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Quantitative determination of amygdalin epimers by cyclodextrin-modified micellar electrokinetic chromatography

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Abstract

A new capillary electrophoresis method was developed for the quantitative determination of the amygdalin epimers, amygdalin and neoamygdalin, which are biologically significant constituents in the crude drugs, namely Persicae Semen and Armeniacae Semen. The effects of surfactants, additives and other analytical parameters were studied. As a result, the resolution of two epimers was performed by cyclodextrin-modified micellar electrokinetic chromatography with a buffer containing α-cyclodextrin and sodium deoxycholate. By the application of this method, a simple, fast and simultaneous quantitative determinations of amygdalin epimers in the crude drugs (Persicae Semen and Armeniacae Semen) and the Chinese herbal prescriptions (Keishi-bukuryo-gan and Mao-to) were achieved.

Keywords: Epimer separation; Persicae Semen; Armeniacae Semen; Keishi-bukuryo-gan; Mao-to; Pharmaceutical analysis; Amygdalin; Neoamygdalin; Sodium deoxycholate; Cyclodextrins

1. Introduction

Amygdalin (D-mandelonitrile-β-D-gentiobioside) (see Fig. 1) is a natural compound with the antitussive and anticancer activities [1]. It is decomposed by the action of β-D-glucosidase to yield hydrocyanic acid which stimulates the respiratory center reflexively and produces a kind of antitussive and antiasthmatic effects [2]. In addition, amygdalin is known to be changed to its epimer, neoamygdalin (L-mandelonitrile-β-D-gentiobioside) (see Fig. 1) in water [3]. Ishihara has reported the decomposition of amygdalin and its epimerization during the decoction of Armeniacae Semen [4]. Therefore, the quantitative measurements of the amygdalin epimers in the crude drugs is a very important step to evaluate the quality of such drugs.

The analysis of the amygdalin epimers by high-performance liquid chromatography (HPLC) has already been reported [5], however, the method has lower efficiency and takes longer analysis time in spite of using huge volumes of solvents. On the other side, the capillary electrophoresis (CE) technique offered an extremely high efficiency within relatively shorter analysis time. It can represent an alternative method to the HPLC for the quality evaluation of such particular herbal drugs. Recently, during our

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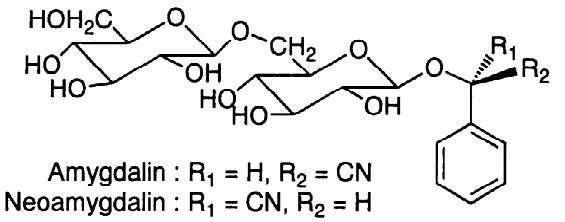

Fig. 1. Chemical structures of amygdalin and neoamygdalin.

manuscript preparation, Kang et al. reported a micellar electrokinetic chromatography (MEKC) for the determination of the amygdalin epimers [6]. However, this technique is not sufficient to analyze the amygdalin epimers in the natural products such as Chinese herbal prescriptions. Therefore, a specific method should be developed to analyze the amygdalin epimers in the natural products.

In a previous paper, the MEKC using bile salts, as chiral surfactants, is known to be applied for chiral separation [7]. And cyclodextrin-modified micellar electrokinetic chromatography (CD-MEKC) is known to improve the selectivity of compounds to form inclusion complexes with cyclodextrin (CD) [8]. In the current paper, we describe the separation of amygdalin epimers in the crude drugs and the Chinese herbal prescriptions. The resolution of amygdalin epimers was improved by CD-MEKC using a chiral surfactant such as sodium deoxycholate (SDOC) and a chiral additive, α-CD. And we established the simple and fast quantitative method for the determination of the amygdalin epimers by CD-MEKC in the crude drugs and the Chinese herbal prescriptions.

2. Experimental

2.1. Materials

Persicae Semen and Armeniacae Semen were obtained in Osaka market, which were of officially approved standards as indicated in Japanese Pharmacopoeia XIII. The seeds were further confirmed by the morphological examination [9]. They were ground finely in a vibrating mill and used in the experiment.

The compositions of the crude drugs in the Chinese herbal prescriptions were as follows: Keishi-bukuryo-gan (Persicae Semen 3; Cinnamomi Cortex 3; Paeoniae Radix 3; Hoelen 3; Moutan Cortex 3) and Mao-to (Armeniacae Semen 5; Ephedrae Herba 5; Cinnamomi Cortex 4; Glycyrrhizae Radix 1.5). The number represents the ratio by mass to prepare the prescription. All the drug materials used in the experiment are deposited for the reference in the specimen room of Kampo & Healthcare Research Labs, Kanebo, Osaka, Japan.

