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Effects of berberine and pomegranate seed oil on plasma phospholipid metabolites associated with risks of type 2 diabetes mellitus by U-HPLC/Q-TOF-MS

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ABSTRACT

A rapid and reliable ultra-performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight mass spectrometry (U-HPLC/Q-TOF-MS) has been firstly used to analyze the changes of plasma phospholipids, in type 2 diabetes mellitus (T2DM) mice after administration of berberine and pomegranate seed oil (PSO). The separation of plasma phospholipids was carried out on an Acquity U-HPLC BEH C18 column (2.1 mm × 50 mm, 1.7 μm, Waters) by linear gradient elution using a mobile phase consisting of 10 mM ammonium formate in water and acetonitrile: isopropanol (1:1, v/v) mixed solution added by 0.25% water and 10 mM ammonium formate. The method demonstrated a good precision and reproducibility. Linear regression analysis showed a good linearity. And potential biomarkers were discovered based on their mass spectra and chemometrics methods. The results demonstrated that the proposed U-HPLC/Q-TOF-MS method was successfully applied to analyze the dynamic changes of phospholipids components in plasma of T2DM mice after drug treatment and could provide a useful data base for meriting further study in humans and investigating pharmacological actions of drugs.

1. Introduction

Diabetes mellitus (DM) is a syndrome of glucose, protein and lipid metabolism disorders caused by deficiency or resistance of insulin in body [1]. Studies have shown that abnormalities of lipid metabolism are prior to diabetes [2]. Phospholipids are the main components of plasma lipoproteins, which assist hydrophobic lipids such as fatty acids and cholesterols transport in blood. Amphiphilic structures of phospholipids make it involved in energy supply, cellular and subcellular partitioning, maintenance of electrochemical gradients, cell signaling and protein trafficking [3]. Study on dynamic changes of phospholipids components is a prospect with great promise in study of etiological approaches to help to identify high risk individuals and investigate pharmacological actions of drugs.

In the process of chromatographic analysis, a large number of phospholipids are not easy to be completely separated for their similar polarities. Mass spectrometry has the ability to separate compounds. Therefore, the combination of chromatography and mass spectrometry, especially liquid chromatography-mass spectrometry (LC–MS) technology, has been used for the separation and identification of phospholipids. With development of the techniques, conventional high performance liquid chromatography (HPLC) has been unable to satisfy the requirement of high throughput samples. HPLC has gradually been replaced by ultra high performance liquid chromatography (U-HPLC) due to its better degree of separation, speed and sensitivity [4,5]. At the same time, high-resolution mass spectrometer, such as quadrupole-time-of-flight mass spectrometry (Q-TOF-MS), has higher resolution and accuracy and further improved the qualitative identification of complex phospholipids. Ultra-performance liquid chromatography coupled with electrospray ionization/quadrupole-time-of-flight mass spectrometry (U-HPLC/Q-TOF-MS) is used to isolate complex lipid samples and provides a possible for the discovery and identification of new lipids. Previous study in our laboratory has demonstrated that a total of 81 phospholipids constituents were identified by fragments information of secondary mass

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spectrometry, elementary composition and database information [6]. However, the dynamic changes of phospholipids in mice plasma after drug intervention have not been taken into account.

Berberine, an isoquinoline derivative alkaloid isolated from herbal plants, is a Chinese medicine traditionally used for the treatment of dysentery, enteritis, chronic diarrhea, cervical erosion, and tapeworm diseases [7]. Pharmacological studies found that berberine had beneficial effects on blood glucose and lipids [8]. Although berberine has effect of lowering blood sugar, the molecular mechanisms governing its repressive influence on hypoglycemic effect is still unclear. Pomegranate (*Punica granatum* L.) is a shrub belonging to family Punicaceae, which has been cultivated and naturalized in many countries including Southeast Asia, tropical Africa, Mediterranean region, and American Southwest. Pomegranate seed oil (PSO), extracted from pomegranate seeds, has received a considerable dietary attention because of its main bioactive component, punicic acid, a *cis*-9, *trans*-11, *cis*-13 conjugated linolenic acid (CLN), which constitutes 64–83% of PSO. Pharmacological studies showed that PSO had anti-inflammatory, anticancer, anti-diabetic, and anti-hyperlipidemic effects [9].

Up to now, there have been no reports of the effects of berberine and PSO on the changes of mice plasma phospholipids of T2DM mice in relevant literatures. In this study, a U-HPLC/Q-TOF-MS analysis method was firstly developed to quickly investigate the changes of plasma phospholipids, in T2DM mice after administration, which could help to comprehend the metabolic alteration of plasma phospholipids in T2DM model mice and further study the pharmacological mechanism of berberine and PSO on T2DM.

2. Materials and methods

2.1. Plant material

Pomegranate seeds were purchased from Guangzhou Qingping Medicine Market, in Guangzhou city, Guangdong Province, China. The plant was authenticated by Prof. Ying Gao, New Medicine Center, Guangzhou University of Chinese Medicine. A voucher specimen with accession No. 20121025 was deposited in School of Chinese Material Medical, Guangzhou University of Chinese Medicine.

