Multiresidue Analysis of Fluoroquinolone Antibiotics in Chicken Tissue Using Automated Microdialysis–Liquid Chromatography

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Abstract

An efficient procedure for the simultaneous extraction and analysis of six fluoroquinolone (FQ) antibiotics is developed using an automated microdialysis–liquid chromatographic (LC) system. In this method, samples extracted from chicken liver and muscle are further purified by microdialysis, separated on an LC column, and the FQs detected by their fluorescence. Recoveries from fortified chicken liver and muscle samples are at least 70% with limits of quantitation (µg/kg) for the FQs in liver (and muscle) as follows: 0.3 (0.4) for danofloxacin, 0.8 (0.2) for desethylene ciprofloxacin, 2 (1) for norfloxacin, 2 (0.8) for enrofloxacin, 3 (1) for ciprofloxacin, and 5 (2) for sarafloxacin. Enrofloxacin and ciprofloxacin are determined in enrofloxacin-incurred chicken liver and muscle samples using this method.

Introduction

The use of fluoroquinolone (FQ) antibiotics in food animals has become a matter of growing concern as reports of microbial resistance in humans increase (1). Although two FQs, sarafloxacin (SAR) and enrofloxacin (ENRO), had been approved for use in chickens in the U.S., approval for SAR has recently been withdrawn. Additional FQs are approved in Europe (2). The U.S. Food and Drug Administration (FDA) has prohibited extra-label use of FQs (3); however, the possibility of further FQ approvals and the potential for misuse warrants the development of efficient multiresidue methods for the determination of these antibiotics in chicken tissues.

Several methods have been developed for the analysis of FQs in chicken tissue (2,4–12). These methods typically use either solid-phase extraction (SPE) for sample cleanup or rely on liquid–liquid extraction. One method uses immunosorbents (11), and another uses microdialysis (10). Most methods then use high-performance liquid chromatography (HPLC) for the separation of FQs, followed by fluorescence detection. One method uses mass spectrometry

(MS)–MS (5), and another uses terbium-sensitized luminescence (9). Difficulties encountered with a number of these methods include a limited analysis of only one or two FQs, relatively low recovery, use of halogenated solvent, or inability to separate all FQs examined with one set of chromatographic conditions. The goal of this work was to develop a procedure that would allow for the simultaneous analysis of ciprofloxacin (CIP), desethylene CIP (DCIP), norfloxacin (NOR), danofloxacin (DANO), ENRO, and SAR from chicken tissue with good recoveries, good reproducibility, and without the use of halogenated solvents.

An automated microdialysis system has considerable potential for use in veterinary drug residue analysis in tissues. With such a system, soluble high-molecular-weight impurities (> 15 kilodaltons) can be efficiently removed by dialysis from an extracted tissue sample, thus providing a cleaner sample. Microdialysis thus provides an alternative to SPE. This approach has been used, for example, for the analysis of flumequine and oxolinic acid in salmon muscle (13); nitrofurans in milk, meat, and eggs (14); and amoxicillin and cefadroxil in bovine serum and muscle (15). Cohen et al. analyzed three quinolones in chicken liver using this technique (10); however, their chromatographic conditions were not suitable for the six FQs in this study and their method utilized double injections of a sample into the microdialysis system, thus necessitating a longer run time. In this study, the basic microdialysis approach was modified for the analysis of a larger group of FQs (six) in more than one tissue (chicken muscle as well as liver). This ultimately involved different chromatographic, microdialysis, and sample preparation conditions. Our recent work that analyzed these six FQs in eggs provided a reasonable starting point for this investigation (16).

Experimental

Chemicals and reagents

DCIP (89.8%), CIP, and ENRO (99.9%) were obtained from Bayer (Kansas City, MO), DANO from Pfizer (Groton, CT), SAR (88.5%) from Abbott (North Chicago, IL), and NOR from Sigma (St. Louis, MO). Acetonitrile (MeCN), methanol (MeOH), hexane,

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and glacial acetic acid were all HPLC grade. Ammonium hydroxide, anhydrous diethyl ether, sodium hydrogen phosphate heptahydrate, sodium dihydrogen phosphate monohydrate, Triton X-100, sodium hydroxide, and sodium chloride were reagent grade. Deionized water prepared with a Barnstead (Dubuque, IA), E-pure system was used to prepare all aqueous solutions. All solutions prepared for HPLC were filtered through a 0.45-μm nylon filter before use.

