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Azithromycin extraction from municipal wastewater and quantitation using liquid chromatography/mass spectrometry

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Abstract

Azithromycin is a broad spectrum antimicrobial agent that is approved in the United States for use in humans. Due to azithromycin's low rate of metabolism it is likely to be found in wastewater treatment plants, where its broad spectrum of antimicrobial activity could lead to development of resistance in bacteria. A liquid–liquid extraction using K_2CO_3 and methyl-*t*-butyl ether (MTBE) was used to extract azithromycin from 10 ml aliquots of wastewater. Liquid chromatography was performed using a Luna C18(2) (30 mm × 2.0 mm) column with a mass spectrometer friendly mobile phase containing 50:24:2:24 acetonitrile, methanol, tetrahydrofuran, and 0.04 M ammonium hydroxide. The mass spectrometer used an electrospray source with positive ionization and an ion trap detector. A linear standard curve from 5 to 200 pg/ml was validated and used to quantitate azithromycin in wastewater.
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Keywords: Azithromycin; Wastewater; Liquid chromatography; Mass spectroscopy

1. Introduction

Azithromycin is a broad spectrum macrolide antimicrobial agent that displays activity against both Gram(+) and Gram(−) bacteria [1,2]. In humans, azithromycin has been approved for the treatment of respiratory, skin, otitis media, and sexually transmitted bacterial infections [1,3]. Some of the characteristics that make azithromycin a useful antimicrobial agent are its high tissue partitioning [4,5], long residence time [2,6,7], acid stability [8], and a relatively low rate of metabolism [9,10]. Most of the azithromycin leaves the body in its unchanged form via feces [9], which suggests that significant amounts of active azithromycin could be introduced into wastewater treatment plants. Furthermore, the slow metabolism of azithromycin indicates possible poor degradation in sewage treatment plants. Presence of the active antimicrobial along with incomplete processing of azithromycin in sewage treatment plants could be a factor in promoting the development of antimicrobial resistance in treatment plants and after its release into the general environment [11–15]. Understanding the disposition of azithromycin in wastewater could lead to better treatment options that would lead to more complete removal of azithromycin, thus reducing its release into the general environment and the potential development of drug resistance. To aid in this understanding, an analytical method that accurately measures low concentrations of azithromycin in wastewater is an essential tool.

Currently published quantitative methods measure azithromycin in plasma, tissues and other bodily fluids, and wastewater. Extractions of azithromycin from plasma matrices are primarily liquid–liquid using MTBE [3,6,10,16–20], but one using diethyl ether [21] and a SPE method [22] have been reported. Extractions of azithromycin from tissue primarily use acetonitrile [17,10], or ethanol [23,24]. Detection

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is generally electrochemical detection [7,17–20,22,25] or MS [1,3,7,10,16,26], although UV has been used with high concentrations in formulation stability testing [8,27,28], fluorescence after derivatization [21], and bioassays have been used [2,6,17,23,24]. The limits of quantitation found using these assays are as low as 10 000 pg/ml [16,21]. The assay from wastewater uses an SPE extraction with mass spectrometer detection and has a limit of quantitation of 2 pg/ml with a sample size of 250 ml [29]. The assay described here is significantly modified from published plasma matrix extractions and the chromatography was also modified [16].

The purpose of this project was to develop and validate an analytical method to quantitate azithromycin in water from wastewater treatment plants using a relatively simple and sensitive extraction method in combination with LC/MS detection. To our knowledge there is only one other reported azithromycin extraction and quantitation methods for water from wastewater treatment plants and no other method that has a limit of quantitation similar to the 4.25 pg/ml extracted from a 10 ml sample described here.

2. Experimental

2.1. Materials

All materials, unless otherwise noted, were obtained from Fisher Scientific (Pittsburg, PA). Acetonitrile, methanol, MTBE and tetrahydrofuran were HPLC grade, 1.0 N ammonium hydroxide was certified A.C.S. The water was deionized and filtered on-site using a Nanopure system (Barnstead/Thermolyne, Dubuque, IA). Azithromycin (917 μg/mg) was purchased from USP (Rockville, MD). The internal standard, erythromycin, had a 98% purity. The HPLC column used was a Luna C18(2) (30 mm × 2.0 mm), 5 μm, 100 Å from Phenomenex (Torrance, CA).

