ELSEVIER

Journal of Chromatography B, 746 (2000) 219–225

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of roxithromycin residues in the flounder muscle with electrospray liquid chromatography–mass spectrometry

Jong-hwan Lim, Beom-su Jang, Rae-kyung Lee, Seung-chun Park, Hyo-in Yun*

Division of Veterinary Pharmacology and Toxicology, Chungnam National University, 220 Kung-dong, Yusong-ku, Taejon, South Korea

Received 23 December 1999; received in revised form 12 May 2000; accepted 31 May 2000

Abstract

A highly sensitive and specific method for the determination of roxithromycin in the flounder muscle by LC–MS was developed and validated. A dichloromethane extract of the sample was separated on C_{18} reversed-phase column with acetonitrile–50 m*M* 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 0.01 and 0.1 ng/g, respectively. Mean recoveries from spiked muscles were 81.1% (ranged from 71.0 to 90.3%) for roxithromycin. The method has been successfully applied to determine roxithromycin in the flounder muscle.

Keywords: Roxithromycin

1. Introduction

Roxithromycin is a semisynthetic macrolide antibiotic derived from erythromycin [1]. Roxithromycin was reported to be absorbed rapidly with the long elimination half time, giving higher plasma levels than erythromycin [2]. Therefore, it can be effective at lower doses with less frequent administration, which is regarded as an advantage in clinical settings. Due to these advantages, it could be applied in human and veterinary medicine [3].

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 quantitation of roxithromycin or other macrolides. UV absorption [5–7], fluorescence [8] and electrochemical detection [9–13] 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 were not suitable to determine low levels of roxithromycin in the biological fluid.

The high-performance liquid chromatography (HPLC) coupled to electrospray mass spectrometric detector could be a powerful technique for separation, identification and quantitation of roxit-

*Corresponding author. Tel.: +82-42-821-6759.

E-mail address: hiyun@hanbat.chungnam.ac.kr (H.-i. Yun).

0378-4347/00/$ – see front matter
PII: S0378-4347(00)00334-0

hromycin. In this study, we developed rapid and sensitive method to determine roxithromycin in the flounder muscle with electrospray LC–MS.

2. Experimental

2.1. Chemical

Roxithromycin was given by Shin-il Chemical (Seoul, Korea). The structural formulae of roxithromycin and erythromycin are shown in Fig. 1.

HPLC grade water, methanol, acetonitrile and dichloromethane were purchased from TEDIA (Fairfileld, OH, USA). Reagent grade ammonium acetate, sodium borate, sodium hydroxide were purchased from SIGMA (St. Louis, MO, USA).

2.2. Instrumentation and chromatographic conditions

Samples were analyzed on a Hewlett-Packard LC/MSD system. It consisted of a G1322A degasser, a G1312A binary pump, a G1315A photo-diode-array detector, 59987A electrospray interface and 5989B mass spectrometer. Separation was achieved on Nova-Pak C_{18} reverse phase column (4 μm, 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 m*M* ammonium acetate and acetonitrile (2:8, v/v).

The pressure of nebulizer was 40 p.s.i. and heated to 350°C. The quadrupole was heated to 100°C. The mass spectrometer was run in the positive mode and selective ion monitoring mode. Since $[M+H]^+$ forms gave the strongest signals, the mass spectrometer was focused on $m/z=$ 837.5 with peak width of 0.12 ms per ion (Fig. 2).

2.3. Sample preparation

Collected fish muscles were stored in the freezer at −20°C and allowed to thaw at room temperature before processing.

Each 1 g muscle sample was added to 2 ml of borate buffer (pH 9.0) and homogenized. Borate buffer was consisted of 0.1*M* boric acid and 0.1*M* potassium chloride and was adjusted with 0.1*M* NaOH to pH 9. Two ml of dichloromethane was added to the homogenates, which was stoppered and shaken for 10 min. The samples were centrifuged at 1300 *g* for 10 min, the upper layer being discarded. The organic phase was transferred into another tube and evaporated to dryness at 30°C under a stream of nitrogen. The residue was reconstituted with 1 ml of acetonitrile and vortexed for 10 s.

2.4. Validation

2.4.1. Calibration curves

Roxithromycin was prepared to construct calibration curves in the range of 0.1–10 ng/g and 10–10 000 ng/g, respectively.

