Milford, Massachusetts, USA) to identify the peak and peak matching, and the method of principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were applied in metabolomic-based drug metabolism studies [44]. To reduce the multi-dimensional data, EZ-info 2.0 software (Waters Corp., Milford, Massachusetts, USA) software modules were used to obtain mass matrix information. According to the metabolic data of urine and different time of urine metabolome analysis, the score plot to reflect the degree of dispersion between groups was depicted, and the OPLS-DA analysis for the control group and the administration groups of SHLI was carried out [45–46]. Cluster analysis was performed in the PCA score plot. According to PCA analysis obtained from projection (loading plot), we looked the biomarkers based on the variation of each component to determine the size of the contribution. Structural analysis was applied to the potential biomarkers of the VIP values exceeding two, to ensure the reliability of the endogenous biomarker. The analysis of secondary mass spectrometry was carried out on the metabolites of the differences, to resolve the biomarkers and related to metabolic pathways. HMDB (<http://www.hmdb.ca/>), METLIN (<http://metlin.scripps.edu/>), Massbank (<http://www.massbank.jp>), and KEGG (<http://www.kegg.com/>), were used as biochemical database to identify the potential markers [47–48].

Results

Biochemical parameters and pathological changes

To evaluate the allergies of SHLI, the serum histamine and tryptase were detected in rats. Compared with the control group (K), the high dose (H) and medium dose (M) groups showed the significantly increased contents of histamine and tryptase ($P < 0.01$), and low dose group (L) showed a slight increase, which was not statistically significant (Fig. 1).

H&E staining was applied to trachea and lungs of the rats. In control group, false ciliated epithelial layer was intact in trachea, with visible collagen and elastic fibers in Lamina propria and visible blood vessels, lymph vessels, and lymph tissue. The ring of cartilage was complete. In the low dose group, the mucosa and submucosa of trachea were thickened, false ciliated epithelial cells and lamina propria were still visible and visible blood vessels, lymph vessels and lymph tissue, lymphocytes were increased, and the ring of cartilage was complete. In the medium dose group, the mucosa and submucosa were further thickened, and most of false ciliated epithelial cells and lamina propria were disappeared. It showed enhanced the basophilic of cells. In the high dose group, the mucosa and submucosa were thickened, false ciliated epithelial cells and lamina propria were disappeared, and basophilic of cells were enhanced, indicating that the levels of lesions were gradually increased as the increases of the dose, as shown in Fig. 2 (1).

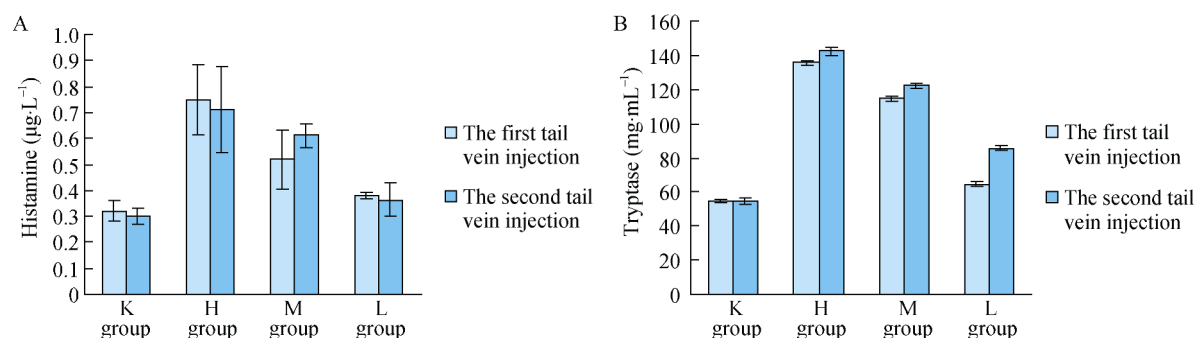


Fig. 1 Comparison serum histamine (A) and tryptase (B) contents in rats of each group (Mean $\pm$ SD, $n = 8$). K: control group; L: low dosage group of SHLI; M: medium dosage group of SHLI; H: high dosage group of SHLI

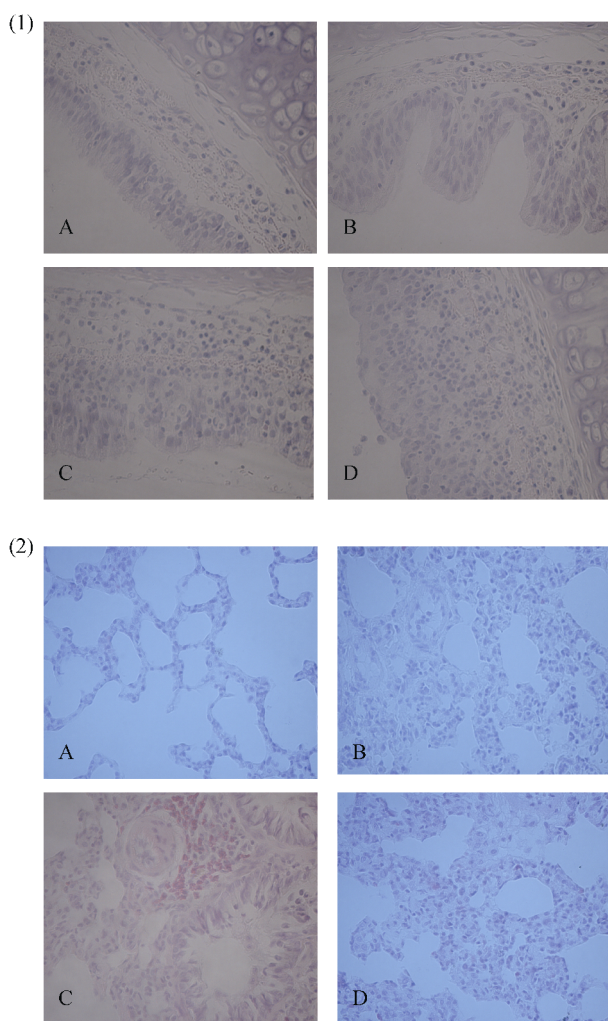


Fig. 2 The tracheal (1) and lung tissue (2) histopathology sections of rats in different groups (H&E, $\times 40$). A, control group; B, low dose group; C, medium dose group; and D, high dose group

As shown in Fig. 2(2), the structures of lung tissue in the control group were intact. In the low dose group, partial atelectasis within the organization was found. In the medium dose group, a local thickening of alveolar septa within the organization,

