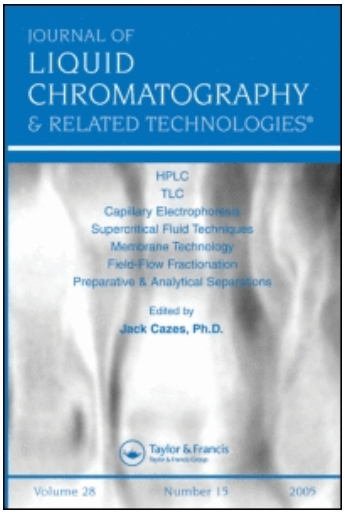

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Abstract: The effects of counter-anions on the reversed-phase HPLC retention of zwitterionic quinolones were studied. Four counter-anions, perchlorate, tetrafluoroborate, trifluoroacetate, and dihydrogenphosphate, were used as mobile phase additives. HPLC analysis was performed at a mobile phase pH 3, to ensure the complete protonation of the piperizine ring and to suppress the ionization of the carboxylic acid groups of the analytes. Results showed that retention factors of all analytes increased to varying degrees with increasing counter-anion concentration. The increase in retention of the analytes is attributed to the chaotropic effect exhibited by the counter-anions. Complete separation of all the studied compounds was achieved by varying the type and amount of counter-anions present in the mobile phase. The changes in selectivity offered by these counter-anions would be beneficial, especially for developing chromatographic methods for the analysis of quinolones in different sample matrices.

Keywords: Quinolones, Chaotropic effect, Counter-anion

INTRODUCTION

The quinolones are a group of antibiotics with a broad spectrum of activity against gram-positive and gram-negative bacteria through inhibition of their DNA gyrase.[1,2] The common structural feature among quinolones is the

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presence of a 1-substituted-1,4-dihydro-4-oxopyridine-3-carboxylic acid moiety combined with an aromatic or heteroaromatic ring. The antibacterial activity of these compounds is increased by the addition of 6-fluoro and 7-piperazinyl groups to the molecule. Quinolones are widely used in human and veterinary medicine for the treatment of pulmonary, urinary, and digestive infections. However, intensive use of these drugs in animals can leave residues in edible animal tissues that can lead to serious problems such as toxicity, drug resistance, and possible allergic reactions in man.[3–5]

High performance liquid chromatography (HPLC) has been used routinely for the quantitative determination of quinolones in biological fluids[6] and animal tissues.[7–10] However, a general problem of HPLC analysis of basic and polar substances like the quinolones, is the severe peak broadening and tailing on reversed-phase columns, due to specific interactions of these analytes with the support.[8] Quinolones containing the basic piperazine group can interact with the residual silanol groups of the reversed-phase column resulting to severe band broadening and tailing.[8] This problem can be overcome by using mobile phase with high acidity and ionic strength or using ion-pairing reagents as mobile phase additives. However, the use of such a mobile phase may rapidly deteriorate the packing materials of the column.[11] Using mobile phases with high acidity promotes the protonation of the basic group of the analyte and prevents the ionization of the residual silanol groups of the column packing material, thus, preventing ionic interaction between the analyte and the stationary phase surface. Ion-pair chromatography on the other hand, uses an ionic organic compound as a mobile phase additive, which forms an ion-pair with the analyte of opposite charge. This ion-pair is a salt, which chromatographically behaves like a non-ionic organic molecule that can be separated in reversed phase chromatography.

HPLC separation of basic analytes can also be achieved by using inorganic counter-anions. Machida et al.[12] reported that the retention of hydrophobic amino compounds could be obtained by the addition of highly polarizable counter-anions such as $H_2PO_4^-$, Cl^-, Br^-, NO_3^-, and I^-. The retention of β-blockers on a C_{18} column was increased in the presence of perchlorate, tetrafluoroborate, hexafluorophasphate, trifluoroacetate, and dihydrogenphosphate, as reported by Jones et al.[13] Trifluoroacetate was also found to affect the reverse-phase separation of triazole derivatives.[14] The influence of these counter-anions on the retention behavior of basic analytes was considered to be the effect of desolvation or chaotropic effect.[13,15] In the presence of a chaotropic anion, the analyte solvation shell is disrupted resulting in an increase in the analyte apparent hydrophobicity and increase in an affinity to the stationary phase. Chaotropic anions as mobile phase additives were found to exhibit a positive influence on chromatographic parameters including loading capacity, peak efficiency, and peak symmetry.[16]

In this study the effect of the type and concentration of counter-anions as mobile phase additives on the retention behavior of zwitterionic quinolones using reversed-phase liquid chromatography was determined. The results

obtained were then used to develop a chromatographic method for the analysis of these analytes in blood serum samples.

EXPERIMENTAL

Chemicals

Enrofloxacin, difloxacin, norfloxacin, oxolinic acid, pipemidic acid, and danofloxacin mesylate were obtained from Sigma-Aldrich (Tokyo, Japan). Gatifloxacin, moxifloxacin, and tosufloxacin were purchased from LKT Laboratories Inc. (Tokyo, Japan). Perchloric acid, trifluoroacetic acid, sodium tetrafluoroborate, sodium trifluoroacetate, and HPLC grade acetonitrile were from Wako Pure Chemical Industries (Osaka, Japan). Sodium perchlorate and phosphoric acid were obtained from Kishida Chemicals (Osaka, Japan). Sodium dihydrogenphosphate was purchased from Merck (Darmstadt, Germany). Water used for mobile phase preparation was purified by a Milli-Q Water Purification System (Millipore, Tokyo, Japan).

