Prevention of Epimerization and Quantitative Determination of Amygdalin in Armeniaceae Semen with Schizandrae Fructus Solution

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(Received July 3, 2006)

Armeniaceae Semen not only contains amygdalin, but emulsin also, which is an enzyme that hydrolyzes amygdalin. The extraction yield of amygdalin from Armeniaceae Semen was low, due to the presence of emulsin, when extracted with water. When Schizandrae Fructus solution was used as the extractant, however, amygdalin was almost completely extracted, regardless of the cutting size, due to the absence of the influence of emulsin. In addition, when the crude powder or small piece forms were used with Schizandrae Fructus solution, on epimerization of the D-amygdalin into neoamygdalin occurred. D-amygdalin and its conversion product, neoamygdalin, were quantitatively analyzed by reverse-phase, high-performance liquid chromatography (HPLC), with an optimized eluent of 10 mM sodium phosphate buffer (pH 2.3), containing 11.5% acetonitrile. The concentration and detector response were linearly correlated over the range 0.05 to 2 mM. The detection limits for both D-amygdalin and neoamygdalin were approximately 5 μM for the amount injected.

Key words: Armeniaceae Semen powder, Schizandrae Fructus solution, D-Amygdalin, Neoamygdalin, Epimerization inhibition, Conversion ratio, Ma-whang-tang

INTRODUCTION

Armeniaceae Semen, the seed of Prunus armeniaca Linne var. arsua Maximowicz, which belongs to the Rosaceae family, contains amygdalin, a fatty oil. This seed has been widely used in oriental medicine for the treatment of coughing, wheezing, sore throat and constipation (Zhu, 1998). Schizandrae Fructus, containing lignan, schizandrin, goisin and organic acid, is the dried fruit of Schizandra chinensis Bailon, which belongs to the Schizandraceae family. This fruit has been widely used in oriental medicine for the treatment of coughing and to improve liver function (Zhu, 1998). D-amygdalin (D-mandelonitrite-β-D-gentiobioside) (Fig. 1) has been reported to selectively kill cancer cells at the tumor site, without systemic toxicity, which is a problem frequently encountered when using general chemotherapeutic agents (Culliton, 1973; Stobbaugh et al., 1978). It was reported that the D-amygdalin in Armeniaceae Semen undergoes a hydrolytic reaction due to emulsin when placed in water, and is almost decomposed when attempts are made for its extraction from the powder form (Heisman et al., 1967; Akahori et al., 1983). It was reported that D-amygdalin in boiling water is epimerized to neoamygdalin (L-mandelonitrite-β-D-gentiobioside) (Fig. 1) (Takayama et al., 1984; Hwang et al., 2002). Because neoamygdalin has no antitumor activity, it is important to suppress the conversion of D-amygdalin into neoamygdalin due to epimerization in water. In the powder form, a method using the addition of acid has been used in order to increase the extraction efficiency as well as to suppress the epimerization (Takayama et al., 1984; Hwang et al., 2002; Koo et al., 2005). However, in traditional oriental preparations, if an acid solution is used as the extractant, changes in major components may arise. Therefore, this study established a method to extract most of the amygdalin using Schizandrae Fructus solution, which is a food and oriental preparation, instead
of an acid solution as the extractant. In addition, the optimal conditions for the suppression of the conversion of D-amygdalin into neoamygdalin and for the hydrolytic reaction due to emulsion were established using Schizandrae Fructus containing many of organic acids.

Gas chromatography (Takayama et al., 1980) and capillary electrophoresis (Isozaki et al., 2001; Kang et al., 2000) methods were used for the practical analyses of amygdalin epimers. High-performance liquid chromatography (HPLC) (Cairns et al., 1978; Smith et al., 1984; Dybowski, 1978) has been reported as a practical method, but could not be used here due to its low efficiency and slow analytical outcome. In our previous paper (Koo et al., 2005); a reverse-phase HPLC method, using a C18 column, for the separation of D-amygdalin and neoamygdalin was developed. In this paper, the efficiency for the quantitative examination of D-amygdalin and neoamygdalin was improved.

