NEWLY DEVELOPED AND VALIDATED METHOD FOR AZITHROMYCIN BY LC/MS-MS

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Summary

A simple, validated stability-indicating liquid chromatographic method was developed for the analysis of azithromycin in human plasma. The chromatograph is equipped with a Qualisil BDS 150 mm × 4.6 mm, 5 µm**.** This new method has been validated in accordance with USP requirements for assay determination, which include accuracy, precision, specificity, linearity and range. This method shows enough selectivity, sensitivity, accuracy, precision, and linearity range to satisfy Federal Drug Administration/International Conference of Harmonization regulatory requirements. The current method demonstrated good linearity over the range of 5.0807 to 1653.8816 ng/mL of azithromycin. The method is sensitive with a detection limit of 5.0807 ng/mL for azithromycin. This developed LC/MS-MS method can be applied for pharmacokinetic studies.

Key words: Azithromycin, Method Development, LC/MS-MS.

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Introduction

Azithromycin is a macrolide antibiotic belonging to the azalide group. Chemically it is (2R,3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S,14S)-11-((2S,3R,4S,6R)-4-(dimethylamino)-3-hydroxy-6-methyltetrahydro-2Hpyran-2-yloxy)-2-ethyl-3,4,10-trihydroxy-13-(2S,4R,5S)-5-hydroxy-4-methoxy-4hyltetrahydro-2H-Pyran-2-yloxy)-3,5,6,8,10,12,14-heptamethyl-1-oxa 6cyclopentade-can-5-one1, used as antibiotic and antibacterial[1]. Azithromycin has been analyzed by HPLC using electrochemical [2,3], fluorescence [4], mass spectrometry [5], and UV [6,7] for detection in bulk material and pharmaceutical forms. This paper describes the new and sensitive method for the estimation of Azithromycin in human plasma.

Materials and Methods

Chemicals and reagents

Reference standards of Azithromycin and Erythromycin were procured from USP and Calyx Chemicals and Pharmaceutical Ltd. respectively. HPLC grade acetonitrile, Ammonium acetate, Sodium carbonate, n-Hexane and other chemicals used were of analytical grade. Purified water from a Milli-Q-system was used throughout the analysis.

Instrumentation

The system (Waters) is equipped with an Auto Sampler and thermo stated column compartment. Mass spectrometric detection was performed in a Quatro Micro triple quadrapole instrument (waters) using multiple reaction monitoring (MRM). A turbo electrospray interface in positive ionization mode was used. Data processing was performed using Mass Lynx 4.1 (2) software.

Chromatographic conditions

Separations were achieved using a Qualisil BDS 150 mm × 4.6 mm, 5 µm. The column and auto sampler tray temperature were kept constant at 30°C and 4°C, respectively. The mobile phase consisted of a mixture of acetonitrile: 20mM ammonium acetate (70:30) and was delivered at a flow-rate of 1.0 mL/min. The sample injection volume was 10 µl.

Mass spectrometric conditions

Samples were ionized by positive-ion electrospray ionization mode. Analysis was carried out using selected ion monitoring (MIM) for specific m/z 749.40 for Azithromycin and m/z 734.37 for oxytetracycline. Peak areas for all components were automatically integrated using LC/MS-MS.

Preparation of stock solution and sample solutions

Stock solution of Azithromycin was prepared by dissolving the accurately weighed reference compound in methanol to give a final concentration of 1 mg/mL, stored at 12°C until it is used. The solution was then serially diluted with water and mixed with blank human plasma to achieve standard working solutions. Internal standard working solution was prepared by diluting the 1 mg/mL stock solution of internal standard with methanol.

Sample preparation
To the vials containing pipetted samples 3mL of organic mixture was added and vortexed. The samples were extracted for 10 min at 2000 rpm in vibromax and centrifuged for 10 min at 4000 rpm at 4°C. 2.4 mL of upper organic layer was transferred to a clean test tube and evaporated with nitrogen at 40° C and 15 psi using LV Evaporator. The dried residue sample was reconstituted with 0.8 mL of mobile phase. The samples were transferred into respectively labeled auto-injector vials. Load the processed samples into LC-MS/MS.

Methods

Sensitivity and specificity
The lower limit of quantification was determined as the minimum concentration that could be accurately and precisely quantified (lowest data point of the standard curve). The specificity of the assay for the analytes versus endogenous substances in the matrix was assessed comparing the lowest concentration in the calibration curves with reconstitutions prepared with drug-free plasma from five different humans. Limit of detection was found to be 5.0807 ng/mL.

Accuracy and precision
The accuracy and precision (presented as relative standard deviation, R.S.D.) of the assay were determined using quality control (QC) samples at 5.1849, 13.6445, 682.2262 and 1240.4112 ng/mL. Accuracy (%) was determined by the percentage ratio of measured over spiked QC concentration (mean of measured/ spiked×100%). Intra-day precision was determined by analyzing replicate aliquots of QCs (n = 6 per each concentration) on the same day. Inter-day precision was determined by repetitive analysis of QC samples (each concentration) on consecutive days.

Recovery and ionization
To investigate the recovery of doxycyline by the LLE method, plasma samples were spiked with Azithromycin at QCS concentration. The resulting peak–area ratios (analyte: internal standard) were compared with that of the standards prepared in suitable solvents to provide the recovery values. Ion suppression of ionization was evaluated by ESI technique, the absolute peak areas of control plasma extracted and then spiked with a known amount of drug, to standards injected directly in the same reconstituted solvent (Mobile phase).

Stability
To evaluate sample stability after three freeze–thaw cycles and at room temperature, five replicates of QC samples at each of the low, medium and high concentrations were subjected to four freeze–thaw cycles or were stored at room temperature for 24 h before sample processing, respectively. Five replicates of QC samples at each of the low and high concentrations were processed and stored under autosampler conditions for 24 h. Stability was assessed by comparing the mean concentration of the stored QC samples with the mean concentration of freshly prepared QC samples.

Method development
Azithromycin is a methanol soluble drug. Hence, an attempt has been made to extract the drug from plasma by precipitating the plasma samples with solvents like acetonitrile, perchloric acid and methanol. It was found that the analyte recovery was

less with these precipitating agents, may be due to decrease in the solubility of Azithromycin in water after the addition of these solvents. Therefore liquid-liquid extraction procedure was tried using tertiary butyl methyl ether: n-Hexane (90:10% v/v) as extracting solvent. It was found that there was no interference from plasma samples when extracted with tert butyl methyl ether: n-Hexane. This proves the satisfactory results with n-hexane. Thus n-hexane was used for sample preparation.

Result and Discussion

Specificity

No additional peaks due to endogenous substances that could have interfered with the detection of the compounds of interest were observed. Representative chromatograms for blank plasma (Figure 1), