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Optimization of capillary electrophoretic separation of quinolone antibacterials using the overlapping resolution mapping scheme

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Abstract

The capillary electrophoretic separation of fourteen quinolone antibacterials, viz., cinoxacin, ciprofloxacin, enoxacin, flumequine, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, oxolinic acid, pefloxacin, pipemidic acid, piromidic acid, rosoxacin and sparfloxacin, was optimized by using the overlapping resolution mapping scheme. Three relevant parameters to run buffer compositions, i.e., the concentrations of sodium cholate and sodium heptanesulfonate, and the volume percentage of acetonitrile, were chosen for optimization. Seven experiments were carried out to obtain the overlapped resolution diagram for locating the area of optimum separations. Following the predicted optimum condition, a baseline separation of the fourteen quinolones was achieved within 8 min.

Keywords: Antibacterial agents; Chemometrics; Overlapping resolution mapping; Pharmaceutical analysis; Quinolones

1. Introduction

Quinolone antibacterial agents, a group of synthetic drugs with bactericidal action, inhibit the bacterial growth by interfering with the bacterial enzyme DNA gyrase needed for its DNA synthesis [1]. Older members of this class, particularly nalidixic acid, have been used for the treatment of urinary tract infections. The more recent introduction of fluorinated quinolones, such as norfloxacin and ciprofloxacin, represents a particularly important therapeutic advance, since these agents have broad antibacterial activity and are orally effective for the treatment of a wide variety of infectious diseases [2].

Numerous high-performance liquid chromatographic methods (HPLC) have been developed for the analysis of these agents, such as in bulky substances [3], pharmaceutical formulations [4] and biological fluids [5–7]. Having carboxylic acid function in their structures, these quinolones should be well amenable to capillary electrophoretic (CE) analysis. However, to date, only one study has reported on the determination of ciprofloxacin and its related impurities [8]. In the present work the optimization of separation of fourteen quinolone antibacterials (Fig. 1), covering the older to the more recent, was investigated.

Many chemometric approaches to the systematic optimization used in HPLC separations have been transferred to CE with success, these including factorial design [9], Plackett–Burman design [10], orthogonal array design [11], overlapping resolution mapping (ORM) scheme [12], simplex algorithm [13] and central composite design [14]. Plackett–Burman design and orthogonal array design, pertaining to fractional factorial designs, cannot determine

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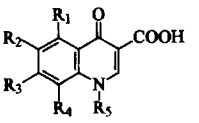
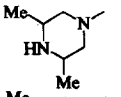
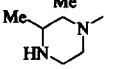
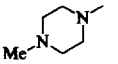
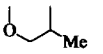
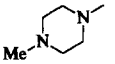
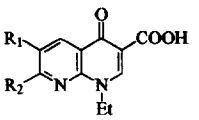
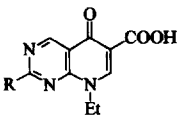


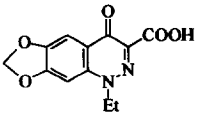
	R_1	R_2	R_3	R_4	R_5
Sparfloxacin (1)	NH_2	F		F	
Lomefloxacin (2)	H	F		F	Et
Norfloxacin (3)	H	F		H	Et
Ciprofloxacin (4)	H	F		H	
Ofloxacin (6)	H	F			
Pefloxacin (8)	H	F		H	Et
Oxolinic acid (9)	H			H	Et
Rosoxacin (10)	H	H		H	Et
Flumequine (12)	H	H	H		





	R_1	R_2
Enoxacin (5)	F	
Nalidixic acid (13)	H	Me

	R
Pipemidic acid (7)	
Piromidic acid (11)	



Cinoxacin (14)

Fig. 1. Structures of the quinolone antibacterials studied in the optimization.

the exact experimental conditions to provide optimum separation. They are suitable as a screening tool to determine the most important factors and the interactions between factors when facing a large number of potential parameters in the initial stage of the experiments. Simplex algorithm and ORM schemes do not require a theoretical model to describe the migration behavior of the solutes and can be used readily for two or three variables. However, the simplex algorithm is susceptible to obtaining a local optimum within the working ranges of the parameters and both the simplex algorithm and ORM scheme suffer from limited use for the optimization of more than three variables, due to difficulties in graphic presentation. Central composite design usually predicts a response surface more accurately than that provided by the ORM approach, but it requires more experiments to be executed.

