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Investigation of enantioselective separation of quinolonecarboxylic acids by capillary zone electrophoresis using vancomycin as a chiral selector

Takashi Arai[a,*], Noriyuki Nimura[b], Toshio Kinoshita[b]

[a]*Production Technology Research Laboratories, Daiichi Pharmaceutical Co., Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 134, Japan*

[b]*School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan*

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Abstract

When a chiral selector that is a pharmaceutical compound is added to the separation buffer in capillary electrophoresis, the enantioselectivity and the mobility of analytes which interact with that chiral selector may be altered. The changes in enantioselectivity and mobility of the analyte are a function of the strength of the affinity interaction, which depends on the structure of each. The macrocyclic antibiotic vancomycin contains a variety of functionalities that are known to be useful for enantioselective interactions (e.g., hydrogen bonding groups, hydrophobic pockets, aromatic groups, amide linkages). Capillary electrophoresis with vancomycin as a buffer additive was used to separate the enantiomers of different compounds. In this study, the chiral separation of quinolonecarboxylic acids that exhibit marked antibacterial activity and of related compounds was achieved by capillary electrophoresis using vancomycin. The correlations between the separation parameters and analyte structures were investigated. The molecular interaction, which is based on the differences of structure, and the effect of experimental parameters on the enantioselective separation between the quinolonecarboxylic acids and vancomycin are discussed.

Keywords: Enantiomer separation; Pharmaceutical analysis; Quinolonecarboxylic acids; Carboxylic acids; Vancomycin; Antibiotics; Ofloxacin; DU-6859

1. Introduction

The increasing need for optically pure substances in the pharmaceutical field has led to the development of several techniques for analytical and preparative chiral separations. Recently, Armstrong and co-workers [1–5] reported that vancomycin could be a widely useful chiral selector for enantioselective separations in a variety of techniques (e.g., CE, HPLC, TLC, supercritical fluid chromatography and GC). Vancomycin is a macrocyclic glycopeptide (M_r 1449) antibiotic produced by the soil bacterium *Streptomyces orientalis*. Its structure is shown in Fig. 1. It has multiple stereogenic centres and also a variety of functional groups that are known to be

* Corresponding author.