Standard solutions

Stock solutions (100 μg/mL) were prepared for each of the six FQs by the addition of the appropriate amount of standard reference material (approximately 5 mg, depending on purity) to a 50-mL actinic volumetric flask. The samples were dissolved and diluted to volume in 0.03M NaOH. These solutions were stored refrigerated and prepared fresh every six months. A fortification solution was prepared by the addition of 1.00 mL of the corresponding stock solutions NOR, CIP, and SAR, a 1:2 dilution of stock solution ENRO, and a 1:10 dilution of stock solutions DCIP and DANO to a 50-mL actinic volumetric flask. The solution was diluted to volume with 0.1M phosphate at pH 9 (buffer A). The fortification solution was stored refrigerated and prepared fresh monthly. Standard curve samples were prepared daily by the dilution of the fortification solution with buffer A to give four solutions encompassing the fortification concentration.

Fortification of tissue samples

Control (antibiotic free) chicken liver and chicken breast (Bell and Evans Brand, Fredericksburg, PA) were purchased fresh, cut into small pieces, and ground into a homogeneous sample using a food processor (Cuisinart mini-prep, Cuisinart, E. Windsor, NJ). This material was then kept frozen at –80°C until use. For fortification, samples of the tissue (1.0 g) were placed in 50-mL disposable centrifuge tubes and an appropriate amount of fortification solution was added to give the desired concentration of FQ. The fortification solution was added to the tissue 1 h before extraction, as will be described.

Incurred tissue samples

Eight chickens were treated with an oral dose of ENRO solution (11 mg/day) for 7 days. Two birds were sacrificed on days 5, 7, 8 (first-day postdose), and 10. Liver and breast muscle samples were harvested and then shipped in dry ice and stored at –80°C. Liver or breast muscle samples from two birds sacrificed at the same time were combined, partially thawed, cut up, and homogenized in a food processor. The homogenized samples were then stored at –80°C. An initial extraction and analysis of a single portion of each sample were performed to determine the approximate ENRO levels. Because of the high level of ENRO incorporation observed, incurred tissue samples were then diluted with the corresponding control tissue to an appropriate level and homogenized with a food processor prior to extraction and analysis.