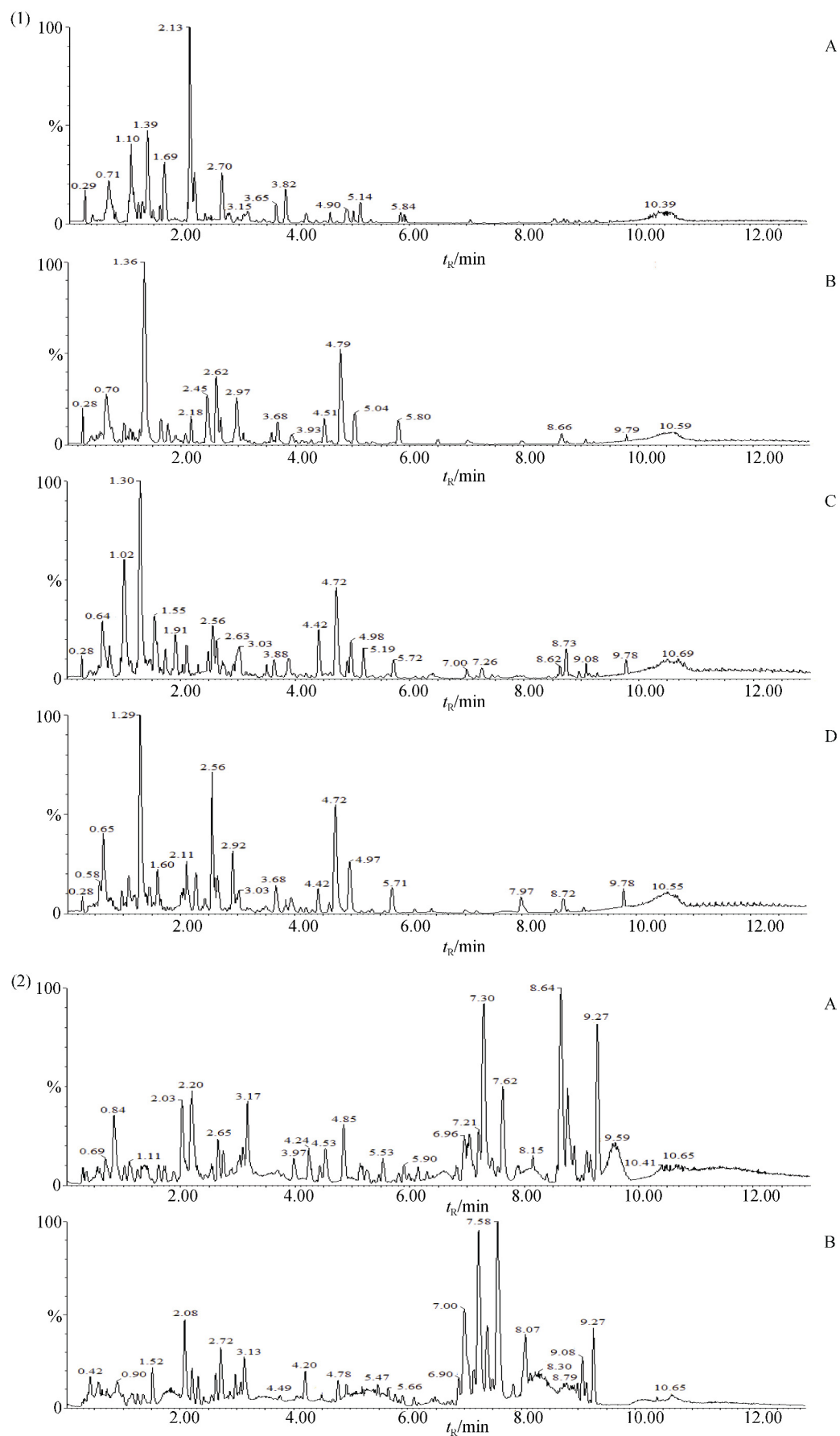
infiltrated of inflammatory cells, and capillary congestion were observed. In the high dose group, alveolar atelectasis in local organizations was obvious, alveolar septa was thickened, capillary congestion was seen in local, and macrophages and eosinophils were increased, indicating that the levels of lesions were gradually increased as the increases of SHLI dose.

Metabonomics analysis

Under the optimal U-HPLC-TOF/MS conditions, in positive and negative scan mode, mass metabolites could get a better separation and effective response effect in a 13-min elution time^[49-51]. Based on peak intensity (BPI), urine samples were collected in positive and negative ion mode, chromatograms are shown in Fig. 3(1), and Fig. 3(2).

PCA analysis

EZinfo software (Waters Corp, Milford, USA) was used to analyze the multivariate data matrix. The unsupervised segregation was checked by principal component analysis (PCA). Both PCA and PLS-DA were used, because of their ability to cope with noisy, highly multivariate, colinear and possibly incomplete data. PCA is an unsupervised pattern recognition method initially used to discern the presence of inherent similarities in spectral profiles. It is a major method to reduce the multi-dimensional data, the process is designed to eliminate more coexistence in the overlapping portion and to extract the main component. According to the structure of the covariance matrix in original data, we looked for a new linear combination of the original variables obtained by the principal component. From the PCA score plots in four samples (Fig. 4), administration of SHLI disrupted the normal urine metabolic profiles of rat to a large extent. The rats in the same group had a good similarity of urinary metabolite profile and different groups could be separated to clusters. The H group was shown to be the furthest away from the control group. These results indicated that the metabolic profiles were greatly changed as a consequence of administration of SHLI in a dose-dependent manner. The endogenous metabolites of rats showed the most significant difference between the control group and the high dose group. These changes in endogenous metabolites may affect the metabolic pathways and networks^[52-56].



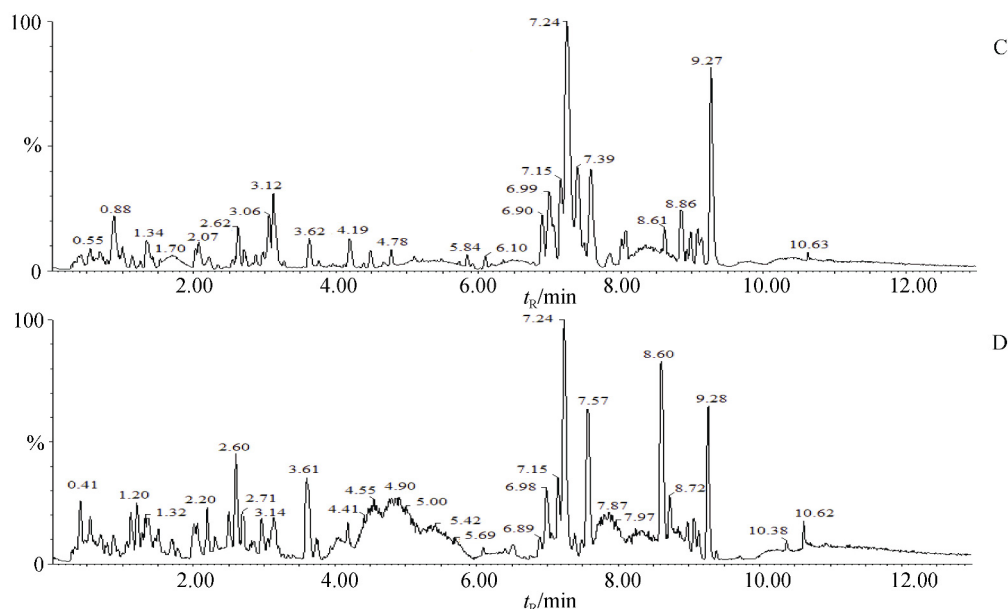


Fig. 3 BPI chromatograms of (A) control group, (B) L group, (C) M group, (D) H group urine samples on the last day analyzed by UPLC-TOF/MS in positive (1) and negative (2) ESI modes

