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Quantitative determination of amygdalin epimers from armeniacae semen by liquid chromatography

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Abstract

D-Amygdalin and its conversion product, neoamygdalin, were quantitatively analyzed on reverse-phase, high-performance liquid chromatography with an optimized eluent of 10 mM sodium phosphate buffer (pH 3.1) containing 8.5% acetonitrile. Linearity between concentrations and detector responses was obtained in the range from 0.05 to 0.5 mM. The detection limits for D-amygdalin and neoamygdalin were approximately 5 μM per injected amount. Armeniacae semen contains not only amygdalin but also emlusin, which is an enzyme that hydrolyzes amygdalin. When extracting amygdalin from a whole piece of armeniacae semen in boiling water, there was almost no influence of emulsin; which increased the extraction efficiency. However, conversion of D-amygdalin into neoamygdalin at high temperature was found. In this report, we solved this problem by using 4% citric acid as an extractant. This solution also prevented the extraction process from being affected by emulsin. In addition, the extraction efficiency remained the same as that when methanol was used as an extractant, regardless of the cutting size.

Keywords: D-Amygdalin; Neoamygdalin; Epimer separation; Armeniacae semen; Citric acid; Epimerization inhibition

1. Introduction

Armeniacae semen is a seed of *Prunus armeniaca* Linne var. *ansu* Maximowicz, which belongs to the Rosaceae family. This seed has been widely used to treat asthma, aplastic anemia and tumors in oriental medicine [1]. It has been reported that D-amygdalin (D-mandelonitrile-β-D-gentiobioside) (Fig. 1) selectively kills cancer cells at the tumor site without systemic toxicity, which is the usual problem when using general chemotherapeutic agents [2–4]. Armeniacae semen contains not only amygdalin but also emlusin, which is an enzyme that hydrolyzes amygdalin. It was reported that D-amygdalin in armeniacae semen undergoes hydrolytic reaction by emulsin when using water, and it is almost decomposed when extracting from powder type [5]. In addition, it was reported that D-amygdalin in boiling water is epimerized to neoamygdalin (L-mandelonitrile-β-D-gentiobioside) (Fig. 1) [6,7]. Since neoamygdalin has no antitumor activity, it is important to prevent D-amygdalin from being converted into neoamygdalin by epimerization in water. Therefore, we established an optimal condition [6,7] to obtain the maximum extraction efficiency by establishing methods to both prevent emulsin from causing the hydrolysis of D-amygdalin in armeniacae semen in water and to prevent D-amygdalin from being converted into neoamygdalin.

Gas chromatography [8] and capillary electrophoresis [9] methods are used as a practical analysis of amygdalin epimers. High-performance liquid chromatography (HPLC) [10–12] is also reported as a practical method, but it could not be used due to low efficiency and the long time required for analysis. In our previous paper [7], we developed a reverse-

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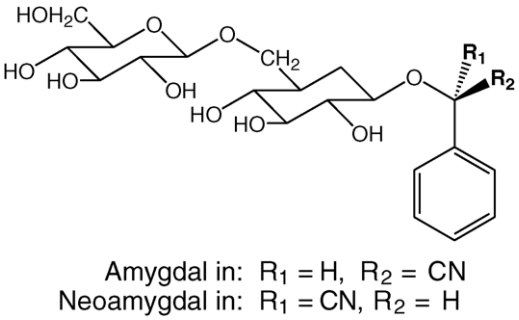

Fig. 1. Chemical structures of D-amygdalin and neoamygdalin.

phase HPLC method using C18 column in order to separate D-amygdalin and neoamygdalin in baseline resolution. In this paper, we improved the efficiency by reducing the analysis time. By this method, we efficiently performed the quantitative analysis of D-amygdalin and neoamygdalin. The aim of this study was to create an analytical method for measuring of D-amygdalin level without converting it to neoamygdalin.

