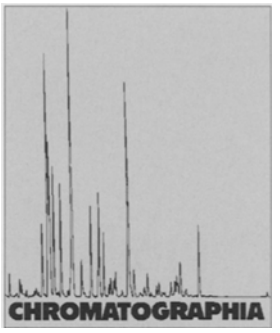
Separation of the Stereoisomers of Racemic Fluoroquinolone Antibacterial Agents on a Crown-Ether-Based Chiral HPLC Stationary Phase



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Key Words

Column liquid chromatography
Enantiomer separation
Chiral stationary phase
Chiral crown ether
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Summary

A chiral stationary phase derived from (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid has been successfully used for the direct separation of the enantiomers of racemic fluoroquinolines containing a primary amino group being investigated as antibacterial agents. The chromatographic resolution behavior was found to depend on the content and the type of acidic and organic modifiers in the mobile phase and on the column temperature.

Introduction

Since norfloxacin was reported to have potent antibacterial activity [1], several quinolone derivatives, e. g. norfloxacin, ofloxacin, enoxacin, ciprofloxacin, lomefloxacin, and fleroxacin, have been developed. Ofloxacin, among others, is chiral and the (–)-*S* enantiomer is more active than the (+)-*R* enantiomer [2]. Recently, fluoroquinolone antibacterial agents containing oxime-substituted 3-amino- or 3-aminomethylpyrrolidines have been developed and some have been found to have potent antimicrobial activity against both Gram-positive and Gram-negative organisms [3, 4]. Because the new fluoroquinolone agents are also chiral we were interested in establishing analytical techniques that distinguish between their enantiomers.

We have recently developed a new HPLC chiral stationary phase (CSP **1**) based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid **2** (Figure 1). CSP **1** is highly effective in the resolution of different racemic compounds containing a primary amino functional group, including α-amino acids, amino alcohols and amines [5, 6]. CSP **1** has also been used to resolve racemic quinolone derivatives containing a primary amino group [7]. In this context, CSP **1** is expected to be useful in the separation of the two enantiomers of new fluoroquinolone antibacterial agents containing oxime-substituted 3-amino- or 3-aminomethylpyrrolidines (Figure 2), because all these compounds contain a primary amino or aminomethyl functional group at the chiral center.

Experimental

Chromatography was performed with HPLC equipment comprising a Waters model 515 HPLC pump, a Rheodyne 7125 injector with 20-μL sample loop, a Younglin M720 absorbance detector (254 nm UV) and a Younglin D520B computing integrator. The column temperature was controlled by means of a Julabo F30 Ultratemp 2000 cooling circulator. The chiral column packed with CSP **1** (150 mm × 4.6 mm i. d. stainless steel) was available from a previous study [6]. Fluoroquinolone antibacterial agents containing oxime-substituted 3-amino- or 3-aminomethylpyrrolidines (Figure 2), prepared by methods reported elsewhere [4], were generously donated by Dr C. Y. Hong (Biotec Research Institute, LG Chem, Taejon, Korea). Sample solutions (1.0 mg mL^{-1}) for chromatographic injection were prepared by dissolving each fluoroquinolone in a 50:50 (*v*/*v*) mixture of methanol and water; approximately 1 μL of these solutions was usually injected. For fluoroquinolone antibacterial agent **5** baseline resolution on CSP **1** was observed for injections ≤ 128 μg (20 μL of 6.4 mg mL^{-1} solution).

Results and Discussion

Results from chromatographic resolution on CSP **1** of the enantiomers of fluoroqui-

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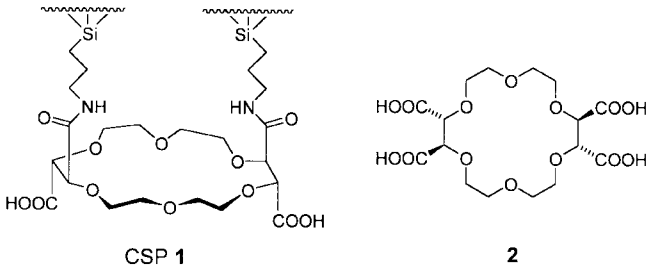

Figure 1. The structures of CSP **1** and (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid **2**.