A triangular ORM scheme was employed in this work to undertake the optimization of separation of the quinolones. During this process three factors relevant to the run buffer compositions were chosen, and only seven experiments were performed to predict the resolution at any point within the triangle.

2. Experimental

2.1. Apparatus

Separations were conducted using a CE system consisting of a Lauer Labs' Prince programmable injector including a 30-kV high-voltage supplier (Emmen, Netherlands), connected with a Dynamax UV-C absorbance detector (Rainin, Emeryville, CA, USA) for UV detection. The electropherograms were recorded with a EZChrom chromatographic data system (Scientific Software, San Ramon, CA, USA) on a 486 DX2 66 PC with an appropriate ADC card and interface. A fused-silica capillary of 50 μm I.D.×375 μm O.D. (Polymicro Technologies, Phoenix, AZ, USA) was used, with total length of 60 cm and detection length of 44 cm. A Mettler delta 320 pH meter with an InLab 410 combination electrode (Essex, England) was used for pH measurement.

The experiments were performed at 30 kV under room temperature (23±2°C). The detection wavelength was set at 260 nm. Samples were injected hydrodynamically at 40 mbar for 6 s.

2.2. Chemicals and reagents

Cinoxacin, flumequine, lomefloxacin, norfloxacin, nalidixic acid, ofloxacin, oxolinic acid, pipemidic acid, quinaldic acid, sodium cholate, sodium deoxycholate, sodium taurocholate and sodium taurodeoxycholate were purchased from Sigma (St. Louis, MO, USA). Ciprofloxacin was supplied by Bayer (Leverkusen, Germany). Pefloxacin was supplied by Rhône-Poulenc Rorer (Vitry, France). Rosoxacin was supplied by Sterling Winthrop (Tansui, Taiwan). Enoxacin, piromidic acid and sparfloxacin were supplied by Dainippon (Osaka, Japan). Sodium tetraborate and sodium dihydrogenphosphate were purchased from Merck (Darmstadt, Germany). Sodium heptanesulfonate was purchased from Fluka (Buch, Switzerland). Methanol and acetonitrile of HPLC grade were purchased from Mallinckrodt (Paris, KY, USA). Water was purified in a Barnstead water purification system (Dubuque, IA, USA).

The run buffers were prepared by mixing stock solutions of sodium borate–sodium dihydrogenphosphate, sodium cholate and sodium heptanesulfonate; after adding acetonitrile, water was finally added to complete the volume. These solutions were filtered through a 0.45 μm filter (Millipore, Bedford, MA, USA) before use. Each run buffer contained 50 m*M* sodium borate–sodium dihydrogenphosphate (7:4, v/v) of pH 7.3.

2.3. Standard solution

Stock solutions of the fourteen quinolone antibacterials, viz., cinoxacin, ciprofloxacin, enoxacin, flumequine, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, oxolinic acid, pefloxacin, pipemidic acid, piromidic acid, rosoxacin and sparfloxacin were prepared at 1 mg ml^{-1} in methanol. A working solution containing each of the above compounds at 25 μg ml^{-1} was prepared by mixing an aliquot of the stock solutions and then diluting with methanol.

2.4. Rinsing of capillary tubes

When a new capillary was used, it was flushed at

2000 mbar with 1.0 *M* sodium hydroxide for 10 min, followed by 0.2 *M* sodium hydroxide for 10 min.

Prior to each analysis, the capillary was flushed with deionized water for 3 min, 0.2 *M* sodium hydroxide for 3 min, water for 3 min, and the run buffer for 4 min successively. With this rinsing procedure, the run-to-run relative standard deviations of actual migration times for all the analytes were found within 1.2% ($n=6$), whereas those of relative migration times with respect to nalidixic acid were within 1.0% ($n=6$).

