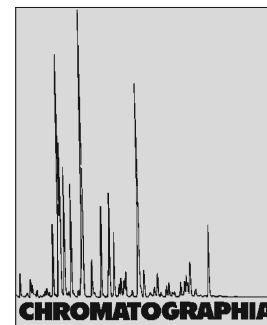# Effective Separation and Simultaneous Determination of Four Fluoroquinolones in Milk by CE with SPE

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Abstract

A simple and sensitive capillary electrophoresis method with solid phase extraction was developed for the determination of sarafloxacin, ciprofloxacin, enrofloxacin and flumequine in milk. Solid-phase extraction with Oasis HLB cartridge column was used for the isolation of four fluoroquinolones in raw milk from a farm and fresh milk sample. Separation conditions of CE, including running buffer, voltage and temperature, were investigated and optimized. Baseline separation was achieved for the four fluoroquinolones under the developed conditions. Correlation coefficients greater than 0.9998 were obtained for all fluoroquinolones with a dynamic range from 1 up to 100 mg L^{-1}. The intra-day precision was less than 5%, and the inter-day precision was less than 6%. The method recoveries of four fluoroquinolones were in the range of 70.9–90.6%. The detection limits for sarafloxacin, ciprofloxacin, enrofloxacin and flumequine was 19.8, 15.2, 13.3 and 15.9 μg kg^{-1}, respectively, which allows positive detection of the fluoroquinolones at the targeted maximum residue levels in milk samples.

Keywords

Capillary electrophoresis
Solid phase extraction
Fluoroquinolones
Milk

Introduction

Quinolones are considered the most important group of synthetic antibacterial drugs, which operate by interfering with DNA synthesis by inhibiting DNA gyrase activity and producing bacterial death [1]. They are widely used in human as well as in veterinary medicine for the treatment of pulmonary, urinary and digestive infections [2]. The wide usage of antibiotics may be responsible for the promotion of resistant stains of bacteria, but the misuse of these medicines and the residues may be responsible for increased concerns on public health, for example, allergic reactions, antibiotic resistance [3]. Owing to their potential impact on human health, the European Union has adopted a maximum residue level (MRL, 100 μg kg^{-1}) for enrofloxacin and its metabolite ciprofloxacin in milk, however, the tolerance limits for sarafloxacin and flumequine in milk has not yet been established by the EU [4]. Some fluoroquinolones have been banned to be used for dairy cows. Therefore, there is a need for the development of a simple and selective method for monitoring their residue levels in edible animal products.

Several analytical techniques have been used for the determination of quinolone residues. Schenck and Callery [5] reviewed the developments in the chromatographic determination of antibiotic residues in milk. Carlucci [6] reviewed the analysis of biological fluids for quinolones by liquid chromatography (LC). Recently, Andreu et al. [7] reviewed the analytical strategies to determine quinolone residues in food and environmental samples. Among them, LC is generally used to determine quinolone residues in biological fluid and animal tissues. Simultaneous determination of enrofloxacin and its primary

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metabolite ciprofloxacin in bovine milk and plasma by ion-pairing LC with a detection limit of 5 μg L^{-1}[8] and enrofloxacin and its metabolite ciprofloxacin in goat milk by LC using diode-array detection with the quantification limit of 20 μg kg^{-1} for both analytes [9].

Chromatographic determination of flumequine in food samples including milk samples by post-column derivatisation with terbium(III) was described [10]. A column-switching LC fluorescence detection method was reported for the determination and on-line clean-up of enrofloxacin, ciprofloxacin, sarafloxacin, oxolinic acid, and flumequine in bovine milk [11]. Recently, liquid chromatography-tandem mass spectrometry (LC-MS-MS) was developed for monitoring quinolone residues in bovine milk with quantification limits of 0.3–1.5 μg L^{-1} [12].

