



Analytical Methods

Development of a single-step precipitation cleanup method for the determination of enrofloxacin, ciprofloxacin, and danofloxacin in porcine plasma

Jeong-Heui Choi^a, M.I.R. Mamun^b, A.M. Abd El-Aty^{c,*}, Jong-Hyouk Park^a, Eun-Ho Shin^a, Ji Yeon Park^a, Soon-Kil Cho^d, Sung Chul Shin^e, Kang Bong Lee^f, Jae-Han Shim^{a,*}^a Natural Products Chemistry Laboratory, College of Agriculture and Life Science, Chonnam National University, 77 Yongbong-ro, Buk-gu, Gwangju 500-757, Republic of Korea^b Department of Chemistry, University of Dhaka, Dhaka 1000, Bangladesh^c Department of Pharmacology, Faculty of Veterinary Medicine, Cairo University, 12211 Giza, Egypt^d National Agricultural Products Quality Management Service, Products Safety Inspection Laboratory, Gwangsan-gu, Gwangju 506-824, Republic of Korea^e Department of Chemistry and Research Institute of Life Science, Gyeongsang National University, Jinju 660-701, Republic of Korea^f Health/Functional Food Standardization Division, Korea Food and Drug Administration, 194, Tongilro, Eunpyeong-gu, Seoul 122-704, Republic of Korea

ARTICLE INFO

Article history:

Received 27 July 2010

Received in revised form 30 January 2011

Accepted 6 February 2011

Available online 26 February 2011

Keywords:

Fluoroquinolone
Porcine plasma
Precipitation cleanup
HPLC-FLD
Activated charcoal
Withdrawal time

ABSTRACT

In this study, we describe a newly developed and simple analytical method using high-performance liquid chromatography coupled to fluorescence detector (HPLC-FLD) for the determination of enrofloxacin, ciprofloxacin, and danofloxacin in porcine plasma. A single-step sample preparation, including extraction with acidic acetonitrile, coagulation with ammonium acetate, and centrifugation made possible the direct analysis of plasma samples without the need for any further cleanup procedure. The developed method was validated with specificity, linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy, and precision. All results were fully adequate. In the study using porcine plasma incurring enrofloxacin, the developed method proved capable of quantifying concentrations below its maximum residue limit, and its time-course residues were excreted within the 10-day withdrawal time.

© 2011 Elsevier Ltd. All rights reserved.

1. Introduction

Population growth, urbanisation, income growth in developing countries, and changes in lifestyle and dietary habits are all factors helping to accelerate a massive global increase in demand for foodstuffs of animal origin (Bruinsma, 2003, chap. 5; Delgado, Rosegrant, Steinfeld, Ehui, & Courbois, 1999, chap. 4). Globally, livestock production currently accounts for some 40% of the gross value of agricultural production, and global meat production has more than trebled since 1960 (Bruinsma, 2003; Speedy, 2003). Intensive farming, particularly in the porcine and poultry industries, has been continually expanding to fulfill an increasing public demand for livestock products, despite the great potential for infectious disease inherent to these industries. Unfortunately, intensive farming has given rise to unsanitary living conditions for food-producing animals, and thus to an increasingly heavy use of veterinary drugs. Fluoroquinolones (FQs), for example, are currently in wide usage as therapeutic and prophylactic synthetic antimicrobial agents in

animal husbandry and aquaculture, as well as in humans, due to their broad-spectrum activity against both Gram-positive and Gram-negative bacteria (Ashwin et al., 2009; Huet et al., 2006). The widespread use of FQs in food-producing animals has resulted in a potential risk of residues in foodstuffs of animal origin and the development of resistant bacterial strains (Huet et al., 2006). As such, these issues that undermine public health may be an inevitable consequence of intensive farming, as well as an increase in the consumption of livestock products and the large-scale use of antimicrobials to treat readily transmissible infections caused by intensive farming.

In the Republic of Korea, the FQs currently licensed for the prevention and treatment of porcine diseases are enrofloxacin (ENRO) and danofloxacin (DANO) (Notification No. 2007-25, 2008). To ensure food safety, maximum residue limits (MRLs) and withdrawal times of ENRO and DANO were established by the Korea Food and Drug Administration (KFDA) and the National Veterinary Research and Quarantine Service (NVRQS), respectively (Table 1). In an effort to ensure overall food safety for consumers, the KFDA mandated that the MRLs of byproducts, including the guts, bones, head, tail, legs, skin, blood, and other edible parts of animals intended for human consumption should be applied to that of the muscles of related animals (Notification No. 2009-24, 2009). The Republic of

* Corresponding authors. Tel.: +82 62 530 2135; fax: +82 62 530 0219 (J.-H. Shim), tel.: +20 2 27548926; fax: +20 2 35725240 (A.M. Abd El-Aty).

E-mail addresses: abdlaty44@hotmail.com (A.M. Abd El-Aty), jhshim@chonnam.ac.kr (J.-H. Shim).