3. Results and discussion

3.1.1. Preliminary test with CZE

The fourteen quinolones can be divided into two groups according to whether or not they carry the piperazinyl moiety in their structures. Compounds carrying piperazinyl substituent, including all the fluoroquinolones (i.e., sparfloxacin, lomefloxacin, norfloxacin, ciprofloxacin, enoxacin, ofloxacin and pefloxacin) and pipemidic acid, are amphoteric. The pK_a values of these compounds range from 7.5–8.5 for the ammonium form and from 5.5–6.0 for the carboxylic function [15]. They are in zwitterionic or unionized form at their isoelectric points which are between pH 6.5–7.5 [16]. The other piperazinyl-noncarrying quinolones (i.e., oxolinic acid, rosoxacin, piromidic acid, flumequine, nalidixic acid and cinoxacin), however, are purely acidic, with their pK_a values due to carboxylic function. According to Consden et al. [17] and Wren [18], the optimum pH of the background electrolyte to separate two weak acids is near to the average of their pK_a values, if the two analytes have similar ionic mobilities. It is therefore reasonable to start with a buffer system (borate–phosphate) with pH range 6.5–7.5 to undertake the preliminary separations of the quinolone mixtures, using the CZE mode. As a result, at pH 7.3 the electropherogram (Fig. 2) showed that the eight piperazinyl-carrying quinolones, as a group crowded together, migrated ahead of the six piperazinyl-noncarrying quinolones which formed an another group of somewhat better-separated peaks. It is noted that in the former group, the peaks of the four monofluorinated quinolones, i.e., norfloxacin, ciprofloxacin, enoxacin and ofloxacin, owing to their greater similarities in molecular structure, were completely coalesced.(Fig. 2)

3.1.2. Use of sodium cholate and choice of parameters

MEKC is generally employed when the simple CZE mode cannot solve the problem. The bile salts, while having been applied to the separation of hydrophobic solutes [19,20] and basic compounds [21], can also be used to separate very polar molecules like simple carboxylic acids [22]. Four bile salts, viz., sodium cholate, sodium deoxycholate, sodium taurocholate and sodium taurodeoxycholate were investigated by their addition to the above CZE buffer which was diluted with a sufficient amount of acetonitrile. No difference in separation selectivities was found among these salts and therefore sodium cholate was chosen for subsequent use based on economic considerations. However, without addition of some modifiers, several peaks were still partially merged. Under such circumstances the ion-pairing agent sodium heptanesulfonate was added, and both selectivities and peak shapes were greatly improved. In this way, sodium cholate (as micelle-forming surfactant) and sodium heptanesulfonate (as ion-pairing agent) were considered as optimization parameters. Acetonitrile, when added in large concentrations to the aqueous phase, could change the partition of solutes between the aqueous mobile phase and the micellar stationary phase. It was chosen as the third parameter for optimization.

Throughout the optimization process the pH value of the run buffer was set at 7.3, chosen after a series of preliminary tests. This value coincided with that in the literature [15] at which the zwitterionic and the unionized forms of the piperazinyl quinolones predominate. These neutral molecules partitioned into micelles more readily than charged species due to less electrostatic repulsions.

3.1.3. Overlapping resolution mapping scheme

In the triangular ORM scheme in this work, seven experiments were conducted at selected points in a triangle. The positions of these seven points are as shown in Fig. 3, with the figures indicating the percentages for the three parameters. The first step in the ORM scheme was to specify the working range of each parameter. Preliminary studies showed that

