High-Performance Liquid Chromatographic Analysis of Saponin Compounds in *Bupleurum falcatum*

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**Abstract**

A mixture of saponin compounds (saikosaponin c, a, and d) in the 70% ethanol extract of a powdered sample of Bupleuri radix are analyzed by an Inertsil ODS-3 C18 column at a flow rate of 1.0 mL/min and detection wavelength of 203 nm. Well resolved chromatograms of saikosaponin c, a, and d are obtained with a gradient elution of acetonitrile–water from 40:60 (v/v) to 50:50 (v/v). The total time required for a single analysis is approximately 20 min. Calibration curves for saikosaponin c, a, and d are linear up to 2.5 mg/mL. The coefficient of variability values for saikosaponins in the extract are below 4%, and the recoveries for saikosaponin c, a, and d are 95.2 ± 1.1, 96.5 ± 0.9, and 96.2 ± 1.0%, respectively. The changes in saikosaponin contents for a two-year growth of *Bupleurum falcatum* are measured by the established high-performance liquid chromatography method.

**Introduction**

Saponins are widely distributed in many plant species and have complex chemical structures consisting of a variety of triterpenoidal or steroidal aglycons and various carbohydrate moieties (1). They are now expected to serve as functional components in food because of their physiological activities (2,3).

The dried roots of *Bupleurum falcatum*, which is a perennial herb growing at the Far Eastern part of Asia, Bupleuri radix, are a well-known crude drug in traditional oriental medicine (4,5). Three major oleananesaponins named saikosaponin c, a, and d, which are the glycosides of pentacyclic triterpenes having the sugar moieties of glucose, fucose, and rhamnose, were isolated from the roots of this herb (6–8), and the chemical structures of them are shown in Figure 1.

Until now, antiinflammatory action, plasma-cholesterol lowering action, hemolytic activity on membrane fluidity, and protective action against hepatic damage by D-galactosamine have been reported for saikosaponin a and d (9,10). Much research has also been done on the separation of the triterpenoidal saponins from Bupleuri radix (6,11–14). For quantitative analysis on saikosaponin a and d by high-performance liquid chromatography (HPLC), a variety of columns and eluents were tested, and a good result was reported by reversed-phase chromatography on a column of octadecysilylated (ODS) silica gel, LS-410, with a mixture of methanol, water, acetic acid, and triethylamine as eluent (6). On the other hand, Kanazawa et al. (15) reported on a simultaneous HPLC determination of the saponin compounds from ginseng and Bupleuri radix using an IPG-ODS column.

These HPLC techniques, however, had the drawback of incomplete separation of saikosaponin c in the extract of *B. falcatum*, primarily owing to their isocratic elution nature. The aim of the present research is to develop a gradient elution technique for simultaneous quantitative determination of saikosaponin c, a, and d by HPLC.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Chemical structures of saikosaponins. 1, 2, and 3 denote saikosaponin a, c, and d, respectively. Symbols: Glc, glucose; Fuc, fucose; Rham, rhamnose.

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Experimental

Herb samples

The roots of *B. falcatum*, which was cultivated for 7 months after planting at Youngchun, Kyungsangpuk-do, Korea, were crushed with a dry mixer (Samsung Electronic Co., Seoul, Korea) after drying in a hot air cabinet dryer at 50°C, and the resulting powder was used as the sample for developing the chromatographic conditions of this study. Various roots of *B. falcatum* under different developmental stages were also harvested and powdered in the same manner and used as the samples for optimizing the harvest time.

Reagents

Standard reference materials of saikosaponin c (mw 927.13), a (mw 780.99), and d (mw 780.99), which have purities greater than 98%, were purchased from Wako Pure Chemical (Osaka, Japan). HPLC-grade acetonitrile, methanol, and water were obtained from Fisher Scientific (Pittsburgh, PA). Ethanol, methanol, and other solvents were of guaranteed reagent grade from various suppliers.

