Research Article

Roles of paeoniflorin and senkyunolide I in SiWu decoction on antiplatelet and anticoagulation activities

Traditional Chinese medicine (TCM) is a complex system, which consists of numerous compounds with related mechanisms to maximize therapeutic efficacy with minimal adverse effects. Some new methods disclosing the contribution of these constituents as well as their relationship in the formula are necessary for elucidating the bio-active constituents and the working mechanisms of TCM. In this study, depletion of target components using preparative HPLC followed by antiplatelet and anticoagulation activities evaluation was first applied to investigate the roles of paeoniflorin and senkyunolide I in a well-known formula, SiWu decoction. The results showed that both paeoniflorin and senkyunolide I not only directly brought about some bio-activities, but also indirectly made the contribution to the total bio-activity reflection of SiWu decoction, especially the latter should deserve to be drawn attention to the research of complicated bio-active constituents of TCM or its formula. So, the significant and effective approach will be very useful for the elucidation of the contribution of each different chemical constituent to the bio-activity of a TCM formula. Furthermore, this study demonstrated the potential utilization of preparative HPLC in the research of TCM.

Keywords: Paeoniflorin / Preparative HPLC / Senkyunolide I / SiWu decoction / Target components depletion

DOI 10.1002/jssc.201000340

1 Introduction

Traditional Chinese medicines (TCMs) are natural therapeutic remedies used under the guidance of traditional Chinese medical philosophy and have been prescribed by TCM practitioners in China and the Chinese community worldwide for thousands of years. Most of the TCM are multi-ingredient formulae, and it is widely accepted that multiple constituents are responsible for their biological activities. Especially, some constituents do not directly bring about some bio-activities, but indirectly make the contribution to the total bio-activity reflection of TCM by possibly improving the absorption, distribution, metabolism and excretion of other bio-active constituents.

Up to now, most TCM researchers usually focused on the total biological activity of TCM extract containing multiple constituents or the activity of purified compounds from TCM. However, it was very difficult to elucidate the actual contribution of every compound for the whole formula. Recently, in the inspiration of gene deletion method, a new approach was conducted to deplete a component from TCM and then examine the changes of activities after depletion [1]. This method was utilized in the study of active principles of Si-ni-san by using immunoaffinity columns, and the results showed that glycyrrhizin and paeoniflorin (PA) were active constituents of Si-ni-san on alleviating the ear inflammation effect [1–3]. Under the enlightenment of the depletion method, a new approach was designed which may be more straightforward and timesaving: we attempted to deplete the target component by using preparative HPLC. As a research model, the method was used to investigate the roles of PA and senkyunolide I (SI) in SiWu decoction (SWD), a well-known formula which has been used as a nourishing and regulating blood prescription to treat gynecologic diseases [4] for almost 1000 years in China and Japan (Japanese name, Shimotsu-to) [5]. The formula consisted of four herbs including Angelicae Sinensis Radix (ASR), Chuanxiong Rhizoma (CR), Paeoniae Radix Alba (PRA) and Rehmanniae Praeparata (RRP) and Senkyunolide I; SWD, SiWu decoction; TCM, traditional Chinese medicine; TT, thrombin time
Radix Praeparata (RRP) at a ratio of 1:1:1:1. Recent study reported that it possessed anticoagulation, vasodilatation and blood circulation activating effects [6–8]. The major bio-active components in the four herbs include phthalides, monoterpenic glycoside, organic acids, and so on [9–13]. PA, a major monoterpenic glycoside in PRA had showed many pharmacological effects, such as anticancer, anti-proliferative and neuroprotective [14, 15]. SI, a phthalide in both CR and ASR, had exhibited the activity of reducing the metamorphose damage of the red blood cell caused by ConA [16]. PA and SI are the major constituents in SWD; however, few studies were reported about their vasodilatation and blood circulation activating effects, and their actual contribution to the whole formula.

