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Metabonomic study on 'Kidney-Yang Deficiency syndrome' and intervention effects of Rhizoma Drynariae extracts in rats using ultra performance liquid chromatography coupled with mass spectrometry

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ABSTRACT

This paper was designed to study metabonomic characters of the 'Kidney-Yang Deficiency syndrome' induced by high dose of hydrocortisone and the therapeutic effects of Rhizoma Drynariae, classic traditional Chinese medicine (TCM) in treating the syndrome. A urinary metabonomics method based on ultra-performance liquid chromatography coupled with mass spectrometry (UPLC/MS) was developed. The significant difference in metabolic profiling was observed from model group (hydrocortisone-induced group) compared with the pre-dose group (rats before hydrocortisone inducing) by using the principal components analysis (PCA). The time-dependent regression tendency in Rhizoma Drynariae treatment group (hydrocortisone-induced rats followed by being administered with Rhizoma Drynariae ethanol extracts) from day 3 to 15 was obtained, indicating the time-dependent recovery effect of Rhizoma Drynariae on 'Kidney-Yang Deficiency syndrome' rats. Some significantly changed metabolites like phenylalanine, phenylacetylglycine, N_2-succinyl-L-ornithine, L-proline, creatinine, hippurate and citrate have been identified. These biochemical changes are related to the disturbance in energy metabolism, amino acid metabolism and gut microflora, which are helpful to further understand the 'Kidney-Yang Deficiency syndrome' and the therapeutic mechanism of Rhizoma Drynariae. The work shows that the metabonomics method is a valuable tool for studying the essence of Chinese medicine's syndrome theory and therapeutic effect mechanism of TCM.

1. Introduction

'Kidney-Yang Deficiency syndrome', recorded firstly in an earliest systematic theoretical monograph existing in China, "Neijing", is characterized by warm dysfunction and metabolic disorder of body fluid, causing aversion to cold, cold limbs, cold of waist and back, ache of waist and knee, tinnitus, impairment of hearing and looseness of teeth [1]. Modern medicine research indicates that the functional disorder with different degree of hypothalamic–pituitary-target gland (adrenal, thyroid and gonad) axis is the significant mechanism for forming 'Kidney-Yang Deficiency syndrome' [2,3]. It is a classic method mimicking the syndrome of Kidney-Yang Deficiency to inject rats a high dose of exogenous glucocorticoid (e.g. hydrocortisone) [4], which leads the decrease of steroid hormone from adrenal cortex. And the animals show signs of exhaustion, such as weight loss, decreased activity, slowed reaction, raritas clothing hair, deplicting, tendency to cluster and dropped appetite. This animal model of 'Kidney-Yang Deficiency syndrome' has been used for the evaluation of therapeutic effect and action mechanism of Kidney-Yang tonifying herbs and preparation.

Rhizoma Drynariae, the dried rhizome of *Drynaria fortunei* (Kunze) J. Sm. (Gu-Sui-Bu in Chinese), is one of the most frequently used traditional Chinese medicines (TCM) which can replenish the kidney, strengthen the bones, promote the healing fracture, and relieve pain [5,6]. For centuries, Rhizoma Drynariae has been effectively used in treating osteoporosis [7–9], bone fracture [10], anti-inflammatory [11], streptomycin ototoxicity [12] and hyperlipemia [13]. Up to now, studies on pharmacological mechanism of Rhizoma Drynariae mostly focus on cell and gene level. There has been no report to demonstrate its mechanism of Kidney-Yang tonifying action. While, little is known about the change of the whole metabolites in an organism treated with Rhizoma Drynariae. Neither has it been experimentally investigated whether nor how this herbal medicine affects the metabolism of whole body.

Metabonomics is an important area of systems biology, defined as the quantitative measurement of the dynamic multiparametric

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metabolic response of living systems to patho-physiological stimuli or genetic modifications [14,15]. Metabonomics, based on the analysis of entire pattern of low molecular weight compounds rather than focusing on individual metabolites, indicates a general procedure that gives information on whole organism functional integrity overtime following exposition of a perturbation. This research strategy is well coincident with the integrity and systemic feature of TCM. The emerging field of metabonomics provides a promising opportunity to generate novel approaches for addressing the therapeutic effect of TCM, molecular mechanism of diverse diseases and TCM, and ultimately towards controlling quality of TCM or exploiting new ideal drugs. Recently, it has been increasingly used as a versatile tool for assessing therapeutic effects of many herbal TCM and TCM prescriptions [16–19].

A number of analytical tools have been currently employed including ^{1}H NMR spectroscopy, HPLC/MS, CE/MS and GC/MS [20–23]. UPLC/MS leads to better chromatographic peak resolution, considerable shorter analysis time and more sensitivity compared to HPLC, has been considered to have a more bright future in the research of metabonomics [24–26].

In the present work, we for the first time studied the urine metabolite profiling of 'Kidney-Yang Deficiency syndrome' induced by hydrocortisone and the positive effect of Rhizoma Drynariae in rats by the metabonomics based on UPLC/MS. The restoration of abnormalities of metabolic pathway in Rhizoma Drynariae treated rats was investigated by comparing the endogenous metabolite profiles using principle component analysis (PCA).

2. Materials and methods

2.1. Chemicals

Hydrocortisone injection was purchased from Zhengzhou Lingrui Pharmaceutical Company (Henan, China). Acetonitrile of HPLC grade was purchased from Tedia (Fairfield, OH, USA). Formic acid of HPLC grade was supplied by Dikma Corp. (Richmond Hill, NY, USA). Water was purified by redistillation and filtered through 0.22 μm membrane filter before use.

2.2. Preparation of ethanol extracts of Rhizoma Drynariae

Rhizoma Drynariae (collection in Hunan, China) was purchased from Chengda Fangyuan Drug Store (Shenyang, China), and authenticated as the dried rhizome of *Drynaria fortunei* (Kunze) J. Sm. by Professor Jincai Lu (School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University, China). Content of naringin (pharmacological active component in Rhizoma Drynariae, 0.84%) was determined referring to the authentic method (pharmacopeia of the People's Republic of China 2010, volume I, page 239).

