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Separation and purification of bovine serum albumin binders from *Fructus polygoni orientalis* using off-line two-dimensional complexation high-speed counter-current chromatography target-guided by ligand fishing

Qi Liu [a], Shuyun Shi [a], Liangliang Liu [a], Hua Yang [a], Wen Su [a], Xiaoqing Chen [a,b,*]

[a] School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China
[b] Key Laboratory of Resources Chemistry of Nonferrous Metals, Ministry of Education, Central South University, Changsha 410083, China

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ABSTRACT

In this study, off-line two-dimensional (2D) complexation high speed counter-current chromatography (HSCCC) was developed for the separation of bovine serum albumin (BSA) binders from the ethyl acetate extract of *Fructus polygoni orientalis*. Target-guided strategy of BSA functionalized iron oxide magnetic nanoparticles coupled with high performance liquid chromatography–tandem mass spectrometry ((BSA-Fe_3O_4 MNPs)-HPLC–MS/MS) experiment was proposed. In the orthogonal separation system, a Normal-Phase HSCCC with 0.01 mol/L copper ion as complexation agent in the aqueous phase was employed for the first dimension and Recycling HSCCC, Reverse-Phase HSCCC with 0.1 mol/L copper ion were used for the second dimension in parallel. Including two pairs of *cis-trans isomers*, seven BSA binders including 3,5,7-Trihydroxychromone (**1**), taxifolin (**2**), *N-cis*-paprazine (**3**), *N-cis*-feruloyltyramine (**4**), *N-trans*-paprazine (**5**), *N-trans*-feruloyltyramine (**6**) and an unidentified compound (**7**) were obtained. The purities of these seven compounds were all over 95.0% as determined by HPLC. The complexation HSCCC behaviors of seven compounds were also investigated by studying their relationship with copper ion. Results showed that the combinative method using (BSA-Fe_3O_4 MNPs)-HPLC and HSCCC is a quick, efficient, and reproductive technique to isolate potentially bioactive compounds from the complex mixture system of natural products. And the usage of off-line 2D-HSCCC and introduction of chelating metal ion into solvent system are effective ways to implement HSCCC separations in complex samples.

1. Introduction

Being one of the most important resources for screening lead compounds, Traditional Chinese Medicines (TCMs) has been developed for various medicinal uses [1]. However, due to the complexity of natural products, the conventional bioassay-guided fractionation of Chinese herbal medicines is a time-consuming, labor-intensive, and inefficient strategy [2], which has seriously hindered the research of new drugs [3]. Therefore, an efficient approach for identifying biologically active compound is required in TCMs. Due to its higher surface area, lower mass transfer resistance and easy separation by a magnetic field [4–7], magnetic nanoparticles have shown great potential applications in many biological and medicinal fields for targets separation recently. In recent years, by immobilizing of the protein on magnetic nanoparticles using adsorption or covalent immobilization, protein-coated magnetic materials used for ligand and protein fishing as well as for the identification of unknown ligands from cellular or botanical extracts is well studied [8]. In Ruin Moaddel's work [9], automated ligand fishing strategy using human serum albumin (HSA)-coated magnetic beads was proposed, the results indicate that "ligand-fishing" technique can be developed using magnetic beads containing an immobilized protein. In our previous work [10], thirteen bioactive compounds were identified from *Puerariae lobata* flower extract by BSA functionalized Fe_3O_4 magnetic nanoparticles (BSA-Fe_3O_4 MNPs), which shows this method has good applications in screening and identifying active compounds in natural products, and also is a potential reference for separation [11–13]. The TCM *Fructus polygoni orientalis*, known as Shui-hong-hua-zi in China, is the fruit of *Polygonum orientale* L. grows all around China. According to zhong-hua-ben-cao (Chinese Materia Medica) [14], it has been used as the potent treatment toward various diseases including bacterial, tumor, hypertension, cardiomyopathy. Our previous

* Corresponding author at: School of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China. Tel.: +86 731 88830833.
E-mail address: xqchen@csu.edu.cn (X. Chen).

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research [15] has identified taxifolin in leaves, woods and seeds of *Polygonum orientale* L. and observed that taxifolin possesses a very potent antioxidant activity, which prompted us to perform a detailed target-guided chemical investigation on ethyl acetate extract of *Fructus polygoni orientalis*. As a result, screening strategy based on BSA-Fe_3O_4 MNPs was proposed.