MATERIALS AND METHODS

Materials
D-amygdalin and methanol were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and Merck (Darmstadt, Germany), respectively. Distilled water was further purified using an Automatic Aquarius AW-1001 (Top Trading Co., Seoul, Korea). HPLC-grade acetonitrile (Merck, Darmstadt, Germany) was used. All other reagents and solvents used were of guaranteed or analytical grade. The crude drugs, such as Armeniacae Semen and Schizandrae Fructus, were purchased from the Kyungdong Market (Seoul, Korea), in accordance with the standards stipulated in Korea Pharmacopoeia (VII). 8-Amino-2-naphthalenesulfonic acid (8, 2-ANS) (Tokyo Kasei Chemical Co., Tokyo, Japan) was used as the internal standard. The composition of the crude drugs in the Chinese herbal prescriptions (known as ma-hwang-tang) is as follows: Armeniacae Semen, 6; Ephedrae Herba, 6; Cinnamomi Cortex, 6; Glycyrrhizae Radix, 3. The numbers represent the mass ratio in the prescription. The organic acids content in the crude drugs were examined (Table I), with the highest content found in Schizandrae Fructus. Therefore, Schizandrae Fructus solution was selected as the extractant.

Instruments and chromatographic conditions
The HPLC system used was comprised of a Nanospace SI-23001 pump (Shiseido, Tokyo, Japan), equipped with a Nanospace SI-23002 UV detector set at 214 nm. The column was either a Hypersil GOLD 3 µ C18 (150 mm x 4.6 mm I.D.; 3 µm, Thermo, Waltham, U.S.A.), with a flow rate of 700 µL/min, or a Capcell Pak C18 MG (250 mm x 4.6 mm I.D.; 5 µm, Shiseido, Tokyo, Japan), with a flow rate of 1.2 mL/min. The injection volume was 10 µL. The column oven was a TOP-101L (Top Trading Co., Seoul, Korea), maintained at either 3 or 8°C. The mobile phase was either a 10 mM sodium phosphate buffer, pH 2.3 or 3.1, containing 11.5 or 8.5% acetonitrile, respectively. A Bruker Advance-500 (600 MHz) NMR Spectrometer was used to obtain the proton NMR.

Preparation of standard solution
A D-amygdalin standard in water was used. Neoamygdalin was prepared from D-amygdalin in aqueous ammonia, as reported by Fischer (Fischer et al., 1985). Neoamygdalin was purified by specific HPLC conditions (Nucleosil 100-5 C18, 250 mm x 10 mm I.D.; mobile phase, 6% acetonitrile; flow rate, 5 mL/min; injection volume, 500 µL; column temperature, ambient) and characterized using FAB-HR mass and 1H-NMR (CD3OD) spectroscopies, according to the standard method (Koo et al., 2005; Takayama et al., 1980; Isozaki et al., 2001; Kang et al., 2000; Cairns et al., 1978). The molecular weights of D-amygdalin and neoamygdalin, as measured by FAB-HR mass spectroscopy, were 458.1672 [M+1] and 458.1674 [M+1], respectively. The NMR spectra confirmed that D-amygdalin and neoamygdalin are epimers of each other, with two distinctly different methine chemical shift values (5.89 ppm for D-amygdalin and 6.07 ppm for neoamygdalin).

Methods for sample preparations
Water as an extractant
Four groups with different cutting sizes of Armeniacae Semen were used: crude powder (passage of mesh 20), small piece (passage of mesh 12), half piece and whole...
piece forms. Five grams of these four groups were prepared from Armeniaca Semen removed from their shells. To each sample, 250 mL of distilled water was added, the mixture extracted under reflux for 3 h and then filtered. An organic solvent, 10 mL n-hexane, was added to a 10 mL- aliquot of this extract solution. The organic layer was removed after partition extraction, followed by the addition of 10 mL of the internal standard solution, containing 0.03 mg of 8, 2-ANS, 8, 2-AN5 was dissolved in 10 mL of 40 mM sodium phosphate buffer (pH 7.2). The solution was filtered through a 0.2 μm membrane filter, and injected onto the HPLC system.

Schizandraceae Fructus solution as an extractant

One gram of Schizandraceae Fructus powder was added to 250 mL of distilled water, and the solution stirred at room temperature for 30 min, filtered and used as the extractant. The following procedure was identical to that described above for the water extractant, except that Schizandraceae Fructus solution was used in place of the distilled water.