Powdered Rhizoma Drynariae (50 g) was extracted with 75% ethanol (600 mL, 3×) under thermal reflux for 1.5 h. After filtration, the ethanol extract was concentrated under reduced pressure. The residue was dissolved in 0.5% sodium carboxyl methyl cellulose to give an extract with a concentration of 2 g/mL (expressed as the weight of raw materials).

2.3. Animal study and sample collection

The study was approved by national legislation of China and local guidelines. A total of 15 male Wistar rats (200–250 g, animal licence No. SCXK-(military) 2007-004) were commercially obtained from Experimental Animal Center of Shenyang Pharmaceutical University (China). The rats were maintained under standard laboratory conditions (temperature of 21–23 °C, relative humidity of 45–65%, and 12 h/12 h light/dark cycle) with food and water freely available. After one-week habituation, all animals were housed individually in metabolism cages and allowed to acclimatize for a further 24 h. Following established protocol [4], 'Kidney-Deficiency syndrome' was induced by injecting intraperitoneally hydrocortisone at a dose of 10 mg/kg (the weight of hydrocortisone/body weight) once daily (8:00–10:00 a.m.) for 15 days. The blood was collected from the suborbital vein before and after hydrocortisone injection at day 15. The biochemical measurements, including triiodothyronine (T_3), thyroxin (T_4), testosterone (T) and estradiol (E_2) in plasma, were determined by radioimmunoassay.

The 15 rats were randomly divided into two groups: treatment group (n = 8) in which Rhizoma Drynariae extract was administered orally at dose of 20 mg/kg body weight once daily between 8:00 and 10:00 a.m. for the following 15 days; recuperation group (n = 7) which was fed naturally without any handle for following 15 days. The blood was collected from the suborbital vein at day 15 of treatment into heparinized tubes and immediately centrifuged at 11,200 × g for 10 min. The plasma was transferred into clean tubes and stored at −80 °C until analysis. Samples of 24-h urine were collected before hydrocortisone injection, at day 15 after hydrocortisone injection and at specific time intervals: treatment group at days 1, 3, 6, 9, 12 and 15 exposed to Rhizoma Drynariae extract. Sodium azide was added to the collection vessels as an antibacterial agent. After centrifugation at 4000 rpm for 10 min to remove residues, the urine samples were immediately stored in aliquots at −80 °C before UPLC/MS analysis.

2.4. Sample preparation

Prior to analysis, urine samples were thawed at room temperature and centrifuged at 11,200 × g for 10 min. The supernatant liquid was diluted at a ratio of 1:1 with water, vortex mixed and filtered through 0.22 μm membrane filter. The filtrate was transferred to autosampler vial kept at 4 °C and an aliquot of 5 μL was injected for UPLC/MS analysis.

2.5. UPLC/MS conditions

The UPLC/MS analysis was carried out using a Waters ACQUITY ultra performance liquid chromatography (UPLC) system (Waters Corp., Milford, USA) coupled with a Micromass Quattro Micro API mass spectrometer (Waters Corp., Milford, MA, USA). The UPLC column used was a 100 mm × 2.1 mm–1.7 μm C_{18} column (Waters Corp., Milford, MA, USA). The column temperature was maintained at 40 °C, the injection volume was fixed at 5 μL. The gradient mobile phase was a mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, which was pumped at the flow rate of 0.25 mL/min without split. The gradient elution program is shown in Table 1. All the samples were kept at 4 °C during the analysis.

An electrospray ionization source (ESI) interface was used, and was set in both positive and negative modes so as to monitor as many ions as possible. The following parameters were employed: source temperature of 120 °C and desolvation temperature of 300 °C, capillary voltage of 3.0 kV and 2.8 kV for positive and negative ionization mode respectively, cone voltage of 25 V for both positive ionization mode negative ionization mode. Nitrogen was

Table 1
Gradient elution program of UPLC–MS.

Time (min)	%A	%B
Initial	95	5
2	95	5
10	70	30
12.5	5	95
13.5	5	95
16	95	5

Table 2
Method validation of radioimmunoassay and sample determination of triiodothyronine (T_3), thyroxine (T_4), testosterone (T) and estradiol (E_2) in rat plasma (mean±SD).

	T_3 (pmol/L)	T_4 (pmol/L)	E_2 (pmol/L)	T (μg/L)
Method validation				
Rang	0.25–8.00	0.75–24.00	2–64	0.1–25.6
Recovery	90–105%	95–102%	94–109%	90–108%
Intra-assay variation	<10%	<10%	<8.0%	<7.4%
Inter-assay variation	<15%	<15%	<7.7%	< 9.5%
Sample determination				
Pre-dose group	3.20±0.40	9.87±0.72	136.6±37.9	4.55±0.86
Model group (Hydrocortisone-induced group)	1.57±0.23**	8.39±1.28**	177.3±40.6**	2.08±0.35**
Recuperation group	1.56±0.34	6.55±1.37	218.3±60.0	1.19±0.13
Rhizoma Drynariae treated group	2.67±0.56##	9.27±1.31	130.0±23.6##	3.76±0.71

$P < 0.01$ (compared with model group).
** $P < 0.01$ (compared with pre-dose group).

used as the desolvation and cone gas with the flow rate of 400 and 30 L/h, respectively. MS data were collected in the full scan mode from m/z 100 to 1000 amu over 0–16 min. Potential biomarkers were analyzed by UPLC/MS/MS. Argon was employed as the collision gas and the collision energy was altered between 5 and 25 eV. NaCsI was used for mass correction before the study. The mass spectrometric data were collected in centroid mode.

2.6. Method validation

The parameters of validation UPLC/MS/MS are same as the parameters given in UPLC/MS/MS conditions. For method validation study, 100 μL each of all the urine samples studied was pooled to generate a pooled quality control (QC) sample containing all the analytes that would be encountered during the analysis [27].