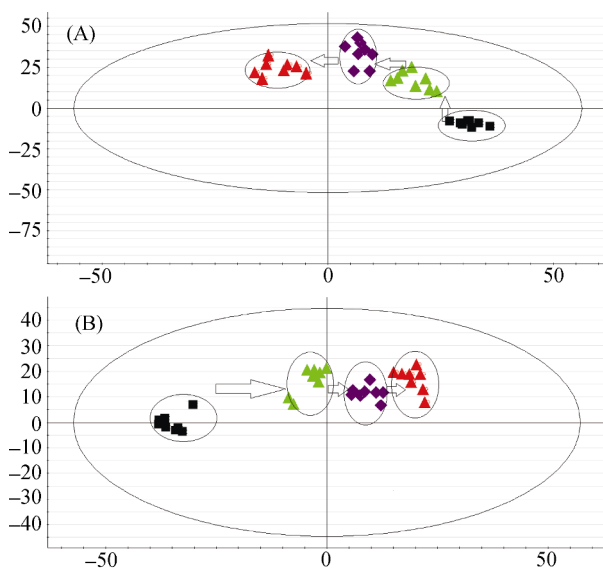


Fig. 4 PCA Score plots of urine samples collected from different groups of rats in positive mode (A) and negative mode (B). K: control group (■); L: low dosage group of SHLI (▲); M: medium dosage group of SHLI (◆); H: high dosage group of SHLI (▲).

OPLS-DA analysis

With the completion of the orthogonal projection to latent structures discriminate analysis (OPLS-DA), we used computational systems analysis with the Metabo Analyst data annotation approach^[57] to distinguish between the control group and the SHL treated groups. PLS-plot has been used in prediction pattern recognition, exploratory data analysis and data mining problems for a long time. In order to obtain a better discrimination among the control and these administration groups, the

OPLS-DA approach was applied to the metabolic profiles. OPLS-DA score plots separated the urine samples into two blocks, showing a clear separation between the control group and high dosage group in both positive and negative ion modes (Fig. 5, $P < 0.05$). The potential markers were extracted based on their contributions to the variations and correlation within the dataset. Based on $VIP > 1$ and the degrees of perturbation in the metabolites evaluated by Student's t -test, the 15 metabolites were identified as potential biomarkers. The found marks differentiating metabolites for the model of allergies reaction and are revealed in serial numbers in Tables 1 and 2.

Potential biomarkers

All the detected ions were arranged in descending order according to their VIP values, and the highest VIP value was 2.16 in the positive mode and 2.41 in the negative mode. Combining the results of the OPLS analysis with the amount variation of ions in each group, 6 ions with VIP values exceeding 2 were selected in the positive mode. At the same time, 9 ions with VIP values exceeding 2 were selected in negative mode. The UPLC-HDMS segregation analysis platform provided the retention time, precise molecular mass and MS/MS data for the structural identification of biomarkers. The precise molecular mass was determined within measurement errors (< 5 ppm) by Q-TOF, and meanwhile, the potential elemental composition, degree of unsaturation and fractional isotope abundance of compounds were obtained. The supposed molecular formula was searched in Chemspider, the Human Metabolome Database, Mass Bank and other related databases to identify the possible chemical constitutions, and the MS/MS data were screened to determine the potential structures of the ions. Thus, taking two ions as examples, the identification procedures were described as follows^[58-59].

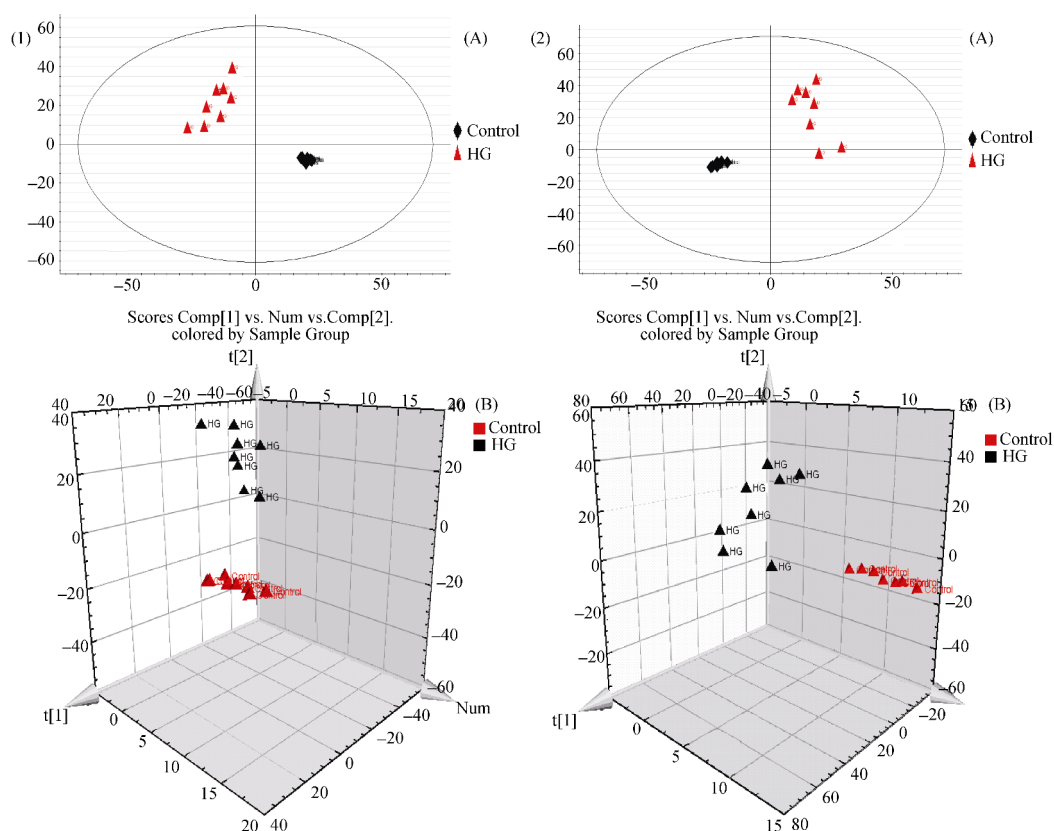


Fig. 5 OPLS-DA model results between the control group and high dosage group of SHLI (HG) in positive (1) and negative (2) mode. (A, 2-D plot; B, 3-D plot)

