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Comparison of ultra-high performance supercritical fluid chromatography and ultra-high performance liquid chromatography for the separation of spirostanol saponins

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ABSTRACT

Spirostanol saponins are important active components of some herb medicines, and their isolation and purification are crucial for the research and development of traditional Chinese medicines. We aimed to compare the separation of spirostanol saponins by ultra-high performance supercritical fluid chromatography (UHPSFC) and ultra-high performance liquid chromatography (UHPLC). Four groups of spirostanol saponins were separated respectively by UHPSFC and UHPLC. After optimization, UHPSFC was performed with a HSS C18 SB column or a Diol column and with methanol as the co-solvent. A BEH C18 column and mobile phase containing water (with 0.1% formic acid) and acetonitrile were used in UHPLC. We found that UHPSFC could be performed automatically and quickly. It is effective in separating the spirostanol saponins which share the same aglycone and vary in sugar chains, and is very sensitive to the number and the position of hydroxyl groups in aglycones. However, the resolution of spirostanol saponins with different aglycones and the same sugar moiety by UHPSFC was not ideal and could be resolved by UHPLC instead. UHPLC is good at differentiating the variation in aglycones, and is influenced by double bonds in aglycones. Therefore, UHPLC and UHPSFC are complementary in separating spirostanol saponins. Considering the naturally produced spirostanol saponins in herb medicines are different both in aglycones and in sugar chains, a better separation can be achieved by combination of UHPLC and UHPSFC. UHPSFC is a powerful technique for improving the resolution when UHPLC cannot resolve a mixture of spirostanol saponins and vice versa.

1. Introduction

Steroidal saponin is a kind of natural products found in the plants of family *Dioscoreaceae*, *Agavaceae*, *Alliaceae*, *Liliaceae* and so on [1]. Steroidal saponins chemically consist of a steroidal aglycone and the linked oligosaccharide moieties. Among them, furostanol saponin contains two sugar chains at positions C-3 and C-26 generally, and spirostanol saponin has one sugar chain at position C-3 and a closed F ring. Spirostanol saponins are known to be formed from furostanol saponins by hydrolysis of glucosyl at C-26 and a subsequent dehydration condensation reaction between the newly formed hydroxyl group at position C-26 and the hydroxyl group at position C-22 [2]. Spirostanol saponins have a lot of pharmacological activities including anti-platelet aggregation and cytotoxicity [3–5]. These activities are strictly dependent on the molecular composition and configuration of saponins [6–8]. Therefore it is essential to separate these various spirostanol saponins and their stereoisomers.

Reversed phase high performance liquid chromatography has been utilized for separations of spirostanol saponins [9–11]. However, it is difficult to separate some of spirostanol saponins, such as 25*R*/*S*-isomers and epimers of sugar parts [12–14]. Ultra-high performance liquid chromatography (UHPLC) using columns packed with porous sub-2 μm particles possesses the advantages

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of high throughput and improved resolution [15]. UHPLC has been widely employed in resolving steroidal saponins and can separate furostanol saponins well [16,17]. However, the separation of spirostanol saponins by UHPLC has not been full characterized.

Thin layer chromatography and silica-gel column chromatography have been used in the separation of spirostanol saponins. However, these normal phase chromatography methods are not performed automatically. In addition, they are environmentally harmful because of the poisonous organic reagents (such as chloroform) used during separation [18–20]. On the contrary, supercritical fluid chromatography (SFC) with CO_2 as the major mobile-phase component is often considered as a "green" technology [21,22]. SFC uses the same columns as standard HPLC systems and is suitable for the analysis of non-polar compounds, and can also be used to separate polar compounds when modifying CO_2 with polar solvents [23,24]. It features high speed, high throughput and improved chromatographic performance [25], and has been successfully used in separating chiral chemicals or enantiomers, such as 25R/S-ergostane triterpenoids [26], spirocyclic terpenoid flavor compounds [27], diketopiperazines [28], 25 *R/S*-spirostanol saponin diastereomers [29].

Ultra-high performance supercritical fluid chromatography (UHPSFC) technology has been developed for several years [30]. However, whether UHPSFC can be well applied in separating spirostanol saponins with structural diversity is still unknown except the report on 25 *R/S*-spirostanol saponin diastereomers [29]. Therefore, in this study, we compared the separation of spirostanol saponins by UHPSFC and by UHPLC. The UHPSFC conditions, including co-solvents, additives and columns were optimized. The advantages and limitations of both UHPSFC and UHPLC are discussed.

2. Materials and methods

2.1. Reagents

HPLC grade methanol (MeOH), ethanol (EtOH) and acetonitrile (ACN) were from Fisher Scientific (USA). Reagent grade formic acid (FA) and ammonium formate (NH_4FA) were from Sigma–Aldrich Fluka (Germany). Analytical reagent ammonium hydroxide (NH_4OH) was from Sinopharm Chemical Reagent Co., Ltd. (China). High-purity CO_2 (≥99.9%) was purchased from Zhenxin Gaisi (China). Ultrapure water was obtained from a Milli-Q RG Purification unit from Millipore (USA).