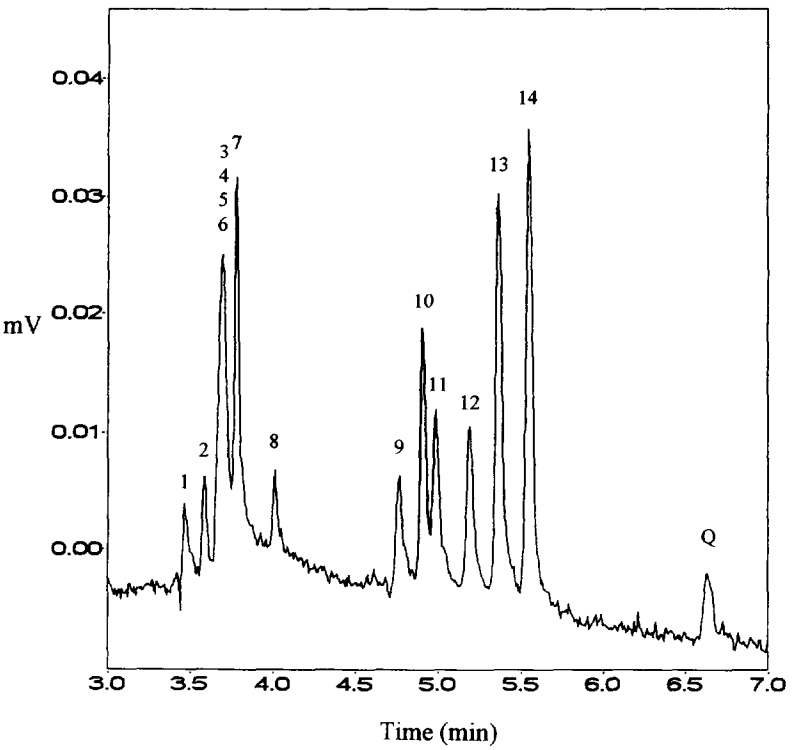


Fig. 2. CZE electropherogram of the quinolone antibacterials. Electrophoretic conditions: 60 m*M* sodium borate–40 m*M* sodium dihydrogenphosphate (pH 7.3); capillary, fused silica, 66 cm (detection length 50 cm)×50 μm I.D.; injection time, 6 s; voltage, 20 kV; temperature, 23°C; UV detection, 260 nm. Compound identities as shown in Fig. 1, Q is quinaldic acid.

at 40 m*M* sodium cholate, 10 m*M* sodium heptanesulfonate and 30% (v/v) acetonitrile a moderate overall separation of the fourteen quinolones was obtained within 8 min. To maintain the analysis time within this period the working ranges were set to 35~45 m*M* for sodium cholate, 5~15 m*M* for sodium heptanesulfonate and 25~35% (v/v) for acetonitrile. From this, the conditions of the seven preplanned experiments were set up as depicted in Table 1 (relation between Fig. 3 and Table 1 was as follows: for sodium cholate, concentrations of 35 m*M* and 45 m*M* in Table 1 were taken as 0% and

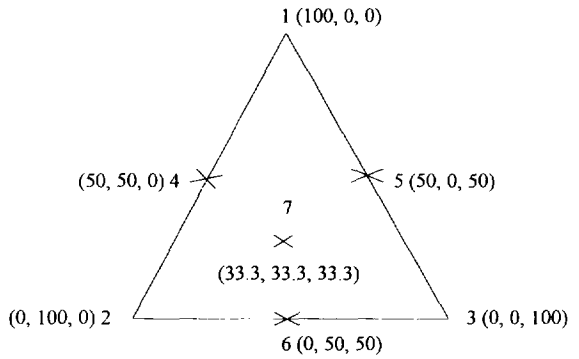


Fig. 3. Experimental design for the seven experiments conducted in the ORM scheme.

Table 1
Experimental conditions for the seven experiments

Exp[a]	Sodium cholate (m*M*)	Sodium hetanesulfonate (m*M*)	Acetonitrile (%, v/v)
1	45	5	25
2	35	15	25
3	35	5	35
4	40	10	25
5	40	5	30
6	35	10	30
7	38	8	28

[a] All experiments were performed at 32 m*M* sodium borate, 18 m*M* sodium dihydrogenphosphate and pH 7.3.

