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Micellar electrokinetic chromatography for the analysis of D-amygdalin and its epimer in apricot kernel

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Abstract

We have developed a simple, rapid and reproducible method for the determination of D-amygdalin and its epimer by using micellar electrokinetic chromatography (MEKC). Separation of D-amygdalin was performed in a 20 m*M* sodium borate buffer (pH 8.5) containing 300 m*M* sodium dodecyl sulfate using a bare fused-silica capillary. The eluates were monitored by the absorbance at 210 nm. The applied electric field was 278 V/cm, and the time needed for the separation of D-amygdalin did not exceed 6 min. The calibration curve for D-amygdalin showed excellent linearity in the concentration range of 5–500 μg/ml. The migration time and the corrected peak area show relative standard deviations ($n=6$) of 0.86% and 1.48%, respectively. The limit of detection ($S/N=3$) for D-amygdalin was 2 μg/ml. Under acidic and neutral conditions, amygdalin exists only as the D-form; however, under basic conditions, it shows both the D- and L-forms with a concentration ratio of 1:1.3 (D-amygdalin/L-amygdalin). Results of HPLC, UV–Vis spectrophotometry, and mass spectrometry reconfirmed the identification of D-amygdalin and its epimer. The number of theoretical plates of D-amygdalin is about 100 000 in MEKC, which is significantly higher than ~8000 of HPLC. This method has been successfully applied to the determination of amygdalin epimers in various apricot kernel extracts and pharmaceutical products. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Amygdalin

1. Introduction

Apricot kernel (apricot; *Armeniacae semen*) extract originates from the semen of *Rosaceae species* (*Prunus armeniaca Linn'e* var. *ansu* Maximowicz) and its related species. It has been prescribed in many oriental medicinal formulations, providing assistance with antifussive, expectorant and laxative functions [1,2]. D-Amygdalin (Fig. 1) is the major component of apricot kernel extract, and the naturally occurring amygdalin possesses only the D-configuration. Recent interest in D-amygdalin has peaked due to the controversy concerning Laetrile, which has been purported to be useful both as an antineoplastic agent and as a secondary cancer chemotherapy agent [3,4].

D-Amygdalin tends to epimerize, particularly under basic conditions, because of the weakly acidic character of the benzylic proton. Since the two

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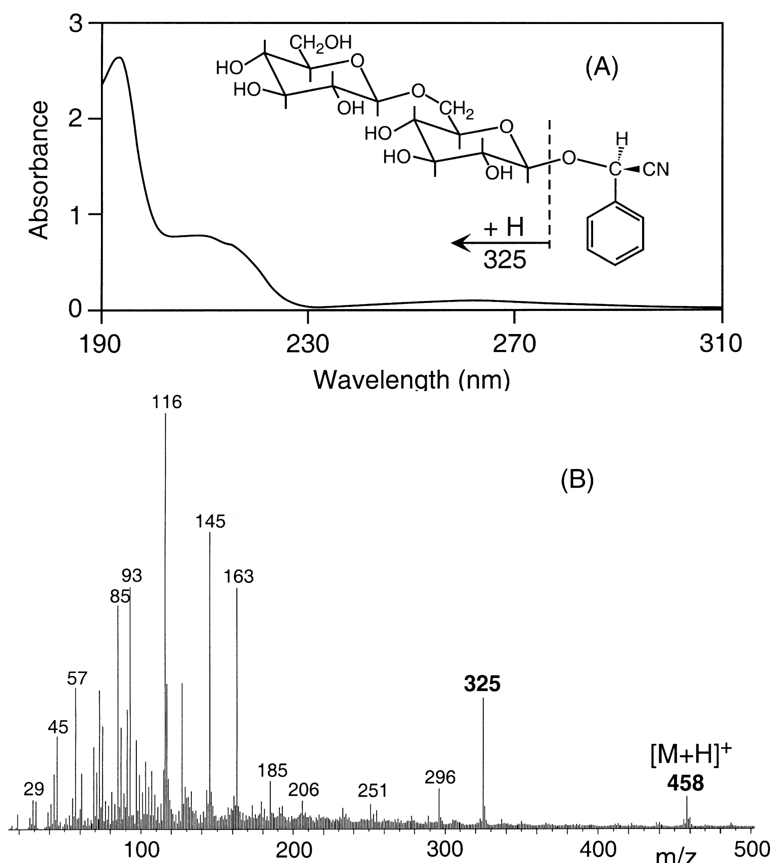