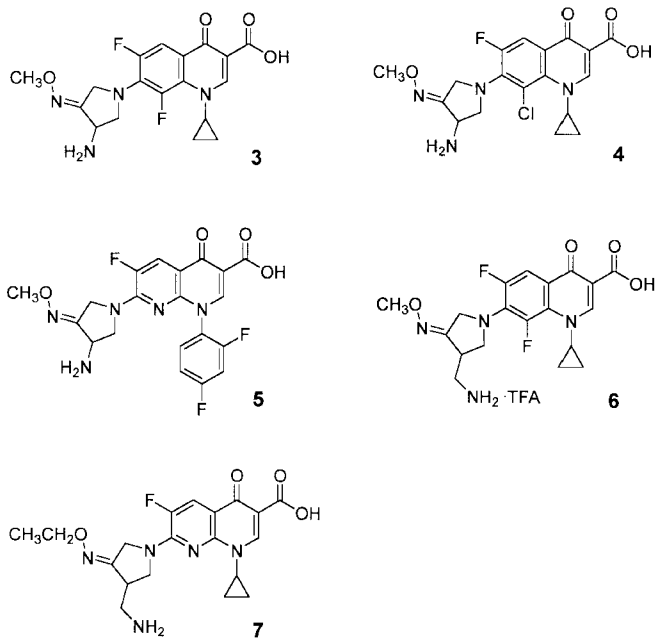

Figure 2. The structures of the racemic fluoroquinolone antibacterial agents investigated in this study.

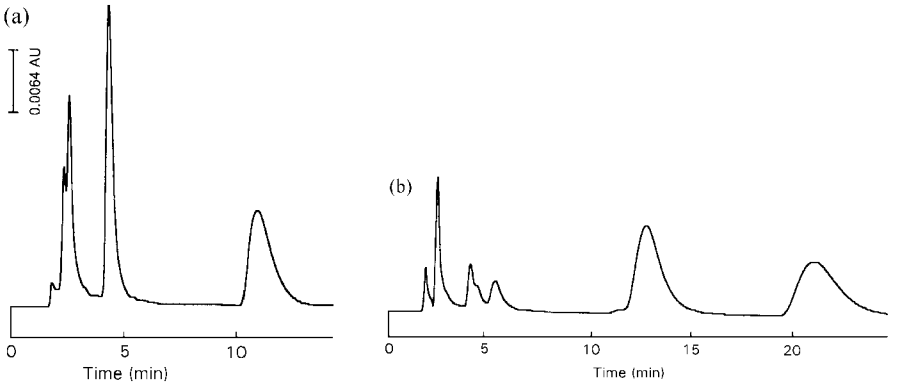

Figure 3. Representative chromatograms illustrating the resolution of fluoroquinolone antibacterial agents **4** (**a**) and **5** (**b**) on CSP **1**. Mobile phase 80% methanol in $H_2O + 10^{-2}$ M H_2SO_4; flow rate 0.8 mL min^{-1}; column temperature 20 °C.

nolone antibacterial agents containing oxime-substituted 3-amino- or 3-aminomethylpyrrolidine groups are summarized in Table I; representative chromatograms are shown in Figure 3. It is apparent that resolution is reasonable, with baseline separation. The chromatographic resolution data listed in Table I were obtained by use of an 80:20 (*v*/*v*) methanol–water mixture containing sulfuric acid (10^{-2} M) as mobile phase; the flow rate was 0.8 mL min^{-1} at 20 °C. It has previously been shown that complexation of primary ammonium ion ($R–NH_3^+$) inside the chiral cavity of the crown ether is essential for chiral recognition of racemic primary amino compounds by chiral crown ethers [8]. In this instance, sulfuric acid added to the mobile phase is believed to protonate the primary amino groups of the fluoroquinolone antibacterial agents shown in Figure 2 and to enhance the formation of a diastereomeric complex inside the chiral cavity of the 18-crown-6 ring of CSP **1**.

Table I. Resolution of the enantiomers of racemic fluoroquinolone antibacterial agents on CSP **1**.

Analyte	k_1	k_2	α	R_S
3	2.34	4.89	2.09	2.17
4	1.60	5.45	3.41	4.86
5	6.14	10.66	1.74	2.55
6	4.41	6.75	1.53	2.50
7	3.10	4.59	1.48	1.64

Chromatography was performed with 80% methanol in water containing sulfuric acid (10^{-2} M) as mobile phase; the flow rate was 0.8 mL min^{-1} and the temperature 20 °C.
k_1 and k_2 are the retention factors of the first- and the second-eluted enantiomers, α is the separation factor, and R_S is the resolution.