Preparation of Standard Solutions

Stock solutions of 1 mg/mL of the quinolones were prepared by dissolving accurately weighed amounts of the analytes in methanol containing 0.02 M NaOH. The solutions were stored in a refrigerator operated at 4°C. Working solutions were prepared daily from stocks at concentration of 25 μg/mL. All solutions were filtered using a 0.2 μm disposable nylon filter (Toyo Roshi Kaisha, Tokyo, Japan) prior to chromatographic analysis.

Preparation of Serum Sample

Preparation of serum samples was done as described by Samanidou et al.[17] Aliquots of 50 μL human blood serum were spiked with 50 μL of a mixture containing all the analytes at two different concentrations: 5 and 2.5 μg/mL. Acetonitrile (400 μL) was added to the mixture to precipitate proteins. After mixing for two minutes, the sample was centrifuged at 3500 rpm for 15 mins and the supernatant was evaporated at 45°C under a nitrogen stream. The residue was dissolved in 20% aqueous methanol and filtered using a 2 μm disposable nylon filter.

Equipment

The chromatograph composed of a JASCO 880-PU Intelligent HPLC Pump (Jasco, Tokyo, Japan) equipped with a Rheodyne 7125 injector (Cotati, CA, USA) with a 20 μL sample loop and a TOSOH UV-8010 uv-visible

detector (Tokyo, Japan). The separation of the analytes was carried out on a Shimpack VP-ODS column (5 μm, 150 × 2 mm i.d.) purchased from Shimadzu (Kyoto, Japan). Column temperature was maintained at 40°C using an oven (TOSOH CO-8000, Tokyo, Japan).

HPLC Measurements

Chromatographic analysis was performed under isocratic conditions at a flow rate of 0.2 mL/min. The mobile phase composed of an aqueous solution of the counter-anion buffered at pH 3.0 and acetonitrile at a ratio of 80:20 unless otherwise specified. The pH of the aqueous phase was maintained at 3.0 by adding phosphoric acid for the experiments employing sodium dihydrogen-phosphate and sodium tetrafluoroborate, while perchloric acid and trifluoroacetic acid were added for those experiments utilizing sodium perchlorate and sodium trifluoroacetate, respectively. Counter-anion concentrations were increased by the addition of the salt of the respective anion. A constant volume of the analytes (20 μL) was injected manually using a glass syringe. Absorbance of the samples was measured at 280 nm. Uracil was used as the unretained marker to measure the void volume. Data acquisition was performed using BORWIN PDA Chromatography Data Handling Software (JASCO, Tokyo, Japan) running on a personal computer.

RESULTS AND DISCUSSION

Effect of Counter-Anion Concentration

The quinolones being studied (Figure 1) have two ionizable functional groups, which means that their acid-base chemistry involves two equilibria, the dissociation of the carboxylic acid group and the deprotonation of the nitrogen of the piperazine ring. In aqueous solutions, these analytes exist in three different forms, which are cationic, zwitterionic, and anionic. The cationic species dominates at pH <5.5, and therefore, the use of mobile phase pH 3.0 would ensure the protonation of the nitrogen in the piperazine ring and also prevent the dissociation of the carboxylic acid group.

Figures 2A–2D show the effect of counter-anion concentration on the retention of eight quinolones. It can be seen from these figures, that the retention factors of the analytes increased with the concentration of the counter-anion. For all the compounds being studied, a characteristic sharp increase in retention factor is observed at the low concentration region (below 10 mM). As the concentration of the counter-anion is increased above 10 mM, very little effect on the retention factor was observed. These observations are in good agreement with the theory of chaotropicity.[18] In aqueous solution, the analytes having basic nitrogen groups, are protonated and solvated. These

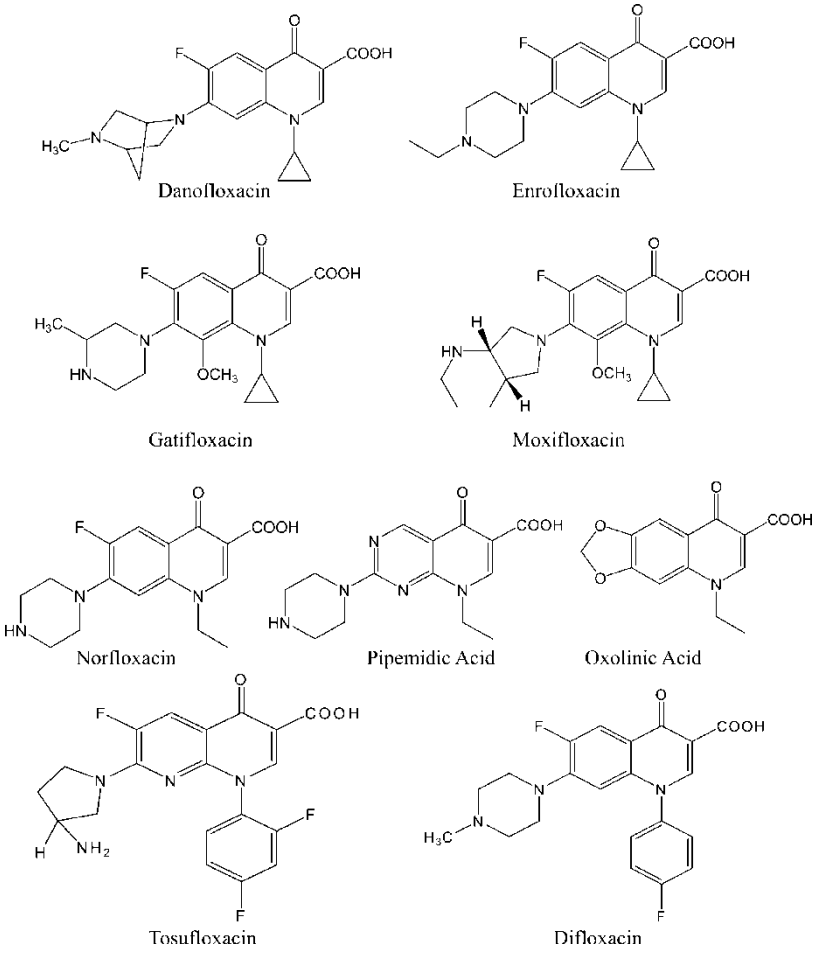

Figure 1. Structures of the zwitterionic quinolones used in this study.