2. Experimental

2.1. Materials

D-Amygdalin and methanol were purchased from Tokyo Kasei Chemical Co. (Tokyo, Japan) and Merck (Darmstadt, Germany), respectively. HPLC-grade acetonitrile (Merck, Darmstadt, Germany) was used. Other reagents and solvents used were of guaranteed grade or analytical grade. Armeniacae semen was purchased from *Kyungdong*, *Daegu*, *Busan* Market in accordance with standards stipulated in Korea Pharmacopoeia (VII). 8-Amino-2-naphthalenesulfonic acid (8,2-ANS) (Tokyo Kasei Chemical Co., Tokyo, Japan) was used as an internal standard.

2.2. Apparatus and chromatographic conditions

The HPLC system used was a Nanospace SI-2/3001 pump (Shiseido, Tokyo, Japan) equipped with a Nanospace SI-2/3002 UV detector set at 214 nm. The column was a Capcell Pak C18 MG (4.6 mm × 250 mm, 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 Nanospace SI-2/3004, maintained at 8 °C or 30 °C. The autosampler was a Nanospace SI-2/3023. The mobile phase was a 10 mM sodium phosphate buffer (pH 3.1) containing 25% methanol or 8.5% acetonitrile.

2.3. Preparation of standard solution

Neoamygdalin was prepared from D-amygdalin in aqueous ammonia as reported by Fischer [13]. One hundred milligrams of D-amygdalin was added to 10 ml of 0.005 M ammonia solution and the mixture was allowed to stand for 2 h at room temperature. During this period, equilibrium between D-amygdalin and neoamygdalin was fully achieved. Neoamygdalin was purified by specific HPLC conditions (Nucleosil 100-5 C18, 250 mm × 10 mm i.d.; mobile phase, 6% acetonitrile; flow rate, 5 ml/min; injection volume, 500 μl; column temperature, ambient) and characterized in FAB-HR mass and ^{1}H-NMR (CD_3OD) spectroscopy by following the standard method [7,9]. The molecular weights of D-amygdalin and neoamygdalin measured by FAB-HR mass spectroscopy were 458.1672 [*M*+1] and 458.1674 [*M*+1], respectively. NMR spectra confirmed that D-amygdalin and neoamygdalin are epimers to each other by showing two distinctly different methine chemical shift values (5.89 ppm for D-amygdalin and 6.07 ppm for neoamygdalin).

2.4. Methods for sample preparations

2.4.1. Methanol as an extractant

Five grams of armeniacae semen powder was added to 250 ml of methanol. The mixture was extracted under reflux for 3 h and filtered. A 10 ml-aliquot of this extract solution was evaporated to dryness. The residue was dissolved in 10 ml of water, followed by the addition of 10 ml of internal standard solution containing 0.03 mg of 8,2-ANS. 8,2-ANS was dissolved in 10 ml of 40 mM sodium phosphate buffer (pH 7.2). The solution was filtered through 0.2 μm membrane filter and was injected in the HPLC system.

2.4.2. Pure water or citric acid solution as an extractant

Five grams of four groups (crude powder, a small piece, a half piece and a whole piece) was prepared from armeniacae semen. To each sample, 250 ml of distilled water or 3–5% of citric acid solution was added. The mixture was extracted under reflux for 3 h and filtered. An organic solvent was added to a 10 ml-aliquot of this extract solution. The organic layer was removed after partition extraction, 10 ml of internal standard solution was added to the aqueous layer, and the solution was filtered and injected for analysis.

3. Results and discussion

3.1. Analysis of D-amygdalin and neoamygdalin

To analyze D-amygdalin and neoamygdalin from the total extract, the mobile phase condition of 10 mM sodium phosphate buffer (pH 3.1) containing 25% methanol was used (Fig. 2(A)). For the quantitative analysis, 10 mM sodium phosphate buffer (pH 3.1) containing 8.5% acetonitrile was applied (Fig. 2(B)). In these HPLC conditions, clear baseline separation of three compounds was obtained. The calibration plots showed a linear range of the UV response to the concentration (0.05–0.5 mM) of D-amygdalin and neoamygdalin, with r^2 values of 0.9998 for D-amygdalin and 0.9994 for neoamygdalin. The detection limits were within 5 μM (S/N = 3).

