J. Vet. Sci. (2003), 4(1), 35-39

JOURNAL OF
Veterinary
Science

Determination of Roxithromycin by Liquid Chromatography/Mass Spectrometry after Multiple-Dose Oral Administration in Broilers

Jong-hwan Lim, Byung-kwon Park and Hyo-in Yun*

Division of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, Chungnam National University, 220 Gung-dong, Yuseong-gu, Daejeon, Korea

Received January 30, 2003 / Accepted March 28, 2003

Abstract

A highly sensitive and specific method for the determination of roxithromycin in broiler tissues by LC/MS was developed and validated. A dichloromethane extract of the sample was separated on C18 reversed-phase column with acetonitrile-50 mM ammonium acetate (80:20, v/v) as the mobile phase and analyzed by LC/MS via atmospheric pressure ionization/electrospray ionization interface. The limit of detection and limit of quantitation were 1 ng/g and 5 ng/g. The method has been successfully applied to determine for roxithromycin in various tissues of broilers. Residue concentrations were associated with administered dose. At the termination of treatment, roxithromycin was found in all collected samples for both dose groups. Liver was detected to have the highest residual concentration of roxithromycin. Residue concentrations of roxithromycin were lower than its LOQ in all tissues from both dose groups 10 days after the treatment of roxithromycin mixed with drinking water at a dose rate of 15 mg/L or 60 mg/L to each broiler for 7 days.

Key words: roxithromycin, broiler, LC/MS, withdrawal time

Introduction

Roxithromycin is a semisynthetic macrolide antibiotic derived from erythromycin [12]. Roxithromycin was reported to be absorbed rapidly with the long elimination half time, giving higher plasma levels than erythromycin [9]. Therefore, it can be effective at lower doses with less frequent administrations, which is regarded as an advantage in clinical settings. Due to these advantages, it could be applied in human and veterinary medicine [8].

Several methods have been reported for determination of roxithromycin in biological fluids. Microbiological assays in plasma, urine and milk have been reported [4]. However, microbiological assays have several disadvantages in terms of the limit of quantitation, specificity and rapidity. Some methods based on the reversed-phase HPLC have been developed for the qunatitation of roxithtomycin or other macrolides. UV absorption [2, 11, 14], fluorescence and electrochemical detection [3, 5, 13, 15] methods have been used, but these methods achieved only relatively high detection limits in the range of several hundred ng/g or ng/ml. They are not suitable to determine low levels of roxithomycin in the biological fluid.

More recently, the advent of mass spectrometer combined with HPLC offers a significant advantage for the absolute confirmation and quantitation of chemicals. The high-performance liquid chromatography (HPLC) coupled to electrospray mass spectrometric detector could be a more powerful technique for separation, identification and quantitation of roxithromycin [4, 7, 10, 6]. Electrospray mass spectrometry and particle beam mass spectrometry have been coupled to LC for the analysis of roxithromycin in biomatrixes [10, 16], as well as for the simultaneous analysis of marcrolides[4, 7].

In this study, a rapid and sensitive method was developed to determine roxithromycin in poultry tissues with electrospray LC/MS and used to evaluate for residue depletion profiles after its treatment with mixed drinking water for 7 days.

Materials and Methods

Chemicals

Roxithromycin was given by Shin-il Chemical (Seoul, Korea). HPLC grade water, methanol, acetonitrile and dichloromethane were purchased from TEDIA (USA). Reagent grade ammonium acetate, sodium borate, sodium hydroxide were purchased from SIGMA (USA).

*Corresponding author: Hyo-in Yun
Division of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, Chungnam National University, 220 Gung-dong, Yuseong-gu, Daejeon, Korea
Tel: +82-42-821-6759; Fax: +82-42-822-5780
E-mail: hiyun@cnu.ac.kr

Chromatographic conditions and extractions

Samples were analyzed on a Hewlett-Packard LC/MSD system. Separation was achieved on Nova-Pak C18 reverse phase column (4 ㎛, 3.9 mm×150 mm I.D., Waters, USA). Flow rate was operated isocratically at 0.4 ml/min. The mobile phase consisted of 50 mM ammonium acetate and acetonitrile (2:8, v/v). The mass spectrometer was run in the positive mode and selective ion monitoring mode focused on m/z = 837.5.

