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Short communication

On-line sample cleanup and chiral separation of gemifloxacin in a urinary solution using chiral crown ether as a chiral selector in microchip electrophoresis

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Abstract

In chiral capillary electrophoresis of primary amine enantiomers using (+)-18-crown-6-tetracarboxylic acid ($18C6H_4$) as a chiral selector, the presence of alkaline metal ions in the sample solution as well as in the run buffer is undesirable due to their strong competitive binding with $18C6H_4$. A channel-coupled microchip electrophoresis device was designed to clean up alkaline metal ions from a sample matrix for the chiral analysis of amine. In the first channel, the metal ions in the sample were monitored by indirect detection using quinine as a chromophore and drained to the waste. In the second separation channel, gemifloxacin enantiomers, free of the alkaline metal ions, were successfully separated using only a small amount of the chiral selector (50 μM $18C6H_4$).

Keywords: Microchip electrophoresis; Chiral crown ether; Chiral separation; Gemifloxacin; Sample cleanup

1. Introduction

As a result of an increasing concern about the physiological, sensory, and toxicological properties of chiral compounds, many analytical methods including high-performance liquid chromatography and capillary electrophoresis (CE) have been applied to separate, identify, and quantify chiral compounds [1,2]. Microchip electrophoresis is based on the same separation principle as CE [3,4] and can be superior to CE in the incorporation of complex channels and parallel processing systems for high throughput analyses owing to modern micromachining technology. For example, precolumn [5], postcolumn [6], or two-dimensional [7] microchip electrophoresis devices could be easily fabricated by coupling microchannels. Very fast separation within a few milliseconds could also be achieved by constructing a very short separation channel of 200 μm [8]. Recently, high speed chiral separation of fluorescein isothiocyanate-labeled amino acids has been demonstrated using cyclodextrin as a chiral selector in microchip electrophoresis with a short separation channel of 7 cm [9].

Chiral crown ether, (+)-18-crown-6-tetracarboxylic acid ($18C6H_4$; Fig. 1a), is quite efficient in resolving chiral primary amines [10–12]. $18C6H_4$ can form diastereomeric complexes with protonated primary amine enantiomers and its chiral recognition stems from the difference in the complex formation energies. Alkaline metals such as sodium and potassium can also form strong complexes with $18C6H_4$, which is detrimental to capillary electrophoretic separation of amine enantiomers [13]. Thus, alkaline metal ions should be avoided as a buffer constituent when $18C6H_4$ is used as

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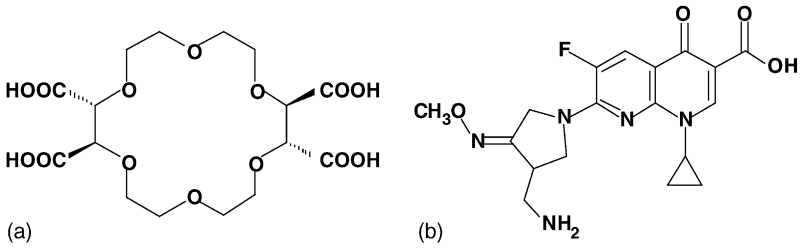

Fig. 1. The structure of (a) 18C6H4 and (b) gemifloxacin.

a chiral selector. Biological samples usually contain sodium and potassium ions at about 1 wt.% concentration [14]. Such metal ions not only in the run buffer but also in the sample solution could spoil the separation efficiency significantly [15]. Furthermore, direct injection of biological samples containing salts into a mass spectrometer for further analysis can cause electrospray instability and sensitivity reduction [16]. Various sample pretreatment methods such as solid phase extraction and dialysis often have to be applied to clean up such salts [16–22].

In this paper, we investigated channel-coupled microchip electrophoresis for the chiral separation of gemifloxacin [15,23,24], which is a primary amine and chiral antibacterial agent as shown in Fig. 1b, in a urinary solution using 18C6H4 as a chiral selector. In the first channel, highly mobile metal ions in a gemifloxacin sample, monitored by indirect detection, were removed to the waste and then gemifloxacin enantiomers were separated using a run buffer containing 18C6H4 in the second channel. Channel-coupled microchip electrophoresis will be suitable for desalting from the sample solution, considering its simplicity, analysis time, and solvent compatibility.

2. Experimental

2.1. Materials

18C6H4 was obtained from RStech (Daejeon, Korea) and Fluka (Buchs, Switzerland). Gemifloxacin was acquired from LG Chemical (Daejeon, Korea). Bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane (Bis–Tris), potassium chloride, and quinine were from Sigma (St. Louis, MO, USA). Sodium chloride was from Junsei Chemical (Tokyo, Japan). Citric acid (CA) was from Yakuri (Osaka, Japan). All reagents were of analytical grade and were used without further purification. Deionized water was obtained from a NANOpure purification system (Barnstead, Dubuque, IA, USA). Human urine was taken from a healthy volunteer and used immediately after collection.