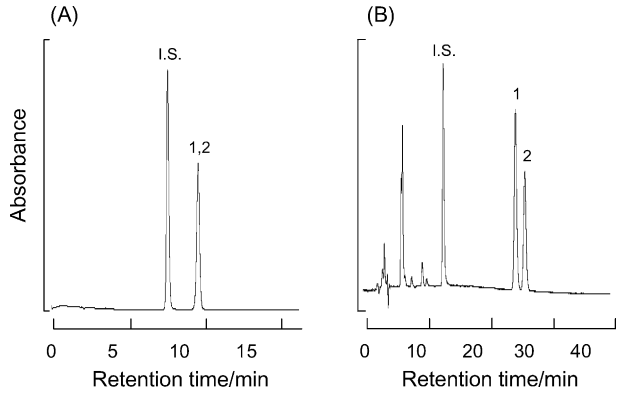

Fig. 2. Reverse-phase HPLC separation of amygdalin by phosphate buffer in either methanol or acetonitrile. (A) 25% methanol (column temperature: 30 °C), (B) 8.5% acetonitrile (column temperature: 8 °C); peaks: IS, 8,2-ANS (internal standard); 1, neoamygdalin; 2, D-amygdalin.

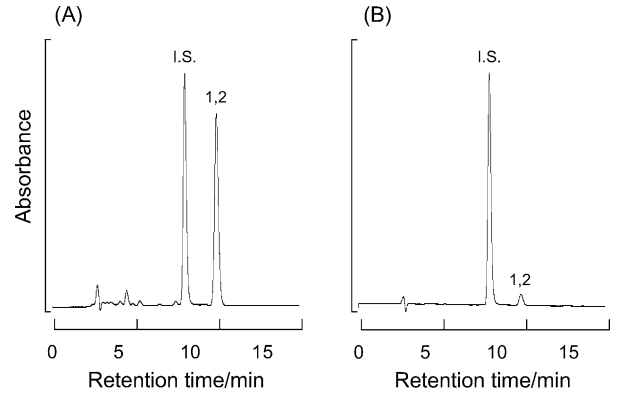

Fig. 3. Influence of emulsin on amygdalin from powder armeniacae semen by the extraction solution (mobile phase, 25% methanol; column temperature: 30 °C). (A) Methanol, (B) water; peaks: IS, 8,2-ANS (internal standard); 1, neoamygdalin; 2, D-amygdalin.

3.2. Influence of emulsin on amygdalin by the extractant

The armeniacae semen powder was extracted with methanol in order to obtain the comparative data not affected by emulsin. Fig. 3 shows the chromatograms of either methanol or water used as extractant. Amygdalin was completely extracted without being hydrolyzed in methanol (Fig. 3(A)). In water extraction, a small part of the amygdalin was extracted and most of this extracted amygdalin was hydrolyzed (Fig. 3(B)). This finding indicates that the moisture content in the solvent has a significant effect on the amygdalin extraction efficiency, because emulsin is a hydrolase.

Table 1
Data on the amygdalin content as a function of the cutting size when extracting amygdalin with water

	Mean ± S.D.			
	Whole[a]	Half[a]	Small[a]	Powder[a]
Amygdalin	45.42 ± 1.21	40.44 ± 0.68	10.18 ± 0.24	1.73 ± 0.14
Neoamygdalin	23.92 ± 0.64	21.32 ± 0.36	5.34 ± 0.13	0.90 ± 0.07
D-Amygdalin	21.50 ± 0.57	19.12 ± 0.17	4.84 ± 0.06	0.83 ± 0.03

[a] Armeniacae semen (mg/g) ($n = 3$).

3.3. Extraction efficiency of amygdalin by cutting size

When extracting amygdalin with water, we used four groups with different cutting sizes of armeniacae semen: crude powder (passage of mesh 20), a small piece (passage of mesh 12), a half piece and a whole piece. Figs. 3(B) and 4 show chromatograms of the extraction efficiencies according to the cutting size. Table 1 summarizes the content of amygdalin by the cutting size, which was 1.73 mg/g in the crude powder, 10.18 mg/g in the small piece, 40.44 mg/g in the half piece and 45.42 mg/g in the whole piece.

