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A Platelet/CMC coupled with offline UPLC-QTOF-MS/MS for screening antiplatelet activity components from aqueous extract of Danshen

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ABSTRACT

Platelets play crucial roles in thromboembolic and cardiovascular disease. The main platelets membrane receptors include adenosine diphosphate receptors, thrombin receptors, thromboxane prostanoid receptors and collagen receptors. In this study, a Platelet/CMC coupled with offline UPLC-QTOF-MS/MS system was built to screen antiplatelet activity components from aqueous extract of Danshen, which serve as an agent of antiplatelet aggregation in Traditional Chinese Medicine. Rosmarinic acid, lithospermic acid, salvianolic acid B, two isomers of salvianolic acid B, salvianolic acid C, salvianolic acid D and salvianolic acid H/I were identified as the potential antiplatelet activity components. Moreover, rosmarinic acid, lithospermic acid, salvianolic acid B, salvianolic acid C and danshensu were tested in platelet aggregation *in vitro* assay. The results suggested their retention time was closely related to the antiplatelet aggregation activities. This study provides a rapid, effective and novel method for screening the potential antiplatelet activity components from Chinese herb medicines.

1. Introduction

Platelets play crucial roles in thromboembolic diseases and cardiovascular disease [1,2], such as myocardial infarction, ischemic stroke and acute coronary syndromes [2,3]. The antiplatelet therapy remains be essential in prevention and treatment. Currently, the therapeutic targets of antiplatelet major include receptors located on platelet membrane (adenosine diphosphate (ADP) receptors (e.g., P2Y purinoceptor 12 ($P2Y_{12}$)), thrombin receptors (e.g., proteinase-activated receptor 1 (PAR1)), thromboxane prostanoid (TP) receptors (e.g., TPα), collagen receptors (e.g., α2β1)) and signaling molecules intracellular (e.g., cyclooxygenase 1 (COX 1), phosphodiesterases) [2]. The antiplatelet drugs developing mainly focus on the above targets. Clopidogrel ($P2Y_{12}$ antagonist) and Aspirin (COX 1 inhibitor) are two quintessential antiplatelet drugs in clinical [2]. Several new drugs in clinical trial are designed as $P2Y_{12}$ antagonist (Ticagrelor) and PAR1 antagonist (Atopaxar) [4,5]. However, a slice of adverse effects of current antiplatelet drugs make it is still necessary to seek new therapeutic agents. Long-term use of Aspirin or clopidogrel could cause drug resistance [6,7].

A multitude of Traditional Chinese Medicines (TCMs) are considered to possess the therapeutic activity of activate blood and remove stasis. It is regarded as a treasure house for new drug discovery of antiplatelet drugs from herbal medicine. It is an effective way. Danshen, the root of *Salvia miltiorrhiza* Bge, is an indispensable herb in the TCM treatment against thromboembolic diseases and cardiovascular disease [8]. The antiplatelet aggregation function of Danshen is demonstrated in a host of pharmacological and clinical studies [9] of activating blood and removing stasis. The chemical constituents of Danshen are divided into two groups: lipophilic components (tanshinone type) and hydrophilic components (phenolic acids) [10]. Recent studies have revealed that quite a few phenolic acids components in Danshen were manifested to have antiplatelet aggregation activity. Salvianolic acid B could interact with collagen receptor α2β1 and inhibit ADP-induced platelet aggregation [11,12]. Salvianolic acid A inhibit platelet activation and arterial thrombosis through the pathway of phosphoinositide 3-kinase [13]. It is paramount to develop an effective strategy to separate and identify single phenolic acids components in herbal medicine.

Cell membrane chromatography (CMC) was first proposed by Professor L.C. He and X.D. Geng in 1996 [14]. CMC has become a rapid and effective method to screen active components from complex systems [15–18]. In this study, a Platelet/CMC coupled with offline an UPLC-QTOF-MS/MS system was built as an rapid, effec-

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tive and novel bio-analytical system for separating and identifying the potential antiplatelet activity components acting on the platelet membrane receptors from aqueous extract of Danshen and others TCMs.

2. Material and methods

2.1. Reagents and chemicals

Ticagrelor was purchased from Selleck Chemicals LLC (Houston, TX, USA). Aspirin, Adenosine 5′-diphosphate (ADP) and 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Thrombin was purchased from Beijing Solarbio Technologies Ltd. (Beijing, China). Danshensu (DSS), rosmarinic acid (RA), lithospermic acid (LA), salvianolic acid B (SAB) and salvianolic acid C (SAC) were purchased from Tianjin Zhong Xin Pharmaceutical Group Co., Ltd. (Tianjin, China). The purity of each reference chemical was above 98%. Silica gel (YQG, 5 μm, 118 Å) was obtained from Qingdao Meigao Chemical (Qingdao, China). Acetonitrile (LC-MS grade) was purchased from Honeywell Burdick & Jackson (Morristown, NJ, USA). Formic acid (HPLC grade) was purchased from Mreda Technology (Columbia, TN, USA). Other reagents used were of analytical grade. Danshen was bought from Boguang Chinese Herbal Medicine Co., Ltd. (Bozhou, China). The ultrapure water used for preparation of all aqueous solutions was produced by Milli-Q water purification system made by Millipore (Bedford, MA, USA). Polytetrafluoroethylene (PTFE) membranes of 0.22 μm used for processing samples were also purchased from Millipore.