The extraction of roxithromyicin in poultry tissues was carried out by the liquid-liquid extraction with borate buffer (pH 9.0) and dichloromethane. In short, each 1 *g* muscle sample was added to 2 ㎖ of borate buffer and homogenized, and then shaken for 10 min. The homogenized sample was added with 2 ㎖ of dichloromethane and vortexed for 5 min. The samples were centrifuged at 1,300 *g* for 10 min, the lower phase being transferred into other tubes and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 1 ml of methanol and vortexed for 30 s. Aliquot of 10 ㎕ was injected after filtration.

Animals

The experiment was conducted in farms housing broilers of around 1 kg body weight. Roxithromycin was given for 7 days in drinking water at a dose rate of 15 mg/L (low dosage) and 60 mg/L (high dosage) to each broiler. Six broilers were taken at random and killed before the start of the experiment and 0, 1, 3, 5, 7 and 10 days after the last dose. Samples of liver, kidney, muscle, adipose tissue and serum were collected and stored in the freezer at -20℃ and allowed to thaw at room temperature before processing.

Data validations

Roxithromycin was used to prepare calibration curves in the range of 0.1 ng/ml~10 ng/ml and 10 ng/ml~10,000 ng/ml, respectively. Recovery and precision were evaluated in accordance with the guideline of residual analysis of veterinary drugs in National Vetrinary Research and Quarantine Service (NVRQS). Limit of detection and limit of quantitation were based on the signal-to-noise ratio based on their areas. The signal-to-noise ratio of 3 was accepted for the limit of detection and that of 10 for the limit of quantiation.

Results

Mass spectra of roxithromycin

The mass spectra of roxithromycin showed that [M+H]+ was the predominant ion (Fig. 2). Each relative abundance of adduct ions, [M+Na]+ or [M+K]+, was less than [M+H]+. The fragment ions were [M-desosamine + H]+, m/z = 679.5; [desosamine + H]+, m/z=419.3; [cladinose-OCH3 + H]+, m/z=115.1. These fragment ions were only detected with fragmentation voltage 100 V. Attempts to increase the abundance of these ions with even high fragmentation voltages resulted in lower molecular weight fragments.

Data validations

As a result of analysis of blank muscle samples, matrix interferences were not detected (Fig. 3) .The suspected peak of roxithromycin was shown about 6.5 min and increased in proportion to concentrations. The liner regression line for roxithromycin showed high correlation coefficients of 0.999. Limit of quantitaion and limit of detection was 5 ng/g and 1 ng/g, respectively. Precision and recovery are shown in Table 1 and was satisfied with the guideline of NVRQS.

Residue concentration in various tissues

Residue concentrations were associated with administered dose (Table 3 and Table 4). At the termination of treatment, roxithromycin was found in all collected samples for both dose groups. Liver was detected to have the highest residual concentration of roxithromycin, followed by skin, kidney, serum, adipose tissue and muscle. Residue concentrations of roxithromycin were lower than its LOQ in all tissues from both dose groups 10 days after the treatment

Discussion

The highly sensitive and specific method for the determination of roxithromycin in the broiler tissues by LC-MS has been established. The limit of detection and limit of quantitation were 1 ng/g and 5 ng/g. These values satisfied the acceptance criteria of the limit of detection and limit of quantitation. The LOQ of this method is more sensitive than other HPLC methods previously reported [2, 2, 3, 11, 13, 14, 15]. South Korea has already set the maximum residue limits (MRLs) for macrolide antibiotics in edible tissues of food-producing animals. The MRLs of erythromycin and tylosin are 0.1 ㎎/kg in cattle and pigs. In case of poultry, those are 0.125 ㎎/kg for erythromycin and 0.1 ㎎ /kg for tylosin. However, there is no legislative framework controlling the use of roxithromycin at the moment. LOD and LOQ in the present studies for roxithromycin were much lower than the MRLs set by the South Korea for other macrolides.