RESULTS AND DISCUSSION

Analysis of D-amygdalin and neoamygdalin

In the separation and quantitative experiment for D-amygdalin and neoamygdalin, the separation patterns of two peaks, according to the HPLC conditions, are shown in Fig. 2. Fig. 2(a) shows the chromatogram obtained using the conditions specified for method described in our previous paper (Koo et al., 2005). The retention times for D-amygdalin and neoamygdalin were 29 min and 31 min, respectively. Fig. 2(b) shows the chromatogram obtained using the conditions of the new method presented in this study. The retention times for D-amygdalin and neoamygdalin were 10.0 min and 12.0 min, respectively. Good column efficiency and a reduced analysis time were accomplished using the Hypersil GOLD 3 μ C18 column, with a smaller particle size and shorter length than the Capcell Pak C18 MG column. The resolution of D-amygdalin and neoamygdalin was dependent on the column oven temperature and pH of the mobile phase. When the column oven temperature and mobile phase pH were lowered, the resolution of the two peaks increased. The analysis time using our new method was reduced to half that of our previous method. A complete baseline resolution was obtained under these conditions, and the rapid separation time made it convenient to use. Quantitative analysis was achieved using the internal standard. The UV response on the calibration plots showed a linear correlation over the concentration range 0.05 to 2.00 mM for both D-amygdalin and neoamygdalin (r² values were 0.9995 for D-amygdalin and 0.9992 for neoamygdalin). The detection limits were within 5 μM (S/N=3).

![Fig. 2. Comparative chromatograms for the amygda1in epimers according to the HPLC conditions. (a): column, 250 mm × 4.6 mm, Capcell Pak C18 MG; mobile phase, 10 mM sodium phosphate buffer (pH 3.1), containing 8.5% acetonitrile; column temperature, 3°C. (b): column, 150 mm × 4.6 mm, Hypersil GOLD 3 μ C18; mobile phase, 10 mM sodium phosphate buffer (pH 2.3), containing 11.5% acetonitrile; column temperature, 3°C. Peaks: I.S., 8, 2-ANS (internal standard); 1, neoamygdalin; 2, D-amygdalin.]
Extraction efficiency of amygdalin by cutting size  
Choosing crude drugs in water and extracting under reflux

For the water extraction of amygdalin, four groups with different cutting sizes of Armeniaceae Semen were used: crude powder, small piece, half piece and whole piece forms. Fig. 3 shows the change in the extraction efficiencies according to the cutting size of Armeniaceae Semen removed from their shells. The extraction efficiency was low for the powder form, tended to increase with increasing cutting size, and was highest for the whole piece form. In general, the extraction efficiency tends to increase with decreasing the cutting size. However, in this case, the results were the opposite, which might have been for the following reasons. Most emulsins, which hydrolyze amygdalin, were present at the seed surface when removed from the shell. When extracting amygdalin from a whole sample of Armeniaceae Semen in boiling water, the emulsion on the seed surface would be extracted and deactivated before the extraction of amygdalin. Therefore, even if amygdalin was extracted, it would not be hydrolyzed by the inactivated emulsion, meaning most of the amygdalin would be extracted without being affected by emulsin.

Conversely, the contact surface would be higher due to the decreased cutting size of the Armeniaceae Semen, i.e. the extraction efficiency of amygdalin would decrease as it would be hydrolyzed before the emulsion became inactivated.

When extracting from the whole piece form, the extraction ratio of D-amygdalin to neoamygdalin of the total amygdalin ranged from 30 to 70%. Thus, because the extraction of neoamygdalin was much higher than that of D-amygdalin, this method would not be suitable as an extraction method of amygdalin.

Putting crude drugs in boiling water and extracting under reflux

For the extraction of amygdalin with boiling water, the same four groups, with different cutting sizes of Armeniaceae Semen, as for the water extraction were used. Fig. 4 shows the changes in the extraction efficiencies according to the cutting size of Armeniaceae Semen removed from their shells. With boiling water, the extraction efficiency of amygdalin increased, regardless of the cutting size, and this increase was especially marked for the powder and small piece forms. With increasing cutting size, the extraction ratio of D-amygdalin of total amygdalin was increased. When extracting from the whole piece form, the extraction ratio of D-amygdalin to neoamygdalin of the total amygdalin ranged from 70 to 30%. Thus, when extracting the whole piece form with boiling water, the extraction efficiency of D-amygdalin was increased.