Table 1 Potential biomarkers identified in positive ESI mode

No.	VIP values	$t_{R_M^+}$	Actual_M	Content variance	$[M + H]^+$	Proposed compound	MS/MS	Losses	Proposed structure
1	2.10	1.92_372.2408	371.2824	↓	372.5561	Docosahexaenoyl ethanolamide $C_{24}H_{37}NO_2$	133 105 147	$-C_{14}H_{25}NO_2$ $-C_{16}H_{29}NO_2$ $-C_{14}H_{27}NO$	
2	2.16	5.66_271.0612	270.0964	↓	271.2420	Aspartyl-Histidine $C_{10}H_{14}N_4O_5$	220 178 177 110	$-H_3O_3$ $-CH_3NO_4$ $-C_2H_6O_4$ $-C_5H_7NO_5$	
3	2.10	1.89_386.2219	383.1907	↓	363.4115	Isopentenyladenine-9-N-glucoside $C_{17}H_{25}N_5O_4$	192 297	$-C_7H_{12}N_2O_3$ $-C_4H_5N$	
4	2.05	1.16_297.1437	296.1624	↑	297.3157	Marasmiol $C_{15}H_{20}O_6$	192 210 126	$-C_4H_9O_3$ $-C_4H_7O_2$ $-C_9H_{15}O_3$	
5	2.03	3.17_234.1306	233.1012	↑	234.2218	Glutaminyl-Serine $C_8H_{15}N_3O_5$	110 84 124	$-C_3H_{10}NO_4$ $-C_3H_6N_2O_5$ $-C_2H_8NO_4$	
6	2.01	3.27_220.1615	219.1120	↑	220.2431	Cis-zeatin $C_{10}H_{13}N_5O$	165 192 135	$-C_2H_3N_2$ $-CH_2N$ $-C_2H_3N_3O$	

Table 2 Potential biomarkers identified in negative ESI mode

No.	VIP values	$t_{R_M^-}$	Actual_M	Content variance	[M – H]	Proposed compound	MS/MS	Losses	Proposed structure
1	2.14	5.68_269.0453	270.0528	↑	269.2369	6-Hydroxydaidzein C ₁₅ H ₁₀ O ₅	162 119	-C ₅ HO ₃ -C ₇ H ₄ O	
2	2.17	1.96_159.0662	160.0484	↑	159.128	Tricholomicacid C ₅ H ₈ N ₂ O ₄	119 160	-C ₂ H ₂ O	
3	2.15	2.51_163.0407	164.0473	↑	163.158	Phenylpyruvic acid C ₉ H ₈ O ₃	119 91	-CO ₂ -C ₂ O ₃	
4	2.32	0.83_173.0705	174.0528	↓	173.1513	2-Isopropyl-3-oxosuccinate C ₇ H ₁₀ O ₅	111 87 85	-C ₂ H ₆ O ₂ -C ₄ H ₆ O ₂ -C ₃ H ₄ O ₃	
5	2.13	3.92_187.0959	188.0797	↓	187.1812	N-acetylglutamine C ₇ H ₁₂ N ₂ O ₄	116 117	-C ₂ HNO ₂ -C ₂ H ₂ N ₂ O	
6	2.30	12.8_201.121	202.0338	↑	201.1249	5-Hydroxy-2-oxo-4-ureido-2,5-dihydro-1H-imidazole-5-carboxylate C ₅ H ₆ N ₄ O ₅	162 119 160	-C ₂ N ₂ O ₂	
7	2.30	4.85_201.1136	202.1116	↑	201.0116	1, 2, 3, 4-Tetrahydro-2-methyl-b-carboline C ₁₂ H ₁₄ N ₂	119 84	-C ₄ H ₆ N ₂ -C ₈ H ₇ N	
8	2.41	2.97_201.1125	202.0954	↑	201.2078	Seriny-Proline C ₈ H ₁₄ N ₂ O ₄	162 119	-C ₃ H ₃ -CH ₆ O ₄	
9	2.18	2.86_211.0602	212.0568	↑	211.2041	Questiomyacin A C ₁₂ H ₈ N ₂ O ₂	142 107 143 108	-C ₃ HO ₂ -C ₆ O ₂ -C ₃ O ₂ -C ₃ O ₂	

In the positive ion mode, the VIP value was 2.16 and the retention time was 5.66 for $[M + H]^+$ 271.0612. The ion contained an even number of nitrogen atoms and an odd number of oxygen atoms, its precise molecular weight was 270.0964, and from the analysis of elemental composition and isotopic abundances its molecular formula was speculated to be C₁₀H₁₄N₄O₅. By the analysis of MS/MS, the main fragment ions were m/z 220, 178 and 110; which could be $[M - H]^-$ lost -H₃O₃, -CH₃NO₄, and -C₅H₇NO₅. At last, according to the database, it was speculated to be Aspartyl-Histidine. Its structure and mass spectrum are shown in Fig. 6. In the negative ion mode, the VIP value was 2.15 and the retention time was 2.51 for $[M - H]^-$ 163.0407. The ion contained an even number of nitrogen atoms and an odd number of oxygen atoms. Its precise molecular weight was 164.0473, and from

the analysis of elemental composition and isotopic abundances, the molecular formula was speculated to be C₉H₈O₃. By the analysis of MS/MS, the main fragment ions were m/z 119, 91, which could be $[M - H]^-$ lost -CO₂, -C₂O₃. Finally, according to the database it was speculated to be phenylpyruvic acid. Its structure and mass spectrum are shown in Fig. 6. All of the identified potential biomarkers are displayed in Tables 1 and 2. And the Arabic numbers in Fig. 6 are compatible with the serial numbers in Tables 1 and 2. In the following discussion section, we further explored the metabolic pathway biomarkers associated with allergic effects [60].

Discussion

SHLI is a famous Chinese patent medicine and in the form of a freeze-dried powder, which was developed by

Harbin Pharmaceutical Group in the 1990s. In recent years, because of its wide distribution and potent biological effects, SHLI has attracted widespread attention and the market demand has increased greatly. Its allergic reactions have emerged, which limits clinical applications. Therefore, we have studied the metabolic profiles for SHLI induced allergic reactions. Our results may provide new ideas and methods to improve the safety and effectiveness of traditional Chinese medicine injection. Urine is an ideal biological medium for disease research and the most common specimen used in metabolomics [61] because it is easy to get without sampling frequency and time constraints or damage to the body. Dynamic detection and analysis could be performed, and therefore, urine samples were used in metabolomics study of SHLI.