Marcrolides are among the safest antibiotics for the treatment of mild-to-moderate community-acquired bacterial infections. Additionally, roxithromycin was regarded as a safe antibiotic compared to erythromycin [8, 9]. Therefore, we assumed that the MRL of roxithromycin was 0.1 ㎎/kg for edible tissues for the calculation of withdrawal time. Due to high interindividual variablility observed in kinetic studies in broilers, statistical approach should be regarded as the method of first choice for the calculation of the withdrawal time, it is important to establish a withdrawal time that guarantees consumer safety. In this study, the withdrawal time of roxithromycin was estimated by the linear regression analysis of the log-transformed tissue concentrations, and was determined at the time when the

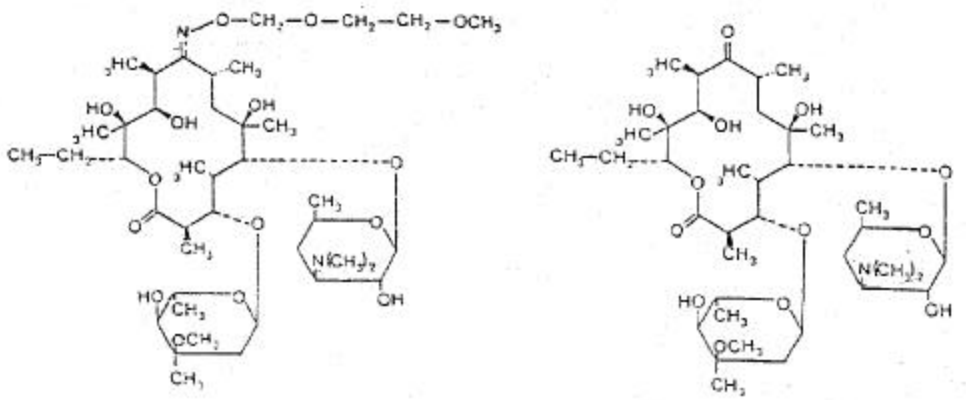

Fig. 1. Structure of roxithromycin (left) and erythromycin (right).