Table 2
Resolutions between adjacent peaks obtained from the seven experiments in Table 1

Exp	Resolution, R_s, for peak pair												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	3.15	2.36	1.44	1.28	4.15	1.43	5.45	8.71	6.57	4.94	2.51	6.13	6.71
2	3.46	1.85	1.45	1.80	3.93	1.86	4.69	9.38	6.57	4.33	2.50	5.49	6.63
3	5.24	0.00	2.24	1.57	5.70	2.56	1.71	5.55	9.47	6.28	1.05	7.19	9.37
4	3.15	1.77	1.29	1.35	3.93	1.50	4.84	8.41	5.90	6.62	2.77	5.35	5.99
5	4.15	0.95	1.61	1.52	4.72	1.64	3.26	3.78	7.82	5.48	2.01	6.45	7.61
6	4.05	0.79	1.56	1.69	4.15	1.82	2.95	6.34	7.71	5.04	2.21	6.31	7.53
7	3.15	1.53	1.57	1.57	5.16	1.63	3.88	7.55	7.05	4.94	2.23	6.74	7.40

100% compositions in Fig. 3, respectively, thus concentration of 40 m*M* corresponded to 50% composition and 38 m*M* corresponded to 33.3% composition; sodium heptanesulfonate and acetonitrile behaved alike). From the seven electropherograms obtained from these experiments, the resolution, R_s, between adjacent peaks were calculated from the equation

$$R_s = \frac{1.18(t_2 - t_1)}{W_{1/2_1} + W_{1/2_2}} \quad (1)$$

where t_1 and t_2 are the migration times and $W_{1/2_1}$ and $W_{1/2_2}$ are the half-height peak widths of two adjacent peaks, respectively. The calculated R_s for all the peak pairs are shown in Table 2. These resolution values were then fitted into a polynomial equation:

$$R_s = a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + a_{123}X_1X_2X_3 \quad (2)$$

where a_i are the coefficients and X_i are the percentages of each parameter as defined in Fig. 3. The values of a_i for each adjacent pair of peaks were determined (Table 3) using the BASIC program developed by Berridge [23]. From Eq. (2) the resolutions for each adjacent pair of peaks could be calculated at any compositions of the run buffer in the triangle. Consequently, a Venn diagram of each adjacent pair of compounds were generated in which the various symbols represented the specified resolution levels [23]. By overlapping all thirteen Venn diagrams and then plotting the symbols representing the lowest resolution among all the individual diagrams, areas defining the composition of buffer which would give the desired resolution among all the peaks in the quinolone mixture were established. The diagram resulting from this overlapping and mapping procedure for the fourteen quinolones is shown in Fig. 4. In this map the regions marked by

Table 3
Coefficients of Eq. (2) for adjacent peak pairs from the seven experiments

Peak pair	Coefficients						
	a_1	a_2	a_3	a_{12}	a_{13}	a_{23}	a_{123}
1–2	3.15	3.46	5.24	−0.62	−0.18	−1.20	−15.60
2–3	2.36	1.85	0.00	−1.34	−0.92	−0.54	11.82
3–4	1.44	1.45	2.24	−0.62	−0.92	−1.14	4.26
4–5	1.28	1.80	1.57	−0.76	0.38	0.02	1.62
5–6	4.15	3.93	5.70	−0.44	−0.82	−2.66	27.06
6–7	1.46	1.86	2.56	−0.58	−1.42	−1.56	2.04
7–8	5.45	4.69	1.71	−0.92	−1.28	−1.00	7.71
8–9	8.71	9.38	5.55	−2.54	−13.40	−4.50	52.41
9–10	6.57	6.57	9.47	−2.68	−0.80	−1.24	1.02
10–11	4.94	4.33	6.28	7.94	−0.52	−1.06	−25.65
11–12	2.51	2.50	1.05	1.06	0.92	1.74	−5.49
12–13	6.13	5.49	7.19	−1.84	−0.84	−0.12	21.09
13–14	6.71	6.63	9.37	−2.72	−1.72	−1.88	14.37

