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Simultaneous determination of benofloxacin, danofloxacin, enrofloxacin and ofloxacin in chicken tissues by high-performance liquid chromatography

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Abstract

A simple, rapid and reliable high-performance liquid chromatographic (HPLC) method for the simultaneous determination of residual fluoroquinolones (benofloxacin, danofloxacin, enrofloxacin and ofloxacin) in chicken has been developed. The drugs were extracted with 0.2% metaphosphoric acid–acetonitrile (7:3, v/v), followed by a Bond Elut C_{18} clean-up procedure. The HPLC separation was carried out on a Wakosil II $5C_{18}$-HG column (150 × 4.6 mm I.D.) with 0.05 *M* phosphate buffer (pH 2.4)–acetonitrile (80:20, v/v) containing 2.5 m*M* 1-heptanesulfonic acid as the mobile phase. A fluorescence detector was used at an excitation wavelength of 295 nm and an emission wavelength of 455 nm. The calibration graphs were linear from 0.1 to 10 ng for danofloxacin and from 1 to 100 ng for benofloxacin, enrofloxacin and ofloxacin. The recoveries of the drugs from tissues fortified at a level of 0.2 μg/g were 81.1–89.6%, and the detection limits were 0.01 μg/g for ofloxacin, danofloxacin and enrofloxacin and 0.02 μg/g for benofloxacin. The time needed per sample was less than 60 min.

1. Introduction

Infectious diseases are a serious problem for the livestock and fish-farm industries; therefore, various kinds of antibiotics and synthetic antibacterials are widely used for prevention and treatment. However, concern has arisen as to the presence of drug residues in livestock products. According to the Japanese Food Sanitation Law, no food should contain antibiotics and, in addition, meat, poultry eggs, fish and shellfish should not contain any synthetic antibacterials. Therefore, a simple and reliable analytical method is required to monitor drug residues in edible tissues of livestock animals.

The new 4-quinolones, or fluoroquinolones, are the most important group of synthetic antibacterial agents since the discovery of sulfonamides. Benofloxacin (BNFX), danofloxacin (DNFX), enrofloxacin (ERFX) and ofloxacin (OFLX) are recently developed fluoroquinolones (Fig. 1). They are highly active against a wide range of Gram-negative and

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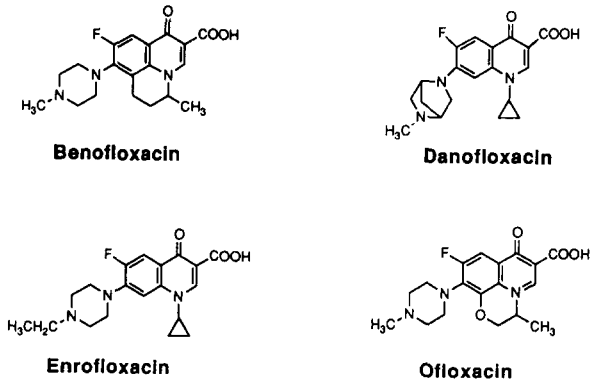

Fig. 1. Structures of benofloxacin, danofloxacin, enrofloxacin and ofloxacin.

Gram-positive bacteria, including those resistant to β-lactam antibiotics and sulfonamides [1,2]. These drugs are absorbed well after oral administration and are distributed extensively in tissues with high tissue/plasma ratios [3]. These characteristics make these drugs suitable for the therapy of a wide range of infections. Therefore, the fluoroquinolones have been used to treat livestock, especially poultry, since 1992 in Japan. Only four kinds of fluoroquinolones are presently approved for use in animals: BNFX, DNFX, ERFX and OFLX. BNFX, DNFX and ERFX are veterinary drugs used solely in livestock farming. Although numerous HPLC methods for the determination of ERFX and OFLX in pharmaceutical preparations, biological fluids and animal tissues have been reported [4–11], there are no methods for the determination of BNFX and DNFX. Thus it seems useful to develop a method for the simultaneous analysis of the four fluoroquinolones remaining in livestock products.

This paper describes a simple, selective and reliable HPLC method for the simultaneous determination of four different fluoroquinolones of which residues could possibly occur in chicken tissues. The technique is based on separation by reversed-phase ion-pair chromatography and sample preparation with Bond Elut C_{18} cartridges.

