Asian Journal of Chemistry; Vol. 24, No. 7 (2012), 3064-3066

ASIAN JOURNAL OF CHEMISTRY

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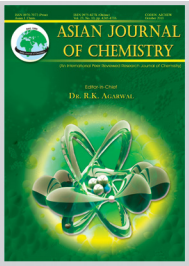

High Performance Liquid Chromatographic Determination of Azithromycin in Animal Feed

SUYING MA* and XIAOJUN SHANG

School of Pharmacy, Xinxiang Medical University, Xinxiang 453003, Henan, P.R. China

*Corresponding author: Fax: +86 373 3029879; Tel: +86 373 3029879; E-mail: suyingma@163.com

(*Received*: 6 July 2011; *Accepted*: 13 February 2012) AJC-11066

A simple, precise, accurate and rapid high-performance liquid chromatography (HPLC) method was developed and validated for the determination of azithromycin in feed. The analyses were separated by the mobile phase 0.1 mol/L KH_2PO_4 (pH 6)-acetonitrile (70:30, v/v) with an SinoChoom ODS-BP C_{18} column (5 μm, 4.6 mm × 200 mm) at a flow rate of 1 mL/min, column temperature 35 ºC and detection wavelength 210 nm. A good linear relationship was obtained in the concentration range 0.5-50 mg/mL (r = 0.9993). The limit of quantification (LOQ) was 100 μg/g and the limit of detection (LOD) was 50 μg/g. Average recoveries ranged from 75.8 to 89.7 % in feed at the concentrations of 0.1-10 mg/g. Intra and interday relative standard deviations were less than 1.6 and 1.4 %, respectively. This HPLC method is simple, reproducible and accurate for determination of azithromycin in feed.

Key Words: Azithromycin, HPLC, Determination, Feed.

INTRODUCTION

The emergence of superbugs such as methicillin-resistant *Staphylococcus aureus* (MRSA) belonging to bacterial skin is causing the growing problem of hospital-acquired infections. It is difficult for common antibiotics to kill the superbugs. It will lead to sepsis, pneumonia, life-threatening complications for pregnant women, old people and young children once infected with this virus. The part reasons of superbug appearance should be attributed to antibiotics abuse.

Azithromycin[1] chemically, (2R,3S,4R,5R,-8R,10R,11R, 12S,13S,14R)-13-[(2,6- dideoxy-3-C-methyl 3-O-methyl-a-L-ribo-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6, 8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethyl-amino) β-D-xylo-hexopyranosyl]oxy]-1-oxa-6-aza-cyclopentadecan-15-one, is a macrolide antibiotic derived from erythromycin. It plays a leading role in the treatment or prophylaxis of several diseases such as opportunistic infections in AIDS, toxoplasmosis, pediatric infections and respiratory tract infections.

Azithromycin wasn't listed in animal antibotics documents in animal husbandry according to the Agriculture Ministry of China. The illogical application and misuse of it will bring about the residues in food. But the application of azithromycin in animal production is not uncommon. For example azithromycin was widely used in animal husbandry and fishery for disease prophylaxis, the residue of azithromycin is easy to cause the appearance of drug resistant strains, so it's necessary to develop a method to determine azithromycin in animal feed[2].

High-performance liquid chromatography (HPLC) with UV detection or fluorescence detection has been widely used to quantitate azithromycin in biological liquids and preparations, but few methods have been developed for the estimation of azithromycin in animal feed[3-11]. The aim of this study was developing and validating a simple, rapid, sensitive and reproducible HPLC method to determine azithromycin in animal feed.

