Haiqiang Jiang[1]
Lei Nie[2]
Yunlun Li[3]
Jun Xie[3]

[1]Experimental center of Shan Dong University of Traditional Chinese Medicine, Jinan, P. R. China
[2]School of Pharmaceutical Sciences, Shandong University, Jinan, P. R. China
[3]Traditional Chinese Medicine Clinical Research Base For Hypertension of Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan, P. R. China

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Research Article

Application of ultra-performance liquid chromatography coupled with mass spectrometry to metabonomic study on spontaneously hypertensive rats and intervention effects of Ping Gan prescription

This study describes the metabonomic characters of the spontaneously hypertensive rats (SHR) and intervention effects of Ping Gan prescription. Ultra performance LC coupled with Q-TOF MS (UPLC/MS-Q-TOF) was employed to develop a metabonomic method of the plasma of SHR. There was a significant difference in metabolic profiling observed between predose group of Wistar Kyoto rats and model group (SHR) by using the principal components analysis (PCA). Some significant changes in metabolites such as LysoPC(22:6), LysoPC(20:4), LysoPC(18:1), cholylglycine, PE(P-16:0e/0:0), sphingosine-1-phosphate, and 2-oxo-4-methylthio butanoic acid were identified. These biochemical changes were associated with the disturbance in sphingolipid metabolism and fat metabolism, which would be helpful to further understand the essence of hypertension and the therapeutic mechanism of Ping Gan prescription. This study suggests that the metabonomic method may be a valuable and feasible tool to explore the therapeutic effect and mechanism of traditional Chinese medicine (TCM).

Keywords: Metabonomics / Ping Gan prescription / UPLC-MS / Spontaneously hypertensive rat
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1 Introduction

Hypertension is a clinical syndrome, which is manifested as arterial hypertension and varying degrees of metabolic disorders. It has a strong hereditary tendency, especially in hypertensive patients of familial aggregation [1, 2]. In the majority of cases, the causes of hypertension are likely to be a complex combination of the genetic, environmental, and other factors. Spontaneously hypertensive rat (SHR) is considered as a good animal model of hypertension because some of the pathophysiological processes in SHR are much similar to those of essential hypertension in human beings [3]. Both neurogenic and non-neurogenic factors contribute to the genesis of hypertension. The most familiar factors include the total peripheral resistance increase, sympathetic nervous system activity, plasma prostaglandin synthesis reduction, and vascular lesion caused by metabolic changes [4].

Metabonomics, based on the analysis of small-molecule compound profiles rather than focusing on individual metabolites, indicates a general procedure that gives information about the whole organism functional integrity overtime after exposition of a perturbation. The research strategy of metabonomics is well fit to the holistic and systemic feature of traditional Chinese medicine (TCM). The emerging field of metabonomics offers a great promise for elaborating the therapeutic effect of TCMs, molecular mechanism of interaction of diverse disease and TCMs, and ultimately toward controlling quality of TCMs or developing new ideal drugs. Recently, as a versatile technology, it has been increasingly employed to evaluate therapeutic effects of many Chinese herbal medicines and TCM prescriptions [5–8].

Many tools of instrumental analysis, such as ^{1}H-NMR spectroscopy, HPLC/MS, CE/MS, and GC/MS, have been often used for the metabolite profiling [9, 10]. Because of better chromatographic peak resolution, much shorter analysis time and higher sensitivity of detection as compared with conventional HPLC, UPLC/MS may be considered as a more promising analytical technique in the research of metabonomics [11–13].