2.2. Preparation of the crude drug extracts

The seed powder of Persicae Semen (0.5 g) or Armeniacae Semen (0.5 g) was treated with 70 ml of methanol and refluxed for 0.5 h. The whole extract material was thereafter ultrasonicated for 5 min. The sample was then filtered and washed by methanol. The filtrates were combined and diluted up to 100 ml in a volumetric flask. A 10-ml aliquot of this extract solution was evaporated to dryness. The residue was dissolved in water (10 ml), followed by the addition of 10 ml internal standard solution (2 mg of methyl 4-hydroxybenzoate in 10 ml of water). The solution was filtered through a 0.2-μm membrane filter.

2.3. Preparation of the Chinese herbal prescription extracts

The Chinese herbal prescription extracts of Keishi-bukuryo-gan (Gui-zhi-fu-ling-wan) and Mao-to (Ma-huang-tang) were spray-dried products. A 0.2 g of Keishi-bukuryo-gan or Mao-to was treated with 10 ml of internal standard solution. The sample was shaken for 15 min, then filtered through a 0.2-μm membrane filter.

2.4. Reagents and chemicals

The separation buffer consisted of 20 m*M* sodium dihydrogenphosphate, 80 m*M* SDOC (Nacalai Tesque, Kyoto, Japan) and 25 m*M* α-CD (Wako, Osaka, Japan) in deionized water, which was adjusted to pH 7.5 with 0.1 mol/l NaOH. All samples and standards were filtered through a 0.2-μm membrane filter before injecting into the capillary. All chemicals used were of analytical reagent grade.

2.5. Standard samples of amygdalin and neoamygdalin

Amygdalin was purchased from Aldrich (Milwaukee, WI, USA). Neoamygdalin was prepared from amygdalin [3], purified by HPLC (YMC D-ODS-5 (250 mm×20 mm I.D.), mobile phase: water–CH_3CN (10:1), flow-rate: 10.0 ml/min, column temperature: ambient) and characterized by ^{1}H-NMR and ^{13}C-NMR [10,11]. The methine chemical shifts for amygdalin (δ_H 5.99) and neoamygdalin (δ_H 6.07) together with all other NMR signals in ^{1}H- and ^{13}C-NMR spectra were corresponded to these epimers as reported previously [11].

2.6. Instrumentation

All chromatographic measurements were carried out with a model P/ACE 5000 system equipped with an UV-absorbance detector (Beckman, Fullerton, CA, USA). The temperature of the capillary tube was maintained at 25°C. Samples were injected by application of pressure for 10 s (0.5 p.s.i. pressure; 1 p.s.i.≡6894.76 Pa) to the anodic end of the capillary. The UV detection was observed at 200 nm. All separations were carried out at a voltage of 20 kV. Data were recorded with the Beckman Gold Station software. Fused-silica capillaries (50 μm I.D.) were from GL Science (Tokyo, Japan). The total length of the capillary was 770 mm and the length up to detector was 700 mm.

Bruker AM-300 (300 MHz) NMR spectrometer was used to obtain ^{1}H- and ^{13}C-NMR spectra and ^{1}H-NMR chemical shift was expressed as δ ppm using tetramethysilane (TMS) as an internal standard.

3. Results and discussion

3.1. Effect of micelle reagents

Preliminary experiments of MEKC were conducted to separate the amygdalin epimers. The MEKC method was not effective enough in our experiment to resolve the amygdalin epimers, using SDS, sodium cholate and some other bile salts as the micelle reagents at 80 m*M* in contrast as reported by Kang et al. [6]. Some of bile salts were used to be as the micelle reagents for the resolution of chiral compounds [12], however, in the present study the resolution was not achieved effectively.

Therefore, CD-MEKC was developed for the resolution of the amygdalin epimers. The effect of SDS, sodium dehydrocholate, sodium cholate, sodium taurocholate and SDOC with α-CD were studied (Fig. 2). In this study, the maximum resolution of the amygdalin epimers was accomplished with the α-CD incorporated SDOC. The differential inclusion-complex formations of the amygdalin epimers with α-CD provided the differential migrations. It should be noted that SDOC has asymmetric structure, but not the SDS, because of this reason the inclusion-complexes of the amygdalin epimers with α-CD could be resolved effectively.