Capillary electrophoresis has the advantage of high separation efficiency, short analysis time, low sample and solvent consumption, low cost of the running and lower matrix effect compared to other separation techniques. In addition, CE can separate compounds in highly polar and water soluble matrices that have been traditionally difficult to handle by chromatographic techniques, for example highly polar and water soluble substances [13]. The major drawbacks in CE are the lower sensitivity and lower repeatability of the identification parameter (migration time) in comparison to the chromatographic techniques. Analysis of antibiotics in biological samples by CE has been reviewed in 2003 [14]. To increase the selectivity and sensitivity of CE, solid phase extraction (SPE) has been combined with CE for the determination of fluoroquinolones in plasma samples with detection limits of 2.2 mg L^{-1} for ciprofloxacin and 1.6 mg L^{-1} for flumequine [15], ciprofloxacin and its impurities with the detection limit of 1 mg L^{-1} [16], flumequine and oxolinic acid in chicken tissues with detection and quantification limits of 10 and 30 μg kg^{-1} for flumequine [17], ciprofloxacin, enrofloxacin and flumequine in pig plasma samples with the detection limits of 70, 85 and 50 μg L^{-1}, respectively [18], and quinolones residues in porcine tissue with the detection limit

of 40 μg kg^{-1} for ciprofloxacin and 35 μg kg^{-1} for enrofloxacin [19]. However there are only few reports of SPE–CE for the detection of quinolone residues in milk samples [20]. A novel analytical method based on capillary zone electrophoresis-MS-MS was proposed for the identification and simultaneous quantification of eight quinolones in bovine raw milk with limits of detection and quantification below 6 and 24 μg L^{-1}, respectively [20]. This method has low detection limits, but requires an expensive instrument.

The purpose of this work is to develop a simple and sensitive method for the determination of four fluoroquinolones in milk by CE combined with SPE. Baseline separation was achieved for the four fluoroquinolones under the developed conditions and the recoveries are in the range of 70.9–90.6%. The intra-day precision is less than 5%, and the inter-day precision is less than 6%. The proposed method has high resolution, speed requires only an extremely small sample volume. It can be used to confirm the presence of the four interested fluoroquinolones in milk samples at the targeted MRL levels.

Experimental

Instrumentation

All experiments were performed with an Agilent 3D CE system with air-cooling and a diode-array detector (Agilent, Waldbronn, Germany). Data were collected with the Agilent Chemstation version A.10.02 chromatographic data system. A 60.9 cm (52.4 cm to the detector) × 50 μm I.D. uncoated fused-silica capillary (Yongnian Optical Fabric Factory, Handan, China) was utilized. Centrifuge (Beijing Jingli Centrifuge Co., Beijing, China), homogenizer (Hengao Instrument Co., Tianjin, China), ultrasonic cleaner (Ultrasonic Instrument Co., Kunshan, China) and PHS-3C pH meter (Shanghai Precision & Scientific Instrument Co., Shanghai, China) were used in sample treatment. Oasis HLB cartridge column (6 mL, 500 mg) from Waters Corporation (Milford, USA) were used for solid phase extraction.

Material and Reagents

Methanol (MeOH), boric acid (H$_3$BO$_3$), sodium tetraborate decahydrate (Na$_2$B$_4$O$_7$·10H$_2$O), sodium hydroxide (NaOH), ethylenediamine tetraacetic acid disodium salt (Na$_2$EDTA), disodium hydrogen phosphate dodecahydrate (Na$_2$HPO$_4$·12H$_2$O) and citric acid monohydrate (C$_6$H$_8$O$_7$·H$_2$O) were obtained from Tianjin Chemical Factory (Tianjin, China).

Sarafloxacin, ciprofloxacin, enrofloxacin and flumequine standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Individual stock solution (1 mg mL^{-1}) of fluoroquinolone was prepared by dissolving 10 mg solid in 0.5 mL 0.01 M NaOH and diluting to 10 mL with double deionized water, and stored at −4 °C. Standard solutions of 1, 2, 5, 10, 20, 50 and 100 μg mL^{-1} were prepared by diluting the stock solution just before use. Double deionized water was used throughout. The suspensions were filtered through a mixed cellulose ester membrane (0.22 μm, Xinya Purification Material Factory, Shanghai, China).