2. Experimental

2.1. Materials and reagents

BNFX, DNFX, ERFX and OFLX were kindly supplied by Takeda Pharmaceuticals (Osaka, Japan), Pfizer Pharmaceuticals (Tokyo, Japan), Bayer Japan (Tokyo, Japan) and Daiichi Pharmaceuticals (Tokyo, Japan), respectively. Bond Elut C_{18} (500 mg) cartridges were purchased from Varian (Harbor City, CA, USA). The cartridges were washed with 5 ml of methanol and 10 ml of distilled water before use. The ion-pairing reagent, 1-heptanesulfonic acid sodium salt, was obtained from Aldrich (Milwaukee, WI, USA). Hyflo Super-Cel was purchased from Johns-Manville (Denver, CO, USA). Mueller–Hinton agar was purchased from Nissui Pharmaceutical (Tokyo, Japan). Other chemicals were of analytical-reagent or HPLC grade. Deionized or distilled water was used in all experiments.

2.2. Preparation of standard solutions

Stock standard solutions of BNFX, DNFX, ERFX and OFLX were prepared by dissolving 20 mg of each compound in 100 ml of acetonitrile. Working standard solutions were prepared by diluting the stock solution with the HPLC mobile phase or 0.05 *M* phosphate buffer (pH 7.0). The standard solutions were stored in the refrigerator in brown glass vessels.

2.3. Apparatus

The HPLC system consisted of a Shimadzu LC-6A solvent-delivery system (Kyoto, Japan), equipped with a Rheodyne 7125 injector (Berkeley, CA, USA), a Model 821-FP intelligent fluorescence detector (Jasco, Tokyo, Japan) operated at an excitation wavelength of 295 nm and an emission wavelength of 455 nm, and a Chromatopak C-R3A data system (Shimadzu). The separation was performed on a Wakosil II $5C_{18}$ HG column (150 × 4.6 mm I.D., Wako, Osaka, Japan) with 0.05 *M* phosphate buffer

(pH 2.4)–acetonitrile (80:20, v/v) containing 2.5 m*M* 1-heptanesulfonic acid as the mobile phase at a flow-rate of 0.6 ml/min. The phosphate buffer was filtered through a Millipore GS 0.22-μm filter (Milford, MA, USA). The chromatograph was operated at ambient temperature.

The other instruments used were a Model 650–40 spectrofluorometer (Hitachi, Tokyo, Japan) and a Model NS-50 Physcotron homogenizer (Niti-on, Chiba, Japan).

2.4. Sample preparation

Sample preparation was based on the method described in a previous paper [12]. A 5-g tissue sample was homogenized at high speed for 2 min, with 100 ml of 0.2% metaphosphoric acid–acetonitrile (7:3, v/v) used as a deproteinizing extractant. The homogenate was filtered through *ca.* 2 mm of Hyflo Super-Cel coated on a suction funnel. The filtrate was evaporated under reduced pressure at 50°C. Evaporation was interrupted when *ca.* 30 ml of solution remained in the flask. The flask contents were applied to a Bond Elut C_{18} cartridge. After washing with 20 ml of distilled water, the cartridge was eluted with 10 ml of methanol. The eluate was evaporated to dryness under reduced pressure and the residue dissolved in 1 ml of HPLC mobile phase. A 10-μl volume of the solution was then injected onto the HPLC system.

2.5. Calibration graphs

Standards at concentrations of 0.01, 0.02, 0.05, 0.1, 0.5 and 1.0 μg/ml of DNFX, and 0.1, 0.2, 0.5, 1.0, 5.0 and 10 μg/ml of BNFX, ERFX and OFLX were prepared from stock standard solutions. A 10-μl volume of these solutions was injected onto the column. Calibration graphs were obtained by measurement of the peak heights.

2.6. Microbiological assay

The antibacterial activities of BNFX, DNFX, ERFX and OFLX were measured by a paper disk method with *Bacillus subtilis* ATCC 6633, *Micrococcus luteus* ATCC 9341 and *Bacillus cereus* ATCC 11778 as test organisms. The assay procedure was carried out according to the official method provided by Ministry of Health and Welfare, Japan [13].

