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Separation and detection of minor constituents in herbal medicines using a combination of heart-cutting and comprehensive two-dimensional liquid chromatography

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ABSTRACT

Herbal medicines contain a large number of minor constituents, which could contribute to their therapeutic effects and provide valuable lead compounds for drug discovery. However, to explore minor constituents from complicated herbal extracts is usually laborious and time-consuming. In order to discover minor novel herbal constituents efficiently, we combined heart-cutting and comprehensive two-dimensional liquid chromatography (HC-2DLC) to remove major components from herbal extracts, and then characterized the minor ones by mass spectrometry. This strategy was employed to analyze *Pueraria lobata* and *Pueraria thomsonii*, the roots of which are used as the Chinese herbal medicine Ge-Gen. Five major compounds in Ge-Gen extract were removed by on-line heart-cutting, and the minor compounds were separated on an RP × RP 2DLC system (1D, Acquity CSH C_{18}, 2.1 × 100 mm, 1.7 μm; 2D, Poroshell Phenyl-Hexyl, 3.0 × 50 mm, 2.7 μm). A synchronized gradient elution program was used to improve chromatographic resolution of the second dimension. By using this 2DLC system, a total of 271 and 254 peaks were separated in *P. lobata* and *P. thomsonii* within 35 min, respectively. The practical and effective peak capacity was 1593 and 677, respectively, and the orthogonality was around 70%. Structures of 12 selected compounds were tentatively characterized by mass spectrometry, and 9 of them were discovered from Ge-Gen for the first time. Contents of these minor compounds in Ge-Gen were preliminarily determined to be 0.01–0.1% (w/w). The HC-2DLC/MS system is a powerful and convenient tool to explore minor novel chemical constituents from complex herbal extracts.

1. Introduction

Although the chemistry of popular herbal medicines has been extensively studied, our understanding on the minor constituents is far from enough. Even from the most popular herbs like *Panax ginseng* (ginseng) and *Glycyrrhiza uralensis* (licorice), new constituents are reported almost each year [1–3]. Recently, a number of minor constituents isolated from Chinese herbal medicines were found to have novel chemical structures and significant biological activities. For instance, a dimeric sesquiterpene lactone from *Inula japonica* could strongly inhibit NO production [4], and triterpenoid-diterpenoid heterodimers from *Pseudolarix amabilis* showed potent cytotoxicities [5]. These minor constituents do not only play an important role in the therapeutic effects of herbal medicines, but also provide valuable lead compounds for drug discovery. Historically, a number of natural drugs like paclitaxel and vincristine were originally isolated from source plants as minor constituents [6,7].

The discovery of novel minor constituents from complicated herbal extracts is laborious, time-consuming, and inefficient [8]. A large amount (>10 kg in most cases) of crude herbal materials are extracted, fractionated, and then purified by repeated column chromatography. By this conventional phytochemical means, it is difficult to predict new compounds before the structures are characterized. The chance of obtaining new compounds may be increased when the separation process is guided by informative analytical techniques such as LC/MS [9]. However, only tens of compounds could be detected from a herbal extract by LC/MS, due to limited chromatographic peak capacity and overlapping of minor compounds by major ones. Although orthogonal column chromatography could improve the separation of herbal extracts,

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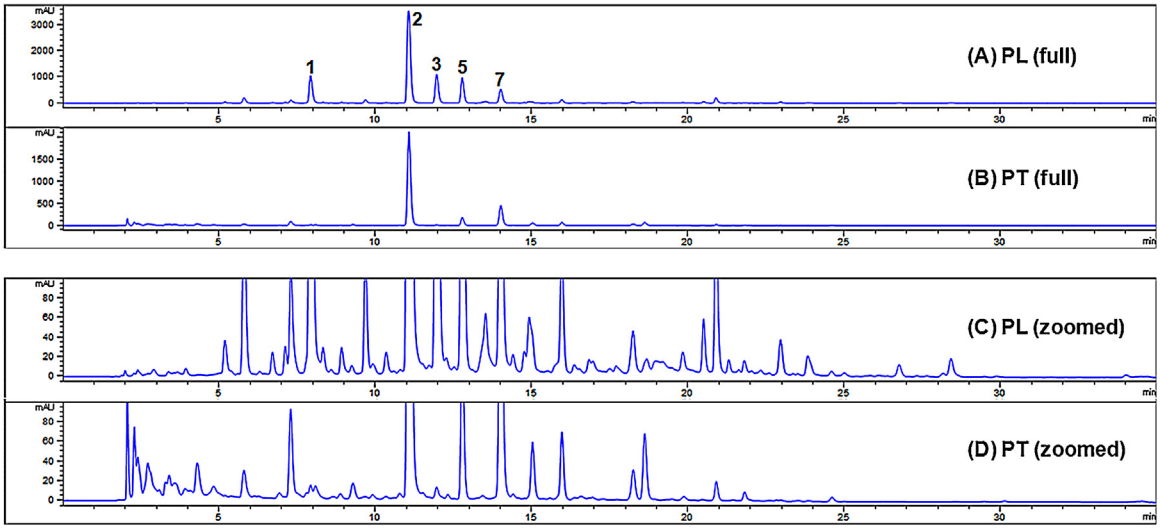

Fig. 1. HPLC chromatograms of *P. lobata* (PL) and *P. thomsonii* (PT) at 250 nm.
The full chromatograms and *y*-axis zoomed-in chromatograms are shown. The major peaks were: 3′-hydroxy puerarin (**1**), puerarin (**2**), 6″-*O*-xylosyl puerarin (**3**), mirificin (**5**), and daidzin (**7**).

and allowed the detection of 334 ginsenosides from *P. ginseng*, this off-line method suffers from poor reproducibility [10,11].

