http://dx.doi.org/10.5012/bkcs.2013.34.3.975

UHPLC Separation of Structurally Diverse Markers in *Fangchi* Species

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Received November 19, 2012, Accepted December 19, 2012

Key Words : Fangchi, Alkaloids, Lignan, Elution patterns, UHPLC-UV

Fangchi, one of the most commonly used traditional herbal medicines, is derived from the rhizoma of *S. acutum* and the radix of *S. tetrandra*. *S. acutum*, and *S. tetrandra* have been widely used for the treatment of rheumatic arthritis.[1,2] The main bioactive components in *S. acutum* are alkaloids and lignan such as sinomenine, isosinomenine, magnoflorine, and syringaresinol;[3] whereas in *S. tetrandra*, tetrandrine and fangchinoline,[2] respectively. Additionally, *C. trilobus* and *A. fangchi* have been also called "Mu fangchi" and "Guang fangchi", respectively. *C. trilobus* has been used in folk medicine as a diuretic, analgesic and an *anti*-inflammatory.[4] The chemical compositions of Fangchi species are slightly different according to their different origins. Thus, the development of a practical method for simultaneous determination of structural-diverse markers is essential for quality control of Fangchi species with different origins.

Fangchi has been studied using several analytical methods, including HPLC-DAD,[5,6] LC-DAD-mass spectrometry (MS),[7,8] LC-DAD-MS/MS,[9] gas chromatography-MS (GC-MS),[10] capillary electrophoresis (CE).[11] Among them, HPLC methods have been popularly applied as an incisive tool for the quality control of herbal medicines. Recently, HPLC assay has been replaced with UHPLC method due to its rapid analysis and high peak capacity. The UHPLC-DAD methods have been used for chemical fingerprinting analysis of specific components in Salviae Miltiorrhizae Radix[12] and Coptidis Rhizoma.[13]

In this study, isolation of marker compounds in Fangchi was performed as described in a previous report.[14] The purity of the isolated compounds was greater than 90% as evaluated by HPLC with UV detection. Protonated, sodium-adducted or intact molecules, $[M+H]^+$, $[M+Na]^+$, or $[M]^+$, of these compounds were detected by FAB-MS in positive ion mode. To elucidate the elemental composition of alkaloids and syringaresinol isolated from *S. Acutum*, exact mass measurements were performed using HRMS at a mass resolution of 10,000. The measured masses of $[M+H]^+$ ions were within 2.6 mmu of the calculated masses, showing excellent agreement.

For simultaneous determination of various markers (alkaloids, nitrophenanthrene carboxylic acid, and lignan) in Fangchi species, the optimization of UHPLC separation conditions was a prerequisite. To optimize separation of these marker compounds, the pH variation, chemical composition, and the ammonium acetate (AmAc) concentration of the mobile phase were investigated. Retention of ionizable compounds with RP-UHPLC is strongly dependent on the pH of the eluent, in addition to the percentage and strength of the organic solvent in the mobile phase.[15,16]

The organic solvents (acetonitrile and methanol) in the mobile phase were also investigated on the effect of the separation and the UHPLC retention time of markers (Fig. 1, see Supplementary Materials for chemical structures of markers). The overall retention time of markers using acetonitrile-water was shorter than when using methanol-water. However, the peak shape for fangchinoline and tetrandrine