McIlvane buffer containing EDTA was prepared by adding 21.5 g of disodium hydrogen phosphate dihydrate, 18.6 g of EDTA and 16.8 g of citric acid monohydrate in 1 L water. The phosphate running buffer was prepared by adding 40 mM sodium tetraborate decahydrate, 42 mM boric acid and 28 mM sodium dihydrogenphosphate in 1 L water. The pH of the McIlvane buffer containing EDTA and the running buffer was adjusted to 4.0 and 9.2, respectively, using 1.0 M phosphoric acid or 1.0 M sodium hydroxide.

Solid Phase Extraction

McIlvane buffer containing EDTA (pH 4.0) was used for the precipitation of protein and extraction of fluoquinolones from raw milk and fresh milk samples. Five grams of milk were accurately weighed and placed in a 100 mL polypropylene centrifuge tubes. The milk and McIlvane buffer was mixed with a mechanical shaker. After shaking for

2 min the mixture was centrifuged at 4,000 rpm for 20 min. The supernatant was then passed through an Oasis HLB cartridge, which was pre-conditioned with 6 mL methanol and 10 mL water. After rinsing with 8 mL water, the fluoroquinolones were eluted with 6 mL MeOH. The collected eluate was evaporated to dryness at 50 °C under a stream of nitrogen, and the residue was re-suspended in 1 mL of 0.01 mol L^{-1} NaOH and analyzed by CE system.

Electrophoresis Conditions

At the beginning of each day, the capillary was conditioned with 0.1 mol L^{-1} NaOH for 5 min, followed by water for 8 min and running buffer for 10 min. In order to equilibrate the capillary and minimize hysteresis effects, the capillary was flushed with running buffer for 4 min between analyses. The phosphate running buffer (pH 9.2) was used for separation of the four fluoroquinolones, which was refreshed after six analyses. Sample introduction was made at the positive side using the pressure of 5 kPa for 8 s. The high-voltage power supply was set to 22 kV. Capillary temperature was kept at 25 °C and the compounds were detected at 275 nm.