2.2. Microchip fabrication

A microchip was fabricated using standard photolithographic, wet etching, and bonding techniques on a quartz substrate [3,6]. A wet-etching mask of a Cr/Au (200 Å/2000 Å) layer was deposited on both sides of a 500-μm thick quartz wafer by thermal evaporation. A positive photoresist (AZ1512; Clariant, Somerville, NJ, USA) was then spin-coated on both sides of the wafer, and the column design (Fig. 2) was transferred to the substrate using a film photomask (Ppmtechnology, Seoul, Korea). The patterned photoresist layer was hardened (110 °C; 1 h) and the unprotected Cr/Au layer was removed. Microfluidic channels on the quartz chip were then isotropically etched in 49 vol.% hydrofluoric acid solution for 20 min at 20 °C (etching rate ∼ 1.5 μm/min). The channels were trapezoidal with a depth of 31 μm and width of 68 and 94 μm at the bottom and top, respectively. Reservoirs were fabricated by a sandblaster using 20-μm sand with a 100-μm thick dry film resist (BF410; Tokyo Ohka Kogyo, Kanagawa, Japan) etch mask. The two wafers were directly bonded at room temperature and then annealed in a furnace (1000 °C, 3 h). Reservoirs were labeled as: (A) run buffer; (B) sample; (C) sample waste; (D) buffer waste; (E) run buffer containing a chiral selector; and (F) buffer waste containing the chiral selector, as shown in Fig. 2. The volume of each reservoir was 80 mm^3.

2.3. Microchip electrophoresis

Microchip electrophoresis was performed using a laboratory-constructed CE system as described in [15]. A He–Cd laser (Omnichrome, Chino, CA, USA) operating at 325 nm was used as a light source for laser-induced fluorescence detection. The fluorescent light was collected by a

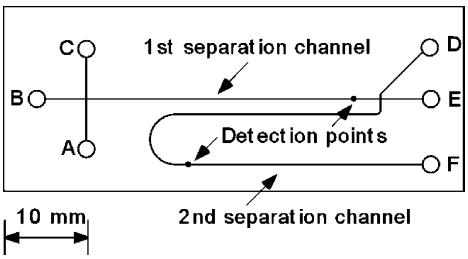

Fig. 2. Schematic layout and dimensions of the microchip. Reservoirs are labeled as: (A) run buffer; (B) sample; (C) sample waste; (D) buffer waste; (E) run buffer containing a chiral selector; and (F) buffer waste containing the chiral selector. The channel depth was 35 μm and width at a half-depth was 60 μm. The dots represent the detection points, which are located at 32 and 38 mm from the first and second injection crosses, respectively.

10× microscope objective (Edmund Optics, Barrington, NJ, USA) through a 405-nm bandpass filter (Melles Griot, Irvine, CA, USA) and was detected with an integrated photomultiplier tube (HC 120-01, Hamamatsu, Bridgewater, NJ, USA). Data acquisition and analysis were performed using an MIO-16-XE DAQ board (National Instruments, Austin, TX, USA) with LabVIEW 5.0.1 (National Instruments). Sample injection and separation on the microchip were performed using a multi-channel high-voltage power supply (Model 1000/3125, Canberra, Meriden, CT, USA), capable of generating 0–5 kV.

The run buffer for indirect detection in the first channel was (50 mM Bis–Tris + 10 μM quinine)/CA of pH 4.0. The chiral run buffer was prepared by dissolving 50 μM $18C6H_4$ in (50 mM Bis–Tris + 10 μM quinine) and then adjusting the pH to 4.0 with CA. The run buffer was pipeted into reservoirs A through D and the chiral run buffer into reservoir F. The first and second channels were filled with the run buffer with quinine and chiral run buffer, respectively, by applying a vacuum to reservoir E for 30 s. Then, reservoirs B and E were refilled with a sample solution and the chiral run buffer, respectively. The voltages applied to reservoirs A through D for the electrophoretic separation in the first channel were 5, 4.8, 4.3, and 3 kV, respectively, with other reservoirs floating. Sample injection in the first separation channel was performed under a gated sample-loading mode [6]; the electrokinetic injection was performed by floating a potential at reservoir A for 1 s, while keeping the potential of the other reservoirs fixed. Gemifloxacin was injected into the second separation channel by floating reservoir D, with reservoir F grounded, for 15 s right after the analyte passed the first detection point to ensure that all gemifloxacin was introduced into the second channel. The chiral separation of gemifloxacin enantiomers in the second channel was conducted by applying 3.0 kV at reservoir E, with reservoir F grounded and all other reservoirs floating. The detection points were located at 32 and 38 mm from the first and second injection crosses, respectively.