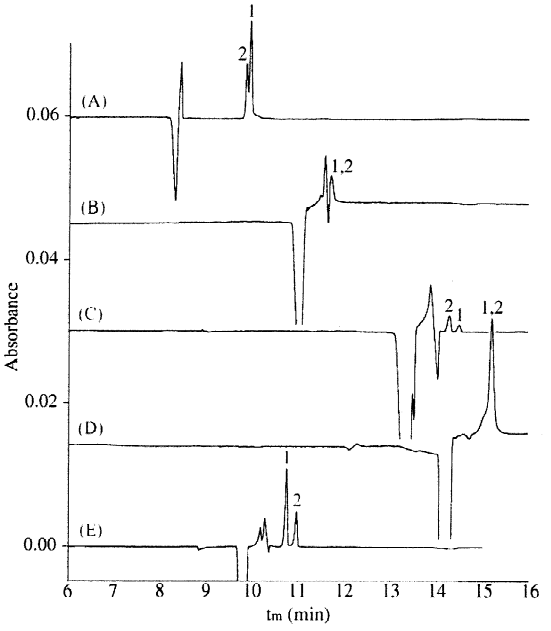

Fig. 2. Electrokinetic chromatograms of amygdalin epimers under CD-MEKC analysis. Run buffer, 20 m*M* Tris–HCl (pH 9.0), 80 m*M* surfactant [(A) SDS, (B) sodium dehydrocholate, (C) sodium cholate, (D) sodium taurocholate, (E) SDOC], 25 m*M* α-CD; separation tube, 770×0.05 mm I.D.; length of the tube used for separation, 700 mm; applied voltage, 20 kV; detection wavelength, 200 nm; temperature, 25°C. Peaks: 1, amygdalin; 2, neoamygdalin.

3.2. Effect of α- and β-CDs

The inclusion-complex formation of the desired components with CD depends on the molecular size and the cavity diameter of CD in addition to the hydrophobicity. The effect of α- and β-CDs on the selectivity of amygdalin and neoamygdalin were shown in Fig. 3. The amygdalin epimers were clearly separated by the addition of α-CD, because of their different response to the α-CD. The asymmetric carbon, which distinguishes the epimers, has unsubstituted phenyl group as a hydrophobic moiety. And α-CD has the 0.47–0.52-nm (diameter) hydrophobic cavity [13], which should be the reason for the presence of specific inclusion of the amygdalin epimers. On the other hand, the amygdalin epimers could not be resolved by the use of β-CD, which has bigger size of cavity than α-CD.

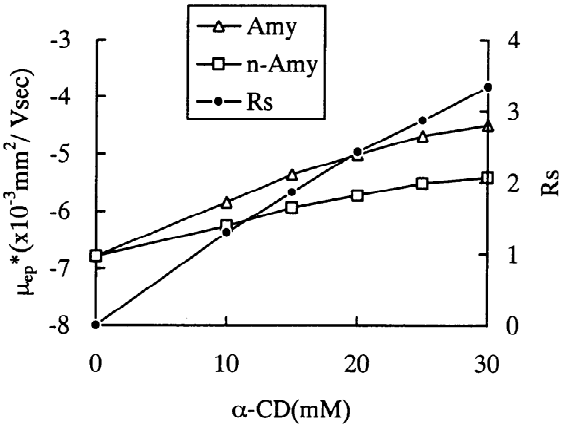

Fig. 4. Effect of α-CD concentration on the resolution of amygdalin epimers. The run buffers were the solution containing 0–30 m*M* α-CD. Other conditions as in Fig. 2. μ^*_{ep}: The effective electrophoretic mobility. R_s: The peak resolution between amygdalin and neoamygdalin.

3.3. Effect of the α-CD concentration

The effective electrophoretic mobility for the amygdalin epimers was found to be dependent on α-CD concentration (Fig. 4). The effective electrophoretic mobility of amygdalin is more influenced than that of neoamygdalin with the concentration of α-CD, since amygdalin could form a stronger complex with α-CD than neoamygdalin. Therefore, the resolution of the amygdalin epimers was increased with an increased α-CD concentration.

