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Multiresidue Determination of Fluoroquinolones in Milk by Ion-Pair High Performance Liquid Chromatography

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A simple, sensitive and reliable ion-pair reverse phase high-performance liquid chromatography method has been developed and validated for the simultaneous determination of five fluoroquinolone residues in milk. The studied fluoroquinolones were ofloxacin, ciprofloxacin enrofloxacin, norfloxacin and sarafloxacin. Chromatographic separation was carried out on a Venusil XBP C_{18} column, in isocratic mode, with 0.025 mol/L H_3PO_4 solution, adjusted to pH 3.2 with 13 mmol/L tetrabutylammonium bromide-acetonitrile (96:4, v/v) as mobile phase. Good linearity over the investigated concentration range was observed, with mean values of correlation coefficients higher than 0.9993 for all the analytes studied. The limit of detection (LOD) and the limit of quantification (LOQ) with acceptable precision were 14.09, 46.97 mg/kg for ofloxacin, 4.39, 14.64 mg/kg for norfloxacin, 10.18, 33.94 mg/kg for ciprofloxacin, 10.53, 35.12 mg/kg for enrofloxacin and 17.44, 58.14 mg/kg for sarafloxacin, respectively. These values are in compliance with requirements for monitoring of maximum residues levels. Overall recoveries from spiked milk samples ranged from 80.7 to 96.92 % with relative standard deviations lower than 4.23 %.

Key Words: HPLC, Ion-pair, Fluoroquinolone, Milk.

INTRODUCTION

Fluoroquinolones (FQs) are antibacterial agents widely used in the treatment of infections in both humans and animals[1]. The most prominent human health risk associated with intensive animal farming and antibiotic use in antimicrobial resistance. In particular, use of fluoroquinolones in animals has generated growing concern because microbial resistance to these drugs has increased. The World Health Organization (WHO)[2] and Food and Drug Administration (FDA)[3] have placed severe restrictions on veterinary use of fluoroquinolones, given the concern about drug-resistant bacteria and the possible failure of human antibiotic therapy[4]. To ensure safety, the Chinese Ministry of Agriculture established maximum residue limits (MRLs) in foodstuffs of animal origin. Ciprofloxacin and enrofloxacin are allowed at concentrations below their maximum residue limits, which is 100 mg/kg for the sum of ciprofloxacin and enrofloxacin[5].

Many analytical methods for determination of fluoroquinolones residue in biological samples, animal derived food and environmental samples are described in the scientific literature, such as microbiological methods[6,7], spectrophotometry[8], capillary electrophoresis[9], chemiluminescence[10] have also been applied. The main analytical methods of fluoroquinolones are based on HPLC with fluorescence[11], ultraviolet[12] or mass spectrometric detection[5]. Some methods have focused on the determination of fluoroquinolone multiresidues in milk[5,6,11]. Clean up from the matrix can be carried out by liquid-liquid extraction[6,13], solid-phase extraction[11,12,14,15], matrix solid-phase dispersion technique[16] and on-line column clean-up coupled to HPLC[17]. The gradient RP-HPLC have been widely used in determination of fluoroquinolone residues in milk samples[12,15,16]. The isocratic RP-HPLC method had been developed for determination of fluoroquinolones in pharmaceuticals and blood serum used a simple mobile phase (CH_3CN-CH_3OH-citric acid 0.4 mol/L, 7:15:78, v/v)[18].

This paper describes an ion-pair reversed-phase high performance liquid chromatographic method for the multiresidue determination of fluoroquinolones in milk. The isocratic HPLC method uses a simple mobile phase, UV-detection and a simple sample-preparation step for milk samples. UV-detection is simple, rapid, selective and reproducible and sensitivity is adequate for routine use. Direct determination of fluoroquinolones in milk can be accomplished by ion-pairing technique, protein precipitation using trichloroacetic acid/acetonitrile and fluoroquinolone extraction using *n*-hexane. The method was

successfully applied to the analysis of fluoroquinolones in milk samples.