Ping Gan prescription was widely used in clinics for hypertension treatment in the Hospital of Shandong University of Traditional Chinese Medicine. Alkaloids were

Correspondence: Dr. Yunlun Li, Traditional Chinese Medicine Clinical Research Base For Hypertension of Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Wenhua Xi Road, Jinan 250011, P. R. China
E-mail: yunlun.lee@hotmail.com
Fax: +86-531-89628203

Abbreviations: eNOS, endothelial NO synthase; **MPP**, mass profiler professional; **PCA**, principal components analysis; **SHR**, spontaneously hypertensive rat; **SPP**, sphingosine-1-phosphate; **TIC**, total ion current; **TCM**, traditional Chinese medicine

the main constituents in Ping Gan prescription ethanol extract, such as rhynchophylline, isorhynchophylline, corynoxeine, hirsutine, hirsuteine, berberine, coptisine, worenine, jatrorrhizine, palmatine, and so on. Triterpenoids were also another important constituents, including alisol A, B, C, alisol A monoacetate, epialisol A. In the present study, the plasma metabolite profiling of SHR and the positive effect of Ping Gan prescription in rats were studied by the UPLC/MS-based metabonomics. The restoration of abnormalities of metabolic pathway in rats treated by Ping Gan prescription was investigated via comparing the profiles of endogenous metabolite using principal component analysis (PCA).

2 Materials and methods

2.1 Chemicals and instrument

Acetonitrile and formic acid of HPLC grade were purchased from Tedia (Fairfield, OH, USA). Water was purified by redistillation and filtered through a 0.22-μm membrane filter before use.

2.2 Preparation of ethanol extracts of Ping Gan prescription

Ping Gan prescription is composed of four materials: *Coptis*, *Uncaria*, *Alismataceae*, and *Aloe*, which were all purchased from Jianlian Traditional Chinese Drug Store (Jinan, China) and authenticated by Professor Fengqin Zhou (Shan Dong University of Traditional Chinese Medicine). *Coptis* refers to the dried rhizome of Ranunculaceae *Coptis chinensis* Franch; *Uncaria* represents the dried hook stems of Rubiaceae *Uncaria rhynchophylla* (Miq.) Jacks; *Alismataceae* indicates the dried tuber of Alismataceae *Alisma orientalis* (Sam) Juzep; and *Aloe* refers to the dried leaves of Liliaceae *Aloe barbadensis* Miller. The certain amount of four materials in Ping Gan prescription, 60 g (*Coptis*), 150 g (*Uncaria*), 60 g (*Alismataceae*), and 5 g (*Aloe*), was weighed, powdered, and mixed sufficiently. Then, the mixture was extracted with 70% 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 water to give an extract with a concentration of 18.336 g raw materials/kg SHR (expressed as the weight of raw materials).

2.3 Animal study and sample collection

This study was approved by national legislation of China and local guidelines. A total of 26 male SHRs (200–250 g) and 9 male Wistar rats were commercially obtained from Experimental Animal Center of Shandong University of Traditional Chinese medicine (China). The rats were maintained under the standard laboratory conditions (temperature, 22°C; relative humidity, 45–65%; 12 h light/12 h dark cycle, 7 am–7 pm/7 pm–7 am) and had free access to standard chow and tap water. After 1-wk habituation, all animals were housed individually in metabolism cages and allowed to acclimatize for a further 24 h. The blood was collected from the abdominal aortic after 15 days. The 26 SHRs were randomly divided into three groups: treatment group ($n = 9$) in which Ping Gan prescription extract was administered orally at a dose of 18.336 g/kg (prescription/body weight), comparison group ($n = 10$) in which captopril was administered orally at a dose of 25 mg/kg body weight, and model group ($n = 7$) in which physiological saline was administered at the equivalent dose. The same treatment in model group was also applied to Wistar rat group. Every group was given once daily between 8 and 10 am for the following 15 days. The blood was collected from the abdominal aortic 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 UPLC/MS analysis.

