Talanta 81 (2010) 766–772

Contents lists available at ScienceDirect

Talanta

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

Effect of traditional Chinese medicine berberine on type 2 diabetes based on comprehensive metabonomics

Yan Gu[a,b,1], Yifei Zhang[c,1], Xianzhe Shi[b], Xiaoying Li[c], Jie Hong[c], Jing Chen[b], Weiqiong Gu[c], Xin Lu[b], Guowang Xu[b,*], Guang Ning[c,**]

[a] Department of Pharmaceutical Analysis, School of Pharmacy, Shenyang Pharmaceutical University, Shenyang 110016, PR China
[b] CAS Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, PR China
[c] Shanghai Clinical Center for Endocrine and Metabolic Diseases, Division of Endocrine and Metabolic Diseases of E-Institutes of Shanghai Universities, Rui-Jin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, PR China

ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 5 January 2010
Accepted 9 January 2010
Available online 18 January 2010

Keywords:
UPLC/MS
Type 2 diabetes
Metabonomics
Berberine
Fatty acids
Dyslipidemia

ABSTRACT

A comprehensive metabonomic method, in combination with fingerprint analysis and target analysis, was performed to reveal potential mechanisms of berberine action in the treatment of patients with type 2 diabetes and dyslipidemia. Serum samples of 60 patients before and after treatment with either berberine or placebo were collected. Ultra-performance liquid chromatography and quadrupole time-of-flight tandem mass spectrometry (UPLC Q-TOF MS) coupled with pattern recognition analysis were used to identify changes in global serum metabolites. Compared with placebo, patients before and after berberine treatment could be separated into distinct clusters as displayed by the orthogonal signal correction filtered partial least-squares discriminant analysis (OSC-PLS-DA) score plot, which indicated changes in circulating metabolites after berberine treatment. Among them, free fatty acids changed markedly. These were further quantified by UPLC combined with single quadrupole mass spectrometry (UPLC SQ MS). There was a highly significant decrease in the concentrations of 13 fatty acids following berberine administration. 10 fatty acids also differed statistically from placebo. These results suggest that berberine might play a pivotal role in the treatment of type 2 diabetes through down-regulating the high level of free fatty acids and that comprehensive metabonomic measurements are potentially very useful for studying the mechanisms of action of traditional Chinese medicines.

1. Introduction

Diabetes mellitus is one of the most common chronic diseases, which is the result of interaction between hereditary and environmental factors. More than 90% of diabetes is type 2 diabetes (non-insulin dependent). Its incidence rate increases steadily together with the improvement of living level and population aging [1]. Long-term high blood sugar is easy to cause a variety of diabetic complications such as hypoglycemic, ketoacidosis, neuropathy, nephropathy, cardiopathy and retinopathy. Type 2 diabetes was recognized as *xiaokezheng* (a disease with symptomatic polydipsia) in ancient China, and traditional Chinese medicine has a long history in the treatment of *xiaokezheng* [2].

Berberine, a natural plant alkaloid isolated from the Chinese herb, Coptis Chinensis (Huanglian), is usually used for diarrhea as antibiotic and antivirus. Recently its potential glucose-lowering effect has been noted [3]. Animal research subsequently showed that berberine has potentially beneficial effects in the treatment of diabetes [4,5]. Some clinical investigators have also confirmed the anti-hyperglycemic effects of berberine [3,6–8]. In our study, we found that, compared with placebo, berberine had a potent glucose-lowering effect by significantly reducing fasting and post-load plasma glucose and HbA1c at 3 months. Furthermore, significant reductions of serum total cholesterol, triglycerides and low-density lipoprotein-cholesterol (LDL-c) were also observed in these patients [8]. Although the therapeutic efficacy of berberine in type 2 diabetes was confirmed by these studies, the precise mechanism of berberine in glucose lowering action is still not fully understood. Furthermore, great efforts were made on in vivo and in vitro studies [9–11], while, less attention has been paid to the impacts of berberine on global metabolism. As type 2 diabetes is a typical metabolic disorder with a chronic perturbation of the metabolic regulatory system, it seems appropriate to perform the metabonomic method to visualize the alteration of global

* Corresponding author. Tel.: +86 411 84379531; fax: +86 411 84379559.
** Corresponding author. Tel.: +86 21 64370045x665344; fax: +86 21 64373514.
E-mail addresses: xugw@dicp.ac.cn (G. Xu), guangning@medmail.com.cn (G. Ning).
[1] These authors contributed equally to this work.

0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.01.015

circulating metabolites and explore the related mechanisms after berberine treatment.

Metabonomics is a new platform of systems biology, defined as the quantitative measurement of the dynamic multi-parametric metabolic responses of living systems to pathophysiological stimuli or genetic modifications [12]. Nowadays, metabonomics has been applied in the fields of therapy monitoring, pharmaceutical discovery and in the evaluation of drug efficacy and toxicity [13–17]. Qualitative and quantitative analysis of the metabonome can estimate metabolic responses to a therapy and reveal the differences in metabolism between various groups. In addition, recognition of the differential metabolites enables us to obtain valuable information concerning drug action on metabolism and as well as to probe molecular mechanisms. The applications of metabonomics in studying effects and mechanisms of drugs on diseases have been adopted [18–20]. These studies reflected the drug-induced effects on global metabolites and obtained the valuable results. In light of this, we performed a comprehensive metabonomic approach in which we combined fingerprint analysis with target analysis to evaluate the metabolic response of berberine in patients with type 2 diabetes and dyslipidemia.