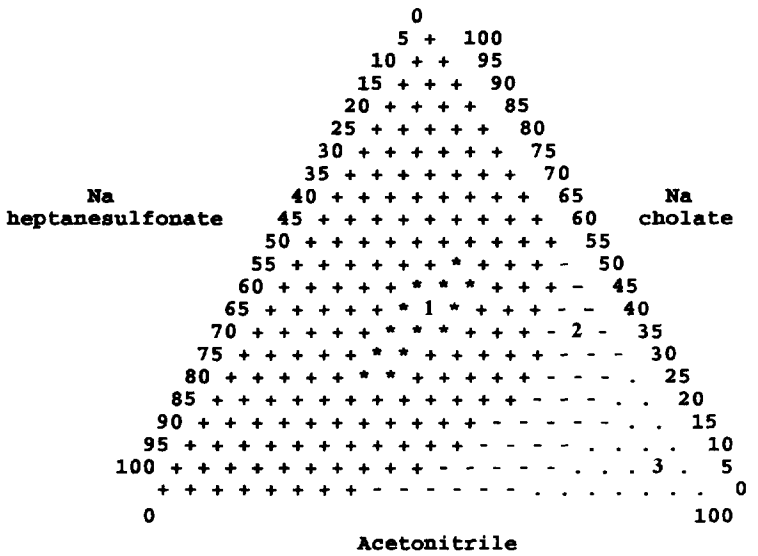


Fig. 4. Overlapped resolution map for the thirteen pairs of peaks. Notation: (●) $R_s<0.5$; (-) $0.5\leq R_s<1.0$; (+) $1.0\leq R_s<1.5$; (*) $R_s\geq 1.5$.

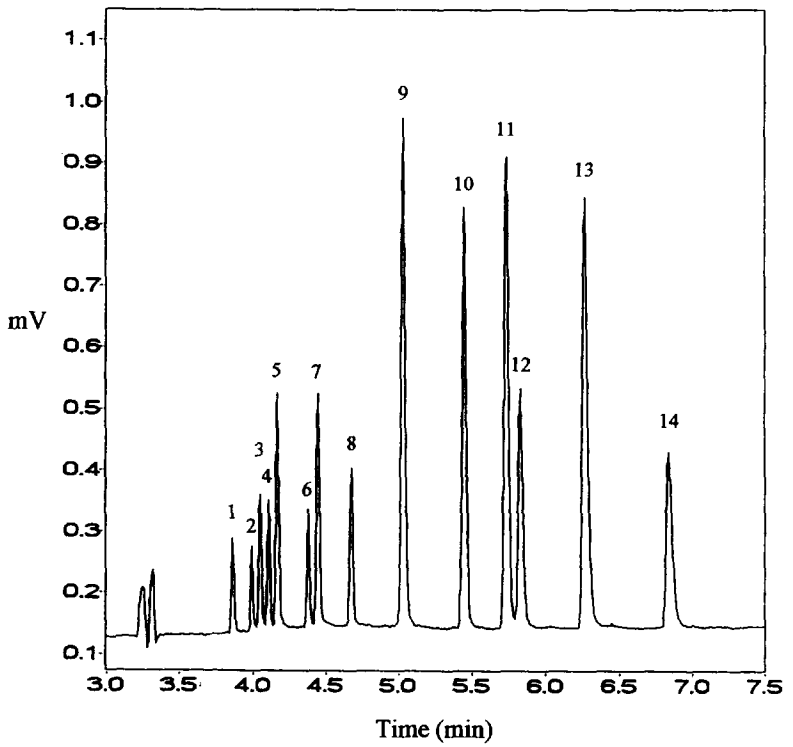


Fig. 5. Electropherogram for the fourteen quinolones using the optimum conditions corresponding to point 1 in Fig. 4. Electrophoretic conditions: 32 m*M* sodium borate, 18 m*M* sodium dihydrogenphosphate, 39 m*M* sodium cholate, 8 m*M* sodium heptanesulfonate, 28% (v/v) acetonitrile (pH 7.3); capillary, fused silica, 59 cm (detection length 43 cm)×50 μm I.D.; injection time, 6 s; voltage, 30 kV; temperature, 23°C; UV detection, 260 nm. Compound identities as shown in Fig. 1.