Increase of extraction efficiency by Schizandraceae Fructus solution

When boiling water was used as the extractant, the extraction efficiency of D-amygdalin from Armeniaceae Semen was increased, regardless of the cutting size. However, the complete inhibition of epimerization was necessary as the conversion ratio of D-amygdalin into neoamygdalin was only 30-50%. For improved efficiency, amygdalin was extracted from Armeniaceae Semen, in the four cutting size forms, using Schizandraceae Fructus solution as the extractant (Fig. 5). In general, the extraction efficiency and extraction ratio of D-amygdalin were improved using the Schizandraceae Fructus solution compared to water, particularly when extracting the crude powder or small piece forms of Armeniaceae Semen. This result indicated that most of the amygdalin could be extracted, without being affected by emulsin, as the organic acids in the
Schizandra Fructus solution not only inactivated the emulsin, but also inhibited the epimerization of D-amygdalin into neoamygdalin.

**Inhibition of the conversion of D-amygdalin into neoamygdalin with Schizandrae Fructus solution**

Table II shows the conversion ratios of D-amygdalin into neoamygdalin according to the cutting size, when the Schizandra Fructus solution was used as the extractant. The epimerization of D-amygdalin was almost completely inhibited, as shown by the conversion ratio of less than 1% when extracting amygdalin from the crude powder or small piece forms of Armeniaceae Semen. This result indicated the organic acids in the Schizandrae Fructus solution inhibited the epimerization of D-amygdalin into neoamygdalin.

However, the conversion ratio increased with increasing cutting size, and exceeded 10% for cutting sizes larger than half the size of Armeniaceae semen.

Thus, when using Schizandrae Fructus solution as the extractant, the highest efficiency and lowest conversion ratio of Armeniaceae Semen were shown when extracting the crude powder or small piece forms of Armeniaceae Semen.

**Application to Chinese herbal prescription**

Ma-whang-tang, a traditional herbal preparation containing Armeniaceae Semen, was applied using 3 approaches. Armeniaceae Semen was used as crude powder. Method 1 (traditional method): putting crude drugs in water, and extracting under reflux for 3 h while increasing the temperature. Method 2 (modified traditional method): putting crude drugs in boiling water, and extracting under reflux for 3 h. Method 3 (our established method): putting crude drugs in boiling Schizandrae Fructus solution, and

**Table II. Conversion ratio of D-amygdalin into neoamygdalin according to the cutting sizes when extracting with Schizandrae Fructus solution**

<table>
<thead>
<tr>
<th>Cutting size</th>
<th>Mean±S.D</th>
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<tbody>
<tr>
<td>Whole</td>
<td>12.50±0.21^a</td>
</tr>
<tr>
<td>Half</td>
<td>10.01±0.47^a</td>
</tr>
<tr>
<td>Small</td>
<td>0.92±0.31^b</td>
</tr>
<tr>
<td>Powder</td>
<td>0.34±0.13^b</td>
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^a Armeniaceae Semen
^b (%) (n=3)
extracting under reflux for 3 h. In method 1, amygdalin was decomposed, and could not be measured. In method 2, the D-amygdalin and neoamygdalin contents in the Ma-whang-tang extract were 15.02 and 7.74%, respectively. In method 3, the D-amygdalin and neoamygdalin contents were 20.13 and 1.48%, respectively. The ratio of neoamygdalin was higher in method 2 (7.74%) than in method 3 (1.48%).

The recovery of the amygdalin epimers was tested by the addition of known amounts of the amygdalin epimers (D-amygdalin 1.5 mg, neoamygdalin 1.0 mg) to an Armeniaceae Semen-blank fraction of ma-whang-tang. The recoveries for D-amygdalin and neoamygdalin in method 3 were 96.8-103.5 and 97.6-102.9%, respectively.

CONCLUSION

Emulsin, an enzyme contained in Armeniaceae Semen, hydrolyzes amygdalin. The extraction yield of amygdalin from Armeniaceae Semen varied according to the cutting size, due to the presence of emulsin when extracting Armeniaceae Semen using water as the extractant.

However, when using Schizandrae Fructus solution as the extractant, the amygdalin in Armeniaceae Semen was almost completely extracted, regardless of the cutting size, with a conversion of D-amygdalin into neoamygdalin in the crude powder or small piece forms of Armeniaceae Semen almost completely inhibited.

The use of a 10 mM sodium phosphate buffer (pH 2.3), containing 11.5% acetonitrile, as the mobile phase for reverse-phase HPLC was effective for the separation and analysis of both D-amygdalin and neoamygdalin.

ACKNOWLEDGEMENTS

This work was supported by a grant (Code #20050301-034-457-105-03-00) from the BioGreen 21 Program, Rural Development Administration, Republic of Korea.

REFERENCES