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Short communication

Determination of quinolones in animal tissues and eggs by high-performance liquid chromatography with photodiode-array detection

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Abstract

A rapid, specific reversed-phase HPLC method is described, with solid-phase extraction, for assaying five quinolones (ciprofloxacin, difloxacin, enrofloxacin, norfloxacin and marbofloxacin) with confirmative diode-array detection in samples of bovine kidney, muscle and eggs. The least efficient extraction was marbofloxacin from kidney tissue (64%). The lower detection limit for each quinolone was: enrofloxacin and ciprofloxacin, 1 ng; norfloxacin and difloxacin, 2 ng; marbofloxacin, 4 ng injected. The intra-day relative standard deviations were lower than 7.9% and lower than 8.6% for inter-day assays. These results indicate that the developed method had an acceptable precision.

Keywords: Quinolones; Antibiotics

1. Introduction

The quinolones are antimicrobial agents used in the treatment of a variety of bacterial infections [1,2]. These compounds act directly on bacterial DNA by inhibiting topoisomerase which leads to cell death, so they are bactericidal.

A significant and progressive increase in the use of quinolones in animal production was noted over the present decade. Thus, residues of these drugs in animal tissues are an important issue.

The 1570/98 Regulation of the European Union [3] established the maximum residues limits for some quinolones (difloxacin and enrofloxacin). These decisions require the development of analytical methods for the detection of these residues in animal tissues.

The methods available for quinolone assays are thin-layer chromatography (TLC)–fluorescence [4–6], capillary electrophoresis–fluorescence [7,8], high-performance liquid chromatography (HPLC)–UV or fluorescence [9–18], HPLC–mass spectrometry (MS) [19,20] or gas chromatography (GC)–MS [21]. The majority of these methods have been developed to analyze biological samples such as serum or urine.

Some analytical methods were designed for residues of quinolones in fish [12–14,22,23] and meat [4,6,13,17,21,24]. Many methods available for animal tissues involve a tedious sample preparation with liquid–liquid extraction and purification steps [4,6,13,21]. Other limitations are the lack of identity confirmation of quinolone.

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Here, we introduce a rapid, specific reversed-phase HPLC method, with solid-phase extraction, for assaying five quinolones (ciprofloxacin, difloxacin, enrofloxacin, norfloxacin and marbofloxacin) with confirmative diode-array detection in samples of bovine kidney, muscle and eggs.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol were HPLC-grade; hydrochloric acid (1 *M*), monopotassium phosphate and orthophosphoric acid were analytical-reagent grade (Merck, Darmstadt, Germany). Sep-Pak C_{18} columns were from Millipore (Waters, Bedford, MA, USA).

2.2. Apparatus

A 2K15 ultracentrifuge with cooling system (Sigma, St. Louis, MO, USA), and a nitrogen evaporation system, with thermostated heating plate (Liebisch, Bielefeld, Germany) were used.

The chromatographic system was of a 2690 Model Alliance. Chromatographic separation was achieved on a C_{18} Hypersil 5 μm BDS (250×4.6 mm) column. Detection was performed with a Model 996 diode array detector, all from Waters. Spectra were measured at 190–350 nm, monitoring wavelength 280 nm. A NEC-Image 466 computer, using Millennium ver. 2.1 software and a HP 560 printer (Hewlett-Packard, Wilmington, DE, USA) were used.

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of 0.1 *M* orthophosphoric acid, pH 3.5–acetonitrile (85:15, v/v). The eluent was carefully degassed with helium and filtered prior to use at a flow-rate of 1 ml/min. The injection volume was 20 μl.

2.4. Standard solutions

Five quinolones were analyzed: enrofloxacin and ciprofloxacin were from Bayer Pharmaceuticals (West Haven, CT, USA), difloxacin, marbofloxacin and norfloxacin from Laboratorios Vetoquinol (Lérida, Spain).

Quinolone stock solutions were prepared in methanol at a concentration of 1 mg/ml. This solution was stored at 4°C for no longer than two months.

Standard work solutions were prepared every day, using methanol as diluent.

Calibration standards were prepared at concentrations from 0.5 μg/ml to 10 μg/ml, for each quinolone, in replicates of three, for three days.

For recovery studies, 5 g of drug-free calf kidney, muscle and internal content of chicken egg samples were spiked by adding different volumes of quinolone standard solutions. The levels assayed were 10, 50 and 100 ng/g, for each quinolone.