HPLC apparatus

A Jasco (Japan) HPLC system (model PV-980) consisting of intelligent pumps (model PV-980), a variable wavelength ultraviolet detector (model UV-975) having a wavelength setting of 203 nm, an intelligent column thermostat (model CO-966), and a 20-µL Rheodyne (Cocati, CA) injection loop were used throughout this study. The columns used were Inertsil ODS-3 C18 (250×4.6-mm i.d., GL Sciences, Japan) having a particle size of 5 µm and Kromasil 4.6-mm i.d., Eka Nobel AB, Sweden). A guard column (GL Sciences) containing the same C18 packing as the Inertsil ODS-3 C18 column was positioned in front of the analytical column to protect it from contamination. Data acquisition was done with Borwin chromatography software (revision 1.2150, Jasco).

Sample preparation for HPLC analysis

Two grams of a Bupleuri radix powder was extracted in an erlenmeyer flask with 70 mL of 70% ethanol in static mode with a reciprocal water bath incubator for 7 h at 45°C. During extraction, the contents of the flask were shaken gently for 1 min twice an hour. After setting at room temperature for 17 h, the contents were filtered through Whatman (Tewksbury, U.K.) #40 filter paper. The residue in the flask was washed with small portions of 70% ethanol and filtered again. The final volume of the combined filtrate was adjusted to 100 mL. It was transferred into a round-bottomed flask and concentrated under reduced pressure with a rotary evaporator (Büchi, Switzerland). The concentrate was then dissolved in 10 mL of HPLC-grade methanol. One milliliter of the resulting solution was filtered through a 0.45-µm PVDF syringe filter (Gelman, Ann Arbor, MI) and used as the sample for HPLC analysis.

Chromatographic procedure by isocratic elution

Isocratic elutions of the HPLC system were done using methanol–water and acetonitrile–water eluents of different compositions. In these cases, the methanol and acetonitrile compositions were changed in the ranges of 70–90% and 40–60%, respectively. Prior to analysis, the column was equilibrated with the corresponding eluent for 1 h at room temperature and then for 30 min at the operating temperature of 35°C. The injection volume and flow rate of the eluent were 10 µL and 1.0 mL/min, respectively.

Chromatographic procedure by gradient elution

Two types of gradient elution concerning the acetonitrile–water system were carried out at the same flow rate and column temperature as the isocratic elution described in the previous section. In one method (method A), the first gradient elution from 40:60 (v/v) acetonitrile–water to 50:50 (v/v) acetonitrile–water was performed for 2 min just after sample loading. Thereafter, elution was maintained isocratically at this eluent composition until the 40 min mark and was followed by the second gradient elution to the initial eluent composition (40:60 acetonitrile–water) until the 45 min mark (refer to A and A’ of Figure 2). In the other method (method B), an isocratic elution of 40:60 (v/v) acetonitrile–water was done during the first 2 min after sample loading. Then, the first gradient elution from 40:60 (v/v) acetonitrile–water to 50:50 (v/v) acetonitrile–water was from 2 to 10 min. Elution was maintained at this eluent composition until the 40 min mark and was followed by the second gradient elution back to 40:60 (v/v) acetonitrile–water until the 45 min mark (refer to B and B’ of Figure 2). In both methods, a reequilibration time amounting to 10 min was required before starting the next measurement.

Results and Discussion

Isocratic elution behavior

Major variables affecting the HPLC separation for specific compounds are reported to be flow rate, eluent composition, and mode of elution (16). We fixed the flow rate at 1.0 mL/min, based on the operating temperature of 35°C. The injection volume and flow rate of the eluent were 10 µL and 1.0 mL/min, respectively.

![Gradient HPLC chromatograms of saikosaponin standards (A and B) and saikosaponins in the Bupleuri radix extract (A’ and B’). Two types of gradient elution (method A and B) using acetonitrile–water were compared. Peaks 1, 2, and 3 are saikosaponin c, a, and d, respectively.](https://example.com/gradient-chromatograms.jpg)
on the isocratic elution exploiting methanol–water (70:30, v/v; data not shown). At this flow rate, isocratic elution behavior was determined by measuring the retention times of saikosaponin standards and saikosaponins in the Bupleuri radix extract at varying eluent compositions of methanol–water and acetonitrile–water (Table I).