2. Materials and methods

2.1. Chemicals

Fatty acids standards were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Nu-Chek Prep (Elysian, MN, USA). HPLC grade acetonitrile was purchased from Merck (Merck, Germany). Water was obtained from a Milli-Q ultra-pure water system (Millipore, Billerica, USA). Ammonium acetate and glacial acetic acid, both of HPLC grade were purchased from Tedia (USA). Methanol and n-Hexane 95% (HPLC/SPECTRO grade) were also purchased from Tedia (USA). Phosphoric acid (analytical grade) was from the Shanghai Chemical Company (Shanghai, China).

2.2. Clinical research design

The clinical component of this study has been described in detail in our recently published data [8]. Briefly, the study was a randomized, double-blind, placebo-controlled and multiple-center trial consisting of a screening visit, a 2-week run-in and a 3-month treatment period. In the study, a total of 116 patients with newly diagnosed type 2 diabetes and dyslipidemia who had not undergone previous treatment for hyperglycemia or dyslipidemia, were randomly allocated to receive either berberine (1.0 g daily) or placebo for 3 months. For our study presented here, we randomly selected 60 of the patients (30 from berberine group and 30 from the placebo group), for comprehensive metabonomic measurement. The study was approved by the Institutional Review Board of Ruijin Hospital, Shanghai Jiao-Tong University School of Medicine and written informed consent was obtained from each patient. The study was conducted in accordance with the principles of the Declaration of Helsinki. The ClinicalTrials.gov registration number is NCT00462046.

2.3. Clinical and biochemical measurements

The clinical and biochemical characteristics of the 60 patients before and after 3 months treatment are shown in Table 1. Clinical data, including date of birth, smoking habits, alcohol consumption, past medical history, height and weight (whilst dressed in light clothes and without shoes), body mass index (BMI), waist and hip circumference and seated blood pressure (measured on the patient's non-dominant arm supported at heart level) were determined by a senior physician. Biochemical measurements, including renal and hepatic function, serum lipid concentrations [total cholesterol, triglycerides, high-density lipoprotein-cholesterol (HDL-c) and low-density lipoprotein-cholesterol (LDL-c)], serum electrolytes, blood counts and urinary analysis, a 75-g oral glucose tolerance test (OGTT), HbA1c and insulin were also performed as previously described [8].

2.4. Sample preparation

Serum samples collected before and after treatment were all stored at −80 °C until use. Prior to fingerprint analysis, the samples were thawed at 4 °C, 400 μL acetonitrile was then added to a 100 μL aliquot of serum samples for protein precipitation. After vortexing for 30 s and centrifuging at 12,000 rpm for 10 min, the supernatant was transferred and dried under vacuum. The dried residue was then dissolved in 100 μL of acetonitrile–water (4:1, v/v) for fingerprint analysis. In target analysis, the stock solutions of fatty acids were prepared in methanol. A series of standard solutions were made by diluting the stock solutions with methanol. The internal standard solution, consisting of C13:0 (475 μg/mL) and C19:0 (50 μg/mL) was also prepared in methanol. All solutions were stored at −20 °C until use. The sample extraction was modified based on the method suggested by Puttmann et al. [21]. Plasma samples were thawed at 4 °C, after which 100 μL of plasma was transferred into a 2-mL polypropylene microcentrifuge vial with 20 μL internal standard solution. After vortexing, 500 μL of the modified Dole's mixture (methanol, n-hexane, 2 M phosphoric acid, 40:10:1, v/v) was added and vortexed. After keeping at room temperature for 10 min, 300 μL of water and 200 μL of n-hexane were added, vortexed for 30 s and centrifuged at 13,000 rpm for 10 min. The upper organic layer was transferred into a 1.5-mL polypropylene microcentrifuge vial, 200 μL of n-hexane was added to the lower layer, vortexed for 30 s and centrifuged for 10 min at 13,000 rpm. The organic phases were combined and evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted with 200 μL of methanol and transferred to an autosampler vial.

2.5. Sample analysis

2.5.1. Fingerprint analysis

Metabonomics analysis was performed using an ACQUITY-ultra-performance liquid chromatography system (Waters Corp., Milford, USA), coupled to a Micromass Q-TOF MS (Waters Corp., Manchester, UK). Separation was achieved on an Acquity UPLC™ BEH C_{18} column (100 mm × 2.1 mm, i.d., 1.7 μm) maintained at 35 °C (Waters Corp., Milford, USA). The gradient program commenced with 95% of a 0.1% formic acid in water for 0.5 min at a flow rate of 0.35 mL/min which then changed to 100% acetonitrile linearly within 24 min. This was then held for 4 min, before finally reverting back to 95% of 0.1% formic acid in water. Once these initial settings were reached, the column was re-equilibrated for 2 min. The injection volume was 5 μL. The ESI ionization source was operated in either positive or negative ion mode. The following parameters were used: capillary voltage, 3100 V; cone voltage, 30 V; collision energy, 6 eV; desolvation gas, 500 L/h; cone gas, 50 L/h; desolvation temperature, 300 °C; and source temperature, 120 °C. Full scan mode was employed in the mass range of 100–1000 amu. Leucine–enkephalin was used as the lock mass. Potential biomarkers were analyzed by LC–MS/MS. The collision energy was set at 20 eV.

2.5.2. Target analysis

Free fatty acids were analyzed using an ACQUITY-ultra-performance liquid chromatography system (Waters Corp., Milford, USA), coupled to an ACQUITY SQ detector (Waters Corp.,

Table 1
Baseline and 3-month clinical characteristics of berberine and placebo groups.