EXPERIMENTAL

High-performance liquid chromatography was performed on Shimadzu HPLC system consisting of LC-20AT pump, SPD 20A UV-Visible absorbance detector, Shimadzu Spin Chrome software with SinoChoom ODS-BP C_{18} column (5 μm, 4.6 mm × 200 mm). Sample injection was performed *via* a Rheodyne syringe. Pure azithromycin were obtained from Yonghe Pharmaceuticals Co. Ltd., Zhengzhou, P.R. China. Acetonitrile of HPLC grade and other chemicals of AR grade were obtained from bafang Chemicals Co. Ltd., Zhengzhou, P.R. China. Deionized water was obtained by using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Chromatographic conditions: The mobile phase was a mixture of 0.1 mol/L KH_2PO_4 (pH 6.0)-acetonitrile (70:30, v/v). The flow rate was 1.0 mL/min. The mobile phase was filtered before use through 0.45 μ membrane filter and degassed for 20 min. The eluents were monitored at 210 nm. The injection volume was 20 μL.

Preparation of standard stock solution and working solution: Standard solution of the pure drug was prepared by dissolving accurately 1250 mg azithromycin in a 25 mL volumetric flask using 20 mL mobile phase. Then the volume was made up to the mark with mobile phase, the obtained concentration is 50 mg/mL. Intermediate and working solutions were prepared by diluting stock solutions with the mobile phase. Calibration standard solutions were prepared at concentrations of 0.5, 1.0, 2.5, 5.0, 12.5, 25.0 and 50.0 mg/mL for azithromycin and injected in replicates of three. The peak area of drug concentration was calculated. The regression of the drug concentration over the peak areas was obtained.

Preparation of sample: Complete formula feed purchased from a local feed additive and animal medicine market was grounded into a homogeneous sample using a mortar. This material was then kept at -4 ºC. Blank feed sample (2 g) was added into 20 mL centrifuge tubes. 100 µL azithromycin work solutions were spiked to each tubes. The homogenized fortified sample was extracted with 5 mL chloroform. The mixture was allowed to stand for 5 min in ultrasonic bath, followed by centrifugation at 3000 rpm for 5 min. The residue was extracted again with 5 mL chloroform. 5 mL of the combined organic layer was evaporated just to dryness at 40 ºC under a stream of nitrogen. The residue was dissolved in 100 µL mobile phase and vortex mixed. 20 µL obtained solution was injected into the HPLC system.

RESULTS AND DISCUSSION

Specificity: According to the ultraviolet spectroscopy, azithromycin has maximum absorbance at 210 nm. Thus, 210 nm was selected as detecting wavelength, under the optimum conditions. Typical chromatograms of azithromycin solutions, blank feed sample and equivalent spiked to a level of 100 µg/g with azithromycin are shown in Fig. 1. The effective baseline separation of azithromycin was observed for spiked feed samples. The chromatograms showed that the components in the feed did not disturb the detection of azithromycin.

Regression equation: Under the optimal conditions, the regression equation A = 17.287C + 9.5154 of azithromycin was established based on the standard samples injected and their peak area in the concentration range of 0.5-50.0 mg/mL with correlation coefficient of 0.9993, where A is peak area based on three parallel measurements and C is the concentration (mg/mL) of azithromycin standard solution.

Limit of detection and limit of quantitation: A series of different concentrations drug solutions were added into blank feed samples. Samples were then prepared by the described procedure. The limit of detection (LOD) are calculated by the ratio of signal to noise (S/N = 3), the lowest level that gave reasonable accuracy and precision was considered to be the limits of quantitation (LOQ). The LOD and LOQ for azithromycin were found to be 50 µg/g and 100 µg/g.

Recovery and precision: The recovery test was carried out by adding azithromycin to the feed samples (three different concentrations of markers: 0.1, 0.5 and 5.0 mg/g). The sample was prepared as above the described procedure and injected for HPLC analysis to calculate the amount of the azithromycin founded. Recovery data from the study was reported in Table-1. The data showed that the recovery of azithromycin ranged from 83.12 to 96.43 %. The measurements of intra and interday variability were utilized to determine the precision of the developed method. The results shown that the relative standard derivations (RSD) of intraday variations was less than 1.6 % and the RSD of interday variations were less than 1.4 %. This method is of high precision.

