# Comparison of Reversed-Phase Liquid Chromatographic Methods for the Separation of New Quinolones

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## Abstract

**Reversed-phase high-performance liquid chromatography is widely used in the analysis of drug substances and their metabolites. The interaction of quinolones with residual silanol in a silica-based $C_{18}$ stationary phase causes peak broadening and bad peak shapes and makes it hard to resolve the peak separations. This unusual interaction is studied and finally can be removed by masking the residual silanol of a silica-based $C_{18}$ stationary phase, then good peak separation is achieved. We have chosen four new quinolones and ciprofloxacin and improved the peak shapes by optimizing the pH of the eluent and the quantity of the additive (*N,N*-dimethyloctylamine, approximately 0–40mM) in the monomeric $C_{18}$ stationary phase. The elution behavior of quinolones in the polymeric $C_{18}$ stationary phase is compared with that in the monomeric $C_{18}$ stationary phase under the same eluent condition. Good peak symmetry and a high plate number are achieved by this technique, which are hardly obtained with the conventional silica-based $C_{18}$ stationary phase. Based on these results, we present data of the influence of the eluent composition such as pH, buffer, and additive concentration on the peak shape.**

## Introduction

The silica-based octadecyl stationary phase is widely used for reversed-phase (RP) high-performance liquid chromatography (HPLC). Silica as a support material has lots of merits. One of these merits is that silica is able to readily modify the surface silanol group and the mechanical stability of silica itself, yet this chemical reactivity is restrictive and some groups remain unreacted silanol (1). This residual silanol causes silanolphilic interaction (such as hydrogen bonding) in the separation of basic compounds, thus generating phenomena such as band broadening, peak tailing, and low plate number (1–3).

The study of using a stationary phase to separate the compound causing the peak tailing in RP-HPLC and additive effects on the stationary phase has progressed much up to now. Hamoir et al. (4) have explained the properties using the several kinds of stationary phase and identified its similar and dissimilar properties, thus interpreting the capacity factor (k') data. Kirkland and Henderson (5) explained the conformational characteristics of the stationary phase using a monomeric $C_{18}$ stationary phase and a polymeric $C_{18}$ stationary phase. Another approach used by Vervoort et al. (6) was the use of principal component analysis for the determination of distinct differences in 14 commercially available RP stationary phases. Ascah and Feibush (1) demonstrated the study of deactivated silica-based RP packing material.

Furthermore, many scientists have proposed the effective eluent system, which uses amine (i.e., tailing suppressor) to improve peak shape. Harmoir et al. (7) in particular experimented by the comparative method using short-chain tertiary amine such as triethylamine (TEA) and long-chain tertiary amine such as *N,N*-dimethyloctylamine (DMOA) as an amine modifier. Although the quantity of DMOA was less than TEA, the peak symmetry and plate number were improved. The silanol-blocking ability of different alkaline earth metal cations and strong amine was studied using basic solutes at pH 7 on a conventional octadecylsilane phase (8). Aliphatic amines have been used to improve the peak symmetry and efficiency of tricyclic antidepressants in capillary electrochromatography (9). Several kinds of amine additive have been employed for the separation of basic antidepressant and antipsychotic drugs on a cyanoalkyl-bonded silica column (10). Engelhardt and Jungheim (11) accounted that a good column is when the peak asymmetry is under 1.2. Vervoort et al. (2) explained that when the asymmetry factor is under 1.5, peak asymmetry is generally accepted in the quantitative analysis.

This study used five different quinolones as a fluoroquinolone antibacterial agent. Ciprofloxacin among them is widely known to represent activity against gram-positive *cocci*. New quinolones (the rest of them) were synthesized in the LG Life Science R&D Institute in LG Chemical Research Park and represent a broad spectrum of antibacterial activity and show far better in vitro activity compared with ciprofloxacin against

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gram-positive pathogens (12). The five different quinolones used in this experiment were compounds that include 3 to 5 nitrogen atoms and had difficulty in the analysis for serious peak tailing. Also, for the most part RP-HPLC separation in the quinolone analysis was unable to obtain good peak shape because of the interaction between the residual silanol and analyte.

The HPLC assay method was able to make a simple and speedy analysis for drugs within serum or urine with good specificity, precision, and accuracy. This method can especially analyze several kinds of drugs simultaneously (13,14). In comparison with the previously mentioned method, the microbiological assay method has adequate sensitivity but is unable to measure the multiple drugs as major disadvantages, which has a possibility of an interference of active metabolites and is time consuming resulting from long incubation (15).

Therefore, establishing a separation condition of quinolones in RP-HPLC and clearing up the interaction mechanism was the purpose of this study.

