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Chiral counter-current chromatography of gemifloxacin guided by capillary electrophoresis using (+)-(18-crown-6)-tetracarboxylic acid as a chiral selector

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Abstract

(+)-(18-crown-6)-tetracarboxylic acid ($18C6H_4$) has been known as a highly efficient chiral selector for resolving primary amine enantiomers in capillary electrophoresis (CE). We investigated the chiral separation of gemifloxacin using $18C6H_4$ in analytical counter-current chromatography (CCC). The separation conditions for CE, including the binding constant, pH, and run buffer constituents, provided a helpful guideline for chiral CCC. A successful separation of gemifloxacin enantiomers could be achieved using a two-phase solvent system composed of 1-butanol-ethyl-acetate-bis(2-hydroxyethyl)aminotris(hydroxymethyl)methane acetate buffer with a small amount of $18C6H_4$. The hydrophobicity of the solvent system and the $18C6H_4$ concentration were varied to optimize the chiral separation.

Keywords: Counter-current chromatography; Enantiomer separation; Crown ethers; Gemifloxacin; Fluoroquinolones; Antibiotics

1. Introduction

Counter-current chromatography (CCC) is a form of support-free liquid–liquid partition chromatography separating analytes by the differences in their partitioning between two immiscible liquid phases [1,2]. Although CCC shows a lower efficiency and requires a longer separation time compared to methods such as analytical HPLC and capillary electrophoresis (CE), it has several merits [1–3]. Since the CCC column requires no solid support, CCC is free from tedious column packing procedures and adsorption of solutes to the column, and the recovery of samples and reagents without contamination or decomposition is possible. As a consequence, the chromatographic parameters of analytical CCC separations are easily applicable to preparative-scale separations [4]. Therefore, one of the most potent applications of CCC could be chiral separation, especially on the preparative-scale, by adding a suitable chiral selector to a two-liquid phase system [2]. Additional benefit is that it should be possible to use the same column repeatedly for different chiral separations. However, examples of successful chiral separation with CCC are rather limited compared to those with other separation techniques such as HPLC [5]. A crucial part of successful enantiomer separation in CCC is the choice of an appropriate chiral selector. One method of finding a suitable chiral selector for CCC is to refer to examples of chiral separations with other separation techniques. *N*-dodecanoyl-L-proline-3,5-dimethyl anilide [6], vancomycin [7], sulfated β-cyclodextrin (S-β-CD) [8], and cinchona alkaloid derivatives [9], which had been well known as effective chiral selectors in HPLC or CE before being used in CCC, were used as chiral selectors in CCC.

CE is a promising technique for analytical chiral analysis [10–12]. It has many advantages including high efficiency, simplicity, and flexibility which make it possible to incorporate various chiral selectors rapidly with consumption of only a small volume of reagents. One shortcoming of CE is the intrinsic difficulty of scaling up. However, for chiral separation using a chiral selector in solution media, CE and CCC are both based on the differences in the binding constants of the two enantiomers with the chiral selector. Thus

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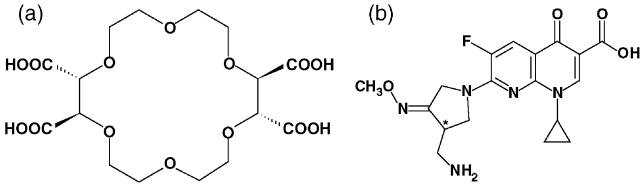

Fig. 1. (a) The structure of 18C6H4. (b) The structure of gemifloxacin. The asterisk denotes the chiral carbon.

the CE information on a chiral selector should be useful for chiral CCC using the same chiral selector.

In this paper, we investigated the separation of gemifloxacin enantiomers with CCC, guided by the results of chiral CE using chiral crown ether, (+)-(18-crown-6)-tetracarboxylic acid (18C6H4; Fig. 1a) as a chiral selector. Gemifloxacin (Fig. 1b) is a fluoroquinolone [13–17] that has broad spectrum antibacterial activity. It has a primary amine group and a chiral center on the pyrrolidine moiety. 18C6H4 has been used as an efficient chiral selector in CE to resolve primary amine enantiomers [11,18]. 18C6H4 has a macrocyclic polyether ring which forms stable inclusion complexes with alkali metals, ammonium ions, or protonated primary amines. In addition to the three hydrogen bonds between the protonated primary amine and the cavity of 18C6H4, secondary interactions between the carboxylic acid groups of 18C6H4 and the groups near the chiral center of the analytes are essential for chiral recognition [19]. Cho and coworkers [20,21] tested several chiral reagents and run buffers of CE and accepted 18C6H4 as the most effective chiral selector for primary amine enantiomers and recommended that the basic constituents of a run buffer should not bind to 18C6H4. The principal parameters and conditions from their CE analyses including the buffer constituents, the buffer pH, binding constants, etc. were helpful in choosing the CCC separation conditions for gemifloxacin enantiomers using 18C6H4.