Results and Discussion

Effect of Running Buffer

The running buffer is an important factor for the separation of fluoroquinolones. The effect of several buffers with different concentration, such as Na$_2$B$_4$O$_7$–NaH$_2$PO$_4$, Na$_2$B$_4$O$_7$–H$_3$BO$_3$, Na$_2$B$_4$O$_7$–H$_3$PO$_4$, Na$_2$B$_4$O$_7$–NaCl and Na$_2$B$_4$O$_7$–H$_3$BO$_3$–NaH$_2$PO$_4$ solutions on the separation of the four analytes were first tried. Among them, the solution of 40 mM Na$_2$B$_4$O$_7$–42 mM H$_3$BO$_3$–28 mM NaH$_2$PO$_4$ has higher ionic strength than that of the others. By using this solution as the running buffer, the baseline separation was observed for the four fluoroquinolones, because increasing the ionic strength of the running electrolyte allowed, via its charge screening effect, the modulation of selectivity thus adjusting the resolution of
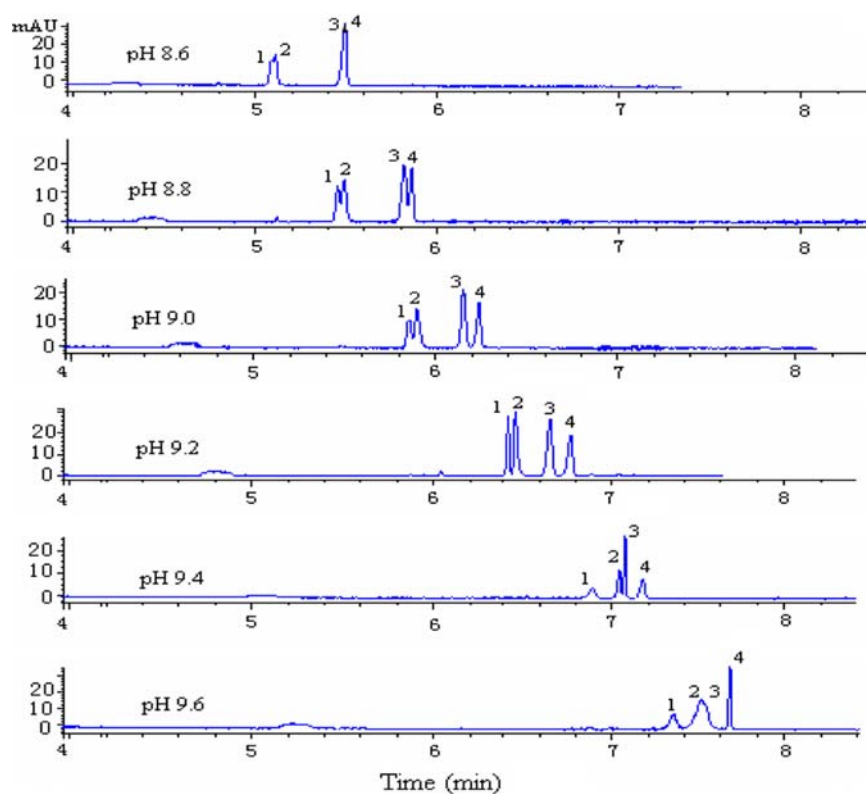
Fig. 1. The effect of pH value on the separation of the four fluoroquinolones 5 mg L^{-1} for each fluoroquinolone; separation voltage, 22 kV; capillary temperature, 25 °C; injection at 5 kPa for 8 s; DAD detection at 275 nm. **1** = sarafloxacin, **2** = ciprofloxacin, **3** = enrofloxacin, **4** = flumequine

closely related fluoroquinolones [21]. Therefore, the system was selected as the running buffer for further experiment.

The pK_1 values of the analytes connected with dissociation of the carboxylic group and and pK_2 values associated with the deprotonation at N4 of the piperazine ring. For sarafloxacin, pK_1 = 5.62 and p$K2$ = 8.18; for ciprofloxacin pK_1 = 5.86 and pK_2 = 8.24; for enrofloxacin, pK_1 = 5.88 and pK_2 = 7.74; for flumequine, pK_1 = 6.65 [22]. Taking into considerations the pK_a values, the effect of pH values from 7 to 9.6 on the separation of the four fluoroquinolones was investigated and the above selected system as the running buffer was further studied. When the pH value of the running buffer was less than 8.6, the four fluoroquinolones could not be separated. The effect of pH values from 8.6 to 9.6 on the separation is depicted in Fig. 1.

When the pH value of the running buffer was higher than 9.4, ciprofloxacin and enrofloxacin could not be separated.

Baseline separation was achieved at pH 9.2 for the four analytes. As seen in Fig. 1, the migration order is sarafloxacin, ciprofloxacin, enrofloxacin and flumequine which, in turn, shows a pK_1 value from low to high. The phosphate running buffer with a pH value of 9.2 was selected for separation of the four fluoroquinolones with higher resolution and without disturbances.

Effect of Separation Voltage

The effect of voltages on the separation of the four fluoroquinolones (5 mg L^{-1} for each fluoroquinolone) was investigated. The peaks of sarafloxacin and ciprofloxacin could not be separated completely at 18 kV, and the sarafloxacin peak was small. The migration times of the analytes were shorter with the increase of the voltage, however, a higher voltage would cause higher current and lead to more Joule heating, which
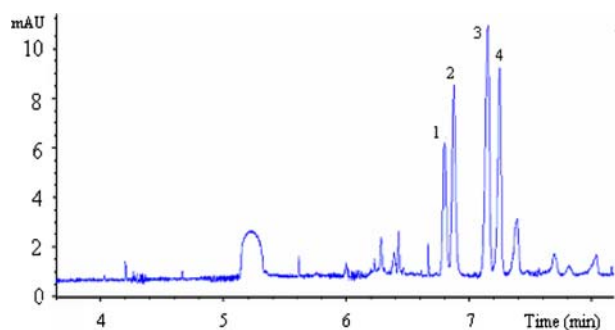
Fig. 2. Electropherogram of fresh milk sample spiked at 2 mg kg^{-1} level for each fluoroquinolone using phosphate running buffer (pH 9.2) and conditions given in Fig. 1. **1** = sarafloxacin, **2** = ciprofloxacin, **3** = enrofloxacin, **4** = flumequine