	Berberine			Placebo			p value[b]
	Before	After	p value[a]	Before	After	p value[a]	
No. (male/female)		30(17/13)			30(19/11)		0.605
Age (year)		51 ± 9			50 ± 10		0.978
Current smokers		9			12		0.425
Alcohol use		3			9		0.055
Body weight (kg)	68.9 ± 10.8	67.2 ± 11.6	0.001	72.7 ± 11.2	71.2 ± 10.8	0.002	0.771
Body mass index (kg/m^2)	25.1 ± 2.9	24.4 ± 3.1	0.001	26.2 ± 3.6	25.7 ± 3.5	0.002	0.592
Waist-to-hip ratio	0.90 ± 0.06	0.89 ± 0.06	0.214	0.92 ± 0.05	0.91 ± 0.05	0.093	0.798
Systolic blood pressure (mmHg)	121 ± 12	115 ± 14	0.064	126 ± 15	123 ± 14	0.164	0.171
Diastolic blood pressure (mmHg)	79 ± 9	77 ± 11	0.263	83 ± 9	80 ± 8	0.037	0.522
Fasting plasma glucose (mmol/L)	6.9 ± 1.0	5.5 ± 0.9	<0.001	6.9 ± 0.9	6.3 ± 1.3	0.019	0.008
Post-load plasma glucose (mmol/L)	11.4 ± 2.5	8.8 ± 2.1	<0.001	12.1 ± 2.3	11.5 ± 2.8	0.254	0.001
HbA1C (%)	7.6 ± 1.1	6.7 ± 0.9	<0.001	7.7 ± 1.4	7.4 ± 1.1	0.203	0.004
Triglyceride (mmol/L)	2.82 ± 2.58	1.59 ± 1.20	0.002	1.92 ± 0.77	2.23 ± 1.41	0.107	0.001
Total cholesterol (mmol/L)	5.21 ± 1.06	4.09 ± 0.85	<0.001	5.38 ± 1.02	5.27 ± 0.82	0.368	<0.001
HDL cholesterol (mmol/L)	1.37 ± 0.61	1.27 ± 0.30	0.273	1.26 ± 0.27	1.21 ± 0.19	0.131	0.623
LDL cholesterol (mmol/L)	3.18 ± 0.89	2.36 ± 0.74	<0.001	3.37 ± 0.78	3.19 ± 0.79	0.129	<0.001
Uric acid (μmol/L)	310.6 ± 86.6	307.4 ± 75.3	0.793	308.6 ± 56.8	322.0 ± 81.0	0.191	0.291
Fasting serum insulin (μIU/mL)	12.1 ± 12.0	10.0 ± 5.9	0.396	10.4 ± 5.7	11.8 ± 8.8	0.397	0.289
Post-load serum insulin (μIU/mL)	56.4 ± 46.8	51.6 ± 29.5	0.555	68.6 ± 40.1	72.8 ± 41.4	0.544	0.078
HOMA-IR (μIU mol/L^2)	3.80 ± 3.97	2.59 ± 1.83	0.102	3.23 ± 2.01	3.45 ± 2.28	0.837	0.135

[a] p value refers to comparison between before *vs.* after treatment within each group.
[b] p value refers to comparison between berberin and placebo groups after treatment using the ANCOVA analysis.

Milford, USA). The chromatographic separation was performed on an Acquity UPLC™ BEH C_8 2.1 mm × 50 mm column with 1.7 μm particles (Waters Corp., Milford, USA) and the temperature was set at 35 °C. The optimal mobile phase consisted of water with 10 mM ammonium acetate (pH5.0) (A) and acetonitrile (B) and the flow rate was set at 0.3 mL/min. Gradient elution was carried out as follows: 50% B at 0–2 min, 50–90% B at 5–10 min, maintained for 3 min, then returned to 50% B and re-equilibrated for 2 min. The injection volume was 5 μL. ESI was used as the ionization source and the analysis was carried out using SIM in the negative mode. The following parameters were used: capillary voltage, 3400 V; cone voltage, 45 V; desolvation gas, 650 L/h; desolvation temperature, 300 °C; and the source temperature, 120 °C.

2.5.3. Data analysis

The UPLC/MS raw data were preprocessed using the Micromass MarkerLynx Applications Manager within the Masslynx software (version 4.0). The area of each peak, after being recognized and aligned, was normalized to the summed total ion intensity of each chromatogram. We first carried out a Wilcoxon paired comparison test to determine whether the global metabolites changed after treatment. The resulting data were then exported into SIMCA-P software (version 11.0, Umetrics, Umea, Sweden) for multivariate statistical analysis, and the partial least-squares discriminant analysis (PLS-DA) model with the orthogonal signal correction (OSC) data filter was constructed to determine how treatment influenced the metabolic pattern of DM-2 patients and to find potential biomarkers. To estimate the predictive ability and robustness of our model, we implemented a validation method using sevenfold cross-validation and response permutation testing. Statistical significance was determined with SPSS 13.0 software (SPSS, Chicago, IL, USA), $p < 0.05$ was considered significant.

3. Results

3.1. Clinical administration

Baseline and 3-month clinical characteristics of berberine and placebo groups are shown in Table 1. The analysis of covariance (ANCOVA) showed a significant improvement in fasting and 2-h OGTT plasma glucose and in HbA1c after berberine treatment compared with the placebo group. Fasting plasma glucose decreased from 6.9 ± 1.0 to 5.5 ± 0.9 mM/L after berberine treatment, while decreasing from 6.9 ± 0.9 to 6.3 ± 1.3 mM/L in the placebo group, with corresponding falls in 2-h glucose from 11.4 ± 2.5 to 8.8 ± 2.1 vs. 12.1 ± 2.3 to 11.5 ± 2.8 and from 7.6 ± 1.1% to 6.7 ± 0.9% vs. from 7.7 ± 1.4 to 7.4 ± 1.1%, in HbA1c for the berberine and placebo groups, respectively, with all parameters differing significantly (Table 1). The ANCOVA analysis also showed that serum total cholesterol, triglycerides and LDL-c concentrations in berberine group were significantly reduced as compared with the changes in the placebo group ($p < 0.0001$, $p = 0.001$, $p < 0.0001$, respectively).