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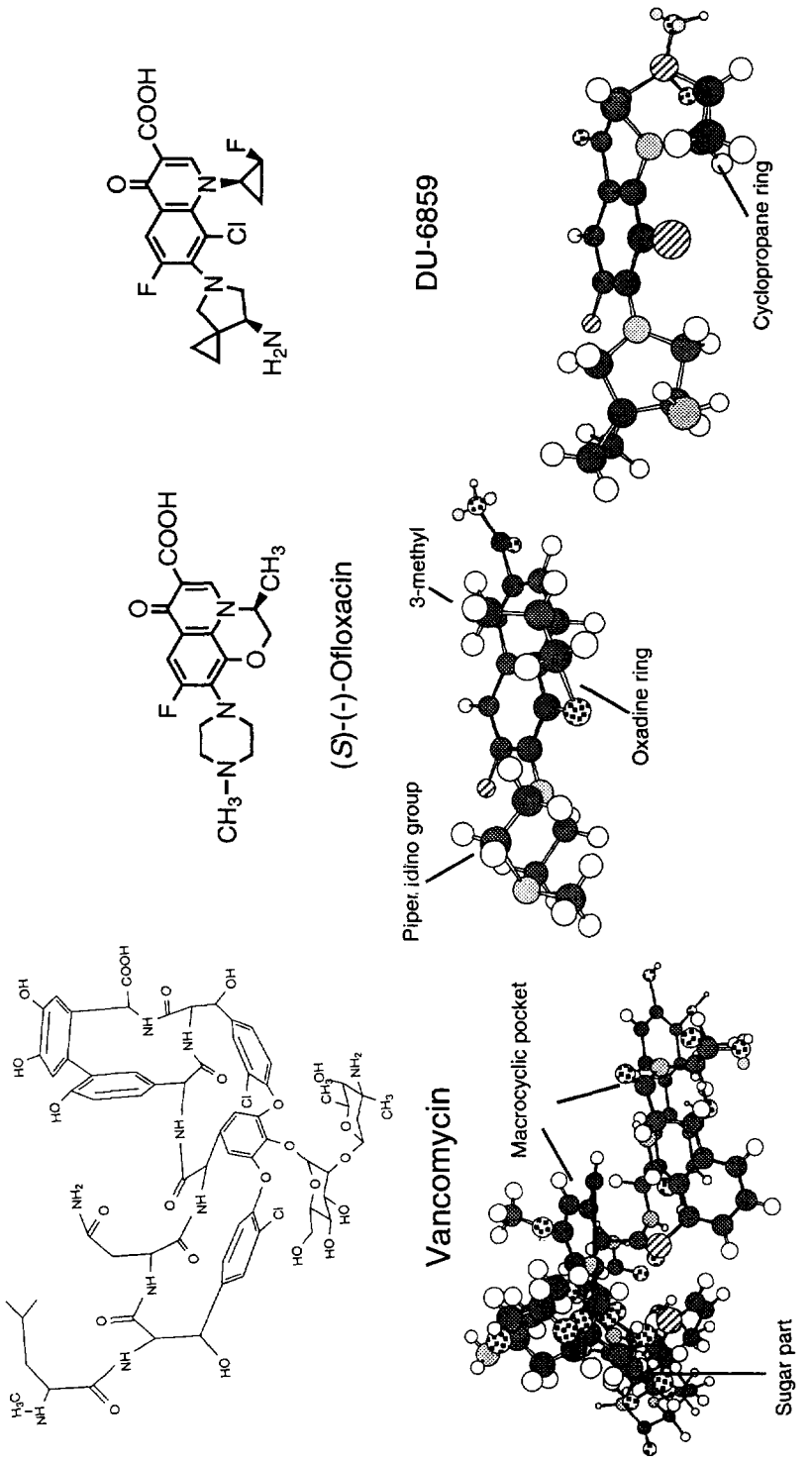


Fig. 1. Structures of chiral selector (vancomycin) and analytes (ofloxacin and DU-6859).

advantageous for stereoselective interactions [2–5].

Recently, quinolone antibacterial agents which have potential similar to or better than that of penicillin and cephalosphorin derivatives have been developed for use in the clinical field. Ofloxacin (OFLX) and DU-6859 are two fluorinated quinolones exhibiting marked bactericidal activity by inhibition of DNA gyrase [6–8]. OFLX has a methyl group at the C-3 position in the oxazine ring and DU-6859 has a fluorocyclopropyl group at the N-1 position and an amino azaspiro group at the C-7 position, and these groups form a stereogenic centre in each compound. In the case of OFLX, it has been shown that the antibacterial activity of (*S*)-(−)-OFLX against Gram-positive and Gram-negative bacteria is 8–128 times greater than that of the (*R*)-(+)-enantiomer and twice as potent as that of (±)-OFLX [9,10]. (*S*)-(−)-OFLX (known as levofloxacin) was developed recently and is widely used in the therapy of various infections. DU-6859 is under development (clinical trials) as a new-generation, optically active quinolonecarboxylic acid. Accordingly, it is important to develop effective analytical procedures for evaluating stereochemical purity, that can be of use in quality control, pharmacokinetic studies and other activities. Previously, we reported chiral separation methods for quinolones by ligand-exchange HPLC [11], counter-current chromatography using bovine serum albumin [12] and capillary affinity electrophoresis [13]. Some of the advantages of CE are (a) unprecedented separation performance at high voltages, (b) wide choice of quantitative detection techniques, (c) the possibility of automation, (d) small sample capabilities, (e) recent successful coupling with mass spectrometry and (f) low running costs (while chiral stationary phases are expensive). Since CE separation is performed in free solution, it is suitable for the observation or simulation of molecular interactions between selectors and analytes.