positively charged species can interact, through electrostatic attraction, with the negatively charged counter-ions present in the mobile phase. The ionic interaction of oppositely charged molecules causes the displacement of the surrounding water molecules as the two ions approach each other. As a result of this desolvation, the apparent hydrophobicity of the analytes increases, therefore increasing the analytes' affinity to the stationary phase.

The mathematical description of the effect of chaotropic anions on the retention of basic analytes on HPLC is described by LoBrutto et al.[15] The equation is:

$$k = \frac{k_s - k_{us}}{K[\mathrm{A}^-] + 1} + k_{us} \tag{1}$$

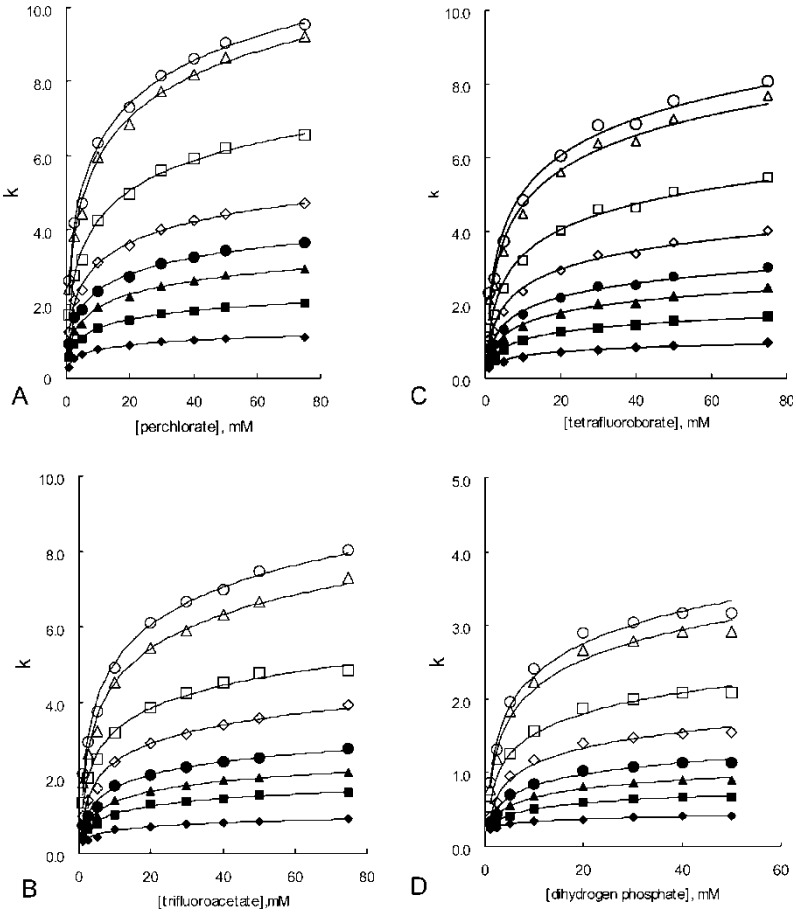

Figure 2. Effect of counter-anion concentration on the retention factor of the analytes. Counter-anions used were: A perchlorate; B trifluoroacetate; C tetrafluroborate; D dihydrogenphosphate. Analytes were: Pipemidic acid (◆); norfloxacin (■); danofloxacin (▲); enrofloxacin (•); gatifloxacin (◇); difloxacin (□); moxifloxacin (△); tosufloxacin (O).

where $[A^-]$ is the concentration of the chaotropic anion, k is the retention factor of the analyte, K is the analyte desolvation parameter, k_s and k_{us} are the analyte limiting retention factors for completely solvated and completely unsolvated forms, respectively. Assuming the absence of analyte-analyte interactions and thermodynamic equilibrium of the chromatographic system, the desolvation parameter K represents the slope of the retention dependence in the region of low counter-anion concentration. A larger value for the constant K indicates that the analyte nears complete desolvation at a low concentration of chaotropic counter-anion.

