

An LLE Based LC- ESI MS/MS Analytical Method Development to Detect Azithromycin Residue in Water to Monitor Contamination Level of River and Fish Farm of Bangladesh

Md Humayun Kabir (✉ h.kabir79@yahoo.com)

Bangladesh Council of Scientific and Industrial Research

Sabina Yasmin

Bangladesh Council of Scientific and Industrial Research

Salma Akter Mou

Bangladesh Council of Scientific and Industrial Research

Bushra Parvin Upoma

Bangladesh Council of Scientific and Industrial Research

Shamim Ahmed

Bangladesh Council of Scientific and Industrial Research

Golam Azam

Bangladesh Council of Scientific and Industrial Research

Tajnin Jahan

Bangladesh Council of Scientific and Industrial Research

Md Saidul Islam

Bangladesh Council of Scientific and Industrial Research

Mohammad Moniruzzaman

Bangladesh Council of Scientific and Industrial Research

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Abstract

The aim of this study to development and validate an analytical method to determine the Azithromycin (AZN) residue in water approach to monitor the contamination level of river and fish farm water of Bangladesh. Azithromycin (AZN) was analyzed using liquid chromatography mass spectrometry (LC-ESI MS/MS). The chromatographic separations of analyte were performed using ZORBAX RRHD Eclipse Plus C18 (2.1×100 mm, particle size 1.8 μm) column and mobile phase was 0.1% formic acid in water and acetonitrile with 50:50. The analyte were detected in positive electron spray ionization mode with multiple reaction monitoring mode (MRM) with mass transition from 749.5 *m/z* to 591 *m/z* and 158 *m/z* as quantifier and qualifier ion respectively. Liquid-liquid extraction method was used for the extraction of AZN residues. The developed method was validated in terms of accuracy, precision, linearity and specificity. The analyte showed a good linear in the range of 0.1-100 μg/L. Three spiking levels (0.25, 0.5 and 1.25 μg/L) was performed for determining accuracy and precision. Recoveries and RSD were in the range of 96.6-101.5% and 3.5-6.3 respectively. The estimated limit of detection and limit of quantification was 0.017 and 0.05 μg/L respectively. Using the developed method, we analyzed 5 different rivers and 5 different fish farm samples. We found no azithromycin residue in rivers water, but we found in one fish farm water azithromycin residue 0.35±0.06 μg/L. The results indicate that we should be concern to use antibiotics in fish farm water in different ways.

Introduction

Azithromycin (AZM) is a macrolide antibiotic and a derivative of erythromycin. Erythromycine methyl substituted nitrogen at position 9 becomes AZM (Mutak, 2007; Xiang et al., 2020) (Fig. 1).

AZM is an antibiotic which is widely used in the treatment of bacterial infections such as streptococcal pharyngitis, pneumonia, diarrhea and intestinal inflammation (Talaiekhosani et al., 2020). It can also create an enhanced spectrum and potency against bacteria compared with other macrolides and superior stability in acidic media (Zeng et al., 2014). AZM not only can be used against atypical pathogen but also be effective against gram positive and gram-negative bacteria (Lalak and Morris, 1993; Retsema et al., 1990; Dunkin et al., 1988).

In this century, Major concerning issues are pharmaceuticals for their widespread use and frequent exposure in the water cycle. They can mix with water bodies from different ways, such as a human consumption, use of wastewater treatment plants sludge as fertilizers, hospitals effluents or unseemly disposal of unused or expired medicines and aquaculture farms (Da Le et al., 2021; Franchi et.al.2011). ("Occurrence and ecological risks of pharmaceuticals in a Mediterranean river in Eastern Spain – Science Direct," n.d.). After wreckage, these residue scan be excreted unchanged, or as metabolites, which might be more toxic substances than native compounds (Lalović et al., 2017). As a result, antimicrobial resistance is a great threat for human being which is caused by antibiotics residue in environment (Hendriksen et al., 2019). Due to the unregulated use of antibiotics in human and animals, lack of wastewater treatment, and poor sanitation, it is estimated that more than 40% death associated with

resistant pathogens in 2050 will be occurred in low and medium-income countries in Asia (Singh et al., 2019; Coyne et al., 2019; Kakkar et al., 2018).