By decreasing the amount of methanol at the flow rate of 1.0 mL/min, the retention times of saikosaponins became longer; however, the resolution of three peaks became better. This phenomenon is typical of a reversed-phase separation (16). Overall, however, the methanol–water mobile phases used (70:30, 80:20, and 90:10, v/v) were not able to adequately resolve saikosaponin c and d in the sample. To improve the resolution of saikosaponin c, a, and d in the Bupleuri radix extract, the acetonitrile–water mobile phases (40:60, 45:55, and 50:50, v/v) were employed instead of methanol–water. The same phenomenon as the methanol–water eluents—that is, an inverse correlation between the amount of organic solvent (acetonitrile) and retention time and resolution—was noticed. Although peak resolution among saikosaponin c, a, and d was improved at the eluent compositions of 40:60 and 45:55 (v/v) acetonitrile–water, the total elution times increased conspicuously (more than 45 min) compared with those obtained with the methanol–water mobile phases. Moreover, a double peak, which possibly suggests an isomeric status of saikosaponin a, was found in the case of standard reference material at these eluent compositions. When the amount of acetonitrile in mobile phase increased to 50% (v), a total running time of less than 30 min was attained, and peak separation of saikosaponin a was not found. In this case, however, the separation of saikosaponin c in the sample was not satisfactory. As shown in Table I, the substitution of acetonitrile–water for methanol–water resulted in better peak resolution and increased analysis time. Hence, a novel method that compromised separation power and analysis time was strongly required.

**Table I. Retention Times of Saikosaponin Standards and Saikosaponins in the Bupleuri radix Extract at Varying Eluent Compositions of Methanol–Water and Acetonitrile–Water**

<table>
<thead>
<tr>
<th>Eluent composition (v/v)</th>
<th>Retention time (min)</th>
<th>Standard reference material</th>
<th>Bupleuri radix extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c</td>
<td>a</td>
<td>d</td>
</tr>
<tr>
<td>CH₃OH–H₂O (70:30)</td>
<td>6.40</td>
<td>10.63</td>
<td>17.03</td>
</tr>
<tr>
<td>CH₃OH–H₂O (80:20)</td>
<td>4.69</td>
<td>5.94</td>
<td>7.29</td>
</tr>
<tr>
<td>CH₃OH–H₂O (90:10)</td>
<td>4.69</td>
<td>5.86</td>
<td>7.18</td>
</tr>
<tr>
<td>CH₃CN–H₂O (40:60)</td>
<td>10.21</td>
<td>21.23</td>
<td>61.25</td>
</tr>
<tr>
<td>CH₃CN–H₂O (45:55)</td>
<td>8.35</td>
<td>16.94</td>
<td>45.94</td>
</tr>
<tr>
<td>CH₃CN–H₂O (50:50)</td>
<td>5.68</td>
<td>8.12</td>
<td>17.29</td>
</tr>
</tbody>
</table>

* Not detected.

**Table II. Retention Times of Saikosaponin Standards and Saikosaponins in the Bupleuri radix Extract at Different Gradient Types of Acetonitrile–Water**

<table>
<thead>
<tr>
<th>Gradient type*</th>
<th>Retention time (min)</th>
<th>Standard reference material</th>
<th>Bupleuri radix extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>c</td>
<td>a</td>
<td>d</td>
</tr>
<tr>
<td>Method A</td>
<td>3.55</td>
<td>7.23</td>
<td>14.89</td>
</tr>
<tr>
<td>Method B</td>
<td>8.16</td>
<td>12.20</td>
<td>19.01</td>
</tr>
</tbody>
</table>

* Described in the Experimental section.