Effect of the Type of Counter-Anion

The retention of the analytes is also dependent on the type of counter-anions present in the mobile phase. Perchlorate, tetrafluoroborate, trichloroacetate, and dihydrogenphosphate affected the retention factors of the compounds at varying degrees as can be seen from Figures 2A–2D. To clearly see the effects of the different types of counter-anions, plots of the retention factor against the concentration of the four counter-anions were made for the least retained (pipemidic acid) and most retained (tosufloxacin) analytes (Figures 3A and 3B). In this figure, it can be seen that regardless of the counter-anion employed in the mobile phase, a consequent increase in the retention of both pipemidic acid and tosufloxacin was observed. However, at each concentration studied, the highest increase in the retention of both analytes were obtained when perchlorate was employed and the lowest when dihydrogenphosphate was used. As explained by Jones et al.[13] the differences observed in the analyte retention may be attributed to the extent of the solvation of the counter-anion used. The least solvated counter-anion has the greatest tendency to interact with the positively charged analytes through ionic interaction. This counter-anion will, therefore, be the most effective in disrupting the analyte solvation shell and, consequently, will lead to an increase in analyte hydrophobicity. In aqueous solutions, perchlorate, tetrafluoroborate, trifluoroacetate, and dihydrogenphosphate have different degrees of solvation. Dihydrogenphosphate anion being able to form hydrogen bonds with water, is highly solvated in aqueous solution. Trifluoroacetate is also capable of acting as hydrogen donor and hydrogen acceptor, but it is not as polar as dihydrogenphosphate because its negative charge is more delocalized due to the fluorine atoms, which exert electron withdrawing effects. Tetrafluoroborate on the other hand, can only act as a very weak hydrogen bond acceptor because the presence of four electron withdrawing fluorine atoms further delocalizes the negative charge. Perchlorate contains four electron withdrawing oxygen atoms. For all the analytes, greater changes in retention were observed when perchlorate was used as compared to tetrafluoroborate. According to Jones et al.[13] this may be attributed to the charge density in which the charge is more delocalized in perchlorate than tetrafluoroborate since the central atom chlorine (Cl), has a greater atomic radius than boron (B).

The effects of the types of counter-anion on the retention of the analytes can also be assessed by comparing the limiting retention factors of the unsolvated forms (k_{us}) and the desolvation parameter K of the analytes obtained for each type of counter anion. Table 1 summarizes the values of k_{us} and K obtained by the curve fitting of equation (1). As can be seen from this table, the k_{us} values for each analyte when perchlorate was used were the highest, while the k_{us} values were the lowest when dihydrogen phosphate was used. In terms of K, the values obtained when dihydrogen phosphate was used were generally higher compared to the values when perchlorate, tetrafluoroborate, or trifluoroacetate were used. The K value is essentially the equilibrium

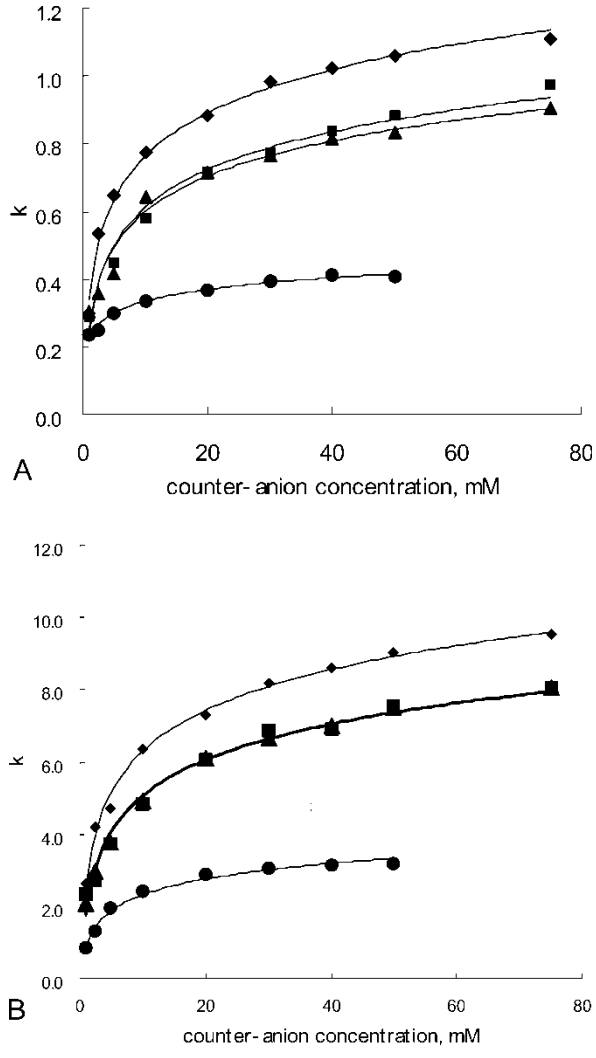

Figure 3. Effect of different types of counter-anion on the retention of (A) pipemidic acid and (B) tosufloxacin. Counter-anions used were: perchlorate (◆); tetrafluoroborate (■); trifluoroacetate (▲); and dihydrogenphosphate (●).

constant of the solvation-desolvation process.[15] This constant also represents the stability of the ion-associated complex.[13] Based on these, it can be said that the stability of ion-associated complexes of dihydrogenphosphate with the studied compounds are more stable than for the other counter-anions.

Figure 4 shows an overlay of the chromatograms for eight zwitterionic quinolones analyzed in the presence of equimolar amounts of the different counter-anions. It is shown that different selectivity can be obtained by

Table 1. Desolvation parameter of the zwitterionic quinolones with different counter-anions

	k_{us}				K			
	ClO_4^-	BF_4^-	CF_3COO^-	$H_2PO_4^-$	ClO_4^-	BF_4^-	CF_3COO^-	$H_2PO_4^-$
Pipemidic acid	1.823	1.168	1.113	0.452	0.159	0.045	0.015	0.089
Norfloxacin	2.151	1.983	1.987	0.829	0.098	0.054	0.056	0.068
Danofloxacin	3.254	2.921	2.625	1.016	0.079	0.049	0.054	0.109
Enrofloxacin	3.919	3.735	3.738	1.279	0.090	0.043	0.036	0.126
Gatifloxacin	5.775	4.800	4.798	1.730	0.079	0.049	0.050	0.140
Difloxacin	7.468	6.467	5.494	2.360	0.072	0.054	0.079	0.129
Moxifloxacin	10.468	9.384	9.076	3.243	0.068	0.044	0.048	0.149
Tosufloxacin	10.942	9.972	9.999	3.561	0.069	0.046	0.050	0.139