The use of antibiotics in Bangladesh is increasing day by day (Angeles et al., 2020), they are not only limited to human healthcare but also widely used in animal husbandry (Roess et al., 2013). Poultry and dairy farming are a common practice in the rural area of Bangladesh, for the lack of knowledge sometimes high dose of antibiotics are applied. The use of antibiotics in animal farming not limit to treat bacterial related disease, but also to enhance the growth and to prevent infections (He et al., 2015; Jechalke et al., 2014; Grijalva et al. 2009). These antibiotics excreted and directly leaching to the water, sometimes the byproducts produced from these farming used as fish feed and there become a great source of water contamination ("Global trends in antimicrobial resistance in animals in low- and middle-income countries | Science," n.d.). So, the continuous impute of antibiotics in the aquatic environment may lead to an adverse effect on aquatic organism (Hernando et al., 2006). Up to 95% administrated antibiotics to human and animal may be excreted to environment without metabolized (Hirsch et al., 1999). In Bangladesh waste water treatment plant not designed yet to remove completely the antibiotics from the industrially produced waste water ("Strategic Survey of Therapeutic Drugs in the Rivers Po and Lambro in Northern Italy | Environmental Science & Technology," n.d.)(*Analysis, Removal, Effects and Risk of Pharmaceuticals in the Water Cycle*, 2007). As Bangladesh is a poor country, its healthcare system also very poor, and over all these the regulatory authorities cannot control the purchase and use of antibiotics both for animal and human, this situation becomes vulnerable for the country to control the antimicrobial resistant pathogens (Hossain et al., 2017; Hossain et al., 2018).

There are some reports already existing for AZM analysis in biological samples, including serum, tissues, and plasma. These methods followed bioassay, which are laborious and time consuming (Riedel et al., 1992). In some reports using High-performance liquid chromatography (HPLC) with ultraviolet detector (UVD) also described (Al-Rimawi and Kharaof, 2010; El-Gindy et al., 2011; Shaikh et al., 2008; Yang et al., 2009). Due to not specific UV chromophore, HPLC-UVD sensitivity not so good, to increase sensitivity, precolumn derivatization followed by fluorescence also used (Bahrami and Mohammadi, 2006; Bahrami et al., 2005). However, derivatization process is laborious, expensive, time consuming and also have the result reproducibility problem. The liquid chromatography couple with mass spectrum (LC-MS) and tandem mass spectrum which is (LC-MS/MS) (Chen et al., 2006; Xu et al., 2008; Shen et al., 2010) the most recent and advantage technique.

The aim of this study is to develop an LLE method to analyze AZM residue using LC-MS/MS, and to monitor the contamination level of river and fish farm water in Bangladesh. To the best of our knowledge there is no LLE method available to analyze AZM residue in water and also no study report yet has been reported about AZM contamination level of Bangladesh River and fish farm water.

Materials And Methods

Chemicals and reagents

Azithromycin (purity 99.5%) were collected from Radiant Pharmaceuticals Ltd, as a generous gift. LC-MS grade Methanol (MeOH), Acetonitrile (ACN), Dichloromethane (DCM) was purchased from AppliChem GmbH, Ottoweg, D-64291 Darmstadt, Germany. Analytical grade Ammonium acetate, Sodium Chloride, Magnesium sulphate was collected from SIGMA-ALDRICH, Germany.

Standard preparation

The stock solution of standard was prepared by weighing 10.01 mg Azithromycin standard, the standard was taken in a 100 mL volumetric flask (Duran group, Wertheim, Germany), and added MeOH up to the mark to make 100 ppm (mg/L) stock solution. The intermediate working solution of 10 ppm was prepared by diluting stock solution with the same MeOH solvent. To construct calibration curve serial dilution was done using same solvent to the concentration 100, 50, 10, 5, 1, 0.5, 0.1 ppb ($\mu\text{g/L}$). All the solution was kept at -4°C prior to analysis.

Sample collection

For method development Milli Q water was used as control sample, the river water was collected from The Padma, The Meghna, The Shitalakkha, The Gomati and The Buriganga river of Bangladesh, the fish farm water was collected from 5 different fish farms located at Chandpur a district of Bangladesh. All the samples are collected in February 2020. The samples were collected using 1 L glass bottle. The samples were carried to laboratory in the same day, using the ice box. Samples are stored in laboratory -4°C refrigerator until analysis.

Sample extraction

100 mL water sample was taken in a 500 mL separatory funnel (Duran group, Wertheim, Germany), 10 g NaCl was added to the water. 70 mL DCM was added to it. The separatory funnel was shaken strongly for 5 minutes with hand to mix the sample properly. Then the separatory funnel was kept untouched for 30 minutes to complete the partition of two aqueous and organic layer. The organic layer was collected in 250 mL round bottomed flask using a funnel where cotton was used to control the leakage of anhydrous Magnesium sulphate. Anhydrous Magnesium sulphate was used to dry/absorb aqueous moisture from organic part. The whole process was repeated using 30 mL DCM to complete the extraction. The collected organic part was dried using vacuum evaporator (Buchi) maintained temperature below 40°C . Then the sample was reconstructed using 10 mL MeOH. Around 1.5 mL was taken through filter using $40\ \mu\text{m}$ syringe filter and taken in 2 mL vial for LC-MS/MS analysis.