2.2. Spirostanol saponins

A total of 20 spirostanol saponins prepared and identified in our lab were used in this study and listed in (Fig. 1). They were saponin Pa (1) [31,32], dioscin (2) [33,34], gracillin (3) [33,34], deltonin (4) [35], saponin Pb (5) [36], mixture of deglucolanatigonin (6a) and desgalactotigonin (6b) [13], gitogenin 3-*O* -α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**7**) [37], 5α-spirostane-25(27)-en-2α,3β-diol 3-*O* -α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (8) [38], 25R-spirostane-5-en-2α,3β-diol 3-*O* -α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (9), ophiogenin 3-*O*-α-L-rhamnopyranosyl-(1→2)-β -D-glucopyranoside (10) [39], saponin Tb (11) [40], 25R-dracaenoside F (12) [41], progenin III (13) [33,34], timosaponin AIII (14) [42], trillin (15) [43], timosaponin AI (16) [44,45], diosgenin (17) [46], sarsasapogenin (18) [47] and tigogenin (19) [48]. Each compound was dissolved in methanol (0.5 mg/mL), and then filtered with 0.22 μm membrane before analysis.

2.3. UHPLC separation

Liquid chromatography was performed on an ACQUITY UPLC™ system (Waters Corporation, Milford, MA, USA), which consisted of an ACQUITY UPLC BEH C18 column (1.7 μm, 100 mm × 2.1 mm), a binary solvent manager, a sample manager with a fixed loop of 10 μL, an external column oven, and an ELSD detector. The elution was performed with H_2O (with 0.1% FA) and ACN. The gradient of ACN was optimized for each separation. The flow rate was 0.5 mL/min with a column temperature of 40 °C. Data acquisition and processing were performed using the software Masslynx 4.1 (Waters Corporation, Milford, MA, USA).

2.4. UHPSFC separation

SFC system was the ACQUITY Ultra Performance Convergence Chromatography™ (UPC^2) system (Waters Corporation, Milford, MA, USA), equipped with a binary solvent delivery pump, a sample manager including partial loop volume injection system, a backpressure regulator, and column manager, as well as a evaporative light scattering detector. The solvent delivery pump was compatible with mobile phase flow rates up to 4 mL/min and pressures up to 41.34 MPa. The whole system was controlled with the Empower™ Pro3 Software (Waters Corporation, Milford, MA, USA). Chromatographic analyses were performed on ACQUITY UPC^2 Torus Diol (1.7 μm, 50 mm × 3.0 mm), ACQUITY UPC^2 Torus DEA (1.7 μm, 50 mm × 3.0 mm), ACQUITY UPC^2 BEH (1.7 μm, 50 mm × 3.0 mm) and ACQUITY UPC^2 HSS C18 SB (1.8 μm, 50 mm × 2.1 mm) columns. The mobile phase was CO_2 with co-solvents. The gradients of the co-solvent were optimized for each group of spirostanol saponins for a better resolution. The backpressure was 13.78 MPa, and the flow rate was 1.2 mL/min with a column temperature of 40 °C.

3. Results and discussion

3.1. Optimization of UHPSFC conditions

According to the structures of steroidal saponins (Fig. 1), they were grouped into 4 groups: Compounds 1–5 (group 1) had the same aglycone and different sugar chains; Compounds 6a and 6b (group 2) had the same skeleton of tigogenin, and were only different in the configuration of one hydroxyl group in the terminal glycosyl; Compounds **7–**13 (group 3) had the same C-3 sugar chain and varied in hydroxyl group at position C-2, C-14 and C-17 or hydrogen bond (double bounds) at position C-5 and C-6; Compounds 13–19 (group 4) had various aglycones. The saponins in each group were mixed respectively and then separated by UHPLC or by UHPSFC to evaluate the separation efficiency.

In order to find an optimal column for each group of spirostanol saponins, 4 achiral columns, HSS C18 SB, BEH, DEA and Diol were evaluated. In all cases methanol was used as co-solvent. Compounds 1**–**5 could be separated into 2, 3, 3 and 5 peaks with HSS C18 SB, BEH, DEA and Diol respectively (Fig. 2A). The Diol column provided a better separation. Similarly, compound **7–**13 could also be separated with column Diol though the resolution was not ideal (Fig. 3A). Therefore, the Diol column was applied in the separation of compounds 1**–**13 by UHPSFC in the following tests. On the contrary, column HSS C18 SB was suitable for separating compounds 13**–**19 (Fig. 2B). With column HSS C18 SB, all 7 compounds could be clearly separated. Among compounds 13**–**19, three compounds (17–19) are non-polar sapogenins and the others have different sugar chains. The HSS C18 SB column contains octadecyl carbon chains bonded silica and residual silanol groups on non-end capped stationary phase [49].The octadecyl carbon chains and