Extraction of FQs

Tissue samples (1.0 g) in 50-mL centrifuge tubes (fortified or incurred) were placed on a mixer (IKA-VIBRAX-VXR, Janke and Kunkel, Cincinnati, OH) for 30 min and then stored in reduced light for an additional 30 min at room temperature. In a fortification experiment, additional controls containing only tissue and a fortification mix (no tissue) were utilized. In an incurred experiment, these controls as well as a fortified tissue control at the expected level were added. Samples were homogenized (Janke and Kunkel Ultra-turrax T-25) with MeCN (3 mL) and concentrated ammonium hydroxide (0.25 mL). The tubes were centrifuged (5 min × 1241 g for liver and 2205 × g for muscle) and the supernatants decanted into fresh 50-mL centrifuge tubes. The pellet was re-extracted with MeCN–NH$_4$OH. Hexane (3 mL), diethyl ether (3 mL), and 1M NaCl (0.25 mL) were added to the combined supernatants. The tubes were mixed with a vortex mixer (15 s) and the upper layer discarded by pipet. The lower layer was evaporated at 40°C under a stream of nitrogen. Additional MeCN was added (1–1.5 mL) to facilitate evaporation. The residues were redissolved in 10.0 mL of buffer A, sonicated for 1 min, and centrifuged (5 min × 1241 g). The resultant solutions were then filtered through a 25-mm, 0.45-μm nylon filter into
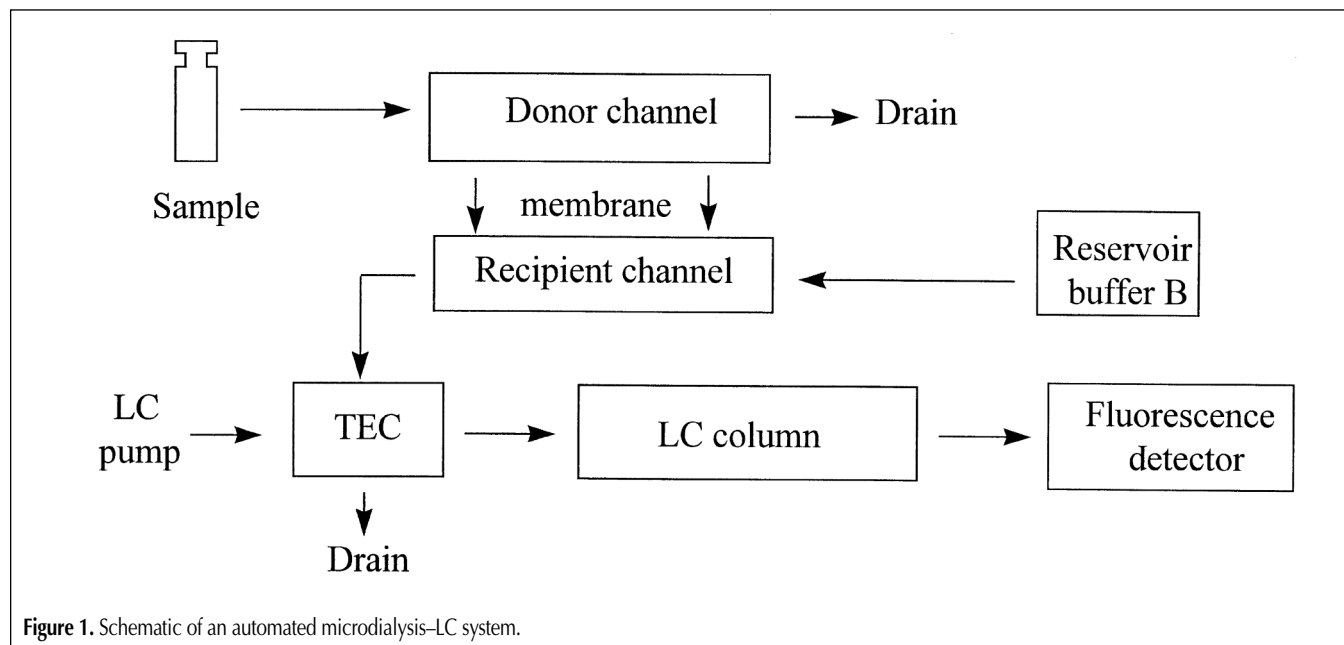

Figure 1. Schematic of an automated microdialysis–LC system.

amber autosampler vials for analysis.

Apparatus

Automated microdialysis was accomplished using the Gilson (Middleton, WI) ASTED XL system equipped with two 401C dilutors, a 370-µL donor channel/650-µL recipient channel dialysis block, a 15-kilodalton cellulose dialysis membrane, and Keypad Version 1.00 software. Prior to initial use, the dialysis membrane was soaked in 1% disodium ethylenediamine tetraacetic acid and then rinsed with water. The ASTED and a Jasco (Easton, MD) FP-1520 fluorescence detector were interfaced via a Hewlett-Packard (HP) 35900E A/D converter to HP Chemstation software controlling a HP 1100 quaternary pump with an online degasser. A Chrontrol (San Diego, CA) XT timer controlled shutdown of the pumps and the ASTED.

Microdialysis

The following sequence was used (Figure 1). Prior to the injection of each sample, the trace enrichment cartridge (TEC, Hypersil C18, 5.8 × 4.6 mm, 10 µ, Keystone Scientific, Bellefonte, PA) was washed with MeCN–H$_2$O (1:1, 970 µL) and conditioned with a 0.02M phosphate buffer at pH 5 (buffer B, 500 µL). A portion of the sample (370 µL) was then injected into the donor channel of the microdialysis block and allowed to equilibrate (5 min). Buffer B was then pulsed through the recipient channel (2 × 650 µL) and the eluate loaded onto the TEC. The TEC was backflushed with buffer B (970 µL) onto the liquid chromatography (LC) column, at which time data acquisition began. The ASTED injection port was rinsed with water (970 µL), the needle was rinsed with water (970 µL inside, 970 µL outside), the dialysis block donor channel was washed with 0.01% Triton X-100 (3700 µL), and the recipient channel washed with buffer B (2700 µL). The entire process (including chromatography, 30 min) took approximately 45 min; however, the ASTED began a new sample while chromatography proceeded. Thus, samples were run every 30 min.

