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Preparative separation of isomeric caffeoylquinic acids from *Flos Lonicerae* by pH-zone-refining counter-current chromatography

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ABSTRACT

This work concentrates on pH-zone-refining counter-current chromatography of two isomeric dicaffeoylquinic acids, 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid along with 3-caffeoylquinic acid, from crude extracts of *Flos Lonicerae*. The elution sequence of the isomeric dicaffeoylquinic acids, the mixing zone and mechanism of separation are discussed. The separation of 2.136 g of the crude sample from *Flos Lonicerae* yielded two isomeric compounds: 0.289 g 3,5-dicaffeoylquinic acid and 0.106 g 3,4-dicaffeoylquinic acid plus 0.690 g 3-caffeoylquinic acid at a high purity of over 92.9%, 94.2% and 97.5%, respectively.

1. Introduction

Flos Lonicerae, the dried buds of four major species of the genus *Lonicera* (Caprifoliaceae), is a traditional Chinese medicinal herb and officially listed in the Chinese Pharmacopoeia [1]. It has been used for the treatment of infection by exopathogenic wind-heat or epidemic febrile diseases at the early stage, sores, carbuncles, furuncles and swelling in traditional Chinese medicine for centuries [2–4]. The major active components of the herb are caffeoylquinic acids (CQAs), including 3-caffeoylquinic acid [5–9], 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid (see Fig. 1) [10–14], which are often used as standards in the quality control of *Flos Lonicerae*. Much attention of quality control and evaluation of *Flos Lonicerae* were paid to 3-caffeoylquinic acid during the previous studies due to its antipyretic and antibiotic property as well as its high content in the herb (not less than 1.5%). However, studies show that dicaffeoylquinic acids including 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid present various kinds of bioactivities and these dicaffeoylquinic acids were also suggested to be the quality control marker of *Flos Lonicerae* recently [10]. Additionally, the reference substances of 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid were unavailable in the chemical market. Method for preparative separation of 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid is warranted for their special role in this plant.

It is difficult to purify each CQAs from plant due to their similar chemical structures. 3,5-Dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid belong to isomeric compounds. Isolation of 3-caffeoylquinic acid (also named chlorogenic acid) by the standard high-speed counter-current chromatography (HSCCC) technique had been reported in the literature [8,9]. However, only 16.9–20.5 mg of 3-caffeoylquinic acid with over 95% purity was obtained during one separation run by standard HSCCC. Analytical separation method of chlorogenic acid by HSCCC was also reported in the literature [15,16]. Isolation of 3,5-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid from other plants by standard HSCCC were also reported in the literature [17,18], but only 17.0–34.6 mg of the dicaffeoylquinic acids were obtained in one HSCCC separation run. Furthermore, it needs to be combined with D101 macroporous resin or AB-8 resin column chromatography, which led to additional loss of the active components due to their adsorption to the solid support. The need for a large quantity of the material required for clinical trial prompts the

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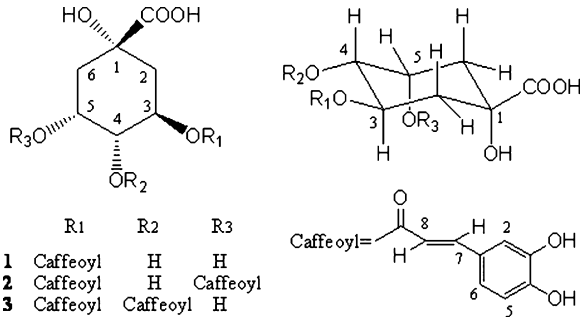

Fig. 1. Chemical structures of caffeoylquinic acids (**1**): 3-caffeoylquinic acid; (**2**): 3,5-dicaffeoylquinic acid; (**3**): 3,4-dicaffeoylquinic acid).

development of a new pH-zone-refining CCC method which provides over a 10-fold increase in sample loading capacity compared with the standard HSCCC technique.

pH-zone-refining CCC, introduced by Ito [19–21], is generally employed as a large-scale preparative technique for separating ionizable analytes. The method elutes highly concentrated rectangular peaks fused together with minimum overlapping while impurities are concentrated and eluted between the outsides of the major peaks according to their pK_a and hydrophobicity [22,23]. Many applications of this technique for purification of ionizable components from natural or synthetic mixtures had been reported [23–29]. This technique has been widely used in the preparation of reference materials needed by some administrations to facilitate quality control by high-performance liquid and thin-layer chromatographic methods of analysis. However, the publications on separation of isomeric and stereoisomeric ionizable components by pH-zone-refining CCC were much fewer than those about separation of non-isomeric compounds by the technique. And all the reports of separation of isomeric and stereoisomeric ionizable components by pH-zone-refining CCC are about the samples from synthetic mixtures [30–37,27], peptide derivatives and amino acids [38–40] and color additives [41–43]. Chiral selectors were added during the separation of stereoisomeric compounds in the literature [36,27,37]. Till now, large-scale separation of isomeric effective constituents from crude extracts of plant by pH-zone-refining CCC has not been reported yet. On the other hand, though lots of reports about application of this technique in separating useful constituents from mixtures, few reports were available about discussing of elution sequence of the analytes and mechanism of separation [30,31,39,42,44,45]. The present paper reports the preparative separation of two isomeric dicaffeoylquinic acids along with 3-caffeoylquinic acid from the crude extract of *Flos Lonicerae* by pH-zone-refining CCC. Meanwhile, the elution sequence of the two isomeric components and possible mechanism of separation were suggested.