In this paper, we demonstrate that the enantiomers of quinolones (OFLX and DU-6859) can be separated by CE using vancomycin as a chiral selector. In addition, good enantioselectivities can be obtained in some cases for OFLX related substances. Furthermore, the correlation between the molecular structure and the chiral separation behaviour of analytes is discussed.

2. Experimental

2.1. Apparatus

Electrophoresis was performed on a Jasco (Tokyo, Japan) CE-800 CE system at 23°C in an untreated fused-silica capillary (50 cm × 50 μm I.D.). The distance from the injection point to the detection point was 45 cm.

A UV absorption detector was connected to a C-R7A integrator (Shimadzu, Kyoto, Japan). Samples were injected electrokinetically at constant voltage (10 kV for 2 s) at the positive side for a fixed period of time. Capillaries were stored overnight filled with water. Each day's operation was started by purging with 0.05 *M* sodium hydroxide solution followed by water.

2.2. Chemicals

OFLX [(±)-OFLX; (*R,S*)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7*H*-pyrido(1,2,3-*de*)-1,4-benzoxazine-6-carboxylic acid], its related substances and DU-6859 [(−)-7-[(7*S*)-amino-5-azaspiro[2,5]heptan-5-yl]-8-chloro-6-fluoro-1-[(1*R*,2*S*)-*cis*-2-fluoro-1-cyclopropyl]-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid] were obtained from Daiichi (Tokyo, Japan). Vancomycin hydrochloride salt was purchased from Sigma (St. Louis, MO, USA). The structures of vancomycin, OFLX and DU-6859) are shown in Fig. 1. Other chemicals were of analytical-reagent grade.

2.3. Buffer preparation

The 0.1 *M* acetic acid–sodium acetate buffer solutions (pH 3.0 and 4.0) were prepared by dissolving 1.36 g of sodium acetate trihydrate in 90 ml of water, adjusting the pH to 3.0 or 4.0 by adding glacial acetic acid dropwise and adding water to 100 ml.

The 0.1 *M* phosphoric acid–sodium phosphate buffer solutions (pH 5.0, 5.5, 6.0 and 7.0) were prepared by dissolving 3.58 g of disodium hydrogenphosphate dodecahydrate in 90 ml of water, adjusting the pH to the desired value by adding phosphoric acid dropwise and adding water to 100 ml.

2.4. Procedures

The acetate and phosphate buffers were used at a concentration of 0.1 *M*. The desired pH values of the buffers were adjusted with acetic or phosphoric acid. The buffer selections used are indicated in the figures and tables. The carrier solutions were prepared by dissolving 5 m*M* vancomycin in the buffers. The two solutions in the two electrode reservoirs were changed after each analysis to avoid changes in pH due to electrolysis of water and decompositions of vancomycin. Sample solutions of ca. 0.1 mg/ml of each compound were prepared by dissolving in water or 0.05 *M* sodium hydroxide solution (compounds **4**, **6** and **8**). The detailed analytical conditions are shown in the figures and tables.

3. Results and discussion

3.1. Chiral separation of OFLX and DU-6859

OFLX and DU-6859 are important pharmaceuticals in the treatment of infectious diseases. Their notable structural characteristics include the ability to form chelates with the carboxylic group and the keto group, their zwitterion formation and their fluorine content. In this study, we attempted the chiral separation of the quinolone compounds by CE using vancomycin as a chiral selector.

Migrations times (t_1, t_2, t_1/t_2), resolutions (R_s) and effective mobilities (μ_{ep}) are given in Table 1; μ_{ep} is given by

$$\mu_{ep} = \frac{lL}{tV} - \mu_{eof}\ (\mathrm{cm^2\ V^{-1}\ s^{-1}})$$

The results indicated that the enantioselectivity for DU-6859 ($R_s = 1.10$) was better than that for OFLX ($R_s = 0.35$), as shown in Fig. 2. One reason may be because OFLX has a relatively planar structure (Fig. 1). Since DU-6859 has three stereogenic centres, a higher molecular mass, primarily owing to the additional Cl and F groups, and a complicated structure, the enantiomers of DU-6859 can be easily resolved by vancomycin.

