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Effective separation and simultaneous determination of seven fluoroquinolones by capillary electrophoresis with diode-array detector

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Abstract

A simple, rapid and accurate method has been developed for effective separation and simultaneous determination of lomefloxacin, gatifloxacin, enoxacin, ciprofloxacin, ofloxacin, enrofloxacin and pefloxacin residues in porcine tissue by capillary electrophoresis with diode-array detector. The separation conditions were investigated and optimized. The sample was extracted with acetonitrile, and a mixture consisted of 25 mM NaH_2PO_4, 25 mM $Na_2B_4O_7$ and 25 mM H_3BO_3 (pH 9.0) was used as a running buffer. A linear relationship between concentration and peak area for each compound was obtained in the concentration range of 0.5–100 mg/L with a correlation coefficient greater than 0.9994. For analysis of porcine tissue, the detection limits of lomefloxacin, gatifloxacin, enoxacin, ciprofloxacin, ofloxacin, enrofloxacin and pefloxacin were 0.013, 0.012, 0.023, 0.040, 0.037, 0.035 and 0.034 mg/kg, respectively. The recoveries are in the range of 72–93%. The intra-day precision is less than 5%, and the inter-day precision is less than 10%. The proposed method has high resolution, speed and the extremely small sample volume required. It can permit to confirm the presence of the studied seven fluoroquinolones in porcine tissue at the required maximum residue limit (MRL) level.

Keywords: Capillary electrophoresis; Simultaneous determination; Seven fluoroquinolones; Porcine tissues

1. Introduction

Fluoroquinolones have been used in the treatment of a variety of bacterial infections in human and veterinary medicine. In infectious diseases the use of these drugs has become a serious problem, as they are substances that leave residues in edible tissue which may be directly toxic or cause resistant human pathogens and possible allergic hypersensitivity reactions in humans [1]. To ensure human food safety, the European Union has set tolerance levels for these compounds in animal products. The maximum residue limit (MRL) for enrofloxacin, ciprofloxacin and its major metabolite is fixed at 100 μg/kg in several edible animal tissues [2]. Since they are widely used to treat and prevent veterinary diseases in food-producing animals, the development or improvement of residues analysis in animal tissues is an important analytical task. Numerous techniques have been developed for their analysis in biological fluids. Most of the analytical methods published are based on high-performance liquid chromatography. CE has become a powerful separation technique because its analysis times are shorter than those of LC and lower volumes of reagents and samples are needed [3–5]. The papers published in 2006 reviewed recent advances of CE [6–10]. Several CE methods have been developed for the determination of some fluoroquinolones in biological fluids, such as moxifloxacin in human plasma [11], ciprofloxacin and its metabolite desethyleneciprofloxacin in human plasma samples [12], enrofloxacin, ciprofloxacin and flumequine in pig plasma samples [13] and 10 quinolones antibiotics in plasma samples [5]. Residue analysis of fluoroquinolones in animal tissues is generally more difficult than in biological fluids [14]. In comparison to liquid matrices, solid samples pose additional challenges for bioanalysis, notably: (a) Solid samples must normally be homogenized to provide a liquid fraction prior to further sample processing. At best, this adds another step to the sample processing and at worst, tissue analysis methods can be complex, tedious and time-consuming;

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(b) supply of control matrix may be extremely limited, or even unavailable; (c) preparation of homogeneous QC pools for validation and routine analysis is problematic for solid samples; (d) estimation of extraction recovery can be difficult, as control samples can only be "spiked" after homogenization and such samples may not fully reflect the performance of incurred samples. A few studies have reported the determination of fluoroquinolones in animal tissues by capillary electrophoresis. The analysis of enrofloxacin and ciprofloxacin in chicken muscle was proposed by capillary electrophoresis using marbofloxacin as internal standard with detection limit of <0.025 mg/kg for the two compounds, but the recoveries for enrofloxacin and ciprofloxacin are only 74% and 54%, respectively [15]. Kowalski et al. described a CE method with UV detection for the respective determination of enrofloxacin, ciprofloxacin, norfloxacin and other five antibiotics and nifursol residues from poultry and porcine tissues using different running buffers [16]. A specific pressure-assisted CE-MS method was described for the analysis of danofloxacin, enrofloxacin, ofloxacin, flumequine and pipemidic acid residues in fortified samples of chicken and fish with a detection limit of 0.020 mg/kg for the five analytes [17]. Most of the papers are developed only for the residue analysis of a small group of fluoroquinolones in animal tissues [16]. The above methods have lower recovery [15] or valuable instruments required [17]. Analytical method needs to be developed to confirm the simultaneous presence of fluoroquinolones in animal tissues at the MRL level. Lomefloxacin, gatifloxacin, enoxacin, ciprofloxacin, ofloxacin, enrofloxacin are widely used to treat and prevent veterinary diseases in food-producing animals. Most of the papers are developed only for the residue analysis of a small group of fluoroquinolones in animal tissues. The commission of Hebei Entry-Exit Inspection and Quarantine Bureau of China spoke for a new method of residue analysis of the seven fluoroquinolones. The purpose of this study was to determine separation conditions and develop a simple and efficient CE method for simultaneous determination of the seven fluoroquinolones in porcine tissue. The effects of the separation conditions on simultaneous determination were investigated and optimized. Under optimal conditions this method has high resolution, speed and the extremely small sample volume required. The recoveries are in the range of 72–93%. The intra-day precision is less than 5%, and the inter-day precision is less than 10%. The proposed methods can permit to confirm the presence of the studied seven fluoroquinolones in porcine tissue at the required MRL level.

2. Experimental

2.1. Instrument

All experiments were performed with an Agilent 3D CE system with air-cooling and a diode-array detector (Waldbronn, Germany). Data were collected with the Agilent Chemstation version A.10.02 chromatographic data system. A 48.5 cm (40.0 cm to the detector) × 50 μm I.D. uncoated fused-silica capillary (Agilent) was utilized. Centrifuge (Beijing Jingli Centrifuge Co. Ltd., Beijing, China), homogenizer (Tianjin, China), ultrasonic cleaner (Ultrasonic Instrument Co. Ltd., Kunshan, China) and PHS-3C pH meter (Shanghai precision & scientific instrument Co. Ltd., Shanghai, China) were used in sample treatment.

2.2. Chemicals and solutions

All chemicals used were of analytical grade. Methanol (MeOH), acetonitrile (ACN), boric acid (H_3BO_3), sodium tetraborate decahydrate ($Na_2B_4O_7{\cdot}10H_2O$), sodium dihydrogenphosphate (NaH_2PO_4), sodium hydroxide (NaOH) and hydrochloric acid (HCl) were obtained from Beijing Chemical Factory (Beijing, China). Lomefloxacin, gatifloxacin, enoxacin, ciprofloxacin, ofloxacin, enrofloxacin and pefloxacin were obtained from National Institute for The Control of Pharmaceutical and Biological Products (Beijing, China).

Individual stock solutions (1000 mg/L) of quinolones were prepared by dissolving 10 mg standards in 0.5 mL 0.1 M HCl and diluting to 10 mL with doubly deionized water, and stored at −4 °C. Standard working solutions were prepared by diluting the standard stock solution just before use. Doubly deionized water was used throughout. The suspensions were filtered through a mixed cellulose ester membrane (0.22 μm, Shanghai, China).

2.3. Samples

Porcine muscle tissue samples were obtained from Fu-market (Baoding, China) and Hebei Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China. Tissue samples were stored at −20 °C until the time of the in vitro study.

2.4. Tissue sample extraction procedure

Approximately 4–6 g of thawed and minced tissue was weighed accurately in a 50 mL tube. Ten milliter acetonitrile was added to the samples. The tube was tightly capped and manually shaken until the tissue was dislodged from the bottom of the tube. The sample was placed on an ultrasonic shaker at high speed for 10 min and centrifuged for 8 min at 4000 rpm. Then the supernatant was decanted into a clean tube and evaporated to dryness under a gentle stream of N_2 at 45 °C. The dry residue was reconstituted in a 0.5 mL of 0.1 M HCl, filtered through a mixed cellulose ester membrane (0.22 μm). The obtained filtrate was injected into the CE system.

2.5. Electrophoresis conditions

The running buffer (pH 9.0) is a mixture of 25 mM NaH_2PO_4, 25 mM $Na_2B_4O_7$ and 25 mM H_3BO_3 unless otherwise stated. The capillary was conditioned at the beginning of each day with 0.1 M NaOH for 5 min, followed by water for 5 min and a running buffer for 10 min. In order to equilibrate the capillary and minimize hysteresis effects, the capillary was flushed with water for 1 min and the running buffer for at least 3 min between analyses. The buffer was refreshed after eight analyses. Sample introduction was made at the positive side using the pressure of 50 mbar for 8 s. The high-voltage power supply was