3.2. Metabonomic fingerprint analysis

The serum metabolic pattern of the study group was analyzed by UPLC Q-TOF MS. Fig. 1 shows the base peak intensity chromatograms (BPC) of a representative serum sample. To verify the reproducibility and reliability of the data, a quality control sample spiked with a solution containing a mixture of standards (4 μg/mL Leucine–enkephalin, 4 μg/mL C12:0 LPC and 5 μg/mL C19:0 LPC) was analyzed after each 10 serum samples throughout the whole analytical workflow. The relative standard deviations (RSDs, %) of the retention time and the peak height of the spiking standard compounds in the quality control sample were 0.05–0.3 and 3.1–6.7, respectively. This showed that the method was robust, with good repeatability and stability.

The Wilcoxon paired comparison test showed that the global metabolites changed after treatment in the berberine group ($p = 0.000$ both in positive and negative ion mode), however, the placebo treatment was not able to significantly ameliorate the perturbed metabolic profiles of DM-2 patients ($p = 0.189$ in positive ion mode and $p = 0.353$ in negative ion mode). To capture the subtle variations, patients in the berberine group were selected to construct a model using PLS-DA with the OSC data filter on the basis of the UPLC Q-TOF MS spectra. As displayed by the PLS-DA score plot, DM-2 patients pre-treated and post-treated with berberine could be separated into distinct clusters (Fig. 2a and c), which indicated the changes of metabolic response in the serum of DM-2 patients after the treatment of berberine. To ensure that the model is reliable and the observed clustering is not due to chance, we implemented

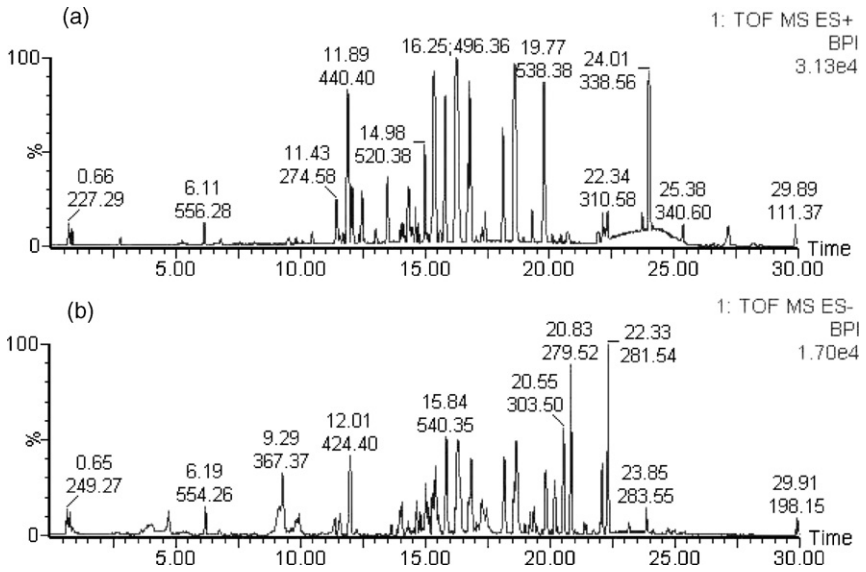

Fig. 1. Representative base peak intensity chromatograms (BPC: positive ion (a) and negative ion (b)) of a serum sample from a DM-2 patient.

a validation method using sevenfold cross-validation, the parameters for the classification from the software were $R^2Y = 0.812$ and $Q^2Y = 0.571$ for ESI^+ and $R^2Y = 0.9$ and $Q^2Y = 0.555$ for ESI^-, which indicated the model had an excellent fitness and prediction. A response permutation test (Y scrambling) showed no overfitting in the model according to Eriksson et al. [22].

The loading plot from the PLS-DA is shown in Fig. 2b and d. The furthest metabolite ions from the origin were potential biomarkers, which are responsible for the separation of DM-2 patients before and after treatment with berberine. Potential biomarkers were identified on the basis of retention time, m/z and product ion scan spectra by comparing with the corresponding standards. Table 2 lists the identification results. But only the metabolites which had statistically significant differences in both the intra-group and inter-group ($p < 0.05$) were meaningful biomarkers. The intensity of detected free fatty acids was found to decrease dramatically after taking berberine and there was a significant difference between the berberine and placebo groups ($p < 0.05$). While no statistical differences were found amongst the other potential biomarkers, either intra-group or inter-group (all $p > 0.05$).