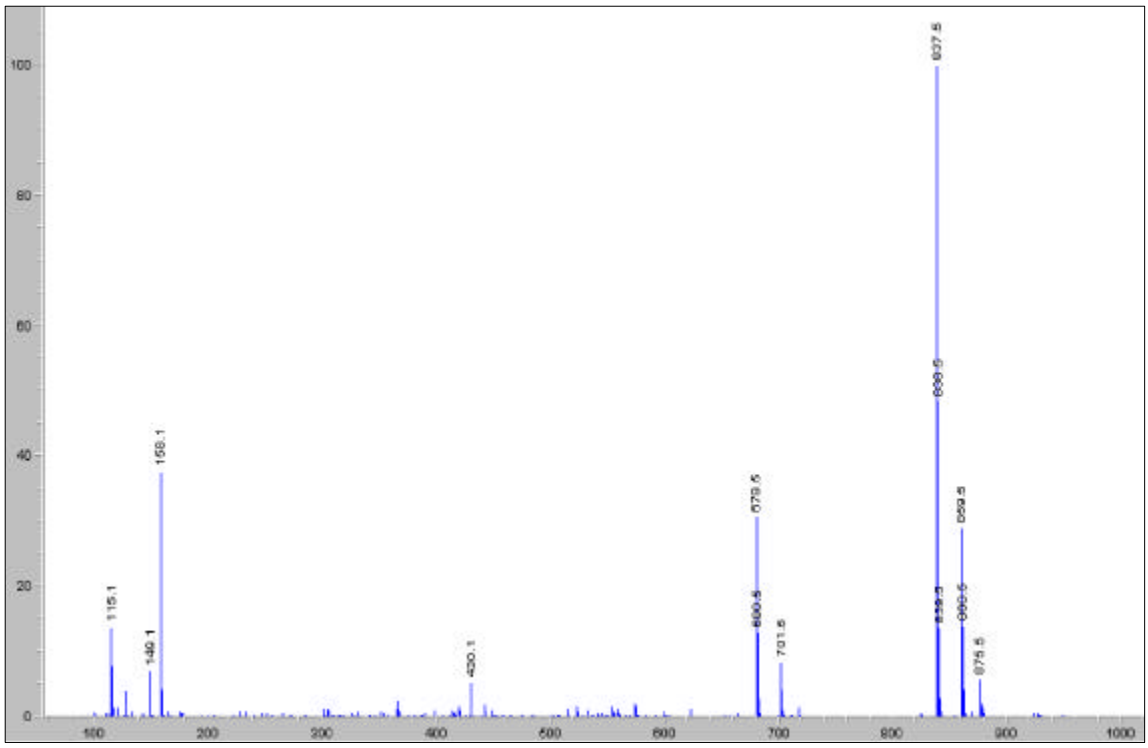

Fig. 2. Representative mass spectrum of roxithromycin (as scan mode from *m/z* 100 to *m/z* 1000).

upper on sided tolerance limit, with a confidence of 95%, was below the MRLs [16]. Roxithromycin was then depleted from edible tissues of broiler up to the concentration of 0.01 ㎎/kg at 5 days after treatment. The withdrawal time of roxithromycin using the statistical method for the calculation of withdrawal times as adopted by Committee for Veterinary Medicinal Products (CVMP) was 4.47 ± 1.18 days in edible tissues of broilers after treatment of roxithromycin mixed with drinking water (15 mg/L) for 7 days.

The LC-MS method has solved previous problems existing in both microbiological and HPLC methods for roxithromycin in biological matrixes [1, 2, 3, 5, 11, 13, 14, 15]: specificity, limit of detection, accuracy, etc. Determination of roxithromycin by HPLC with UV detector has been developed [2, 11, 14], but these methods are difficult to detect roxithormycin due to its weak UV absorbance. The fluorimetric detection with precolumn derivatization procedures requires long separation times and is less sensitive than LC/MS. In addition, fluorimetric detection is limited for simultaneous determination because of the different derivatization method of each drug [15]. Many researchers have reported the determination methods of erythromycin and roxithromycin using HPLC with electrochemical detector, which is more sensitive than UV detector [3, 5, 13]. But, these methods are