2.2. Chromatography

The mobile phase used contained 50% acetonitrile, 24% methanol, 2% tetrahydrofuran, and 24% 0.04 M ammonium hydroxide. The ammonium hydroxide solution was made by combining 1 part 1 M ammonium hydroxide to 23 parts water. With a flow rate of 0.3 ml/min the approximate retention times for azithromycin were 2.3 min and for erythromycin were 1.3 min. The total run time, including time for the autosampler to load the sample was a little over 6 min. Because the apparent pH of 10.8 for this solution is above the manufacturer's recommendations for the column used in this assay, the condition of the column should be monitored during use, 2 months of method development and assaying under these conditions did not cause problems, the low concentration of the buffer reduces potential problems with the high pH. Azithromycin has pK_a's of 8.7 and 9.5, no ionization problems were found in the mass spectrometer.

2.3. Instrumentation

The LC/MS system (ThermoFinnigan, San Jose, California) was comprised of a P4000 narrow-bore quaternary pump with a vacuum degasser, AS3000 auto sampler, UV6000 photo diode array detector, and a LCQ_{DUO} ion trap mass spectrometer. For this study the MS instrument parameters were optimized for azithromycin; those parameters were: ionization source, electrospray ionization (ESI) set for positive ionization; source voltage, 5.0 kV; sheath gas flow rate, 90 arbitrary units; auxiliary gas flow rate, 15 arbitrary units; capillary voltage, 5.0 V; capillary temperature, 285 °C; tube lens offset, −16 V; intermultipole lens voltage, −20 V; multipole 1 offset, −3. V; multipole 2 offset, −6.0 V. Nitrogen gas was used for sheath gas and auxiliary gas. The ion for azithromycin was monitored at 749.3 *m*/*z* and the ion for the internal standard, erythromycin was monitored at 734.2 *m*/*z*. The MS was set to scan a range of 748.5–753.0 *m*/*z* and then perform MS/MS (normalized collision energy, 30%; isolation width 3 *m*/*z*) on detected ions at 749.3. After that a range of 730.0–740.0 *m*/*z* was scanned and MS/MS was performed on detected ions at 734.2 *m*/*z*. The software used for instrument control and data processing was the Xcalibur software suite v.1.2 (ThermoQuest, San Jose, California, USA).

2.4. Preparation of standard and quality control (QC) samples

Stock solutions of azithromycin and erythromycin were made in acetonitrile. The stock solutions were used to make solutions in deionized water used to spike the standard curve and QC samples. Standards, in deionized water, were made fresh each day that samples were extracted and ranged from 5 to 200 pg/ml. The QC samples were prepared at the concentrations of 12.5, 40, and 125 pg/ml in deionized water, in bulk, prior to validation and then aliquoted into 10 ml samples and stored at −20 °C until used. For the intra-day assay validation, five QC samples were randomly selected from each of the three different concentrations. After that, for each assay, two QC samples at each concentration were selected at random and extracted with the standard curve and unknown samples.

2.5. Extraction method

Unknown samples and QCs were allowed to thaw and the unknown samples, standards, and QCs extracted were 10 ml aliquots of water that were placed in 50 ml glass centrifuge tubes. To each tube, 50 μl of internal standard (1 μg/ml erythromycin in water), 0.5 ml of 0.5 M K_2CO_3 and 12 ml methyl-*t*-butyl ether were added. The tubes were capped with caps containing Teflon liners, vortexed vigorously for 30 s, and then centrifuged for 10 min at 1000 × *g*. The supernatant (methyl-*t*-butyl ether) was transferred to a disposable 15 ml glass centrifuge tube, then dried under N_2 in a 25 °C water bath. The samples were then reconstituted with 100 μl of

mobile phase, vortexed for 1 min, centrifuged for 10 min and transferred to an auto sampler vial, and 40 μl was injected onto the LC/MS system. During method development vortexing time and drying temperature were tested and found to need to be changed from prior requirements. Both of these aspects of the extraction were tested by putting samples through the entire extraction while changing only the tested aspect. The changes were then compared against each other to see which gave the best results.

2.6. Quantitation

Using the product ion at *m*/*z* 591 for azithromycin the product ion at *m*/*z* 734.2 for erythromycin, peak area ratio of analyte:internal standard was plotted and a linear regression equation obtained for quantitation. This standard curve was weighted 1/*x* and was linear from 5 to 200 pg/ml. An LOQ of 4.25 was chosen based on the lowest point of the standard curve minus the acceptable error of 15%, the limit of detection was not determined.

