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< Short Communication >

The development and validation of a novel liquid chromatography tandem mass spectrometry (LC-MS/MS) procedure for the determination of fluoroquinolones residues in chicken muscle using modified QuEChERS (quick, easy, cheap, effective, rugged and safe) method

Sunjin Park, Hyobi Kim, Byungkook Choi, Chung-Oui Hong, Seon-Young Lee, Inhae Jeon, Su-Young Lee, Pilhee Kwak, Sung-Won Park, Yong-Sang Kim, Kwang-jick Lee*

Veterinary Drugs & Biologics Division, Animal and Plant Quarantine Agency (APQA) Gimcheon 39660, Korea

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Abstract

A novel rapid procedure with liquid chromatography tandem mass spectrometry (LC-MS/MS) detection has been developed by changing various conditions including sample preparation such as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) methodology. This work has been involved the optimization and validation of detection method for fluoroquinolones which are widespread used in livestock especially in the chicken. Five grams of homogenized chicken muscle were extracted with QuEChERS EN and acetonitrile containing 5% formic acid and cleaned with anhydrous magnesium sulfate and C_{18} sorbent. The separation was performed on Acquity UPLC HSS T3 (2.1 mm×100 mm, 1.8 μm) column. The mobile phase A and B were composed of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid, respectively. Flow rate was 0.25 mL/min and column temperate was 40°C. LC-MS/MS with multiple reaction monitoring has been optimized for ten fluoroquinolones (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, marbofloxacin, norfloxacin, ofloxacin, orbifloxacin, pefloxacin and sarafloxacin). The method developed in this study has been presented good linearity with correlation coefficient (R^2) of 0.9971～0.9998. LOD and LOQ values ranged from 0.09 to 0.76 ppb and from 0.26 to 2.29 ppb, respectively. The average recoveries were from 77.46 to 111.83% at spiked levels of 10.0 and 20.0 μg/kg. Relative standard deviation (%) ranged 1.28～11.90% on intra-day and 3.10～8.38 % on inter-day, respectively. This analysis method was applicable to the livestock residue laboratories and was expected to be satisfactory for the residue surveillance system.

Key words : Fluoroquinolones, QuEChERS, Chicken, Muscle, LC-MS/MS

INTRODUCTION

Fluoroquinolones (FQs) are a group of antimicrobials broadly used in the treatment of bacterial disease of food producing animals and humans especially on *Salmonella* and *Campylobacter*, food poisoning bacteria. But the use of FQs for veterinary purposes could lead to the emergence of FQ-resistant bacterial strains in humans (Zweerink and Edison, 1986; EC, 2005; Fabrega et al, 2008).

*Corresponding author: Kwang-jick Lee, Tel. +82-054-912-0577, Fax. +82-054-912-0583, E-mail. leekwj@korea.kr

Because of the emergence of resistance of *Campylobacter* to fluoroquinolones, the use of enrofloxacin for poultry was withdrawn by the U.S. Food and Drug Administration in 2005 (Okeke et al, 2005). Japan and Korea permit the use of enrofloxacin only for the treatment of chickens other than laying hens and the expression of resistance of enrofloxacin in meats is very serious stage in Korea. In a 2007 CVMP report, EMA reported that human infection of resistant bacteria of fluoroquinolones were mostly associated with fluoroquinolones used in farm animals, especially poultry.

To protect human health, The EU and other regulatory agencies around the world have established safe maximum residue limits (MRLs) for these drugs. In Korea, MRL for the sum of enrofloxacin and ciprofloxacin was set to 0.1 mg/kg for porcine, cattle and poultry muscles but should not be detected in egg. Other fluoroquinolones as norfloxacin, ofloxacin and pefloxacin should not be detected in food producing animals which are banned for use and sale as these are commonly used in human and livestock (U.S. Food and Drug Administration, 2005).