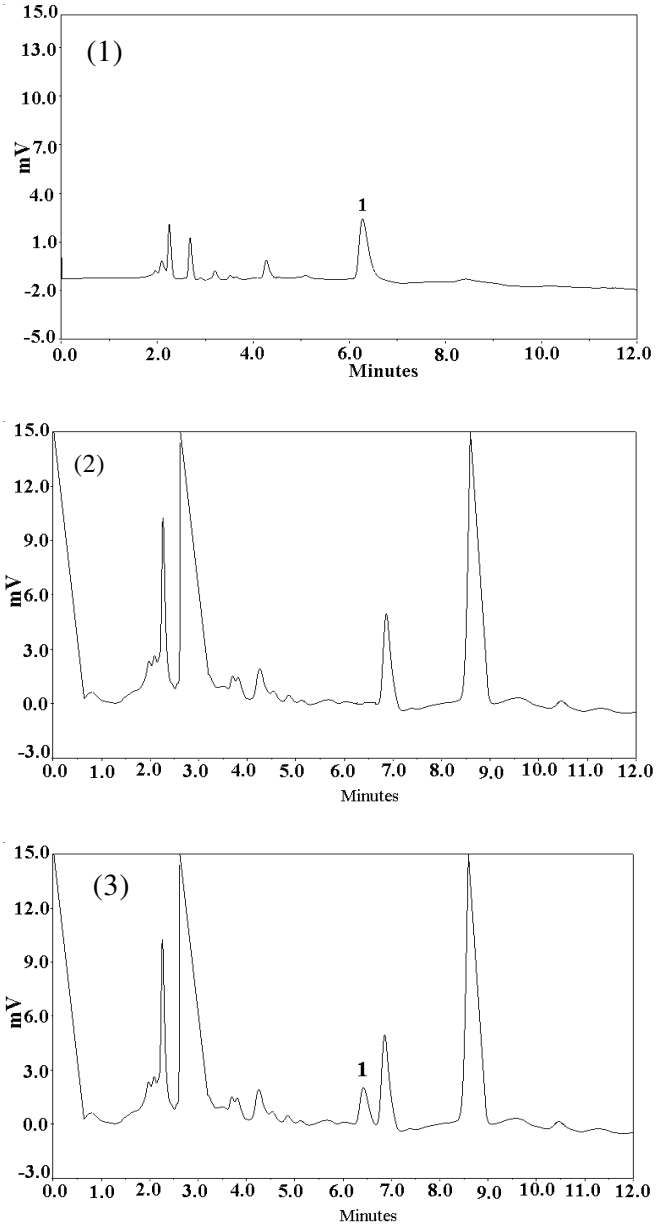

Fig. 1. Chromatograms of azithromycin (1); blank feed sample (2); blank feed spiked with azithromycin (3)

TABLE-1
INTRA AND INTERDAY PRECISION STUDIES

Drug	Concentration (µg/g)	Intra-day		Inter-day	
		Mean*	RSD (%)	Mean*	RSD (%)
Azithromycin	100	85.10	1.51	90.08	1.33
	500	452.05	1.48	455.35	1.07
	5000	4780.11	1.42	4823.64	1.12

*Mean of five determinations

Selection of extraction solvent: Azithromycin were soluble in organic solvent, the main extraction solvents included methanol, chloroform and acetone. The matrix of feed samples is relatively complicated. The use of chloroform as extraction reagent provided minor impurities for its good degreasing effect and lower boiling point. The extraction effect of chloroform, methanol and acetone had been evaluated. After the comparation, it had been found that chloroform gives the least impurities. Only 5 mL of the total 10 mL combined organic layer was evaporated for further analysis, the operation reduced the impurities from the feed samples remarkably and it also shortened the analysis time.

Conclusion

In this work, the use of chloroform as an extraction reagent is effective for extraction of azithromycin in animal feed. The method has some excellencies, such as higher recovery, lower detection limit, effective baseline separation, lesser reagent to be required. The limit of quantification was 100 µg/g with good intra and interday precision. It is one most rapid method for chromatographic analysis of azithromycin in animal feed sample. In conclusion, this method is rapid, high sensitive and provides good reproducibility and accuracy for the quantification of azithromycin in animal feed sample.

ACKNOWLEDGEMENTS

The authors acknowledged the financial support from Xinxiang Medical University.

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