Fig. 1. (A) UV absorption spectrum for aqueous solution and (B) positive-ion mass spectrum obtained by fast atom bombardment of D-amygdalin.

epimers of amygdalin may have different physiological properties, it is important to develop a method for the quantitative analysis of amygdalin epimers. Cairns et al. employed various analytical methods for the identification and quantitative determination of amygdalin epimers [5]. However, they failed to obtain a full quantitative determination of D-amygdalin and L-amygdalin. Smith and Weber developed a method for the preparative and analytical separation of amygdalin and its related compounds by reversed-phase high performance liquid chromatography (HPLC), in which, however, the baseline resolution of amygdalin epimers was not satisfactory either [6]. Other analytical methods such as ^{13}C nuclear magnetic resonance (NMR), thin-layer chromatography (TLC), mass spectrometry (MS), HPLC, gas chromatography (GC), GC–MS, and an indirect determination using enzymatically-derived benzaldehyde have also been developed [3,6–10]. Unfortunately all these methods have shortcomings due to the difficulties in the sample pre-treatment, poor reproducibility, low efficiencies and long separation times, not to mention the fact that they fall well short of quantitatively determining the epimers of amygdalin.

In this report, we introduce a micellar electrokinetic chromatography (MEKC) method using sodium dodecyl sulfate (SDS) for the determination of D-amygdalin and its epimer in apricot kernel extract even without any pre-treatment. HPLC, UV–Vis spectrophotometry and MS reconfirmed the identification of D-amygdalin and its epimer.

2. Experimental

2.1. Chemicals

D-Amygdalin, SDS and Sudan III were purchased from Sigma (St. Louis, MO, USA). β-Cyclodextrin, HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Apricot kernel extract was provided by ALPS Pharmaceutical (Gifu, Japan). A 10-kg amount of apricot kernel was extracted three times with 30% (v/v) aqueous ethanol under standing at ambient for 3 days. The filtered extract was then concentrated in a vacuum at 40°C to give an aqueous ethanol extract of 1.0 kg. Water was purified with a Milli-Q TM/Milli-RO Water System (Millipore, Bedford, MA, USA). All other chemicals were reagent grade and used without further purification.

2.2. Sample preparation

The stock solution of standard D-amygdalin (1 mg/ml) and apricot kernel extract (1 mg/ml), prepared every week by dissolving in water, was stored at 4°C in the dark. As required, appropriate dilutions were made by the addition of water. In capillary electrophoresis (CE), the diluted solution of apricot kernel extract was filtered with a 0.45-μm membrane filter (Sartorius, Göttingen, Germany) just before the introduction into the CE system. In HPLC, the apricot kernel extracts were re-extracted three times with 50% (v/v) aqueous ether in order to eliminate any impurities. The aqueous layer was collected and cleaned up by use of C_{18} or NH_2 Sep-Pak cartridge (Millipore) before it was injected into the HPLC system. For the comparison of the configuration of amygdalin at various pH conditions, the amygdalin sample solutions were prepared in three different pH conditions. Sample I was aqueous D-amygdalin solution (100 μg/ml) whose pH was adjusted to 1.6–2.0 by adding of 630 μl of 1 *M* HCl to 10 ml solution. Sample II was 100 μg/ml aqueous D-amygdalin solution. Sample III was aqueous D-amygdalin sample solution (100 μg/ml) whose pH was adjusted to 10.9–11.2 by adding 100 μl of 20% (v/v) aqueous ammonia to 10 ml solution.

2.3. Micellar electrokinetic chromatography

All MEKC analyses were carried out using a P/ACE 5500 (Beckman, Fullerton, CA, USA) instrument, equipped with a diode-array detector monitoring at 210 nm. A bare fused-silica capillary (Polymicro Technologies, AZ, USA) of 27 cm (effective length 20 cm)×50 μm I.D. was kept at 25°C, and a voltage of 7.5 kV was applied along the capillary. The run buffer was a 20 m*M* sodium borate buffer (pH 8.5) containing 300 m*M* SDS. Samples were introduced with low pressure (0.5 p.s.i.$=3.4\cdot10^3$ Pa) for 3 s at the anodic end of the capillary. Methanol and Sudan III were used as the electroosmotic flow (EOF) marker and the micellar marker, respectively.