3.4. Effect of the SDOC concentration

The effect of the SDOC concentration on the effective electrophoretic mobility of the amygdalin epimers was studied (Fig. 5). The effective electrophoretic mobility of neoamygdalin was influenced

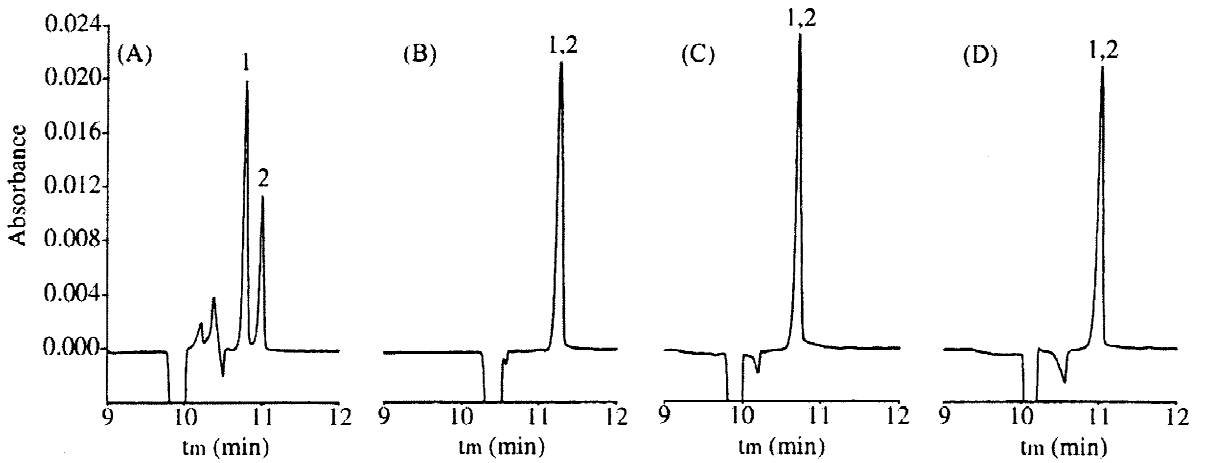

Fig. 3. Effects of α-CD and β-CDs on the resolution of amygdalin epimers. The run buffers were the solutions containing 25 m*M* cyclodextrin [(A) α-CD, (B) β-CD, (C) heptakis (2, 6-di-*O*-methyl)-β-CD, (D) heptakis(2, 3, 6-tri-*O*-methyl)-β-CD]. Other conditions as in Fig. 2.

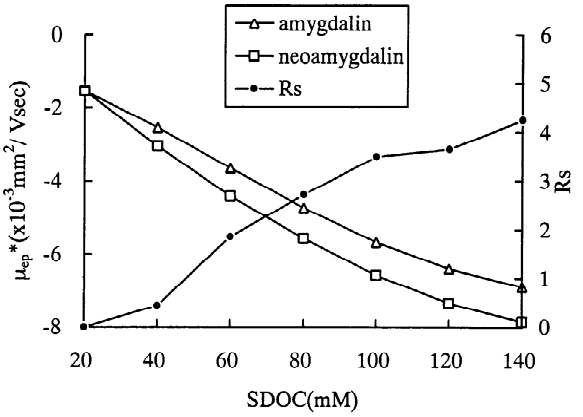

Fig. 5. Effect of SDOC concentration on the electrophoretic mobilities of amygdalin epimers. The run buffers were the solutions containing 20–140 m*M* SDOC. Other conditions as in Fig. 2. μ_{ep}^{*}: The effective electrophoretic mobility. R_s: The peak resolution between amygdalin and neoamygdalin.

more than that of amygdalin, because of its stronger interaction with SDOC micelle. As a result, the resolution of the amygdalin epimers was increased with the increased SDOC concentration, markedly less than 80 m*M* SDOC.

It is reported that CD-MEKC is useful for the separation of electrically neutral and highly hydrophobic compounds [8]. In this study, the CD-MEKC method is also found to be quite suitable for the separation of electrically neutral and highly hydrophilic compounds, such as amygdalin epimers.

3.5. Analysis of amygdalin and neoamygdalin in the crude drugs and the Chinese herbal prescriptions

Persicae Semen and Armeniacae Semen extracts were subjected to the CD-MEKC under the same conditions as the standard samples of the amygdalin epimers. An excellent resolution was achieved for both drugs with the α-CD incorporated SDOC buffer system (Fig. 6). Other constituents in the crude drug extracts did not interfere the mobility of the amygdalin epimers in this condition. The content of amygdalin and neoamygdalin in Armeniacae Semen was 3.2 and 0.15%, respectively, and in Persicae Semen was 4.4 and 0.20%, respectively.