A QC samples was run prior to the start of the main analytical run for six times to 'condition' the system. The method repeatability was evaluated by analysis of six replicates of QC samples on one day. The post-preparative stability of sample was tested by analyzing six prepared QC samples left at autosampler (maintained at 4 °C) for 24 h with the fresh-prepared QC samples ($n = 6$) continuously in a single batch. The stability of QC samples following the freeze (−80 °C)–thawing (room temperature) process was evaluated by analyzing the QC samples undergone from 1 to 3 freeze thaw cycles together with the freshly thawed QC samples ($n = 6$) in a single batch. The system stability was carried out by injecting a QC sample every 10 samples during the whole sample analysis.

2.7. Data analysis

All the collected urine samples were analyzed with the validated method. Each sample was represented by a total ion current (TIC) chromatogram. The raw data were processed using the Micromass MarkerLynx Applications Manager version 4.0 (Waters Corp., Milford, USA). This applications manager incorporates a peak deconvolution package that allows detection of the mass, retention time and intensity of the peaks eluting in each chromatogram. The area of each peak, after being recognized and aligned, was normalized to the summed total ion intensity of each chromatogram. The resulting three-dimensional data, peak number (RT–m/z pair), sample name, and normalized ion intensity were introduced to SIMCA-P 11.0 software package (Umetrics, Umea, Sweden) for principal component analysis (PCA). Mean centered was used for data scaling and centering. ANOVA was performed in succession to reveal the statistical differences for the variables among pre-dose group, model group and Rhizoma Drynariae treatment group. The homogeneity of the variance was tested before ANOVA analysis. For identification of potential markers, the following databases have been used: HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/), Massbank (http://www.massbank.jp), PubChem (http://ncbi.nim.nih.gov/) and KEGG (http://www.kegg.com/). The significance of variation between groups in data of biological parameters was determined using paired-sample t-test by Excel 2003 (Microsoft, USA). P-values less than 0.05 were considered significant and values less than 0.01 were considered highly significant.

3. Results and discussion

3.1. Hydrocortisone-induced 'Kidney-Yang Deficiency syndrome' and the treatment with Rhizoma Drynariae extract

To validate the establishment of animal model, the content of T_3, T_4, T and E_2, which were widely admitted as diadynamic criteria of 'Kidney-Yang Deficiency syndrome' [28], were determined in rats plasma. The results of method validation of radioimmunoassay and sample determination are given in Table 2. The T_3, T_4 and T concentrations were significantly decreased ($P < 0.01$) in model rats. Supplementally, during the disposal with hydrocortisone, the model rats gradually lost weight, became emaciated, depilation, coat in abdomen wetted, and exhibited symptoms, such as languorous, sluggish and crouched. All these results indicated that the rats presented the typical pathological features of 'Kidney-Yang Deficiency syndrome'. After normal feeding for 15 days, the plasma contents of T_3, T_4 and T of recuperation group rats were still lower than those of pre-dose group rats, and no significant differences were found between recuperation group and model group. This indicates that in this period thyroid gland and gonad are still in restrained state, suggests that the recuperation group's rats are still in Kidney-Yang Deficiency state after stopped feeding hydrocortisone.

For metabonomics study, where the assay aims to provide a global profile of metabolites in urine via a non-targeted approach, further evaluation of the stability of this animal model was undertaken using PCA, a non-biased statistical technique. Fig. 1 depicts the PCA result obtained from the UPLC/MS data of the model group rats versus the recuperation group rats which were fed naturally without any handle for 15 days after hydrocortisone induced. No obvious evidence of differences was observed. It was clear that the rats fed naturally without any handle for 15 days were still at the pathological state of 'Kidney-Yang Deficiency syndrome', and this animal model could be employed for study on the therapeutic effects of Rhizoma Drynariae on the syndrome.

Treatment group rats have recuperated after orally administered with Rhizoma Drynariae. The extract obviously improved the numeral value of E_2 and T_3 ($P < 0.01$), as well as T_4 and T showing a trend of enhancement. Rhizoma Drynariae presented therapeutic effects on 'Kidney-Yang Deficiency syndrome'.

3.2. 3.2. Method validation

Extracted ion chromatographic peaks of 13 ions were selected according to their chemical polarities and m/z values. The paired retention time-m/z of these ions are 0.98–113.8, 2.39–120.2,

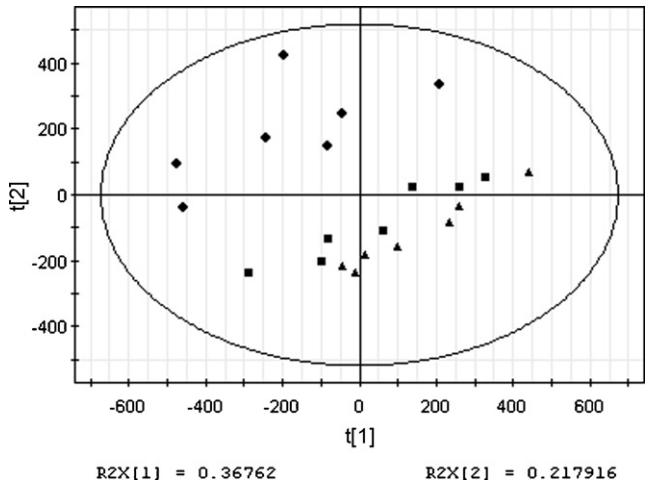

Fig. 1. PCA score plot in positive mode based on the urinary metabolic profiling of the pre-dose rats (▲), hydrocortisone-induced rats at day 15 (■) and recuperation group rats at day 15 (●).

4.32–188.0, 5.75–180.4, 6.67–194.4, 9.40–176.0, 13.10–332.7 in positive ion mode and 1.25–191.4, 5.75–178.4, 6.74–192.4, 7.65–283.5, 9.35–187.5, 13.3–295.7 in negative ion mode with retention times covering the whole analytical time. Method repeatability (RSD %) of these selected peaks was 0.3–3.7% for retention times and 5.2–12.4% for peak areas. System stability (RSD %) of retention times and peak areas of 13 selected ions was 0–4.1% and 5.4–13.2%, respectively. Furthermore, PCA analysis was applied to project the complex data and extract information from the whole metabolic profile in QC samples. The first two injection samples were separate from the other four samples, while the latter were tight clustered, which gives an indication of the reliability of the data (shown in Fig. 2). As the same as the reference [27] noted, the UPLC/MS system could provide the reproducible retention times after six repeated injections of one QC sample. PCA was also performed on data of post-preparative stability and freeze–thaw stability. No significant difference was found from the PCA score plot, which demonstrated that the urine samples were stable at 4 °C for 24 h and after three freeze–thaw cycles. All the results indicated the method was robust with good repeatability and stability.