3.2. Possible separation mechanism

According to Armstrong et al. [5], the electrical charge of vancomycin under these conditions fluctuates, because it has the six reported pK_a values (ca. 2.9, 7.2, 8.6, 9.6, 10.5 and 11.7). Under the experimental operating conditions, we speculate that the charge of vancomycin may be positive and its effective mobility is ca. +3.0 $\mathrm{cm^2\ kV^{-1}\ min^{-1}}$ (pH ≤ 5.5) or more. Also, the electroosmotic flow at pH 3–4 is very slow, so the migration time depends on the flow of vancomycin. Actually, the effective mobilities (μ_{ep}) at lower pH values were increased (Table 1).

The reason why, on decreasing the pH, Armstrong et al. obtained shorter migration times, although at the lowest pH (pH 3.0) this parameter slightly increased, is not clear. However, at the lowest pH, which is near the lowest pK_a (2.9) of vancomycin, the charge of vancomycin and its migration time are variable.

3.3. Examination of enantioselective separation conditions

A number of attempts to improve the separation efficiency were made and the results examined. The electric voltage, buffer concentrations and vancomycin concentration were initially fixed at the values mentioned in the reports of Armstrong and co-workers [1–5].

The effects of pH on electrophoretic behaviour are shown in Table 1. The chiral separations of

Table 1
Effect of pH on the migration times and resolution of quinolonecarboxylic acid enantiomers with vancomycin as the chiral selector[a]

Compound	Parameter	pH 7.0	pH 5.5	pH 4.0	pH 3.0
OFLX	$t_{(eof)}$ (min)[b]	38.5	44.0	89.0	93.0
	t_1 (min)[c]	56.5	44.7	31.4	41.1
	μ_{e1} ($cm^2 V^{-1} s^{-1}$)[d]	$-3.1 \cdot 10^{-5}$	$-0.13 \cdot 10^{-5}$	$7.7 \cdot 10^{-5}$	$5.1 \cdot 10^{-5}$
	t_2 (min)[c]	56.5	44.7	32.2	41.1
	μ_{e2} ($cm^2 V^{-1} s^{-1}$)[d]	$-3.1 \cdot 10^{-5}$	$-0.13 \cdot 10^{-5}$	$7.4 \cdot 10^{-5}$	$5.1 \cdot 10^{-5}$
	t_2/t_1	1.00	1.00	1.03	1.00
	R_s	0.00	0.00	0.35	0.00

		pH 7.0	pH 6.0	pH 5.0	pH 4.0	pH 3.0
DU-6859	$t_{(eof)}$ (min)[b]	38.5	42.5	47.0	89.0	93.0
	t_1 (min)[c]	37.0	41.6	30.1	35.8	45.2
	μ_{e1} ($cm^2 V^{-1} s^{-1}$)[d]	$0.4 \cdot 10^{-5}$	$0.2 \cdot 10^{-5}$	$4.0 \cdot 10^{-5}$	$6.3 \cdot 10^{-5}$	$4.3 \cdot 10^{-5}$
	t_2 (min)[c]	37.0	41.6	30.5	37.5	45.2
	μ_{e2} ($cm^2 V^{-1} s^{-1}$)[d]	$0.4 \cdot 10^{-5}$	$0.2 \cdot 10^{-5}$	$3.8 \cdot 10^{-5}$	$5.8 \cdot 10^{-5}$	$4.3 \cdot 10^{-5}$
	t_2/t_1	1.00	1.00	1.01	1.03	1.00
	R_s	0.00	0.00	0.36	1.10	0.00

[a] Separations at pH 3.0 and 4.0 were performed using 5 m*M* vancomycin in 0.1 *M* acetate buffer. Other separations were performed using 5 m*M* vancomycin in 0.1 *M* phosphate buffer. The run voltage for all separations was +10 kV. See Experimental for further details.
[b] The time corresponding to the electroosmotic flow (EOF), $t_{(eof)}$, is given with the methanol peak. Note that a decrease in the EOF velocity results in an increase in $t_{(eof)}$.
[c] Migration times of enantiomers, t_1 and t_2.
[d] Effective mobilities, μ_{e1} and μ_{e2}, are defined in the text.

quinolonecarboxylic acids are best achieved at lower pH values.