2.4 Sample preparation

For sample preparation, plasma samples were thawed at room temperature before analysis. Two hundred microliters of plasma was placed into a 1.5-mL conical plastic test tube and 400 μL of acetonitrile was added. The test tubes were capped, vortex-mixed vigorously for 30 s, and centrifuged at 13 000 × *g* for 10 min. The supernatant was pipetted out and lyophilized. After filtered through a 0.22-μm nylon filter film to eliminate particulate matter, 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 plasma analysis was carried out by ultra performance LC (UPLC) system (Agilent, USA) equipped with a Q-TOF mass spectrometer (Agilent). The column used was a 150 mm × 2.1 mm, 3.5 μm ZORBAX SB-C18 column (Agilent, USA). The column temperature was set at 40°C. 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 0.20 mL/min without split. The gradient elution program used is summarized in Table 1. The injection volume was fixed at 5 μL. All the samples were incubated at 4°C during the analysis. An electrospray ionization source (ESI) interface was used and set in positive mode. The following parameters were employed: source temperature of 120°C and desolvation temperature of 325°C, capillary voltage of 3.0 kV, cone voltage of 25 V. Nebulizer: 15 psi, dry gas flow: 5 L/min. MS data were collected in the full-scan mode from m/z 100 to 1000 amu over 0–20 min. Potential biomarkers were analyzed by UPLC/MS/MS. Helium 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 MS data were collected in centroid mode.

Table 1. Gradient elution program of UPLC-MS

Time (min)	Acetonitrile (0.1% formic acid) (%)	Water (0.1% formic acid) (%)
0	5	95
20	100	0

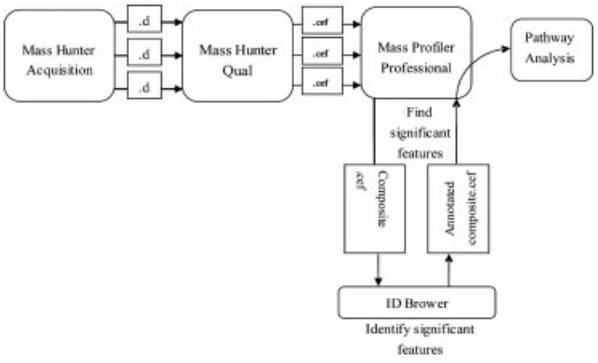

Figure 1. Data analysis procedure by MPP.

2.6 Data processing

Each sample was represented by a total ion current (TIC) chromatogram. First, the raw data of samples stored as ''∗.d'' format were converted into ''∗.cef'' format by Mass Hunter Qual (Agilent), and then were processed using the Mass Profiler Professional (MPP, Agilent) for PCA and found the significant features. Data analysis procedure is shown in Fig. 1. MPP software allows to calculate the detection and retention time alignment of the peaks in each chromatogram. The peaks in chromatograms of different samples were first aligned according to the same mass/retention time pair together, and then the corresponding peak areas were normalized to the summed total ion intensity of each chromatogram. After alignment and normalization of peaks for each sample chromatogram, a single data set stored as a matrix was formed. This data set was then analyzed by using PCA. The significant features were found from the S-plots of PCA by MPP software. The potential markers were identified by Agilent METLIN Database and other metabonomic database, such as the HMDB database.

3 Results and discussion

3.1 The change of blood pressure from different groups

The rat systolic blood pressure was measured by a tail-cuff method. Table 2 summarizes the changes of blood pressure in different groups. Compared with the model group, the rat systolic blood pressure in the comparison group or in the treatment group was significantly reduced after continuous treatment with Ping Gan prescription or captopril, respectively, ($p<0.05$). From the view of lowering the blood pressure, the therapeutic effect on hypertension of Ping Gan prescription was similar with that of captopril. Then metabonomic was used to explore the intervention effects on SHR of Ping Gan prescription.