High Speed Counter-Current Chromatography (HSCCC) has been applied in the separation of natural products [16–21] since 1970s. As a newly developed HSCCC separation technology, complexation HSCCC has provided good separation results for structurally similar compounds [22,23]. Complexation HSCCC is a highly selective separation technology, which is based on different chelating abilities between receptor and ligand. So coefficient parameters of target compounds can be altered by adding complexation agent to the aqueous phase of two solvent systems. What's more, hydroxylflavonoids possessing the 3-hydroxy-4-keto, the 5-hydroxy-4-keto or ortho-dihydroxyl groups have the ability to chelate with a variety of metal ions such as Cu(II), Fe(III) and Al(III) [24]. Satisfactory peak resolution of *cis-trans* isomers [25] and 5-hydroxyisoflavone [23] isomers had been obtained by using complexation agent such as, silver ion and copper ion in HSCCC. Because two pairs of enantiomers and other structurally similar compounds with hydroxyl and carbonyl groups were identified in the ethyl acetate extract of *Fructus polygoni orientalis* by HPLC–MS/MS, complexation HSCCC was considered in our separation. And copper ion as a complexation agent would be an excellent alternative in our HSCCC separation process. As is well known, it is comparatively difficult to isolate all constituents in single HSCCC operation mode because there are many constituents with similar structures and largely differed polarities present in natural herbs [26]. Recent advances in the application of HSCCC such as recycling and reverse operation mode has afforded researchers new strategies to isolate and identify compounds from complex samples. Recycling elution mode of HSCCC, ordinarily used in the separation of isomers and enantiomers [27,28], has good application prospect in separation. Nevertheless, due to the limited peak capacity of 1D-HSCCC, structurally similar compounds are usually eluted simultaneously in one peak during HSCCC separation [12], which prevented their differentiation by retention time (Rt). Since Rt in HSCCC was directly decided by the *K*-value in the solvent system [29], Rt changes of target compounds in HSCCC could be achieved by modifying the solvent system. Those co-eluted compounds could be separated in the second dimensional HSCCC, in which a different solvent system was used. Accordingly, 2D-HSCCC associated with enhanced peak capacity and selectivity seemed to be a potentially suitable approach for the separation of complex samples. Recently, on-line 2D-HSCCC has been developed by Pan and coworkers [30,31], and successfully applied to purification oridonin, ponicidin and prenylflavonoids from *Rabdosia rubescens* and *Artocarpus altilis*, however, this method could not be adopted extensively because it needs two HSCCC and other special equipments for analysis. Thus, a relatively simple off-line 2D-HSCCC strategy employing different kinds of HSCCC operation modes was developed for the separation in this study, and the chemical structures of these identified compounds were shown in Fig. 1. A roadmap of the whole work is given in Fig. 2.

2. Experimental

2.1. Apparatus

The preparative HSCCC was performed using a model TBE-300B HSCCC (Shanghai Tauto Biotechnique Co. Ltd., Shanghai, China). The apparatus consisted of an upright coil type-J planet centrifuge with three multilayered coils connected in series (diameter of tube, 1.6 mm, total capacity 260 mL) and a 20 mL manual sample loop. The rotation speed was adjustable, ranging from 0 to 1000 rpm. An optimum speed of 850 rpm was used in the experiment. The HSCCC system was equipped with a TBP-1002 pump, a TBD-2000 UV detector, a HX-1050 constant temperature regulator (Beijing Boyikang Lab Implement Co. Ltd., Beijing, China) and a WH V4.0 workstation (Shanghai Wuhao Information Technology Co. Ltd., Shanghai, China). In the separation process, the temperature of separation columns was maintained at 25 °C, and the effluent was collected and determined at 254 nm.

HPLC apparatus (Dionex Ultimate 3000, USA) and a reversed phase SunFire™ C18 (250 mm × 4.6 mm i.d., 5 μm, Waters, Milford, MA, USA) column were employed for analysis. Dionex Ultimate 3000 system is comprised of a DGP-3600RS binary pump, WPS-3000RS auto sampler, TCC-3000 RS thermo stated column compartment and a DAD-3000RS multiple wavelength detector (Dionex, Sunnyvale, CA, USA). MS/MS data were acquired in the negative ion mode from a Micromass® Quattro micro™ API mass spectrometer (Waters Corp., Milford, MA, USA) with an ESI interface. The morphology of amino-functionalized Fe_3O_4 MNPs was investigated using transmission electron microscopy (TEM) (JEM-2100F, JEOL, Japan). The powder X-ray diffraction allowed the identification of the crystalline structure of Fe_3O_4 MNPs, using powder X-ray Diffractometer (Rigaku RINT 2500, Rigaku Corporation, Japan) with Cu/Ka radiation at 30 mA, 40 kV. Magnetization was measured at room temperature in a vibration sample magnetometer (VSM7307, Lake Shore, USA). NMR experiments were performed on a Bruker-400 (Bruker Corporation, Germany) NMR spectrometer. Deuterated DMSO was used as the solvent, and the reference compound tetramethylsilane (TMS) was used as internal standard for the determination of chemical shifts.

2.2. Reagents

The dried *Fructus polygoni orientalis* were purchased from Bozhou TCM exchanger center (Anhui, China), and identified by Prof. Mijun Peng from Key Laboratory of Hunan Forest Products and Chemical Industry Engineering, Jishou University. BSA and glutaraldehyde (25% (w/v) aqueous solution) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Petroleum ether (60–90 °C), *n*-hexane, ethyl acetate, *n*-butanol, 95% ethanol and methanol used for active fractions preparation and HSCCC separation were of analytical grade and obtained from Chemical Reagent Factory of Hunan Normal University (Hunan, China). Acetonitrile used for HPLC were of chromatographic grade (Merk, Darmatadt, Germany). Copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) with a purity of 99% was purchased from Tianjin hengxing chemical reagent manufacturing Co. Ltd. (Tianjin, China). Ultrapure water (18.2 MΩ resistivity) used in the present work was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Preparation of crude sample

The dried and chopped *Fructus polygoni orientalis* (80 g) were extracted twice (2 h each) with 480 mL of 95% ethanol each. Then the extracts were combined, filtered and evaporated to dryness (3.25 g) by rotary evaporation at 55 °C under reduced pressure. The concentrated residue was diluted with 0.5 L of H_2O and extracted successively with petroleum ether (0.72 g), ethyl acetate (0.48 g), and *n*-butanol (0.35 g). As there were little peaks in petroleum ether extract and *n*-butanol part, the ethyl acetate extract of *Fructus polygoni orientalis* was considered for ongoing research. And the concentrated ethyl acetate extract was stored in a refrigerator (4 °C) and subjected for subsequent HPLC analysis and HSCCC separation.