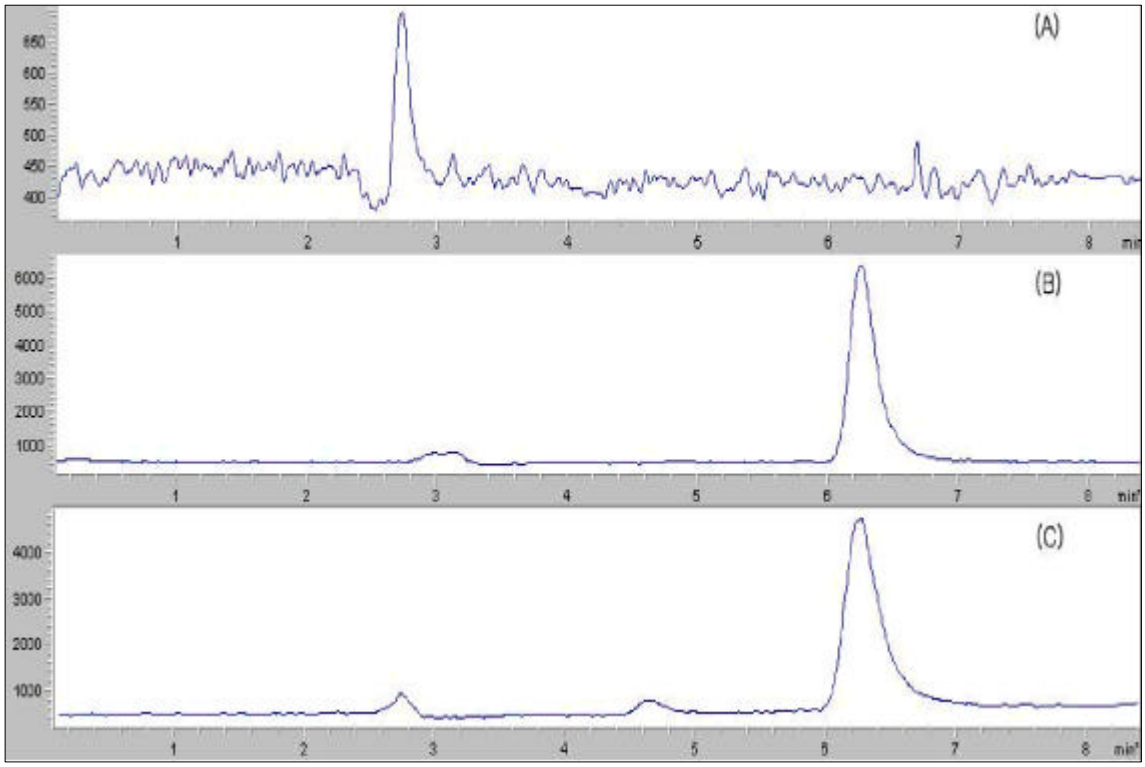

Fig. 3. Representativc total ion chromatogram (as SIM at m/z=837.5) of roxithromycin for blank muscle (A), standard solution with roxithromycin at 10 ng/g (B), and spiked muscle with roxithromycin at 10 ng/g (C).

Table 1. Recovery and precision of roxithromcyin in various tissues

Spiked tissues	Spiked conc. (μg/ml)	Detected conc. (μg/ml)	Recovery (%)	Precision (%)
Liver	0.01	0.074±0.002	74.4±5.6	7.5
Kidney	0.01	0.073±0.010	73.1±9.0	12.3
Serum	0.01	0.082±0.015	82.0±6.7	8.2
Muscle	0.01	0.075±0.006	75.1±8.7	11.6
Skin	0.01	0.075±0.016	75.1±8.3	11.1
Adipose tissue	0.01	0.070±0.021	70.3±7.3	10.47
Intestine	0.01	0.077±0.013	77.2±5.4	7.0

Table 2. Concentration of roxithromycin in various tissues obtained at different time points after treatment of roxithromcyin mixed with drinking water (15 mg/L) for 7 days

Mean ± S.D. (μg/g)

Tissues	0 day	1 day	3 day	5 day	10 day
Liver	0.74±0.53	0.32±0.10	0.07±0.05	0.01±0.0	−
Kidney	0.30±0.11	0.15±0.10	0.02±0.04	−	−
Small intestine	0.05±0.02	0.03±0.02	0.01±0.03	−	−
Skin	0.79±0.24	0.32±0.18	0.05±0.02	−	−
Muscle	0.11±0.07	0.08±0.05	0.02±0.01	−	−
Adipose tissue	0.16±0.05	0.07±0.02	0.03±0.02	−	−
Serum	0.22±0.13	0.05±0.01	0.01±0.02	−	−

−, not detected or under LOQ.

Table 3. Concentration of roxithromycin in various tissues obtained at different time points after treatment of roxithromycin mixed with drinking water (60 mg/L) for 5 days

Mean ± S.D. (μg/g)

Tissues	0 day	1 day	3 day	5 day	10 day
Liver	2.98±0.85	1.08±0.54	0.08±0.02	0.02±0.01	−
Kidney	0.73±0.38	0.31±0.12	0.05±0.03	−	−
Small intestine	0.32±0.17	0.16±0.06	0.01±0.03	−	−
Skin	0.90±0.42	0.39±0.19	0.07±0.02	0.01±0.03	−
Muscle	0.42±0.30	0.210±0.13	0.06±0.01	0.02±0.03	−
Adipose tissue	0.55±0.42	0.16±0.15	0.04±0.03	−	−
Serum	0.56±0.09	0.15±0.05	0.04±0.01	−	−

−, not detected or under LOQ.

difficult to set up analytic condition because the determination methods by electrochemical detection are very sensitive to environmental condition.

In conclusion, LC/MS with electrospray is a simple, rapid and effective technique for the determination of roxithromycin in broiler tissues. The optimal withdrawal time of roxitrhomycin for edible tissues of broiler is suggested to be 7 days after treatment of roxithromycin mixed with drinking water at a dose rate of 15 mg/L.