3. Results and discussion

3.1. Chromatographic conditions

It is generally known that quinolone derivatives give severely tailing peaks in reversed-phase chromatography [12,14,15]. BNFX, DNFX, ERFX and OFLX also appeared as strongly tailing peaks in reversed-phase chromatography using conventional C_{18} (ODS) columns. Residual silanol groups and metal impurities in column packing materials are known to be the cause of tailing in reversed-phase chromatography [16,17]. This can be reduced by using mobile phases which have a high ionic strength or high acidity and which contain modifiers such as citric acid, perchloric acid or tertiary amine salts [14,15]. In a previous paper [18], we reported that the use of oxalic acid in the mobile phase could mask metal impurities and inhibit tailing.

Highly purified silica gel with a low content of metal impurities has recently been developed as a packing material for HPLC columns, and has been applied for the separation of coordination compounds [17,19]. Thus, the conditions for separation of the four drugs have been examined using pure silica-based ODS columns: Wakosil II $5C_{18}$ HG, Inertsil ODS-II, Puresil $5C_{18}$, TSK-GEL ODS 80_{TS} and L-column ODS. The drugs were adsorbed on the column or showed severe tailing when conventional C_{18} columns were used. However, when pure silica gel was used as the column packing material, broad tailing of the peaks could be prevented. Peak shapes were optimal with Wakosil II $5C_{18}$ HG, which was thus chosen for this study.

BNFX, DNFX, ERFX and OFLX are highly polar compounds due to the presence of two

ionizable groups, carboxylic acid and piperazine. Without the addition of ion-pairing reagents to the mobile phase, separation of the drugs could not be achieved by manipulation of the pH, the ionic strength of the buffer or by changing the organic component. Therefore, ion-paired reversed-phase chromatography was utilized to enhance the separation of the drugs. The influence of the counter ion, 1-heptanesulfonic acid, on the separation was studied. For all compounds, capacity factors (k') increased with increasing counter-ion concentration (Fig. 2), and the separation of the drugs improved.

The effect of mobile-phase pH on the peak shapes and separation of the drugs was also studied. The asymmetry of the peaks increased with increasing pH in the range pH 2.0–4.5. As a result, 0.05 *M* phosphate buffer (pH 2.5)–acetonitrile (80:20, v/v) containing 2.5 m*M* 1-heptanesulfonic acid was chosen as the mobile phase. Under these conditions, the retention times of the four drugs fell in the range 9.0–16.0 min.

The drugs showed strong fluorescence in acidic aqueous solutions. After characterization of the absorption spectra of BNFX, DNFX, ERFX and OFLX in the mobile phase, detection was performed with excitation at 295 nm and emission at 455 nm, which are the common maximum wavelengths of these compounds.

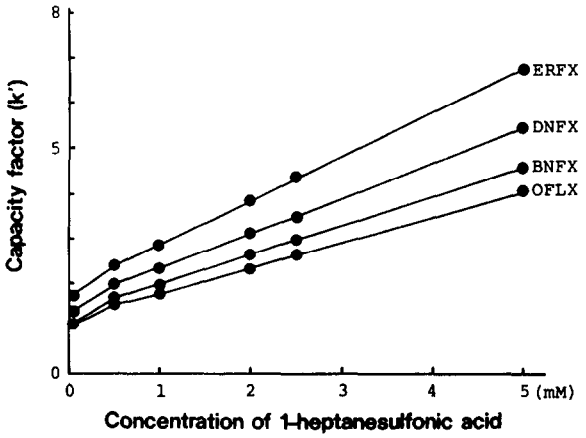

Fig. 2. Effect of the concentration of 1-heptanesulfonic acid on the capacity factor (k') of benofloxacin, danofloxacin, enrofloxacin and ofloxacin. LC conditions: column, Wakosil II $5C_{18}$ HG; mobile phase, 0.05 *M* phosphate buffer (pH 2.4)–acetonitrile (80:20, v/v) containing 0–5.0 m*M* 1-heptanesulfonic acid; flow-rate, 0.6 ml/min; detection, FL (ex 295/em 455 nm).