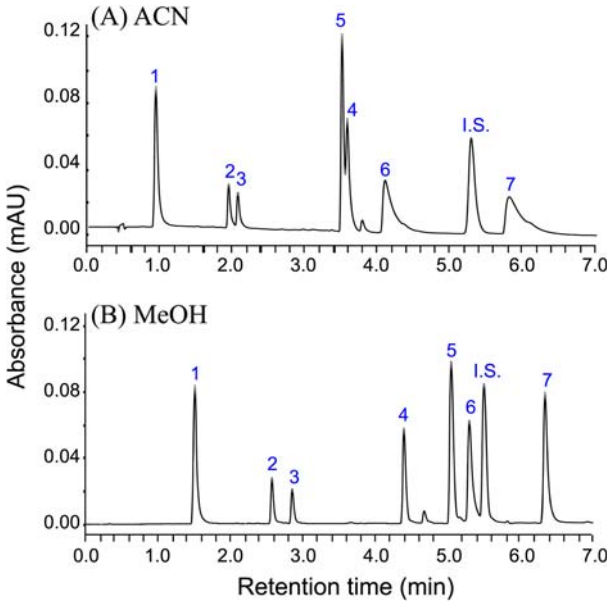

Figure 1. UHPLC chromatograms of standard mixtures using different organic solvents: (A) ACN and (B) MeOH. Peak identities: 1: magnoflorine; 2: sinomenine; 3: isosinomenine; 4: syringaresinol; 5: aristolochic acid I; 6: fangchinoline; 7: tetrandrine; and I.S.: propyl 4-hydroxybenzoate. The UHPLC conditions were: columns: WATERS Acquity UPLC® BEH C18 columns (50 × 2.1 mm, i.d., 1.7 μm) connected to Acquity UPLC® BEH C18 VanGuard™ pre-columns (5 × 2.1 mm, i.d., 1.7 μm); mobile phase: (a) 20 mM ammonium acetate pH 6.0 adjusted by acetic acid, and (b) methanol; the gradient elutions: (A) 10-33% ACN for 0-2 min; 33% ACN for 2-4 min; and 33-50% ACN for 4-6 min; and (B) 10-50% MeOH for 0-3.5 min and 50-80% MeOH for 3.5-7 min.

[a]These authors contributed equally to this work.

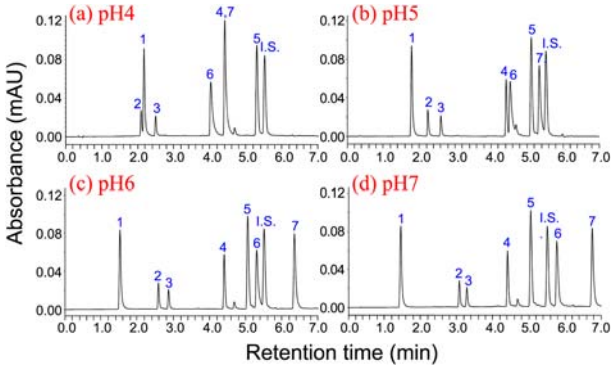

Figure 2. UHPLC chromatograms of standard mixtures according to changing pH: (a) pH 4.0; (b) pH 5.0; (c) pH 6.0; and (d) pH 7.0. Peak identities are the same as in Figure 1.

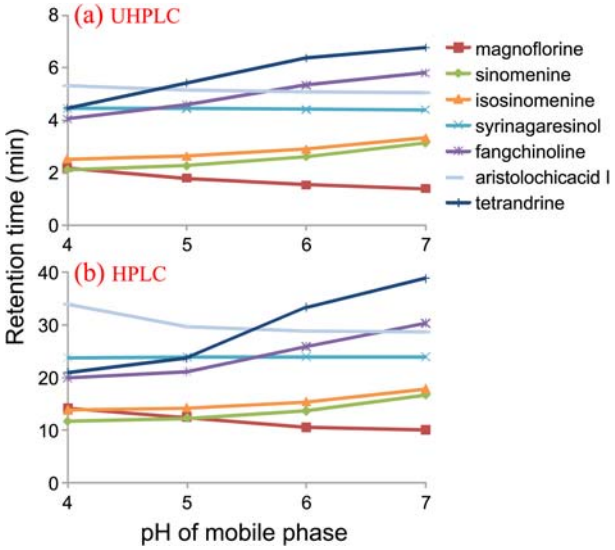

Figure 3. Profiles of retention times of bisbenzylisoquinoline, aporphine, morphine alkaloids, lignan, and aristolochic acid I on C18 column according to change of mobile phase pH values.

showed peak-tailing. Otherwise, when using methanol, the overall peak shape of markers had a symmetric distribution, even though the overall retention times of markers were longer.