2.2. Reagents and chemicals

Metformin (purity ≥90%) was purchased from Hubei Xingyihe Chemical Co., Ltd (Hubei, China). Streptozotocin (STZ, NO. S0130) was supplied by Sigma Corporation (St Louis Missouri, USA). Heparin (NO. 1208111) was purchased from Jiangsu Wanbang Biochemical Pharmaceutical Co., Ltd. (Jiangsu, China). Kits including glucose (Glu) kits (NO. 20130402147), total cholesterol (TC) kits (NO. 20130502146), and triglyceride (TG) kits (NO. 20130301121) were obtained from Shanghai Rongsheng Biological Pharmaceutical Co., Ltd. (Shanghai, China). Citric acid and sodium citrate (NO. 20101201-2, Analytical grade) were from Tianjin Damao Chemical Instrument Factory (Tianjin, China). Methanol, acetonitrile, and isopropanol (HPLC-grade) were from Merck (Darmstadt, Germany). Ammonium formate (HPLC-grade) was from Aladdin Chemistry Co., Ltd. (Shanghai, China). BHT (Analytical grade) was from Sigma–Aldrich (Steinheim, Germany). Chloroform (Analytical grade) was from Fuyu Fine Chemical (Tianjin, China). Formic acid (HPLC-grade) was from Dikma Co. (Beijing, China). Leucine-enkephalin was obtained from Sigma (St Louis Missouri, USA). All phospholipid standards including PE (18:0/0:0), PC (O-18:1/18:1), SM (d18:0/12:0), LPC (16:0), and PS (16:0/18:1) were purchased from Avanti Polar Lipids Co. (Alabama, USA).

2.3. Berberine and PSO samples

Berberine ($C_{20}H_{18}NO_4$) is an isoquinoline derivative alkaloid in herbal plants. Berberine chloride was purchased from Pi & Pi Technology Inc (Guangzhou, Guangdong). The purity of berberine was ≥98%. Pomegranate seeds were extracted by using supercritical CO_2 fluid extraction (SC-CO_2), which was performed in a HA-121-50-01 supercritical fluid CO_2 extraction apparatus (Jiangsu Nantong Huaan Supercritical Fluid Extraction Inc., Jiangsu, China). Briefly, the degree of crushed pomegranate seeds was 40 mesh, weigh accurately 2.0 kg pomegranate seeds sample and placed into a 5 L extraction vessel. After the extraction vessel was tightly sealed, carbon dioxide was pressurized through the extraction vessel from bottom to top, then the extractors and separators were given temperature and pressure. The determined conditions for cupercritical-CO_2 extraction were conducted at an extraction pressure of 29–30 MPa and a temperature of 45 °C for 2 h, and the flow rate of CO_2 was 15 L/h. The extracted oil (0.96 kg) was collected and then weighed to obtain the yield. PSO yield was expressed as the ratio of the weight (g) of PSO to that of seed (g) placed into the extraction vessel.

2.4. Determination of punicic acid in PSO [10]

Concentration of punicic acid in PSO was examined by pre-column derivatization HPLC method. In brief, the analysis of punicic acid was performed using an Agilent Shimadzu LC-10AT vp plus equipment. Punicic acid was derivatized with ω-bromoacetophenone as a derivative reagent and triethanolamine as a catalyst. Using HPLC reversed phase column (Diamonsi C18 column, 250 mm × 4.6 mm, 5 m), detection wavelength was set at 272 nm, column temperature 30 °C, with methanol-acetonitrile-water (68.5:20.0:11.5) as mobile phase, flow rate was 1.0 mL/min. The content of punicic acid in PSO was about 55%.

2.5. Animal experiment design

Male C57BL/6J mice (7 weeks, SPF grade) were purchased from Medical Laboratory Animal Center of Guangdong Province (NO. SCXR 2008-0002, Guangzhou, China). All animals were raised in SPF grade animal housing, experimental animal center of Guangdong Pharmaceutical University and kept under the conditions at a room temperature of 24 ± 2 °C, a humidity of 50–70 % with a 12-h day–night cycle, free to eat and drink. Experimental mice model of T2DM was built by the method of disposable injection of low-dose STZ and high-fat chow inducing mice [11]. After one week adaptation period, the mice were randomly divided into normal control group (NG, 8 mice) and diabetic model group (32 mice), and fed with regular chow for three weeks. The diabetic group mice were injected with STZ freshly prepared in citric acid-sodium citrate buffer (0.1 mol/L, PH 4.4) at a single dosage of 120 mg/kg after fasting for 4 h. NG was injected with sodium citrate buffer in parallel. Three weeks later, after fasting for 12 h, the animals were anesthetized with diethyl ether, and immediately bled using retro-orbital venous plexus puncture to collect blood. Plasma was obtained after centrifugation (3000 g for 15 min at 4 °C). Value of fasting plasma glucose (FPG) and random plasma glucose (RPG) were measured to determine whether meet the diagnostic criteria of diabetes. Then the established diabetic groups were randomly divided into four groups, model group (MG), positive control group (Metformin group, CG), PSO group (PG) and berberine group (BG), each group of at least 6 mice. NG was fed with regular chow. The rest of groups were fed with high-fat chow (32.06% lard, 26.17% casein, 16.35% maltodextrin, 9.00% sucrose, 6.54% cellulose, 4.58% Minerals AIN-93, 3.27% soya-bean oil, 1.31% vitamin AIN-93, 1.00% cholesterol, 0.39% L-cystine, 0.33% choline chloride, 0.15% cholate).