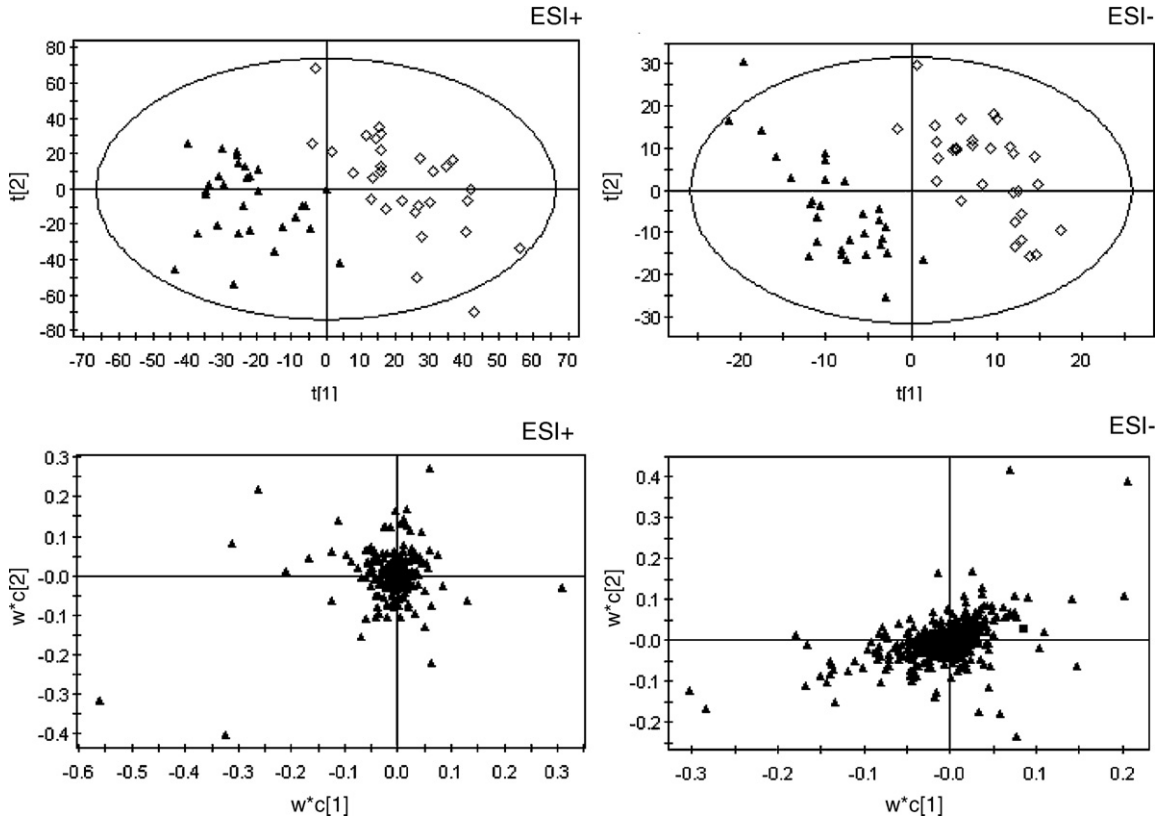

Fig. 2. (a) Score (◊: DM-2 patients pre-treated with berberine, ▲: DM-2 patients post-treated with berberine) and (b) loading plot from OSC-PLS-DA model in ESI^+ mode; (c) score (◊: DM-2 patients pre-treated with berberine, ▲: DM-2 patients post-treated with berberine) and (d) loading plot from OSC-PLS-DA model in ESI^- mode.

Table 2
Identification results of potential biomarkers.

Retention time (min)	Mass (*m*/*z*)	Identification result
Positive ion mode		
0.83	132.1	Leucine/isoleucine
2.65	205.1	Tryptophan
1.68	166.1	Phenyalanine
16.26	496.3	C16:0 LPC
15.35	520.3	C18:2 LPC
18.59	524.3	C18:0 LPC
Negative ion mode		
11.59	448.3	Glycochenodeoxycholic acid
22.01	255.2	C16:0 FFA
16.30	540.3	C16:0 LPC
20.77	279.2, 280.2[a]	C18:2 FFA
22.25	281.2, 282.2[a]	C18:1 FFA
15.38	564.3, 504.3[b]	C18:2 LPC
20.13	327.2	C22:6 FFA
20.77	329.2	C22:5 FFA
20.22	253.2	C16:1 FFA

[a] Isotope ion.
[b] Fragment ion.

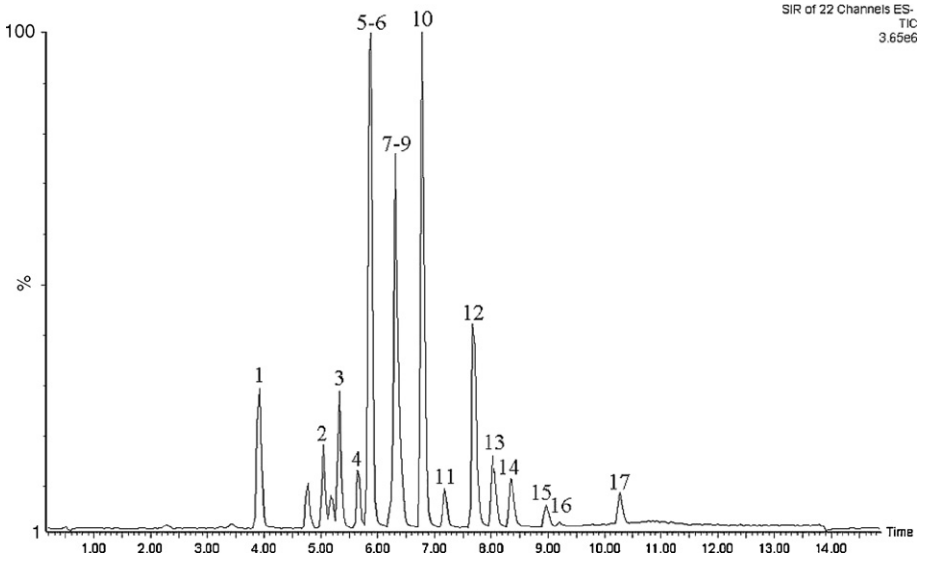

Fig. 3. A representative total ion chromatogram of 15 fatty acids and 2 internal standards using SIM mode. 1:C13:0, 2:C20:5, 3:C16:1, 4:C22:6, 5: C20:4, 6: C18:2, 7:C22:5, 8:C16:0, 9:C20:3, 10:C18:1, 11:C20:2, 12:C18:0, 13:C20:1, 14:C19:0, 15:C20:0, 16:22:1, 17:24:1.

Table 3
Free fatty acid quantification in the serum of healthy controls and DM-2 patients (μg/mL).