Ge-Gen (kudzu root) is a popular traditional Chinese herbal medicine used for the treatment of cardiovascular diseases. It is derived from the roots of *Pueraria lobata* (Willd.) Ohwi and *Pueraria thomsonii* Benth [12,13]. It contains five predominant chemical constituents, i.e. 3′-hydroxy puerarin (**1**), puerarin (**2**), 3′-methoxy puerarin (**3**), mirificin (**5**), and daidzin (**7**), which account for more than 98% of peak area in the HPLC fingerprint (Fig. 1). Nonetheless, when the chromatogram is zoomed for 30 folds, a lot of minor peaks could be observed. Little is known on the structures of these minor constituents due to their low amounts and insufficient separation in single-dimension chromatography. In fact, no more than 48 compounds have been detected from *P. lobata*, so far [14–16].

Two-dimensional liquid chromatography (2DLC) is a powerful technique to improve separation capability [17]. Combination of cation-exchange, size exclusion, or hydrophilic interaction chromatography (HILIC) with reversed-phase chromatography (RP) could generate desirable orthogonality and high peak capacity, and has been used to separate complex herbal extracts [18–22]. For instance, Liang et al. used HILIC × RP 2DLC to separate 543 peaks from *Scutellaria barbata*, and the peak capacity was 2879. Due to differences between the first and second dimensions in mobile phase and sampling frequency, most of these 2DLC methods were operated in the off-line mode, and required long analysis time (>60 min). RP × RP provides better compatibility than the other 2DLC modes in terms of mobile phase. Moreover, reversed-phase mode is the most powerful and frequently used mechanism to separate herbal extracts, which usually contain a group of small molecules with very similar molecular weight, polarity, and structural scaffold. Dugo et al. established a comprehensive RP × RP 2DLC method to analyze extracts of *Ilex paraguariensis*. Although different separation selectivities of the two dimensions yielded high peak capacity, the 2DLC system did not show particular advantage over 1D-HPLC, probably due to the presence of a few predominant components [23].

In this study, we combined heart-cutting and comprehensive two-dimensional liquid chromatography (HC-2DLC) to separate minor constituents in an aqueous methanol extract of Ge-Gen. On-line heart-cutting was used to remove major compounds, while comprehensive RP × RP 2DLC could efficiently separate the minor ones. A total of 271 and 254 peaks were detected from *P. lobata* and *P. thomsonii*, respectively. The structures of 12 selected unknown compounds were tentatively characterized by on-line coupled mass spectrometry, and nine of them were discovered from Ge-Gen for the first time.

2. Experimental

2.1. Chemicals and reagents

Methanol, isopropanol, acetonitrile (Fisher) and formic acid (Sigma–Aldrich, MO, USA) were of HPLC grade. De-ionized water was prepared using a Milli-Q water purification system (Millipore, MA, USA). High-purity nitrogen (99.9%) and helium (99.99%) were from Gas Supply Center of Peking University Health Science Center (Beijing, China). Reference standards were isolated from *P. lobata* (Willd.) Ohwi by the authors, including 3′-hydroxy puerarin (**1**), puerarin (**2**), 6″-*O*-xylosyl puerarin (**3**), 3′-methoxy puerarin (**4**), mirificin (**5**), 3′-methoxy mirificin (**6**), daidzin (**7**), 3′-methoxy daidzin (**8**), genistein 8-*C*-[apiosyl-(1→6)]-glucoside (**9**), daidzein 4′-*O*-glucoside (**10**), calycosin 7-*O*-glucoside (**11**), (4*S*)-puerol A 2″-*O*-glucoside (**12**), (4*R*)-puerol A 2″-*O*-glucoside (**13**), 3′-methoxy daidzein4′-*O*-glucoside (**14**), genistin (**15**), 4′-methoxy puerarin (**16**), formononetin 8-*C*-[apiosyl-(1→6)]-glucoside (**17**), 3′-hydroxy daidzein (**18**), (4*S*)-puerol B 2″-*O*-glucoside (**19**), ononin (**20**), and daidzein (**21**). Biochanin A (**22**) was isolated from *G. uralensis* by the authors. Purity of all the standards was above 98% by HPLC/UV analysis. Their structures are given in Fig. 2.

2.2. Crude drugs and sample preparation

Dried roots of *P. lobata* (PL) and *P. thomsonii* (PT) were purchased as the traditional Chinese medicine Ge-Gen from drug stores in Shandong Province. Voucher specimens were deposited at the authors' laboratory. The crude drug materials were powdered, and an aliquot of 1 g was extracted with 5 mL of 70% methanol in an ultrasonic bath for 30 min. The solutions of PT and PL were diluted with water by 3-fold and 30-fold, respectively, given their different concentrations of chemical constituents. The samples were filtered through 0.22 μm membranes before use. A volume of 1 μL