2.5. Sample preparation

A 5-g amount of sample was transferred to a 30-ml centrifuge tube, 20 ml of 1 *M* HCl was added and the mixture sonicated for 5 min. The tube was subsequently stoppered and centrifuged for 5 min at 5600 *g*.

The supernatant liquid was taken after centrifugation and Sep-Pak C_{18} cartridges, previously conditioned, are then used for purification. The cartridges were washed with 10 ml of water and the elution of quinolones was performed with 4 ml of monopotassium phosphate (1 m*M*), pH 2.5–methanol (1:1) mixture.

The purification residues were evaporated in a nitrogen stream at 35°C to 200 μl. From this 200 μl, 20 μl were taken to be injected into the chromatographic system.

3. Results and discussion

3.1. HPLC profiles

The pH of the mobile phase was a critical factor in achieving the chromatographic separation of the five quinolones studied. This also has been noted by other authors for quinolones [10–12,14,15,20]. No ion pairing reagent in the mobile phase (essential in other works [10,15]) was necessary to obtain acceptable peak shapes without tailing, using the reversed-phase column chosen. Adequate chromatographic

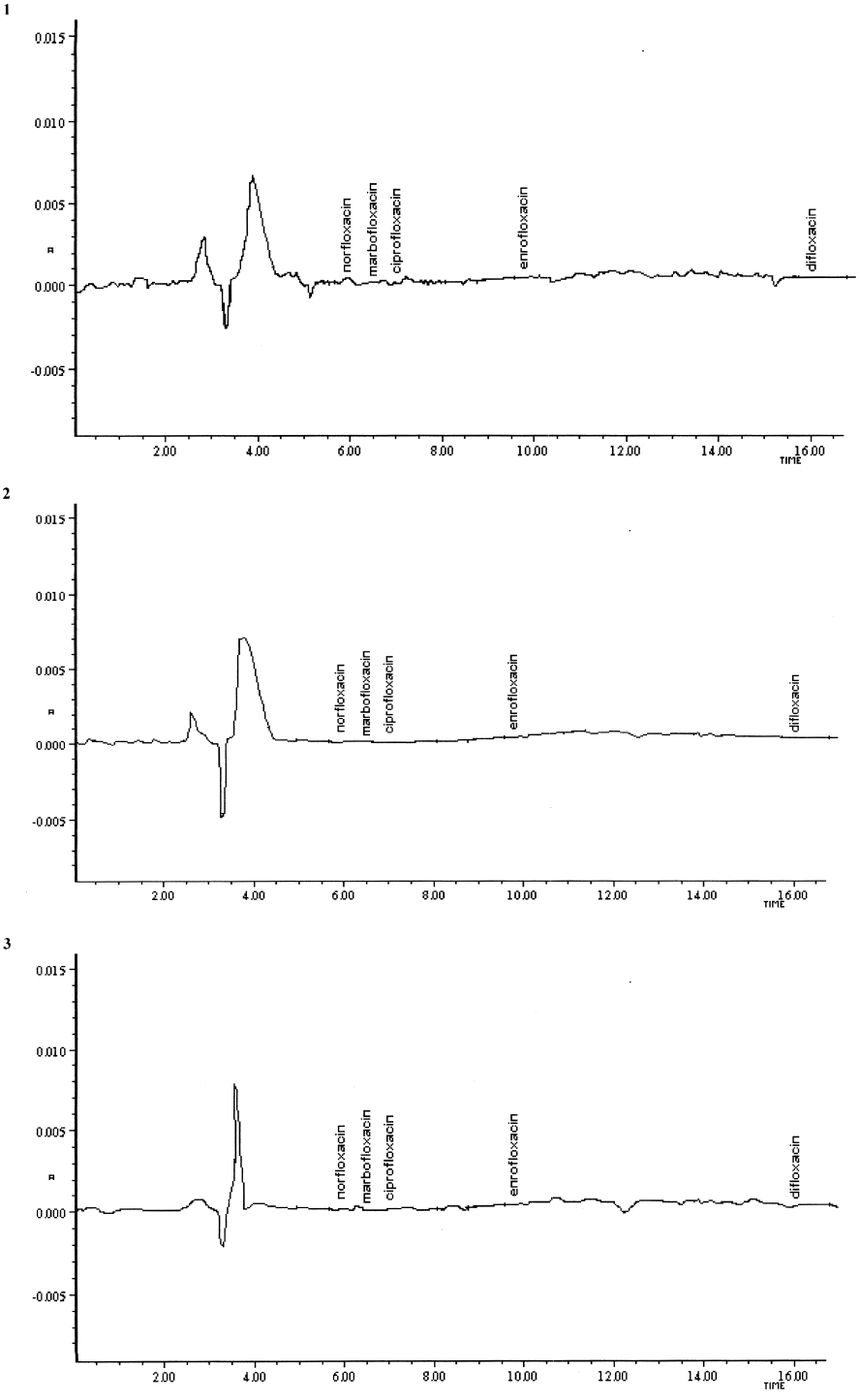

Fig. 1. Chromatogram of analysis of drug-free egg (1), kidney (2) and muscle (3).