Table 1
Maximum residue limits (MRLs)^a and guidelines^b for the safe use of ENRO and DANO.

Compound	Species	MRL value (mg/kg or mg/L)				Guideline for safe use			
		Muscle ^c	Liver	Fat	Kidney	Species	Dose	Administration	Withdrawal time (day)
ENRO	Cattle	0.1	0.3	0.1	0.2	Cattle ^e	5 mg/kg/day	Intramuscular or hypodermic injection	20
	Pig	0.1	0.2	0.1	0.3				
	Sheep	0.1	0.3	0.1	0.2				
	Goat	0.1	0.3	0.1	0.2				
	Rabbit	0.1	0.2	0.1	0.3	Pig	5 mg/kg/day	Intramuscular or hypodermic injection	20
	Poultry	0.1	0.2	0.1	0.3				
	Fish	0.1							
	Eggs	ND ^d					150 g/t feed	Oral	10
	Milk	0.05							
	Crustacean	0.1							
DANO	Cattle	0.2	0.1	0.1	0.4	Cattle ^e	1.25 mg/kg/day	Intramuscular or hypodermic injection	5
	Pig	0.1	0.05	0.1	0.2				
	Poultry	0.2	0.4	0.1	0.4	Pig	1.25 mg/kg/day	Intramuscular or hypodermic injection	25
	Milk	0.03							
						Chicken and duck ^f	5 mg/kg/day	Oral with water	5

^a Established by the Food and Drug Administration, Republic of Korea (Notification No. 2009-24).

^b Established by the National Veterinary Research and Quarantine Service, Republic of Korea (Notification No. 2007-25).

^c Additional MRL for guts, bones, head, tail, legs, skin, blood, and other edible parts of related animal.

^d Non-detection.

^e Excluding when milking.

^f Excluding when laying.

Korea, along with other countries in Southeast Asia, have traditionally utilised a variety of byproducts (legs, blood, guts, etc.) as food supplies. In particular, blood is frequently consumed by Koreans due to its high nutritional value; additionally, blood is distributed relatively rapidly throughout markets, due to its short storage time. Thus, it will be important to fundamentally implement the above-mentioned recommendations not only to ascertain the proper usage of veterinary drugs, and to monitor their residues regularly via a simple, rapid, and standardised analytical method, but also to determine the appropriate withdrawal time so that blood can be safely used as a food product or component.

There are some simple and cost-effective methods for the screening of FQs, including the microbial inhibition-based method (Ashwin et al., 2009), enzyme-linked immunosorbent assays (Huet et al., 2006), and the spectrofluorometric method (Chen & Schneider, 2003). However, these screening methods are appropriate only for the exposure of veterinary drug residue violations above a given tolerance level; they have questionable utility as a quantitative method, due to potential variability in control samples (Chen & Schneider, 2003). Although high-performance liquid chromatographic (HPLC) techniques coupled to a universal ultraviolet or fluorescence detector (FLD) can be effectively employed for quantitative analysis, these techniques also frequently necessitated the use of solid-phase extraction (SPE) as a cleanup procedure (Christodoulou, Samanidou, & Papadoyannis, 2007; Hermo, Nemetlu, Barbosa, & Barrón, 2010; Herrera-Herrera, Hernández-Borges, & Rodríguez-Delgado, 2009).

This paper describes the development, validation, and application of a HPLC-FLD analytical method, in which sample preparations are simplified with precipitation cleanup and centrifugation to detect and quantify enrofloxacin (ENRO), ciprofloxacin (CIP), and danofloxacin (DANO) in porcine plasma. A commercial ENRO product developed for oral treatment was administered, and its residues were quantified from incurred porcine plasma via the method described herein. Additionally, the effect of activated charcoal on ENRO residues was evaluated via coadministration with ENRO, and the residual duration in blood was compared with the withdrawal time of ENRO established by the NVRQS.

2. Experimental

2.1. Chemicals and reagents

Pure standard ENRO (purity, 99.9%) and DANO (98.4%) were purchased from Riedel-de Haën (Sigma-Aldrich GmbH, Seelze, Germany). A CIP standard (purity, 98.0%) was generously provided by the CJ Cheiljedang Corporation, Seoul, Republic of Korea. Analytical-grade sodium sulphate monobasic, phosphoric acid, and ammonium sulphate were acquired from Sigma-Aldrich (Missouri, USA). Methanol and acetonitrile were of HPLC grade and were supplied by Merck KGaA (Darmstadt, Germany). All other chemicals and reagents used throughout the study were of analytical grade, unless stated otherwise.