In order to identify and determine D- and L-amygdalin as separate entities in the electropherogram of the apricot kernel extracts, the MEKC system was readjusted with a longer bare fused-silica capillary [67 cm (effective length 60 cm)×50 μm I.D.] using a run buffer of 20 m*M* sodium borate buffer (pH 8.5) containing 150 m*M* SDS. When the long capillary of 67 cm was used, quantitative analysis of the baseline separated D-amygdalin was possible at a lower SDS concentration of 150 m*M*. The separation voltage was 18 kV and the operating temperature was set to 20°C. For the application of the MEKC method to the analysis of pharmaceutical formulations, we selected a sample, Tusna syrup (Hanil Pharmaceutical, Seoul, South Korea) which has been sold as an antifussive and expectorant drug for children. A 1-ml volume of the drug was diluted with 1 ml of water. The diluted-solution was filtered through a 0.45-μm membrane filter and was directly introduced into the MEKC system. The sample is composed of 12 ingredients, including apricot kernel extract. The MEKC separation temperature was increased to 25°C to reduce the viscosity of pharmaceutical formulation with sugar and other components.

2.4. Identification of epimers by HPLC, UV–Vis and MS

For the identification of D-amygdalin and its epimer, the separated peaks of the sample were collected by a HPLC system, and their UV spectra

and mass spectra were checked. The UV spectrum of D-amygdalin was obtained from a HP 8453 UV–visible spectrophotometer (Hewlett-Packard, Germany), whose results were compared with the spectra obtained by both the MEKC and the HPLC methods with a diode-array detector.

Liquid chromatography was performed by using a Waters HPLC system (Milford, MA, USA). We tried a number of HPLC separation conditions to effectively determine D-amygdalin in apricot kernel extract. One such condition involved a column (25 cm×4.3 mm I.D., particle size 5 μm) of LiChrosorb RP-18 (Merck) at 30°C with aqueous 10% methanol as the mobile phase (flow-rate 1.0 ml/min). The other involved a water–acetonitrile mixture as the mobile phase (flow-rate 1.2 ml/min). For the identification of D-amygdalin and its epimer by MS, the sample fraction was collected with a fraction collector.

The fast atom bombardment (FAB) tandem mass spectra were taken with a JMS-AX505 WA spectrometer (JEOL, Tokyo, Japan). The ion source was operated at 10 kV accelerating voltage in the positive mode with a mass resolution of 1000 (10% valley). The ions were produced by FAB using a cesium ion gun operated at 22 kV (filament current: 2.5 A). Aqueous samples were mixed with glycerol in the positive mode on the FAB probe tip.

3. Results and discussion

3.1. Optimization of MEKC

At pH 6.5–8.5, D-amygdalin exists in a neutral form with the absorption maximum at 200±10 nm in aqueous solution (Fig. 1A). In order to analyze D-amygdalin as a non-ionic form, we tried several sodium borate buffers with acetonitrile and anionic surfactant SDS at different pH values. At pH≥9.0, the peak of D-amygdalin was split into two, implying that that the aglycone entity or non-sugar derived chiral center of mandelonitrile is susceptible to epimerization under basic conditions. The epimerization would be promoted by the presence of the weakly acidic the benzylic proton [5]. Meanwhile, at lower pH (pH<5.0), SDS can not be used because the EOF could be drastically reduced or reversed [11]. So we chose pH 8.5 for the determination of D-amygdalin in order to avoid the stability problem of D-amygdalin. Addition of an organic solvent such as acetonitrile or β-cyclodextrin into the run buffer system did not improve the resolution of the D-amygdalin peak. We found a 20 m*M* sodium borate buffer (pH 8.5) with 300 m*M* SDS to be the optimal run buffer. As we varied the applied voltage in the range 5–15 kV along the 27 cm (effective length 20 cm)×50 μm I.D. capillary, we found the optimal condition at 278 V/cm. A diode-array detector was used to measure the UV absorption spectrum of each separated peak in order to confirm the peak identity. The UV spectra of these two peaks were found to be very similar to each other. In particular, in apricot kernel extracts, both the matched migration times and the similar UV spectra indicate that they are the same compounds.