2.7. Validation

The azithromycin assay was considered valid if the intra-day accuracy and precision values of the QC samples were within 15% of the expected values for each of the three QC concentrations. The equation used to calculate accuracy was $\%\,\text{accuracy} = ((C_{\text{intended}} - C_{\bar{x}})/C_{\text{intended}}) \times 100$ and the equation for precision was $\%\,\text{precision} = (C_{\text{SD}}/C_{\bar{x}}) \times 100$; where C_{intended} was the intended concentration, $C_{\bar{x}}$ was the average measured concentration, and C_{SD} was the standard deviation of the measured concentrations. An assay was rejected if more than two of the six QCs for a given standard curve were >±15% from their intended value or if both QCs at a single concentration were >±15% from their intended concentration. A rejected assay was reextracted using fresh unknowns, standards, and QCs.

2.8. Recovery

Recoveries were measured by comparing the peak areas from the extracted QC samples to solutions containing the expected amounts of drug. Recovery solutions were made by combining the equivalent amount of azithromycin that was in a 10 ml aliquot of QC sample with 50 μl internal standard, then diluting with mobile phase to a 100 μl volume.

2.9. Wastewater sample collection

Wastewater samples were collected from four northeast Kansas municipal wastewater treatment plants. The samples were collected from the influent, effluent and intermediate points such as the aeration and sedimentation tanks in the wastewater treatment process, and transported to the laboratory under ice. Wastewater samples were stored at −70 °C until extraction.

Table 1
Validation statistics

	Concentration (pg/ml)		
	12.5	40	125
Intra-day			
Precision (%)	6.2	6.3	6.1
Accuracy (%)	7.1	2.4	3.0
Inter-day			
Precision (%)	10.6	8.8	7.4
Accuracy (%)	1.5	0.4	0.4
Recovery (%)	65	57	77

Precision and accuracy data are presented as percentage from expected value, recovery as percent of the total. The intra-day $n = 5$, and inter-day $n = 17$.

3. Results and discussion

The validation statistics for the azithromycin assay are presented in Table 1. The assay validated within the standards set for this lab as described above. The values (mean ± SD) obtained for the linear regression equation (area ratio = (concentration in pg/ml × *m*) + *b*) were: $m = 0.000448 \pm 0.000125$ and $b = -0.00121 \pm 0.00222$, $R^2 = 0.9777 \pm 0.0134$. A representative standard curve is shown in Fig. 1. The limit of quantitation for this method was 4.25 pg/ml. This method provided adequate sensitivity to detect azithromycin in all wastewater samples tested.

The chromatography of azithromycin and the internal standard (erythromycin) following extraction from wastewater is shown in Fig. 2. Some temporal overlap of signal existed, but the individual ions were easily distinguished with no interference at their individual *m*/*z*. Erythromycin was chosen as an internal standard because it is chemically closely related to azithromycin and was not expected to be in the wastewater.

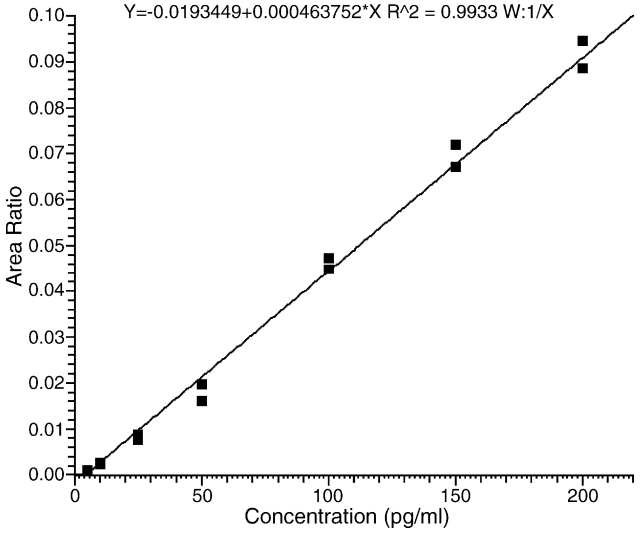

Fig. 1. Representative graph of standard curve for azithromycin extracted from water.