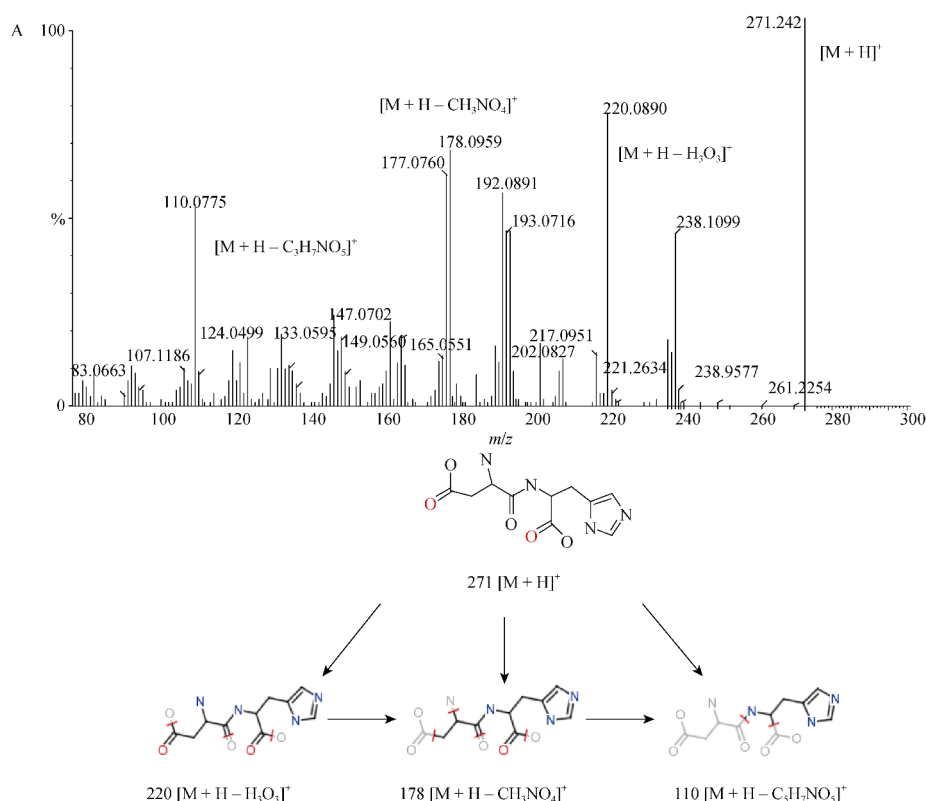
In the present study, the allergy model was produced by administration of different doses of SHLI in rats. UPLC-TOF/MS was used to study the urinary metabolic perturbations associated with allergy induced by SHLI and to identify the relations between the injections and alters of metabonomic profiles, and then to discover potential biomarkers of SHLI in allergies.

The results from the present study suggested that the metabonomic spectra of post-treatment were greatly changed in the SHL groups and the profiles was deviated significantly. These changes were dose-dependent among the groups treated with various doses of SHLI. By the analysis of pattern recognition, we found 15 potential biomarkers related to allergy.

In the following sections, we would like to discuss the biological significance of the major biomarkers found in the present study. Docosahexaenoic acid (DHA) is an essential

fatty acid in humans, and is the most abundant of ω -3 fatty acids in neural tissues. Docosahexaenoyl ethanolamide (DHEA) is ethanolamine amide of DHA. It has been reported that the concentrations in brain and retina are similar to that of arachidonoyl ethanolamide (AEA). Docosahexaenoic acid is the differential metabolite of SHLI induced allergic reactions and is closely related to inflammatory mediators. The possible reason was related to elevate free fatty acids (FFAs) and endothelial dysfunction. Docosahexaenoic acid has an important biological significance in inflammation. And by related analysis of metabolic pathway, its impact is mainly on the metabolic pathway of linolenic acid.

Phenylpyruvic acid is catabolic byproduct of phenylalanine metabolism or an intermediate of a keto-acid, and it has a slight odor of honey. The amount of phenylpyruvate is usually very low in urine or blood, and it can be found at high levels of phenyl pyruvate in the urine of individuals with phenylketonuria (PKU). The lack of phenylalanine hydroxylase (PAH) is the main reason of PKU, so that phenylalanine is converted to phenylpyruvic acid rather than tyrosine, especially, by a transaminase pathway route involving glutamate, and excess phenylalanine can be metabolized into phenylketones. The metabolites of the transamination reaction include phenethylamine, phenylacetate, and phenylpyruvate. In persons with PKU, dietary phenylalanine is either converted to phenylpyruvic acid or accumulated in the body. In the present study, the content of phenylketonuria was increased in urine, suggesting that it is involved the metabolic disorders of phenylalanine.



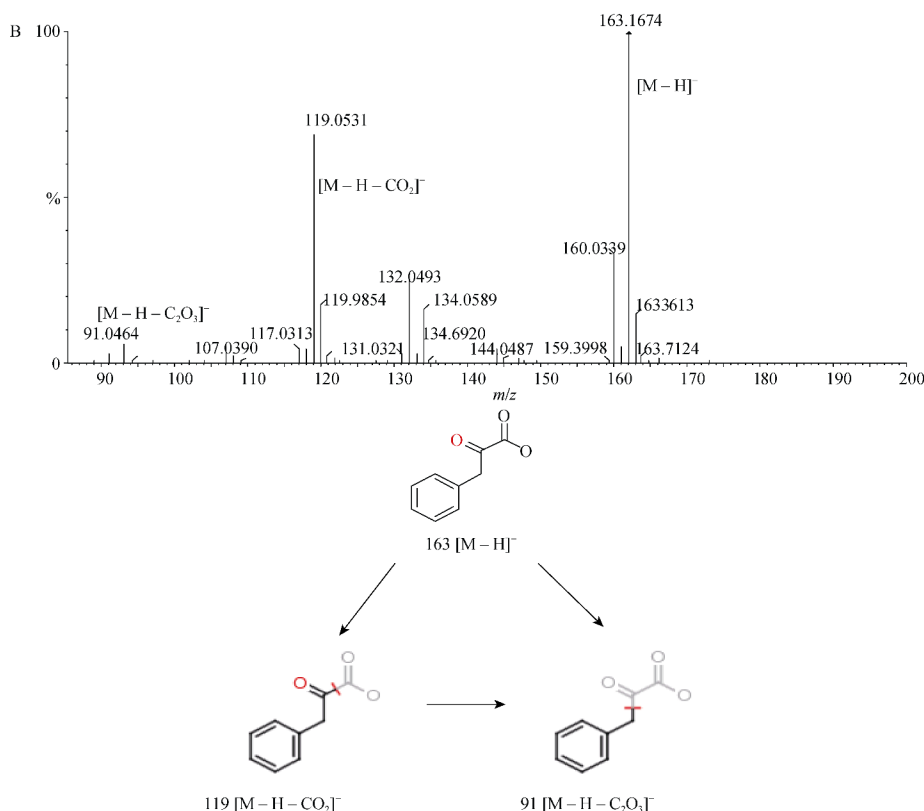


Fig. 6 Chemical structure and mass fragment information of aspartyl-histidine in positive (A) and negative ESI (B) mode