In general, the extraction efficiency tends to increase as the cutting size decreases. However, in this case, the result was opposite which might be due to the following reasons. Most emulsins, which hydrolyze amygdalin, were present at the seed surface removed from the shell. When extracting amygdalin from a whole sample of armeniacae semen in boiling water, the emulsin on the seed surface would first be extracted and deactivated before the extraction of amygdalin. Therefore, even if amygdalin were extracted, it would not be hydrolyzed with the inactivated emulsin, which means that most of the amygdalin would be extracted without being affected by emulsin.

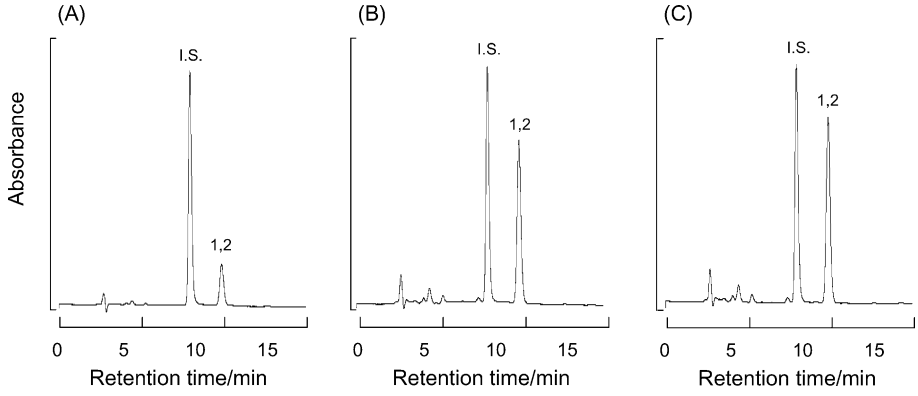

Fig. 4. Comparative chromatogram on the water extraction efficiency of amygdalin by cutting size (mobile phase, 25% methanol; column temperature: 30 °C). (A) Small piece, (B) half piece, (C) whole piece; peaks: IS, 8,2-ANS (internal standard); 1, neoamygdalin; 2, D-amygdalin.

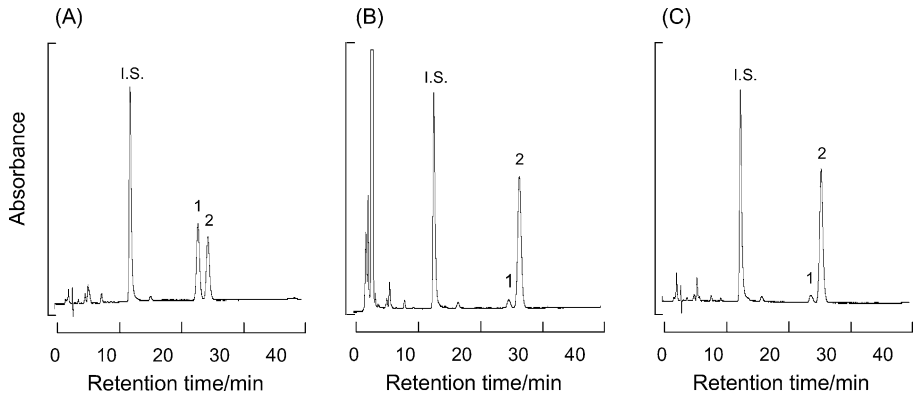

Fig. 5. Inhibition of the conversion of D-amygdalin into neoamygdalin by 4% citric acid (mobile phase, 8.5% acetonitrile; column temperature: 8 °C). (A) Pure water, (B) 4% citric acid, (C) methanol peaks: IS, 8,2-ANS (internal standard); 1, neoamygdalin; 2, D-amygdalin.