2.2. Standard solutions

Stock solutions of ENRO, DANO, and CIP were prepared in a mixture of 0.04 M phosphate buffer (pH 3.0) and methanol (25:75, v/v) at a concentration of 100 µg/mL. Intermediate standard solutions were prepared via serial dilutions of the stock solutions with the same solvent at concentrations of 8, 4, and 2 µg/mL. Working standard solutions were prepared in blank plasma extracts at seven different concentrations (0.025, 0.05, 0.1, 0.2, 0.25, 0.5, and at 1 µg/mL for ENRO and CIP, and (0.005, 0.01, 0.02, 0.04, 0.05, 0.1, and 0.2 µg/mL) for DANO; these solutions were subsequently utilised for calibration curves. Standard solutions were stored at -24 °C in amber bottles pending analysis.

2.3. Blank and incurred porcine plasma

Twenty fattening pigs (aged 6 weeks and weighing 56 kg) without any previous history of FQ treatment were employed in the experiments conducted in this study. The animals were fed on antibiotic-free feed and water *ad libitum*. The pigs were allocated into two groups. The first group was treated with ENRO and the second group received ENRO coupled with activated charcoal.

Enril Capsule (CTCbio Co., Seoul, Republic of Korea) was mixed with feed (Pig solution I, Cargill Incorporated, Minnesota, USA) at the manufacturer's recommended dosage of active ingredient (a.i.) 150 g/t feed for seven consecutive days. Charcoal activated for 12 h in an oven at 130 °C was mixed at 1% (w/w) with feed previously homogenised with Enril Capsule at the recommended dosage. Two pigs were slaughtered on days 1, 2, 3, 5, and 7 after the final administration of the drug, and the incurred porcine plasma was prepared and stored at –24 °C pending analysis. Animal welfare and experimental procedures were conducted in accordance with the guidelines of the Ethical Committee (Chonnam National University, Republic of Korea).

Blank blood samples were exsanguinated from the jugular vein prior to drug administration, and all plasma samples were separated via 10 min of centrifugation at 450g with heparin.

2.4. Sample preparation

Exactly 200 µL of plasma sample was withdrawn in a microcentrifuge tube, to which 0.02 g of ammonium sulphate and 800 µL of 0.04 M phosphate buffer (pH 1.5)–acetonitrile (8:2, v/v) were added. The mixture was vortex-mixed for at least 1 min and maintained for 10 min in a freezer at –24 °C followed by 5 min of centrifugation at 2200g. The supernatant was decanted into another microcentrifuge tube and recentrifuged as before after adding an identical amount of ammonium sulphate (0.02 g). The final supernatant was filtered through a membrane filter polyvinylidene fluoride (PVDF), (0.20 µm, Woongki Science Co., Ltd., Seoul, Republic of Korea) and subsequently injected into an HPLC-FLD system. Fortified plasma samples were prepared by adding 50 µL of mixed working standard solutions to the samples, which resulted in final concentrations that ranged from 0.05 to 0.2 µg/mL. The fortified samples were left to stand for 30 min prior to extraction in order to allow the spike solution to penetrate into the matrix, then the extraction procedure was performed as described above.

2.5. High-performance liquid chromatography

The HPLC system employed in this study consisted of a Shimadzu (Kyoto, Japan) Model SCL-10A VP system controller, a model LC-6 AD binary pump, a CTO-20A column oven, a Spectrofluorometric detector (RF-10 A) and the CLASS VP computing program. Analytical separation of the FQs from plasma extracts was conducted on an Apollo C₁₈ column (250 × 4.6 mm i.d., 5 µm, Alltech, Illinois, USA), protected by an Apollo C₁₈ guard column (4.6 × 7.5 mm, 5 µm, Alltech, Illinois, USA). These columns were maintained in an oven at 40 °C. The mobile phase of 0.04 M phosphate buffer (pH 3.0)–acetonitrile (82:18, v/v) was flowed at a rate of 1 mL/min. The FQs were detected using a fluorescence detector at an excitation wavelength of 278 nm and an emission wavelength of 450 nm.

3. Results and discussion

3.1. Chromatographic conditions

It has been generally recognised that many fluoroquinolones, including ENRO, DANO, and CIP, generate significant peak tailing on reversed-phase (RP) chromatography (Horie, Saito, Nose, & Nakazawa, 1994; Vybíralová, Nobilis, Zoulova, Květina, & Petr, 2005). Residual silanol groups and metal impurities in column packing materials constitute key factors causing tailing in RP chromatography (Horie et al., 1994; Kimata, Tanaka, & Araki, 1992; Ohhira, Ohmura, & Hanai, 1989). A variety of efforts have been made to minimise the secondary interaction of free silanol groups

with polar FQs, including the use of end-capped columns, inactivation of the active residual silanol groups with base-competing reagents (triethylamine, tetrabutylammonium acetate, etc.), the formation of ion pairs with the protonated analytes by ion-pairing reagents (heptanesulphonate, sodium dodecyl sulphate, etc.), and the use of an acidic mobile phase with citric and phosphoric acids in order to attenuate silanol ionisation (Gigosos et al., 2000; Vybíralová et al., 2005).