The pH value of the mobile phase is the most important factor in the separation of alkaloids because alkaloids with one or two nitrogen atoms are generally basic and nucleophilic compounds. When alkaloids are ionized under acidic or basic medium conditions they become less hydrophobic. Alkaloids tend to easily gain protons and become ionized under acidic conditions. Also, due to higher stability in acidic rather than in alkaline solutions of the phenolic groups present in some alkaloids the separation was performed in acidic buffers.[17] In addition to alkaloids, the retention behaviors of syringaresinol and aristolochic acid I were also investigated on the pH of mobile phase.

To find the optimal pH conditions of the mobile phase, four pH values (pH 4.0, 5.0, 6.0 and 7.0) were examined for the retention behavior of alkaloids, aristolochic acid I and syringaresinol on C18 column. Figure 2 shows UHPLC

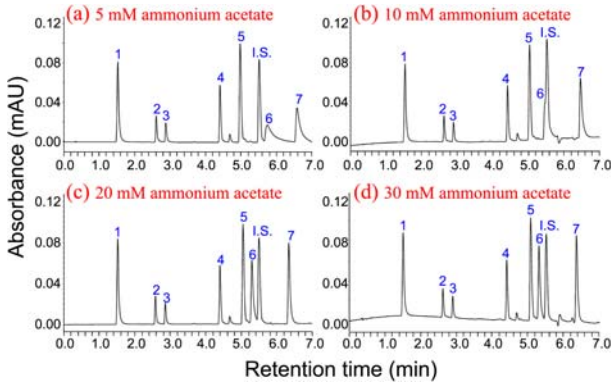

Figure 4. UHPLC chromatograms of standard mixtures according to changing buffer concentrations: (a) 5 mM; (b) 10 mM; (c) 20 mM; and (d) 30 mM ammonium acetate. Peak identities are the same as in Figure 1.

chromatograms of seven compounds under different pH conditions of the mobile phase. The overall retention times of alkaloids were dramatically changed as the pH varied in the mobile phase, which means that the pH value is a very important factor for separation of alkaloids. Magnoflorine and sinomenine could not be separated at pH 4.0, even though the strength of the organic solvent changed. Seven marker compounds and I.S. were successfully separated above pH 5.0. Among the four pH conditions, pH 6.0 was chosen as the optimal pH value for resolution and sensitivity.

The retention time profiles of aporphine, morphines, bisbenzylisoquinolines, syringaresinol, and aristolochic acid I on C18-UHPLC according to pH variations are depicted in Figure 3(a). The retention times of morphines (sinomenine and isosinomenine) and bisbenzylisoquinolines (fangchinoline and tetrandrine) alkaloids were greatly increased as pH values increased. Generally, the pK_a values for nitrogen atoms are 7.33-8.52 for morphine alkaloids (8.27 for sinomenine and 8.32 for isosinomenine) and for bisbenzylisoquinoline alkaloids (7.33 and 8.33 for fangchinoline, 7.87 and 8.52 for tetrandrine).[18] Therefore, these compounds could be converted into their protonated forms below pH 6.0. The increase in retention times with increasing pH was due to formation of the free (non-protonated) forms of the alkaloids, which resulted from increased hydrophobicities. The retention times of bisbenzylisoquinolines were more drastically increased than those of morphine alkaloids as the pH increased from 4.0 to 7.0, because of high basicity of bisbenzylisoquinolines.