3. Results and discussion

The cleanup of metal ions was performed using the first separation channel including reservoirs A, B, C, and D. Monitoring of metal ions was conducted with an indirect detection method. A mixture of 50 mM KCl, 50 mM NaCl, and 100 μM gemifloxacin was separated with the run buffer of (50 mM Bis–Tris + 10 μM quinine)/CA of pH 4.0. Bis–Tris was used as a run buffer component to prevent the competitive binding with the chiral selector $18C6H_4$ [13]. 10 μM quinine was added to the run buffer as a chromophore for the indirect detection of metal ions in microchip electrophoresis. Two negative peaks and one positive peak were observed as shown in Fig. 3. The first negative peak at 0.3 min was identified as a potassium ion peak, and the second peak at 0.5 min as a sodium ion peak by spiking. Peak fronting was observed since metal ions have higher mobility than the run buffer cations [25]. The positive peak was assigned as a gemifloxacin racemate peak. Gemifloxacin could be successfully separated from metal ions since the latter have much higher mobilities than the former. The run-to-run reproducibility in the gemifloxacin peak was higher than 99% and further monitoring of the gemifloxacin peak in the first separation channel was not necessary for the later chiral separation of gemifloxacin in the second channel after initial confirmation. The migration time of gemifloxacin was 1.5 ± 0.1 min for multi-day runs.

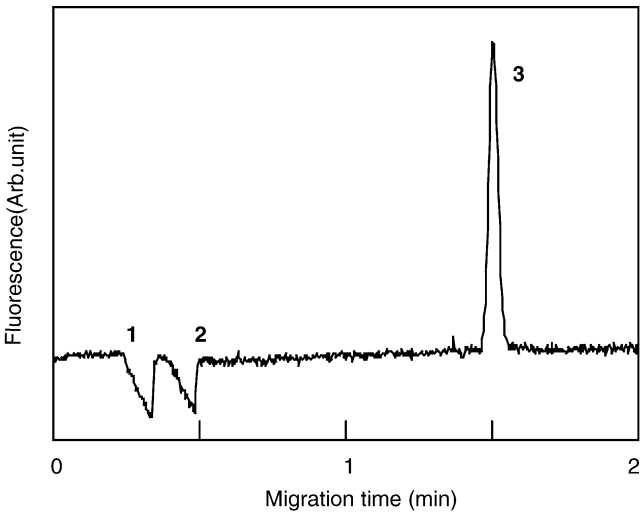

Fig. 3. Simultaneous detection of metal ions and gemifloxacin in microchip electrophoresis. Peaks: (1) K^+, (2) Na^+, (3) gemifloxacin racemate; run buffer: (50 mM Bis–Tris + 10 μM quinine)/CA of pH 4.0; sample solution: (50 mM NaCl + 50 mM KCl + 100 μM gemifloxacin) in the run buffer; light source: He–Cd laser (325 nm); indirect laser-induced fluorescence detection at 405 nm using a photomultiplier tube. The applied voltages applied to reservoirs A through D for the electrophoretic separation were 5, 4.8, 4.3, and 3 kV, respectively, with other reservoirs floating. Sample injection was performed by floating a potential at reservoir A for 1 s, while keeping the potential of the other reservoirs fixed.

In the previous study, the chiral separation of gemifloxacin dissolved in a highly saline solution was investigated using $18C6H_4$ as a chiral selector in CE and microchip electrophoresis [15]. The resolution between two gemifloxacin enantiomers was reduced from 4.0 to 1.5 in the presence of 20 mM sodium ion in the sample solution. Sodium ion binds to $18C6H_4$ more strongly than gemifloxacin ion [13]. Until the zones of sodium ion and gemifloxacin are well separated, sodium ion significantly inhibits gemifloxacin from binding to $18C6H_4$. Since $18C6H_4$ is highly negatively charged at pH 4.0, the complex formation with $18C6H_4$ decreases the mobility of sodium ion. This gives more time for sodium ion to interfere with the binding of gemifloxacin with $18C6H_4$, which leads to a reduction in resolution. Thus, the removal of sodium ions from the sample solution is essential for the efficient chiral separation of gemifloxacin.

