On-Line Micellar Electrokinetic Chromatography-Electrospray Ionization Mass Spectrometry for the Direct Analysis of Amygdalin Epimers

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D-Amygdalin (Figure 1) is one of cyanoglycosides and the major component of apricot kernel extract, providing anti-tussive, expectorant, laxative activities[1,2] and also useful as both an antineoplastic agent and 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.[5] Since the amygdalin epimers may have different physiological properties, it is significant to develop a method for the direct identification and quantitative analysis of amygdalin epimers.

Various methods such as high-performance liquid chromatography (HPLC), carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectroscopy, chemical ionization mass spectrometry and gas chromatography with flame ionization detection (GC-FID) have been employed for the identification and quantitative determination of amygdalin epimers.[6-13] Recently, capillary electrophoresis (CE) methods have been developed for the determination of amygdalin epimers.[14-16] Surprisingly, although the amygdalin coexisted in D- and L- amygdalin forms at neutral or basic conditions, only the D-isomer exists under acid conditions.[14,16] This phenomenon suggests that the dominant form of amygdalin epimer could be easily changed during the sample pretreatment process for isolating each amygdalin epimers. However, present methods including our previous scheme focus only on the quantitative determination of amygdalin epimers.[14-16] These methods also required time consuming complicated procedures to prepare the sample. Therefore, there is a need to develop a direct on-line method for rapid and simple identification of amygdalin epimers in small sample volumes. Coupling micellar electrokinetic chromatography (MEKC) with mass spectrometry (MS) may solve the problem with respect to identification of the unknown epimer compounds. MS provides important information not only about the mass, but also about the structure of the separated compounds.[17-19] In this study, MEKC was coupled to ESIMS for the direct identification of amygdalin epimers in apricot kernel extract without any sample pretreatment. Only by controlling fundamental parameters, the amygdalin epimers were identified within 35 min.

Material and Methods

Chemicals. D-Amygdalin, sodium dodecyl sulfate (SDS), formic acid, ammonium acetate, acetic acid, sodium borate, boric acid and Sudan III were purchased from Sigma (St. Louis, MO, USA). Apricot kernel extract was provided by ALPS Pharmaceutical Ind. Co., Ltd. (Gifu, Japan). β-Cyclodextrin, HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, FR, Germany). Water was purified with a Milli-Q TM/Milli-RO® Water System with a 0.22 μm-membrane filter Millipak® 40 (Bedford, MA, USA). All other chemicals were of reagent grade.

Sample preparation. The amygdalin sample solution and apricot kernel extract were prepared by using a procedure previously described by Kang and coworkers.[16] As required, appropriate dilutions were made by addition of water. For MEKC, the apricot kernel extract was filtered through a 0.45-μm membrane filter (Sartorius Göttingen, Germany) just prior to its introduction into the CE system. For the on-line MEKC-ESIMS, the stock solutions of D-amygdalin (100 μg/mL), which were prepared weekly by dissolving in the aqueous ammonium acetate buffer (pH 7.0) and SDS, were stored at 4 °C in darkness. Appropriate dilutions were made as required daily. The sample of amygdalin epimer was aqueous D-amygdalin solution (100 μg/mL) whose pH was adjusted to 11 by adding 100 μL of 20% (v/v) aqueous ammonia to 10 mL of the stock solution of D-amygdalin.

Direct infusion and on-line MEKC-ESIMS. The on-line MEKC-ESIMS separations were performed with an ATI Unicam Model 200 Crystal CE system (Boston, MA, USA). The CE capillary was a bare fused-silica capillary with dimensions of 50 μm i.d. and 360 μm o.d. The running buffers contained ammonium acetate buffer (pH 7.0) and SDS. The applied voltage was 20-30 kV. The analytes were injected hydrodynamically at 250 mbar (= 25×10^3 Pa) for 3-5 s. The CE system was coupled to a Micromass Quattro II triple quadrupole mass spectrometer equipped with an electrospray ionization source (Altrincham, WA, UK). The Micromass Quattro II electrospray adapter kit containing both gas and liquid sheath tubes was used for the direct infusion. A solution (49.5/50/0.5, v/v/v = methanol/water/formic acid) containing D-amygdalin (1 ng/mL) was directly infused at 10 μL/min using a Hewlett-Packard HPLC system (Palo Alto, CA, USA). By applying a 3 kV electric potential at the electrospray needle, the first quadrupole was scanned from *m/z* 100 to 1100 with the scan rate of 2.0 s/scan for detection of D-amygdalin. For the integration of MEKC with ESIMS, a fused-silica capillary with 50 μm i.d. and 360 μm o.d. was mounted within the electrospray probe. The sheath