On the other hand, the retention times of aristolochic acid I and magnoflorine were decreased as pH increased. The HPLC retention time of aristolochic acid I was decreased until pH 5.0, and then was constant in the range of pH 5.0-7.0 (Fig. 3(b). This can be explained because most of the aristolochic acid I may be present in the ionized form above pH 5. However, the UHPLC retention time of aristolochic acid I did not significantly decrease according to pH increases because the overall retention times of UHPLC are much shorter than the retention times of HPLC. For magnoflorine with a quaternary amine group, its partial charge on nitrogen

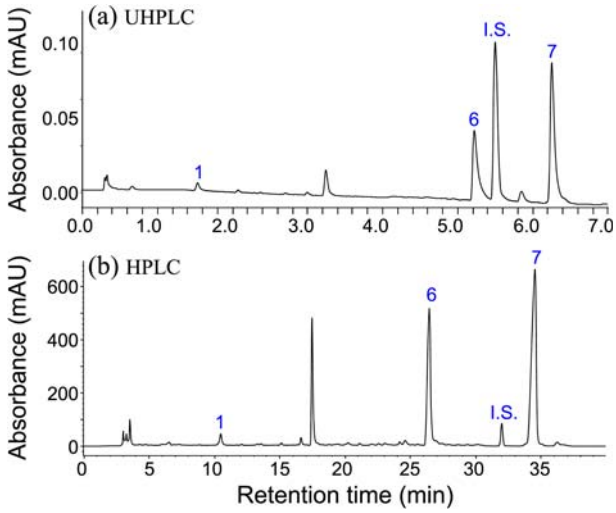

Figure 5. Comparison of UHPLC and HPLC chromatograms of maker compounds in *Stephania tetrandra*. Peak identities are the same as in Figure 1.

atom might be slightly decreased as pH decreases, resulting in increasing its hydrophobicity. In contrast, in our previous studies,[19,20] the HPLC retention behavior of quaternary protoberberine alkaloids did not greatly influence in acidic and neutral media due to intact cation on their nitrogen atoms. The retention time of syringaresinol did not change with respect to pH variations due to its less ionizability.

Additionally, to investigate the optimal concentration of AmAc in the mobile phase, different concentrations (from 5 to 30 mM) at pH 6.0 were tested, as shown in Figure 4. The concentration of AmAc did not influence the retention times of the marker compounds, except for bisbenzylisoquinolines (fangchinoline and tetrandrine). The peak shapes of bisbenzylisoquinolines were greatly improved above 15 mM AmAc, providing the improvement of detection sensitivity and the reduction of retention times for the bisbenzylisoquinolines. This may be attributed to the fact that silanol interactions (hydrogen bonding or ion-exchange) of basic compounds and residual free silanols of silica based stationary phases lead to retention increases and peak tailings. Silanol interactions can be reduced by blocking of ionized silanol groups by amine or ammonium additives in the mobile phases.[21,22]

Taking into consideration the chromatographic separation and detection sensitivities of seven markers and an appropriate buffer concentration for LC columns and pump systems, 20 mM AmAc (adjusted with acetic acid to pH 6.0) -methanol and 235 nm wavelength were chosen for UHPLC analysis. Under these optimized UHPLC conditions, seven marker compounds were successfully separated within 7 min and shown above 2.60 resolutions even for the closest peaks aristolochic acid I and fangchinoline.

Optimized separation conditions of UHPLC were compared with HPLC method for analyzing markers in Fangchi species. It is not possible to provide a strict comparison of the HPLC and UHPLC systems, because it is impossible to connect both columns to the same chromatographic system. On the other hand, the mobile phase constituents were the same and the gradient programs were constructed with respect to analysis speed and good resolution in both cases. Generally, both analytical methods showed good results, but the UHPLC system appeared to be superior. By comparing the data and chromatograms generated from UHPLC and HPLC, the advantages of UHPLC over HPLC can be summarized as follows (Fig. 3 and 5): (1) the single running time for UHPLC (7 min) was about five times shorter than that of HPLC (35 min), which may be the most important factor for high throughput analysis; (2) a slower flow rate of 0.4 mL/min reduced solvent consumption to only 2.8 mL (more ecological and lower analysis costs), while solvent usage for a single run in HPLC was up to 28 mL; (3) the injection volume of UHPLC (0.5 μL) was 20 times smaller than that of HPLC (10 μL); (4) the width of fangchinoline peak, which has the widest peak by UHPLC, was only 12 s, whereas in the HPLC method it was approximately 70 s that means UHPLC suitable for separating marker compounds from crude extract with complicated matrix; (5) UHPLC showed 1.5-4.4 times higher sensitivities, as the ranges of limits of detection (LOD) and limits of quantitation (LOQ) were 0.01-0.05 μg/mL and 0.05-0.2 μg/mL for UHPLC, and 0.04-0.10 μg/mL and 0.13-0.34 μg/mL for HPLC, respectively.