## Experimental

### Apparatus

Chromatography was carried out on a Waters HPLC system

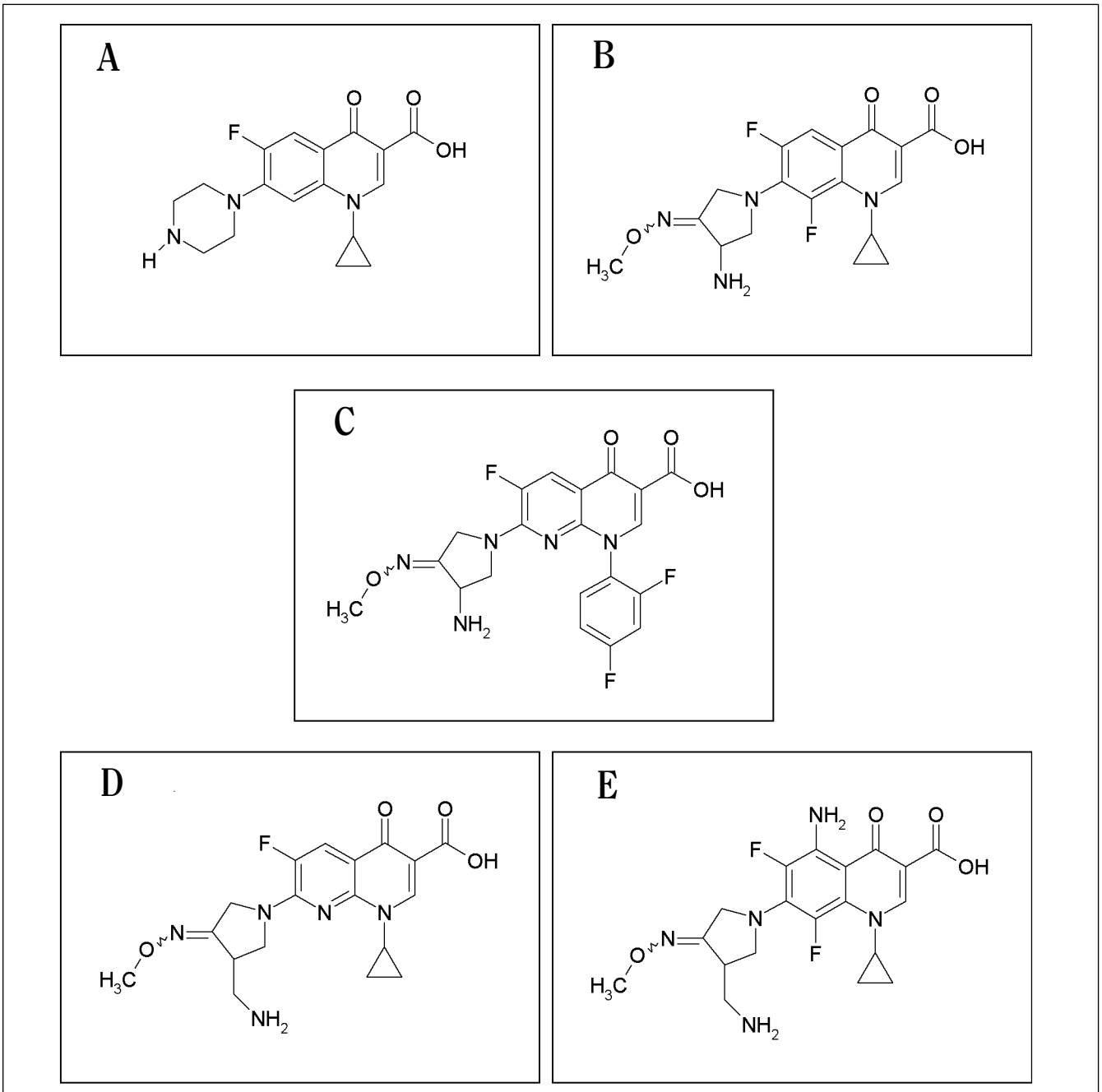


**Figure 1.** Structures of quinolones used in this study: (A) ciprofloxacin, (B) LB20277, (C) LB20296, (D) LB20304, and (E) LB20325.

**Table I. Eluent Composition Used in This Study**

| Stationary phase | pH of the eluent | Additive | Ciprofloxacin | LB20277 | LB20296 | LB20304 | LB20325 |
|---|---|---|---|---|---|---|---|
| *Monomeric $C_{18}$ stationary phase* | pH 2 | without | 34% MeOH–phosphate | 40% MeOH–phosphate | 45% MeOH–phosphate | 40% MeOH–phosphate | 45% MeOH–phosphate |
| | | with | 24% MeOH–phosphate–30mM DMOA | 28% MeOH–phosphate–30mM DMOA | 36% MeOH–phosphate–30mM DMOA | 28% MeOH–phosphate–30mM DMOA | 40% MeOH–phosphate–30mMDMOA |
| | pH 4 | without | 36% MeOH–phosphate | 42% MeOH–phosphate | 48% MeOH–phosphate | 42% MeOH–phosphate | 48% MeOH–phosphate |
| | | with | 30% MeOH–phosphate–30mM DMOA | 30% MeOH–phosphate–30mM DMOA | 38% MeOH–phosphate–30mM DMOA | 30% MeOH–phosphate–30mM DMOA | 38% MeOH–phosphate–30mM DMOA |
| *Polymeric $C_{18}$ stationary phase* | pH 4 | without | 30% MeOH–phosphate | 36% MeOH–phosphate | 45% MeOH–phosphate | 36% MeOH–phosphate | 45% MeOH–phosphate |
| | pH 2 | TFA | 15% ACN–0.1% TFA | 20% ACN–0.1% TFA | 20% ACN–0.1% TFA | 20% ACN–0.1% TFA | 20% ACN–20.1% TFA |