3.3. Analysis of metabolic pattern and identification of potential biomarkers

Typical UPLC/MS positive and negative ion base peak intensity (BPI) chromatograms of rat urine from the pre-dose group, model group and Rhizoma Drynariae treatment group are shown in Fig. 3. Although some differences could be visually noted among the three sets of detail lustrated in Fig. 3, more subtle changes could be found using a pattern recognition approach, such as PCA. Score plots from PCA (Fig. 4) showed obvious separation between the pre-dose group and model group in both positive and negative ion modes, which suggests that urinary biochemical perturbation significantly happened in model group.

Corresponding loading plots are shown in Fig. 5. The ions furthest away from the origin contribute significantly to the clustering of the two groups and may be regarded as the potential biomarkers for hydrocortisone-induced 'Kidney-Yang Deficiency syndrome'. Structure identification was performed according to their molecular ion masses and MS/MS product ion analysis comparing with authentic standards or database resources. Based on the relative intensities of the metabolites from the normalized spectrum, ANOVA was used to reveal the significant differences of identified metabolites between the model group and pre-dose group, as well

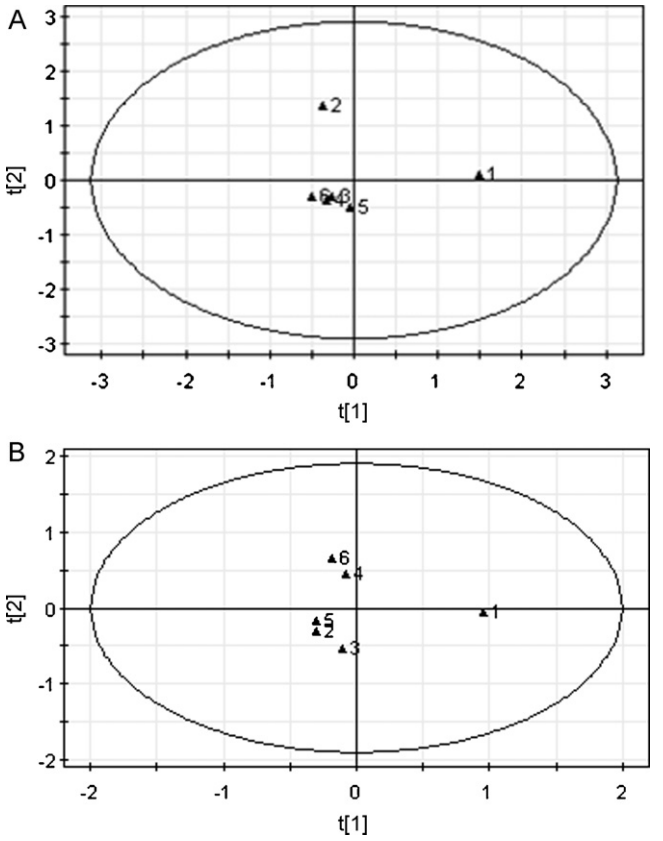

Fig. 2. System equilibration test using PCA analysis based on the urinary metabolic profiling of QC samples in (A) positive ion mode and (B) negative ion mode.

as between the model group and treatment group. The significant variables detected in the positive ion and negative ion modes are summarized in Table 3. The related pathway of each biomarker was also given by searching HMDB and KEGG database.

Take the biomarker at *m/z* 194 in positive ion mode as an example to illustrate the identification process. In positive ion spectrum (Fig. 6A), besides the base peak ion at *m/z* 194.4, the ions at *m/z* 216.5 and 387.5 were found. Thus, we infer that the quasi-molecular ion is *m/z* 194.4 ($[M+H]^+$) and the ions at *m/z* 216.5 and 387.5 are the adduction $[M+Na]^+$ and $[2M+H]^+$, respectively. The ions at *m/z* 192.4 ($[M-H]^-$) and *m/z* 385.7 ($[2M-H]^-$) in negative ion mode (Fig. 6B) further validated the metabolite has a molecular mass of 193.4 Da. Furthermore, its fragmentation (Fig. 6C) from tandem MS in positive ion mode was investigated. Two major fragment ions were found at *m/z* 75.5 and *m/z* 90.6, which represent the fragments of $[HOCOCH_2NH_2]^+$ and $[C_6H_5CH_2]^+$, respectively. Finally, it was identified as phenylacetylglycine by comparing with the fragmentation pattern of compound (HMDB00821) in HMDB database. Some unknown metabolites with retention time and *m/z* pairs of 6.0_327.6, 7.0_415.6, 7.4_459.6 in the positive ion mode and 6.7_338.5, 7.7_165.4 in the negative ion mode also showed a significant variance and their identifications are still in progress.

3.4. Time-dependent effect of Rhizoma Drynariae extract on the urinary metabolic pattern of hydrocortisone-induced 'Kidney-Yang Deficiency syndrome'

In order to evaluate time-dependent effect of Rhizoma Drynariae extract on the urinary metabolic pattern of hydrocortisone-induced 'Kidney-Yang Deficiency syndrome', a PCA model was constructed to analyze all the data acquired from pre-dose group, model group and treatment group at days 3, 6, 9, 12 and 15. It can

Table 3
Identification results of varying ions and their change trend.