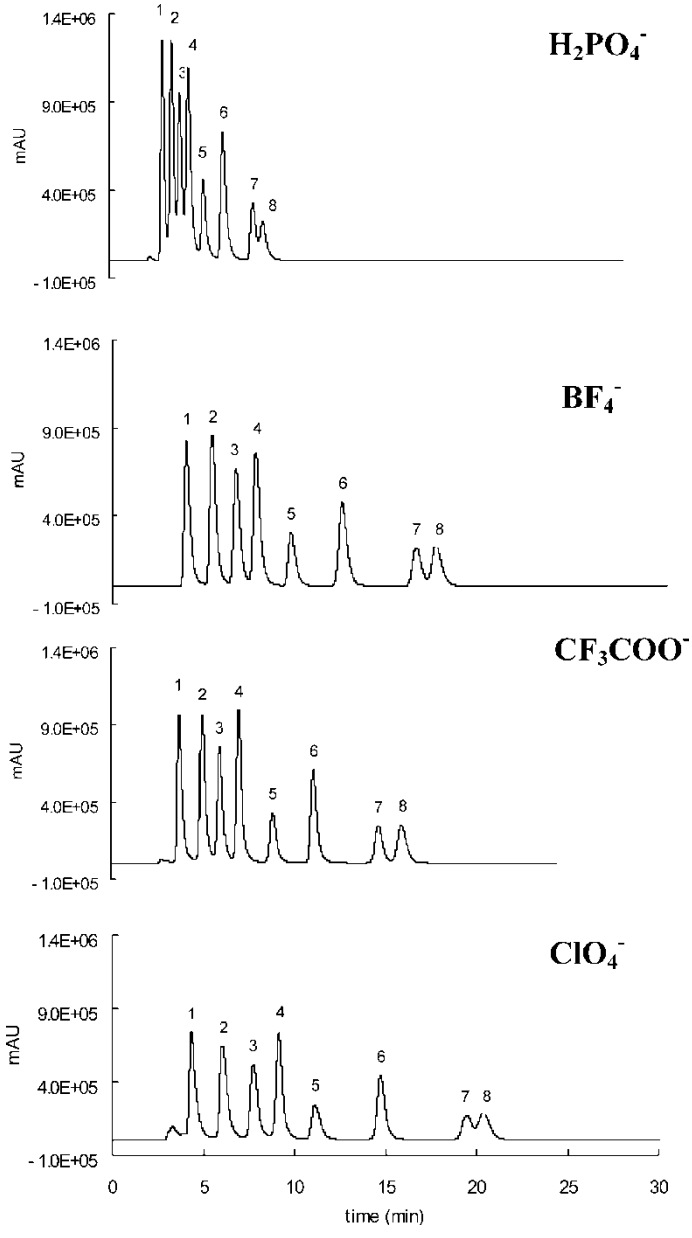

Figure 4. Separation of zwitterionic quinolones using different types of counter-anion. Peaks: (1)-pipemidic acid; (2)-norfloxacin; (3)-danofloxacin; (4)-enrofloxacin; (5)-gatifloxacin; (6)-difloxacin; (7)-moxifloxacin; (8)-tosufloxacin. Chromatographic conditions: mobile phase- aqueous solution of counter anion at pH 3.0 plus acetonitirle (80:20); flow rate – isocratic at 0.2 mL/min; column temperature −40°C; UV detection- 280 nm; injected sample volume −20 μL; sample concentration −25 μg/mL.

changing the type of counter-anion employed. The analytes were not separated by using dihydrogenphosphate as a mobile phase additive as indicated by the overlapping peaks. Good resolutions were obtained by using trifluoroacetate, tetrafluoroborate, and perchlorate anions, but peaks 7 and 8, corresponding to moxifloxacin and tosufloxacin, respectively, were not perfectly resolved. On the other hand, Figure 5 shows the effect of trifluoroacetate on the retention