In this study, the pH and the organic modifier content of the mobile phase were evaluated on a pure silica-based and end-capped Apollo C₁₈ column for peak sharpness and separation of the analytes; the results are shown in Fig. 1. In the beginning, 0.2 M formic acid (pH 3.5) was mixed with acetonitrile at 80:20 (v/v). However, peak tailing and low sensitivity were shown in the chromatogram. In order to overcome the problem of poor resolution, 0.04 M phosphate buffer (pH 3.5) was mixed with acetonitrile at 80:20 (v/v), resulting in a considerable increase in the peak symmetry and sharpness of the three FQs. The pH 3.0 of the 0.04 M

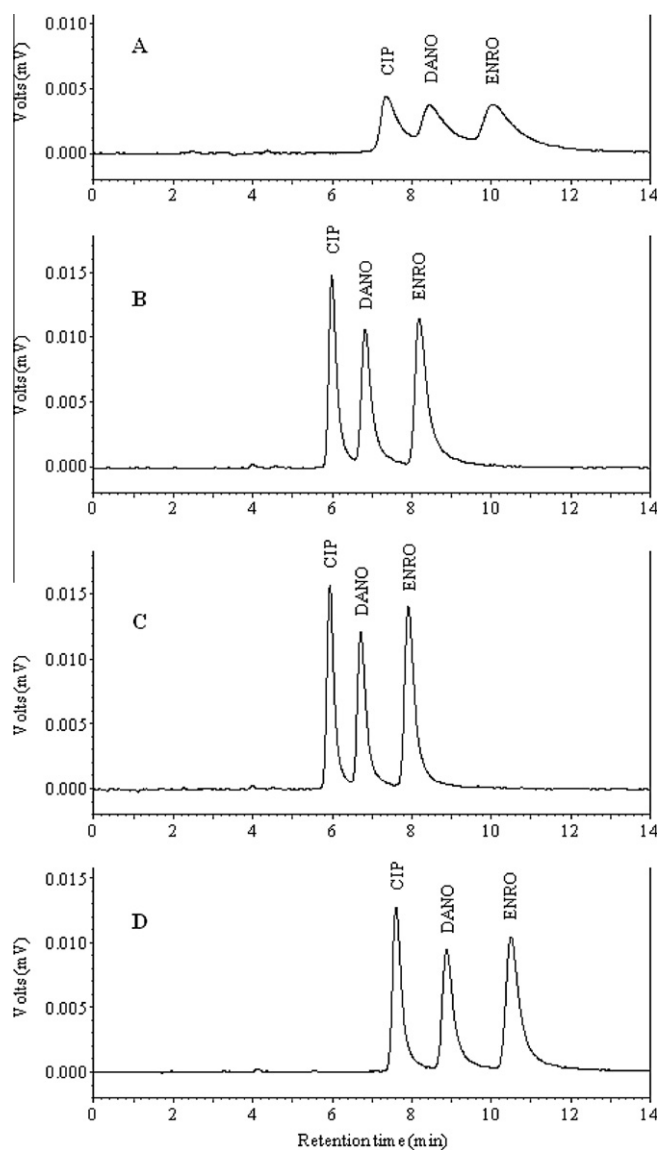


Fig. 1. Optimisation of peak sharpness and separation of three FQs on an Apollo C₁₈ with different mobile phases: (A) 0.2 M formic acid (pH 3.5)–acetonitrile (80:20, v/v); (B) 0.04 M phosphate buffer (pH 3.5)–acetonitrile (80:20, v/v); (C) 0.04 M phosphate buffer (pH 3.0)–acetonitrile (80:20, v/v); (D) 0.04 M phosphate buffer (pH 3.0)–acetonitrile (82:18, v/v).

phosphate buffer resulted in a slight increase in peak height; however, excellent peak separation was achieved when the volume of acetonitrile was decreased [0.04 M phosphate buffer (pH 3.0)–acetonitrile (82:18, v/v)], despite a reduction in the peak height (Fig. 1). Based on the above experiments, optimal peak shape and separation of the FQs were achieved with a mixture of 0.04 M phosphate buffer (pH 3.0)–acetonitrile (82:18, v/v), which was selected as a mobile phase in this study.

3.2. Optimisation of sample preparation

A previous SPE for the chromatographic detection of FQs in plasma has generally been utilised not only to purify but also to concentrate sample extracts (Garcés, Zerzaňová, Kučera, Barrón, & Barbosa, 2006; Tasso & Costa, 2007). Generally, SPE requires conditioning and equilibration steps for maximum capability. In other words, SPE is time-consuming, uneconomical, and may not always be sufficiently effective. Additionally, frequent sample transfers can result in sample contamination and wasted time. Measures to minimise sample handling, such as evaporation and extract transfer steps, would serve to reduce the potential exposure of an analyte to contaminants (Anastassiades & Lehotay, 2003). This study was focused on simple extraction and cleanup within one step to diminish sample preparation steps. The reconstitution solvent used in our previous study, 0.04 M phosphate buffer (pH 1.5)–acetonitrile (8:2, v/v), was used in this study as a trial extraction solvent to separate the analytes from porcine