As is apparent from Table I, enantioselectivity, denoted by separation factors, α, varies with the structure of the fluoroquinolone compounds (shown in Figure 2). One interesting observation is that the enantiomers of fluoroquinolone compounds containing a 3-aminopyrrolidine group (**3**, **4**, and **5**) are better resolved than those of compounds containing a 3-aminomethylpyrrolidine group (**6** and **7**). The primary amino group of compounds **3**, **4**, and **5** is directly attached to the chiral center. In contrast, in fluoroquinolone compounds **6** and **7** the primary amino group is separated from the chiral center by one methylene unit. Complexation of the primary ammonium ion ($R–NH_3^+$) of fluoroquinolone compounds inside the cavity of chiral 18-crown-6 ring of the CSP is expected to discriminate between the two enantiomeric compartments more significantly when they are closer to the primary ammonium ion. Consequently, fluoroquinolone compounds containing a 3-aminopyrrolidine ring in which the primary amino group is adjacent to the chiral center are better resolved than those containing a 3-aminomethylpyrrolidine ring. The effect of other structural variations in solutes on enantioselectivity is, however, not yet clear.

The content and the type of acidic mobile-phase modifier might influence chromatographic behavior in the resolution of fluoroquinolone antibacterial agents on CSP **1**. The effects on chromatographic behavior of varying the mobile phase sulfuric acid content in the resolution of fluoroquinolone antibacterial agents **4**

Table II. Effect of varying the identity and amount of acidic mobile-phase modifier, at constant methanol content (80 %), on the resolution of racemic fluoroquinolones **4** and **6** on CSP **1**.

Acidic modifier	Concn (mM)	**4**			**6**		
		k_1	α	R_S	k_1	α	R_S
H_2SO_4	1	2.89	3.49	6.25	8.47	1.49	2.00
H_2SO_4	5	1.84	3.51	6.17	5.09	1.52	3.10
H_2SO_4	10	1.60	3.41	4.86	4.41	1.53	2.50
H_2SO_4	20	1.57	3.43	5.83	4.32	1.54	2.80
CF_3CO_2H	10	0.73	3.25	4.00	1.80	1.47	1.56
$HClO_4$	10	2.85	3.40	6.80	6.36	1.56	2.92

The flow rate was 0.8 mL min^{-1} and the temperature 20 °C.
k_1 is the retention factor of the first-eluted enantiomer, α is the separation factor, and R_S is the resolution.

Table III. Effect of varying the identity and amount of organic modifier in the aqueous mobile phase, at constant concentration (10^{-2} M) of sulfuric acid as acidic modifier, on the resolution of the enantiomers of racemic fluoroquinolone compounds **4** and **6** on CSP **1**.

Organic modifier	Concn (%)	**4**			**6**		
		k_1	α	R_S	k_1	α	R_S
CH_3OH	20	0.70	2.56	2.75	2.16	1.29	0.60
CH_3OH	50	0.76	2.83	3.72	1.93	1.42	1.72
CH_3OH	80	1.60	3.41	4.86	4.41	1.53	2.50
CH_3OH	100	4.16	4.05	6.37	14.09	1.54	2.64
C_2H_5OH	20	0.62	2.61	2.50	1.79	1.32	0.86
C_2H_5OH	50	0.97	2.97	3.40	1.74	1.43	1.40

The flow rate was 0.8 mL min^{-1} and the temperature 20 °C.
k_1 is the retention factor of the first-eluted enantiomer; α is the separation factor and R_S the resolution.

Table IV. Effect of varying the column temperature on the resolution of the enantiomers of racemic fluoroquinolone compounds **4** and **6** on CSP **1**.

Temperature (°C)	**4**			**6**		
	k_1	α	R_S	k_1	α	R_S
15	2.00	3.62	4.63	5.78	1.56	2.45
20	1.60	3.41	4.86	4.41	1.53	2.50
25	1.30	3.11	5.33	3.36	1.50	2.80

Mobile phase 80% CH_3OH in H_2O + H_2SO_4 (10^{-2} M); flow rate 0.8 mL min^{-1}.
k_1 is the retention factor of the first-eluted enantiomer; α is the separation factor and R_S the resolution.

and **6** on CSP **1** are summarized in Table II. Perchloric and trifluoroacetic acids were also investigated as acidic mobile-phase modifiers and their effects on resolution were compared with that of sulfuric acid (Table II). As shown in the table, the retention factors (k) decrease continuously as the amount of sulfuric acid in the mobile phase is increased, although no significant trends are observed for the separation factors (α) and the resolution (R_S). When trifluoroacetic acid was used instead of sulfuric acid, retention factors and resolution were substantially reduced whereas separation factors did not change significantly. The use of perchloric acid increased retention factors substantially but α and R_S did not change significantly.