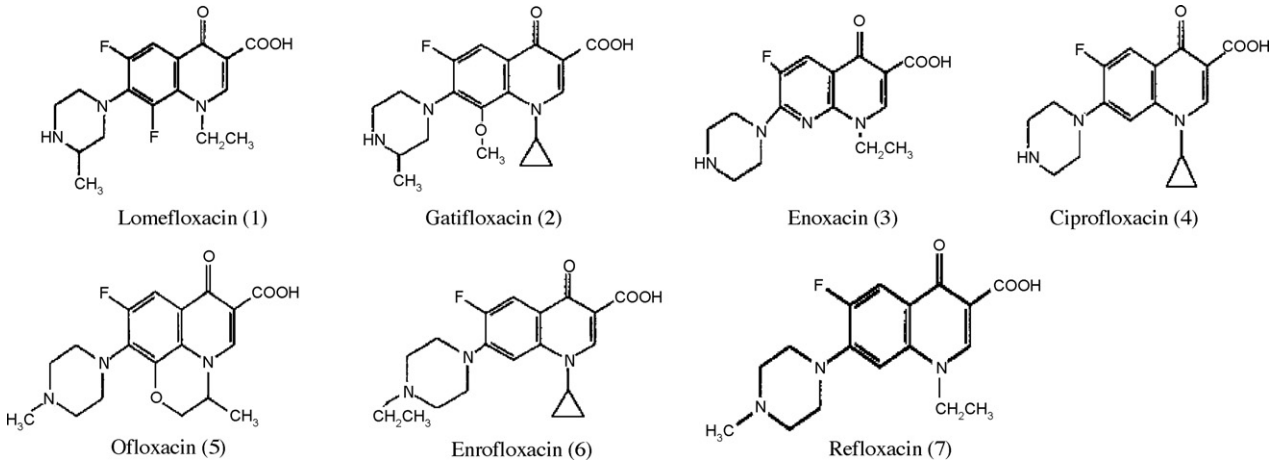

Fig. 1. The chemical structures of fluoroquinolones studied.

set to 22 kV. Capillary temperature was kept at 20 °C, and the compounds were detected at 280 nm.

3. Results and discussion

3.1. Tissue extraction sample optimisation

Acetonitrile is an effective precipitating agent for deproteinization. So extracting compounds with acetonitrile from tissue can decrease the protein interference. Hexane was used usually as the extracting solvent to remove fat. The extraction effect was investigated by comparing the electropherograms with and without hexane. The same result was obtained. Considering simplifying the sample pre-treat process and saving organic solvent, we would not add hexane for removing fat, and only use acetonitrile. The results showed that the procedure of sample pre-treat is feasible.

3.2. Effect of running buffer

The chemical structures of fluoroquinolones studied are shown in Fig. 1.

The effect of pH value from 7 to 11 on separation of the seven fluoroquinolones with negative electric charge was investigated with sodium tetraborate decahydrate and boric acid as the running buffer. From Fig. 2 it is indicated that the studied fluoroquinolones were separated only at pH 9. In order to improve the resolution, further separation was tested by adding methanol, acetonitrile and SDS, separately, but no improvement was observed. It is showed in Figs. 3–5, respectively.

The effects of buffer concentration from 20 mM to 60 mM on resolution were further tested at pH 9. The result is listed in Table 1.

All neighbouring two peaks can be separated effectively with the resolution of >1.5 only when a 40 mM sodium tetraborate decahydrate was used, but for separation of the analysts in the spiked porcine tissue sample, there was co-migration between fluoroquinolones and impurity component in the sample, and the disturbances were very serious. Sodium dihydrogenphosphate was used for the separation of ciprofloxacin [16]. We attempted to use it to resolve the above co-migration and disturbances problems. So the effect of sodium dihydrogenphosphate concentrations on separation was further investigated. When different concentrations (10, 20, 25, 30, 40 mM) of sodium dihydrogenphosphate were added into the running buffer, the concentration