Aspartyl-histidine is a dipeptide made up of aspartate and histidine. Glutamyl-Serine is a dipeptide made up of glutamine and serine. It is an incomplete breakdown product of protein catabolism or protein digestion. The metabolic pathways are involved in the metabolism of amino acid. 2-Isopropyl-3-oxosuccinate is an intermediate in leucine biosynthesis, as the third step in leucine biosynthesis after the fork from valine synthesis, and can be generated from (2R, 3S) - 3-Isopropylmalate. It is a reaction of oxidative decarboxylation. Leucine biosynthesis involves a five-step conversion process beginning with the valine precursor 2-keto-isovalerate. In this pathway, the final step is catalyzed by two transaminases of broad specificity involving Branched-chain amino acid transferase (IlvE) and Tyrosine aminotransferase (TyrB). Through the spontaneous reaction, 2-isopropyl-3-oxosuccinate is converted to 4-methyl-2-oxo-valeric acid, and in this pathway, specifically involved is the metabolism of 2-oxo-acid.

N-Acetylglutamine (GlcNAc) is an amino acid (an acetylated analogue of glutamine) that is modified, and a metabolite exist in normal human urine. By NMR and HPLC-MS, the decomposition products of GlcNAc have been identified as N-(2, 6-dioxo-3-piperidiny)l acetamide, N-acetyl-L-glutamic acid, glutamic acid, pyroglutamic acid, and glutamine. GlcNAc is the source of glutamine for parenteral nutrition, as glutamine is too unstable, but GlcNAc is very stable. In the patients treated with glycopeptides and/or aminoglyco-

sides, the urine levels of GlcNAc indicates the degree of renal tubular injury. We have detected the high amounts of N-acetylated amino acids (i.e.: N-acetylglutamine) in the patient with aminoacylase I deficiency (EC 3.5.1.14, a homodimeric zinc-binding metalloenzyme located in the cytosol) [62].

5-Hydroxy-2-oxo-4-ureido-2, 5-dihydro-1H-imidazole-5-carboxylate is a portion of the metabolism pathway of purine.

All the results from the present study suggested that the metabolic profiles of healthy rats were disturbed by administration of SHLI. The potential biomarkers identified may provide the evidence of allergy induced by SHLI.

Conclusion

In the present study, on the analysis of metabolic pathways based the UPLC/ESI-TOF-MS technique combined with chemometrics method was used to demonstrate the urinary metabolic characteristics in rats treated with SHLI. The principal component analysis was used as an example of pattern recognition techniques to apply the chromatographic data to develop a distinct clustering of the three groups, based on their urine samples. With the OPLS analysis, 15 biomarkers related to SHL allergy were identified rapidly and precisely. Importantly, we discovered the metabolic pathways were associated with allergic reactions of SHLI. We were the first to study the mechanism of SHLI induced allergic reactions at the metabolic level. These results indicated that metabonom-

ics is a powerful tool for allergy study to gain more insights into the mechanism of allergy at the metabolic level. In conclusion, our results from the present study suggested that metabonomic methods not only were high sensitive and specific, but also could benefit further understanding of disease mechanisms, developing biomarkers for earlier detection. The strategy could provide a practical method for assessment of drug allergy in traditional Chinese medicines.