2. Experimental

2.1. Reagents

(+)-(18-crown-6)-tetracarboxylic acid was purchased from RStech (Taejeon, South Korea) and Fluka (Buchs, Switzerland). Gemifloxacin was obtained from LG Chemical (Taejeon, South Korea). 1-Hexane, methanol (MeOH), 1-butanol (BuOH), 2-BuOH, ethyl acetate (EtOAc), chloroform, and bis(2-hydroxyethyl)aminotris(hydroxymethyl)-methane (Bis–Tris) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The organic solvents were of glass-distilled HPLC grade. Citric acid (CA) was from Yakuri (Osaka, Japan). Glacial acetic acid (HOAc) was from Merck (Darmstadt, Germany). Water was purified with a NANO pure II system (Barnstead, Dubuque, IA, USA) and a 0.22 μm Millipak 40 filter (Millipore, Bedford, MA, USA).

Each solvent mixture was degassed by vacuum filtration and thoroughly equilibrated in a separatory funnel. The two phases were separated shortly before use. The upper (more organic) phase was used as the mobile phase (MP) and the lower (more aqueous) phase as the stationary phase (SP). In order to reduce the consumption of 18C6H4, the chiral SP was prepared by dissolving a desired amount of 18C6H4 in 10 ml of the lower phase, which is slightly larger than one column volume (7.4 ml). At times helium gas was used for further degassing. Several buffers with different pH values and acid components were tested. Each buffer was prepared by titrating Bis–Tris aqueous solution with an acidic counterpart, such as CA or HOAc, to the desired pH. After adding 18C6H4, the pH of the lower phase changed by −0.1 to −0.2.

2.2. Counter-current chromatography

CCC analyses were performed using a PTR TCC-1000 (PharmaTech Research, Baltimore, MD, USA). It is a compact table top unit (30 cm × 30 cm × 40 cm) with a toroidal coil separation column. The separation column was made of 0.4 mm i.d. polytetrafluoroethylene (PTFE) tubing having a doughnut-shaped configuration (toroidal coil) around a cylindrical centrifuge bowl (12 cm in diameter and 5 cm in height). The volume of the column was 7.4 ml. The ends of the toroidal coil were connected to flow lines made of thick walled PTFE tubing (0.5 mm i.d.) which can be connected to other peripherals. Since these flow tubes are twist-free when the column is rotating, no rotary seals were needed. The TCC-1000 was equipped with a Rheodyne 7125 injector (Rohnert Park, CA, USA) having a 20 μl loop, a Pharmacia LKB HPLC pump 2248 (Uppsala, Sweden) for delivering the SP and a Sykam S1121 pump (Gauting, Germany) for the MP. By using a Rheodyne 5011 low-pressure rotary valve for solvent selection, the two pumps were connected to the column in turns. The column outlet was connected to an Orom FL-300 fluorescence detector (Seoul, Korea). The detection wavelengths were set at 270 nm for excitation and at 406 nm for emission. A back-pressure regulator (Supelco, Bellefonte, PA, USA) was connected to the detector outlet for preventing bubbles and improving baseline stability. Data acquisition and analysis were performed using a PCI-1200 DAQ board (National Instruments, Austin, TX, USA) controlled by a program written with LabVIEW 5.0.1 (National Instruments).

Following the treatment of Breinholt et al. [8] for the separation of enantiomers, the upper phase of the each selected solvent system was used as the MP and the lower phase as the SP. The separation column was first flushed with the SP without 18C6H4 and then filled with the 18C6H4-containing SP. While the column was being rotated at about 770 rpm, the MP was pumped into the column at a flow rate of 0.2 ml/min. The 18C6H4-containing SP, displaced by pumping the MP into the column, was collected in order to recover 18C6H4. After reaching equilibrium in the column, a sample solution,

prepared by dissolving 0.2 mg of gemifloxacin in 1 ml MP of each solvent system followed by 30–50 times dilution by the MP, was injected. For further studies, the effluent was collected at the detector outlet. After each run, the column contents were expelled into a graduated cylinder by applying nitrogen gas to one end of the column. The retention volume of the SP estimated from the two-phase liquid in the cylinder was 2.0–2.3 ml, which is in accordance with the low retention of the SP generally encountered for a solvent system containing viscous components such as 1-BuOH [22].