	Healthy controls (n = 20)[a]	DM-2 patients						p value[c]
		Berberine group (n = 30)			Placebo group (n = 30)			
		Before	After	p value[b]	Before	After	p value[b]	
C16:1	1.91 ± 0.31	2.87 ± 1.35	2.19 ± 1.04	0.002	2.59 ± 0.95	3.37 ± 2.59	0.065	0.003
C16:0	48.2 ± 12.5	66.3 ± 21.8	47.1 ± 15.1	<0.001	56.3 ± 14.5	60.3 ± 23.1	0.285	0.001
C18:2	16.9 ± 3.65	42.7 ± 16.5	29.8 ± 12.9	<0.001	26.5 ± 9.18	32.3 ± 16.0	0.034	0.012
C18:1	23.8 ± 5.64	63.6 ± 26.6	48.3 ± 23.3	0.003	40.7 ± 13.2	46.9 ± 22.9	0.106	0.104
C18:0	27.9 ± 3.00	31.9 ± 8.99	24.1 ± 5.78	<0.001	33.9 ± 5.83	34.7 ± 8.31	0.517	<0.001
C20:4	1.78 ± 0.49	3.63 ± 1.66	2.71 ± 1.00	0.008	1.98 ± 0.91	2.23 ± 1.45	0.194	0.834
C20:5	0.24 ± 0.11	0.31 ± 0.20	0.25 ± 0.11	0.218	0.25 ± 0.18	0.45 ± 0.64	0.355	0.532
C20:3	0.23 ± 0.06	0.61 ± 0.30	0.41 ± 0.21	0.001	0.35 ± 0.17	0.46 ± 0.36	0.055	0.285
C20:2	0.38 ± 0.04	0.59 ± 0.28	0.45 ± 0.17	0.006	0.56 ± 0.21	0.68 ± 0.36	0.086	0.001
C20:1	0.30 ± 0.03	0.66 ± 0.36	0.49 ± 0.23	0.006	0.59 ± 0.25	0.71 ± 0.37	0.090	0.002
C20:0	0.23 ± 0.05	0.30 ± 0.11	0.22 ± 0.06	<0.001	0.24 ± 0.05	0.27 ± 0.09	0.160	0.005
C22:6	1.13 ± 0.15	1.70 ± 0.75	1.20 ± 0.56	<0.001	1.12 ± 0.46	1.29 ± 1.02	0.270	0.020
C22:5	0.31 ± 0.07	0.59 ± 0.36	0.42 ± 0.21	0.003	0.35 ± 0.14	0.47 ± 0.43	0.130	0.071
C22:1	0.26 ± 0.08	0.90 ± 0.58	0.70 ± 0.46	0.068	1.11 ± 0.86	1.47 ± 1.61	0.266	0.023
C24:1	0.08 ± 0.01	0.15 ± 0.05	0.11 ± 0.04	<0.001	0.16 ± 0.07	0.17 ± 0.08	0.276	<0.001

[a] Free fatty acids in serum of 20 healthy controls were simultaneously analyzed with the same method.
[b] p value refers to comparison between before *vs*. after treatment within each group.
[c] p value refers to comparison between berberin and placebo groups after treatment using the ANCOVA analysis.

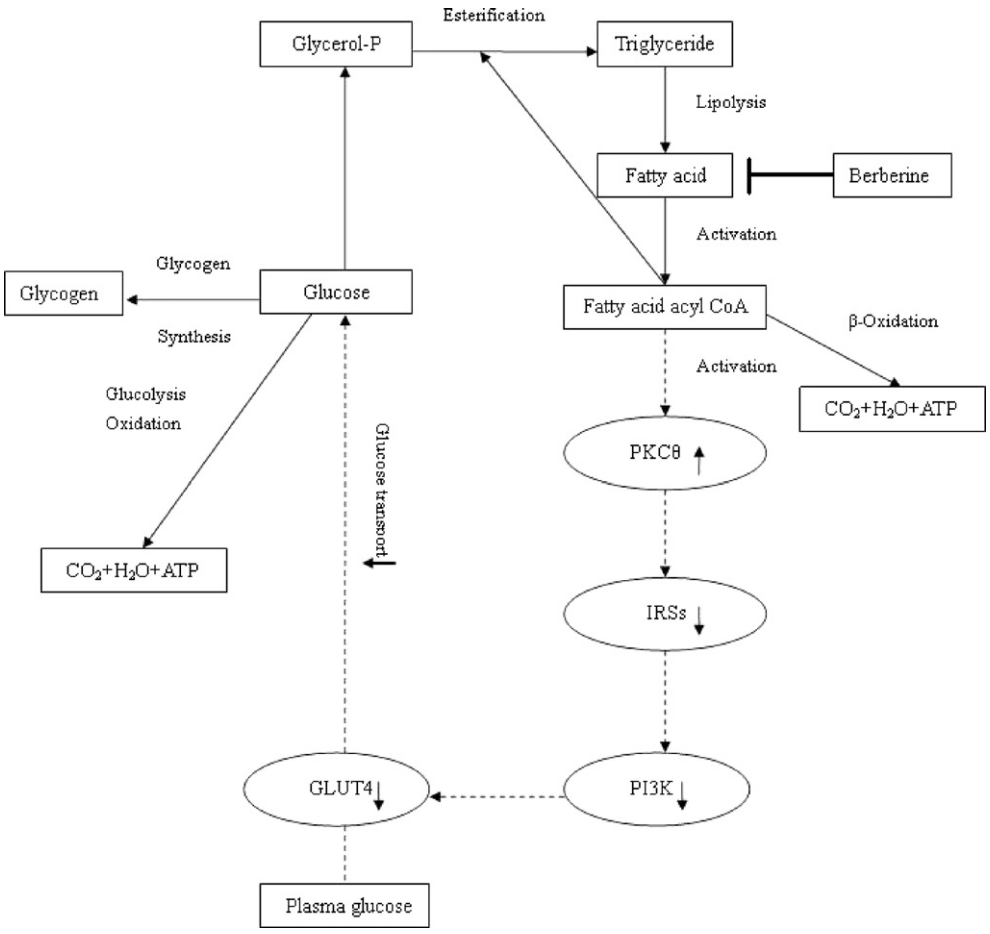

Fig. 4. Correlation between fatty acids, insulin resistance and glucose. Solid arrows represent the major metabolic pathways of fatty acids and glucose. Dotted arrows show the negative effect of high circulating free fatty acids on the insulin signaling pathway. The inhibition may be alleviated by berberine through down-regulating the high level of free fatty acids.