EXPERIMENTAL

Ofloxacin, ciprofloxacin, enrofloxacin, norfloxacin and sarafloxacin were obtained from Beijing Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). The structures of these molecules are shown in Fig. 1. Methanol, acetonitrile and hexane HPLC-grade were obtained from Tianjin Kermel Chemical Reagent Co., Ltd. (Tianjin, China). Tetrabutyl ammonium bromide, trichloroacetic acid and anhydrous sodium sulfate and acetic acid were obtained from Tianin Reagent Chemicals Co., Ltd. (Tianjin, China). All other chemicals and solvents were of analytical grade. Solutions prepared for HPLC were through a 0.45 mm PVDF syringe filter before use. Doubly deionized water (DDW) was used throughout. Commercial whole milk were purchased from market.

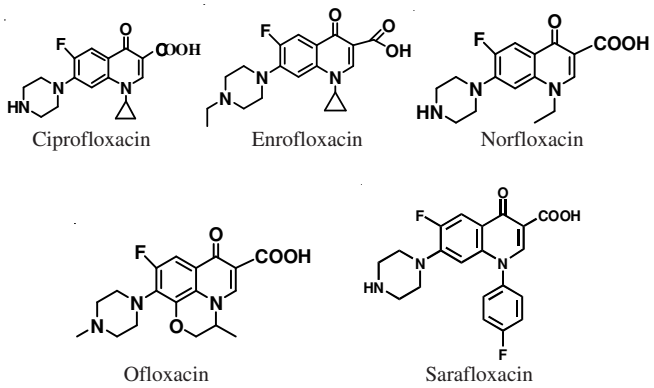

Fig. 1. Molecular structures of different fluoroquinolones

A SK-1 vortex mixer (Shanghai Shangdeng Experimental Facility Co., Ltd) was used to mix and homogenise milk samples during pretreatment. For milk defatting and protein removal, an YXJ-2 Ultracentrifuge (Jintan Jingbo Experimental Instrument Factory), KH-250DE Ultrasonic Cleaner (Kunshan Ultrasonic Equipment Co., Ltd) was employed. The pH of the mobile phase solutions and samples was adjusted with a PHS-2C Precision pH/mV Meter (Shanghai LIDA Instrument Factory).

Standard preparation: Standard stock solutions (250 μg/mL) were prepared in methanol and stored at 4 ºC for no longer than 1 month. Working solutions (5 μg/mL) were prepared daily by diluting with the mobile phase. Calibration standards were prepared at concentrations of 0.05, 0.1, 0.2, 0.5, 2.0 μg/mL for each fluoroquinolone and injected in replicates of three.

Chromatographic conditions: Chromatographic separation of the fluoroquinolones was performed using an HPLC (LC-6A, Shimadzu Corporation, Kyoto, Japan) equipped with a LC-6A pump and UV3000 detector (Beijing Tong Heng Innovation Technology Co., Ltd) at a wavelength of 277 nm. The samples were separated on C18 stainless column (Venusil XBP C18, 4.6 mm × 150 mm, 5 μm) and eluted with a mobile phase consisting of a mixture of 13 mM tetrabutylammonium bromide-acetonitrile (96:4, v/v) at pH = 3.2. The flow rate was 1.0 mL/min at ambient temperature, the injection volume 20 μL.

Sample preparation[16]: Purchased samples were stored at -20 ºC. A measure of 2 g milk samples were transferred to a 50 mL polypropylene centrifuge tube and mixed. After a 10 min equilibration period, 7 mL of 2.5 % trichloroacetic acid/acetonitrile (25:75, v/v) was added, vortex-mixed (30 s) and left undisturbed for 10 min. A quantity of 4 g of anhydrous sodium sulfate was added thereafter, thoroughly mixed for 15 s and maintained for 5 min. The samples were centrifuged at 3000 rpm for 20 min.