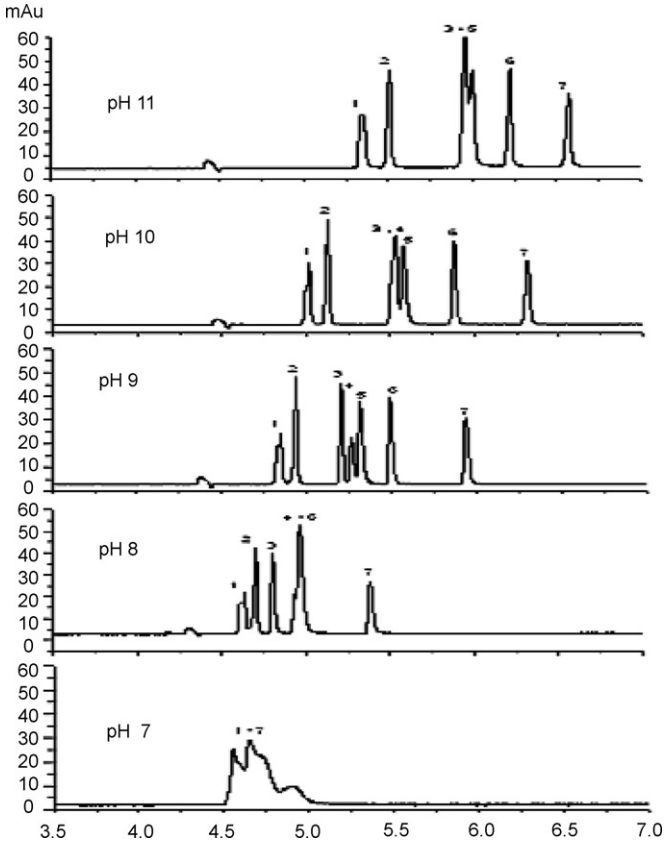

Fig. 2. The effect of pH value on separation of the seven fluoroquinolones. Running buffer, sodium tetraborate decahydrate (30 mM)–boric acid; injection, 50 mbar for 8 s; separation voltage, 20 kV; capillary temperature, 25 °C; DAD detection at 280 nm; 1, lomefloxacin; 2, gatifloxacin; 3, enoxacin; 4, ciprofloxacin; 5, ofloxacin; 6, enrofloxacin; 7, pefloxacin.

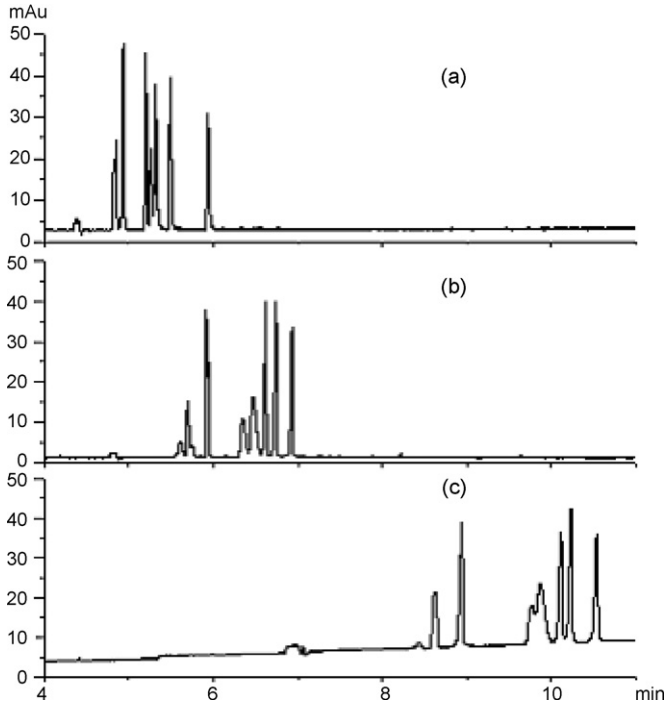

Fig. 3. The effect of methanol in the running buffer. Running buffer, sodium tetraborate decahydrate (30 mM)–boric acid (pH 9.0) with (a) 0% methanol, (b) 5% methanol and (c) 10% methanol; injection, 50 mbar for 8 s; separation voltage, 20 kV; capillary temperature, 25 °C; DAD detection at 280 nm.

of sodium tetraborate decahydrate needs to be adjusted in order to make the running buffer to be pH 9. The effective separation of the peaks of fluoroquinolones from the peaks of impurity was observed in the presence of 25 mM sodium tetraborate decahydrate and 25 mM sodium dihydrogenphosphate, as shown in Fig. 6. Therefore, a mixture of 25 mM sodium tetraborate decahydrate–25 mM boric acid–25 mM sodium dihydrogenphosphate (pH 9) was selected for separation of the seven fluoroquinolones with higher resolution and without disturbances.

3.3. Effect of separation voltage

The effect of different voltages on separation of the seven fluoroquinolones was investigated. The result is shown in Fig. 7.