3.3. Metabonomic target analysis

The above results, show that berberine mainly affect the metabolism of free fatty acids in type 2 diabetes mellitus. To investigate the metabolic state more comprehensively and accurately, a target analysis with some pretreatment techniques to remove disruptors was further employed. In the target analysis, 17 free fatty acids were separated over 15 min, except for C18:2 and C20:4, C20:3, C22:5 and C16:0, which were completely resolved by selected ion monitoring (Fig. 3). The method validation including linearity, limits of detection, accuracy, precision, and matrix effect were also performed (detailed in supplementary data). The results indicate that our method is suitable for quantifying endogenous free fatty acids in serum. As shown in Table 3, the concentrations of 13 fatty acids (C16:0, C16:1, C18:0, C18:1, C18:2, C20:0, C20:1, C20:2, C20:3, C20:4, C22:5, C22:6 and C24:1) in patients after 3 months berberine administration decreased significantly compared with those before treatment and some components differed from placebo statistically (all $p < 0.05$). Some fatty acids, for example, C16:0 and C18:0, had a similar concentration to that in the healthy group after berberine treatment.

4. Discussion

The glucose-lowering effects of berberine have recently been recognized. This discovery has been accompanied by many studies attempting to elucidate its potential mechanism. Lee et al. [4] reported that berberine can activate AMP-activated protein kinase (AMPK) in 3T3-L1 adipocytes and L6 myotubes and facilitate GLUT4 translocation in L6 myotubes. Yin et al. [9] and Zhou et al. [10] reported that berberine promoted glucose uptake in HepG2 and 3T3-L1 cells independent of insulin action, Yin et al. [11] also found berberine enhanced glucose metabolism by stimulation of glycolysis. Deng et al. [23] reported that berberine improved insulin sensitivity by increasing the protein tyrosine kinase activity of membrane-bound insulin receptors from type 2 diabetes. However, since an imbalance between carbohydrates and lipids metabolism is involved in the pathophysiology of type 2 diabetes, and berberine can both normalize the hyperglycemia and dyslipidemia, it seems necessary to perform a metabonomic analysis to visualize the alteration of global circulating metabolites after berberine treatment. Therefore, in our present study, we carried out a comprehensive metabonomic analysis combined with fingerprint analysis and target quantitative analysis based on our recent clinical work. We showed that berberine could significantly down regulate the serum concentrations of 13 saturated and unsaturated fatty acids in the type 2 diabetes patients, where-as, no clear difference was found in the placebo patients.

Free fatty acids are synthetic donors of lipid structure in membranes and prostaglandins, and known to be an important energy source in the body. They are derived mainly from the adipolysis of triglycerides in subcutaneous and visceral tissue. Under physiological conditions, fatty acids can supply basal energy of liver, skeletal muscle, myocardium and brain, and also protect protein from decomposition under fasting conditions. Moreover, fatty acids can promote insulin secretion in the basal or glucose stimulated

state, and are essential substances of stimulus-secretion coupling in β-cells [24,25]. However, it has been documented in the literature, that the long-term high level of free fatty acids can induce or aggravate insulin resistance and contribute to the development and progression of type 2 diabetes [26–30]. Extensive studies have been undertaken to show that a high level of circulating free fatty acids can inhibit insulin receptor substrates (IRSs) function, which occurs after increasing phosphorylation of serine residues in IRSs protein, and IRSs-associated phosphatidylinositol 3-kinase activity (PI-3K) [31–33].

In our present study, the significant decrease of fatty acids levels observed, were in accordance with the significant decrease of glucose and lipid concentrations (including fasting and post-load glucose, HbA1c, total cholesterol, triglycerides and LDL-c) in our clinical work. Although no significant decreases in fasting and post-load serum insulin concentrations or HOMA-IR values were found when comparing berberine and placebo groups after 3 months, a trend towards a decrease in these values could nevertheless be found in the berberine group, while no change was seen in the placebo group. Regarding the changes in biochemical values and serum metabolites, berberine may mediate glucose metabolism via participation in lipid metabolism and regulation of insulin secretion and insulin sensitivity by down-regulating fatty acids metabolism, thereby enhancing glucose utility and glycolysis. Fig. 4 shows the correlation between fatty acids, insulin resistance and glucose.

5. Conclusions

In the current study, a metabonomics method combined with fingerprint analysis and target quantitative analysis was employed to study the changes of metabolites in type 2 diabetes patients after treatment with berberine. The results demonstrated that berberine mainly affects the metabolism of free fatty acids in type 2 diabetes. As highlighted above, we suggest that fatty acids as potential pharmaceutical targets for the treatment of diabetes hold great promise, and that berberine might play a role in the treatment of type 2 diabetes via down-regulating the high levels of free fatty acids in patient serum, thus mediating glucose and lipid metabolism. Given the benefits of a comprehensive metabonomic analysis in this study, we speculate that such analyses may be useful in revealing the complex mechanism of drug action and presents a useful tool for probing mechanisms of action of traditional Chinese medicines.

Conflicts of interest

The authors declared that they have no conflicts of interests.