Instrumentation

An Agilent LC module (1290 Infinity II) coupled with triple quadruple mass spectrometer (6420LC/TQ) was used for sample analysis. A ZORBAX RRHD Eclipse Plus C18 (2.1X100 mm, $1.8\ \mu\text{m}$ particle size) was used for analyte separation. The binary mobile phase consists of 0.05 M Ammonium acetate in water: acetonitrile (ACN), 90:10 (A) and 0.05 M Ammonium acetate in water: Methanol (MeOH), 10:90 (B). A linear isocratic mobile phase of 70% A and 30% B was used with a total flow of 0.2 mL/minute. The

analyte was analyzed in LC-MS/MS positive electron spray ionization mode (ESI⁺). Multiple reaction monitoring (MRM) with mass transition from 749.5 *m/z* to 591 *m/z* and 158 *m/z* as quantifier and qualifier ion respectively. The dwell and fragmentor voltage, and collision energy are shown in table 2.

Method validation

The Specificity linearity, limit of detection and quantification (LOD and LOQ), precision, and accuracy was taken as parameter of analytical method validation. The specificity of the method was established by comparing the control sample, spiked sample, and real sample chromatogram. The linearity of the method was determined by constructing a calibration curve of concentration against peak area of different concentration. The LOD was explained as the minimum amount that can be determined using the instrument with signal-to-noise ratio (S/N=3) and LOQ explained as the minimum amount that can be quantified using the instrument with signal-to-noise ratio (S/N=10). Relative standard deviation (RSD) was assessed to determine the precision of the analytical method. Accuracy of the method was determined using the recovery study, at two different spiking level recovery study was done. During the recovery study optimized sample extraction method was followed.

Results And Discussion

Method optimization

To obtain good extraction *n*-Hexane and dichloromethane (DCM) both the solvent was tested. DCM found better extraction performance over *n*-hexane, so DCM was taken as the extraction solvent. Using DCM as solvent salting out extraction was also performed. To optimize the method sodium chloride (NaCl) salt was used and we perform extraction using 5, 10, and 15% NaCl salt in the sample. The result of these experiment showed 10% NaCl and DCM solvent is the best optimized method (Table. 1). In extraction DCM solvent amount also optimized. We tried extraction using three different solvent amounts, the amount of DCM solvent was experiment (50 +30) = 80 mL, experiment (70+30) =100mL and experiment (100+30) =130 mL. Among the three experiment, extraction with (50+30=80mL) solvent, cannot extract the analyte properly, but extraction with (70+30=100mL) and (100+30=130mL) solvent showed similar performance of extraction. Considering the solvent amount extraction with (70+30=100 mL) was taken as the optimized method (Table 1).

Method Validation

Specificity

The specificity of the method was determined by comparing the control sample chromatogram, real sample chromatogram with standard chromatogram. No unwanted peak was observed at the retention time of analyte peak, which determine the specificity of the method.

Linearity

Linearity was checked by constructing a linear curve range of concentration level (0.1-200 ppb). The linear graph was obtained with the regression equation $Y=45423.1299x+64.2074$ and coefficient $R^2=0.9994$ (Table 2)

Limit of detection and quantification (LOD and LOQ)

The estimated LOD and LOQ was 0.017 and 0.05 µg/kg respectively, this amount is very low considering the others method. Xiang et.al. reported that the LOD and LOQ of AZM in feather sample were 0.5 and 2.0 µg/kg respectively (Xiang et.al. 2020).

Accuracy and precision

To obtain accuracy and precision recoveries studies was done in triplicate by spiking standard in three concentration level. The spiking level was 0.5, 0.25, and 1.25 µg/kg and the recovery were in the range (96.6-101.5) % and RSD (3.5-6.3) (Table 2). These recoveries and RSD were in the acceptable range (70–120%) which are recommended by SANTE/11945/2015 (2015)

Residue amount in real sample

AZM residue determined in the river and farm water sample. As the representative of the total Bangladesh river five important and big river was selected. To represent the farm water condition five different farm water was collected. Following the developed and validated method. We found no residue in river water but we found in one farm water residue as 0.35 (µg/L) (Table 3). In some reports we found the reported value of positive detection of AZM with other pharmaceutical drugs. Le et.al. reported the residue amount of AZM in different river water of Vietnam in the range of (235-780) ng/L (Da Le et al., 2021). Hernández et al. reported the occurrence of AZM in waste water and sea water of antarctic in the range of 0.1-1.0 µg/L (Hernández F et al, 2019). Thai et al. reported the presence of AZM in effluents of pharmaceutical manufacturers and other sources around Hanoi, Vietnam (Thai PK et.al. 2018)