The high-voltage power supply influences migration speed of solute, which influences separation effect. An electrophoresis current increases with the increase of voltage, so that

Table 1
Resolution of analytes using different concentration of sodium tetraborate decahydrate

Concentration (mM)	R_1	R_2	R_3	R_4	R_5	R_6
20	3.30	4.08	1.75	1.96	1.04	1.41
30	0.674	2.58	1.36	2.91	2.13	2.35
40	3.77	2.28	2.38	3.54	1.57	2.15
50	3.11	4.28	0.86	1.81	1.56	2.33
60	3.00	3.15	0.621	0.588	0.696	2.34

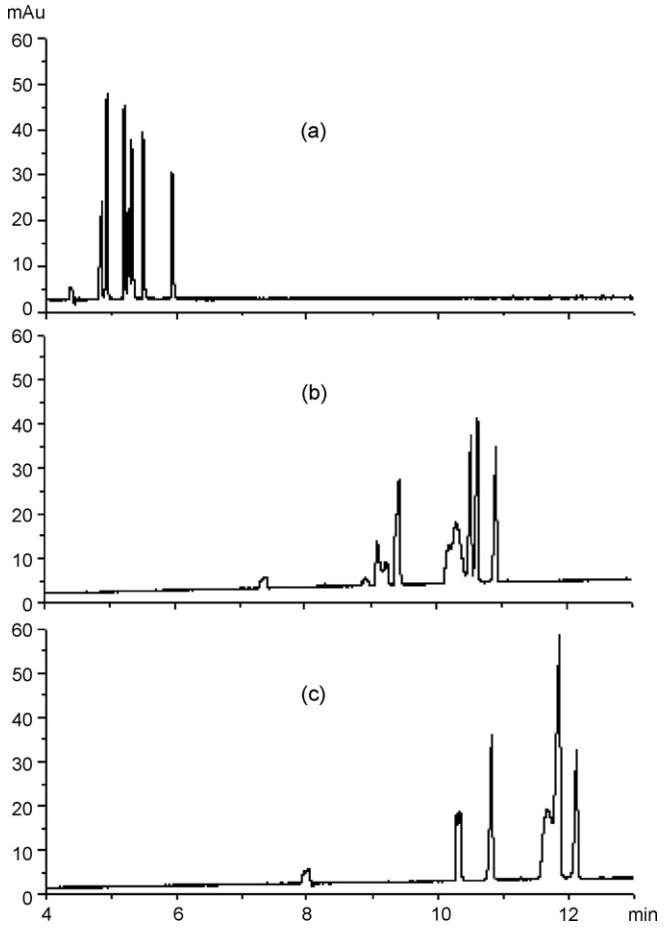

Fig. 4. The effect of acetonitrile in the running buffer. Running buffer, sodium tetraborate decahydrate (30 mM)–boric acid (pH 9.0) with (a) 0% acetonitrile, (b) 5% acetonitrile and (c) 10% acetonitrile; injection, 50 mbar for 8 s; separation voltage, 20 kV; capillary temperature, 25 °C; DAD detection at 280 nm.

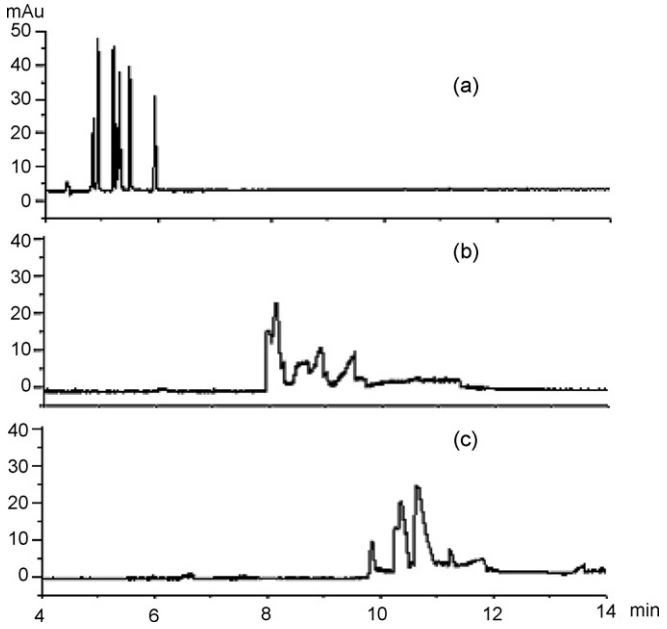

Fig. 5. The effect of SDS in the running buffer. Running buffer, sodium tetraborate decahydrate (30 mM)–boric acid (pH 9.0) with (a) 0 mM SDS, (b) 10 mM SDS and (c) 20 mM SDS; injection, 50 mbar for 8 s; separation voltage, 20 kV; capillary temperature, 25 °C; DAD detection at 280 nm.