3. Results and discussion

3.1. Chromatographic parameters for chiral separation in CCC

CCC is based on the partitioning of an analyte between two immiscible liquid phases. The most important parameter to control in CCC is the distribution ratio, defined as:

$$D = \frac{\text{Total concentration of solute in SP } (= C_{SP})}{\text{Total concentration of solute in MP } (= C_{MP})} \quad (1)$$

the separation factor (α) and resolution (R_S) for the two enantiomers A_+ and A_- eluting, respectively, earlier and later are given by:

$$\alpha = \frac{V_- - V_{MP}}{V_+ - V_{MP}} = \frac{D_-}{D_+} \quad (2)$$

$$R_s = 2\left(\frac{V_- - V_+}{W_- + W_+}\right) \quad (3)$$

where V and W are the retention volume and base width of the peak indicated by the subscript, respectively. For a column with a separation efficiency N, the resolution for the two enantiomers can be expressed as [23]:

$$R_s = \frac{(\alpha - 1)D_+\sqrt{N}}{2(\alpha + 1)D_+ + 4(1 - S_F)/S_F} \quad (4)$$

where S_F is the fraction of the SP in the column. Note that R_S is a monotonically increasing function of α, D_+, and S_F. Assuming that a chiral selector, CS, is only soluble in the SP, CSA_+ and CSA_- complexes exist only in the SP in equilibrium. The distribution ratios for the A_+ and A_- isomers can be expressed as:

$$D_\pm = D_0(1 + K_\pm[CS]_{SP}) \quad (5)$$

where D_0 is the distribution ratio in the absence of CS and $K_\pm$ is the binding constant of the corresponding CSA complex given by:

$$K_\pm = \frac{[CSA_\pm]_{SP}}{[CS]_{SP}[A_\pm]_{SP}} \quad (6)$$

then the separation factor becomes

$$\alpha = \frac{1 + K_-[CS]_{SP}}{1 + K_+[CS]_{SP}} \quad (7)$$

which has a minimum value of 1 with no separation and increases with the chiral selector concentration up to the value K_-/K_+, in principle.

3.2. Selection of a suitable solvent system

The solvent systems used for CCC should have a reasonably short settling time and sufficient sample solubility without causing decomposition, denaturation, or emulsification. Satisfactory retention of the SP is also an important criterion [3]. For a reasonable analysis time and solvent consumption, two-phase solvent systems yielding distribution ratios of 0.5–2 are desirable for CCC. Although higher distribution ratios (>3) provide higher resolutions, they lead to an increased run time and peak broadening. With $D < 1$, faster separation is possible at the cost of resolution [24].

Breinholt et al. [8] successfully separated the enantiomers of 7-*des*-methylormeloxifene using *S*-β-CD as a chiral selector with the solvent system EtOAc/MeOH/triethylammonium acetate buffer (pH 5.2) in CCC. $18C6H_4$ has similar properties to *S*-β-CD in that both are negatively charged and have good water solubility. However, gemifloxacin is more hydrophilic than 7-*des*-methylormeloxifene and is known to be well soluble in acidic buffer solution. Thus, to increase the solubility of gemifloxacin in the MP, a more polar organic solvent is needed than that used in the separation of 7-*des*-methylormeloxifene. The initial search for a suitable solvent system was carried out by comparing D_0 values of solvent systems composed of various volume ratios of 1-hexane, EtOAc, MeOH, 1-BuOH, and aqueous run buffers prepared with 20 mM Bis–Tris aqueous solution by adjusting the pH with CA. Bis–Tris was chosen as the base constituent in order not to hamper the chiral selecting capability of $18C6H_4$ as suggested by the CE studies [21]. Since the presence of $18C6H_4$ can shift the partitioning of gemifloxacin more toward the SP, a solvent system yielding a D_0 value smaller than 0.5 was desirable. In solvent systems containing 1-hexane, gemifloxacin hardly distributed into the MP when the buffer pH was below 7 (in order to have the amine group of gemifloxacin protonated), and thus 1-hexane was ruled out. As the proportion of 1-BuOH was increased, the partitioning of gemifloxacin into the MP also increased, but insufficiently. In order to make the solvent system more polar, HOAc was added to a 1-BuOH (20 mM Bis–Tris-CA) (pH 7) system followed by an increase in the buffer concentration. When the volume of HOAc was reduced to 0.5 vol% of the total solvent system, the D_0 values were still within the desirable range. Thus HOAc was chosen as the acid constituent of the buffer instead of CA and further optimization was based on the solvent system, 1-BuOH/20 mM Bis–Tris acetate buffer) (1/1, v/v).