In order to investigate the effects of chelate formation on the separation, the effect of the addition of 10 m*M* of copper ion ($CuSO_4$) to the carrier solution on chiral separation of OFLX at 4.0 was examined (experimental conditions as in Table 1). The enantioselectivity disappeared with the addition of copper ion.

The addition of 10% of 2-propanol at pH 4.0, which was reported to have an effect in some cases [5], caused a decrease in the separation of enantiomers. These results suggest that interactions such as hydrogen bonding between the analytes and the chiral selector play an important role in the enantioselectivity of quinolones.

According to the data on the addition of $CuSO_4$, chelation is not necessary for performing enantioselectivity.

3.4. Analytical parameters to determine the enantiomer of DU-6859

Detection limit, linearity of the calibration graph and repeatability were evaluated in this analytical system for the determination of DU-6859 enantiomer. The limit of quantification was about 0.5%, and a good correlation between peak-height ratio (y) and concentration (x, %) of enantiomer was obtained ($y = 0.010x + 0.003$, $r = 0.998$). The repeatability (R.S.D.) was 7.1% with a 1.0% spiked sample ($n = 6$).

This method is applicable for determining trace amounts of DU-6859 enantiomer.

3.5. Correlation of structures and separations

In order to investigate the correlation between the structures of OFLX-related substances

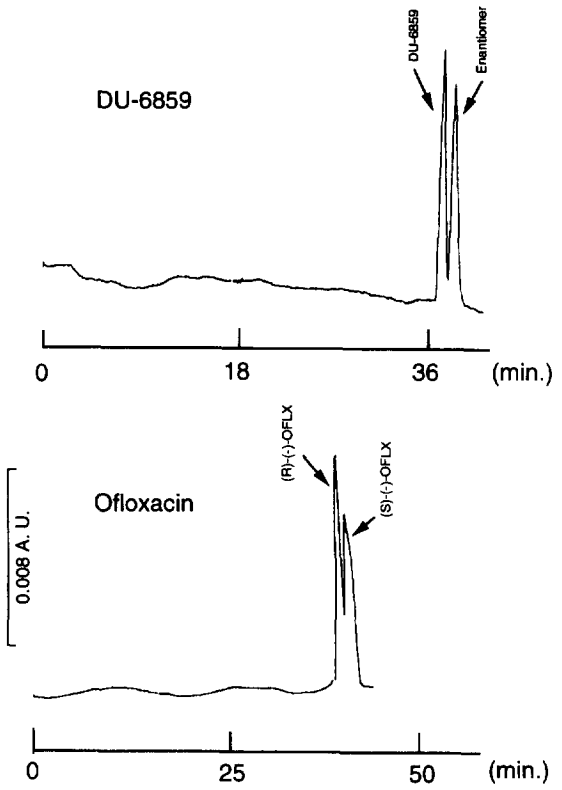


Fig. 2. Enantioselective electropherograms of OFLX and DU-6859 using vancomycin as a chiral selector. Electrophoretic conditions: run buffer, 0.1 *M* acetate buffer (pH 4.0) with 5 m*M* vancomycin; applied voltage, 10 kV (driving current, 20 μA); detection wavelength, 300 nm. For other conditions, see Experimental.

(Table 2) and chiral separation behaviour, a comparison of retention and enantioselectivity was made. This is discussed below. The compound numbers correspond to those in Table 2, and typical electropherograms of OFLX-related substances are shown in Fig. 3.