The samples were then extracted once again with 7 mL of 2.5 % trichloroacetic acid/acetonitrile (25:75, v/v) and maintained at room temperature for 10 min. The organic layers were recovered after 20 min centrifugation at 3000 rpm. *n*-Hexane (10 mL) was then added to the combined supernatant. After 15 s vortex mixing, the upper layer was discarded after 10 min of centrifugation at 3000 rpm. The lower layers were transferred to glass tubes and concentrated under a stream of nitrogen at 45-50 ºC. The concentrated residues were then dissolved in 1 mL of mobile phase (13 mM tetrabutyl ammonium bromide (pH 3.2)-acetonitrile (96:4, v/v)), filter it with 0.45 μm filter membrane, then 20 μL of the filtrate was injected into the HPLC system.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions: The chromatographic separation was achieved on a C18 with the acetonitrile-phosphoric acid/triethylamine buffer, as mobile phase[19], the effect is not ideal, norfloxacin and ciprofloxacin can not be completely separated. While acetonitrile-methanol-Britton Robinson (BR) buffer was used as mobile phase[20], although they can be completely separated, but BR buffer's ingredient is complex that is the mixture of phosphoric acid, acetic acid and boric acid. Crystallize was shaped to obstruct the fluid penetration when the BR buffer mixes with methanol and acetonitrile. This is because of the presence of piperazinyl and carboxylic acid groups in fluoroquinolone structures. These groups are responsible for chemical tailing during liquid chromatographic analysis, because of interactions with the stationary phase. The pH and organic modifier content of the mobile phase used for HPLC analysis of fluoroquinolones are known to effect the capacity factors of these compounds[21]. The combination of an acidic eluent that consists of methanol or acetonitrile, water and an ion-pairing reagent of the tetrabutyl ammonium type, enables elution of the fluoroquinolones with good peak shape[22].

In order to optimize the separation of the analytes, the constituents and the relative percentages of the isocratic mobile phase were tested. The effect of tetrabutyl ammonium bromide concentration varied in the range 10-15 mM on the fluoroquinolones separation was investigated. With eluents containing larger tetrabutyl ammonium bromide concentrations the retention times and the number of theoretic plates decreased. The best compromise in terms of resolution, run time, efficiency and pressure of HPLC system was found to be 13 mM in tetrabutyl ammonium bromide concentration. The addition of acetonitrile as modifier decreased the retention was varied in the range 2-10 % (v/v) and finding out acetonitrile at 4 % concenration produced the best well-shaped peaks.

TABLE-2
PRECISION, RECOVERY (%), LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ) OF FLUOROQUINOLONES IN FORTIFIED MILK (n = 5)

Analyte	Found (μg/kg)	Spiked level (μg/kg)	Detected (μg/kg)	Recoveries (%)	RSD (%)	LOD (μg/kg)	LOQ (μg/kg)
Ofloxacin	174.22	500	577.73	80.70	2.96	14.09	46.97
Norfloxacin	42.11	500	526.69	96.92	3.37	4.39	14.64
Ciprofloxacin	497.80	1000	1368.69	87.10	3.87	10.18	33.94
Enrofloxacin	0	1000	925.01	92.50	4.17	10.53	35.12
Sarafloxacin	0	1000	935.47	93.55	4.23	17.44	58.14

[a]LOD calculated as three times the signal-to-noise ratio; [b]LOQ calculated as 10 times the signal-to-noise ratio.