In this study, depletion of target component using preparative HPLC followed by antiplatelet and anti-coagulation activities test was successfully applied to the investigation of the roles of two major components, PA and SI in SWD (Fig. 1). This study provided a new and effective approach to clarify the contribution of every compound in TCM formula to the total bio-activity reflection of TCM, and it also extended the ways of exploring useful components in TCM formula. Furthermore, this study demonstrated the potential utilization of preparative HPLC in the characterization of the roles of multi-ingredients in TCM formula.

2 Materials and methods

2.1 Plant materials

ASR, CR, PRA and RRP were purchased from Minxian (Gansu province), Pengzhou (Sichuan province), Hailaer (Inner Mongolia) and Jiaozuo (Henan province), respectively. All crude plants were identified as Angelica sinensis (Oliv.) Diels, Ligusticum chuanxiong Hort., Paeonia lactiflora Pall. and Rehmannia glutinosa Libosch. by the corresponding author. The voucher specimens (No. NJUTCM-20060818-20060821) were deposited in Nanjing University of Chinese Medicine.

2.2 Instrumentation

The Waters AutoPurification™ system including 2545 Binary Gradient Module, 2767 Sample Manager, column fluidics Organizer, 2489 UV/Visible Detector, Fractionlynx™ Software and Sunfire™ prep C18 OBD™ column (30 × 150 mm, 5 μm); the analytical HPLC apparatus including Waters 2695 separations module, 2996 DAD and Sunfire™ C18 column (4.6 × 250 mm, 5 μm) (Waters, Milford, MA, USA). Platelet aggregation and blood coagulation factors analyzer (LG-PABER-I, Steellex, Beijing, China).

2.3 Chemicals and kits

PA (purity ≥98%) was supplied by Research Center of Standardization of Chinese Medicines (011002, Shanghai, China). SI (purity ≥98%) was isolated from Ligusticum chuanxiong Hort. by using Zhang’s method [17] in our laboratory and its structure was confirmed based on 1H NMR and ESI-MS analysis. Adenosine diphosphate (ADP), thrombin, Tris-HCL and kits for prothrombin time (PT) (ISI 1.0) and activated partial thromboplastin time (APTT) assay were commercial reagents from Sun Biochemical Co. Ltd. (Shanghai, China). Methanol was obtained from Tedia (Fairfield, OH, USA). Other reagents used in the experiment were of analytical grade.

2.4 Animals

Male New Zealand white rabbits (QingLongshan Laboratory Animal, Nanjing, China) weighing 1.9–2.1 kg were used. They were housed in a conventional animal facility with free access to food and water where the environmental temperature and relative humidity were monitored and controlled (22 ± 1°C, 55 ± 5% relative humidity, 12 h light/12 h dark cycle). All animals received humane care with the guide for the care and use of laboratory animals (National Research Council of USA, 1996) and the related ethical regulations of our university.

2.5 Preparation of SWD

The 400 g of mixed crude herbs (ASR, CR, PRA and RRP) at the weight ratio of 1:1:1:1 were crushed into small pieces, and then extracted twice in 4 and 3.2 L of water, with

Figure 1. Chemical structures of PA (A) and SI (B).
refluxing times of 2 and 1.5 h, respectively. The decoction was combined and the solvent was removed below 65°C till certain volume at the ratio of 1:1 (w/w, weight of all herbs and the extracted filtrates) under vacuum, and then ethanol was added slowly with churning all the time until the ethanol content reached 80%. After being deposited for 24 h, the solution was filtered to dispose of the deposition and concentrated to a certain concentration under vacuum below 65°C, the sample of SWD was obtained (0.34 g was equivalent to 1.00 g crude drugs of the formula).