Monitoring of these residues is necessary to ensure food safety. Current method for FQs analysis in food producing animals are based on high performance liquid chromatography (HPLC) with ultraviolet (Maraschiello et al, 2001; Hermo et al, 2006; Hermo et al, 2008) and fluorescense detection (Cohen et al, 1999; Yorke and Froc, 2000; Schneider and Donoghue, 2003) or mass spectrometric detection. Mass spectrometry combined with HPLC provides very high sensitivity and selectivity with capabilities of simultaneously analyzing multiresidues. LC-MS/MS is widely used in many methods in this respect (Schneider and Donoghue, 2003; Toussaint et al, 2005; Van Hoof et al, 2005; Hermo et al, 2006; Granelli and Branzell, 2007; Hermo et al, 2008). Sample pretreatment is important in LC-MS/MS to increase analytical efficiency including analytical time, recovery of analytes and so on. The method referred to as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) was introduced in extraction of pesticide residues in fruits and vegetables with potential application in other matrices (Anastassiades et al, 2003). Several modifications including the addition of organic acids into the buffered extraction phase and the use of other sorbents have been proposed in order to improve extraction efficiency and reduce matrix interference (Aguilera-Luiz et al, 2012; Lombardo-Agui et al, 2012; Sun et al, 2012; Zhao and Stevens, 2012; Zhang et al, 2015; Zhang et al, 2016). Many studies have shown sample pretreatment in various matrix including milk, honey, poultry muscle, plasma and liver using QuEChERS methodology (Lombardo-Agui et al, 2012; Lopes et al, 2012; Lucatello et al, 2015; Rocha et al, 2015; Zhang et al, 2015). There are many studies that used QuEChERS AOAC (American Standard) or EN (European Standard) in extraction salts to increase extraction efficiency (Lombardo-Agui et al, 2012; Lopes et al, 2012; Zhao and Stevens, 2012; Lucatello et al, 2015). To decrease matrix interference there are many studies that have proposed various sorbents including molecularly imprinted solid-phase extraction (MISPE) method (Stubbings and Bigwood, 2009; Lombardo-Agui et al, 2010; Lee et al, 2013; Zhao and Lucas, 2015; Guo et al, 2016). The purpose of this work is the development and validation of QuEChERS methodologies by LC-MS/MS to determine FQs in poultry muscle. The extraction step is optimized in order to achieve better extraction efficiency for 10 FQs studied. The method developed are used to determine FQs in poultry muscle in the inspection laboratories and slaughterhouse below MRLs.

MATERIALS AND METHODS

Chemicals and reagents

Fluoroquinolones were analytical standard grade and were purchased from Sigma (Sigma-Aldrich, St. Louis, USA). These FQs were included marbofloxacin, orbifloxacin, enrofloxacin, sarafloxacin, ciprofloxacin, danofloxacin, difloxacin, ofloxacin and norfloxacin. Acetonitrile (ACN), methanol (MeOH) and water of HPLC grade were obtained from J.T.Baker (Phillipsburg, NJ). Formic acid was supplied by Merck (Darmstadt, Germany). Potassium dihydrogen phosphate was obtained from Aldrich. 0.1% formic acid in water and 0.1% formic acid in acetonitrile were purchased from Fisher chemical for mobile phase. For the QuEChERS extraction, QuEChERS AOAC (6 g magnesium sulfate, 1.5 g sodium acetate) and QuEChERS EN (4 g magnesium sulfate, 1g sodium chloride, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) were supplied by Bekolut. Magnesium sulfate and octadecylsilane (C_{18}) in 15 mL dispersive tube were used for clean-up procedure and obtained from Bekolut. Filters of 4 mm with 0.22 μm PVDF membrane (Millex®) were used for filtration of the final extracts before analysis.

Instrumentation

Separation was performed on Nexera X2 liquid chromatography system (two pumps, oven, auto sampler, degasser unit) from Shimadzu. Mass spectrometer measurements were performed on AB SCIEX QTRAP 6500 with electrospray ionization. Instrument data were collected using Analyst® Software version 1.6.3 and processed using MultiQuant™ 3.0.1 Software. A pH-meter (Orion verastar, Thermo scientific), Avanti® J-E centrifuge (Beckman coulter), evaporator system (EvaT-0200, Goojung engineering) and vortex-2 genie (Scientific industries) were also used.