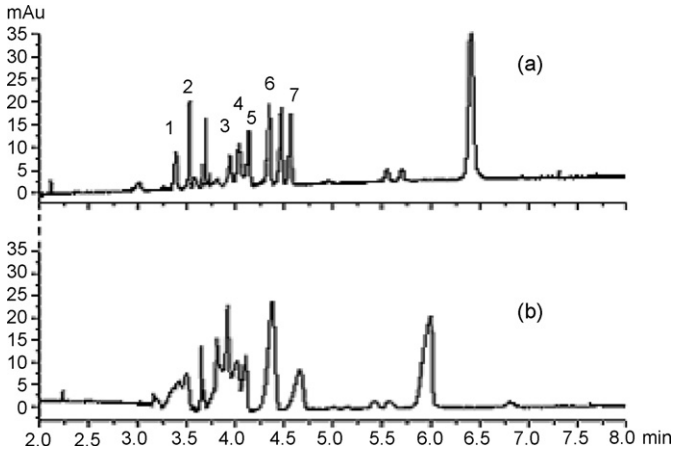

Fig. 6. Electropherograms of seven fluroquinolones in porcine tissue sample at pH 9: (a) Running buffer, 25 mM sodium tetraborate decahydrate–boric acid–5 mM sodium dihydrogenphosphate; (b) 40 mM sodium tetraborate decahydrate–boric acid; injection, 50 mbar for 8 s; separation voltage, 22 kV; capillary temperature, 25 °C; DAD detection at 280 nm.

migration time decreases, but Joule heat increases, which produces radial temperature gradient and decreases separation efficiency. The height of the peaks of fluoroquinolones at 12 kV is lower, especially the peaks of lomefloxacin and enoxacin. The electropherograms obtained under different voltages in the range of 18–30 kV are shown in Fig. 7. The six peaks and seven peaks were obtained at 18 kV and 22 kV, respectively. An electrophoresis current was increased, and resolution was decreased with the increase of voltage. The peaks couldn't be separated absolutely when voltages were beyond 22 kV. A 22 kV voltage was selected for capillary electrophoresis in this work.

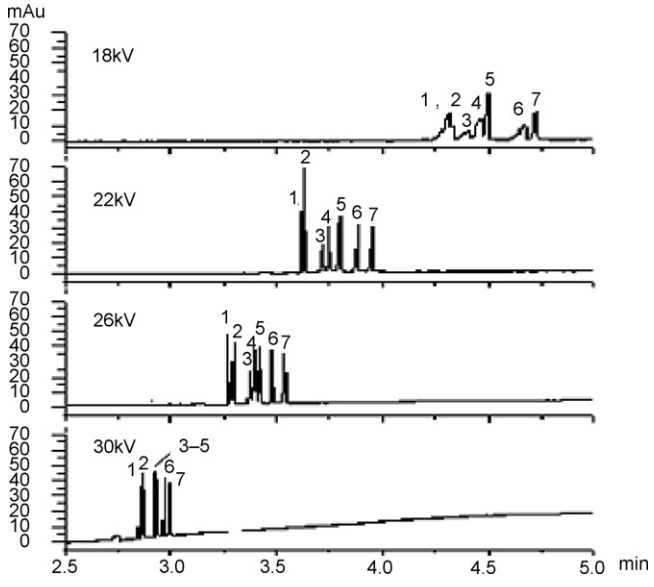

Fig. 7. Effect of different voltage on separation of the seven fluoroquinolones. Running buffer, 25 mM sodium tetraborate decahydrate–25 mM boric acid–25 mM sodium dihydrogenphosphate; injection, 50 mbar for 8 s; capillary temperature, 25 °C; DAD detection at 280 nm.