The resolution (R_s) of the apricot kernel extract from a nearby unknown peak is largely dependent on the concentration of SDS. At below 250 m*M* SDS in the run buffer (pH 8.5), the peak of D-amygdalin overlaps with the unknown peaks. Meanwhile, with 350 m*M* SDS, the baseline resolution increases, while the analysis time is significantly prolonged. Fig. 2 shows that the value of R_s is increased as the SDS concentration. In fact, the maximum value of R_s can be obtained when the electrophoretic mobility (μ_{eo}) is just balanced by the EOF as $\mu_{eo} \approx -\mu$ (mobility of component). This means that a long analysis time is required in order to obtain the highest resolution. The resolution was 1.62 at 300 m*M* SDS, for which the conditions were considered to be optimal for the quantitative analysis. When we calculated the efficiency (N) for the electropherogram of D-amygdalin obtained at the selected MEKC separation condition, it showed a value of ~100 000. Fig. 2 also shows the effect of the SDS concentration on the capacity factor (k'). As the SDS concentration increases, k' increases monotonically. If D-amygdalin does not interact with the micelle at all ($k'=0$), the migration time of D-amygdalin would be equal to t_0. It is to be noted that the migration time window was limited between t_0 (migration time of methanol) and t_{mc} (migration time of Sudan III) in our experiment.

3.2. Linearity, limit of detection and relative standard deviation

The calibration curve was obtained by using the

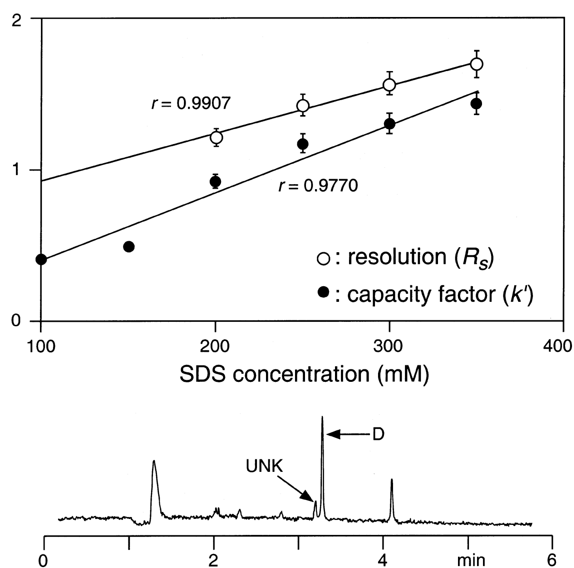

Fig. 2. Effects of SDS concentration on the capacity factor (k', ●) and the resolution (R_s, ○) of D-amygdalin in the MEKC method. Lines represent least-squares fitting to the data. The vertical bars represent the standard deviations ($n=3$). $k'=(t-t_0)/[t_0\{1-(t/t_{mc})\}]$, where t_0 and t_{mc} represent the migration times of methanol and Sudan III, respectively. MEKC conditions: run buffer, a solution of 20 mM sodium borate buffer (pH 8.5) and 300 mM SDS; applied voltage, 7.5 kV at 25°C; a bare fused-silica capillary of 27 cm (effective length 20 cm)×50 μm I.D.; hydrodynamic injection for 3 s at 0.5 p.s.i. *D: D-Amygdalin. UNK: Unknown peak.

standard solutions of D-amygdalin. The concentration versus the velocity-corrected peak area (=peak area· migration velocity) was plotted, and its regression line was used for the determination of sample concentrations. The calibration curve for D-amygdalin in the MEKC was linear over the concentration range 5–500 μg/ml. The regression curve was given by $y=357.1429x+1.2857$ (the linear correlation coefficient, $r=0.9992$), where y is the concentration (μg/ml) of D-amygdalin and x is the velocity-corrected peak area (10^{-6}·absorbance·cm). The relative standard deviations (RSDs) of the migration time and the corrected peak area for D-amygdalin were also obtained by analyzing the results on the standard solutions six times. The limit of detection ($S/N=3$) for D-amygdalin was 2 μg/ml and RSD ($n=6$) of the migration time and the corrected peak area were 0.86% and 1.48%, respectively.

3.3. Epimers of amygdalin

Under acidic and neutral conditions (samples I and II in Section 2.2), amygdalin showed only the D-form (Fig. 3A and B). However, under the basic condition (sample III), amygdalin showed both forms (D- and L-form) with a clear baseline resolution (Fig. 3C). The epimeric pair was completely analyzed within 6 min and both forms could be determined by use of D-amygdalin as the standard.