the symbol * should give a minimum resolution of 1.5 between all the adjacent peak pairs. To test the validity of this optimization scheme, three points were selected from the diagram to perform the experiments. Point 1 (as the optimum), 2 and 3 represent three different resolution levels predicted, viz., greater than 1.5, between 0.5 and 1.0 and less than 0.5, respectively. The three electropherograms obtained by following the conditions predicted (for example, point 1 representing 39 m*M* sodium cholate, 8 m*M* sodium heptanesulfonate and 28% (v/v) acetonitrile) are shown in Figs. 5–7 respectively. In Fig. 5, the electropherogram obtained by using the optimum conditions predicted at point 1 indicates that all fourteen peaks are nearly baseline resolved and the total analysis time is less than 8 min. The comparisons between the experimentally determined resolutions and those predicted from Eq. (2) are shown in Table 4 and except two peak pairs (pair 1–2 and 6–7), the deviations from agreement for all other peak pairs were found to be less than 7%.

The similarities in the relative positions of the quinolone peaks between the electropherograms of MEKC (Fig. 5) and CZE (Fig. 2) caused speculation as to the exact nature of the separation mechanism at work. At such high concentrations of acetonitrile as presently used (around 30%), it is questionable whether micelles of the bile salt could be formed [10]. Even if the micelles were formed, it is likely that under these conditions only a minor portion of the solutes would interact with them, so that it was probably the electrophoretic migration which dominated the separation of solutes. It was shown that around pH 7.3, the less (net) negatively charged piperazinylquinolones (compounds 1~8) migrated before the more negatively charged non-piperazinyl

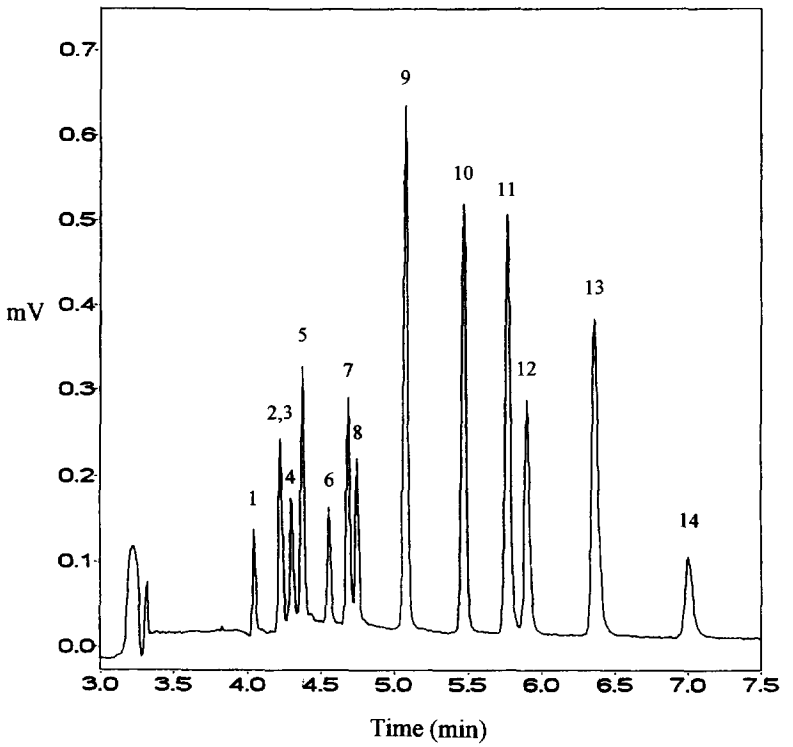


Fig. 6. Electropherogram for the fourteen quinolones using the conditions corresponding to point 2 in Fig. 4. Electrophoretic conditions: 32 m*M* sodium borate, 18 m*M* sodium dihydrogenphosphate, 38.5 m*M* sodium cholate, 5.5 m*M* sodium heptanesulfonate, 31% (v/v) acetonitrile (pH 7.3); other conditions as in Fig. 5. Compound identities as shown in Fig. 1.