t_R_m/z	Metabolite identification	Relative intensity in predose group	Relative intensity in model group	Relative intensity in recuperation group	Relative intensity in treatment group	Homogeneity of the variance (*P*-value)		ANOVA analysis (*P*-value)		Change trend of model group vs. predose group	Change trend of treated group vs. model group	Related pathway
						Model vs. predose	Treatment vs. model	Model vs. predose	Treatment vs. model			
Positive mode												
1.0_114	Creatinine	353.2 ± 23.1	293.6 ± 51.1	289.6 ± 52.0	359.7 ± 32.8	0.111	0.385	9.41E−03	8.26E−03	↓	↑	Arginine and proline metabolism
5.7_105	Hippurate fragment	436.3 ± 44.4	310.1 ± 34.6	297.1 ± 24.6	371.3 ± 24.2	0.606	0.231	1.83E−05	1.09E−03	↓	↑	Phenylalanine metabolism
5.7_180	Hippurate	148.2 ± 10.2	105.8 ± 11.1	94.8 ± 10.1	122.3 ± 10.2	0.685	0.658	1.49E−06	7.84E−03	↓	↑	Phenylalanine metabolism
6.7_194	Phenylacetyl-glycine	110.9 ± 11.1	150.3 ± 10.1	149.2 ± 9.4	131.7 ± 4.8	0.430	0.118	3.24E−06	3.2E−04	↑	↓	Phenylalanine metabolism
6.7_216	$[M+Na]^+$ of phenylacetyl-glycine	49.2 ± 4.7	65.8 ± 6.8	63.2 ± 3.7	54.3 ± 5.2	0.13	0.243	5.55E−05	1.88E−03	↑	↓	Phenylalanine metabolism
1.4_116	L-Proline	38.3 ± 6.2	50.7 ± 7.6	51.3 ± 5.6	40.9 ± 3.5	0.826	0.102	2.97E−03	5.07E−03	↑	↓	Arginine and proline metabolism
2.4_120	Phenylalanine fragment	32.4 ± 4.0	52.4 ± 9.1	50.2 ± 4.8	36.5 ± 4.6	0.127	0.178	5.67E−05	5.88E−04	↑	↓	Phenylalanine, tyrosine and tryptophan biosynthesis
7.6_233	N_2-succinyl-L-ornithine	24.3 ± 4.7	37.8 ± 4.2	35.2 ± 2.4	24.8 ± 2.8	0.591	0.069	3.14E−05	4.31E−06	↑	↓	Arginine and proline metabolism Urea cycle and metabolism of amino groups
Negative mode												
1.3_191	Citrate	65.7 ± 6.1	56.3 ± 5.0	54.3 ± 4.6	66.3 ± 5.5	0.342	0.817	4.89E−03	2.05E−03	↓	↑	Citrate cycle (TCA cycle)
5.7_178	Hippurate	430.5 ± 37.5	319.6 ± 31.8	320.4 ± 39.4	371.4 ± 34.1	0.241	0.906	1.71E−05	7.16E−03	↓	↑	Phenylalanine metabolism
9.6_212	Indoxyl sulfate	78.9 ± 4.2	67.0 ± 5.4	65.6 ± 4.7	78.3 ± 5.4	0.415	0.912	2.47E−03	8.27E−03	↓	↑	Metabolic pathways
11.6_187	Cresol sulfate	33.4 ± 3.3	37.3 ± 2.6	39.0 ± 2.9	33.8 ± 2.9	0.341	0.376	1.99E−02	2.26E−02	↑	↓	Metabolic pathways
6.7_192	Phenylacetyl-glycine	111.2 ± 10.7	144.0 ± 11.6	147.5 ± 10.8	124.1 ± 3.1	0.867	0.072	4.09E−05	3.52E−04	↑	↓	Phenylalanine metabolism
1.2_167	Uric acid	32.3 ± 2.9	34.9 ± 3.2	35.2 ± 3.5	45.7 ± 5.7	0.448	0.428	1.19E−01	3.32E−04	↑	↓	Metabolic pathways

"↑" and "↓" represent the compound is up- and down-regulated. The significance level (*P*-value) was set at 0.05 in homogeneity of the variance and ANOVA analysis.