2.4.2. Specificity

The lack of matrix interference was established by

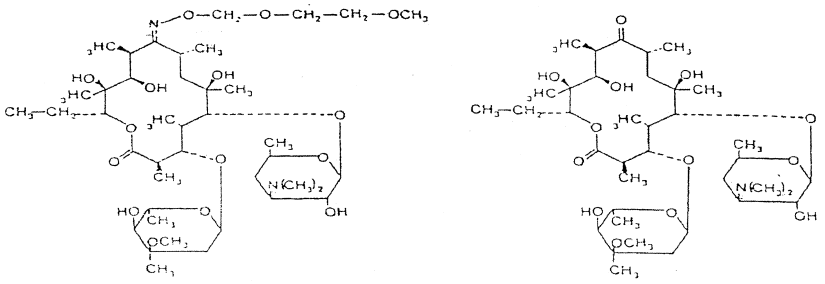

Fig. 1. Structure of roxithromycin (left) and erythromycin (right).

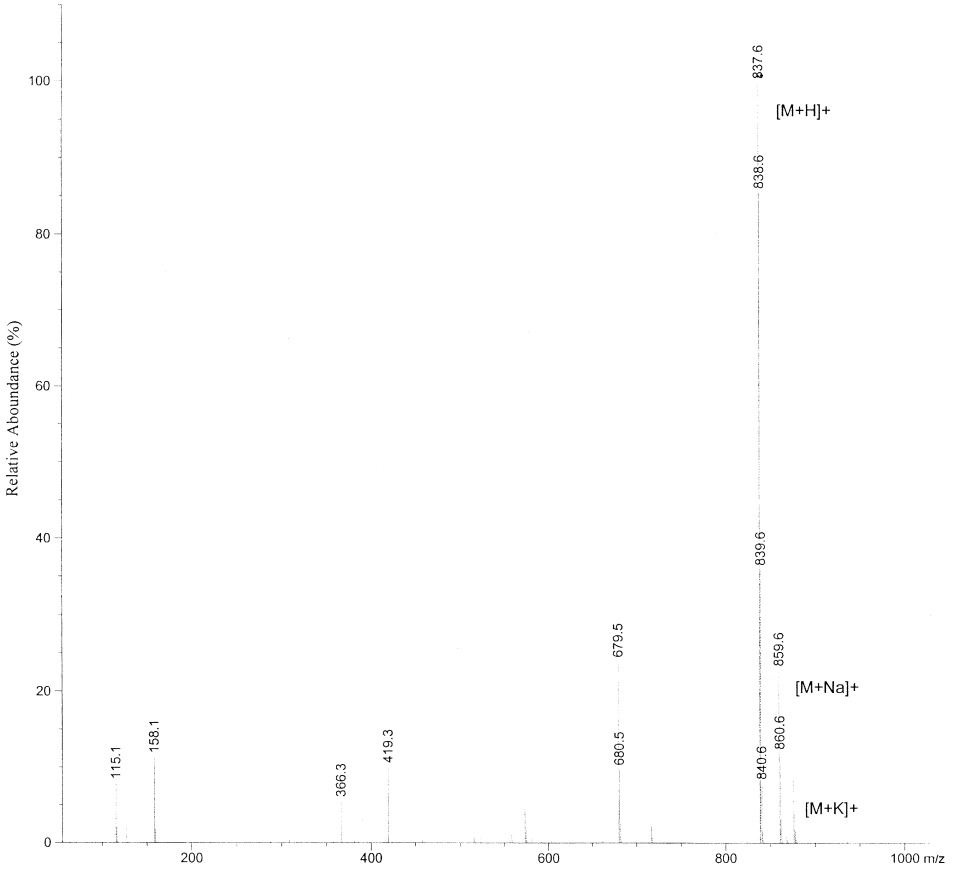

Fig. 2. Representative mass spectrum of roxithromycin (as scan mode from m/z 100 to m/z 1000).

the analysis of blank muscle samples ($n=6$). The chromatograms were visually inspected for peaks from endogenous substances. Except for [M+H]+, other fragment ions ($m/z=679.5$, $m/z=158.1$, $m/z=115.1$) were detected for confirmation of roxithromycin.