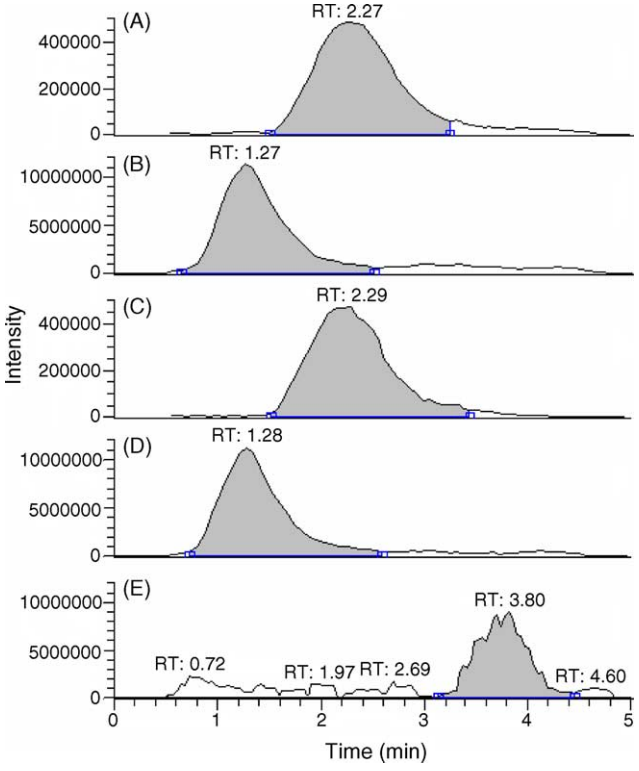

Fig. 2. Chromatograms of azithromycin extracted from water. (A) Mid-range standard (100 pg/ml). (B) Internal standard (erythromycin) for A, spiked concentration of 5 ng/ml. (C) Wastewater sample, 1 ml wastewater and 9 ml deionized water to make up 10 ml extraction, calculated concentration, as is, was 97 pg/ml. (D) Internal standard for C, spiked concentration of 5 ng/ml. (E) Wastewater (10 ml, influent) extraction with no erythromycin added.

The early development of this method used oleandomycin as the internal standard. Oleandomycin was originally desired as the internal standard due to its similarity to azithromycin and because it has a very low probability of being seen in the samples due to its not being used in medicine or manufacturing. However, when wastewater samples were assayed, it was noted that the signal from the oleandomycin was suppressed in some samples, it is not known if the suppression was from poor extraction from the wastewater matrix or ion suppression at the mass spectrometer. When erythromycin was used, this difference in the internal standard signal was not present. Even though erythromycin is commonly used in human medicine, it is metabolized, primarily to anhydroerythromycin, prior to its arrival at the wastewater treatment plant [30], so no or very little unchanged erythromycin should be seen in the wastewater. A close look at the signal obtained from wastewater samples without added erythromycin at the retention time and *m/z* of erythromycin confirmed the absence of interference which allowed us to use erythromycin as an internal standard.

The MS/MS spectra for azithromycin obtained on this system were similar to those found previously [1,26,31]. The product ions and their percent relative abundance compared to the major product ion found for azithromycin were: 591 (100%), 592 (29%), 573 (10%), and 434 (8%) *m/z*. The product ions and their percent relative abundance to the major product ion found for erythromycin were: 576 (100%), 522 (46%), 716 (43%), 558 (34%), 698 (23%), and 540 (16%) *m/z*, which is similar to other published product ion spectra [32]. The product ions and their percent relative abundance to the major product ion found for oleandomycin were: 544 (100%), 585 (6%), 601 (3%), 543 (2%), 495 (2%), and 302 (1%) *m/z*. The proposed fragments for some of the product ions of each agent are shown in Fig. 3. Loss of the cladinose sugar in the mass spectrometer is a common indicator for all of these macrolides. Erythromycin and oleandomycin also showed many product ions that are 18 *m/z* apart and likely relate to the loss of H_2O from the ions.