The binding constants between $18C6H_4$ and the two gemifloxacin enantiomers obtained from CE with a 20 mM TEA–CA buffer (pH 3) were [20,21]

$$K_+ = 1020 \pm 110, \quad K_- = 1320 \pm 80 \quad (8)$$

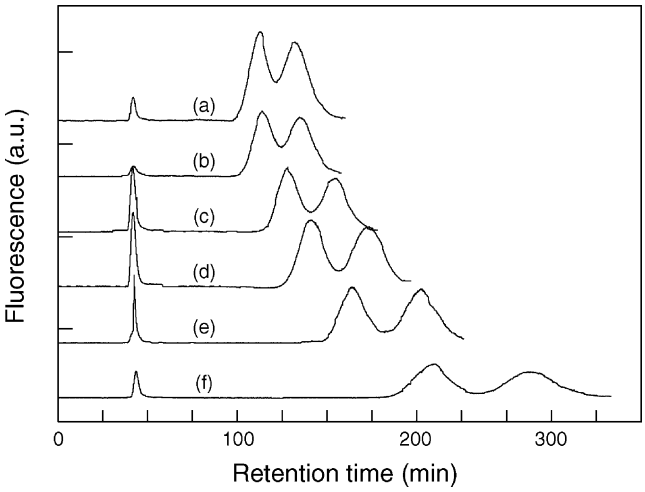

Fig. 2. CCC chromatograms of gemifloxacin. Effects of the hydrophobicity of the solvent system on the separations of gemifloxacin enantiomers are demonstrated. The enantiomer assignment has not been performed. Experimental conditions: 1-BuOH–EtOAc (20 mM Bis–Tris acetate buffer) (pH 6) of (a) (10:1:10, v/v) (b) (9:2:10, v/v) (c) (8:3:10, v/v) (d) (7:4:10, v/v) (e) (6:5:10, v/v) and (f) (5:6:10, v/v); $[18C6H_4]_{SP} = 1$ mM; the first eluting peak is unknown; the flow rate of the MP, 0.15 ml/min; 770 rpm; $S_F = 0.33$.

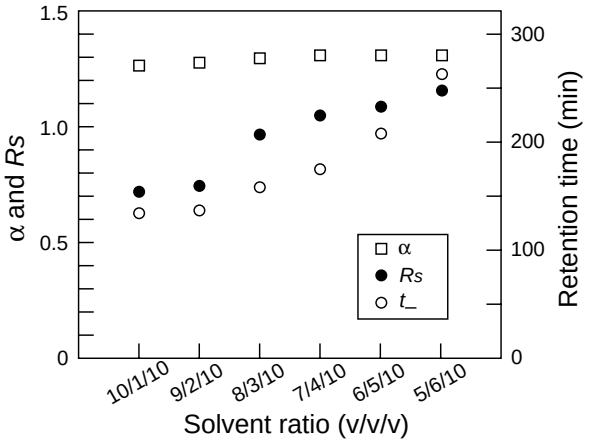

Fig. 3. Effects of the solvent hydrophobicity on the separation factor (α), resolution (R_S), and retention time of the later eluting peak (t_-). The solvent systems were 1-BuOH–EtOAc (20 mM Bis–Tris acetate buffer) (pH 6) at various volume ratios as described in the graph. Other conditions as in Fig. 2.

For a two-phase solvent system without $18C6H_4$ composed of 1-BuOH–EtOAc (20 mM Bis–Tris acetate buffer) (pH 6) 6:5:10, v/v, D_0 was 1.1. By rearranging Eq. (5), the CS concentration giving $D_+ = 2$ can be chosen as:

$$[CS]_{SP} = \frac{(D_+/D_0 - 1)}{K_+} = \frac{(2/1.1) - 1}{1020\,M^{-1}} \approx 1\,mM \quad (9)$$

from Eq. (6), the initial chiral selector concentration is given by

$$[CS]_{initial} = [CS]_{SP}(1 + K_+[A_+] + K_-[A_-]) \quad (10)$$

In this paper, both $K_+[A_+]$ and $K_-[A_-]$ were much smaller than 1 and the initial concentration yielding $D_+ = 2$ is estimated to be 1 mM. Then the separation factor of gemifloxacin in CCC with 1 mM $18C6H_4$ becomes:

$$\alpha \approx 1.1 \pm 0.1 \quad (11)$$

Thus the concentration of $18C6H_4$ was chosen at 1 mM for the initial optimization of separation conditions, after which the $18C6H_4$ concentration was optimized. Note that a higher value of $[CS]_{initial}$ should be used for a higher concentration of the analyte according to Eq. (10).