2.6 Preparation of SWD depleted of PA or SI

The SWD extract was diluted with methanol in ultrasonic machine, and filtered through a 0.45 μM membrane before injection. Its concentration was almost 300 mg/mL. The injection volume was 120 μL; the mobile phase was water (A)/methanol (B) at a flow rate of 30 mL/min; the detection wavelength was set at 232 nm (λmax of PA) and 277 nm (λmax of SI). Based on the analytical method of the sample, the preparative HPLC gradient was chosen as follows: 0 min – 35% B; 7 min – 35% B; 8 min – 40% B;
13 min – 42% B; 14 min – 52% B; 20 min – 60% B; 22 min – 100% B; 27 min – 100% B and 28 min – 35% B. Fractions were collected according to the retention time of PA and SI with 10 mL per fraction (tube). The remnant solutions were collected in a 5-L flask, and then the solvent was removed at 60°C under vacuum, respectively. So, two new samples were obtained, one was SWD depleted of PA (SWD-PA), another was SWD depleted of SI (SWD-SI).

2.7 Evaluation of the antiplatelet and anticoagulation activities

Blood was collected through a polyethylene cannula placed in the common carotid artery of male New Zealand white rabbits by a 10 mL plastic flask containing 3.8% sodium citrate (1:9, v/v). Platelet-rich plasma (PRP) was prepared by low-speed centrifugation of the blood at 800 rpm for 10 min and further centrifuged at 3000 rpm for 10 min to prepare platelet-poor plasma (PPP) [18, 19].

Platelet aggregation test (PAGT) was performed by the turbidimetric method of Born and Cross using a four channel platelet aggregation and blood coagulation factors analyzer according to the manufacturer’s instructions. In brief, 280 μL PRP with 10 μL sample solution was incubated at 37°C for 3 min in the analyzer before the addition of 10 μL ADP (5 μM), then, changes in light transmission were recorded for 6 min, and the percentages of aggregation were calculated.

The anticoagulation activity was measured by clotting assay of thrombin time (TT), PT and APTT according to the methods provided by the biological reagents provider (Sun Biochemical). Briefly, TT was measured by incubating 50 μL PPP with 10 μL sample solution for 3 min at 37°C, then 50 μL bovine thrombin (10 U/mL) which had been preincubated for 5 min at 37°C was added and the clotting time was recorded; PT was measured by incubating 50 μL PPP with 10 μL sample solution for 5 min at 37°C, then adding 100 μL warmed thromboplastin agent and the clotting time was recorded; APTT was measured by incubating 50 μL PPP with 10 μL sample solution for 3 min at 37°C followed by adding 100 μL warmed 20 mM CaCl2 and the clotting time was recorded.

PAGT and anticoagulation test of each sample were conducted three to five times in four channels. All the samples were dissolved in DMSO (control) to obtain stock solutions. In order to eliminate the effect of the solvent on the aggregation, the final concentration of DMSO was fixed at 0.5% [20]. Student’s t-test was used to test the significance of differences between the tested samples and control.

3 Results and discussion

3.1 The depletion results of preparative HPLC

Analytical HPLC was used to check the depletion results. Based on the comparison with standard substances and related articles [5, 21–24], the two peaks (Peak I containing PA; Peak II containing SI) depleted can be identified as the target component, PA (tR = 18.80 min) and SI (tR = 31.12 min), and their purity was validated with wavelength scanning by analytical HPLC and 1H NMR (the details can be found in Supporting Information Fig. S1A–C and Fig. S2A–C). As shown in Figs. 2A and B, PA or SI was successfully depleted from SWD, respectively, and exclusively. Using this method, two samples SWD-PA (SWD depleted of PA) and SWD-SI (SWD depleted of SI) were obtained. Thereafter, the antiplatelet aggregation and anticoagulation bioactivities of SWD, PA, SI, SWD-PA and SWD-SI were evaluated.