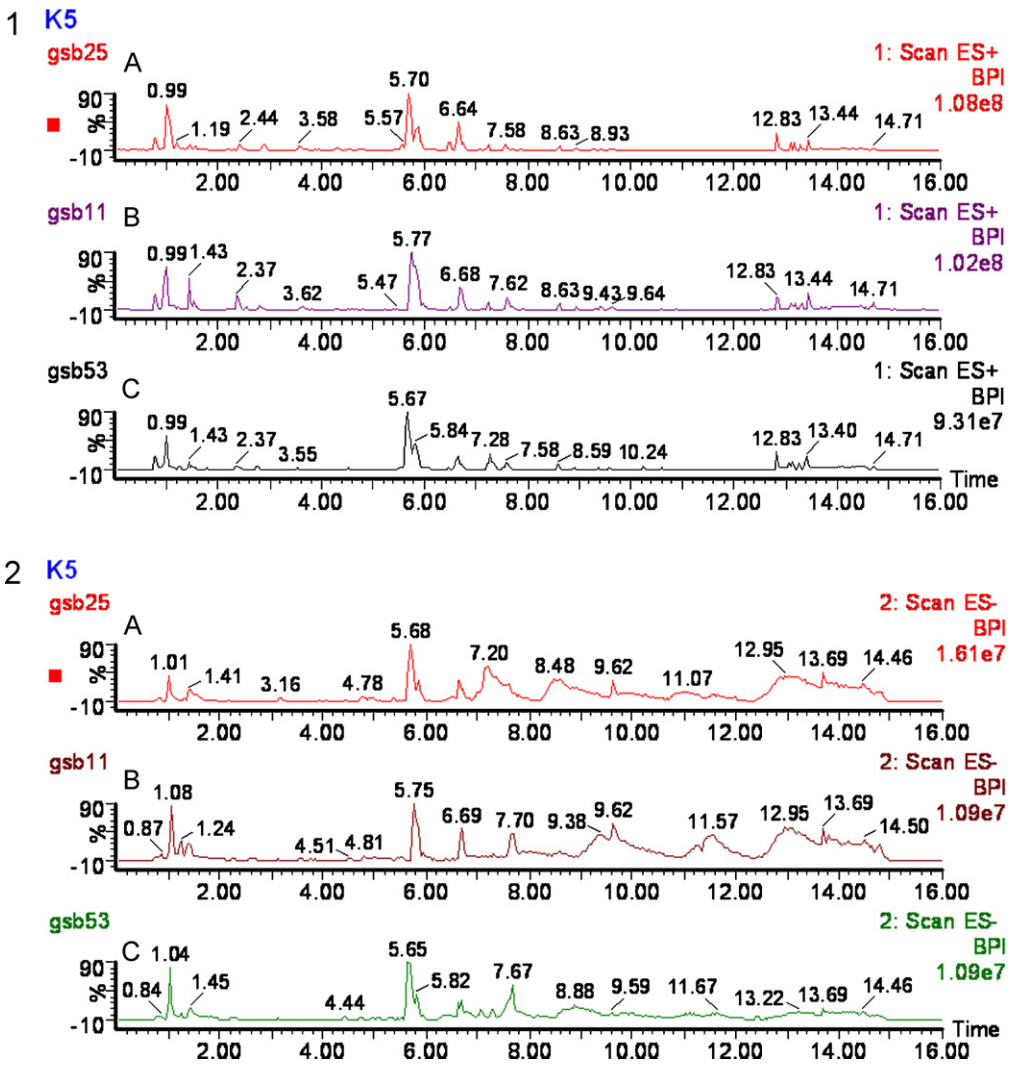

Fig. 3. Typical base peak intensity (BPI) chromatograms obtained from rat urine in (A) pre-dose group, (B) model group and (C) treatment group in positive mode (1) and negative mode (2).

be seen that from the score plot (Fig. 7A), a separation of the model group and pre-dose group was clearly achieved, while the Rhizoma Drynariae treatment group was mainly located between the model group and pre-dose group. The Rhizoma Drynariae treatment group is much closer to the pre-dose group than the model group along by the treatment time, showing that the urinary metabolic pattern was significantly changed with the treatment of Rhizoma Drynariae. To better understand the time-related trajectory of metabolic pattern with the treatment of Rhizoma Drynariae from day 3 to 15, PCA scores based on the average peak area percentage in UPLC/MS spectra of the model group and treatment group were illustrated in Fig. 7B. Transient shift in the trajectory plot revealed the dynamic progress of the metabolic network during the Rhizoma Drynariae treatment time. The spot of model group is obviously deviated from that of pre-dose group, suggesting high fluctuations happened in the metabolic regulatory network, which called 'Kidney-Yang Deficiency syndrome'. The spot of treatment group at day 3 is close to that of model group, indicating the dominant 'Kidney-Yang Deficiency syndrome' state. The spots of treatment group at days 6–12 clustered near the center of the plot with a tendency back to pre-dose group, which might be an indication of accumulated effect of Rhizoma Drynariae. The spot of treatment group at day 15 ultimately approached the pre-dose state, suggesting that Rhizoma Drynariae had some recovering or therapeutic effects on the rats exposed to hydrocortisone. The regression tendency of metabolic pattern in rats of treatment group indicated that the Kidney-Yang tonifying effect of Rhizoma Drynariae was time dependent.

Table 3 gives the change trends of the metabolites identified. The results effectively indicated that these metabolites may be the biomarkers of kidney-tonifying effects of Rhizoma Drynariae, which were related to the action mechanism of Rhizoma Drynariae.

In the model group, phenylalanine and phenylacetylglycine were significantly increased compared with that in the pre-dose group. Phenylalanine is an essential amino acid, phenylacetylglycine is the metabolites of phenylalanine by the gut microflora. Phenylalanine is the precursor of the amino acid, tyrosine. Significant fate of tyrosine is a conversion to the catecholamine, such as dopamine, norepinephrine and epinephrine [29]. It was reported that after a period of increased sympathetic tone in stress, sympathetic excitability gradually decreased due to weariness, leading to a rise in sympathetic tone as a result of mutual inhibition and the physiopathologic state of 'Kidney-Yang Deficiency syndrome' can be established as parasympathetic hyperfunction goes up to a threshold value [30]. An increase in plasma concentration of phenylalanine (leading the increased level of phenylalanine in urine, determined in our study) or phenylalanine/tyrosine ratio can cause an inhibition in DA and NE synthesis in the brain [31]. Thus, the decreased DA and NE levels in the brain would be the result of

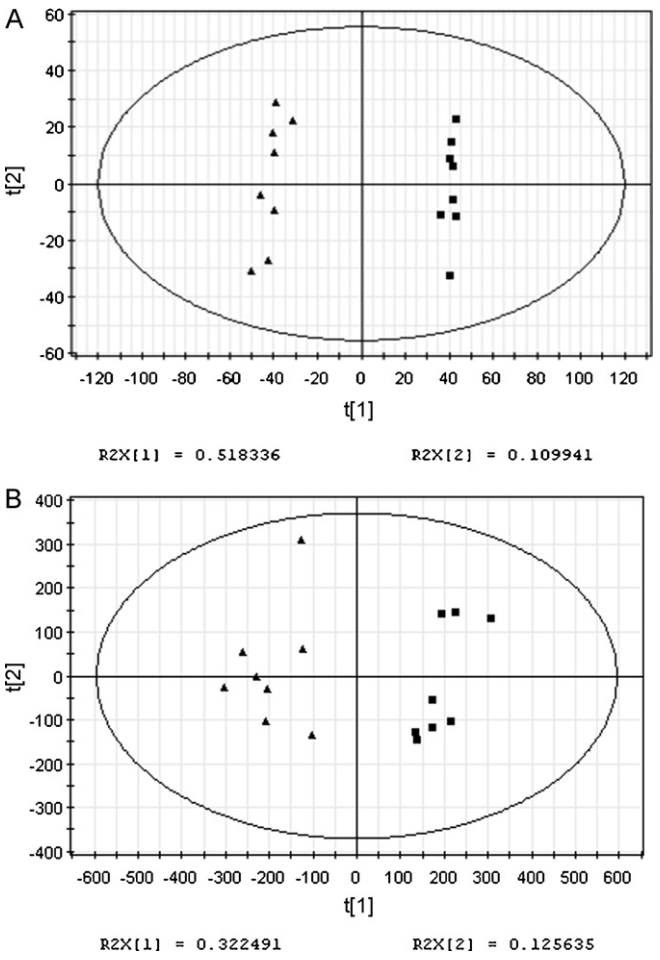

Fig. 4. Score plots ((▲) pre-dose group rats, (■) model group rats) in (A) positive ion mode and in (B) negative ion mode from a PCA model.