3.5.1. Effect of carboxylic group on chiral separation

In order to observe the effect of a carboxylic group at the C-6 position, the separation behaviour of OFLX and the decarboxylic compound **1** were compared. Enantioselective separation of **1** could not be achieved in this separation system. This result suggests that a carboxylic group is needed to achieve the separation. It is presumed that keto and carboxylic interactions, except for chelate formation, are involved in the enantioselectivity of quinolone compounds using vancomycin as a chiral selector.

3.5.2. Effects of functional group at the C-10 position on chiral separation

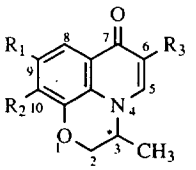
In order to observe the effects of the piperadino group and its alkyl chain length ($-C_2H_5$, CH_3, H) on enantioselectivity, the separation behaviour of OFLX and **2** and **3**, related compounds substituted at the C-10 position, were examined.

When the separation effectiveness of analogous compounds with piperidino-H ($R_s = 0.45$), piperidino-CH_3 (OFLX) ($R_s = 0.35$) and piperidino-C_2H_5 ($R_s = 0.20$) at the C-10 position as substituents (R_2) is compared, it is seen to decrease in that order. This order corresponds to the order of their basicity. Also regarding the C-10 position, the N-oxide form (**5**) could not be resolved and substituents which had weakly basic groups, such as fluorine and dimethylamino (**4** and **6**), could not be eluted in this system. It can therefore be concluded that the presence of a basic group at the C-10 position is essential for enantioselective separation.

3.5.3. Effects of functional groups at the C-9 position on chiral separation

In order to observe the effects of Cl, F and H groups at the C-9 position on enantioselectivity, the separation behaviour of OFLX and the substituted compounds **7** and **8** was examined. When a comparison is made of the separation effectiveness of compounds with the substituents Cl ($R_s = 0.95$), F (OFLX) ($R_s = 0.35$) and H ($R_s = 0.00$) at C-9 (R_1), it decreases in order of decreasing electronegativity: Cl > F > H. The enantioresolution at pH 4.0 decreases in that order, according to the electronegativity of the substituent. It is presumed that these electronegative groups affect the neighbouring group (C-10 position) and act as proton acceptors. This interaction may result in an increase in the basicity of the C-10 group.

Table 2
Enantioselective migration behaviour of OFLX-related substances



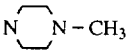
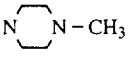
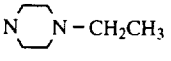
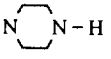
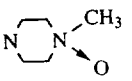
Compound	Structure	t_1 (min)	t_2 (min)	t_2/t_1 (R_s)
OFLX	R_1 = F	31.4	32.2	1.03
	R_2 = 	(*R*)-(+)-	(*S*)-(−)	(R_s: 0.35)
	R_3 = COOH			
1	R_1 = F	30.0	30.0	1.00
	R_2 = 			(R_s: 0.00)
	R_3 = H			
2	R_1 = F	38.5	39.5	1.03
	R_2 = 			(R_s: 0.20)
	R_3 = COOH			
3	R_1 = F	35.0	36.3	1.04
	R_2 = 			(R_s: 0.45)
	R_3 = COOH			
4	R_1 = F R_2 = F R_3 = COOH	Not eluted (>100 min)	Not eluted (>100 min)	——
5	R_1 = F	60.0	60.0	1.00
	R_2 = 			(R_s: 0.00)
	R_3 = COOH			

Table 2 (*Continued*)

Compound	Structure	t_1 (min)	t_2 (min)	t_2/t_1 (R_s)
6	$R_1 = F$ $R_2 = N(CH_3)_2$ $R_3 = COOH$	Not eluted (>100 min)	Not eluted (>100 min)	–
7	$R_1 = H$ $R_2 =$  $R_3 = COOH$	36.8	36.8	1.00 (R_s: 0.00)
8	$R_1 = Cl$ $R_2 =$  $R_3 = COOH$	31.8	33.3	1.05 (R_s: 0.95)
9	$R_1 =$  $R_2 = F$ $R_3 = COOH$	40.4	40.4	1.00 (R_s: 0.00)

All separations were performed using 5 m*M* vancomycin in 0.1 *M* acetate buffer (pH 4.0). See Experimental and Fig. 2 for further details.

