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Analytical Methods

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

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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.

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 prophalytic 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

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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	Pig	5 mg/kg/day	Intramuscular or hypodermic injection	20
	Rabbit	0.1	0.2	0.1	0.3				
	Poultry	0.1	0.2	0.1	0.3				
	Fish	0.1					150 g/t feed	Oral	10
	Eggs	ND[d]							
	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				
	Milk	0.03				Pig	1.25 mg/kg/day	Intramuscular or hypodermic injection	25
						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, Nemutlu, 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.