Standard solutions

Individual stock solutions (100 mg/L) were prepared by dissolving the compounds with methanol (in ciprofloxacin, water:methanol (10:90, v/v) and were then stored at −20°C in darkness. Mixture working solutions (10 mg/L, 1 mg/L) were diluted from stock solutions weekly. Thirty mM phosphate buffer solution (pH 7.0) was prepared by dissolving 2.04 g of potassium dihydrogen phosphate in 500 mL of water and the pH was adjusted with 5N NaOH solution.

LC-MS/MS analysis

UHPLC separation were performed in Acquity UPLC HSS T3 column (2.1 mm i.d.×100 mm, 1.8 μm) using a mobile phase consisting of 0.1 % formic acid in water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B) at flow rate of 0.25 mL/min. The gradient was 10 % B for 0～1 min, 10～70 % B for 1～10 min, 70～100 % B for 10～11 min, 100 % B for 11～14 min, 100～10 % B for 14～15 min and then maintained for another 5 min. The temperature of the column was 40°C and the injection volume was 10 μL. The mass spectrometer was working with an electro spray ion source (ESI) in positive mode under the multiple reaction monitoring (MRM) conditions shown in Table 1. The ionization source parameters were curtain gas (nitrogen) 20 psi, ion spray voltage 5,500 V, source temperature 650°C and nebulizer gas and heater gas (both of them nitro-

Table1. Instrument acquisition data for the analysis of FQs by LC-MS/MS

Analyte	Precursor ion (m/z)	DP*	Quantification ion (m/z)†	Identification ion (m/z)†	RT (min)
Ciprofloxacin	332.1	56	314 (29)	231 (49) 148 (69)	4.50
Danofloxacin	358.1	81	340 (31)	82 (71) 255 (49)	4.68
Difloxacin	400.1	86	356.1 (25)	382 (29) 299 (39)	5.26
Enrofloxacin	360.1	96	342 (29)	316 (27) 286 (45)	4.80
Marbofloxacin	363.1	61	72 (59)	70.1 (61) 42 (63)	4.22
Norfloxacin	320.1	66	302 (27)	231 (53) 189 (65)	4.34
Ofloxacin	362.1	46	318 (27)	261 (39) 58 (63)	4.39
Orbifloxacin	396.1	56	295 (33)	378 (27) 266.9 (49)	4.91
Pefloxacin	334.1	71	316 (27)	290 (23) 233 (35)	4.40
Sarafloxacin	386.1	96	367.9 (31)	299 (39) 255 (67)	5.14

*Declustering potential (DP).
†Collision energy (eV) is given in parentheses.

gen) were set to 30 psi and 80 psi respectively.

Sample preparation and clean-up

Chicken muscle parts were finely milled with a grinder. 5 g of ground sample was weighed into a 50 mL conical tube. Then 5 mL of 30 mM KH_2PO_4 buffer (pH7.0) were added and homogenized in vortex for 1 min. 15 mL of 5% formic acid in ACN were added and the mixture was homogenized in vortex for 1 min. QuEChERS EN [4 g magnesium sulfate ($MgSO_4$), 1 g sodium chloride (NaCl), 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate] kit supplied by Bekolut was added and the tube was shaken vigorously for 1 min. The sample was centrifuged at 5,000 rpm for 10 min and 6 mL of supernatant was transferred into the 15 mL dispersive tube contained with 900 mg $MgSO_4$ and 150 mg C_{18}, stirred in vortex for 1 min and centrifuged at 5,000 rpm for 10 min. An aliquot of 3 mL of supernatant was transferred to a 15 mL conicaltube, dried under a stream of nitrogen and the residue was redissolved with 1 mL of H_2O/ACN (90/10), filtered with 0.2 μm PVDF and analyzed by LC-MS/MS.