Most of the amount of BNFX, DNFX and OFLX taken orally is known to be distributed to each tissue in an unchanged form [20–22]. Therefore, the HPLC method proposed here is useful for the evaluation of the fluoroquinolones remaining in chicken tissues. On the other hand, the fluoroquinolones are known to be metabolized into N-desmethyl (or desethyl) and N-oxide analogues. Some metabolites of the drugs show antibacterial activities. Therefore, the development of an analysis method which enables the simultaneous measurement of the fluoroquinolones and their primary metabolites (N-desmethyl and N-oxide derivatives) is extremely important for pharmacokinetics research. In the future, we will examine the development of a method for analyzing residues including the principal metabolites.

3.2. Clean-up

In a previous paper [12], we reported a simultaneous determination of quinolone antibacterials (nalidixic acid, oxolinic acid and piromidic acid) in cultured fish where samples were extracted with metaphosphoric acid–methanol and the clean-up procedure used prepacked C_{18} cartridges. Therefore, we evaluated the use of such a method for the simultaneous determination of the four fluoroquinolones. Table 1 shows the results of recovery experiments on chicken muscle tissues fortified with 0.2 μg/g of each drug. An increase in methanol content resulted in improved recovery; however, the recoveries of each drug were below 70%. Therefore, we used acetonitrile instead of methanol for sample extraction. An increase in the acetonitrile content also resulted in improved recovery, but more interfering substances were eluted as well. Acetonitrile at 30% provided good recoveries, while minimizing the elution of contaminants.

Table 2 shows the effect of the metaphosphoric acid concentration on the recovery of BNFX, DNFX, ERFX and OFLX. Extraction

Table 1
Effect of organic solvent content in the extracting solvent on the recovery of ofloxacin, benofloxacin, danofloxacin and enrofloxacin from chicken muscle

Extracting solvent	Recovery (%)			
	OFLX	BNFX	DNFX	ERFX
0.2% MPA[a]	44.5	40.1	41.6	35.4
0.2% MPA–methanol (8:2)	67.1	59.6	60.7	56.5
0.2% MPA–methanol (7:3)	72.1	68.1	68.1	61.0
0.2% MPA–methanol (5:5)	69.5	67.3	66.4	63.8
0.2% MPA–acetonitrile (9:1)	69.5	63.6	61.7	59.3
0.2% MPA–acetonitrile (8:2)	74.6	71.2	70.8	70.0
0.2% MPA–acetonitrile (7:3)	83.3	83.7	81.1	82.2
0.2% MPA–acetonitrile (6:4)	83.1	80.1	82.3	83.0
0.2% MPA–acetonitrile (5:5)	79.8	77.3	75.8	80.3

Samples were spiked with 0.2 μg/g of each drug. Mean results of three replicates.
[a] Metaphosphoric acid.

with 0.1% metaphosphoric acid–acetonitrile (7:3, v/v) was not effective enough with regard to deproteinization. Based on the above experiments, 0.2% metaphosphoric acid–acetonitrile (7:3, v/v) was used as the extraction solvent.

Next, we tested the capacity of a commercial C_{18} cartridge to retain BNFX, DNFX, ERFX and OFLX. The drugs (2 μg each) were added to a chicken muscle extract which was prepared as described above, and the retention capacity for each drug on the different C_{18} cartridges was compared. The results are shown in Table 3. Generally, the drugs are strongly adsorbed on the C_{18} cartridges except for one cartridge, and part of the loaded compounds was not eluted. This is believed to be caused by the quinolones interacting with metal impurities and residual silanol in the packing material. However, when Bond Elut C_{18} (200 mg) was used for clean-up, the drugs slightly eluted (1–4%) from the cartridge. Therefore, Bond Elut C_{18} (500 mg) was used for clean-up. Fig. 3 shows chromatograms of chicken tissue extracts obtained under the established conditions. There were no interfering peaks on the HPLC chromatograms from both tissue samples.