2. Experimental

2.1. Apparatus

The counter-current chromatography apparatus used in the present study is a TBE-300A multilayer coil planet centrifuge for performing standard HSCCC (made in 2006 by Shanghai Tauto Biotechnique, Shanghai, China), equipped with three preparative multilayer coils (column total volume 250 ml, wound with 1.6 mm I.D. PTFE tubing). The β values of this column range from 0.46 to 0.73 ($\beta = r/R$, $R = 6.5$ cm, where r is the distance from the coil to the holder shaft, and R, the revolution radius or the distance between the holder shaft and central axis of the centrifuge). The revolution speed of the apparatus can be regulated with a speed controller in the range between 0 rpm and 1000 rpm where an optimum speed of 800 rpm was used in the present studies. The separation columns were installed in a vessel that was retained at 25 °C by a Model HX-1050 constant-temperature controller (Beijing Boyikang Lab Instrument Co., Beijing, China). The solvent was pumped into the column with a Model NS-1007 constant-flow pump (Beijing Shengyitong Technique Co., Beijing, China). Continuous monitoring of the effluent was achieved with a Model UV-II detector (Shanghai Institute of Biochemistry, Chinese Academy of Sciences, Shanghai, China) at 254 nm. SEPU3000 workstation (Hangzhou PuHui Technology, Hangzhou, China) was employed to record the chromatogram. Eluate was collected with a Model BSZ-160 fractions collector (Shanghai Huxi Tech, Shanghai, China).

The HPLC system used was a CLASS-VP Ver.6.1 system (Shimadzu, Japan) comprised of a Shimadzu SPD10Avp UV detector, a Shimadzu LC-10ATvp Multisolvent Delivery System, a Shimadzu SCL-10Avp controller, a Shimadzu LC pump, and a CLASS-VP Ver.6.1 workstation.

2.2. Reagents and materials

All organic solvents used for pH-zone-refining CCC were of analytical grade. Trifluoroacetic acid (TFA) and ammonia were of reagent grade. Acetonitrile (ACN) used for HPLC analysis was of chromatographic grade. Methyl *tert*-butyl ether (MtBE) was redistilled before use. The reagents were purchased from Hangzhou HuiPu Chemical Factory, Hangzhou, China.

2.3. Extraction of crude sample from Flos Lonicerae

Three hundred grams of the dried matrix of *Flos Lonicerae* was extracted with about 3.0 l of 70% ethanol at pH 4 for three times (1-week period each time) under room temperature. The ethanol extracts were combined and evaporated under reduced pressure to about 50 ml and 100 ml of water was added to the concentrated solution. Then the combined extract was extracted again with 1.0 l ethyl acetate for five times. The ethyl acetate extracts were combined and evaporated under reduced pressure to give 22.0 g residue. The residue was dissolved in 200 ml water and the insoluble sludge was filtered. The 200 ml filtrate was lyophilized, yielding a 7.25 g of crude sample.

2.4. Preparation of two-phase solvent system and sample solution

For the present study, we selected a two-phase solvent system composed of MtBE–ACN–water (2:2:3, v/v/v). The solvent mixture was thoroughly equilibrated in a separation funnel and the two phases separated shortly before use. Then, TFA (retainer) was added to the upper organic stationary phase to obtain a final concentration of 10 mM with pH 2.6 and NH_4OH (eluter) was added to the lower aqueous mobile phase to obtain a final concentration of 8 mM NH_4OH with pH 10.1.

The crude sample solution was prepared by dissolving 2.136 g sample in 20 ml of the stationary phase (10 mM TFA) and 10 ml water.

2.5. Separation procedure

The column was first entirely filled with the organic stationary phase containing TFA at 10 mM. This was followed by sample injection. Then the aqueous phase containing NH_4OH (eluter base) at 8 mM was pumped into the inlet of the column at a flow-rate of 2.0 ml/min in the head-to-tail elution mode, while the column was rotating at 800 rpm. The effluent from the outlet of the column was continuously monitored at 254 nm and collected into test tubes