RESULTS AND DISCUSSION

Optimization of clean-up

For sample preparation, QuEChERS methodology was modified with several factors based on the previous literature (Narayana and Ganni, 2014). In this study we intended to keep focused on the clean-up procedure for the good recoveries of 10 fluoroquinolones. The 2 g sample was used and added with 10 mL of water. After vortexing for 60 sec, we added 5% formic acid in acetonitrile and vortexed for 60s. After liquid-liquid partition by AOAC QuEChERS salt mixture, 6 mL of upper acetonitrile layer were transferred to dispersive SPE tubes. PSA, C_{18} and graphite carbon black (GCB) were selected for the separation of co-extracted compounds from the organic layer usually. We used various d-SPEs which were contained with different kinds and quantities of sorbents (Fig. 1). We confirmed that d-SPE containing GCB had very poor recoveries. Ciprofloxacin, marbofloxacin and norfloxacin had poor recoveries in all d-SPEs. So we selected d-SPE 10 containing 150 mg C_{18} and 900 mg $MgSO_4$ for better recoveries in ciprofloxacin, marbofloxacin and norfloxacin.

Optimization of extraction and liquid-liquid partition

For optimization of sample preparation, quantity of sample was increased from 2 g to 5 g. We compared

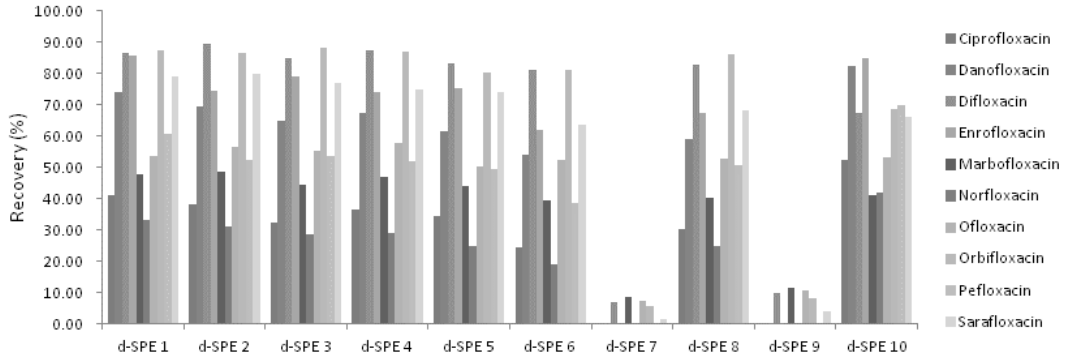

Fig. 1. Effect of various sorbents in clean up step, in the QuEChERS procedure for chicken samples. †d-SPE1: 150 mgPSA, 900 mg$MgSO_4$/d-SPE2:150 mgPSA,150 mgC_{18}, 900 mg$MgSO_4$/d-SPE3:150 mgPSA, 15 mgGCB, 900 mg$MgSO_4$/ d-SPE4:150 mgPSA, 45 mgGCB, 900 mg$MgSO_4$/d-SPE5: 400 mgPSA,1,200 mg$MgSO_4$/d-SPE6: 400 mgPSA, 45 mgGCB, 400 mgC_{18}, 1,200 mg$MgSO_4$/d-SPE7: 400 mgPSA, 400 mgGCB, 400 mgC_{18}, 1,200 mg$MgSO_4$/d-SPE8: 400 mgPSA, 400 mgC_{18}, 1,200 mg$MgSO_4$/d-SPE9: 400 mgPSA, 400 mgGCB, 1,200 mg$MgSO_4$/d-SPE10: 150 mgC_{18}, 900 mg$MgSO_4$. PSA: Primary-secondary amine, GCB: graphized carbon black, C_{18}: octadecylsilane.

water with 30 mM KH_2PO_4 in homogenizing sample and QuEChERS AOAC with QuEChERS EN in extraction salts. The 10 fluoroquinolones were spiked with 20 μg/kg in sample and added with 5 mL of water or KH_2PO_4. Extraction solvents were used with 10 mL acetonitrile containing 5% formic acid. Extraction salts were used with QuEChERS AOAC or QuEChERS EN. We selected 30 mM KH_2PO_4/QuEChERS EN resulting in good recoveries (Fig. 2). But ciprofloxacin and nor floxacin had more poor recoveries than any other compounds. To increase recoveries of two compounds we had tried optimization of liquid-liquid partition. We considered the ratio of quantity of acetonitrile per KH_2PO_4. So the ratios (30 mM KH_2PO_4:5% FA in ACN) were 1:1 (10 mL:10 mL), 1:2 (5 mL:10 mL), 1:3 (5 mL:15 mL). The ratio of 1:3 had good recoveries in all fluoroquinolones (Fig. 3).