consisting of two 510 HPLC pumps, a gradient controller, a Rheodyne injector, and a 490E programmable multiwavelength detector. A TCM column oven (Waters, MA) was used to regulate the temperature of the column. Data integration was performed on a Model 746 integrator (Waters, MA). The HPLC columns used were a Microsorb $C_{18}$ (monomeric $C_{18}$ stationary phase, 250- × 4.6-mm i.d., 5 µm) (Rainin, CA) and a Capcell-Pak $C_{18}$ (polymeric $C_{18}$ stationary phase, 250- × 4.6-mm i.d., UG, 5 µm) (Shiseido, Tokyo, Japan).

### Chemicals and reagents

New quinolones were synthesized by the LG Life Science R&D Institute (Figure 1). Ciprofloxacin was purchased from Sigma (St. Louis, MO). All solvents were of HPLC-grade acetonitrile (ACN) and methanol (MeOH) (J.T. Baker, Phillipsburg, NJ). Potassium dihydrogen phosphate ($KH_2PO_4$) and *o*-phosphoric acid ($H_3PO_4$) were purchased from J.T. Baker. DMOA was purchased from Fluka (Industriestrasse, Budis, Switzerland).

### Chromatographic conditions

An aqueous phosphate buffer was prepared by dissolving 50mM $KH_2PO_4$. $H_3PO_4$ was added until the desired pH of eluent was reached. In the case of amine modifier, the desired pH was adjusted after additive was mixed with the eluent. Before chromatography, the eluent was filtered through a 0.45-µm membrane filter. The proportion of organic solvent in the eluent was designed to have a similar retention time of analytes being performed by the comparative experiment. The eluents used in this study are shown in Table I. The amount of sample injected was 10 µL of a 0.5-mg/mL solution in eluent (40% MeOH–phosphate, pH 4). The change of column efficiency was checked during all experiments, and the test solution used was a mixture of urasil, acetophenone, anisole, and toluene. The composition of the eluent used in the test of column efficiency was 60% MeOH–phosphate buffer (pH 4). The column temperature was 40°C, and detection was carried out at the maximum wavelength of analyte. The flow rate was 1.0 mL/min in all of the experiments.

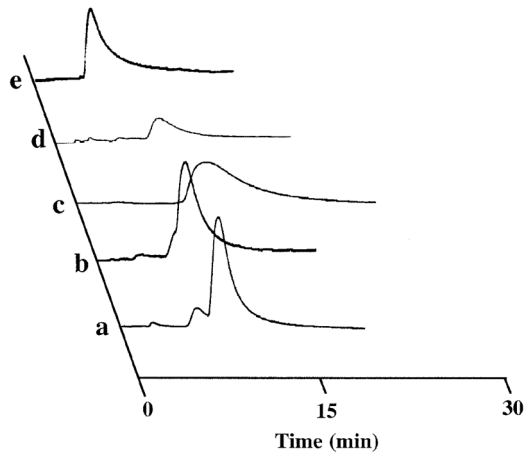


**Figure 2.** Chromatograms of quinolones on the monomeric $C_{18}$ stationary phase with the MeOH–phosphate eluent without additive (pH 4): LB20277, a; LB20296, b; LB20304, c; LB20325, d; and ciprofloxacin, e.

### Framework of experimental procedure

The experiments were divided into six parts. In the first part, the monomeric $C_{18}$ stationary phase was used and a set of five quinolones was chromatographed on the MeOH–phosphate eluent without additive (pH 4). The quinolones had 3 to 5 nitrogen atoms as shown in Figure 1. These nitrogen atoms were interacted with the residual silanol of the monomeric $C_{18}$ stationary phase. Thus, the elution behavior of quinolones by interaction with the residual silanol of the monomeric $C_{18}$ stationary phase was investigated.

In the second part, in order to show the silanol effect of the silica-based monomeric $C_{18}$ stationary phase, a comparative

experiment was performed by increasing DMOA to an eluent condition of the first part.

In the third part, as another method to prove a silanol effect, the polymeric $C_{18}$ stationary was used and experimented in the eluent composition of the first part.

In the fourth part, to examine the pH effect of residual silanol, the elution behavior of the quinolones was studied using acidic eluent without an additive in the monomeric $C_{18}$ stationary phase.

In the fifth part, the improvement of the peak shape by masking the silanol under acidic eluent including the additive was investigated.

In the sixth part, we proposed eluent composition in liquid chromatography (LC)–mass spectrometry (MS) coupling using the polymeric $C_{18}$ stationary phase, based on the previously mentioned results.

**Calculation**

The k' value was measured by:

$$k' = (t_r - t_o) / t_o \quad \text{Eq. 1}$$

where $t_r$ is the retention time of the compound of interest and $t_o$ is the column void time measured using the solvent peak obtained when trace amounts of ACN were injected onto the column.