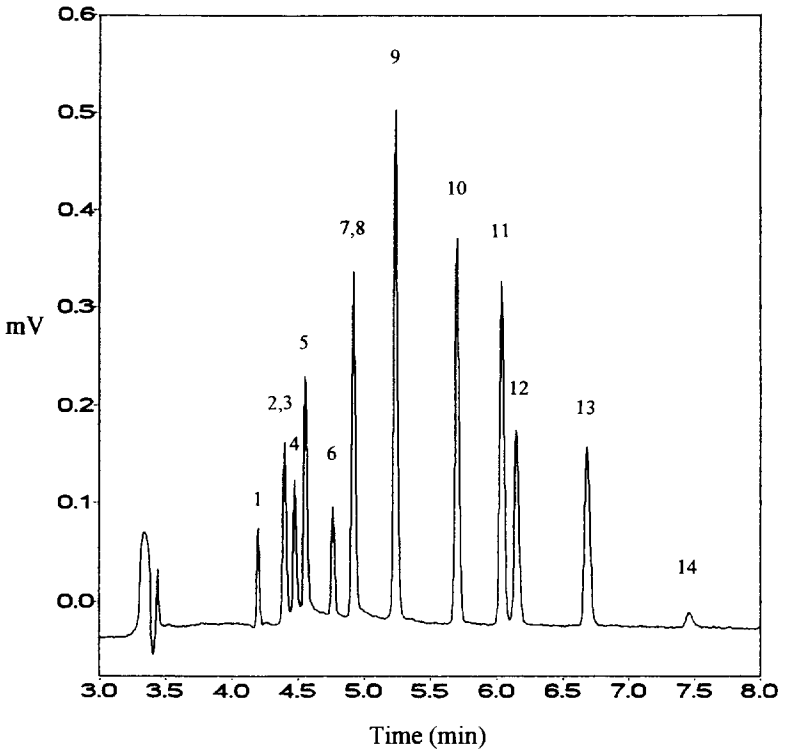


Fig. 7. Electropherogram for the fourteen quinolones using the conditions corresponding to point 3 in Fig. 4. Electrophoretic conditions: 32 m*M* sodium borate, 18 m*M* sodium dihydrogenphosphate, 35.5 m*M* sodium cholate, 5.5 m*M* sodium heptanesulfonate, 34% (v/v) acetonitrile (pH 7.3); other conditions as in Fig. 5. Compound identities as shown in Fig. 1.

Table 4
Experimental and predicted resolutions of adjacent peak pairs under optimum conditions

Peak pair	Resolution	
	Expt	Pred
1–2	4.08	3.10
2–3	1.52	1.60
3–4	1.49	1.55
4–5	1.48	1.54
5–6	4.79	5.13
6–7	1.79	1.59
7–8	4.00	4.02
8–9	7.45	7.53
9–10	7.08	6.95
10–11	4.89	5.03
11–12	2.28	2.27
12–13	6.49	6.68
13–14	7.10	7.30

quinolones (compounds 9~14), as in the case of CZE.

4. Conclusions

In this study, the triangular ORM scheme has been successfully used for the optimization of separation for fourteen quinolone antibacterials. Three relevant parameters to the buffer media, i.e., the concentrations of sodium cholate and sodium heptanesulfonate, and the volume percentage of acetonitrile, were chosen and seven experiments were performed to realize the optimization.

The ORM scheme is straightforward and rapid in itself, however, some preliminary works are still very important, especially in the determination of CE

mode and the choice of relevant parameters. For the latter, some fractional factorial designs, such as Plackett–Burman design [10] and orthogonal array design [11], could be helpful.

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