The same method was applied to the Chinese herbal prescriptions, Keishi-bukuryo-gan containing

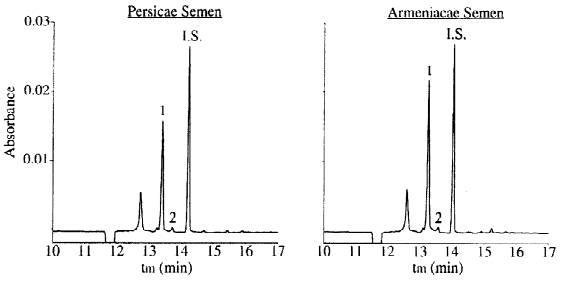

Fig. 6. Electrokinetic chromatograms of extractions of commercially available Persicae Semen and Armeniacae Semen. Peaks: 1, amygdalin; 2, neoamygdalin; I.S., internal standard.

Persicae Semen and Mao-to containing Armeniacae Semen. The chromatograms of the amygdalin epimers in the extract of Keishi-bukuryo-gan and Mao-to were shown in Fig. 7. In this method, the amygdalin epimers were clearly separated with other constituents in the complex mixture of the extract. The content of amygdalin and neoamygdalin was 3.2 and 1.3% in Keishi-bukuryo-gan extraction and 7.2 and 2.6% in Mao-to extraction, respectively. The increase of neoamygdalin ratio in the Chinese herbal prescriptions, in comparison with in the crude drugs, showed the epimerization of amygdalin during the decoction.

Quantitative analysis was achieved by using the internal standard. The calibration graphs for amygdalin and neoamygdalin showed good linearity in the concentration range, 102–406 mg/l and 49.6–199 mg/l, respectively.

For the regression equation $y = ax + b$, where x is the mass ratio of the amygdalin epimers vs. internal standard and y is the peak area ratio, correlation coefficients of peak area ratio (r) were as follows: for amygdalin, $y = 0.301x - 0.0225$ ($r = 0.9998$); for neoamygdalin, $y = 0.299x - 0.0121$ ($r = 0.9999$). The coefficients of variation of peak area ratios for amygdalin and neoamygdalin (six injections) were 1.15 and 1.76%, respectively. The recovery of the amygdalin epimers was tested by the addition of known amounts of the amygdalin epimers (amygdalin: 2.03 mg, neoamygdalin: 0.993 mg) to a Persicae Semen-blank fraction of Keishi-bukuryo-gan. The recoveries for amygdalin and neoamygdalin (the results of three injections) were 97.3–103% and 96.5–102%, respectively, in this method. These

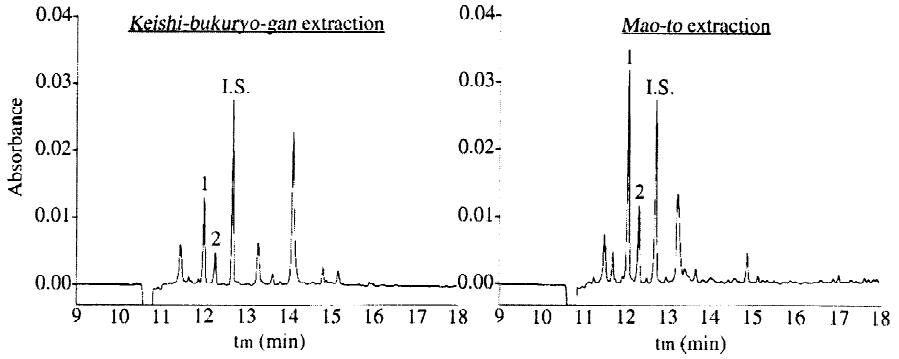

Fig. 7. Electrokinetic chromatograms of Keishi-bukuryo-gan extraction and Mao-to extraction. Peaks: 1, amygdalin; 2, neoamygdalin; I.S., internal standard.

results show that the CD-MEKC is sufficiently sensitive for the reproducible determination of the amygdalin epimers in the crude drugs and the Chinese herbal prescriptions.

4. Conclusions

We developed a CE method for the quantitative analysis of electrically neutral and highly hydrophilic epimers such as amygdalin and neoamygdalin, using SDOC micelle and the α-CD incorporated buffer system. The analysis can be completed within a short time in comparison to HPLC, without the use of huge volumes of toxic solvents. Because of specific resolution by CD-MEKC, especially for the analysis of the natural products or the complex mixture of herbal prescriptions containing the amygdalin epimers, this method is more significantly useful than MEKC. Consequently, this CD-MEKC is considered to be a very useful technique for the analysis of the amygdalin epimers in the crude drugs and the Chinese herbal prescriptions.

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