The peak asymmetry factor ($F_{asy}$) was calculated by drawing a perpendicular line from the apex of the peak to the baseline and measuring the front (a) and back (b) widths of the peak at 10% height (16).

$$F_{asy} = b / a \quad \text{Eq. 2}$$

The chromatographic efficiency was determined from:

$$N = (41.7 \times (t_r / W_{0.1})^2) / (b / a + 1.25) \quad \text{Eq. 3}$$

where $W_{0.1}$ represents the peak width at 10% of its height (17).

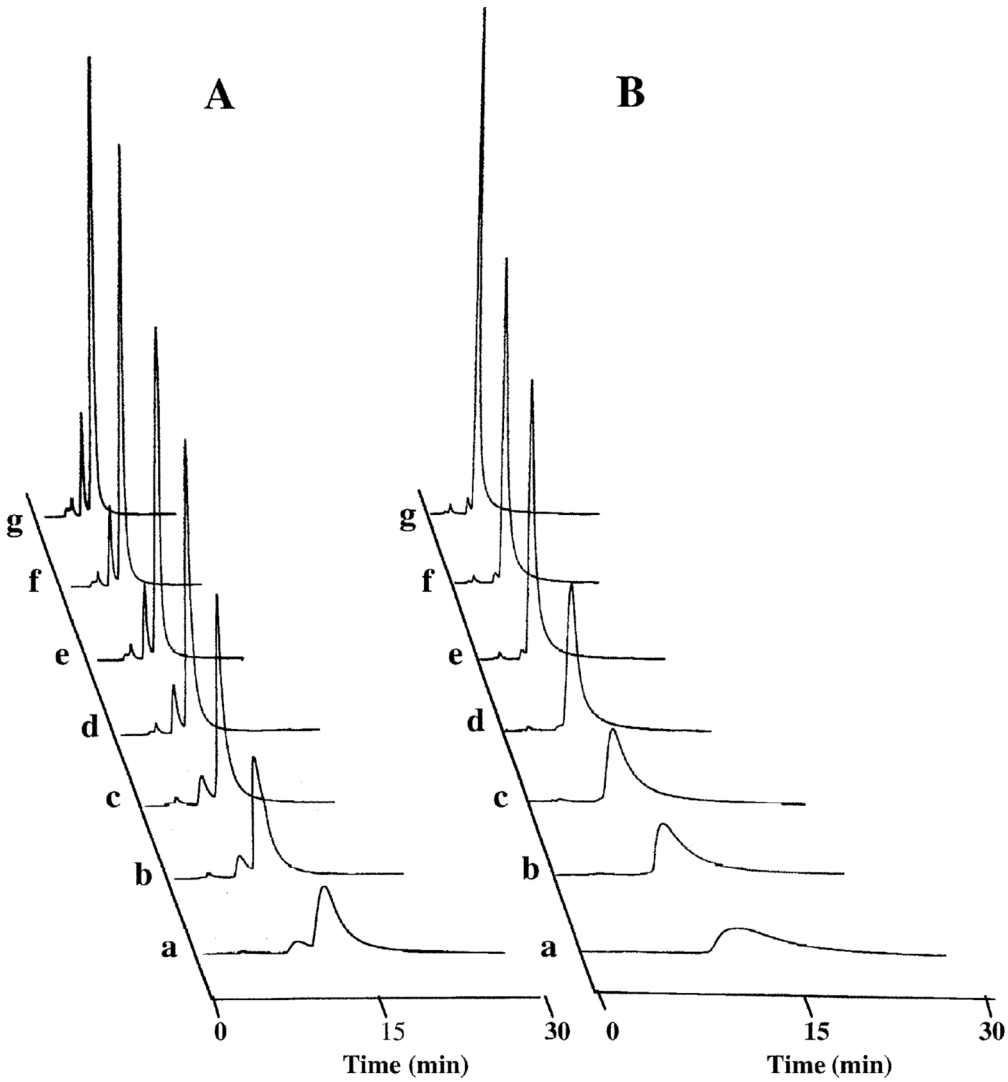


**Figure 3.** Comparison of two chromatograms ((A) LB20277 and (B) LB20304) for the peak shape of quinolones in the monomeric $C_{18}$ stationary phase. The concentrations (mM) of DMOA were: 0, a; 2, b; 5, c; 10, d; 20, e; 30, f; and 40, g.

## Results and Discussion

### Effect of the residual silanol on the monomeric $C_{18}$ stationary phase

In order to study the effect of the residual silanol of the $C_{18}$ stationary phase, the experiment was performed without additive eluent, which did not mask the silanol group (presented in Table I).

In all quinolones, the hydrogen bonding (i.e., interaction of the nitrogen atom of quinolones with the silanol group was caused) represented the band broadening, peak tailing, and low plate number. Chromatograms of five quinolones are shown in Figure 2. As can be seen in Figure 2, quinolones showed poor resolution, bad peak shape, and an asymmetry factor of more than 3.