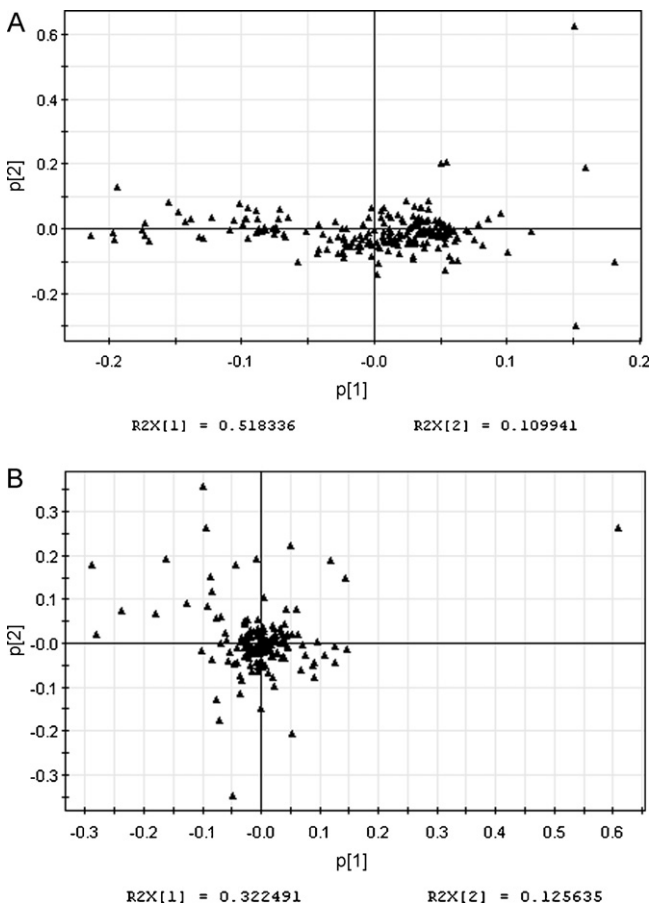

Fig. 5. Loading plot in (A) positive ion mode and in (B) negative ion mode.

elevated plasma concentration of phenylalanine observed in the model group at day 15, and in turn contribute to inhibition of parasympathetic tone and a rise in sympathetic tone, resulting in the physiopathologic state of 'Kidney-Yang Deficiency syndrome'. Furthermore, phenylalanine could suppress appetite via stimulating the production of cholecystokinin (a peptide hormone of the gastrointestinal system responsible of stimulating the digestion of fat and protein and also acting as huger suppressant). The increase of phenylalanine in hydrocortisone induced rat results in a reduction of dietary consumption, which presumably explains the rats loss of appetite during the modeling.

N_2-succinyl-L-ornithine and L-proline also contributed to the separation between the model group and the pre-dose group. N_2-succinyl-L-ornithine is an arginine degradation product by the gut microflora. N_2-succinyl-L-ornithine and L-proline are all in the arginine succinyltransferase pathway. In our study, high dose of hydrocortisone caused the elevated level of N_2-succinyl-L-ornithine and L-proline, which showed that 'Kidney-Yang Deficiency syndrome' are involved in amino acid metabolism.

In the model group, creatinine and citrate were significantly decreased. These metabolites are associated with energy metabolism. Creatinine is a nonenzymatic breakdown product of creatine and phosphocreatine, and the creatine–phosphocreatine system is crucial for cellular energy transportation. Citrate is a major intermediate in TCA cycle. The decreased level of urine citrate suggests weaken TCA cycle due to the defect in mitochondria dysfunction in the model group of 'Kidney-Yang Deficiency syndrome'. Mitochondria is a cellular organ related with energy metabolism, which supply energy for cellular activity via oxidative phosphorylation in the process of energy conversion. The decreased level of creatinine and citrate indicates the 'Kidney-Yang Deficiency syndrome' is related with energy metabolism dysfunction. As a result, the rats in the model group showed the signs of cold limbs, cold body.

Hippurate is another potential biomarker for the separation of pre-dose group and model group. Hippurate is metabolic product of phenylalanine by the gut microflora. It can protect nephridial tissue through interference of hydroxylation [32]. The decrease level of hippurate, observed in urinary metabolite profiles of model group compared with pre-dose group, is a cue of kidney impaired in rats with 'Kidney-Yang deficiency syndrome'.

The changes of phenylacetylglycine, N_2-succinyl-L-ornithine and hippurate are associated with the changes in gut microflora, because they are considered the metabolites of gut microflora. The 'Kidney-Yang Deficiency syndrome' might cause the alteration of intestinal flora [33], and the changes in phenylacetylglycine, N_2-succinyl-L-ornithine and hippurate.

It was reported that Rhizoma Drynariae, as the 'Yang-invigorating' Chinese tonifying herbs might speed up ATP generation by increasing mitochondrial electron transport to promote cellular activities and body function [34], resulting in safeguarding mitochondria and other organelle, which might be responsible for therapeutic effect of Rhizoma Drynariae. In our study, the up-regulation of creatinine and citrate was observed in the Rhizoma Drynariae treatment group compared with model group (see Table 3). Rhizoma Drynariae could elevate the energy metabolism and energy storage by up-regulating the level of creatinine and citrate and enhance the mitochondria function. The down-regulation of the phenylalanine, phenylacetylglycine, N_2-