3.5.4. Effects of piperidino group at C-9 and C-10

In order to observe the effect of the piperidino group and fluorine at the C-9 and C-10 positions, respectively, on enantioselectivity, OFLX and compound **9** were compared. The separation effectiveness of **9** is poorer than that of OFLX. This implies that the positions of these groups are important for chiral discrimination.

3.5.5. Summary of essential structure for chiral separation in this system

These interactions are considered to be related to the variety of functionalities of vancomycin (e.g., hydrogen bonding, hydrophobic pockets, aromatic groups, amide linkages). The essential functions of OFLX derivatives for chiral separation are shown in Fig. 4. The presumed interactions between vancomycin and OFLX derivatives are (1) hydrogen bonding and amide linkages may occur with piperidino, keto and carboxylic groups, (2) the molecular sizes of the quinolones fit the hydrophobic pockets and (3) aromatic interaction may occur with the naphthyl ring of the quinolones.

4. Conclusions

Enantioselective separations of quinolone antibacterial reagents were achieved by capillary zone electrophoresis using vancomycin as a chiral selector. Separations were optimized by adjusting the pH of the run buffer. In this separation system, essential components of the structures of the OFLX derivatives are (1) keto and carboxylic functions at the C-6 and C-7 positions, (2) basic functional group at the C-10 position and (3) an electronegative functional group at the C-9 position.

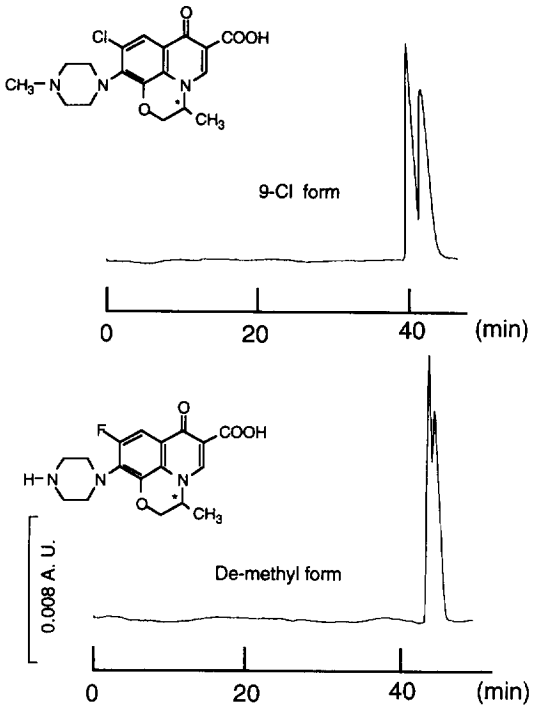


Fig. 3. Electropherograms of OFLX derivatives (C-9 chloride form and C-10 demethyl form) using vancomycin as a chiral selector. Electrophoretic conditions as in Fig. 2.

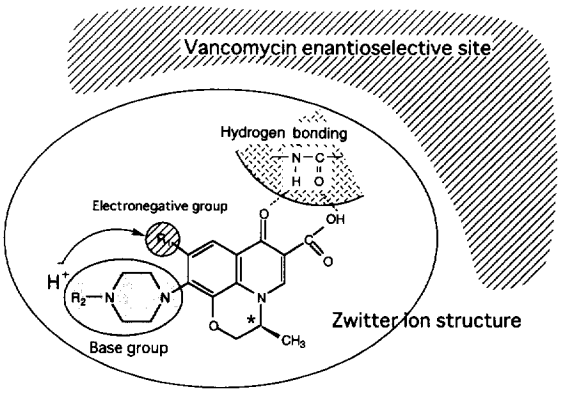


Fig. 4. Speculative diagram of the separation scheme between the chiral selector of vancomycin and quinolonecarboxylic acids.

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