In summary, the UHPLC method had advantages over HPLC in terms of time saving, solvent saving, performance, and efficiency. Moreover, this method showed good resolution, high sensitivity, and a short analysis time that resulted in higher sample throughput, less solvent consumption, and less sample injection volume than HPLC.

In this study, a rapid and simple UHPLC-DAD method was established for the simultaneous analysis of five alkaloids, aristolochic acid I, and syringaresinol in different Fangchi species. UHPLC-DAD could be a useful and practical tool for the quality control of Fangchi species with different origins.

Experimental

Materials and Reagents. Sinomenine, isosinomenine, magnoflorine, and syringaresinol in Fangchi species were isolated by a previously reported method.[14] Tetrandrine (purity ≥ 90%) was purchased from Sigma-Aldrich (Milwaukee, WI, USA). Fangchinoline and Aristolochic acid (purity > 98%) were purchased from Chengdu Biopurify Phytochemicals Ltd (Sichuan, China). Propyl-4-hydroxybenzoate, which was used as an internal standard (I.S.), was purchased from Daejung (Korea, purity > 99%).

Preparation of Crude Drug Extracts. One gram of pulverized Fangchi was placed into 20 mL of methanol and extracted for 30 min in an ultrasonic bath at room temperature. After extraction, the extract was centrifuged twice at 3,000 rpm for 10 min The supernatant was collected and filtrated through a 0.22 μm membrane filter, and propyl-4-hydroxybenzoate (I.S.) was added to the extract solution to obtain a final solution of 5 mg/mL *S. acutum*, and 100 μg/

mL propyl-4-hydroxybenzoate before injecting into UHPLC system.

UHPLC and HPLC conditions. UHPLC analysis was performed using a WATERS Acquity UPLC system (Waters, Milford, MA) equipped with a quaternary solvent delivery manager, a column manager, a sample manager, and a diode array detector (DAD). The chromatographic separation analysis was carried out on a WATERS (Milford, MA, USA) Acquity UPLC® BEH C18 column (50 × 2.1 mm, i.d., 1.7 μm) connected to an Acquity UPLC® BEH C18 VanGuard™ pre-column (5 × 2.1 mm, i.d., 1.7 μm). The mobile phases consisted of solvent A (20 mM AmAc at pH 6.0 adjusted by acetic acid) and solvent B (methanol). The gradient elution mode was programmed as follows: 10-50% B for 0.0-3.5 min and 50-80% B for 3.5-7.0 min. The UV detection wavelength was set at 235 nm. The flow rate and injection volume were set at 0.4 mL/min and 0.5 μL, respectively.

HPLC analysis was performed on an Agilent Series 1100 HPLC system consisting of a quaternary delivery system, an auto-sampler and a DAD. The chromatographic separation analysis was carried out on a Shiseido UG 120 C18 (250 × 4.6 mm, i.d., 5 μm) column. The mobile phases and detection were the same as used in UHPLC system. The gradient elution mode was programmed as follows: 10-55% B for 0-20 min and 55-75% B for 20-40 min. The flow rate and injection volume were set at 0.8 mL/min and 10 μL, respectively.

Acknowledgments. This research was supported by a grant from the Korea Food and Drug Administration (2011) for "Studies on the standardization of herbal medicine" and from the National Research Foundation of Korea (No. 2011-0012671).

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