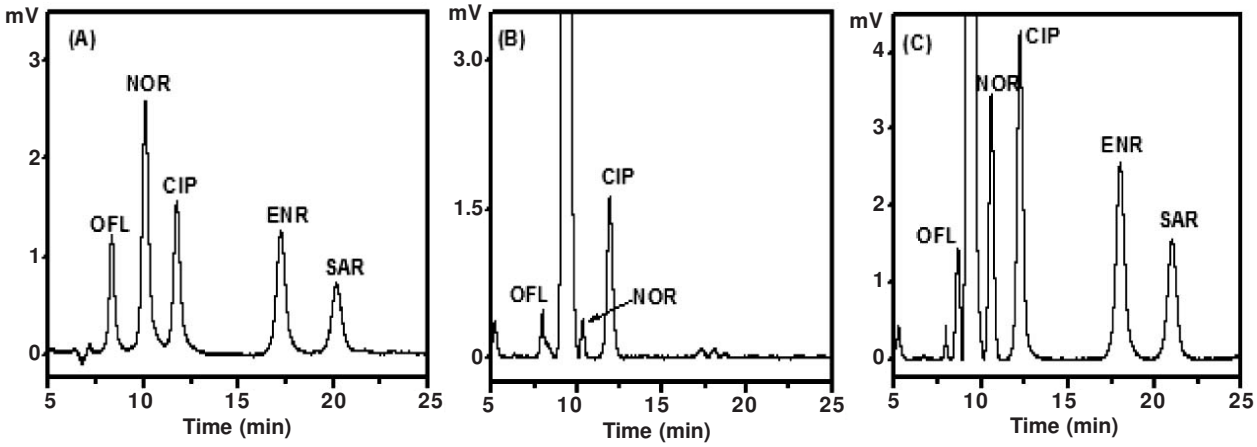

Fig. 2. Chromatogramof (A) the standard solution (1 μg/mL), (B) the milk samples and (C) the milk sample spiked with the fluoroquinolones (concentration: 0.50 mg/kg of ofloxacin and norfloxacin, 1.0 mg/kg of ciprofloxacin, enrofloxacin and sarafloxacin). Mobile phase: 13 mM tetrabutylammonium bromide (pH 3.2)-acetonitrile (96:4, v/v). Flow rate: 1 mL/min, Detection: 277 nm

pH is one of the most powerful tools for optimizing the separation of analyte mixtures. To optimize the pH of the mobile phase, the influence of pH on the separations of the five drugs in different acidity condition (pH = 2.2, 2.8, 3.0, 3.2, 3.4), adjust pH by phosphoric acid, were investigated. The results suggest that the best separation of the analytes was obtained at pH 3.2. Therefore, this pH value was chosen as the optimum for present evaluations. The flow rate employed was 1.0 mL/min with a UV detection at 277 nm.

Analytical parameters: The calibration curve was calculated by line regression of the measured peaks areas and the corresponding concentrations of the calibration standard solutions described in standard solutions. In the range of 0.05-2 μg/mL, the calibration curve of each fluoroquinolone showed good linearity with correlation coefficient (R) more than 0.9988. The instrument detection limits for fluoroquinolones were calculated three times of the signal noise ratio according to the lowest concentration point in standard curve and shown in Table-1.

TABLE-1
LINEAR REGRESSION EQUATIONS, CORRELATION COEFFICIENT (r) AND INSTRUMENT DETECTION LIMITS FOR FLUOROQUINOLONES

Analyte	Linear equation	R	Instrument detection limits (μg/L)
Ofloxacin	$Y = 488 + 2.51 \times 10^4 C$	0.9988	2.63
Norfloxacin	$Y = 209 + 6.52 \times 10^4 C$	0.9999	1.01
Ciprofloxacin	$Y = -299 + 3.97 \times 10^4 C$	0.9995	1.66
Enrofloxacin	$Y = 215 + 4.77 \times 10^4 C$	0.9997	1.38
Sarafloxacin	$Y = 498 + 2.67 \times 10^4 C$	0.9990	2.47

Application and validation of the method: The fortified milk samples with spiked levels at 0.5 and 1.0 mg/kg had been analyzed by HPLC in triplication. According to the spiked level, the limit of detection (LOD) and the limit of quantification (LOQ) for the method were calculated three times and ten times of the signal noise ratio, respectively. The fortified recoveries were 80.70-96.92 % with RSD no more than 4.23 %. The LOD and LOQ were 4.39-17.44 μg/kg and 14.64-58.14 μg/kg, respectively (Table-2). Fig. 2 shows the representative HPLC-UVD chromatograms for the standard solution, the milk sample and the spiked milk sample at 0.5 and 1.0 mg/kg levels. The results show the samples contained fluoroquinolone residues, The residues concentrations of ofloxacin and ciprofloxacin were found to exceed the maximum residues level; norfloxacin was below the maximum residues level; enrofloxacin and Sarafloxacin were not found in all samples.

Conclusion

In this study, an analytical protocol involving the determinations of fluoroquinolone residues by the ion-pair RP-HPLC was successfully developed. The developed method was validated in terms of precision, accuracy, linearity, sensitivity, selectivity and stability and is suitable for the determination of the residues of these compounds in milk samples with a high degree of sensitivity.

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