Method validation

Performance characteristics of the method were linearity, intra and inter-day precision, limits of detection and quantification. Matrix-matched calibration curves were established at eight concentration level diluted with two-fold in sample fortifying at 100 μg/kg. Limit of detection (LOD) and limit of quantification (LOQ) were determined as follow: LOD=3.3×SD/S; LOQ=10×SD/S, where SD is the standard deviation of y-intercepts and S is the slope obtained from the calibration curve prepared for matrix. Correlation coeffients, LODs and LOQs were shown in Table 2. Calibration curves showed good linearity with correlation coefficients (R^2) higher than 0.997 in all the cases. Recovery and precision were determined by processing spiking sample at two levels (10, 20 μg/kg) over three different days (Table 3). Mean recov-

Table 2. Linearity evaluation and sensitivity data for the FQs detected in this study

Analyte	R^2	LOD (μg/kg)	LOQ (μg/kg)
Ciprofloxacin	0.9971	0.76	2.29
Danofloxacin	0.9974	0.52	1.59
Difloxacin	0.9991	0.13	0.39
Enrofloxacin	0.9994	0.13	0.39
Marbofloxacin	0.9997	0.09	0.26
Norfloxacin	0.9974	0.15	0.44
Ofloxacin	0.9986	0.40	1.21
Orbifloxacin	0.9998	0.17	0.52
Pefloxacin	0.9993	0.22	0.67
Sarafloxacin	0.9985	0.18	0.55

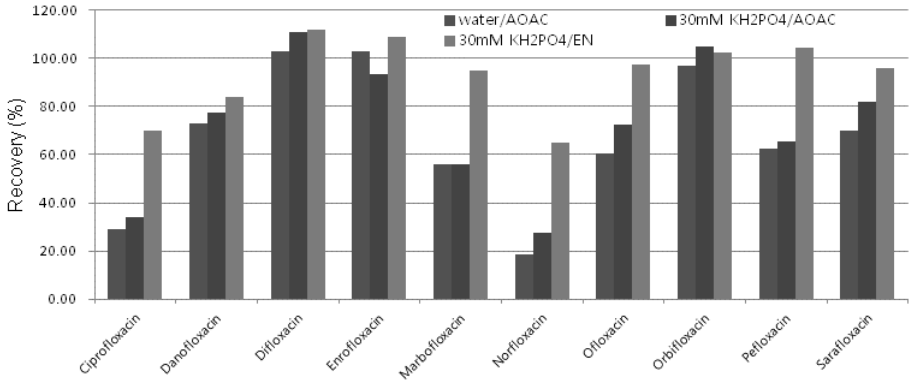

Fig. 2. Effect of various homogenizing solvent and extraction salts in extraction step, in the QuEChERS procedure for chicken samples.

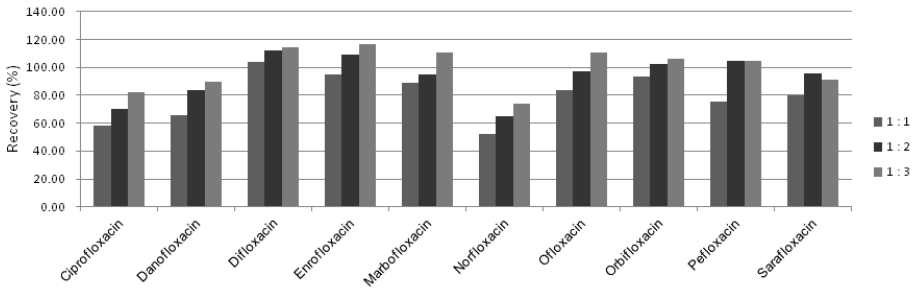

Fig. 3. Effect of the ratio of quantity of acetonitrile per KH_2PO_4 in liquid-liquid partition step, in the QuEChERS procedure for chicken samples (KH_2PO_4: ACN=1:1, 1:2, 1:3).

eries of all analytes were in the range of 77.46% (norfloxacin) to 111.83% (danofloxacin). The intra-day and inter-day precision were lower than 11.9% and 8.38%, respectively, indicating good repeatability and reproducibility.