3.2 Analysis of metabolic pattern

The small-molecule metabolites in rat's plasma were detected using UPLC-Q-TOF-MS. In the positive mode, typical TICs are shown in Fig. 2. Some differences in TICs of different groups were observed. More subtle changes can be found by the pattern recognition approach, such as PCA. Score plots of PCA (Fig. 3) show obvious separation among the treatment group, model group, comparison group, and Wistar rats group. The comparison group was far away from the remaining three groups, indicating that changed metabolic pattern resulted from captopril may be significantly different from others. The position of treatment group was near the middle of the model group and Wistar rats group, suggesting that changed metabolic pattern was caused by Ping Gan prescription. After treatment with Ping Gan prescription, SHR had a conversion to that of Wistar rats group, indicating that a recuperated trend occurred in treatment group. The results manifest that Ping Gan prescription could change the abnormal metabolic status and may have a different treatment mechanism with captopril.

3.3 Identification of potential biomarkers

MPP software was employed to search the small-molecule metabolites of significant differences (*T*-test, $p<0.05$). The potential markers were identified by using the ''ID browser'' to search in Metlin database and compare with the accurate mass charge ratio in some databases, including HMDB, KEGG, METLIN, LIPID MAPS, and PUBCHEM [14]. The related metabolic pathway was searched in KEGG database. The identification results are summarized in Table 3. Three LysoPCs and PE(16:0) were found. All the compounds had the interrelation with sphingolipid metabolism.

The m/z value of 380.2557 was selected in positive ion mode as an example to show the process of biomarker identification. The ion at m/z 380.2557 is an $[M+H]^+$ ion, which is shown in Fig. 4 as a base peak. Its molecular weight was obtained by MPP. The potential biomarker was first searched in Agilent METLIN Database according to the molecular weight. And then, the m/zs of potential biomarkers were compared with the accurate m/z in some database, including HMDB, KEGG, LIPID MAPS, and PUBCHEM. After search and comparison mentioned above, the compound was finally identified as sphingosine-1-phosphate (SPP). In the MS shown in Fig. 4, the ions at m/z 333.1669, 316.2837, and 298.2731 represent $[M\text{-}P{=}O]^+$, $[M\text{-}POOH]^+$, and $[M\text{-}PO_3H_2]^+$, respectively.

Table 2. Changes in systolic blood pressure (mm Hg) before and after treatment

Group	No.	Dose (1 kg/day)	Before the treatment	After 1 wk of the treatment	After 2 wk of the treatment
Wistar rats group	9	12 mL NS	120.760 ± 4.562	120.359 ± 5.560	121.059 ± 2.687
Model group	7	12 mL NS	181.260 ± 2.818	181.055 ± 2.118	181.160 ± 2.395
Comparison group	10	24.96 mg	180.799 ± 2.969	163.831 ± 1.439[a)]	165.673 ± 2.527[a)]
Treatment group	9	18.336 g	182.449 ± 1.586	164.609 ± 2.460[a)]	166.467 ± 2.684[a)]

a) Compared with the model group $p < 0.05$.