### Masking effect of the residual silanol by the increasing DMOA concentration

In order to prevent the interaction of the residual silanol with ionic solutes, DMOA as a masking agent was added to the eluent. DMOA concentration was increased to the range of 0 to 40mM. Two representative chromatograms are shown in Figure 3. As the additive increased, the peak shape was sharpened. The change of retention time (log k'), $F_{asy}$, and plate number is presented in Figure 4. As the additive increased (such as Figure 4A), the retention time of quinolones decreased gradually. Adding DMOA to the eluent caused competitive exchange interaction between the ionic solute and additive, which interact with the silanol site, thus the retention time came to be reduced by degrees. When additive mostly fills the

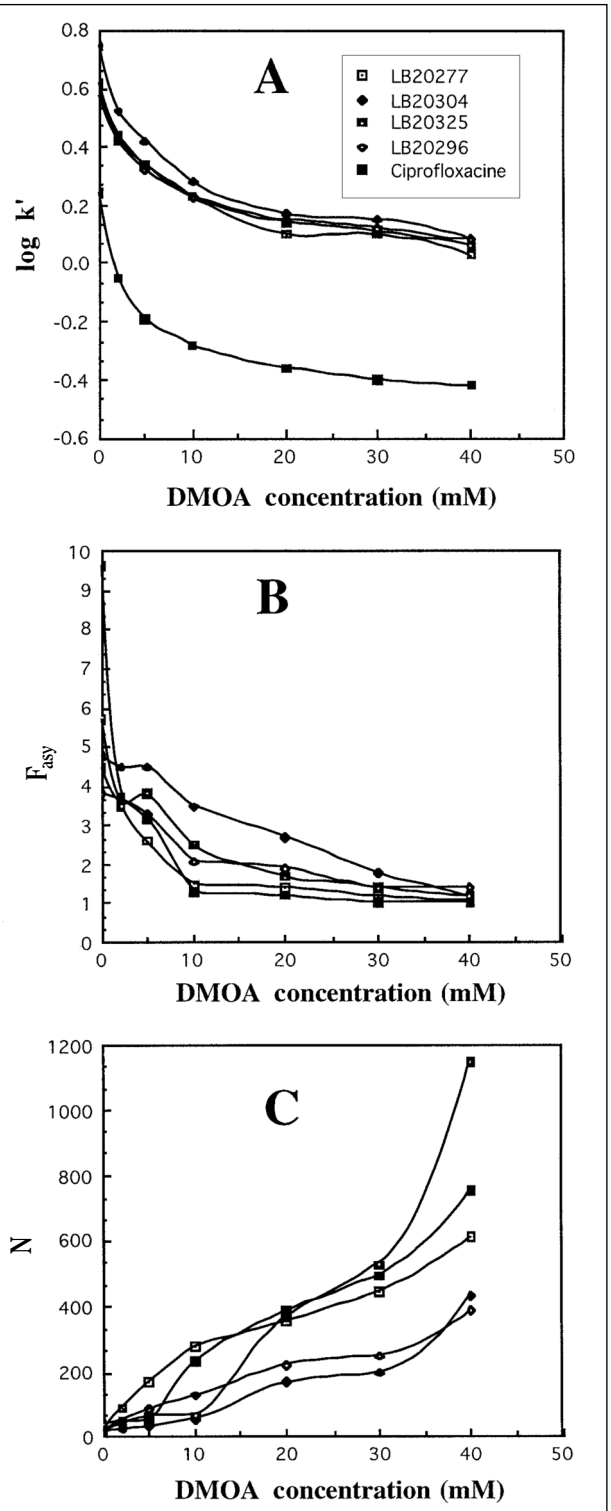


**Figure 4.** The comparative data of retention and peak shape by the change of DMOA concentration.

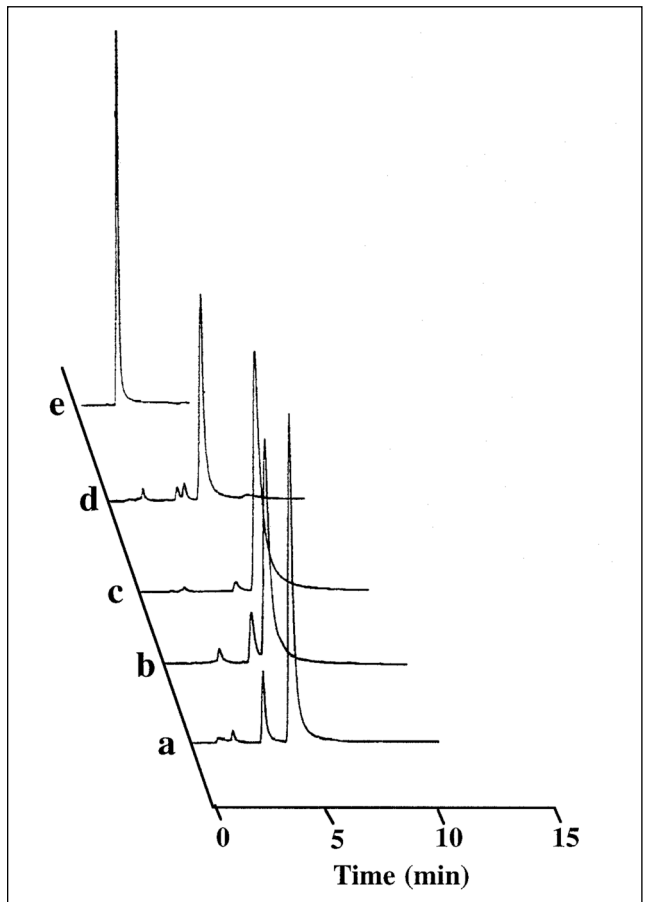


**Figure 5.** Chromatograms of quinolones on the monomeric $C_{18}$ stationary phase with the MeOH–phosphate eluent with additive (pH 4): LB20277, a; LB20296, b; LB20304, c; LB20325, d; and ciprofloxacin, e.

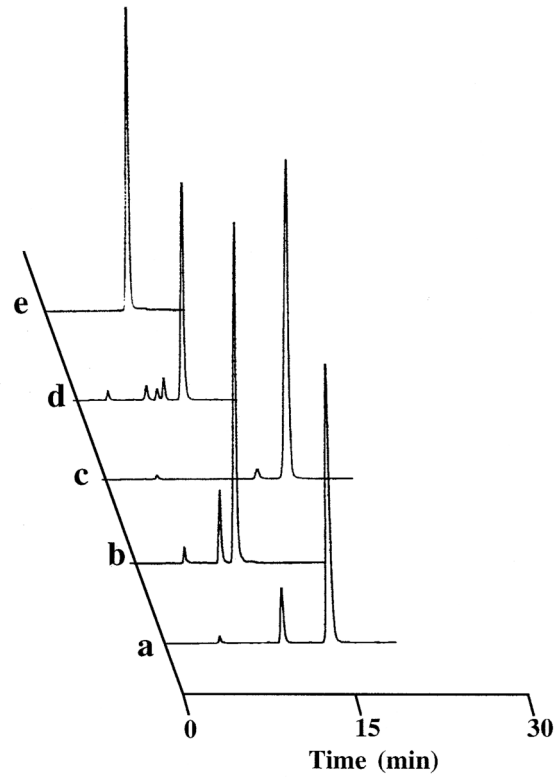


**Figure 6.** Chromatograms of quinolones on the polymeric $C_{18}$ stationary phase with the MeOH–phosphate eluent without additive (pH 4): LB20277, a; LB20296, b; LB20304, c; LB20325, d; and ciprofloxacin, e.

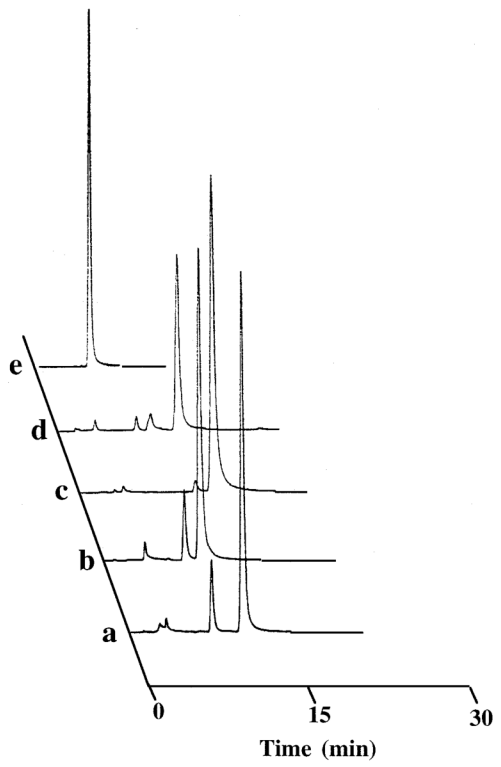