The solvent hydrophobicity greatly affects the partition ratios of the enantiomers and their resolution [6]. In order to control the hydrophobicity of the solvent system, EtOAc, a more hydrophobic and less viscous organic solvent than 1-BuOH, was added to 1-BuOH (20 mM Bis–Tris acetate buffer) (pH 6) at various volume ratios. Fig. 2 shows a set of chromatograms of gemifloxacin enantiomers, which are arranged according to the hydrophobicity of the solvent system. The separation factor and resolution for these separations are shown in Fig. 3. As the solvent system became more hydrophobic, the resolution improved. On the other hand, the separation factor showed a slight increase, up to the 1-BuOH–EtOAc ratio of 8:3, and thereafter remained flat. The retention time also increased with the hydrophobicity of the solvent system, since gemifloxacin is less soluble in the more hydrophobic MP. Although the resolution may be further improved by increasing the proportion of EtOAc, the separation time would be over 5 h. Note that $18C6H_4$ could be partitioned in the MP of high polarity even though $18C6H_4$ was assumed to be soluble only in the SP. In that case, the chiral discrimination ability of $18C6H_4$ would decrease.

3.3. Effects of the buffer pH

In the CE study by Cho and coworkers [20] the maximum resolution between the two enantiomers of gemifloxacin using $18C6H_4$ as a chiral selector was obtained at pH 4.5. As the buffer pH increases up to pH 7 where the primary amine group of gemifloxacin ($pK_{a2} = 8.93$) remains protonated, $18C6H_4$, having four carboxylic acid groups with pK_a values of 2.13, 2.84, 4.29, and 4.88, becomes more negatively charged and the binding with positively charged gemifloxacin enantiomers becomes stronger. In the meantime, the increase in pH is followed by an increase in electroosmotic mobility due to the ionization of silanol groups on the inner wall of a fused silica capillary and the resolution in CE will decrease as [25]:

$$R_S \propto \frac{\mu_+ - \mu_-}{\sqrt{\mu_{ave} + \mu_{EOF}}} \quad (12)$$

where μ_+ and μ_- are respectively the electrophoretic mobilities of A_+ and A_-, and μ_{ave} the average of μ_+ and μ_-. Therefore, the pH value of 4.5 for the maximum resolution in CE was a compromise between the positive effect of an increased binding constant and the negative effect

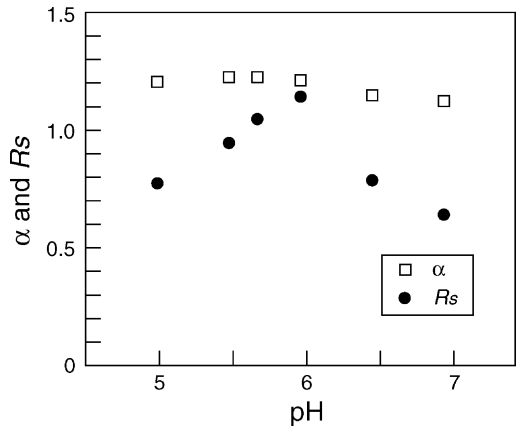

Fig. 4. Effects of the buffer pH. Experimental conditions: 1-BuOH–EtOAc (20 mM Bis–Tris acetate buffer) (6:5:10, v/v); $[18C6H_4]_{SP} = 1$ mM; the flow rate of the MP, 0.2 ml/min; 770 rpm; $S_F = 0.31$.