**Optimization by gradient elution**

To assay saponin compounds in *B. falcatum* with good resolution and a reasonable elution time, two types of gradient elution (method A and B) of acetonitrile–water were performed as described in the Experimental section, and the elution patterns and retention times of saikosaponin standards and saikosaponins in the Bupleuri radix extract are shown in Figure 2 and Table II. When method A (in which gradient formation was done) was initially used, the resolution of saikosaponin standards was good. However, saikosaponins in the sample (saikosaponin c in particular) were not adequately resolved, primarily owing to interferences caused by solvent peaks (A and A' of Figure 2). Thus, it was necessary to delay the elution of saikosaponin c to obtain a good separation. For this purpose, we chose method B, in which gradient formation started at 2 min after sample injection. This kept the organic solvent fraction in mobile phase low (in comparison with method A) during the initial phase of analysis, resulting in a delayed elution and good resolution of saikosaponins in the standard and sample (B and B' of Figure 2) (16). In this situation, the retention times of standard saikosaponin c, a, and d were 8.16, 12.20, and 19.01 min, respectively, and those of saikosaponins in the sample were 8.44, 12.27, and 19.50 min, respectively. The elution times of saikosaponins obtained using method B were longer than those of method A by 4–5 min. This fact clearly implied that a good HPLC separation of saikosaponin c, a, and d is obtained by adjusting the gradient type without a conspicuous increase in analysis time.

**Comparison of columns**

From the above experiments on isocratic and gradient elution, it was found that the gradient elution according to method B is...
the best way for separating saponin compounds in the extract of Bupleuri radix. As previously noted, chromatographic columns can affect separation power and elution patterns when the flow rate, eluent composition, and mode of elution are optimized (12). Thus, we analyzed the performance of the Kromasil column, which has similar dimensions to Inertsil ODS-3 C18 columns, by the gradient elution of method B at a flow rate of 1.0 mL/min (Figure 3). It was demonstrated that the first peak in the sample (saikosaponin c), which was well separated with Inertsil ODS-3 C18 column, was not clearly resolved with this column. Moreover, the baseline of the sample chromatogram obtained with this column was drifting more. This fact meant that Inertsil ODS-3 C18 column is more suitable for a specific saikosaponin analysis employing the analytical conditions described here. Hence, further experiments were done with the Inertsil ODS-3 C18 column at the gradient elution according to method B.

**Calibration curves**

Figure 4 shows the calibration curves for saikosaponin c, a, and d prepared by an external standard method. As shown in Figure 4, the calibration curves were linear and reproducible, as evidenced by the correlation coefficients $r$ of 0.995, 0.995, and 0.996 for saikosaponin c, a, and d, respectively. Saikosaponin d was the most responsive at a fixed concentration, followed by saikosaponin a and c. The upper limit of linear responses was normally obtained at 2.5 mg/mL for all saikosaponins. The lower limit of linear responses, which normally represents sensitivity or detection limit, was attained at 0.2–0.5 mg/mL, depending on the types of saikosaponin.

The coefficient of variability (standard deviation/mean x 100, %) values for the saponin compounds in the Bupleuri radix extract were below 4%, which represents a good reproducibility of the present HPLC assay (16,17). On the other hand, the recoveries for saikosaponin c, a, and d in the present analysis were 95.2 ± 1.1, 96.5 ± 0.9, and 96.2 ± 1.0%, respectively. The present analysis shows a similar recovery of analysis in comparison with previous research (16,17).

**Estimation of harvest time for B. falcatum**

Based on the resolution and the reproducibility and recovery of analysis, the HPLC method established in the present study was presumed to be a reliable method for analyzing the contents of saikosaponins in B. falcatum and food products containing these compounds.

It has been reported that saikosaponin d shows the strongest physiological activity and thus correct determination of the harvest time for B. falcatum is important to maximize the contents of active compounds and economic profit (14). Table III shows the changes in saikosaponin contents measured by this method for a two-year growth of B. falcatum. The contents of saikosaponin c reached a plateau value of 142.75 mg/100 g sample after 111 days, and those of saikosaponin a gradually increased during the whole period. On the other hand, the contents of saikosaponin d increased to the maximum value of 247.25 mg/100 g sample and decreased thereafter. From these results, it was estimated that the optimum harvest time for B. falcatum lies between 145 days after growth and one year of growth.

**Conclusion**

HPLC separation of saponin compounds (saikosaponin c, a, and d) in Bupleurum falcatum was performed on a reversed-phase (ODS C18) column, and the chromatographic selectivity according to mobile phase and mode of elution was evaluated. The results show that a good separation (concerning resolution and analysis time) is possible by gradient elution employing acetonitrile-water. Considering reproducibility and recovery of analysis, this method might be a routine analytical tool for quantitative analysis of saponin compounds in B. falcatum and food products containing these compounds.

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References


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