Chromatographic conditions

The LUNA phenylhexyl HPLC column (250 × 4.6 mm, 5 µ) was obtained from Phenomenex (Torrance, CA). All chromatographic gradients utilized MeCN–MeOH (1:1) as the organic phase and 2% acetic acid (pH 3) with ammonium hydroxide as the aqueous phase. Mobile phase solutions were prepared fresh daily. The chromatographic gradient for liver samples was 20% organic (10 min), 20–35% organic (8 min), 35% organic (2 min), 35–20% organic (2 min), and 20% organic (2 min). The chromatographic

gradient for muscle samples was 16% organic (5 min), 16–32% organic (13 min), 32% organic (4 min), 32–16% organic (2 min), and 16% organic (2 min). The column was maintained at 30°C with a column heater, and the flow rate was 1 mL/min. The fluorescence detector was set at a 278-nm excitation wavelength and a 440-nm emission wavelength. Quantitation was achieved with an external standard curve generated by the ASTED-LC and measurement of the peak height.

Results and Discussion

The first priority was to establish an effective method for the extraction of FQs from chicken tissue. Several solvent combinations were compared in their ability to extract FQs from chicken liver, including MeCN–NH$_3$, 1% acetic acid–MeOH, and 1% acetic acid–MeCN. The Holtzapple approach involving extraction with 0.1M NaOH, neutralization with phosphoric acid, and dilution with phosphate-buffered saline (pH 7)–10% MeOH was also investigated (11). Ammoniacal MeCN, the extraction combination used with eggs (16), produced comparable or better recoveries than the other combinations, generally with a lower level of matrix contaminants. Extracts produced were defatted using ether–hexane. Evaporation of the lower MeCN layer gave a residue that was dissolved in buffer A. Because chicken muscle and liver generally produced more problematic matrix contaminants than we had experienced in our previous work with eggs, the procedure was modified to take the final evaporated residue up to 10 mL in buffer A rather than 2 mL. Increasing the gain on the fluorescence detector readily compensated for this increased dilution, which was designed to prolong the lifetime of the dialysis membrane. Liver extracts appeared to provide longer membrane life than muscle extracts, possibly because of muscle protein precipitation during dialysis.

The samples produced using this method still contained tissue matrix peaks that interfered with the FQ analysis. The use of a lower molecular weight cutoff membrane to eliminate these conflicting materials was tested by filtration of the sample through an Amicon centricon centrifugal filter (Millipore Corporation, Bedford, MA) (MW cutoff of 3000). The resultant filtrate still contained the same level of matrix interference. Resolution of this difficulty was next attempted by changing chromatographic conditions. Solvent and gradient variations using a LUNA C18(2) column, the column used previously for the egg study (16), were unsuccessful in separating tissue matrix peaks from those of the FQs. However, a change of column chemistry was successful. A

Table I. FQ Recovery from Fortified Chicken Liver

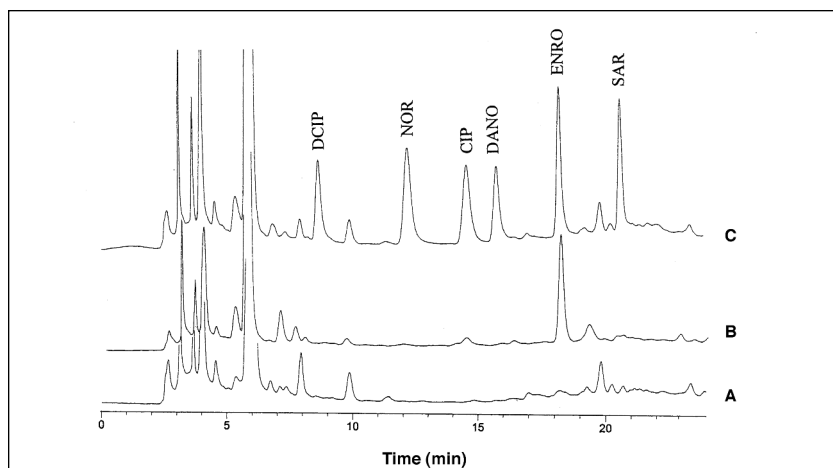| Fortification level (µg/kg)	%Recovery* (RSD)			Fortification level (µg/kg)	%Recovery* (RSD)	Fortification level (µg/kg)	%Recovery* (RSD)	
	NOR	CIP	SAR		ENRO		DCIP	DANO
100	75.1 (4.0)	74.7 (3.6)	78.3 (4.2)	50	87.5 (6.6)	10	77.4 (6.8)	90.6 (8.8)
50	92.0 (8.3)	91.0 (8.6)	92.0 (6.6)	25	111 (7.3)	5	96.5 (12.7)	114 (11.6)
20	82.8 (7.3)	78.7 (7.1)	65.7 (5.6)	10	90.4 (8.7)	2	80.8 (12.2)	98.4 (11.0)