separation was obtained using the above method. The retention times for each quinolone (mean±SD, $n=10$) were: marbofloxacin, 6.36±0.21 min; norfloxacin, 5.73±0.14 min; ciprofloxacin, 6.99±0.24 min; enrofloxacin, 9.54±0.43 min; difloxacin, 14.44±0.45 min. Using the sample preparation procedure described, the blank chromatogram for the three matrices did not contain peaks at the retention times corresponding to any of the quinolones. Figs. 1 and 2 show the chromatogram from the analysis of blank egg and muscle blank spiked with 100 ng/g of each of five quinolones.

3.2. Calibration, limit of detection

A calibration curve described by the equation $y=mx+b$, where y represents the response value of the analyte in the sample (peak area), and x the number of ng of each quinolone on column, was generated in triplicate for each day, over three days. The mean correlation coefficient, intercept and slope values for all five quinolones tested are indicated in Table 1. The chromatographic method was demonstrated to be linear from 4 to 100 ng of each quinolone injected ($r\geq 0.9994$).

The lower detection limit for each quinolone was made by analyzing calibration standards from 0.5 to 4 ng of each quinolone injected (enrofloxacin and ciprofloxacin, 1 ng; norfloxacin and difloxacin, 2 ng; marbofloxacin, 4 ng).

3.3. Intra-day and inter-day repeatability

Analysis of the calibration standards was used to determine the intra-day (three repetitions of each concentration) and inter-day repeatability (three repe-