Analysis of actual samples

Twenty chicken samples of 3 brand were obtained from the several market to validate the method and apply to the actual samples. These parameters including retention time and ion ratio were compared in samples. Most ciprofloxacin were detected under limit of detection (LOD). A-1 was detected under LOD and A-2 was detected under limit of quantitation in enrofloxacin. Residues of enrofloxacin were detected in sample A-3, A-4, B-1, B-2 and B-4 at 0.659, 1.107, 0.415, 1.008 and 0.578 μg/kg, respectively (Table 4). Whereas other fluoroquinolones were not detected in other samples. Maximum residue level of enrofloxacin (combined with ciprofloxacin) in Korea was 100 ppb in chicken muscle. So chicken muscles purchased in market were under the MRL, indicating chicken in market is guaranteed for food safety on chemical residues.

For the more applications, thirty samples were collected in the slaughterhouse located in three areas. Enrofloxacin were found in 7 samples of I area and 3 samples of C area. I-4 and I-10 were detected over 10 μg/kg in enrofloxacin (Table 5). But they were below the MRL of enrofloxacin.

CONCLUSION

In this study, a validated, simple, fast, reproducible and sensitive method for simultaneous analysis of ten fluoroquinolones in chicken muscle by LC-MS/MS using QuEChERS procedure was developed. For good re-

Table 3. Validation parameter of the optimized LC-MS/MS method

Analyte	Recovery (%)		Intraday precision (RSD%)*		Interday precision (RSD%)†	
	10 (μg/kg)	20 (μg/kg)	10 (μg/kg)	20 (μg/kg)	10 (μg/kg)	20 (μg/kg)
Ciprofloxacin	78.11	83.25	11.90	1.84	7.98	6.35
Danofloxacin	109.21	111.83	6.23	3.53	8.38	5.04
Difloxacin	99.63	95.63	2.58	1.11	3.10	4.54
Enrofloxacin	109.71	107.03	10.53	4.15	8.05	6.17
Marbofloxacin	92.23	95.08	9.09	3.93	5.96	4.86
Norfloxacin	77.46	78.20	4.10	3.95	6.19	3.60
Ofloxacin	93.56	99.19	4.53	1.51	4.72	5.71
Orbifloxacin	88.24	86.04	3.54	2.13	6.32	6.65
Pefloxacin	102.82	104.26	1.92	2.05	3.69	4.79
Sarafloxacin	94.01	90.92	4.60	1.22	5.99	5.83

*Number of replicates=3.
†Number of replicates=9 in 10 (μg/kg) and 11 in 20 (μg/kg) for inter-day precision.

Table 4. Concentration of FQs (μg/kg) found in real samples in market

Analyte	A-1	A-2	A-3	A-4	B-1	B-2	B-3	B-4	C-2	C-3
Ciprofloxacin	-	-	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	-
Enrofloxacin	<LOD	<LOQ	0.659	1.107	0.415	1.008	-	0.578	-	<LOD

Table 5. Concentration of FQs (mg/kg) found in real samples in slaughterhouse

Analyte	I-11	I-2	I-3	I-4	I-5	I-9	I-10	C-3	C-7	C-8
Enrofloxacin	5.402	3.104	0.701	10.701	1.904	6.164	13.615	0.798	0.706	<LOQ

coveries, sample preparation procedures were optimized including liquid-liquid partition and clean-up with d-SPE. Satisfactory validation parameters were obtained for linearity, recovery, precision, LODs and LOQs. Therefore, this method is suitable for identification and quantification of fluoroquinolones in chicken muscle. We will apply this method on routine analysis of residual fluoroquinolones in edible tissues furthermore.

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