plasma; however, no precipitation or coagulation was generated in this case (Choi et al., 2009). However, the addition of ammonium sulphate (0.02 g) to the plasma diluted with the extraction solvent resulted in a turbid extract, and precipitation readily emerged via centrifugation after 10 min of cooling at -24°C . Additionally, the precipitate generated via centrifugation was higher with twice-repeated cycles with ammonium sulphate than when a twofold amount (0.04 g) of ammonium sulphate was used in one shot. No precipitate remained at the third addition of ammonium sulphate (the precipitate was not quantitatively measured). The extraction of the FQs with 0.04 M phosphate buffer (pH 1.5)–acetonitrile (8:2, v/v) and coagulation with ammonium sulphate in a single-step procedure enabled rapid sample preparation.

3.3. Method validation

3.3.1. Specificity

The representative chromatograms of a standard mixture, blank, and fortified plasma samples were compared such that the interference of endogenous compounds could be assessed at the elution times of the analytes in the same chromatographic run as shown in the blank chromatogram. The retention times of the analytes at the fortified plasma samples were matched with those of the standard solutions although a ghost peak was noticed next to CIP (Fig. 2). As good separation was also achieved between the FQs on the HPLC system used herein, our method proved able to detect ENRO, DANO, and CIP simultaneously and selectively in porcine plasma.

3.3.2. Linearity

The linearity of the developed method was evaluated using the squared correlation coefficients (r^2) of seven-point matrix-matched calibration curves obtained via the analysis of blank plasma extracts with the analytes in a range from 0.025 to 1 $\mu\text{g}/\text{mL}$ for ENRO and CIP, and in a range from 0.005 to 0.2 $\mu\text{g}/\text{mL}$ for DANO, in triplicate. Favourable linearity was achieved within the concentration range, with a correlation coefficient (r^2) of 0.9998 for ENRO and DANO, and 0.9999 for CIP (Table 2). Additionally, calibration with matrix-matched standard solutions can result in the minimisation of the difference in the signal output between the analyte in a pure solvent and those in the sample extract.

3.3.3. Limit of detection and quantitation

The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the standard deviation of blank samples' responses (σ) and the slope of the calibration curve (S), which were calculated by multiplying σ/S by 3.3 and 10, respectively (ICH, 1996). The LODs of ENRO, DANO, and CIP in porcine plasma were 0.007, 0.001, and 0.008 $\mu\text{g}/\text{mL}$, and the LOQs were 0.023, 0.004, and 0.025 $\mu\text{g}/\text{mL}$, respectively (Table 2). Consequently, the sensitivity of the method developed herein was adjudged reasonable for the determination of MRL values of ENRO and DANO residues in porcine plasma.

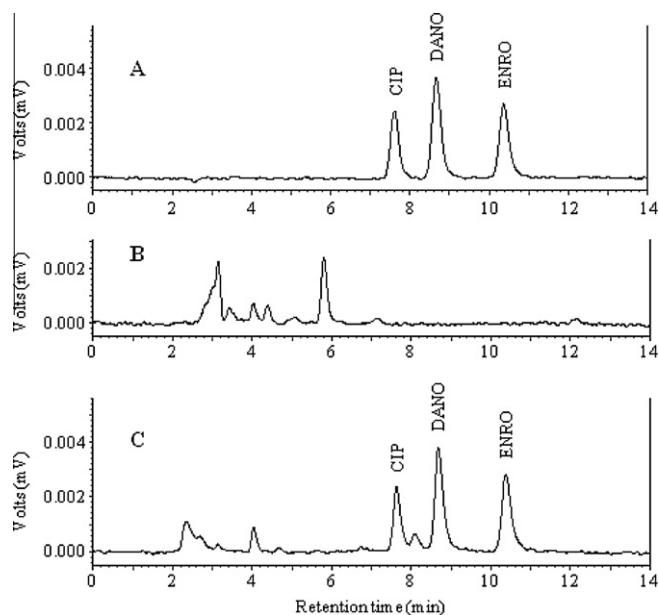


Fig. 2. Typical chromatograms for specificity of the developed method: (A) a standard mixture of three FQs; (B) blank porcine plasma; (C) porcine plasma spiked at 0.05 $\mu\text{g}/\text{mL}$ (ENRO), 0.02 $\mu\text{g}/\text{mL}$ (DANO), and 0.1 $\mu\text{g}/\text{mL}$ (CIP).

Table 2

Calibration curve, linearity (correlation coefficient, r^2), LOD, and LOQ of ENRO, DANO, and CIP in porcine plasma by HPLC-FLD following precipitation cleanup.