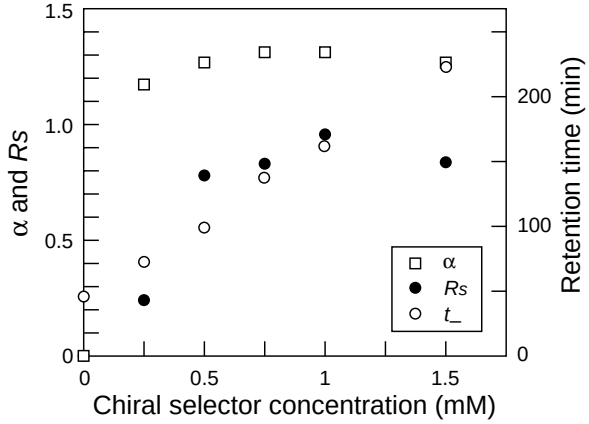

Fig. 5. Effects of the chiral selector concentration. The retention times of the later eluting peak (t_-) are also shown. Experimental conditions: 1-BuOH–EtOAc (20 mM Bis–Tris acetate buffer) (pH 5.7) (6/5/10, v/v); the flow rate of the MP, 0.2 ml/min; 770 rpm; $S_F = 0.31$.

of an increased μ_{EOF}. In the absence of μ_{EOF}, the maximum CCC resolution for the gemifloxacin enantiomers was thus expected at a pH higher than 4.5. The separation results of enantiomers in CCC, expressed as the separation factor (α) and resolution (R_S) at various buffer pH values, are shown in Fig. 4. The solvent system was composed of 1-BuOH–EtOAc (20 mM Bis–Tris acetate buffer 6:5:10, v/v). The maximum resolution was obtained around pH 6, which is higher than the maximum pH of 4.5 in CE. At pH 6, the fraction of the fully deprotonated $18C6H_4$ calculated using the pK_a values of the four carboxylic acid groups is 93% and the calculated fraction of gemifloxacin with the primary amine group protonated is practically 1 [26]. Thus gemifloxacin can form a strong complex with $18C6H_4$ at pH 6. However, the pH value of 5.7 was chosen as optimal for further investigation considering the analysis time.

3.4. Effects of the chiral selector concentration

The effect of the $18C6H_4$ concentration on the separation of gemifloxacin was investigated by varying the amounts of $18C6H_4$ in the SP at pH 5.7. Fig. 5 illustrates the effects of the concentration of $18C6H_4$ on α and R_S. The presence of $18C6H_4$ in the aqueous phase greatly enhanced the partition of gemifloxacin into the SP due to the high binding affinity between $18C6H_4$ and gemifloxacin. The retention time of gemifloxacin was 45 min in the absence of $18C6H_4$, whereas the retention time of the later eluting enantiomer was 223 min with 1.5 mM $18C6H_4$ in the SP.

The separation factor steadily increased with the concentration of $18C6H_4$ and reached a maximum value of 1.3 around 1 mM of $18C6H_4$. This maximum value is slightly larger than the value 1.1 ± 0.1 estimated from Eq. (11) with the binding constants obtained by CE. The resolution also had a maximum value of 0.96 with 1 mM of $18C6H_4$, where the efficiency of the first eluted peak was 330. Note that solvent compositions for CE and CCC were not identical. At pH 5.7, the binding constant K_+, calculated from the measured values $D_0 = 1.5$ and $D_+ = 8.4$, was 4600 which is larger than that at pH 4.5 due to the increased electrostatic interactions between $18C6H_4$ and gemifloxacin. As shown in Fig. 5, the increase in the $18C6H_4$ concentration required a longer elution time, causing peak broadening. When the concentration of $18C6H_4$ was 1.5 mM, the efficiency for the first eluted enantiomer was 270. Although the difference in elution times of the two enantiomers was 43 min ($t_+ = 180$ and $t_- = 223$ min) and $\alpha = 1.3$, the resolution decreased to 0.84. In this case, the value of N required from Eq. (4) for a baseline separation ($R_S = 1.5$) is 700. Due to the generally low efficiency of a CCC device compared to HPLC, peak broadening resulted in partially separated peaks.

4. Conclusions

We have investigated the chiral separation of gemifloxacin using $18C6H_4$ as a chiral selector in analytical CCC guided by the information obtained from CE. As the solvent hydrophobicity increased, the resolution of gemifloxacin enantiomers also increased. Since gemifloxacin is quite hydrophilic, a polar solvent system composed of 1-BuOH/EtOAc/20 mM Bis–Tris acetate buffer was found to be suitable. Since a higher pH was preferred for a stronger interaction with gemifloxacin, in which polyprotic $18C6H_4$ can be fully deprotonated, the best resolution was obtained at pH 6, which is higher than the optimum pH of 4.5 for CE. The $18C6H_4$ concentration was also optimized to improve the resolution. The maximum resolution was observed for 1 mM of $18C6H_4$, which happened to be the initial value chosen from the binding constant information from CE.

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