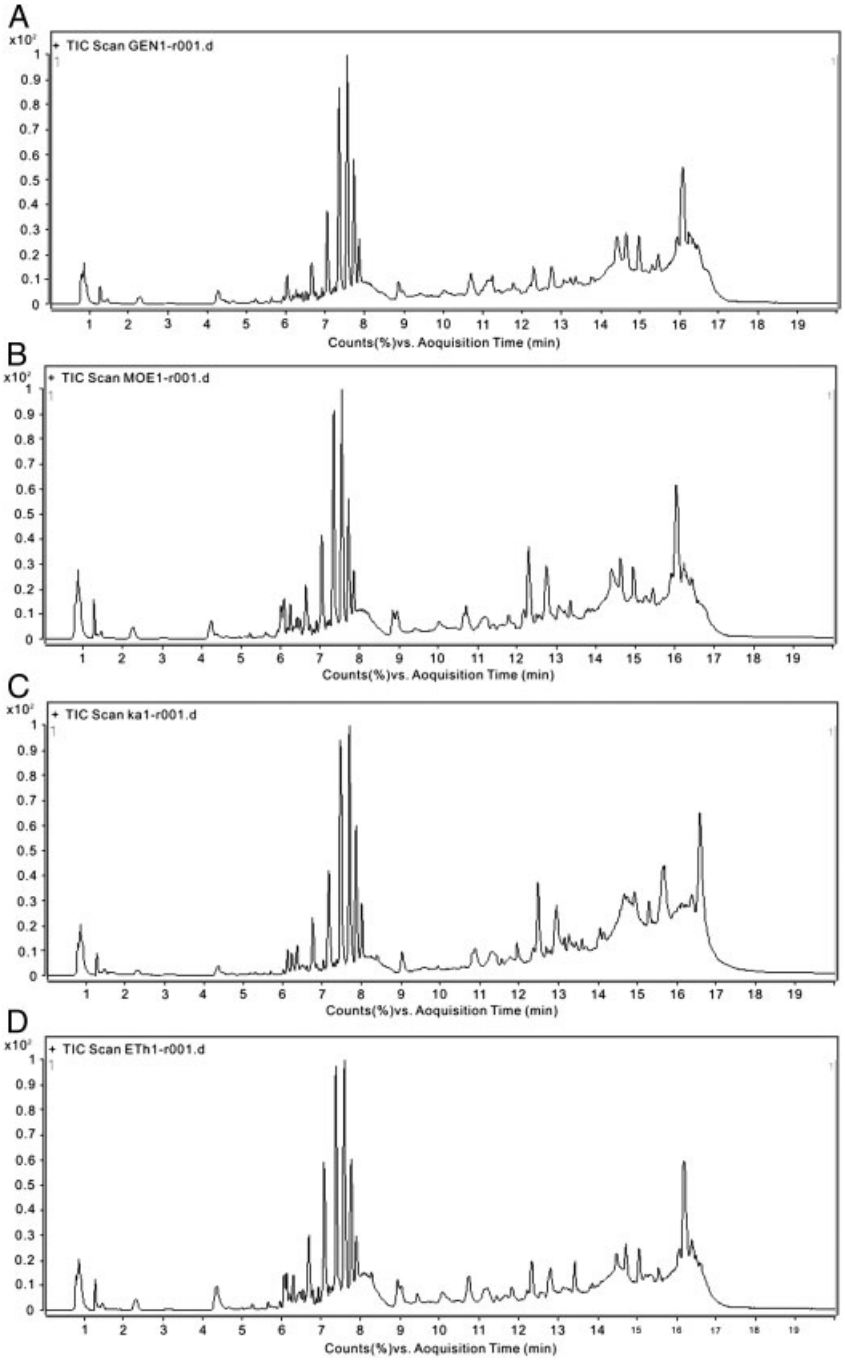

Figure 2. UPLC-MS TICs of plasma samples from the Wistar rats group (A), the model group (B), the comparison group (C), and the treatment group (D).

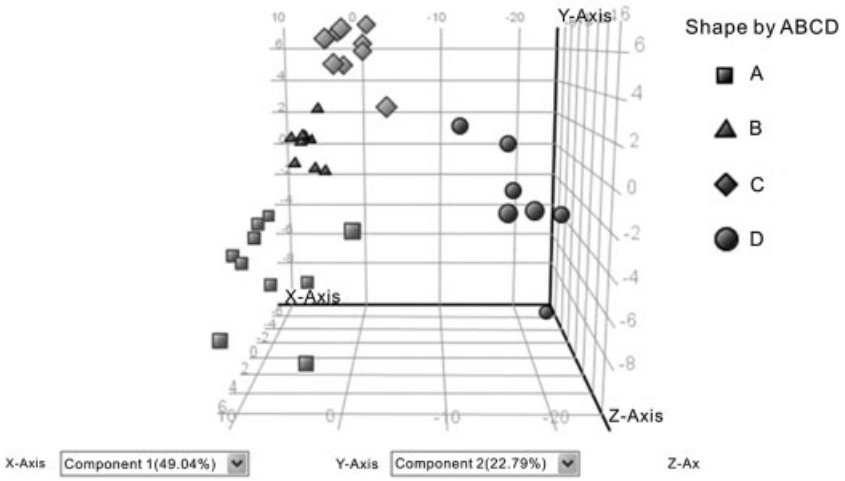

Figure 3. Three-dimensional score scattering plot of PCA for plasma samples (The letters A, B, C, and D refer to comparison group, treatment group, Wistar rats group, and model group, respectively).