3.2 Effect of ADP-induced platelet aggregation

Platelets were blood cells that participate in the primary hemostatic process; they needed to be activated to perform all of their functions. The inhibition of platelet function represented a promising approach for the prevention of thrombosis and its recurrence. ADP was the oldest and one of the most important agonists of platelet activation [25]; therefore, ADP was chosen as the agonist for the in vitro PAGT. Figure 3A shows that the order of potency of inhibition of ADP-induced aggregation is SWD > SWD-SI > SWD-PA > PA > SI and the former three showed an apparent concentration-dependent manner. SWD exhibited the most significant antiplatelet aggregation ability in comparison with SWD-SI and SWD-PA, which showed that the depletion of PA or SI from SWD led to noteworthy reduction of SWD’s activity in ADP-induced aggregation (p<0.01). The result demonstrated that PA and SI were indispensable components in SWD on the effect of antiplatelet aggregation. Interestingly, although SI did not show any effect on ADP-induced platelet aggregation, the activity decreased when it was depleted from SWD, which indicated that SI may act as a role of promoting the antiplatelet activity of other components in SWD. Meanwhile, PA exhibited some effects on antiplatelet aggregation, and the activity of SWD also decreased without PA in it, which showed that PA was an effective constituent of SWD on antiplatelet aggregation effect.

3.3 Effect of anticoagulation

The in vitro anticoagulation activities were measured by TT, PT and APTT, and the results of clotting time assays are shown in Figs. 3B–D.

TT reflected the blood coagulation status that transformed fibrinogen into fibrin by interaction with thrombin. Prolongation of TT may indicate the inhibition of thrombin activity or fibrin polymerization [26]. Fig. 3B shows that the order of the prolongation of TT is SWD > SWD-SI > SWD-PA with concentration-dependent manner. PA and SI did not show any effect on TT in the tested concentration range,
but the absence of them (SWD-PA, SWD-SI) led to the shortening of TT, which indicated that the two compounds were indispensable components of SWD on the prolongation effect of TT.

PT was used to characterize the extrinsic coagulation factors. Prolongation of PT indicated the inhibition of extrinsic part of coagulation [26]. As shown in Fig. 3C, the order of PT prolongation effect of SI, SWD-SI, SWD-PA and PA was as follows: SI > SWD-SI, SWD-PA > PA. In the concentration ranging from 3C/5 to C/20, SI showed more potential PT prolongation activity than SWD, which indicated that SI was an active compound on PT prolongation, but its activity was depressed in the whole formula by the interaction of other components.

APTT was used for the evaluation of coagulation factors in the intrinsic blood coagulation pathway. Prolongation of APTT suggested an inhibition of the intrinsic and/or common system. Figure 3D shows that PA and SI had no effect on
APTT prolongation, but when they were depleted from SWD, respectively, the APTT of SWD was remarkably shortened. So, the results revealed that PA and SI were indispensable components in SWD for promoting other components in the formula to exhibit the APTT prolongation effect.

According to the above study and result analysis, both PA and SI not only directly brought about some bio-activities, but also indirectly made the contribution to the total bio-activity reflection of SWD, especially the latter should deserve to be drawn attention in the research of complicated bio-active constituents of TCM or its formula.

4 Concluding remarks

In the study, a new, convenient and effective approach was established for studying on complicated bio-active constituents of TCM formula with preparative HPLC followed by the bio-activity evaluation. It was an effective extension of the application scope of preparative HPLC to the research of TCM. The direct and indirect contribution of the chemical constituents to the total bio-activity reflection of the formula can be demonstrated by using this method.

In our coming research, more components will be depleted, respectively, or simultaneously from the SWD, and more efficiency evaluation tests will be designed and carried out. So, the significant and effective approach will be very useful for the elucidation of the contribution of each different chemical constituent to the total bio-activity reflection of a TCM formula.

This research was financially supported by Key Research Project in Basic Science of Jiangsu College and University (06KJA36022, 07KJA36024), 2009’ Program for New Century Excellent Talents by the Ministry of Education (NCET-09-0163), National Natural Science Foundation of China (30873235), Natural Science Foundation of Jiangsu Province, China (BK2008455), 2007–2008’ Jiangsu Science and Technology Research Project in Chinese Medicine (H207047), 2006’ and 2007’ Project for Supporting Jiangsu Provincial Talents in Six Fields (06-C-020, 07-C-010), Fundamental Research and Important Incubation Project by Nanjing University of Chinese Medicine (08XPY03), The Project Sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, Nanjing University of Chinese Medicine. We are also pleased to thank Waters China Ltd. for technical support.

The authors have declared no conflict of interest.

5 References