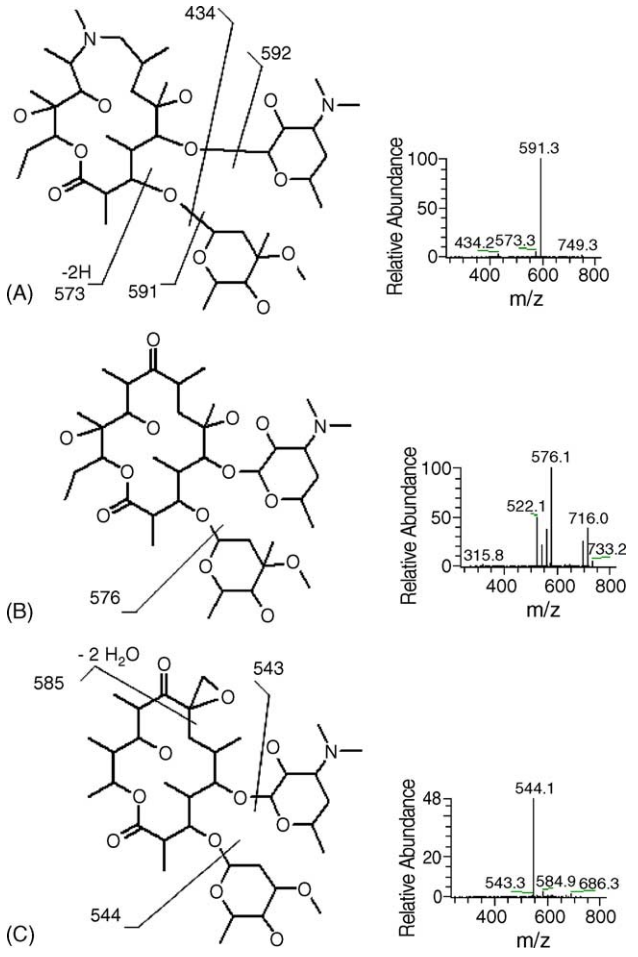

Fig. 3. Azithromycin (A), erythromycin (B), and oleandomycin (C) molecules showing spectra obtained from MS/MS and proposed fragmentations that produce the major product ions. In azithromycin (A), the 573 *m/z* fragment is caused by the loss of the cladinose sugar along with its glycosidic oxygen atom and two hydrogen atoms from the aglycone ring. Erythromycin's (B) product ion consists of the loss of the cladinose sugar without its glycosidic oxygen atom. The 585 *m/z* fragment from oleandomycin (C) is equivalent to a fragment of 619 *m/z* minus $2 \times H_2O$. The other ions described for oleandomycin and erythromycin are equivalent to the parent ion or the product ions shown minus 1, 2, or 3 sets of H_2O (18 *m/z* each).

Table 2
Detected azithromycin with varying vortexing times ($n = 3$)

Vortexing time (min)	Peak height (ion count $\times 10^5$)	
	Average	SD
0	22	4.6
0.5	60	8.8
1	55	11
2	50	19
5	44	20
10	36	11

Table 3
Detected azithromycin with varying drying temperatures ($n = 3$)

Drying temperature (°C)	Peak height (ion count $\times 10^5$)	
	Average	SD
25	79	13
30	43	20
35	50	9.4
40	36	26
45	12	12
50	23	17
55	9.2	7.5

It was found to be important to use Teflon liners in the centrifuge caps. Rubber cap liners, which came with the centrifuge tubes originally, appeared to sorb some of the macrolides and when MTBE came in contact with the rubber liner, it was apparently dissolved into the MTBE solution which caused a sticky precipitate to form when the supernatant was dried off of the extracted sample. The use of Teflon cap liners solved both of these issues.

Azithromycin was adequately mixed following 30 s of vortexing. Longer vortexing times appeared to result in a reduction of detectable azithromycin (Table 2). Drying temperatures above 25 °C also appeared to cause reductions in the amount of detectable azithromycin in this assay (Table 3).

4. Conclusion

The sensitivity and specificity of a LC/MS/MS system combined with a simple liquid–liquid extraction allowed the quantitation of very low concentrations of azithromycin in wastewater. The extraction used K_2CO_3 and MTBE to partition azithromycin from the wastewater, the MTBE was dried off to concentrate the azithromycin. Chromatography was performed using a mass spectrometry friendly mobile phase containing acetonitrile, methanol, tetrahydrofuran, and ammonium hydroxide and a Luna C18(2) (30 mm × 2.0 mm) column. A linear standard curve from 5 to 200 pg/ml was validated and used to quantitate azithromycin. Azithromycin concentrations in the northeast Kansas municipal wastewater samples were found to range between 430 ± 50 and $14{,}900 \pm 1700$ pg/ml.

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