References

1. **Barry, A. and Packer, R.** Roxithromycin bioassay procedures for human plasma, urine and milk specimens. Eur. J. Clin. Microbiol. 1986, **5(5)**, 536-540.
2. **Cachet, T., Kibwage, I., Roets, E., Hoogmartens, J. and Vanderhaeghe, H.** Optimization of the separation of erythromycin and related substances by high-performance liquid chromatography. J. Chromatogr. 1987, **409**, 91-100.
3. **Croteau, D., Vallee, F., Bergeron, M. and Lebel, M.** High-performance liquid chromatographic assay of erythromycin and its esters using electrochemical detection. J. Chromatogr. 1987, **419**, 205-212.
4. **Daisci, R., Palleschi, L., Ferretti, E., Achene, L. and Cecilia, A.** Confirmatory method for macrolide residues in bovine tissues by micro-liquid chromatography-tandem mass spectrometry. J. Chromatogr. A. 2001, **926(1)**, 97-104.
5. **Demotes-Mainaird, F., Vincon, G., Jarry, C and Albin, H.** Micro-method for the determination of roxithromycin in human plasma and urine by high-performance liquid chromatography using electrochemical detection. J. Chromatogr. 1989, **490(1)**, 115-123.
6. **EMEA.** Note for guidance: approach towards harmonization of withdrawal periods. 1995, EMEA/CVMP/036/95.
7. **Hwang, Y. H., Lim, J. H., Park, B. K. and Yun, H. I.** Simultaneous determination of various macrolides by liquid chromatography/mass spectrometry. J. Vet. Sci. 2002, **3(2)**,103-108.
8. **Kirst, H. A. and Sides, G. D.** New directions for macrolide antibiotics: structural modifications and in vitro activity. Antimicrob. Agents Chemother. 1989, **33(9)**, 1413-1418.
9. **Lassman, H. B., Puri, S. K., Ho, I., Sabo, R. and Mezzino, M. J.** Pharmacokinetics of roxithromycin (RU 965). J. Clin. Pharmacol. 1988, **28(2)**, 141-152.
10. **Lim, J. H., Jang, B. S., Lee, R. K., Park, S. C. and Yun, H. I.** Determination of roxithromycin residues in the flounder muscle with electrospray liquid chromatography-mass spectrometry. J. Chromatogr. B Biomed. Sci. Appl. 2000, **746(2)**, 219-225.
11. **Macek, J. Ptacek, P. and Klima, J.** Determination of roxithromycin in human plasma by high-performance liquid chromatography with spectrophotometric detection. J. Chromatogr. B Biomed. Sci. Appl. 1999, **723(1-2)**, 233-238.
12. **Markham, A. and Faulds, D.** Roxithromycin. An update of its antimicrobial activity, pharmacokinetic properties and therapeutic use. Drugs. 1994, **48(2)**, 297-326.
13. **Nilsson, L., Walldorf, B. and Paulsen, O.** Determination of erythromycin in human plasma, using column liquid chromatography with a polymeric packing material, alkaline mobile phase and amperometric detection. J. Chromatogr. 1987, **423**, 189-197.
14. **Stubbs, C., Haigh, J. and Kanfer, I.** Determination of erythromycin in serum and urine by high-performance liquid chromatography with ultraviolet detection. J. Pharm. Sci. 1985, **74(10)**, 1126-1128
15. **Torano, J. S. and Guchelaar, H. J.** Quantitative determination of the macrolide antibiotics erythromycin, roxithromycin, azithromycin and clarithromycin in human serum by high-performance liquid chromatography using pre-column derivatization with 9-fluorenylmethyloxycarbonyl chloride and fluorescence detection.Volume. J. Chromatogr. B Biomed. Sci. Appl. 1998, **720 (1-2)**, 89-97
16. **Zhong, D., Chen, X., Gu, J., Li, X. and Guo, J.** Applications of liquid chromatography-tandem mass spectrometry in drug and biomedical analyses. Clin. Chim. Acta. 2001, **313(1-2)**, 147-50.