A typical positive-ion FAB mass spectrum of D-amygdalin displays abundant protonated species ($[M+H]^+$, m/z 458) (Fig. 1B). The peak at m/z 325 corresponds to the diglucoside ion generated by the loss of DL-mandelonitrile. The mass spectra of the two epimers were nearly identical. By the conventional HPLC method, the epimers were separated within about 140 min for the standard amygdalin sample solution whose pH was adjusted to 10.9–11.2 by adding of 20% (v/v) aqueous ammonia (Fig. 4C). In the MEKC method, the identification of the amygdalin epimers was reconfirmed by the spiking of L-amygdalin collected by the HPLC method using the modified method of Cairns et al. [5]. The ratio of D-amygdalin-to-L-amygdalin was 1:1.3 in the basic condition (Table 1). The result was the same as in the HPLC method, and reconfirmed that the aglycone or non-sugar derived chiral center of mandelonitrile is susceptible to epimerization in the basic condition because of the weakly acidic character of the

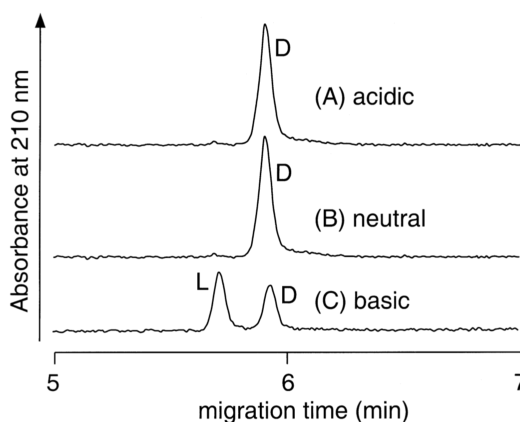

Fig. 3. Epimerization of amygdalin at various pH. (A) pH 1.6–2.0 (sample I), (B) pH 6.5–7.0 (sample II), and (C) pH 10.9–11.2 (sample III). MEKC conditions as in Fig. 2. *D: D-Amygdalin. L: L-Amygdalin.

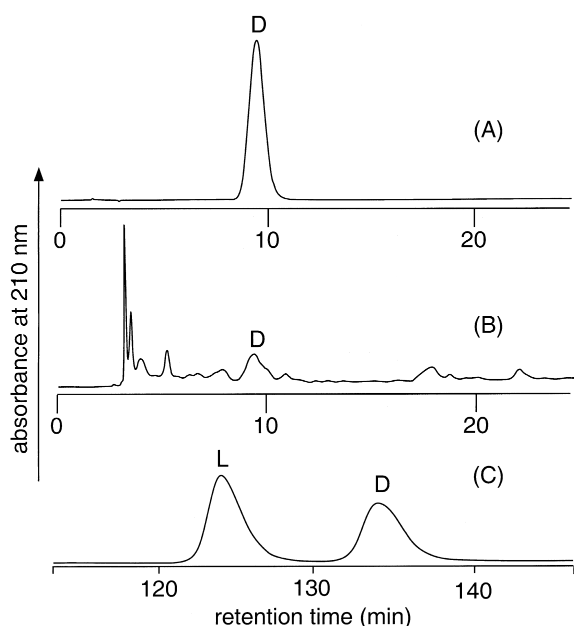

Fig. 4. HPLC chromatograms of (A) standard D-amygdalin, (B) apricot kernel extract, and (C) the epimers of amygdalin. HPLC conditions for (A) and (B): column, LiChrosorb RP-18 (25 cm× 4.3 mm I.D., particle size 5 μm) at 30°C; mobile phase, aqueous 10% methanol; flow-rate, 1.0 ml/min; detection at 210 nm. HPLC conditions for (C): mobile phase, aqueous 2% acetonitrile; flow-rate, 1.2 ml/min; other HPLC conditions were the same as for conditions (A) and (B). *D: D-Amygdalin. L: L-Amygdalin.

benzylic proton. The concentrations of D-amygdalin in various apricot kernel extracts were measured in the range 119.0–138.7 mg/ml and the mean±SD (n=6) was 126.9±7.85 (Table 2). These results indicate that the content of D-amygdalin in apricot kernel is about 1.2–1.4% because the apricot kernel extract used in our experiment was 30% (v/v) ethanol extract (apricot kernel:apricot kernel 30%, v/v, ethanol extract=10:1, w/w).

Fig. 5C shows an electropherogram obtained by applying the MEKC method in a pharmaceutical formulation. Although the sample contained 12 components, the separation of D-amygdalin showed a baseline resolution in the MEKC condition. We could easily determine D-amygdalin in the pharmaceutical formulation by control of temperature, capillary length and applied electric field. These results show that the determination of D-amygdalin in apricot kernel extracts, its standardization, and quality control in pharmaceutical plants or bulky samples are possible by the MEKC method. Especially, quality control in the pharmaceuticals is important because D-amygdalin is a component of a natural product,

Table 1
The epimer and ratio of amygdalin at various pH conditions

Sample	Epimers	Ratio D-form:L-form
I (pH 1.6–2.0)	D-Amygdalin	1:0
II (pH 6.5–7.0)	D-Amygdalin	1:0
III (pH 10.9–11.2)	D-Amygdalin, L-amygdalin	1:1.3[a]

[a] This result is the same as in the HPLC method.