Table 3. Hypertension potential biomarkers and metabolic pathway

RT[a)]	*m/z*	Metabolites	Metabolic pathway
12.266127	567.332	LysoPC(22:6)	Sphingolipid metabolism
12.308451	543.3319	LysoPC(20:4)	Sphingolipid metabolism
13.091862	521.3479	LysoPC(18:1)	Sphingolipid metabolism
11.427721	379.2483	SPP	Sphingolipid metabolism
14.038425	331.3232	Unknown	
9.78	436.4320	Glycocholic acid	Primary bile acid biosynthesis
12.89323	437.2901	PE(16:0)	Sphingolipid metabolism
9.799055	465.3082	2-Oxo-4-methylthio butanoic acid	

a) RT refers to retention time.

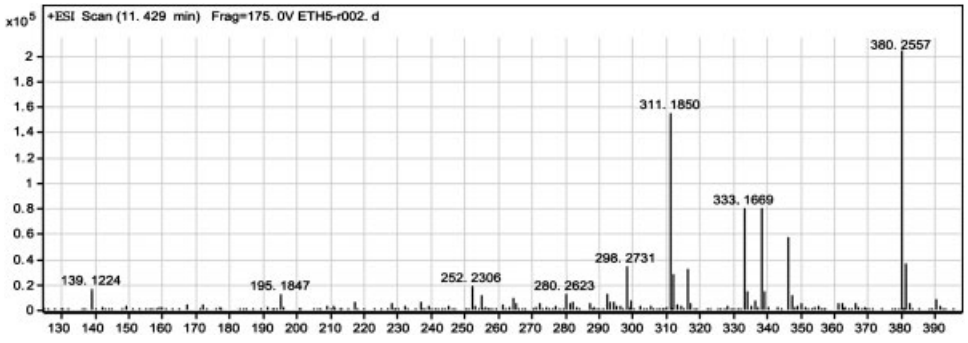

Figure 4. The MS of SPP.

3.4 Study of the changed metabolic trend

The changed metabolic trend is the terminal behavior of changed metabolic pattern induced by hypertension. In the present study, seven potential biomarkers were identified (Table 3), including five phospholipids, bile acid, and fatty acid. Their relative intensities are shown in Fig. 5. The four biomarkers such as LysoPC(22:6), LysoPC(20:4), LysoPC(18:1), and cholylglycine had an increasing trend from Wistar rats group to model group. After treatment with Ping Gan prescription, a clear tendency of these four biomarkers to return to normal in treatment group was observed. However, no obvious changes occurred in comparison group as compared with the model group. SPP had also a similar direction to normal, whereas the degree was only slight. The change of 2-oxo-4-methylthio butanoic acid was reduced from Wistar rats group to model group. Treatment group and comparison group both had a trend back to Wistar rats group, but the degree to recuperation in comparison group was relatively clear. This suggested that intervention effects of Ping Gan prescription and captopril on SHR may have the different metabolic patterns.