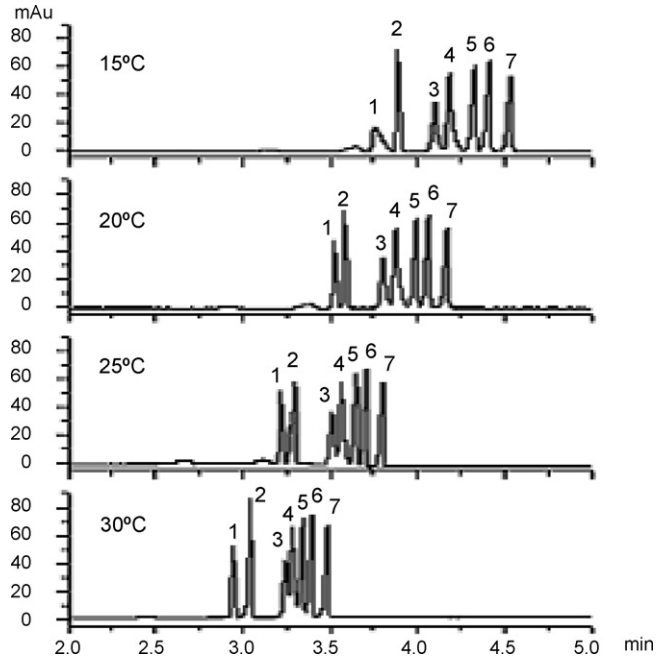

Fig. 8. Electropherograms under different capillary temperature. Running buffer, 25 mM sodium tetraborate decahydrate, 25 mM boric acid and 25 mM sodium dihydrogenphosphate; injection, 50 mbar for 8 s; separation voltage, 22 kV; DAD detection at 280 nm.

3.4. Effect of separation temperature

The effect of the capillary temperature on separation was investigated. Electropherograms and resolution under different capillary temperature are shown in Fig. 8 and Table 2, respectively.

It can be seen that migration time and resolution increase with the decrease of capillary temperature. The resolutions in 15 °C and 20 °C are beyond 1.5. The resolutions between peaks 3 and 4, and peaks 5 and 6 are less than 1.5 in 25 °C. The resolutions between peaks 3 and 4, peaks 4 and 5, and peaks 5 and 6 are less than 1.5 in 30 °C. The resolution was best when the temperature was set at 15 °C, but the shape of the first peak was bad. So column temperature was set at 20 °C for separation with a resolution of >1.5 and good shape of the peaks.

3.5. Linearity, detection limits and precision

The linearity for analysis of the studied fluoroquinolones was evaluated with concentrations of calibration standards against measured peak areas under the optimal conditions. The equa-

Table 2
Resolution under different column temperatures

Temperature (°C)	R_1	R_2	R_3	R_4	R_5	R_6
15	1.66	4.28	1.70	2.92	1.66	2.42
20	1.54	4.47	1.57	2.59	1.56	2.22
25	1.85	4.25	1.00	1.61	1.26	1.95
30	3.14	4.51	0.736	1.34	1.23	1.92

Table 3
Linearity of the calibration curves

Analyte	Concentration range (mg/L)	Regression equations	Correlation coefficient (r)
Lomefloxacin	0.5–100	$Y = 0.6311X + 0.2804$	0.9996
Gatifloxacin	0.5–100	$Y = 0.9739X - 0.2502$	0.9999
Enoxacin	0.5–100	$Y = 0.6686X - 0.3573$	0.9999
Ciprofloxacin	0.5–100	$Y = 1.1211X - 0.6461$	0.9998
Ofloxacin	0.5–100	$Y = 0.9443X - 0.1304$	0.9997
Enrofloxacin	0.5–100	$Y = 1.1056X - 0.8625$	0.9996
Pefloxacin	0.5–100	$Y = 0.9345X - 0.6612$	0.9994

tions of calibration curves obtained based on three parallel measurements for standard solution are listed in Table 3. It can be seen that the linearity is satisfactory with a correlation coefficient (r) greater than 0.9994.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise [1], and the limit of quantification (LOQ) was calculated that produced a peak with 10 times the signal-to-noise ratio. The determined LOD values for lomefloxacin, gatifloxacin, enoxacin, ciprofloxacin, ofloxacin, enrofloxacin and pefloxacin in porcine tissue based on 10 parallel measurements were 0.013, 0.012, 0.023, 0.040, 0.037, 0.035 and 0.034 mg/kg, respectively, and LOQ values were 0.043, 0.040, 0.075, 0.098, 0.093 and 0.090 mg/kg, respectively. The obtained detection limits of the fluoroquinolones studied permit the detection of porcine tissue samples at the required MRLs.

3.6. Determination of spiked porcine tissue

The recoveries of the studied fluoroquinolones in porcine tissue added with 10 mg/kg analyte were examined. The recoveries are shown in Table 4 along with the relative standard deviation. The recoveries were in the range of 72–93%. The relative standard deviation (R.S.D.) is less than 5%.

There was a variation between the recoveries for different analytes. It is because the recovery is possibly related with the structure of analyte and its combinative intensity with porcine tissue.