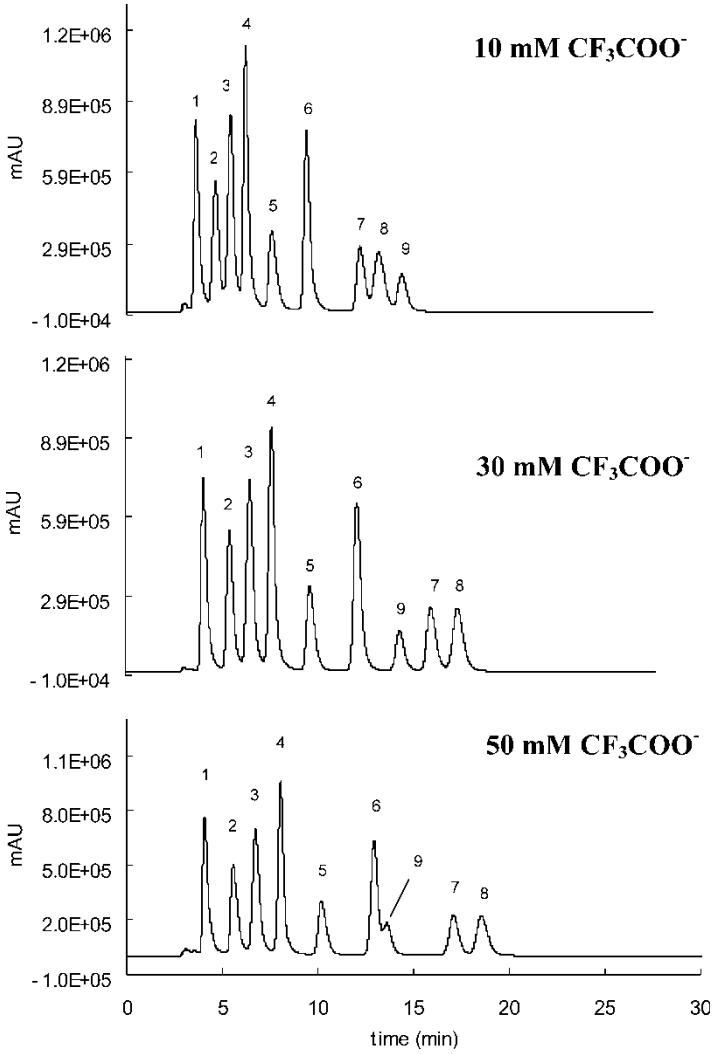

Figure 5. Separation of zwitterionic quinolones using varying concentrations of trifluoroacetate. Peaks: (1)-pipemidic acid; (2)-norfloxacin; (3)-danofloxacin; (4)-enrofloxacin; (5)-gatifloxacin; (6)-difloxacin; (7)-moxifloxacin; (8)-tosufloxacin; (9)-oxolinic acid. Chromatographic conditions are the same as in Figure 4.

of the analytes. In this figure, increasing the concentration of the trifluoroacetate also resulted in different selectivities. Peak number 9 corresponds to oxolinic acid. The difference of this analyte to the rest is that it does not contain a basic functional group, therefore, its retention should not be affected by the presence of counter-anion. It must be noted that the retention of oxolinic acid was not affected by the increase in trifluoroacetate concentration as indicated by almost constant retention time in all trifluoroacetate concentrations. As can be seen in Figure 5, the order of retention of the analytes was changed as a result of the chaotropic effect of trifluoroacetate. At 10 mM trifluoroacetate, oxolinic acid was the last to be eluted but as the concentration increased to 30 mM, moxifloxacin and tosufloxacin became more retained as compared to oxolinic acid. At 50 mM trifluoroacetate, the peak for difloxacin almost overlapped with that of oxolinic acid. Figures 4 and 5 clearly reveal the significant effects of the type and concentration of counter-anions on the retention of zwitterionic quinolones.

Effect of pH

The effect of counter-anions on the retention of compounds having basic functional groups also depends on pH.[14] Ionic interaction between the basic analytes and the counter-anion must occur in order for desolvation to take effect. Efficient ionic interaction will take place if the basic analytes are completely ionized or protonated. Complete ionization of such compounds is achieved at acidic pH. As mentioned earlier, the quinolones being analyzed in this study contain both basic amino groups and carboxylic acid groups. The degree of ionization of these functional groups will dictate the total charge of the molecules and the strength of their interactions with the counter-anion. Figure 6 shows the effect of varying the pH of the aqueous component of the mobile phase on the retention of the quinolone analytes in the presence of 30 mM trifluoroacetate. At pH 3.0, all the analytes are assumed to be positively charged hence, the chaotropic effect of the counter-anion was clearly observed. Increasing the pH to 3.5 and 4.0 resulted in tailing and overlapping of some peaks. At these pH values, the carboxylic acid groups are ionized to a certain extent, although the amino groups of the analytes are still protonated. The negatively charged carboxylic acid groups within the zwitterionic quinolones presumably repelled the negatively charged trifluoroacetate anion, and prevented to some degree, the chaotropic effect exhibited by the counter-anion. Oxolinic acid (peak 9), which does not contain a basic amino group, was not affected at all by trifluoroacetate.

Application to Method Development

The increase in retention of the studied quinolones in the presence of counter-anions can result in significant changes in selectivity as discussed earlier. This