3.3. Recovery

Linear calibration graphs were obtained from 0.1 to 10 ng (equivalent to 0.002–0.2 μg/g) for DNFX standard, 1 to 100 ng (equivalent to 0.02–

Table 2
Effect of concentration of metaphosphoric acid in the extracting solvent on the recovery of ofloxacin, benofloxacin, danofloxacin and enrofloxacin from chicken muscle

Extracting solvent	Recovery (%)			
	OFLX	BNFX	DNFX	ERFX
0.2% MPA–acetonitrile (7:3)	83.3	83.7	81.1	82.2
0.5% MPA–acetonitrile (7:3)	79.5	77.1	77.0	79.3
1.0% MPA–acetonitrile (7:3)	80.3	76.9	76.7	81.1
2.0% MPA–acetonitrile (7:3)	81.0	79.0	77.4	80.2

Details as in Table 1.

Table 3
Influence of extraction cartridges on the recovery of fluoroquinolone antibacterials from chicken muscle extract

Cartridge		Recovery (%)			
		OFLX	BNFX	DNFX	ERFX
Bond Elut C_{18}	(200 mg)	95.1	95.6	96.8	97.3
Baker C_{18}	(200 mg)	80.5	79.7	72.0	90.7
Toyo-Pak ODS	(300 mg)	30.5	27.0	25.7	45.7
Sep-Pak C_{18}	(400 mg)	98.9	96.7	95.0	94.9
Bond Elut C_{18}	(500 mg)	98.8	96.9	95.5	96.5
Baker C_{18}	(500 mg)	3.7	4.4	0.4	23.1
Easy Chromato C_{18}	(500 mg)	60.5	60.4	18.2	72.5

Average of results of three replicates. Samples were spiked with 0.4 μg/g of each drug. The drugs were added to the already extracted tissue.

2.0 μg/g) for BNFX, ERFX and OFLX standards. Table 4 summarizes the recoveries of the drugs from commercial samples of chicken muscle and liver fortified with 0.2 μg/g of each drug. Greater than 80% overall mean recoveries and 5% standard deviations were obtained with both samples. The detection limits of the method were 0.01 μg/g for OFLX, DNFX and ERFX and 0.02 μg/g for BNFX in chicken muscle and liver (see Fig. 3C and E).

3.4. Comparison of HPLC and bioassay

The microbiological assays tended to lack specificity. Therefore, these assays are not suitable for the identification of residual antibacterials. However, the microbiological assays perform very well as a qualitative method for the screening of remaining amounts of antibacterial substances. Therefore, the antibacterial activities of the quinolones were examined using *Bacillus*

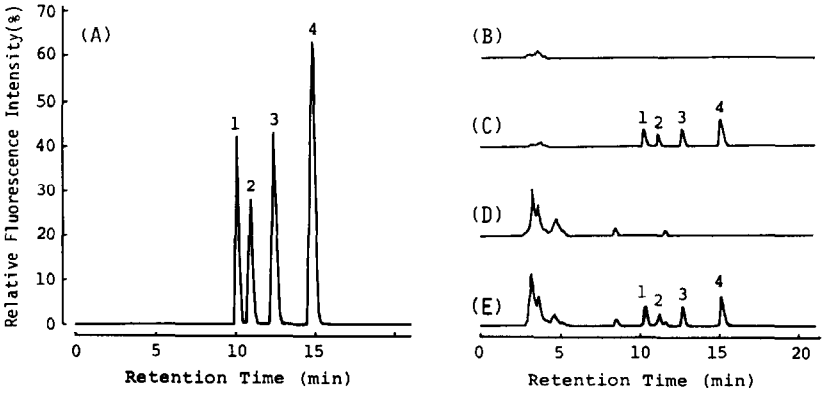

Fig. 3. Typical chromatograms of extracts of chicken tissues. (A) Standard mixture. Peaks: 1 = ofloxacin (10 ng); 2 = benofloxacin (20 ng); 3 = danofloxacin (1 ng); 4 = enrofloxacin (10 ng). (B) Chicken muscle extract. (C) Extract of chicken muscle fortified with 0.002 μg/g of danofloxacin, 0.02 μg/g of ofloxacin and enrofloxacin and 0.04 μg/g benofloxacin. (D) Chicken liver extract. (E) Extract of chicken liver fortified with 0.002 μg/g of danofloxacin, 0.02 μg/g of ofloxacin and enrofloxacin and 0.04 μg/g benofloxacin. LC conditions: mobile phase, 0.05 *M* phosphate buffer (pH 2.4)–acetonitrile (80:20, v/v) containing 2.5 m*M* 1-heptanesulfonic acid. Other conditions as in Fig. 2.