In the resolution of α-amino acids, amines, and amino alcohols on CSP **1**, retention factors have been reported to increase continuously as the amount of sulfuric acid in the mobile phase is increased, because of increased complexation by the primary ammonium group ($R–NH_3^+$) inside the cavity of the 18-crown-6 ring of the CSP [5, 6]. In contrast, as shown in Table II, the retention factors of the fluoroquinolones decrease continuously as the amount of sulfuric acid in the mobile phase is increased. The compounds contain several basic tertiary amine sites in addition to the primary amino group, in contrast with α-amino acids, amines, and amino alcohols. Consequently, at higher concentrations of the acidic modifier the additional basic tertiary amine sites of the fluoroquinolone compounds are expected to be extensively protonated. The ionic strength of the mobile phase also increases as the amount of acidic modifier is increased. In this instance, the retention of the protonated (or charged) fluoroquinolone antibacterial agents decreases as the amount of sulfuric acid in the mobile phase is increased.

The effect of the organic mobile-phase modifier on chromatographic behavior in the resolution of the fluoroquinolone antibacterial agents on CSP **1** was investigated by changing the amount of methanol or ethanol in the mobile phase at constant sulfuric acid concentration (10^{-2} M) at 20 °C. As shown in Table III, retention, separation, and resolution (k, α, and R_S) all increase continuously as the organic-modifier content of the mobile phase is increased. When ethanol was used as organic modifier increasing the content beyond 50% was not useful because of the high back-pressure developed during chromatography, possibly because of the high viscosity of the mobile phase.

The column temperature might be the another factor influencing chromatographic behavior in the resolution of fluoroquinolone antibacterial agents on CSP **1**. Table IV shows that although retention and separation factor (k and α) increase as column temperature is reduced, resolution (R_S) usually decreases. At lower temperatures formation of the two diastereomeric complexes by the enantiomers inside the chiral cavity of 18-crown-6 ring of CSP **1** is expected to be favored. This is expected to be more significant for the more stable diastereomeric complex. Consequently, retention and separation factors increase as column temperature is reduced. At lower temperatures, however, the rate of formation of the diastereomeric complexes is expected to be slower. In this instance, the chromatographic peaks of the two enantiomers become broader at lower temperatures and, as a consequence, resolution decreases as the column temperature is reduced.

The mechanism of chiral recognition in the resolution of the fluoroquinolone antibacterials shown in Figure 2 on CSP **1** is not yet clear. In addition to complex formation by the primary ammonium group ($R–NH_3^+$) of the analytes inside the cavity of the 18-crown-6 ring of CSP **1** mentioned above [8], interactions between the carboxylic acid groups of the CSP and the side groups of the analytes might be necessary for chiral recognition. The two side carboxylic acid groups of the chiral selector of CSP **1** can act as steric barriers or as hydrogen-bonding donor or acceptor

groups. Further studies are needed to discover the exact role of these two carboxylic acid groups of CSP **1**.

In the absence of acidic modifier in the mobile phase (neutral pH), retention of fluoroquinolone antibacterial agents on the column was too long to be observed. For example, in the absence of acidic modifier fluoroquinolone **4**, which had shortest retention time and was completely eluted from the column within 15 min by the mobile phase shown in Table I, was not eluted from the column even though elution was monitored for 180 min. This long retention might be expected to result from the highly lipophilic interaction between the relatively large analytes and the stationary phase or from electrostatic interaction between the carboxylic acid groups of the CSP and the ammonium groups of the analytes. We do not, however, yet have any evidence to rationalize the long retention of the analytes at neutral pH, and further studies are needed.

In summary, we have shown that CSP **1**, based on (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid **2**, is very useful in the separation of the enantiomers of racemic fluoroquinolones containing a primary amino functional group being investigated as antibacterial agents. It has been shown that the chromatographic resolution behavior is to some extent affected by changing the amount and/or type of organic and acidic modifiers in the mobile phase and the column temperature. It is, in general, concluded that 80:20 methanol–water containing sulfuric acid (10^{-2} M) as acidic modifier, at 20 °C, are conditions most broadly applicable to the resolution on CSP **1** of racemic fluoroquinolone antibacterial agents containing a primary amino functional group.

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