**Figure 7.** Chromatograms of quinolones for an investigation of the influence of pH in the monomeric $C_{18}$ stationary phase (pH 2): LB20277, a; LB20296, b; LB20304, c; LB20325, d; and ciprofloxacin, e.

silanol site by competitive interaction, the retention time of quinolones is kept constant.

In routine analysis, peak asymmetry within proper ranges can be allowed. However, in quantitative analysis, $F_{asy}$ below 1.5 can be accepted (2).

In all quinolones, as the additive increases, $F_{asy}$ decreases below 1.5 (Figure 4B) and the plate number (Figure 4C) increases. The chromatograms of quinolones in the eluent condition that added 30mM of DMOA in pH 4 are presented in Figure 5. It is known that all quinolones showed good peak shape.

### Elution behavior in the silica-based polymeric $C_{18}$ stationary phase

As another way to prove that quinolones show poor peak shape and low plate number for interaction by residual silanol, the quinolones were made to be eluted on the silica-based polymeric $C_{18}$ stationary phase Capcell-Pak $C_{18}$. The packing materials of Capcell-pak $C_{18}$ were silicone-coated silica gels modified with octadecyl. Silicone coating protects silanol groups on the silica gel surface; therefore, the stationary phase overcomes unusual interaction by residual silanol. The composition of the eluent was made to show a similar retention time of the quinolone in the monomeric $C_{18}$ stationary phase, and the additive was not used.