Table 4
Recoveries of ofloxacin, benofloxacin, danofloxacin and enrofloxacin from chicken muscle and liver

Sample	Recovery (mean ± S.D., $n = 5$) (%)			
	OFLX	BNFX	DNFX	ERFX
Intra-assay				
Chicken muscle	83.3 ± 1.9	83.7 ± 1.7	81.1 ± 1.1	82.2 ± 0.9
Chicken liver	89.6 ± 3.3	84.7 ± 3.4	82.8 ± 1.9	84.7 ± 1.5
Inter-assay				
Chicken muscle	84.6 ± 2.9	85.0 ± 2.9	81.5 ± 1.0	83.3 ± 2.3
Chicken liver	87.0 ± 4.2	85.2 ± 4.1	82.7 ± 1.6	85.1 ± 1.7

Samples were spiked with 0.2 μg/g each drug.

subtilis ATCC 6633, *Micrococcus luteus* ATCC 9341 and *Bacillus cereus* ATCC 11778 as test organisms. These bacteria are frequently used to test the presence of residual antibiotics in animal and meat. As shown in Table 5, the antibacterial activities of the four fluoroquinolones for the test organisms were extremely strong, and the sensitivity of the drug detection was sufficient.

Chicken muscle samples fortified with ERFX were then analyzed by the HPLC method and the bioassay method in which *Bacillus subtilis* ATCC 6633 was used as the test organism. The concentrations obtained with the two methods showed a linear correlation (Fig. 4). The equation of the fitted curve was $y = 1.04x - 0.02$ ($n = 21$; $r = 0.98$). Similar excellent correlations were

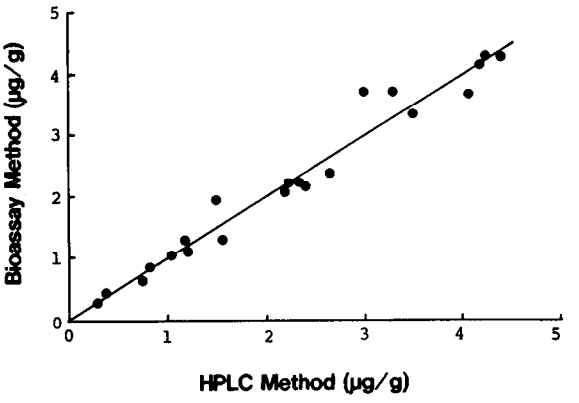

Fig. 4. Correlation between HPLC and bioassay methods for enrofloxacin in chicken muscle. Pregression equation: $y = 1.04x - 0.02$ ($r = 0.98$; $n = 21$).

Table 5
Antibacterial activities of fluoroquinolone antibacterials

Organism	Detection limit (μg/ml)					
	OFLX	BNFX	DNFX	ERFX	OXA[a]	NA[b]
B. subtilis ATCC 6633	0.20	0.39	0.10	0.20	1.56	12.5
B. cereus ATCC 11778	1.56	1.56	0.78	0.78	6.25	50
M. luteus ATCC 9341	12.5	25	6.25	12.5	>50	>50

Each drug was dissolved in acetonitrile or 0.02 *M* sodium hydroxide–acetonitrile (2:8, v/v) at a concentration of 200 μg/ml. These stock solutions were diluted with 0.05 *M* phosphate buffer (pH 7.0) before use.
[a] Oxolinic acid.
[b] Nalidixic acid.

obtained for chicken muscle samples fortified with BNFX, DNFX and OFLX, respectively.

Residual antibacterials presently causing food sanitation problem in Japan are the unchanged parent compounds administered to livestock. As mentioned above, most of the fluoroquinolones which have been taken orally are known to be distributed to each tissue as the unchanged parent compounds. The detection limits of the method were below 0.02 μg/g for each drug in chicken muscle and liver, and the time required for the analysis of one sample was less than one hour. Therefore, the method proposed here is useful for evaluation of the residues of the four fluoroquinolones remaining in chicken tissues.

4. Acknowledgements

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