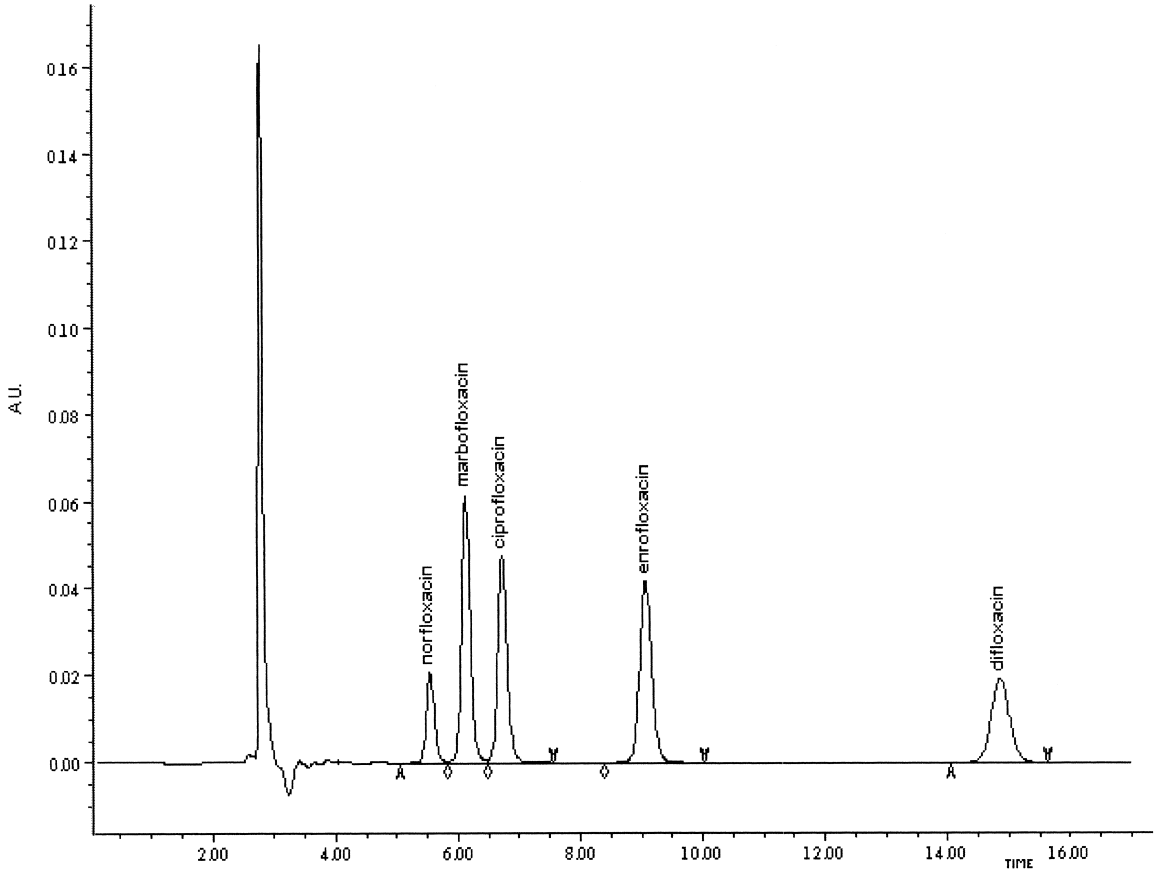

Fig. 2. Chromatogram of drug-free muscle tissue spiked with 100 ng/g of each quinolone.

Table 1
Correlation values of quinolones

Quinolone	Calibration graph	Slope RSD (n=9) (%)	Correlation coefficient
Marbofloxacin	y=7670x+159	4.50	0.9995
Norfloxacin	y=6524x+810	4.71	0.9994
Ciprofloxacin	y=6392x+630	6.94	0.9996
Enrofloxacin	y=6700x+630	9.15	0.9996
Difloxacin	y=4895x+102	2.32	0.9997

titions of each concentration, three days, with different analysts and reagents). The results (for three levels) are shown in Table 2. The intra-day relative standard deviations (RSDs) were lower than 7.9% and lower than 8.6% for inter-day assays. These results indicate that the method developed had acceptable precision.

3.4. Recovery

When samples, after extraction, were taken to dryness the percentage recovery for the quinolones decreased markedly and better recoveries (%) were obtained when they were taken to 200 μl. Calculation of extraction recovery for each quinolone was done by comparison with the respective mean calibration curve values for each with the unextracted standards. The samples (kidney, egg and muscle) were spiked with 50, 70 and 100 ng/g of each quinolone and subjected to the sample preparation procedure explained above (n=6). The results are shown in Table 3. The extraction recoveries of quinolones were similar in the different matrices. The extraction of quinolones from the kidney are

Table 2
Intra-day and inter-day repeatability

Amount injected (ng)	Intra-day repeatability RSD (n=3) (%)	Inter-day repeatability RSD (n=9) (%)
Marbofloxacin		
4	6.69	7.00
40	4.09	4.80
100	3.12	3.81
Norfloxacin		
4	7.8	8.55
40	3.11	3.23
100	0.58	2.15
Difloxacin		
4	2.12	3.58
40	1.87	2.55
100	0.85	1.60
Ciprofloxacin		
4	3.39	3.96
40	1.87	2.20
100	0.85	1.01
Enrofloxacin		
4	2.57	3.56
40	2.87	3.85
100	1.87	2.64

Table 3
Percentage recoveries of five quinolones in kidney, muscle and egg samples

Tissue source	Quinolone	Spiked amount					
		50 ng/g		70 ng/g		100 ng/g	
		Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
Muscle	Marbofloxacin	74	11.4	72	8.7	79	1.66
	Norfloxacin	98	2.15	99.5	0.71	98	9.3
	Ciprofloxacin	76	7.4	70	3.05	70	1.0
	Enrofloxacin	82	3.4	74	5.7	73	2
	Difloxacin	70	4.1	65	6.7	72	2
Kidney	Marbofloxacin	64	12.3	65	9.0	64	4.5
	Norfloxacin	88	5.6	98.5	3.1	77	5.3
	Ciprofloxacin	69	6.7	76	4.05	76	5.1
	Enrofloxacin	81	5.2	72	6.3	75	2.3
	Difloxacin	69	5.3	65	6.7	73	3.4
Egg	Marbofloxacin	72	9.5	76	8.7	79	1.66
	Norfloxacin	99	3.5	99	0.71	98	9.3
	Ciprofloxacin	78	6.6	77	3.5	77	2.1
	Enrofloxacin	82	6.5	87	6.4	85	2.2
	Difloxacin	75	4.4	88	3.1	89	2.6

more difficult than for the muscle or egg. Norfloxacin recoveries were always higher than the recoveries for the other drugs. The least efficient extraction was marbofloxacin from kidney tissue (64%).

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