The chromatograms are shown in Figure 6. All quinolones in the figure did not show peak tailing and band broadening. Consequently, it was known that the bad peak shapes of quinolones were the influences by residual silanol in the monomeric $C_{18}$ stationary phase.

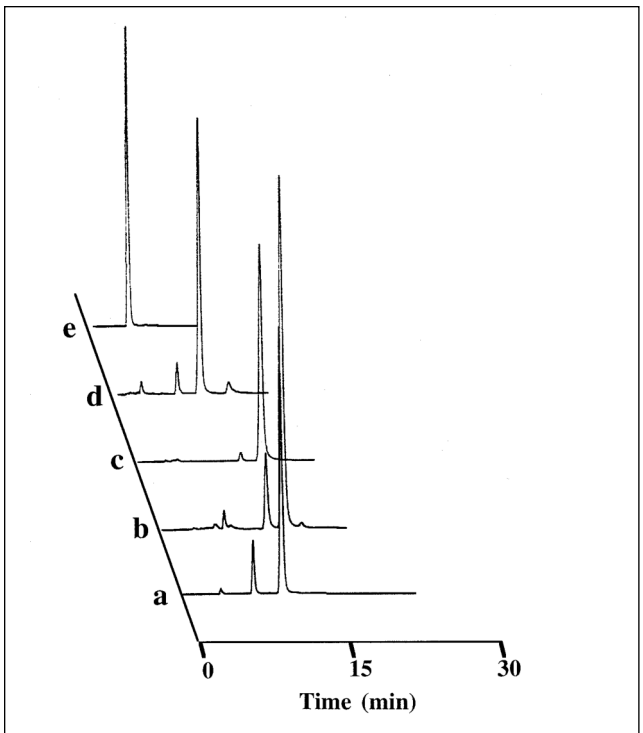


**Figure 8.** Chromatograms of quinolones on the monomeric $C_{18}$ stationary phase with the MeOH–phosphate eluent with additive (pH 2): LB20277, a; LB20296, b; LB20304, c; LB20325, d; and ciprofloxacin, e.

### pH effect of the residual silanol on the monomeric $C_{18}$ stationary phase

The residual silanol is shielded in the acidic condition of the eluent. Therefore, interaction between the analyte and residual silanol can be decreased or removed. In order to investigate the effect of pH with residual silanol, the quinolones were made to be eluted under the eluent of pH 2 without additive. The basic functional groups of quinolones and silanol become protonation in the eluent condition of pH 2, and the interaction becomes decreased as well as peak asymmetry. A chromatogram is shown in Figure 7. All quinolones represented a little better peak shape in comparison with the chromatogram of Figure 5 in the eluent condition of pH 4 including the additive.

In order to investigate the interaction of the additive with the silanol group at pH 2, 30mM DMOA was added to the eluent at pH 2. As shown in Figure 8, we could obtain a good chromatogram in which tailing was eliminated such as that of the polymeric $C_{18}$ stationary phase.

### Comparison of the chromatogram concerning the improvement process of the peak shape

The elution behavior of the quinolones investigated up to this point is represented in Figure 9. It is known that by the method of adding additive, the protonation of silanol sites, or both, peak shape can be improved. Also, the best peak shape was seen in the condition of acidic eluent (pH 2) including the additive.

### Eluent composition available in the LC–MS analysis of quinolones

The determination of a drug and metabolite of trace-amount extract within blood or urine is a critical and analytical issue. The amines as additive or inorganic species could not be used in the eluent of LC–MS. Therefore, the eluent with additive used in the monomeric $C_{18}$ stationary phase of this study could not be used. As a consequence, in order to eliminate silanol interaction as mentioned previously, the polymeric $C_{18}$ stationary phase and ACN–trifluoroacetic acid (TFA) system as an eluent were used.

In all quinolones, good resolution and peak shape were shown. The chromatogram of five quinolones is illustrated in Figure 10.