Compound	Linear range ($\mu\text{g}/\text{mL}$)	Calibration equation ^a	r^2	LOD ^b ($\mu\text{g}/\text{mL}$)	LOQ ^c ($\mu\text{g}/\text{mL}$)
ENRO	0.025–1.0	$y = 352098x - 661.94$	0.9998	0.007	0.023
DANO	0.005–0.2	$y = 1669790x + 849.77$	0.9998	0.001	0.004
CIP	0.025–1.0	$y = 206264x + 359.41$	0.9999	0.008	0.025

^a Calibration equation was created by plotting peak area (y) against a concentration (x) of analyte.

^b Limit of detection.

^c Limit of quantitation.

3.3.4. Accuracy and precision

Accuracy and precision were evaluated via intra- and inter-day analyses, all of which were conducted in a single laboratory. Precision was calculated in terms of intra-day repeatability and inter-day reproducibility. Accuracy was expressed as a percentage of recovery and precision as a relative standard deviation (RSD). Intra- and inter-day analyses were conducted using fortified porcine plasma at three different concentrations (0.05, 0.1, and 0.2 µg/mL). Intra-day analysis was conducted in six replicates at each concentration level, whereas inter-day analysis was performed for three consecutive days in triplicate at the same concentrations. Table 3 shows the results regarding the accuracy and precision of the developed method. The averages of intra- and inter-day accuracy ranged from 90.2% to 106.6% and from 86.5% to 103.6%, respectively. The intra-day repeatability of the developed method was measured in a range of 1.6–10.2%, whereas inter-day reproducibility ranged between 2.1% and 9.9%. These accuracy and precision values were consistent with the ranges listed in the Codex guidelines (Codex, 1993), and thus the method described herein can be considered excellent as a reliable, reproducible, and accurate routine analytical method.

3.4. Quantitative assay of ENRO in incurred porcine plasma

ENRO residues were then evaluated via the developed and validated method, taking into account variations in the time-course residues of incurred porcine plasma. Because CIP is a metabolite of ENRO, the total residual concentration of ENRO was the sum of the CIP and ENRO residues (Table 4). The ENRO residues remaining after oral administration were quantified for at least 3 days and were maximal at day 1, regardless of the addition of activated charcoal. Meanwhile, the ENRO residue on day 1 was higher in the activated charcoal-treated group than in the ENRO-treated group. The

Table 3
Intra- and inter-day accuracy and precision of ENRO, DANO, and CIP in porcine plasma.

Compound	Concentration (µg/mL)	Accuracy (%)	Precision (RSD, %)
<i>Intra-day assay (n = 6)</i>			
ENRO	0.05	90.2	8.7
	0.1	93.2	10.2
	0.2	90.7	2.9
DANO	0.05	99.7	2.7
	0.1	101.2	1.6
	0.2	106.6	1.7
CIP	0.05	91.6	9.4
	0.1	97.3	4.5
	0.2	101.1	2.4
<i>Inter-day assay (n = 3 × 3 days)</i>			
ENRO	0.05	86.5	3.4
	0.1	87.0	5.0
	0.2	90.2	4.3
DANO	0.05	98.9	2.8
	0.1	98.2	3.9
	0.2	103.6	2.1
CIP	0.05	89.8	9.9
	0.1	92.8	7.5
	0.2	97.3	3.3

Table 4
Time course residues^a (mean ± SD, µg/mL) of ENRO in incurred porcine plasma.

Compound	1 day	2 days	3 days	5 days	7 days
ENRO	0.279 ± 0.002	0.023 ± 0.001	0.042 ± 0.005	T ^b	T
ENRO with activated charcoal	0.455 ± 0.005	0.028 ± 0.001	0.025 ± 0.008	T	T

^a Total residue of ENRO calculated as follows: A = B + C × (MW of ENRO/MW of CIP) where A = total residual concentration of ENRO, B = residual concentration of ENRO, and C = residual concentration of CIP.

^b Trace.

concentration at day 3 in the ENRO-treated group was slightly higher than that noted at day 2, which was probably caused by sample mishandling. Indeed, such higher concentrations were non-significant because they were still substantially lower than MRL. In both treatment groups, ENRO residues were sharply reduced to less than the MRL at day 2.

Activated charcoal is an important material employed for the clinical management of drugs or chemical overdoses. Because of its large surface area, it has the ability to effectively bind to administered compounds, thereby preventing systemic uptake in the gastrointestinal tract (Minton, Gluchman, & Henry, 1995; Stass, Kubitz, Möller, & Delesen, 2005). The coadministration of activated charcoal was intended as an effective antidote to prevent unnecessary overdoses of ENRO in the present study; however, contrary to expectations, unexpected high plasma ENRO contents were detected in the group to which activated charcoal was coadministered. An objective comparative analysis between our findings and previous results was not possible, because there have been no prior reports thus far concerning the coadministration of activated charcoal with ENRO. Stass et al. (2005) reported a marked reduction in plasma moxifloxacin concentrations of males, after the coadministration of activated charcoal. However, Torre, Sampietro, Quadrelli, Bianchi, and Maggiolo (1988) previously determined that the coadministration of activated charcoal with CIP exerted no effects on the relevant pharmacokinetic parameters in *in vivo* human studies. Different physicochemical properties were noted between FQs at various pH conditions pig; pH 3.4–4.8 in the stomach, (Yi & Kornegay, 1996); 6.4–7.2 in the small intestine, (Risley, Kornegay, Lindemann, Wood, & Eigel, 1992; Yi & Kornegay, 1996); 5.6–6.7 in the large intestine, (Hansen et al., 2010; Shim, Williams, & Versteegen, 2005) of the gastrointestinal tract, variations in drug concentrations in the same organ, and variations in the rate of absorption in different species (Siefert et al., 1999), might cause higher ENRO concentrations in animal to which activated charcoal was coadministered than in those receiving ENRO. These debates, however, will require additional research in the future.