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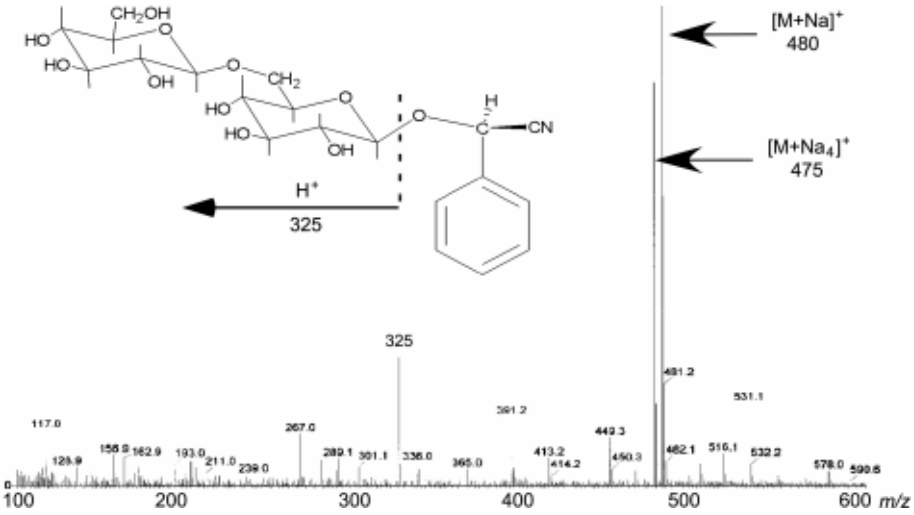

Figure 1. Structure and positive ion mode electrospray ionization mass spectrum of 1 ng/mL D-amygdalin.

capillary was fixed ~0.5 mm outside the probe tip, and the CE capillary was extended 0.2 mm beyond the sheath capillary. The inlet reservoir containing SDS and ammonium acetate buffer was maintained at the same height as the electrospray needle. The sheath liquid was made up of 49.5/50/0.5 (v/v/v) methanol/water/formic acid, and was delivered to the probe tip at the rate of 10 μL/min by a Hewlett-Packard HPLC pump. High-purity N_2 gas was supplied at the rate of ~20 L/h as the sheath gas for the coaxial probe. The drying gas was maintained at 175 L/h for liquid flow rates through the probe up to 50 μL/min. The ESI voltage of +3.0 kV was applied to the capillary tip, and the cone voltage was set at 20 V for the full-scan analysis.

Results and Discussion

A positive ion mode electrospray ionization mass spectrum of 1 ng/mL of D-amygdalin by direct infusion is shown in Figure 1. The peak at *m/z* 480 corresponds to the $[M+Na]^+$ and *m/z* 475 corresponds to the $[M+NH_4]^+$. The peak at *m/z* 325 corresponds to the diglucoside ion generated by the loss of DL-mandelonitrile. For the long capillaries in the MEKC-ESIMS experiments, application of pressure with voltage helped to accelerate the elution of the analyte (Figure 2). When we applied 28.4 kV of applied voltage with 0.5 psi (= 3.4 kPa) pressure, the resolution of epimers was decreased to R = 1.2. However the migration time also decreased to about 10 min, and the identification of amygdalin epimers remained possible. Figure 3 shows typical MEKC-ESIMS electropherogram. The amygdalin epimers were eluted within 35 min and monitored by the ESIMS. The peaks at *m/z* 480 and 475 were observed at the separated peaks in MEKC-ESIMS. Although increasing the concentration of SDS decreased the sensitivity and the efficiency of the MEKC-ESIMS, the 30-mM SDS was enough to obtain baseline separation of amygdalin epimers at the MEKC-ESIMS system without significant decreasing of ESIMS sensitivity. The amygdalin epimers showed baseline separation with a resolution (R) of 1.5 and eluted within 35 min.

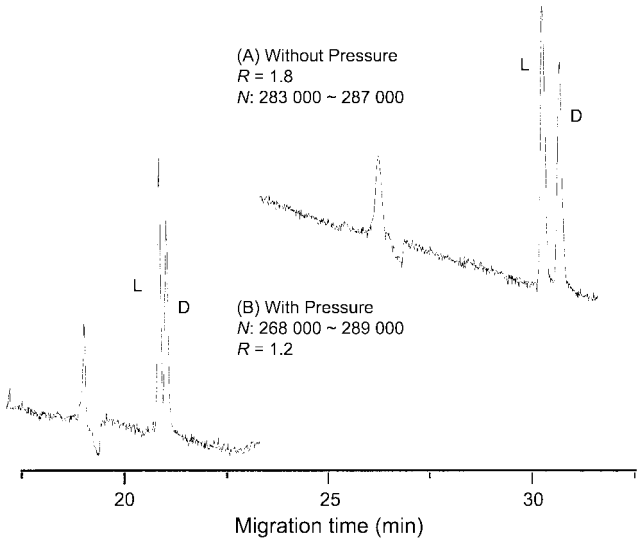

Figure 2. MEKC electropherograms demonstrating the pressure effect in MEKC-UV system. (A) Applied voltage of 28.4 kV without pressure at 25 °C. (B) Applying voltage of 28.4 kV and low pressure of 0.5 psi at 25 °C. Others MEKC conditions: running buffer, 20 mM ammonium acetate buffer (pH 7.0) and 30 mM SDS; a bare fused-silica capillary of 127 cm long (effective length 120 cm) × 50 μm i.d.; hydrodynamic injection for 5 s at 0.5 psi. Sample: 100 μg/mL D-amygdalin dissolving in solution of pH 11.0. *Indicates: N = Efficiency. R = Resolution.