According to the tissue sample extraction procedure, all seven fluoroquinolones in spiked porcine tissue were determined under the optimal CE conditions. The electropherograms of analyte-free porcine tissue sample (blank sample) and the porcine tissue sample spiked with fluoroquinolones are shown in Fig. 9.

Table 4
Recoveries of the fluoroquinolones in porcine tissue

Analyte	Added (mg/kg)	Found (mg/kg)			Average recovery (%)	R.S.D. (%)
Lomefloxacin	10	8.42	8.52	8.80	85.79	2.30
Gatifloxacin	10	9.52	9.26	9.20	93.25	1.82
Enoxacin	10	7.33	7.48	6.86	72.23	4.48
Ciprofloxacin	10	8.21	8.52	8.42	83.84	1.89
Ofloxacin	10	7.65	7.77	7.87	77.64	1.42
Enrofloxacin	10	8.27	8.74	8.96	86.58	3.71
Pefloxacin	10	8.83	8.99	8.95	89.24	0.93

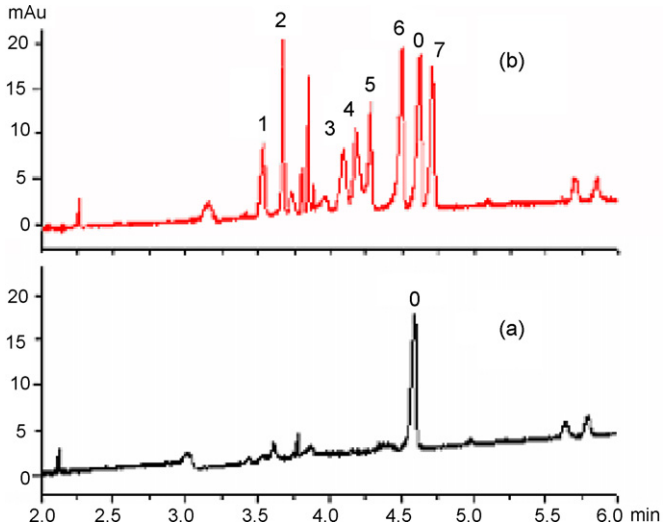

Fig. 9. Electropherogram of (a) analyte-free porcine tissue sample and (b) porcine tissue sample spiked with 10 mg/kg of fluoroquinolones. Running buffer, 5 mM sodium tetraborate decahydrate–5 mM boric acid–25 mM sodium dihydrogenphosphate (pH 9.0); injection, 50 mbar for 8 s; separation voltage, 22 kV; capillary temperature, 20 °C; DAD detection at 280 nm; 0, impurity; 1, lomefloxacin; 2, gatifloxacin; 3, enoxacin; 4, ciprofloxacin; 5, ofloxacin; 6, enrofloxacin; 7, pefloxacin.

Table 5
Reproducibility of the fluoroquinolones in porcine tissue

Analyte	Intra-day		Inter-day	
	R.S.D. (%, time)	R.S.D. (%, area)	R.S.D. (%, time)	R.S.D. (%, area)
Lomefloxacin	1.00	3.76	4.89	7.36
Gatifloxacin	1.30	0.53	3.62	6.34
Enoxacin	3.17	2.01	4.62	8.02
Ciprofloxacin	3.43	3.65	3.67	4.15
Ofloxacin	3.71	3.49	4.55	6.71
Enrofloxacin	0.81	4.01	4.27	6.53
Pefloxacin	1.41	3.53	5.09	9.46

It can be seen that the seven fluoroquinolones were separated very well. The peak 0 between peak 6 and peak 7 was from an impurity in analyt-free porcine tissue samples; it doesn't interfere the determination of the analytes. The intra-day data of 10 mg/kg fluoroquinolones were determined in replicate ($n = 6$) on a single day. The inter-day data were obtained from the analysis of samples at a concentration of 10 mg/kg with three assays on separate days. The R.S.D. of the areas and the migration time were displayed in Table 5.

4. Conclusion

The optimal conditions for effective separation and simultaneous determination of the studied fluoroquinolones in porcine tissue are 25 mM NaH_2PO_4–25 mM $Na_2B_4O_7$–25 mM H_3BO_3 buffer (pH 9.0), 20 °C of capillary temperature, 22 kV of applied voltage and 280 nm of detection wavelength. The analysis for the CE part is achieved in 5 min. This method has high resolution, speed and the extremely small sample volume required.

The proposed method can be applied for routine determination of the seven fluoroquinolones in porcine tissue and biological samples.

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