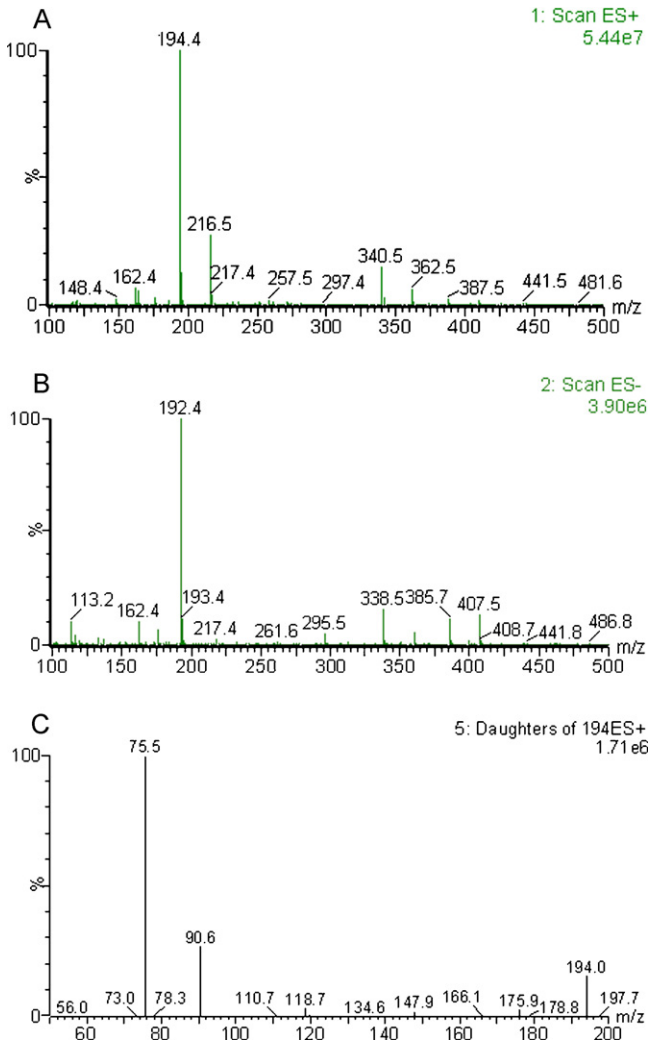

Fig. 6. Mass spectra of biomarker at (A) *m*/*z* 194 in positive ion mode, (B) *m*/*z* 192 in negative ion mode, (C) product ion scan spectrum of the biomarker in positive ion mode.

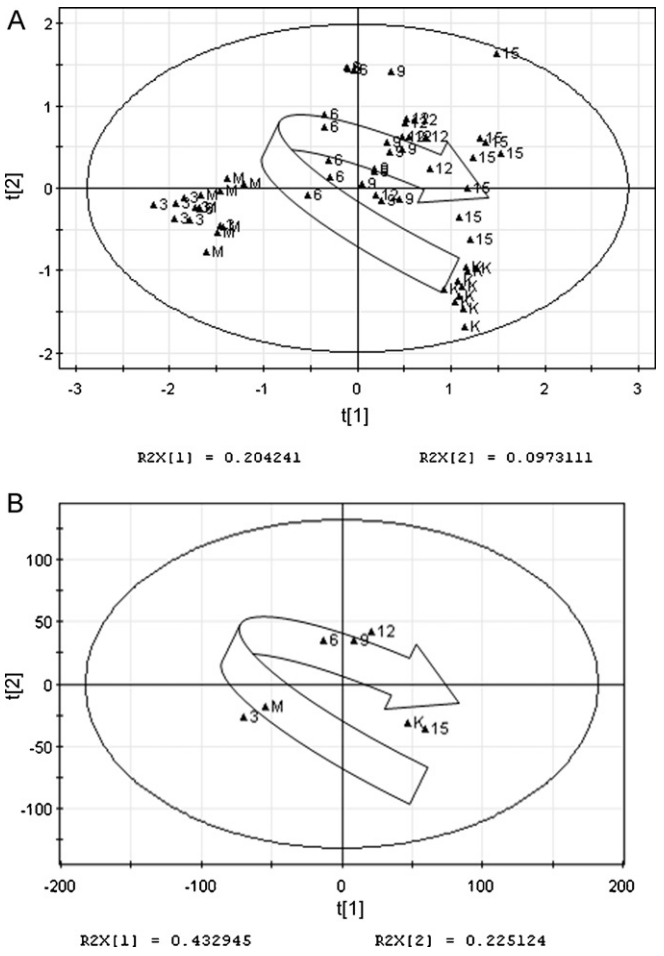

Fig. 7. (A) Score plot and (B) mean trajectory of PCA score in positive ion mode from PCA model classifying the state of rats at different time points (*k*: pre-dose group rats; *m*: model group rats; 3, 6, 9, 12 and 15: the Rhizoma Drynariae treatment group rats at days 3, 6, 9, 12, 15, respectively).

succinyl-L-ornithine and L-proline, the up-regulation of hippurate was also observed in the Rhizoma Drynariae treatment group compared with model group. The mean levels of each biomarker were reversed at different degrees after taking Rhizoma Drynariae ethanol extract. These results combined with the assay of biological parameters suggest that Rhizoma Drynariae extracts have unique characteristics for the treatment of 'Kidney-Yang Deficiency syndrome' induced by hydrocortisone. The biomarker revealed metabolic pathways (amino acid metabolism, energy metabolism and gut microflora) might be involved in the therapeutic mechanism of Rhizoma Drynariae.

4. Conclusions

A metabonomics method based on UPLC/MS has been developed to study the specific physiopathologic state named 'Kidney-Yang Deficiency syndrome' in TCM, induced by a high dose of hydrocortisone in rats, and therapeutic effects of Rhizoma Drynariae. With pattern recognition analysis (PCA), a clear separation of model group and pre-dose group was achieved. The time-dependent regression tendency in Rhizoma Drynariae treatment group from day 1 to 15 was obtained, which provide a visual, overall and dynamic progress and the recovery effect of Rhizoma Drynariae on 'Kidney-Yang Deficiency syndrome' rats. Some potential biomarkers like phenylalanine, phenylacetylglycine, L-proline, N_2-succinyl-L-ornithine, creatinine, hippurate, etc., have been found and identified. Combined with the result of biological parameters assay, these changes in urinary metabolites suggest that the disorders of amino acid metabolism, energy metabolism and gut microflora are related to 'Kidney-Yang Deficiency syndrome' induced by high dose of hydrocortisone and the potential effect of Rhizoma Drynariae on all the three metabolic pathways. This work demonstrated that metabonomics method is a potentially powerful tool to study the essence of Chinese medicine's syndrome theory and therapeutic effect mechanism of TCM.

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