Conclusion

An LLE based analytical method development to analyze azithromycin antibiotics residue in river and fish farm water of Bangladesh. The new developed and validated method is used to analysis the AZM residue in water to find out the contamination level of river and fish farm water. Though the river water of Bangladesh have the chance to be contaminated with various antibiotics but we did not find any AZM residue in any river water of Bangladesh. In fish farm water we found 0.35 µg/L residue only in one fish farm water among the five different fish farm water. The residue may be come from fish feed or from the nearby dairy farm as that the farm located closely. We can suggest from the findings of our experiment, the river water may still safe from pharmaceuticals contaminants and need to monitor the fish feed used in fish farming need to monitor the contamination level, the dairy farm wastage also need to control to minimized the contamination nearby fish farm water.

Declarations

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Author's contribution:

Md Humayun kabir: Conceptualization, design, execution, writing and supervision of the work. Sabina Yasmin: Data analysis, writing, editing and execution, Salma Akter Mou: Experiment execution, Bushra parvinn Upoma: Experiment execution. Shamim Ahmed: data analysis. Golam Azam: Experiment execution. Tajnin jahan: Experiment execution. Md. Saidul Islam: Data analysis, review and editing, Mohammad Monizzaman: Data analysis and editing.

Availability of data and materials

Not applicable

Ethics declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no competing interests.

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Tables

Table 1. Experiment on different solvent, solvent amount and NaCl amount

Experiment No.	Solvent Name	Solvent amount (mL)	NaCl %	Recovery %
1	<i>n</i> -hexane	100+30	-	05
2	DCM	100+30	-	45
3		100+30	5	70
4		100+30	10	95
5		100+30	15	96
6		50+30	10	78
7		70+30	10	97
8		100+30	10	97

Table 2. Linear equation, average recovery (\pm RSD), LOD, LOQ

Analyte	Linear equation(R^2)	Spiking level ($\mu\text{g}/\text{kg}$)	Average recovery \pm RSD	LOD ($\mu\text{g}/\text{kg}$)	LOQ ($\mu\text{g}/\text{kg}$)
Azithromycin	Y=45423.1299x+64.2074	1.25	96.6 \pm 6.3	0.017	0.05
		0.25	99.1 \pm 4.4		
		0.5	101.5 \pm 3.5		

Table 3. Residue amount of azithromycin in river and farm water

Sample id*	Azithromycin Residue amount± RSD(µg/L)
SR1	ND**
SR2	ND
SR3	ND
SR4	ND
SR5	ND
SF1	ND
SF2	ND
SF3	0.35±0.06
SF4	ND
SF5	ND

*SR= River sample SF= Farm sample, **ND= Not detected

Figures

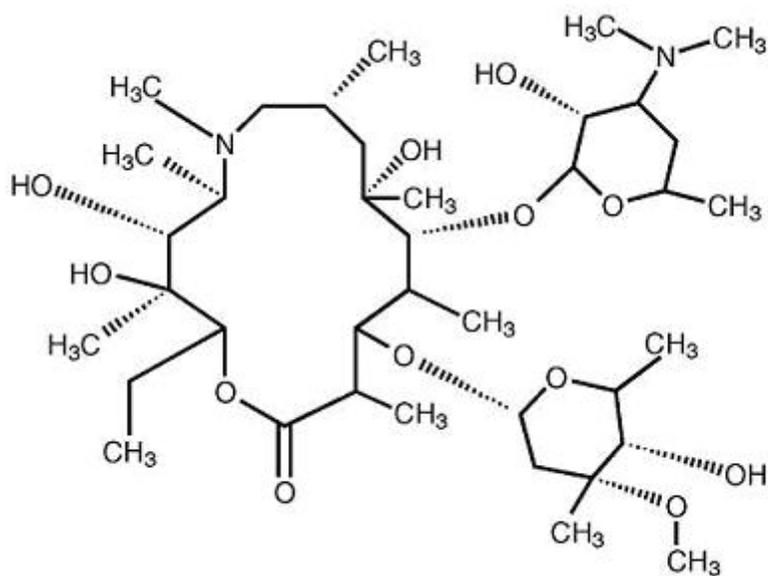


Figure 1

Chemical structure of Azithromycin