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Combination of off-line two-dimensional hydrophilic interaction liquid chromatography for polar fraction and two-dimensional hydrophilic interaction liquid chromatography × reversed-phase liquid chromatography for medium-polar fraction in a traditional Chinese medicine

Zheng Liang, Kuiyong Li, Xinliang Wang, Yanxiong Ke, Yu Jin*, Xinmiao Liang*

Engineering Research Center of Pharmaceutical Process Chemistry, Ministry of Education, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

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ABSTRACT

Two-dimensional liquid chromatography (2-D LC) has been widely used for the analysis of complex samples owing to its great improvement in separation selectivity and peak capacity. However, one 2-D LC system may not be enough to meet the separation requirements due to the complexity of certain samples and respective limitations of two separation modes. In this work, water extract of *Scutellaria barbata* D. Don, a traditional Chinese medicine, was fractionated into polar fraction and medium-polar fraction by means of solid phase extraction (SPE). The fraction preparation made it easy to select the corresponding combination of 2-D LC method from hydrophilic interaction chromatography (HILIC) and reversed-phase liquid chromatography (RP-LC). An off-line 2-D HILIC × HILIC to analyze the polar fraction and an off-line 2-D HILIC × RP-LC to analyze the medium-polar fraction were developed, respectively. In total, 749 peaks were detected: 206 peaks from the polar fraction by the 2-D HILIC × HILIC and 543 from the medium-polar fraction by the 2-D HILIC × RP-LC. The practical peak capacities obtained in both systems were 2698 and 2879, and the orthogonality reached 63.18% and 90.62%, respectively. The results demonstrated that the two systems were both highly orthogonal, and the peak capacities greatly increased.

1. Introduction

Traditional Chinese medicine (TCM) commonly contains numerous components with diverse polarity, which often represents a great challenge for chromatographic analysis. Though high performance liquid chromatography (HPLC) is one of the most effective separation methods, its limitations in performance, selectivity and peak capacity are gradually found for the analysis of complex samples.

At present, comprehensive analysis of TCM increasingly relies on two-dimensional liquid chromatography (2-D LC) technique, an effective method to increase peak capacity and resolution. For a 2-D LC separation, the maximum theoretical peak capacity is the product of the peak capacities obtained on each of the two dimensions [1]. Besides, orthogonal separation based on different separation mechanisms could improve separation selectivity and facilitate the analysis of much more components in TCM. Different separation modes can be combined to construct a 2-D LC method according to the sample properties. Two-dimensional reversed-phase liquid chromatography/reversed-phase liquid chromatography (2-D RP × RP-LC) [2–7] has been the wide mode for TCM. For better orthogonality with traditional C18, some new stationary phases have been prepared and applied in 2-D RP × RP-LC systems. A novel click oligo (ethylene glycol) (Click OEG) stationary phase was combined with C18 to develop a 2-D RP × RP-LC system for analyzing *Lignum Dalbergiae Odoriferae* [2]. A highly orthogonal off-line 2-D RP × RP-LC system developed from Click dipeptide stationary phase and C18, was used to analyze *Rheum Palmatum* L. [4]. Although 2-D RP × RP-LC is very effective in the characterization of medium and weak polar components in TCM, it is invalid for polar components since these are poorly eluted under RP-LC mode. Thus the information of these polar components is frequently ignored when a 2-D RP × RP-LC method is employed. Recently, hydrophilic interaction liquid chromatography (HILIC) has been steadily gaining interest and widely used for the analysis of polar compounds [8–10]. 2-D HILIC × HILIC systems were also conducted to improve the separation for polar components in TCM. Xu et al. developed a comprehensive HILIC × HILIC–MS system to separate and identify saponins from *Quillaja saponaria* [11]. Subsequently, Liu et al. developed two different off-line 2-D HILIC × HILIC systems for the analysis of polar components in *Carthamus tinctorius* Linn. [12]. HILIC offers selectivity complementary to traditional RP-LC since

* Corresponding authors. Tel.: +86 021 64250633; fax: +86 021 64250622.
E-mail addresses: Jiny@ecust.edu.cn (Y. Jin), liangxm@ecust.edu.cn (X. Liang).

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their good orthogonality [13–16]. Liu et al. developed an off-line RP-LC × HILIC system based on C18 and Click β-cyclodextrin (β-CD) stationary phases, showing excellent abilities for the separation of medium-polar components in *C. tinctorius* Linn. [13]. Even though the 2-D RP-LC × HILIC system could separate medium-polar and weak-polar components in the first dimension (RP-LC), medium-polar and polar components in the second dimension (HILIC) and could solve the problem of peak capacity and separation selectivity to some extent, there were still certain deficiencies by any single 2-D LC for comprehensive characterization of TCM samples due to limitations of separation modes and complexity of samples. The aim of this study was to divide a TCM extract into several fractions according to chromatographic polarity, followed by the development of an appropriate 2-D LC system for each fraction. The described combination of 2-D LC systems represents a more promising approach for the analysis of complex TCM samples.

Scutellaria barbata D. Don has been used in TCM for a long time for the treatment of tumors, hepatitis, cirrhosis, and other diseases. Flavonoids are main bioactive components in *S. barbata* [17]. In this work, water extract of *S. barbata* D. Don was divided into two fractions with solid phase extraction (SPE) according to corresponding chromatographic polarity. Subsequently, an off-line 2-D HILIC × HILIC and an off-line 2-D HILIC × RP-LC were developed to analyze the two fractions. Such double off-line 2-D LC systems enabled a comprehensive study of the components in *S. barbata* D. Don.

2. Experimental

2.1. Apparatus and chemicals

The HPLC system (Agilent 1200, Agilent Technologies, Waldbronn, Germany) consists of a G1312B binary pump, a G1367C autosampler, a G1379B degasser, a G1316B automatic thermostatic column compartment and a G1315C diode array detection (DAD) system.