Among the biomarkers summarized in Table 3, five phospholipids were identified. Phosphate is a kind of amphiphilic substance which consists of the hydrophilic head composed of phosphoric acid substituent group and the hydrophobic head constituted by fatty acid chain. It can

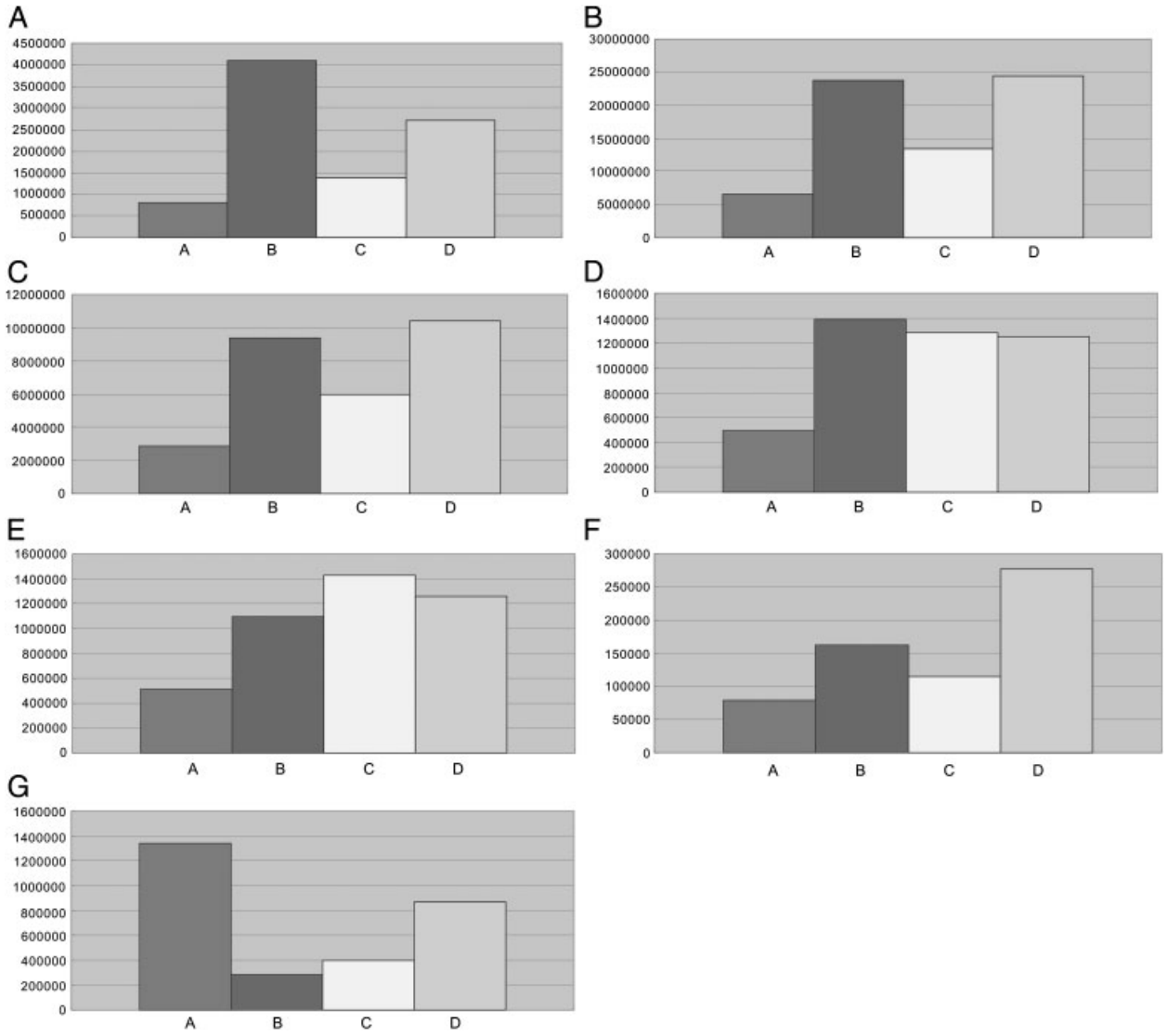

Figure 5. Changed metabolic trend of potential biomarkers 1–7 in different groups (The letters A, B, C, and D refer to Wistar rats group, model group, treatment group, and comparison group, a–g refer to LysoPC(22:6), LysoPC(20:4), LysoPC(18:1), cholylglycine, PE(P-16:0e/0:0), SPP, and 2-oxo-4-methylthio butanoic acid.).

dissolve fatty precipitation in plasma by emulsification and prevent hypertension caused by narrowed vessel wall [15]. Here, five phospholipids associated with phosphate were found, indicating that the abnormality of phosphate metabolism would occur, and phosphate may serve as the biomarker of hypertension.