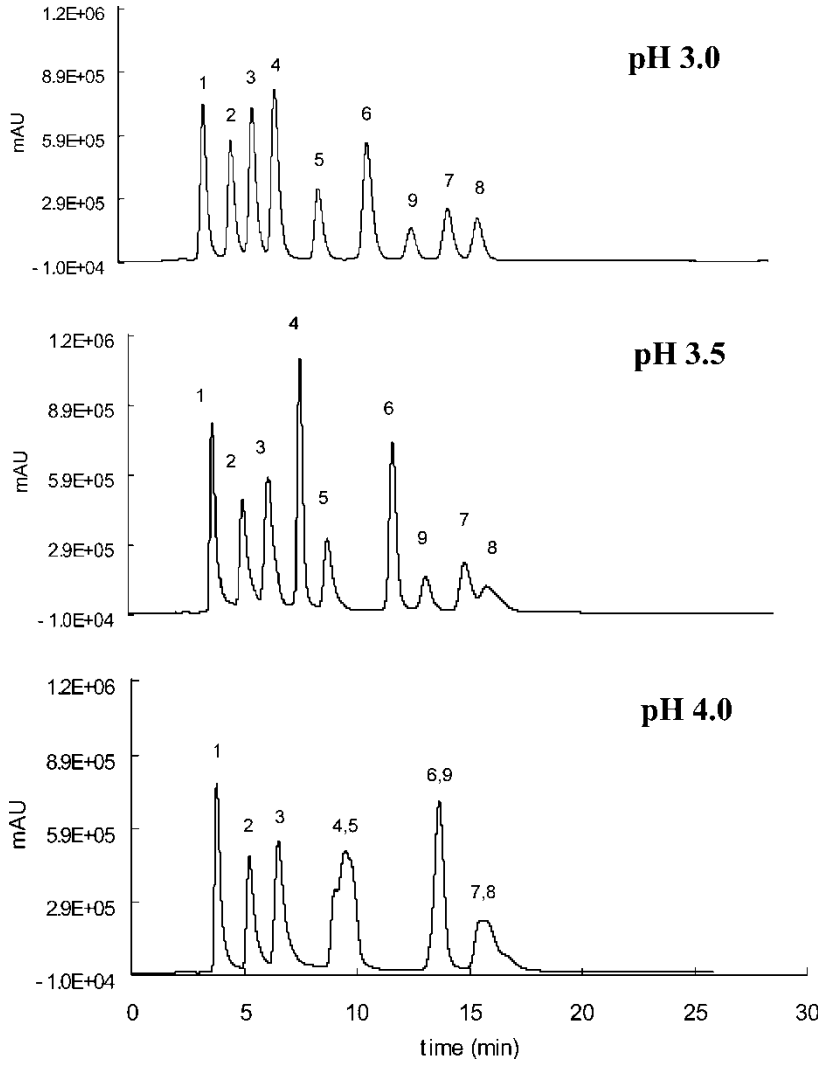

Figure 6. Effect of pH on the separation of zwitterionic quinolones using 30 mM trifluoroacetate as mobile phase additive. Peaks: (1)-pipemidic acid; (2)-norfloxacin; (3)-danofloxacin; (4)-enrofloxacin; (5)-gatifloxacin; (6)-difloxacin; (7)-moxifloxacin; (8)-tosufloxacin; (9)-oxolinic acid. Chromatographic conditions are the same as in Figure 4.

effect is of practical importance, especially in developing HPLC methods for the separation of these analytes. For this part of the study, the determination of the optimum separation condition for the nine quinolones was carried out. Trifluoroacetate was chosen as the counter-anion because of its compatibility with LC-MS, which will be the subject of our future studies. In order to

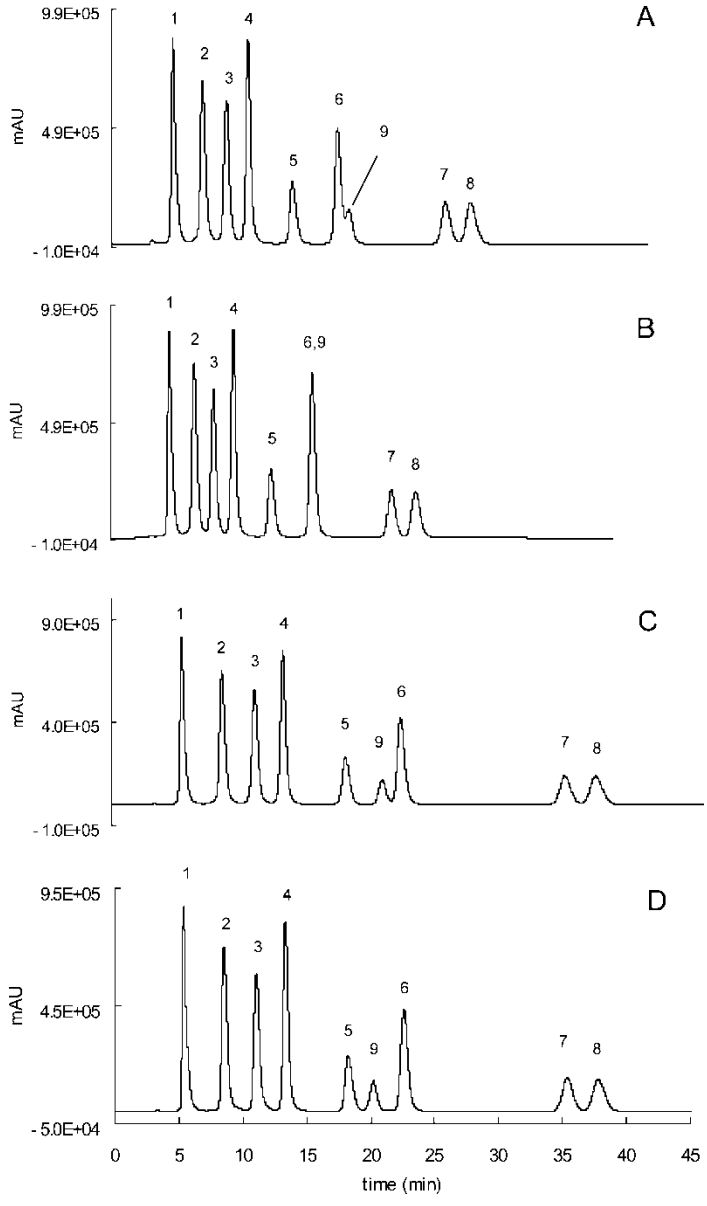

Figure 7. Separation of zwitterionic quinolones in the presence of trifluoroacetate using different mobile phase compositions. The mobile phase composed of an aqueous solution of trifluoroacetate at pH 3 and acetonitrile. The concentration of trifluoroacetate and the ratio of the aqueous component to acetonitrile were varied as follows: (A) 30 mM trifluroacetate, (82:18); (B) 40 mM trifluoroacetate, (83:17); (C) 50 mM trifluoroacetate, (81–19); (D) 50 mM trifluoroacetate, (83:17). Peak identity and other chromatographic conditions are the same as in Figure 5.