The Mass Spectrometry (MS) determination was performed on a Waters ACQUITY UPLC™ system equipped with a binary solvent manager and autosampler. The UPLC was interfaced through an electrospray ionization (ESI) ion source to quadrupole time-of-flight (Q-TOF) Premier™ mass spectrometer operating in positive mode (Waters MS Technologies, Manchester, UK).

Acetonitrile (HPLC grade) from TEDIA (OH, USA) was used for HPLC analysis. Formic acid (HPLC grade) was purchased from ACROS Organics (NJ, USA). Ammonium formate was purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol used for sample preparation was obtained from YuWang Chemical Reagent Factory (Shandong, China). Milli-Q ultrapure water (Billerica, MA, USA) was used throughout for solution preparation unless stated. *S. barbata* D. Don was collected in Henan province.

2.2. Sample preparation

15 g sample was ground into powder and decocted in 100 mL of water for 120 min. The decoction was filtrated out and the residue was decocted in another 100 mL of water for 90 min. The decoctions were combined and concentrated, and then methanol was added until the concentration of methanol reached 75%. After standing for 24 h in refrigerator at 4 °C, the supernatant was filtered. The filtrate was evaporated and the residue was dissolved in 10 mL of water. After centrifugation, the supernatant was filtered through a 0.45 μm hydrophilic membrane prior to the injection for HPLC analysis.

Oasis® HLB cartridge (Waters, Milford, MA, USA) was first activated with methanol and equilibrated with water. Sample solution (0.5 mL) was loaded onto cartridge and eluted with 10% methanol (5 mL) for polar fraction and 80% methanol (10 mL) for medium-polar fraction in sequence. This step was repeated four times and the respective fractions were combined. Then the combined fractions were evaporated to dryness and dissolved in 1 mL of water and 1 mL of CH_3OH/H_2O (50:50, v/v), and denoted as samples I and II, respectively. The samples were stored at 4 °C before use.

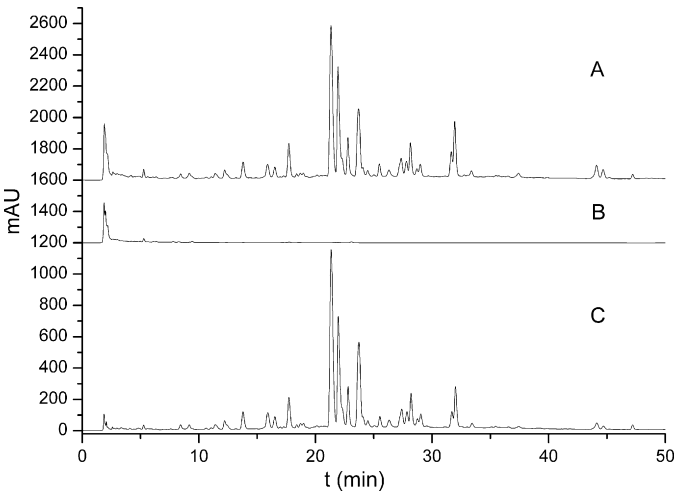

Fig. 1. The HPLC chromatograms of samples under RP-LC mode: (A) water extraction; (B) sample I (polar fraction); (C) sample II (medium-polar fraction). Column: XUnion C18 column (5 μm, 150 mm × 2.1 mm i.d.); Mobile phase: (A) water with 0.1% formic acid (B) acetonitrile with 0.1% formic acid; gradient: 0–40 min, 10–30% B, 40–50 min, 30–65% B; flow rate: 0.2 mL/min; temperature: 30 °C; UV detection: 280 nm.

2.3. Chromatographic and MS conditions

In the 2-D HILIC × HILIC analysis, an Atlantis HILIC Silica column (250 mm × 4.6 mm i.d., 5 μm particle size, Waters) was used as the first dimensional column. The corresponding mobile phase A was water with 5 mM ammonium formate (pH = 2.69), the mobile phase B was ACN/water (95:5, v/v) with 5 mM ammonium formate. The linear gradient was from 5% A to 40% A in 30 min at a flow rate of 1.0 mL/min. Fractions were collected manually from 3 to 20 min at 1 min intervals and they were denoted as fractions 1–18 in order. The fractions were evaporated to dryness under a nitrogen stream and the residue was dissolved in 200 μL of ACN/water (50:50, v/v). The second dimensional analysis was performed on a XAmide column (150 mm × 4.6 mm i.d., 5 μm particle size, Sipore Co. Ltd.). The mobile phases and linear gradient used in the two dimensions were same. The injection volume was 20 μL for the first dimension and 10 μL for the second dimension, respectively.

In the 2-D HILIC × RP-LC analysis, a XAmide column was taken as the first dimensional column. The corresponding mobile phase A was water with 0.1% (v/v) formic acid, and the mobile phase B was ACN with 0.1% (v/v) formic acid. The linear gradient was from 5% A to 40% A in 30 min at a flow rate of 1.0 mL/min. Fractions were collected manually from 2 to 21 min at 1 min intervals and they were denoted as fractions 1–20 in order. The fractions were evaporated to dryness under a nitrogen stream and the residue was dissolved in 200 μL of CH_3OH/water (50:50, v/v). The second dimensional analysis was performed on a XUnion C18 column (150 mm × 2.1 mm i.d., 5 μm particle size, Sipore Co. Ltd.). The mobile phases used in the two dimensions were same. The linear gradient was from 10% B to 30% B in 40 min, and then reached 65% B at 50 min. The flow rate was 0.2 mL/min. The injection volume was 10 μL for the first dimension and 5 μL for the second dimension, respectively.