* n = 6.

Figure 2. Liquid chromatograms of an extract of (A) control chicken liver; (B) ENRO-incurred chicken liver, day 7 (x 0.6); and (C) fortified chicken liver (50 µg/kg NOR, CIP, and SAR; 25 µg/kg ENRO; and 5 µg/kg DCIP and DANO).
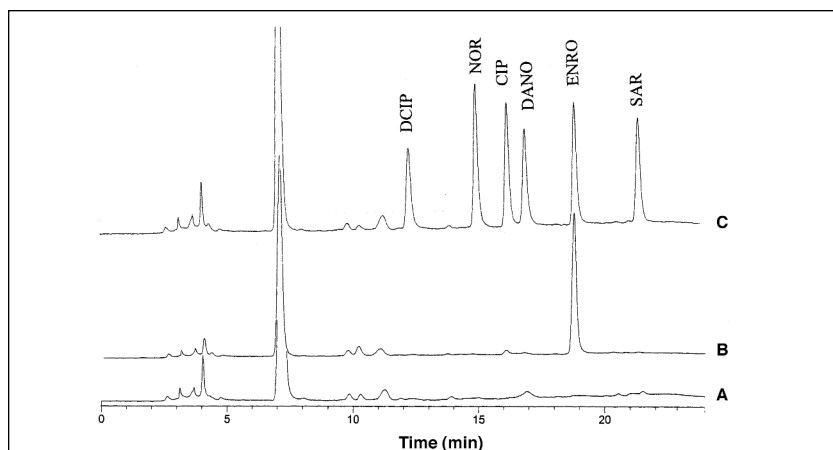

Figure 3. Liquid chromatograms of an extract of (A) control chicken muscle; (B) ENRO-incurred chicken muscle, day 7 (x 0.6); and (C) fortified chicken muscle (50 µg/kg NOR, CIP, and SAR; 25 µg/kg ENRO; and 5 µg/kg DCIP and DANO).

LUNA phenylhexyl column with a 20–35% MeOH–MeCN (1:1) gradient in 2% acetic acid (pH 3) provided excellent separation of the six FQs from the interference of the matrix peaks in chicken liver.

After the establishment of an effective extraction and analysis method, fortified chicken liver was investigated. Chicken liver was fortified at three different levels and the FQs analyzed. The results (Table I) showed good recoveries and relative standard deviations (RSDs). Sample chromatograms are shown in Figures 2A and 2C. The limits of quantitation (µg/kg) in liver were determined by ten times the root mean square of the noise divided by the slope of the standard curve and are as follows: 0.3 for DANO, 0.8 for DCIP, 2 for NOR and ENRO, 3 for CIP, and 5 for SAR. The standard curve was observed to be linear ($R^2 > 0.999$) from 0.2 to 20 µg/mL SAR.