2.4.3. Accuracy and precision

Roxithromycin was spiked in a blank muscle samples (0.1 ng/g, 10 ng/g, 1 μg/g) and assayed to determine the accuracy expressed as mean relative error (R.E.) and the precision expressed as coefficient of variation (C.V.). Each sample was assayed to determine the inter-day accuracy and precision of roxithromycin.

2.4.4. Recovery

The recovery of roxithromycin was assessed in triplicate determinations at spiked muscles (0.1 ng/g, 10 ng/g and 1 μg/g).

The responses from the spiked sample were compared with those from the blank muscle sample.

2.4.5. Limit of quantitation

Limit of detection and limit of quantitation were determined 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 quantitation.

3. Results

The mass spectra of roxithromycin showed that $[M+H]^+$ was the predominant ion (Fig. 2). Each

relative abundance of adduct ions, $[M+Na]^+$ and $[M+K]^+$, was less than 5% of $[M+H]^+$. The fragment 1 ion of roxithromycin (m/z=679.5, $[M-$ desosamine$+H]^+$) is corresponding to the loss of sugar moiety from $[M+H]^+$. The fragment 2 ion (m/z=419.3) is 20 membered macrolide ring with the loss of sugar moieties and etheroxime side chain from roxithromycin. The fragment 3 ion (m/z= 158.1, [desosamine–$OH+H]^+$) is corresponding to desosamine, a sugar moiety in roxithromycin. The fragment 4 ion (m/z=115.1, [cladinose–$OCH_3+H]^+$) is another sugar moiety in roxithromycin. 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. Roxithromycin eluted from the analytical column with a retention time of 5.6 min.