The ratio of D-amygdalin to L-amygdalin was 1 : 1.3, which was same as the previous result obtained at UV detection.[16] When we applied 30 kV of applied voltage with 34-mbar (= 3.4 kPa) pressure, the resolution of epimers was decreased to $R \approx 1.2$. However the migration time also decreased, and the identification of amygdalin epimers was also possible. Thus, the author suggests that the co-application of applied voltage with the low pressure (< 3.4 kPa) could be a good technique to decrease the migration time when the long capillary is employed in CE-ESIMS. In the earlier MEKC-UV detections for the identification of amygdalin epimers, the separated peaks of the sample were collected by a HPLC system, and their UV spectra and mass spectra were check-

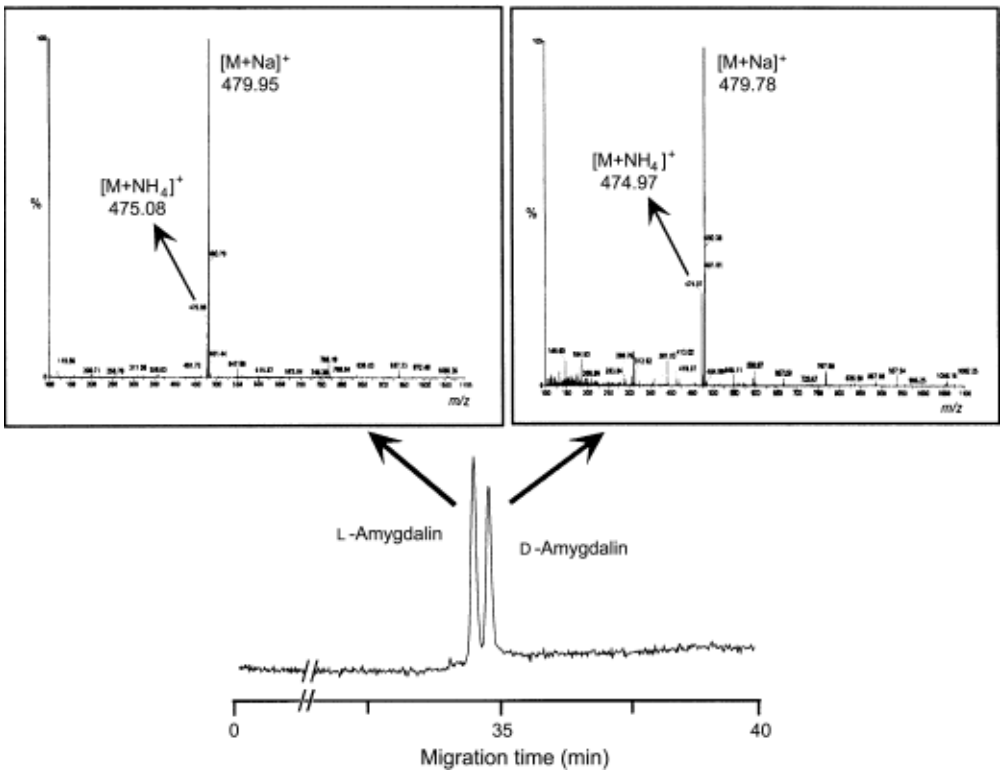

Figure 3. Total ion current electropherogram of the amygdalin epimers in a positive mode of MEKC-ESIMS system. Running buffer, 20 mM ammonium acetate buffer (pH 7.0) and 30 mM SDS running buffer; 50 μm i.d. × 135 cm total length capillary; 30 kV-applied voltage; hydrodynamic injection for 3 s at 250 mbar (= 25 kPa); sheath liquid, methanol/water/formic acid (49.5/50/0.5, v/v/v); ESI voltage +3.0 kV and cone voltage 20 V. Sample is same in the Figure 2. *Insets: Mass spectrum of its peak.

ed.[16] These techniques are still relatively time-consuming (~several hours). The dominant form of amygdalin epimer can be also changed during the collecting step of each separated peaks. Ideally, one would like to directly identify and quantitative low volume levels of the amygdalin epimers, so that the sample can be assayed quickly, avoiding the time consuming pretreatment steps. The MEKC-ESIMS provided a direct on-line method for rapid and simple identification of amygdalin epimers in small sample volumes (< 0.1 μL) without any pretreatment. In conclusion, on-line MEKC-ESIMS method was readily adopted for rapid identification and determination of amygdalin epimers without tedious pretreatment steps. The coupling of MEKC to ESIMS analysis solved the identification problems related to unknown epimer compounds without any pretreatment. Even though the separation time of amygdalin epimers in the MEKC-ESIMS method were relatively long time compared with the MEKC-UV method, the total analysis time of D-amygdalin and its epimer including the sample pretreatment was much shorter.

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