affected the separation of the tested analytes [19]. When the voltage was set at 22–30 kV, baseline separation of the four fluoroquinolones was achieved. As higher sarafloxacin and ciprofloxacin peaks were obtained at 22 kV, a 22 kV voltage was selected for CE in this work.

Effect of Separation Temperature

The effect of the capillary temperature on the separation of fluoroquinolone (5 mg L^{-1} for each fluoroquinolone) was investigated. The migration time decreased with the increase of capillary temperature. Under different capillary temperatures, the resolutions are given in Table 1.

When the temperature was set at 25 °C, CE separation has higher resolutions with 2.66 ($R_{1–2}$), 7.30 ($R_{2–3}$) and 3.62 ($R_{3–4}$). Therefore, a column temperature of 25 °C was used for optimized separation and symmetric peaks.

Extraction and Clean-Up

Most reported extraction procedures were based on the removal of milk proteins with acidic solutions or organic solvents followed by sample enrichment and clean-up with SPE. Because the milk matrix contains interferences such as proteins, lactose, and inorganic ions, the success of the extraction procedure depended on the effective deprotenization and washing steps. Dichloromethane has

been used to extract ciprofloxacin and enrofloxacin from chicken muscle tissue samples with reported recoveries of 74 and 54% for enrofloxacin and ciprofloxacin, respectively [23]. A two-step solid-phase extraction procedure was developed by Lara et al. [20] using Oasis MAX and HLB cartridges without protein precipitation. In our previous work, acetonitrile was used for protein precipitation and extraction of seven quinolones in porcine tissue [19]. In this work, McIlvane/EDTA solution (pH 4.0) and Oasis HLB cartridges were used for the precipitation of protein and the extraction of fluoquinolones from milk samples. McIlvain buffer containing EDTA is a mild acidic solvent, it helps chelating metals and prevents them from binding to the adsorbing sites resulting in extraction efficiency of the analytes to be improved.

Since the fluoroquinolones were soluble in water and dissoluble in organic solvents, water was used for washing the SPE material after sample application to wash off the solvents and avoid loss of analytes. Methanol was used as eluting solvent to improve the recoveries of these fluoroquinolones.

The effect of different eluting volumes (4, 5, 6, 7, 8 mL) of methanol on recoveries was also investigated. The obtained recoveries were in the range of 60.5–70.6% for sarafloxacin, 66.5–78.4% for ciprofloxacin, 74.5–90.0% for enrofloxacin, and 74.8–89.6% for flumequine. With a selected 6 mL methanol elution, recoveries from 70.1 to 90.0% were obtained for the four fluoroquinolones. Under the optimal conditions the effective separa-

tion of the four fluoroquinolones in spiked fresh milk samples without interference were observed, as shown in Fig. 2.

Validation Results

The linearity of calibration curves was evaluated with measured peak areas against concentrations of calibration standards under the optimal conditions. The equations of calibration curves obtained based on three replicate measurements of standard solution were as follows: $A = 1.7172C + 0.7522$, $r = 0.99989$ for sarafloxacin; $A = 2.4033C + 0.8112$, $r = 0.99994$ for ciprofloxacin; $A = 2.9206C + 0.9624$, $r = 0.99991$ for enrofloxacin; $A = 1.9616C + 1.6897$, $r = 0.99989$ for flumequine. Correlation coefficients (r) greater than 0.9998 were obtained for all fluoroquinolones with a dynamic range from 1 up to 100 μg mL^{-1}.