Enantiomers of gemifloxacin in a urinary solution were separated on a coupled-channel microchip with a run buffer of (50 mM Bis–Tris + 10 μM quinine)/(50 μM $18C6H_4$ + CA) of pH 4.0 (Fig. 4). First, metal ions were separated from

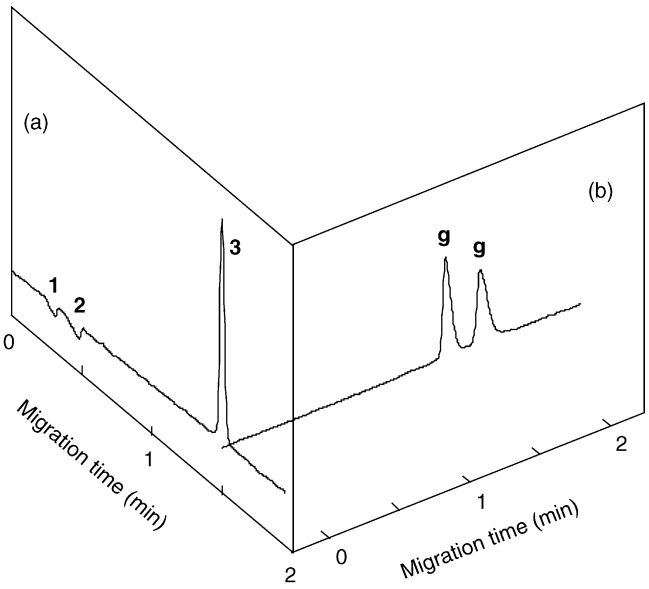

Fig. 4. Chiral separation of gemifloxacin dissolved in a urinary solution with microchip electrophoresis. Peaks: (1) K^+; (2) Na^+; and (3) gemifloxacin racemate. Peaks for gemifloxacin enantiomers are denoted by 'g'. (a) The removal of metal ions was performed in the first separation channel. (b) The chiral separation was performed in the second separation channel using a run buffer of 50 mM Bis–Tris/CA containing 50 μM 18C6H$_4$ (pH 4.0). A urinary solution was five-fold diluted using 50 mM Bis–Tris/CA (pH 4.0). Gemifloxacin was 100 μM in five-fold diluted urinary solution. The sample injection and electrophoretic separation in the first channel were performed as in Fig. 3. Gemifloxacin was injected into the second separation channel by floating reservoir D, with reservoir F grounded, for 15 s right after the analyte passed the first detection point to ensure that all gemifloxacin was introduced into the second channel. For the analysis in the second channel, the applied voltage was 3.0 kV at reservoir E with reservoir F grounded and all other reservoirs floating. Analytes were detected at 32 and 38 mm from the first and second injection crosses, respectively.

gemifloxacin racemate in the first separation channel to avoid the undesirable effects of metal ions (Fig. 4a). A successful chiral separation of gemifloxacin enantiomers was achieved in the second separation channel, which was filled with a run buffer containing only 50 μM 18C6H$_4$ (Fig. 4b). Quinine, which was used to monitor the elution of sodium ions, had a negligible effect on this chiral separation since it is a tertiary amine and hardly binds to 18C6H$_4$. Regardless of whether the second channel was filled with run buffer with or without 18C6H$_4$, the change in migration of gemifloxacin in the first channel was negligible, indicating that the diffusion of 18C6H$_4$ into the first channel is not problematic. The migration times for the gemifloxacin enantiomers were 1.6 ± 0.1 and 1.8 ± 0.1 min after the sample introduction into the second channel. Thus, the total analysis time was less than 4 min. The area ratios of the earlier to the later eluting gemifloxacin peaks were 50:50 and the resolution was 2.0, which is the same as when metal ions were not included in the sample solution using only the chiral buffer system and an effective separation channel length of 3.8 cm. Previously, gemifloxacin enantiomers dissolved in a urinary solution could be separated with a resolution of 2.0 on a polydimethylsiloxane microchip using a run buffer of 50 mM Bis–Tris/(15 mM EDTA + 0.5 mM 18C6H$_4$ + CA) of pH 4.0 and effective length of 9.4 cm [15]. Adding EDTA to a run buffer improved the separation efficiency of the gemifloxacin enantiomers, at the expense of resolution. By removing metal ions using a channel-coupled microchip, however, a similar resolution could be obtained using a smaller amount of 18C6H$_4$ (50 μM) and a shorter effective length of 3.8 cm.

4. Conclusions

The chiral separation of gemifloxacin in saline solution using 18C6H$_4$ as a chiral selector was investigated using a channel-coupled microchip electrophoresis device. Metal ions hampering the complex formation of primary amine analytes with 18C6H$_4$ were removed in the first separation channel using the difference in electrophoretic mobilities of metal and amine ions. In the second separation channel the enantiomers of gemifloxacin were successfully separated with a run buffer of (50 mM Bis–Tris + 10 μM quinine)/(50 μM 18C6H$_4$ + CA) of pH 4.0. By desalting the sample solution using a channel-coupled microchip electrophoretic device, we could avoid the undesirable competitive binding effect of the alkaline ions and significantly reduce the consumption of the chiral selector 18C6H$_4$.

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