ENRO-incurred chicken liver samples were also investigated (the results are shown in Table II and a sample chromatogram in Figure 2B). The "corrected" values in the last two columns of Table II represent the actual levels in the original sample after taking the dilution into account. The levels of ENRO remained high during days 5 and 7 of dosing and the first-day postdose, then dropped precipitously by the third-day postdose, keeping with what would be expected for the required 2-day withdrawal period for the use of ENRO in chickens (17). The levels of ENRO found in these liver samples were much higher than those found previously in eggs, and it decreased postdose more rapidly (16). It is interesting to note that the ENRO metabolite CIP was also detected and quantitated in these samples using this

Table II. FQ Levels from Incurred Chicken Liver

Day	Measured ENRO* (µg/kg) (RSD)	Measured CIP* (µg/kg) (RSD)	Dilution	Corrected ENRO (µg/kg)	Corrected CIP (µg/kg)
5	30.8 (7.1)	5.10 (10.2)	1:200	6160	1020
7	33.4 (12.6)	5.78 (11.8)	1:200	6680	1160
8	34.8 (14.4)	14.2 (12.2)	1:100	3480	1420
10	28.4 (5.5)	10.2 (4.4)	1:5	142	51.0

* $n = 6$.

Table III. FQ Recovery from Fortified Chicken Muscle

Fortification level (µg/kg)	%Recovery* (RSD)			Fortification level (µg/kg)	%Recovery* (RSD)	Fortification level (µg/kg)	%Recovery* (RSD)	
	NOR	CIP	SAR		ENRO		DCIP	DANO
100	70.6 (10.5)	71.8 (10.7)	77.6 (9.4)	50	80.7 (11.3)	10	72.7 (13.4)	83.0 (13.5)
50	70.1 (12.7)	71.6 (12.6)	76.1 (8.8)	25	80.9 (10.5)	5	72.4 (16.6)	82.2 (13.3)
20	83.3 (6.2)	83.9 (6.3)	85.4 (4.4)	10	88.7 (7.4)	2	85.4 (9.3)	90.7 (9.4)

* $n = 6$.

method. The presence of CIP in ENRO-incurred samples has been noted previously in our work with eggs (16) and that of Gorla et al. (18).

For the analysis of chicken muscle, a slightly modified gradient (16–32%) with the phenylhexyl column was used to ensure the resolution of the FQs from the matrix peaks. The analysis of chicken muscle fortified at three levels is shown in Table III. Again, good recoveries and RSDs were obtained. Sample chromatograms are shown in Figures 3A and 3C. The limits of quantitation (µg/kg) in muscle were calculated in the same way as for liver: 0.4 for DANO, 0.2 for DCIP, 1 for NOR and CIP, 0.8 for ENRO, and 2 for SAR.

Results from a study of incurred chicken muscle are shown in Table IV. The "corrected" values are calculated by taking sample dilution into account. Levels of ENRO detected in muscle are much lower than liver, but follow the same pattern by dropping off significantly as of 3-days postdose. The level of ENRO in these muscle samples is less than that found in eggs with a more rapid decrease postdose (16). The metabolite CIP is again detected using this approach. A sample chromatogram is shown in Figure 3B.

One potential complication in the use of the ASTED system is the possibility for sample carryover. Carryover was observed with all six FQs used in this study, although it was particularly noticeable with DCIP and DANO. Attempts to eliminate carryover by employing additional washing of the membrane using pH 3 phosphate buffer in place of buffer B or the incorporation of 5% MeOH into the buffer B solution were not completely successful. The ASTED was most easily used with diluted samples in which extensive flushing of the system to completely remove FQ background was not required. For cases in which a low level of FQ background would not be problematic (such as the routine monitoring of samples at regulatory tolerance levels), this system would be very effective.

Conclusion

The automated microdialysis–LC system utilized in this work provided an efficient method for the simultaneous analysis of six FQs. The FQs were extracted with good recoveries and reproducibility and with little organic solvent. High sensitivity was achieved because of low matrix interference and the use of the fluorescence detector. The automated method is rapid and capable of analyzing up to 48 samples in 24 h.

Acknowledgments

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Table IV. FQ Levels from Incurred Chicken Muscle

Day	Measured ENRO* (µg/kg) (RSD)	Measured CIP* (µg/kg) (RSD)	Dilution	Corrected ENRO (µg/kg)	Corrected CIP (µg/kg)
5	52.2 (13.1)	2.63 (14.6)	1:25	1300	65.8
7	40.8 (9.8)	1.81 (16.1)	1:40	1630	72.4
8	34.7 (9.5)	2.08 (12.1)	1:40	1390	83.2
10	19.1 (8.2)	0.48 (27)	1:2	38.2	0.97

* $n = 6$.

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