Because no detectable ENRO concentrations were noted on day 5 post-treatment, we suggest that the complete depletion of ENRO from plasma is possible in that approximate time period. The coadministration of activated charcoal exerted no detectable effects on the duration of ENRO in the blood, and the withdrawal time prior to slaughter (10 days) as established by the NVRQS was fully adequate.

4. Conclusion

In this study, a simple and rapid HPLC-FLD analytical method was developed to allow for the separation of ENRO, DANO, and CIP from porcine plasma. The sample preparation technique includes a single-step extraction and centrifugation without the need for any further cleanup procedure. The developed method was not only verified by reliable validation results, but also proved able to quantify ENRO residues at concentrations lower than its MRL (0.1 mg/L) in incurred porcine plasma. A precipitation cleanup for the single-step

extraction enabled the developed method to be simpler, faster, and more economical than SPE cleanup methods. Additionally, the current technique is environmental-friendly because it does not utilise SPE cartridge and/or solvents. Co-administration of activated charcoal induced an increase in early ENRO concentration, but did not delay the pattern of ENRO disappearance. The withdrawal time of ENRO (10 days for oral administration) was considered sufficient for the purpose of ensuring food safety of blood.

Acknowledgement

This study was financially supported by the Agricultural R&D Promotion Center, Ministry for Food, Agriculture, Forestry and Fisheries (Grant No. 204152-3), Republic of Korea.