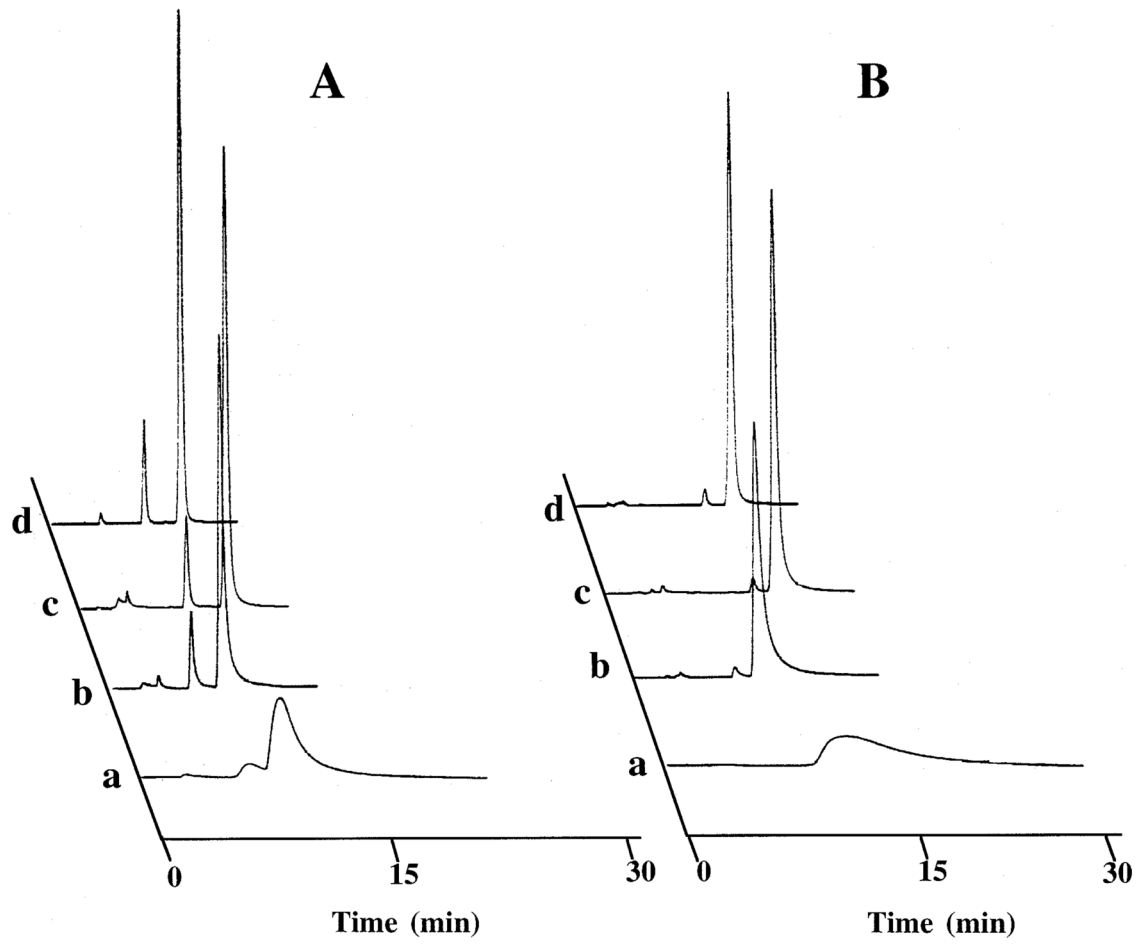


**Figure 9.** Comparison of two chromatograms ((A) LB20277 and (B) LB20304) for the improvement process of the peak shape on the monomeric $C_{18}$ stationary phase: (a) without additive (pH 4), (b) with additive (30mM DMOA, pH 4), (c) without additive (pH 2), and (d) with additive (30mM DMOA, pH 2).

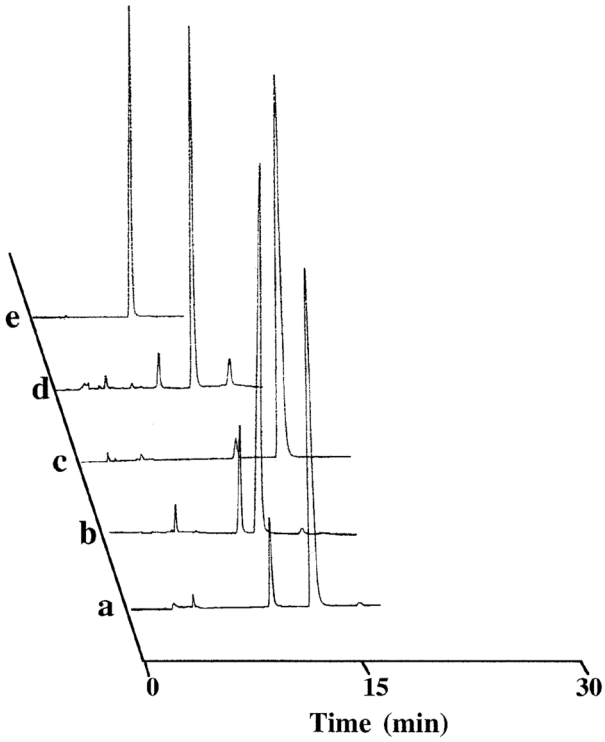


**Figure 10.** Chromatograms of quinolones in the ACN–TFA system: LB20277, a; LB20296, b; LB20304, c; LB20325, d; and ciprofloxacin, e.

## Conclusion

The elution behavior of quinolones in the $C_{18}$ stationary phase, so far, has been studied. The important things in the serious interaction mentioned are probably the hydrogen bonding of the analyte and the silanol site. It is known that the strong tailing and low plate number shown by interaction with the residual silanol of a silica-based monomeric $C_{18}$ stationary phase depend on the structural parameter of the analyte.

Peak shape can be improved by the protonation of silanol sites, adding additive, or both. Another method used to obtain good peak shape is using the polymeric stationary phase, in which silanol sites are eliminated.

Using LC–MS is essential to the analysis of drug metabolites involved with quinolones, thus it is suggested that the polymeric $C_{18}$ stationary phase and ACN–TFA system as the eluent should be used.

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