The limit of detection (LOD) was determined as the sample concentration that produces a peak height of three times the level of the baseline noise. The limit of quantification (LOQ) was defined as the sample concentration that produced a peak height of ten times the signal-to-noise ratio [19, 23]. For the determination of sarafloxacin, ciprofloxacin, enrofloxacin and flumequine standard solution, LODs are 14.2, 12.0, 12.0 and 14.4 μg L^{-1}, and for the analysis of the spiked milk sample the LODs are 19.8, 15.2, 13.3 and 15.9 μg kg^{-1}, respectively, and the LOQs are 66.0, 50.7, 44.3 and 53.2 μg kg^{-1}, respectively, for the same fluoroquinolones. The detection limits of the proposed method were lower than those for LC [9] and CE [15, 16, 18, 19], and higher or close to those for LC-MS-MS [12] or SPE-LC-MS-MS [20]. The obtained detection limits of the fluoroquinolones studied permit the detection in milk samples at the targeted MLRs. The recoveries of the targeted fluoroquinolones from spiked raw milk from farms and spiked fresh milk samples were investigated. The recovery of the analyte at a spiked level of 2 mg kg^{-1} for raw milk samples from farms was 70.9% for sarafloxacin, 78.0% for ciprofloxacin, 89.3% for enrofloxacin and 90% for flumequine, and the relative standard deviation (RSD)

Table 1. Resolution under different column temperatures

Temperature (°C)	Resolution[a]		
	R_{1-2}	R_{2-3}	R_{3-4}
15	1.85	9.45	3.81
20	2.35	6.62	3.65
25	2.66	7.30	3.62
30	2.63	5.49	3.57

[a] Sarafloxacin(1), ciprofloxacin(2), enrofloxacin(3), and flumequine(4)

Table 2. Recoveries and precision for four fluoroquinolones in spiked fresh milk sample

Analyte	Added (mg kg^{-1})	Recovery[a] (%)	RSD[a] (%)	Intra-day[b] RSD(%)	Inter-day[c] RSD(%)
Sarafloxacin	2	71.6	1.51	4.31	4.46
Ciprofloxacin	2	78.3	1.45	4.72	5.37
Enrofloxacin	2	90.3	1.53	3.35	4.67
Flumequine	2	90.6	1.32	2.51	3.07

[a] $n = 3$
[b] $n = 4$
[c] $n = 6$

was in the range of 1.75–2.23%. For fresh milk samples, the recoveries are provided in Table 2, along with the intra- and inter-day precisions RSD.

The method recoveries range from 70.9 to 90.6% for the targeted fluoroquinolones with RSDs <2.3%. The reason(s) for the relatively low recoveries observed with sarafloxacin and ciprofloxacin are not known and possibly related to their molecular structures and combinative intensity with milk matrix.

The RSD of peak areas was used to express intra- and inter-day precision. As shown in Table 2, the milk samples spiked at the 2 mg kg^{-1} level of each fluoroquinolone were analyzed in four replicates on a single day, an intra-day precision ($n = 4$) of <5% and an inter-day precision ($n = 6$) of <6% were achieved.

Conclusion

This work has shown that McIlvaine buffer containing EDTA (pH 4.0) is an effective solvent for extracting fluoroquinolones in milk samples, methanol is an efficient solvent for eluting the analytes from the Oasis HLB cartridges. Optimal separation was achieved with the use of the phosphorate running buffer (pH 9.2) as the running buffer. Successful baseline separation and quantitation of four fluoroquinolones in milk samples was achieved with the use of Oasis HLB cartridges as SPE for interferences—free CE analysis. The proposed method has the advantages of short analysis time, simple clean-up system, high detection capability, and can be applied to monitor structurally related novel fluoroquinolone antibiotics in milk samples.

Acknowledgments

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