Acknowledgments

We are indebted to all the patients who participated in this study and thank the kind proof reading by Christopher Pynn. This study is supported by the grant from 973 Project of the State Ministry of Science and Technology of China (Nos. 2006CB503902 and 2006CB503904), the China International Science and Technology Cooperation Program (2007DFA31060), National Natural Science Foundation of China (Nos.30700383, 30725037, 20835006, and 20805045), and the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX1-YW-02).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2010.01.015.

References

[1] International Diabetes Federation, Diabetes Atlas, 3rd ed., 2006, Brussels.
[2] G. Ning, J. Hong, Y.F. Bi, W.Q. Gu, Y.F. Zhang, Z.G. Zhang, Y. Huang, W.Q. Wang, X.Y. Li, J. Diabetes 1 (2009) 163–172.
[3] Y.X. Ni, Zhong Xi Yi Jie He Za Zhi 8 (1988) 711–713 (article in Chinese).
[4] Y.S. Lee, W.S. Kim, K.H. Kim, M.J. Yoon, H.J. Cho, Y. Shen, J.M. Ye, C.H. Lee, W.K. Oh, C.T. Kim, C. Hohnen-Behrens, A. Gosby, E.W. Kraegen, D.E. James, J.B. Kim, Diabetes 55 (2006) 2256–2264.
[5] S.H. Leng, F.E. Lu, L.J. Xu, Acta Pharmacol. Sin. 25 (2004) 496–502.
[6] P. Xie, H. Zhou, Y. Gao, Chin. J. Clin. Healthc. 8 (2005) 402–403 (article in Chinese).
[7] J. Wei, J. Wu, J. Jiang, S. Wang, Z. Wang, Zhong Xi Yi Jie He Ganbing Za Zhi 14 (2004) 334–336 (article in Chinese).
[8] Y.F. Zhang, X.Y. Li, D.J. Zou, W. Liu, J.L. Yang, N. Zhu, L. Huo, M. Wang, J. Hong, P.H. Wu, G.G. Ren, G. Ning, J. Clin. Endocrinol. Metab. 93 (2008) 2559–2565.
[9] J. Yin, R. Hu, M. Chen, J. Tang, F. Li, Y. Yang, J. Chen, Metabolism 51 (2002) 1439–1443.
[10] L. Zhou, Y. Yang, X. Wang, S. Liu, W. Shang, G. Yuan, F. Li, J. Tang, M. Chen, J. Chen, Metabolism 56 (2007) 405–412.
[11] J. Yin, Z. Gao, D. Liu, Z. Liu, J. Ye, Am. J. Physiol. Endocrinol. Metab. 294 (2008) E148–156.
[12] J.K. Nicholson, J.C. Lindon, E. Holmes, Xenobiotica 29 (1999) 1181–1189.
[13] J.K. Nicholson, J. Connelly, J.C. Lindon, E. Holmes, Nat. Rev. Drug Discov. 1 (2002) 153–161.
[14] J.L. Griffin, M.E. Bollard, Curr. Drug Metab. 5 (2004) 389–398.
[15] P.Y. Yin, X.J. Zhao, Q.R. Li, J.S. Wang, J.S. Li, G.W. Xu, J. Proteome Res. 5 (2006) 2135–2143.
[16] M.J. Chert, M.M. Su, L.P. Zhao, J. Jiang, P. Liu, J.Y. Cheng, Y.J. Lai, Y.M. Liu, W. Jia, J. Proteome Res. 5 (2006) 995–1002.
[17] D.I. Ellis, W.B. Dunn, J.L. Griffin, J.W. Allwood, R. Goodacre, Pharmacogenomics 8 (2007) 1243–1266.
[18] X.J. Zhao, Y. Zhang, X.L. Meng, P.Y. Yin, C. Deng, J. Chen, Z. Wang, G.W. Xu, J. Chromatogr. B 873 (2008) 151–158.
[19] Y.Q. Bao, T. Zhao, X.Y. Wang, Y.P. Qiu, M.M. Su, W.P. Jia, W. Jia, J. Proteome Res. 8 (2009) 1623–1630.
[20] T.G. Huo, S. Cai, X.M. Lu, Y. Sha, M.Y. Yu, F.M. Li, J. Pharm. Biomed. Anal. 49 (2009) 976–982.
[21] M. Puttmann, H. Krug, E.V. Ochsenstein, R. Kattermann, Clin. Chem. 39 (1993) 825–832.
[22] L. Eriksson, E. Johansson, N. Kettaneh-Wold, S. Wold, Multi- and Megavariate Data Analysis, Umetrics Academy, 2001, pp. 43–56.
[23] X.L. Deng, X.R. Li, C.G. Tian, J. Nanjing Med. Univ. 19 (2005) 181–186.
[24] G. Boden, X.H. Chen, N. Iqbal, Diabetes 47 (1998) 1609–1612.
[25] R.L. Dobbins, M.W. Chester, M.B. Daniels, J.D. McGarry, D.T. Stein, Diabetes 47 (1998) 1613–1618.
[26] R.H. Unger, Diabetes 44 (1995) 863–870.
[27] G. Boden, Diabetes 46 (1997) 3–10.
[28] J.D. Mcgarry, R.L. Dobbins, Diabetologia 42 (1999) 128–138.
[29] J.D. McGarry, Diabetes 51 (2002) 7–18.
[30] G. Boden, G.I. Shulman, Eur. J. Clin. Invest. 32 (2002) 14–23.
[31] M.E. Griffin, M.J. Marcucci, G.W. Cline, K. Bell, N. Barucci, D. Lee, L.J. Goodyear, E.W. Kraegen, M.F. White, G.I. Shulman, Diabetes 48 (1999) 1270–1274.
[32] N. Houstis, E.D. Rosen, E.S. Lander, Nature 440 (2006) 944–948.
[33] S. Schinner, W.A. Scherbaum, S.R. Bornstein, A. Barthel, Diabet. Med. 22 (2005) 674–682.