On the other hand, the contact surface would be higher because the cutting size of the armeniacae semen would decrease, i.e. the extraction efficiency of amygdalin would decrease because amygdalin was hydrolyzed before the emulsin is inactivated.

3.4. Epimerization of D-amygdalin by heating

As shown in Table 1, when extracting the whole piece in water, although the extraction efficiency was higher, D-amygdalin was converted into neoamygdalin through epimerization by heating. Fig. 5(A) shows a chromatogram, indicating that D-amygdalin was converted into neoamygdalin when extracting amygdalin in water. The content of neoamygdalin and D-amygdalin in armeniacae semen was 23.88 and 21.54 mg/g, respectively.

3.5. Inhibition of the conversion of D-amygdalin into neoamygdalin with citric acid

Citric acid solution (3–5%) was used as an extractant for armeniacae semen to inhibit the conversion of D-amygdalin into neoamygdalin. As can be seen in Fig. 5(B), which shows the chromatogram when extracting with 4% citric acid solution, the conversion of D-amygdalin into neoamygdalin was inhibited. The content of D-amygdalin and neoamygdalin was 44.80 and 2.20 mg/g, respectively, which are comparable values to that of methanol (Fig. 5(C)).

3.6. Increase of extraction efficiency by citric acid solution

In water extraction, a larger cutting size showed higher efficiency. However, two problems were encountered: rapid conversion of D-amygdalin into neoamygdalin and a big difference in the extraction efficiency depending on the cutting size. In particular, the problem of the considerably low extraction efficiency when extracting from powder had to be solved first. We therefore compared 4% citric acid solution as an extractant with water on the four cutting-size groups. The results showed that the quantities of extracted amygdalin did not vary regardless of the cutting size when using 4% citric acid, whereas the extraction efficiency in water was reduced by the cutting size (Fig. 6). The recovery of amygdalin epimers was tested by adding known amounts of the amygdalin epimers (D-amygdalin 1.0 mg, neoamygdalin 1.0 mg) to a whole piece extracted in the 4% citric acid solution. The recoveries for D-amygdalin and neoamygdalin were 98.0–102.6% and 97.2–102.5%, respectively.

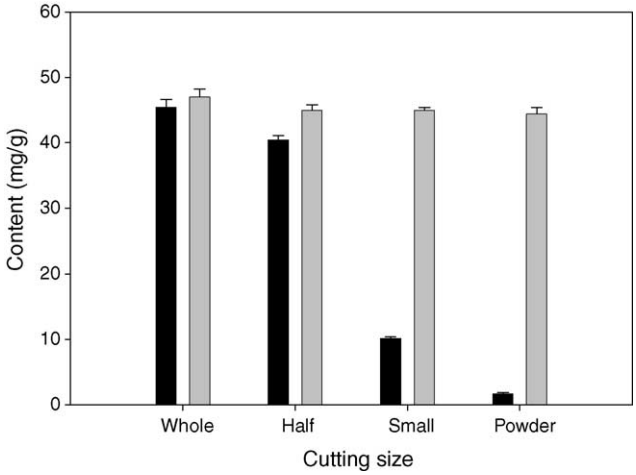

Fig. 6. Extraction yield of four different cutting sizes by water or 4% citric acid: ■ water 4%, □ citric acid.

4. Conclusion

The emulsin present in armeniacae semen is an enzyme that hydrolyses amygdalin. When extracting the amygdalin with water, a larger cutting size results in a higher extraction efficiency. When extracting amygdalin from a whole sample, even though most of the amygdalin was extracted without being affected by the emulsin, D-amygdalin was converted into neoamygdalin through epimerization by heating.

However, extraction process was not affected by emulsin when using a 3–5% aqueous citric acid solution as an extractant, and epimerization as a result of heating did not occur. Based on the experimental results, there was no difference in the quantity of extracted amygdalin regardless of the cutting size. The use of a 10 mM sodium phosphate buffer (pH 3.1) containing 8.5% acetonitrile as a mobile phase in reversed-phase HPLC was effective in separating and analyzing amygdalin and neoamygdalin.

Acknowledgements

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