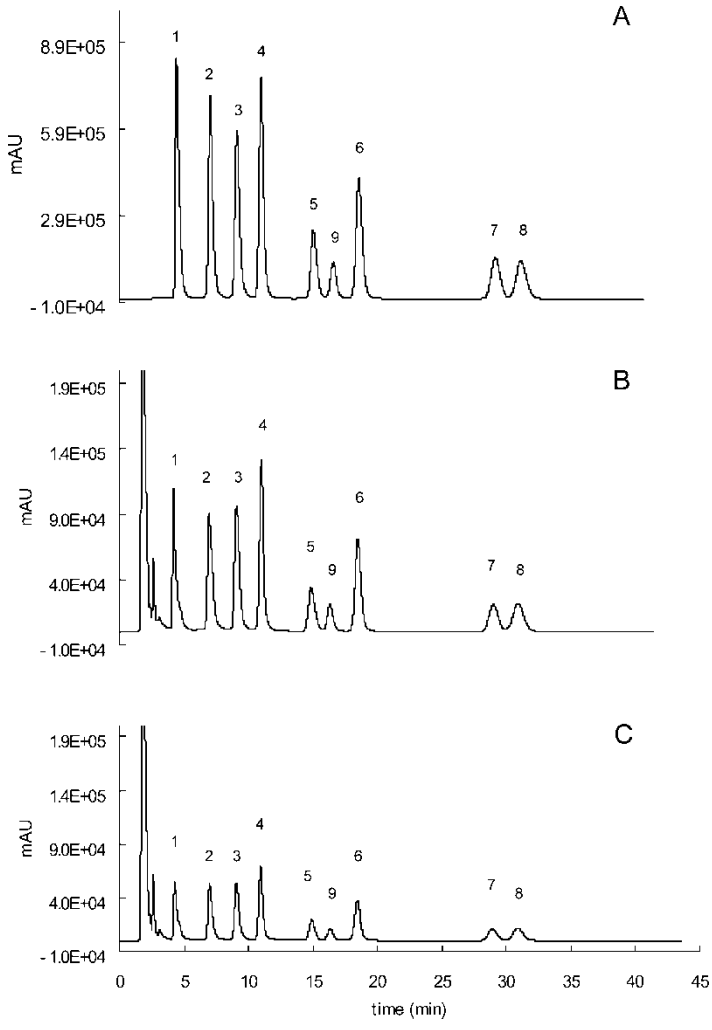

Figure 8. Separation of zwitterionic quinolones using the optimized chromatographic conditions. (A) standard samples; (B) blood serum spiked with 5.0 μg/mL of the analytes; (C) blood serum spiked with 2.5 μg/mL of the analytes. Chromatographic conditions: mobile phase- 50 mM trifluoroacetate at pH 3.0 plus acetonitirle (83 : 17); flow rate – isocratic at 0.25 mL/min; column temperature −40°C; UV detection- 280 nm; injected sample volume −20 μL. Peak identity is the same as in Figure 5.

find the optimum separation condition, the mobile phase composition was changed with respect to the amount of trifluoroacetate and percentage of acetonitrile. Figures 7A–7D show the chromatograms obtained at different mobile phase compositions. As can be seen from these figures, different

retention selectivity can be obtained by varying the concentration of trifluoroacetate and percentage of acetonitrile in the mobile phase. Based on these results, the mobile phase containing 83% of 50 mM trifluoroacetate pH 3.0 and 17% acetonitrile was found to be optimum (Figure 7D). Increasing the flow rate to 0.25 mL/min using the optimized mobile phase composition resulted in faster separation without peak overlapping. This flow rate was, therefore, used for the succeeding analyses. The chromatogram of the nine quinolones using the optimized conditions is shown in Figure 8A. Complete separation of the nine quinolones was achieved in less than 35 minutes. Figures 8B and 8C on the other hand, show the chromatograms of blood serum samples spiked with known concentrations of the nine analytes. These figures show the applicability of the optimized condition for the analysis of these quinolones on blood samples.

CONCLUSION

The effect of chaotropic anions namely perchlorate, tetrafluoroborate, trifluoroacetate, and dihydrogenphosphate, as mobile phase additives on the reversed-phase separation of zwitterionic quinolones was studied. It was found that the retention factors of all studied quinolones increased with increasing amount of counter-anions in the mobile phase. This behavior is consistent with the theory of chaotropicity. The effect of the counter-anion concentration followed the general equation derived by Jones et al.[13] Using this equation, the desolvation parameter and the limiting retention factor for the completely unsolvated form were computed. Analysis of these parameters revealed similar effects of all types of counter-anions studied on the retention of the zwitterionic quinolones.

A difference in the analyte retention was also observed when different types of counter-anions were employed. The chaotropic effect of the counter-anions was found to be related to the anion's solvation and charge delocalization properties. Dihydrogenphosphate, which is the most polar and most solvated among the anions used exhibited the least chaotropic effect.

The chaotropic effect exhibited by trifluoroacetate was found to be dependent on the pH of the mobile phase. Since the studied analytes contain both basic and acidic functional groups, the degree of their ionization is affected by the pH of the mobile phase. At pH 3.0, where the analytes exist in completely protonated forms, the chaotropic effect exhibited by the counter-anions was found to be the most effective. The increase in pH led to a decrease in chaotropic effect as a result of the deprotonation of the carboxylic acid group of the analytes.

The effect of the types and concentration of the counter-anions on the retention of zwitterionic quinolones can be useful in terms of developing methods for the chromatographic separation of these analytes. The addition of chaotropic anions as mobile phase additives resulted in changes in

selectivity and resolution, leading to effective separation of the analytes being studied. The mobile phase containing trifluoroacetate was found to be effective for the analysis of blood serum spiked with the quinolones. Further quantitative analyses employing these counter-anions for the separation of quinolones on different sample matrices are now being conducted.

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