References

- Anastassiades, M., & Lehotay, S. J. (2003). Fast and easy multiresidues method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. *Journal of AOAC International*, 86, 412–431.
- Ashwin, H., Stead, S., Caldow, M., Sharman, M., Stark, J., Rijk, A., et al. (2009). A rapid microbial inhibition-based screening strategy for fluoroquinolones and quinolone residues in foods of animal origin. *Analytica Chimica Acta*, 637, 241–246.
- Bruinsma, J. (2003). *World agriculture: Towards 2015/2030. An FAO perspective*. London: Earthscan Publication Ltd. Available from: <http://www.fao.org/DOCREP/005/Y4252E/y4252e00.htm>. Accessed 14.07.10.
- Chen, G., & Schneider, M. J. (2003). A rapid spectrofluorometric screening method for enrofloxacin in chicken muscle. *Journal of Agricultural and Food Chemistry*, 51, 3249–3253.
- Choi, J. H., Mamun, M. I. R., Abd El-Aty, A. M., Kim, K. T., Koh, H. B., Shin, H. C., et al. (2009). Inert matrix and Na₄EDTA improve the supercritical fluid extraction efficiency of fluoroquinolones for HPLC determination in pig tissues. *Talanta*, 78, 348–357.
- Christodoulou, E. A., Samanidou, V. F., & Papadoyannis, I. N. (2007). Validation of an HPLC-UV method according to the European Union Decision 2002/657/EC for the simultaneous determination of 10 quinolones in chicken muscle and egg yolk. *Journal of Chromatography B*, 859, 246–255.
- Codex. (1993). *Codex guidelines for the establishment of a regulatory programme for control of veterinary drug residues in food, CAC/GL 16*. Available from: http://www.euro.who.int/document/fos/convet_e.pdf.
- Delgado, C., Rosegrant, M., Steinfeld, H., Ehui, S., & Courbois, C. (1999). *Livestock to 2020: The next food revolution*. Washington: International Food Policy Research Institute. Available from: <http://www.fao.org/ag/aginfo/resources/documents/lvst2020/2020.pdf>.
- Garcés, A., Zerzañová, A., Kučera, R., Barrón, D., & Barbosa, J. (2006). Determination of a series of quinolones in pig plasma using solid-phase extraction and liquid chromatography coupled with mass spectrometric detection. Application to pharmacokinetic studies. *Journal of Chromatography A*, 1137, 22–29.
- Gigosos, P. G., Revesado, P. R., Cadahía, O., Fente, C. A., Vazquez, B. I., Franco, C. M., et al. (2000). Determination of quinolones in animal tissues and eggs by high-performance liquid chromatography with photodiode-array detection. *Journal of Chromatography A*, 871, 31–36.
- Hansen, C. F., Phillips, N. D., La, T., Hernandez, A., Mansfield, J., Kim, J. C., et al. (2010). Diets containing inulin but not lupins help to prevent swine dysentery in experimentally challenged pigs. *Journal of Animal Science*. doi:10.2527/jas.2009-2719.
- Hermo, M. P., Nemutlu, E., Barbosa, J., & Barrón, D. (2010). Multiresidue determination of quinolones regulated by the European Union in bovine and porcine plasma. Application of chromatographic and capillary electrophoretic methodologies. *Biomedical Chromatography*. doi:10.1002/bmc.1483.
- Herrera-Herrera, A. V., Hernández-Borges, J., & Rodríguez-Delgado, M. Á. (2009). Fluoroquinolone antibiotic determination in bovine, ovine and caprine milk using solid-phase extraction and high-performance liquid chromatography-fluorescence detection with ionic liquids as mobile phase additives. *Journal of Chromatography A*, 1216, 7281–7287.
- Horie, M., Saito, K., Nose, N., & Nakazawa, H. (1994). Simultaneous determination of benfloxacin, danofloxacin, enrofloxacin and ofloxacin in chicken tissues by high-performance liquid chromatography. *Journal of Chromatography B*, 653, 69–76.
- Huet, A. C., Charlier, C., Tittlemier, S. A., Singh, G., Benrejeb, S., & Delahaut, P. (2006). Simultaneous determination of (fluoro)quinolone antibiotics in kidney, marine products, eggs, and muscle by enzyme-linked immunosorbent assay (ELISA). *Journal of Agricultural and Food Chemistry*, 54, 2822–2827.
- ICH (1996). ICH harmonised tripartite guideline, Q2B. Validation of analytical procedures: Methodology.
- Kimata, K., Tanaka, N., & Araki, T. (1992). Suppression of the effect of metal impurities in alkylsilylated silica packing materials. *Journal of Chromatography*, 594, 87–96.
- Minton, N. A., Gluchsmann, E., & Henry, J. A. (1995). Prevention of drug absorption in simulated theophylline overdose. *Human and Experimental Toxicology*, 14, 170–174.
- Notification No. 2007-25 of the National Veterinary Research and Quarantine Service (2008). Standards for the safe use of veterinary drugs, Republic of Korea.
- Notification No. 2009-24 of the Korea Food and Drug Administration (2009). Maximum residue limits of veterinary drugs, Republic of Korea.
- Ohhira, M., Ohmura, F., & Hanai, T. (1989). Inertness of bonded silica gel packings. *Journal of Liquid Chromatography*, 12, 1065–1074.
- Risley, C. R., Kornegay, E. T., Lindemann, M. D., Wood, C. M., & Eigel, W. N. (1992). Effect of feeding organic acids on selected intestinal content measurements at varying times postweaning in pigs. *Journal of Animal Science*, 70, 196–206.
- Shim, S. B., Williams, I. H., & Verstegen, M. W. A. (2005). Effects of dietary fructo-oligosaccharide on villous height and disaccharidase activity of the small intestine, pH, VFA and ammonia concentrations in the large intestine of weaned pigs. *Acta Agriculturae Scand Section A*, 55, 91–97.
- Siefert, H. M., Domdey-Bette, A., Henninger, K., Hucke, F., Kohlsdorfer, C., & Stass, H. H. (1999). Pharmacokinetics of the 8-methoxyquinolone, moxifloxacin: A comparison in humans and other mammalian species. *Journal of Antimicrobial Chemotherapy*, 43, 69–76.
- Speedy, A. W. (2003). Global production and consumption of animal source foods. *The Journal of Nutrition*, 133, 4048S–4053S.
- Stass, H., Kubitz, D., Möller, J. G., & Delesen, H. (2005). Influence of activated charcoal on the pharmacokinetics of moxifloxacin following intravenous and oral administration of a 400 mg single dose to healthy males. *British Journal of Clinical Pharmacology*, 59, 536–541.
- Tasso, L., & Costa, T. D. (2007). High performance liquid chromatography for quantification of gatifloxacin in rat plasma following automated on-line solid phase extraction. *Journal of Pharmaceutical and Biomedical Analysis*, 44, 205–210.
- Torre, D., Sampietro, C., Quadrelli, C., Bianchi, W., & Maggiolo, F. (1988). Effects of orally administered activated charcoal on ciprofloxacin pharmacokinetics in healthy volunteers. *Chemioterapia*, 7, 382–386.
- Vybíralová, Z., Nobilis, M., Zoulova, J., Května, J., & Petr, P. (2005). High-performance liquid chromatographic determination of ciprofloxacin in plasma samples. *Journal of Pharmaceutical and Biomedical Analysis*, 37, 851–858.
- Yi, Z., & Kornegay, E. T. (1996). Sites of phytase activity in the gastrointestinal tract of young pigs. *Animal Feed Science Technology*, 61, 361–368.