References

- [1] Wang XJ, Lv HT, Sun H, et al. Metabolic urinary profiling of alcohol hepatotoxicity and intervention effects of Yin Chen Hao Tang in rats using ultra-performance liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry [J]. *J Pharm Biomed Anal*, 2008, **48**(4): 1161-1168.
- [2] Chen Y, Tang Y, Zhang YC, et al. A metabolomic study of rats with doxorubicin-induced cardiomyopathy and shengmai injection treatment [J]. *PLoS One*, 2015, **10**(5): e0125209.
- [3] Wang P, Sun H, Lv HT, et al. Thyroxine and reserpine-induced changes in metabolic profiles of rat urine and the therapeutic effect of Liu Wei Di Huang Wan detected by UPLC-HDMS [J]. *J Pharm Biomed Anal*, 2010, **53**(3): 631-645.
- [4] Sun H, Wang HY, Zhang AH, et al. Berberine ameliorates nonbacterial prostatitis via multi-target metabolic network regulation [J]. *OMICS*, 2014, **19**(3): 186-195.
- [5] Wrona M, Mauriala T, Bateman KP, et al. 'All-in-one' analysis for metabolite identification using liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry with collision energy switching [J]. *Rapid Commun Mass Sp*, 2005, **19**(18): 2597-2602.
- [6] Zhao YY, Cheng XL, Vaziri ND, et al. UPLC-based metabonomic applications for discovering biomarkers of diseases in clinical chemistry [J]. *Clin Biochem*, 2014, **47**(15): 16-26.
- [7] Bateman KP, Castro-Perez J, Wrona M, et al. MS^E with mass defect filtering for *in vitro* and *in vivo* metabolite identification [J]. *Rapid Commun Mass Sp*, 2007, **21**(9): 1485-1496.
- [8] Zhao YY, Lin RC. UPLC-MS^E application in disease biomarker discovery: the discoveries in proteomics to metabolomics [J]. *Chem Biol Interact*, 2014, **215**(1): 7-16.
- [9] Yang Z, Hou JJ, Qi P, et al. Colon-derived uremic biomarkers induced by the acute toxicity of Kansui radix: A metabolomics study of rat plasma and intestinal contents by UPLC-QTOF-MS^E [J]. *J Chromatogr B*, 2016, **1026**: 193-203.
- [10] Zhao YY, Cheng XL, Wei F, et al. Intrarenal metabolomic investigation of chronic kidney disease and its TGF- β 1 mechanism in induced-adenine rats using UPLC Q-TOF/HSMS/MS^E [J]. *J Proteome Res*, 2013, **12**(2): 692-703.
- [11] van Dyk M, Mangoni AA, McEvoy M, et al. Targeted arginine metabolomics: A rapid, simple UPLC-QToF-MS^E based approach for assessing the involvement of arginine metabolism in human disease [J]. *Clin Chim Acta*, 2015, **447**: 59-65.
- [12] Zhao YY, Zhang L, Long FY, et al. UPLC-Q-TOF/HSMS/MS^E-based metabonomics for adenine-induced changes in metabolic profiles of rat faeces and intervention effects of ergosta-4, 6, 8(14), 22-tetraen-3-one [J]. *Chem Biol Interact*, 2013, **201**(1-3): 31-38.
- [13] Wesseling H, Want EJ, Guest PC, et al. Hippocampal proteomic and metabonomic abnormalities in neuro- transmission, oxidative stress, and apoptotic pathways in a chronic phencyclidine rat model [J]. *J Proteome Res*, 2015, **14**(8): 3174-87.
- [14] Zhao YY, Shen XF, Cheng XL, et al. Urinary metabonomics study on the protective effects of ergosta-4, 6, 8(14), 22-tetraen-3-one on chronic renal failure in rats using UPLC Q-TOF-MS and a novel MS^E data collection technique [J]. *Process Biochem*, 2012, **47**(12): 1980-1987.
- [15] Zhao YY, Cheng XL, Wei F, et al. Application of faecal metabonomics on an experimental model of tubulointerstitial fibrosis by ultra performance liquid chromatography/high-sensitivity mass spectrometry with MS^E data collection technique [J]. *Biomarkers*, 2012, **17**(8): 721-729.
- [16] Zhang ZH, Chen H, Vaziri ND, et al. Metabolomics signatures of chronic kidney disease of diverse etiologies in the rats and humans [J]. *J Proteome Res*, 2016, **15**(10): 3802-3812.
- [17] Zhao YY, Liu J, Cheng XL, et al. Urinary metabonomics study on biochemical changes in an experimental model of chronic renal failure by adenine based on UPLC Q-TOF/MS [J]. *Clin Chim Acta*, 2012, **413**(5-6): 642-649.
- [18] Shi J, Cao B, Wang XW, et al. Metabolomics and its application to the evaluation of the efficacy and toxicity of traditional Chinese herb medicines [J]. *J Chromatogr B*, 2016, **1026**: 204-216.
- [19] Chen DQ, Chen H, Chen L, et al. Metabolomic application in toxicity evaluation and toxicological biomarker identification of natural product [J]. *Chem Biol Interact*, 2016, **252**: 114-130.
- [20] Zhang ZH, Vaziri ND, Wei F, et al. An integrated lipidomics and metabolomics reveal nephroprotective effect and biochemical mechanism of *Rheum officinale* in chronic renal failure [J]. *Sci Rep*, 2016, **6**: 22151.
- [21] Zhang ZH, Wei F, Vaziri ND, et al. Metabolomics insights into chronic kidney disease and modulatory effect of rhubarb against tubulointerstitial fibrosis [J]. *Sci Rep*, 2015, **5**: 14472.
- [22] Lin SH, Chan W, Li JH, et al. Liquid chromatography/mass spectrometry for investigating the biochemical effects induced by aristolochic acid in rats: the plasma metabolome [J]. *Rapid Commun Mass Sp*, 2010, **24**(9): 1312-1318.
- [23] Chen H, Cao G, Chen DQ, et al. Metabolomics insights into activated redox signaling and lipid metabolism dysfunction in chronic kidney disease progression [J]. *Redox Biol*, 2016, **10**: 168-178.
- [24] Zhao YY, Tang DD, Chen H, et al. Urinary metabolomics and biomarkers of aristolochic acid nephrotoxicity by UPLC-QTOF/HDMS [J]. *Bioanalysis*, 2015, **7**(6): 685-700.
- [25] Zhao YY, Wang HL, Cheng XL, et al. Metabolomics analysis