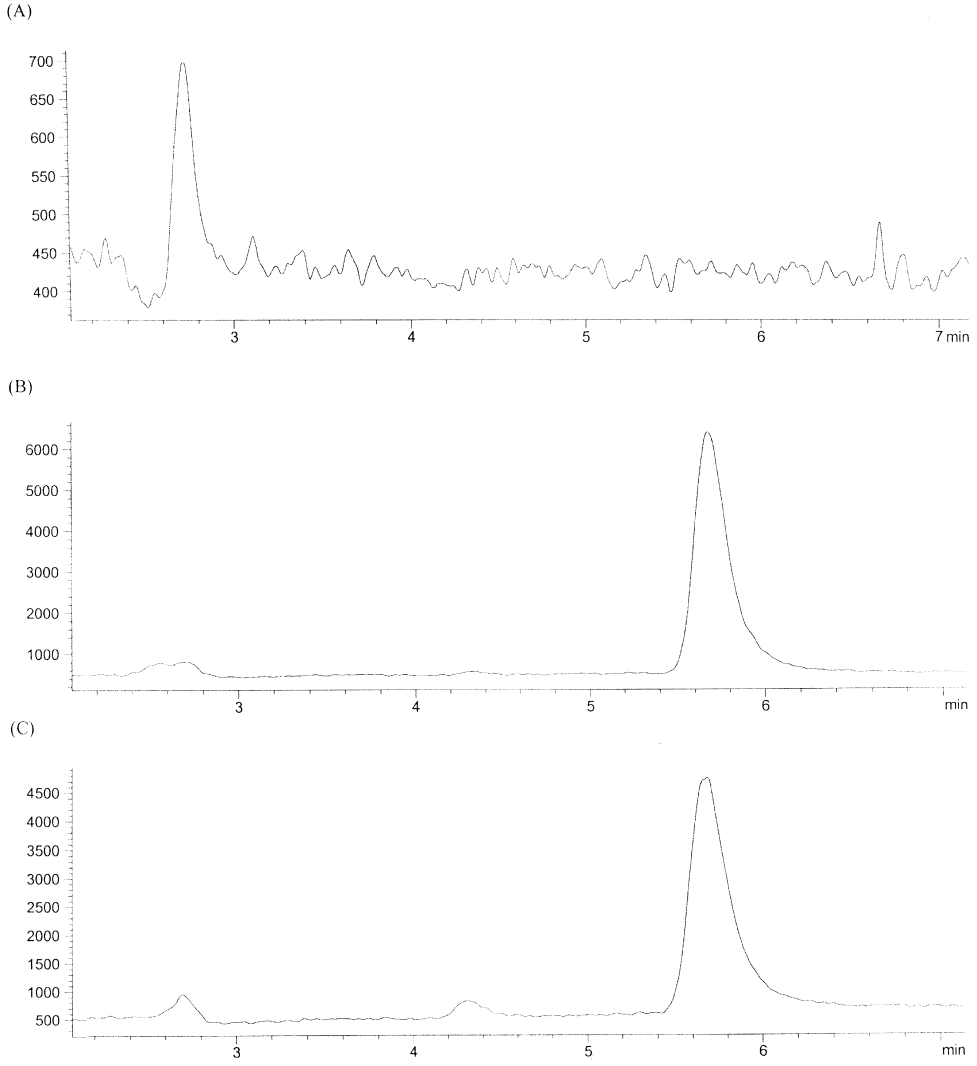

Fig. 3. Representativc total ion chromatogram (as SIM at m/z=837.5) of roxithromycin for blank flounder muscle (a), standard solution with roxithromycin at 1 ng/g concentration (b), and spiked flounder muscle with roxithromycin at 1 ng/g concentration.

Table 1
Reproducibility on the calibration curve

Nominal concentration (ng/ml)	Observed concentration (ng/ml) Mean±SD	C.V. (%)	R.E. (%)
0.1	0.1±0.01	4.0	−4.3
1	1.1±0.08	2.6	6.2
10	10.3±0.7	6.24	5.4
Nominal concentration (μg/ml)	Observed concentration (μg/ml) Mean±SD	C.V. (%)	R.E. (%)
0.1	0.1±0.01	7.6	−5.6
1	1.0±0.03	3.3	2.4
10	10.0±0.4	4.0	−2.8

3.1. Specificity

As a result of analysis of blank muscle samples, matrix interference was not detected (Fig. 3). The suspected peak of roxithromycin was shown about 5.6 min and increased in proportion to concentrations. Electrospray mass spectrometry is a suitable method for determination of roxithromycin in the flounder muscle.

3.2. Linearity and reproducibility

The linear regression line for roxithromycin in the range of 0.1–10 ng/g and 10–10 000 ng/g showed high correlation coefficients (r) of 0.999 and 0.996, respectively. The C.V. and R.E. of observed at 0.1 ng/g were 4.0 and 4.3%, respectively. The C.V. and R.E. at 1–10 000 ng/g were not more than 8% and within ±6%, showing high reproducibility (Table 1).

Table 3
Recovery of roxithromycin

	Spiked concentration (ng/ml)	Recovery (%) Mean±SD
N=8	0.1	70.9±14.5
N=12	10	82.0±9.1
N=9	1000	90.2±12.1

3.3. Accuracy and precision

Limit of quantitation and limit of detection was 0.1 and 0.01 ng/g, respectively. Precision and accuracy are shown in Table 2. The C.V. at 0.1, 10 and 1000 ng/ml ranged from 4.3 to 10.7%, and the R.E. ranged from −4.5% −25.7% for intra-day. The C.V. at 0.1, 10 and 1000 ng/ml ranged from 7.5 to 31.7%, and the R.E. ranged from −15.4 to −24.6%.

Table 2
Accuracy and precision of roxithromycin in the flounder muscle

	Target conc. (ng/ml)	Detected concentration (ng/ml) Mean±SD	C.V. (%)	R.E. (%)
Intra-day	0.1	0.07±0.01	10.7	−25.7
(n=7)	10	8.87±0.37	4.3	−10.2
	1000	994.4±80.0	8.0	−4.6
Inter-day	0.1	0.07±0.02	31.6	−24.6
(n=18)	10	7.65±0.64	8.4	−19.0
	1000	811.3±60.9	7.5	−15.4

3.4. Recovery

The recovery mean of roxithromycin in the flounder muscle was 70.4±14.5% for 0.1 ng/g samples, 81.4±9.1% for 10 ng/g samples, 90.3±12.1% for 1 μg/g samples (Table 3).

4. Discussion

The highly sensitive and specific method for the determination of roxithromycin in the flounder muscle by LC–MS has been established. The limit of detection and limit of quantitation were 0.01 ng/g and 0.1 ng/g, respectively. These values satisfied the acceptance criteria of the limit of detection and limit of quantitation. The C.V. for LOQ and LOD were 28.3 and 21.9%, respectively. The LOQ of this method is more sensitive than other HPLC methods previously reported [5–13].