2.2. Instrument configuration and conditions

Agilent 1260 Infinity Quaternary HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA) was adopted in the CMC. The HPLC system comprised of a quaternary pump (G1311C), an autosampler (G1329B), a thermostat (G1330B), a thermostatted column compartment (G1316A) and a diode-array detector (G1315D). Data collection and processing were performed on Agilent 1200 Chemistation. ODS columns (4.6 mm × 12.5 mm I.D., 5 μm, Welch Materials, shanghai, China) were used to collect the retained fractions.

Waters ACQUITY™ Ultraperformance LC (UPLC) system (Waters Corporation, Milford, MA, USA) was used to separate and identify the components in retained fractions. The UPLC system was equipped with a PDA detector, a column compartment, a sampler manager and a binary solvent manager, and connected to a Xevo™ G2-S QTOF mass spectrometer. The separation was carried out on an ACQUITY UPLC™ BEH C_{18} column (2.1 mm × 50 mm, I.D., 1.7 μm, Waters Corporation) at 30 °C with a flow rate of 0.3 mL/min. Mobile phase was a mixture of 0.1% formic acid–water (A) and acetonitrile (B). The gradient program of mobile phase for Ticagrelor was as follows: 0-10 min, 5-95% B. The gradient program of mobile phase for aqueous extract of Danshen was as follows: 0–3 min, 2% B; 3–5 min, 2–5% B; 5–9 min, 5–7% B; 9–10 min, 7–10% B; 10–20 min, 10–12% B; 20–21 min, 12–13% B; 21–26 min, 13–15% B; 26–30 min, 15–18% B; 30–36 min, 18–20% B; 36–38 min, 20% B; 38–41 min, 20–95% B. The injection volume was 5 μL and the PDA detection was performed in the range of 210–400 nm. The MS was operated in negative mode, with capillary to 2500 V, the cone set to 40 V, and source offset to 80 V. The desolvation gas was maintained at 600 L/h at a temperature of 400 °C. The cone gas was 50 L/h with a source temperature of 100 °C. The scan range was 100–1100 Da.

Platelet aggregation assay used FlexStation 3 (Molecular Devices Corp., Sunnyvale, CA, USA).

2.3. Preparation of samples and standard solutions

The aqueous extract of Danshen was prepared as follows: 50 g of Danshen was refluxed with 400 mL water for 2 h and filtered. The filtrate was concentrated to a volume of 70 mL under reduced pressure and vacuum, and then injected into preparative HPLC to remove most SAB, which was located by comparing the retention time with the standard compound. The prepared aqueous extract was concentrated and then dried by vacuum freeze-drying. 10 mg aqueous extract of Danshen (AED) dissolved in 10 mL 50% methanol–water (v/v) and then stored at −80 °C in the dark before use.

Ticagrelor, Aspirin, DSS, RA, LA, SAB and SAC standard solutions (1 mg/mL) were freshly prepared in 50% methanol–water (v/v) every week and stored at −80 °C in the dark.

For CMC analysis, all the stored solutions were diluted to suitable concentrations by mobile phase. For UPLC-QTOF-MS/MS analysis, Ticagrelor and Aspirin stored solutions were diluted with 50% methanol–water (v/v), respectively. DSS, RA, LA, SAB and SAC stored solutions were diluted with 25% methanol–water (v/v), respectively. The enriched Ticagrelor by ODS column in CMC was dissolved in 50% methanol–water (v/v). The enriched AED by ODS column in CMC was dissolved in 25% methanol–water (v/v).

All the injecting samples were filtered through a 0.22 μm PTFE membrane before analysis.

2.4. Platelet extract and preparation for the Platelet/CMC system

Ten male Sprague-Dawley (SD) rats (250–280 g bodyweight) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). In asepsis condition, rats were anesthetized by 10% chloral hydrate and draw blood from abdominal aorta. The whole blood and ACD (38 mM Citric acid, 75 mM sodium citrate, 124 mM dextrose) 20:3 (V/V) mixture was centrifuged at 200 × *g* at room temperature for 10 min. The supernatant platelet-rich plasma was collected and centrifuged at 800 × *g* at room temperature for 10 min. The pellet was re-suspended in buffer A (pH 7.40, 130 mM NaCl, 10 mM sodium citrate, 9 mM $NaHCO_3$, 6 mM dextrose, 0.9 mM $MgCl_2$, 0.81 mM KH_2PO_4, 10 mM Tris-base) and then centrifuged again at 800 × *g*, 4 °C for 10 min to obtain pure platelet. The pure platelet was re-suspended in 50 mM Tris–HCl (pH 7.40) to be ruptured by ultrasonicating for 30 min, and centrifuged at 1000 × *g*, 4 °C for 10 min. The supernatant was centrifuged twice at 12,000 × *g*, 4 °C for 20 min to get platelet membrane pellet. The platelet membrane pellet was suspended in 5 mL ultrapure water. A total of 50 mg silica gel was activated at 105 °C for 30 min. The activated silica gel was slowly added into the platelet membrane suspension under vacuum at 4 °C. The mixture was subsequently agitated for 30 min with a magnetic stirrer and let stand for 12 h. The silica gel surrounding by platelet membrane was packed into the CMC column (10 mm × 2.0 mm I.D.) by a wet packing method to obtain a platelet cell membrane stationary phase column.

2.5. System suitability of the Platelet/CMC offline UPLC-QTOF-MS/MS system

In order to verify the system suitability of the Platelet/CMC column, the specificity, selectivity and reproducibility were tested. After optimizing the chromatographic conditions, ultrapure water was chose as the mobile phase with the flow rate of 0.1 mL/min, the detection wavelength was 254 nm, 280 nm and 293 nm, the column temperature was 37 °C ± 0.5 °C. Then the column was attached to HPLC system, equilibrated 80 min.