- reveals the association between lipid abnormalities and oxidative stress, inflammation, fibrosis, and Nrf2 dysfunction in aristolochic acid-induced nephropathy [J]. *Sci Rep*, 2015, **5**: 12936.
- [26] Johnson CH, Patterson AD, Krausz KW, et al. UPLC-ESI QTOFMS-based metabolomics for urinary biomarker discovery in gamma-irradiated rats [J]. *Radiat Res*, 2011, **175**(4): 473-484.
- [27] Dong H, Zhang A, Sun H, et al. Ingenuity pathways analysis of urine metabolomics. phenotypes toxicity of Chuanwu in Wistar rats by UPLC-Q-TOF-HDMS coupled with pattern recognition methods [J]. *Mol Biosyst*, 2012, **8**(4): 1206-1221.
- [28] Yan G, Zhao Y, Deng P, et al. Investigation of toxicological effects of Shuanghuanglian injection in Beagle dogs by metabolomic and traditional approaches [J]. *Exp Biol Med (Maywood)*, 2010, **235**(11): 1356-1364.
- [29] Zhang AH, Sun H, Dou SS, et al. Metabolomics study on the hepatoprotective effect of scoparone using ultra-performance liquid chromatography/electrospray ionization quadruple time-of-flight mass spectrometry [J]. *Analyst*, 2013, **138**(1): 353-361.
- [30] Zhang AH, Sun H, Han Y, et al. Urinary metabolic biomarker and pathway study of hepatitis B virus infected patients based on UPLC-MS system [J]. *PLoS One*, 2013, **8**(5): e6381.
- [31] Tong X, Sun H, Yan GL, et al. Evaluation study on urine metabolomics in yinhuang rat model induced by triplet factors of rhubarb, ethanol, and α -naphthylisothiolcyanate [J]. *Chin J Integr Med*, 2011, **17**(5): 369-375.
- [32] Zhang XM, Clausen M, Zhao XL, et al. Enhancing the power of liquid chromatography-mass spectrometry-based urine metabolomics in negative ionmode by optimization of the additive [J]. *Anal Chem*, 2012, **84**(18): 7785-7792.
- [33] Huang Y, Tian Y, Li G, et al. Discovery of safety biomarkers for realgar in rat urine using UFLC-IT-TOF/MS and ^1H NMR based metabolomics [J]. *Anal Bioanal Chem*, 2013, **405**(14): 4811-4822.
- [34] Lu F, Cao M, Wu B, et al. Urinary metabolomics study on toxicity biomarker discovery in rats treated with *Xanthii Fructus* [J]. *J Ethnopharmacol*, 2013, **149**(1): 311-320.
- [35] Wu B, Cao M, Liu SM, et al. Experiment study on hepatotoxicity induced by the water extract of cocklebur fruit in rats [J]. *Adverse Drug React J*, 2010, **12**: 381-386.
- [36] Wen CC, Zhang ML, Ma JS, et al. Urine metabolomics in rats after administration of ketamine [J]. *Drug Des Devel Ther*, 2015, **9**: 717-722.
- [37] Gao XY, Guo MX, Li Q, et al. Plasma metabolomic profiling to reveal antipyretic mechanism of Shuang-huang-lian injection on yeast-induced pyrexia rats [J]. *PLoS One*, 2014, **9**(6): e10001.
- [38] Zhang A, Sun H, Han Y, et al. Urinary metabolic biomarker and pathway study of hepatitis B virus infected patients based on UPLC-MS system [J]. *PLoS One*, 2013, **8**(5): e64381.
- [39] Xue CS, Zhang AH, Sun H, et al. An improved ultra-performance liquid chromatography-electrospray ionization/ quadrupole-time-of-flight high-definition mass spectrometry method for determining ingredients of herbal *Fructus corni* in blood samples [J]. *Pharmacogn Mag*, 2013, **10**(40): 422-425.
- [40] Miao WJ, Wang Q, Bo T, et al. Rapid characterization of chemical constituents and rats metabolites of the traditional Chinese patent medicine Gegen-Qinlian-Wan by UHPLC/DAD/qTOF-MS [J]. *J Pharm Biomed Anal*, 2013, **72**(2): 99-108.
- [41] Kong J, Chalcraft K, Mandur TS, et al. Comprehensive metabolomics identifies the alarmin uric acid as a critical signal for the induction of peanut allergy [J]. *Allergy*, 2015, **70**(5): 495-505.
- [42] Park CH, Noh JS, Kim JH, et al. Evaluation of morroniside, iridoid glycoside from corni fructus, on diabetes-induced alterations such as oxidative stress, inflammation, and apoptosis in the liver of type 2 diabetic db/db mice [J]. *Biol Pharm Bull*, 2011, **34**(10): 1559-1565.
- [43] Hagel AF, Layritz CM, Hagel WH, et al. Intravenous infusion of ascorbic acid decreases serum histamine concentrations in patients with allergic and non-allergic diseases [J]. *Naunyn Schmiedebergs Arch Pharmacol*, 2013, **386**(9): 789-793.
- [44] Zhang AH, Sun H, Han Y, et al. Exploratory urinary metabolic biomarkers and pathways using UPLC-QTOF-HDMS coupled with pattern recognition approach [J]. *Analyst*, 2012, **137**(18): 4200-4208.
- [45] Herberth G, Offenberk K, Rolle-Kampeczyk U, et al. Endogenous metabolites and inflammasome activity in early childhood and links to respiratory diseases [J]. *J Allergy Clin Immunol*, 2015, **136**(2): 495-497.
- [46] Wang SP, Liu L, Wang LL, et al. Screening and analysis of the multiple absorbed bioactive components and metabolites in rat plasma after oral administration of Jitai tablets by high-performance liquid chromatography/diode array detection coupled with electrospray ionization tandem mass spectrometry [J]. *Rapid Commun Mass Sp*, 2010, **24**(11): 1641-1652.
- [47] Loeser RF, Pathmasiri W, Sumner SJ, et al. Association of urinary metabolites with radiographic progression of knee osteoarthritis in overweight and obese adults: an exploratory study [J]. *Osteoarthr Cartilage*, 2016, **24**(8): 1-8.
- [48] Xiong YK, Lin X, Liang S, et al. Identification of senkyunolide I metabolites in rats using ultra performance liquid chromatography/quadrupole-time-of-flight tandem mass spectrometry [J]. *J Pharm Biomed Anal*, 2013, **81-82**(6): 178-186.
- [49] Xia XL, Jin HZ, Yan SK, et al. Analysis of the bioactive constituents of *ChanSu* in rat plasma by high performance liquid chromatography with mass spectrometric detection [J]. *J Pharm Biomed Anal*, 2010, **53**(3): 646-654.
- [50] Hu YH, Jiang P, Wang SP, et al. Plasma pharmacochimistry based approach to screening potential bioactive components in Huang-Lian-Jie-Du-Tang using high performance liquid chromatography coupled with mass spectrometric detection [J]. *J Ethnopharmacol*, 2012, **141**(2): 728-735.
- [51] Zhao Y, Lei P, Chen DQ, et al. Renal metabolic profiling of

- early renal injury and renoprotective effects of poria cocos epidermis using UPLC Q-TOF/HSMS/MS^E [J]. *J Pharm Bio-med Anal*, 2013, **81-82**(1): 202-209.
- [52] Wu R, Wu ZM, Wang XH, *et al.* Metabolomic analysis reveals that carnitines are key regulatory metabolites in phase transition of the locusts [J]. *Proc Natl Acad Sci USA*, 2012, **109**(9): 3259- 3263.
- [53] Zeng F, Chen HQ, Yang JR, *et al.* Development and validation of an animal model of prostate inflammation-induced chronic pelvic pain: evaluating from inflammation of the prostate to pain behavioral modifications [J]. *PLoS One*, 2014, **9**(5): e96824.
- [54] Noecker C, Eng A, Srinivasan S, *et al.* Metabolic model-based integration of microbiome taxonomic and metabolomic profiles elucidates mechanistic links between ecological and metabolic variation [J]. *mSystems*, 2016, **1**(1): e00013-e00015.
- [55] Chen JH, Wen H, Liu J, *et al.* Metabonomics study of the acute graft rejection in rat renal transplantation using reversed-phase liquid chromatography and hydrophilic interaction chromatography coupled with mass spectrometry [J]. *Mol Biosyst*, 2012, **8**(3): 871-878.
- [56] Dove AD, Leisen J, Zhou M, *et al.* Biomarkers of whale shark health: a metabolomic approach [J]. *PLoS One*, 2012, **7**(11): e49379.
- [57] Xia J, Wishart DS. Web-based inference of biological patterns, functions and pathways from metabolomic data using Metabo-Analyst [J]. *Nat Protoc*, 2011, **6**(6): 743-760.
- [58] Spagou K, Wilson ID, Masson P, *et al.* HILIC-UPLC-MS for exploratory urinary metabolic profiling in toxicological studies [J]. *Anal Chem*, 2011, **83**(1): 382-390.
- [59] Roux A, Lison D, Junot C, *et al.* Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: A review [J]. *Clin Biochem*, 2011, **44**(1): 119-135.
- [60] Manna SK, Patterson AD, Yang Q, *et al.* UPLC-MS-based urine metabolomics reveals indole-3-lactic acid and phenyl-lactic acid as conserved biomarkers for alcohol-induced liver disease in the Ppara-null mouse model [J]. *J Proteome Res*, 2011, **10**(9): 4120-4133.
- [61] Bujak R, Daghir E, Rybka J, *et al.* Metabolomics in urogenital cancer [J]. *Bioanalysis*, 2011, **3**(3): 913-923.
- [62] Van Coster RN, Gerlo EA, Giardina TG, *et al.* Aminoacylase I deficiency: a novel inborn error of metabolism [J]. *Biochem Biophys Res Commun*, 2005, **338**(3): 1322-1326.

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