Numerous studies indicate that ox-LDL is the independent risk factor for hypertension and Lyso-PC is the major lipid component of OX-LDL that damages endothelial diastolic function by affecting the synthesis and release of NO [16]. Endothelial vasodilator dysfunction resulted from NO reduction plays an important role in the process of hypertension. First, Lyso-PC depresses the activity of endothelial cells to release NO by inhibiting Gi protein interaction with its receptor. Second, Lyso-PC could decrease the activity of endothelial NO synthase (eNOS) through reducing mRNA levels, whereas eNOS could catalyze L-arginine oxidation to generate NO. Because of the activity of eNOS reduced by Lyso-PC, the release of NO via L-arginine oxidation could be reduced [17]. As vascular endothelial factor, NO reduction can lead to vascular endothelial substances to the low reactivity. Then, vasodilation was weakened, peripheral resistance was increased, and hypertension was developed finally [18]. As shown in Fig. 5A–C, it is clear that the high level in model group and low level in treatment group of LysoPC, and the treatment group had the near level with the normal. But the comparison group had little effect for the LysoPC metabolism. This suggested that LysoPC may be regarded as the biomarker of hypertension and explain the treatment effect of Ping Gan prescription.

Cholylglycine also had a regress trend from model group to Wistar rats group after the treatment of Ping Gan

prescription compared with captopril (Fig. 5D). Cholylglycine is one of the components of bile acid, and bile acid can consume the fat. The increased cholylglycine resulted from the rise of fat, which could lead to the rapid arterial baroreceptor resetting, baroreflex sensitivity reduction, and increased blood pressure [19, 20]. Cholylglycine reduction observed after the treatment of Ping Gan prescription suggested that abnormal fat metabolism may be involved in the pathway of hypertension.

SPP had two effects in cardiovascular endothelial cells. On one hand, it could induce endothelial nitric oxide which was used for diastolic vessel [21]. It could also induce coronary smooth muscle contraction through the SPP/Rho pathway. Under the circumstances of hypertension with injured vascular endothelia, SPP could not induce endothelial cells to produce NO for diastolic vessel, but still made the coronary artery smooth muscle contraction [22]. The increase of SPP in model group and decrease of SPP in treatment group is shown in Fig. 5F. We found the tendency of returning to normal from SHR and the comparison group had no change. Therefore, increased SPP would happen with elevating blood pressure by coronary smooth muscle contraction. It may be considered the potential biomarker of hypertension.

4 Concluding remarks

A metabonomic method based on UPLC/MS combined with pattern recognition has been developed to study the SHR plasma analysis. From the viewpoint of blood pressure change, the treatments of captopril and Ping Gan prescription have almost no difference in lowering blood pressure. But there were clear differences in metabolites among the four groups, suggesting that there may be a different treatment mechanism between captopril and Ping Gan prescription. According to the results, the potential biomarkers including LysoPC(22:6), LysoPC(20:4), LysoPC(18:1), cholylglycine, PE(P-16:0e/0:0) SPP, and 2-oxo-4-methylthio butanoic acid were identified. The related metabolic pathways including sphingolipid metabolism and fat metabolism to these potential biomarkers were also elaborated. The present study demonstrated that metabonomic method could be a potentially powerful tool to study the therapeutic effect and the mechanism of Ping Gan prescription.

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The authors have declared no conflict of interest.

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