Table 2
Contents of D-amygdalin in various apricot kernel extracts

Sample[a]	D-Amygdalin[b] (mg/ml)	Mean±SD[c]	RSD[d] (%)
I	138.7		
II	119.9		
III	120.2	126.9±7.85	6.19
IV	130.2		
V	125.3		

[a] Sample: Different apricot kernel extracts.
[b] Average content (n=6) of D-amygdalin in apricot kernel extracts (mg/ml).
[c] SD: Standard deviation.
[d] RSD: Relative standard deviation.

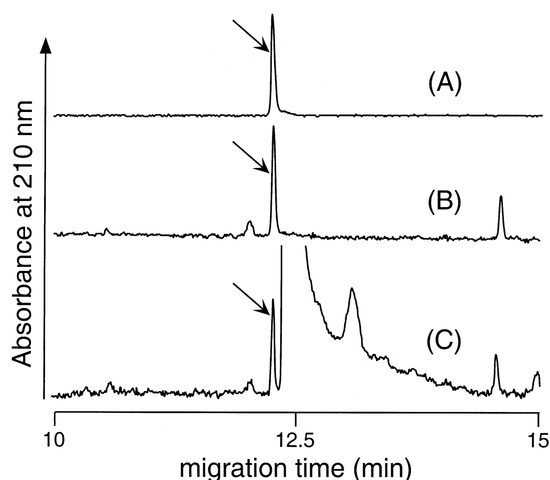

Fig. 5. MEKC electropherograms of (A) standard D-amygdalin, (B) apricot kernel extract, and (C) a pharmaceutical formulation. MEKC conditions: run buffer, a solution of 20 mM sodium borate buffer (pH 8.5) and 150 mM SDS; applied voltage, 20 kV at 25°C; a bare fused-silica capillary of 67 cm (effective length 60 cm)×50 μm I.D.; hydrodynamic injection for 3 s at 0.5 p.s.i. *Arrow: D-Amygdalin.

apricot kernel extract, which contains a lot of other components.

3.4. Comparison of HPLC and MEKC

Compared with the HPLC method, the MEKC method was much simpler, faster and more efficient. In the HPLC method, the separation time of the standard D-amygdalin is about 10 min with the efficiency approaching 8000 (Fig. 4A). However, the peak of D-amygdalin could not be perfectly separated in apricot kernel extracts at the HPLC condition (Fig. 4B). As the ratio of water in the mobile phase increased, the baseline separation showed a better resolution; however, its separation time was greatly increased (about 140 min, Fig. 4C). When the HPLC method was applied to the apricot kernel extracts, we did not obtain reproducible results. Meanwhile, in the MEKC method, the separation time of D-amygdalin was within 6 min and *N* was about 100 000. This means that the efficiency is 13-times higher and the separation time is 23-times faster in the MEKC than in the HPLC method. In general HPLC methods for the determination of natural products, the sample should be pre-treated by solvent extraction or cleaned up by using a C_{18} or NH_2 Sep-Pak cartridge. However, in spite of the pre-treatment, we could not quantitatively determine the D-amygdalin in apricot kernel extracts because the sensitivity and reproducibility of the measurements gradually decreased during the analysis owing to the adsorption of sample on the column. Meanwhile, in the MEKC, washing the capillary with NaOH and water after each injection recovered the reproducibility.

4. Conclusion

Our results have demonstrated that the MEKC method can be readily used to determine D- and L-amygdalin in various apricot kernel extracts and pharmaceutical formulations. Compared with the HPLC method, the MEKC analysis was much faster and more efficient. The main advantage of the MEKC over the HPLC method is the superior resolution, which enables the quantitative analysis of samples that can not be analyzed by HPLC. Considering the results of this study, the MEKC method should be highly suitable for the quality control of apricot kernel extracts. It can be also applied to pharmaceutical formulations. In the basic condition, the ratio of D-amygdalin-to-L-amygdalin is 1:1.3, suggesting that L-amygdalin is more stable than D-amygdalin in the basic condition.

Acknowledgements

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