The LC–MS method has solved previous problems existing in both microbiological and HPLC methods for roxithromycin in biological matrixes [4–13]: specificity, limit of detection, accuracy, etc.

The proposed method was applied to the determination of roxithromycin in the flounder muscle for the estimation of withdrawal time. The experiment was conducted in water tanks housing flounders of around 1 kg body weight. The temperature was maintained at 18°C, an optimum temperature for flounders. Roxithromycin was given for 7 days as a mixed feed at a daily dose rate of 7.2 mg/kg b.w. and 28.8 mg/kg b.w. to each flounder. Seven flounders were taken at random and killed before the start of the experiment and 0, 1, 5, 10, 20, 40 days after the last dose.

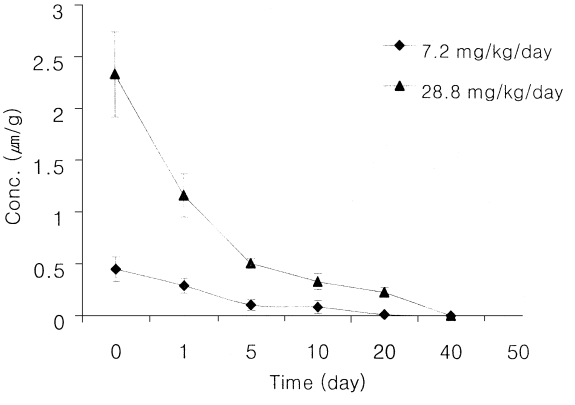

Fig. 4. Muscle concentration vs. depletion time curve obtained from flounder muscle after oral administration of roxithromycin for 7 days as a mixed feed at a dose rate of 7.2 mg/kg/day and 28.8 mg/kg/day to each flounder.

Fig. 4 shows the representative muscle concentration vs. depletion time curve after oral administration of roxithromycin for 7 days as a mixed feed at a dose rate of 7.2 mg/kg b.w. and 28.8 mg/kg b.w. to each flounder. Muscle concentrations of roxithromycin after treatment were declined in the course of time and not detected in 40 days after treatment. The method has been successfully applied to determine roxithromycin concentration in the flounder muscle for the estimation of the withdrawal time.

5. Conclusion

LC–MS is a simple, rapid and effective technique for the determination of roxithromycin in the flounder muscle. Chromatographic separation and method development time are minimized when confirming the molecular identities of the target substance. The precision and accuracy developed in this method are suitable and sensitive to determine the concentration of roxithromycin for establishing its withdrawal time in flounders.

References

[1] A. Markham, D. Faulds, Drug 48 (1994) 297.
[2] H. Lassman, S. Puri, I. Ho, R. Sabo, M. Mezzino, J. Clin. Parmacol. 28 (1988) 141.
[3] H. Kirst, G. Sides, Antimicrob. Agents. Chemother. 33 (1989) 1419.
[4] A. Barry, R. Packer, Eur. J. Clin. Microbiol. 5 (1986) 536.
[5] T. Cachet, I. Kibwage, E. Roets, J. Hoogmartens, H. Vanderhaeghe, J. Chromatogr. 409 (1987) 91.
[6] C. Stubbs, J. Haigh, I. Kanfer, J. Pharm. Sci. 74 (1985) 1126.
[7] J. Macek, P. Ptacek, J. Klima, J. Chromatogr. B 723 (1999) 233.

[8] J. Torano, H. Guchelaar, J. Chromatogr. B 720 (1998) 89.
[9] M. Chaem, W. Chiou, J. Chromatogr. 278 (1983) 91.
[10] D. croteau, F. Vallee, M. Bergeron, M. Lebel, J. Chromatogr. 419 (1987) 205.
[11] G. Duthu, J. Liq. Chromatogr. 7 (1984) 1023.
[12] L. Nilsson, B. Walldorf, O. Paulsen, J. Chromatogr. 423 (1987) 189.
[13] F. Demotes-Mainaird, G. Vincon, C. Jarry, H. Albin, J. Chromatogr. 490 (1989) 115.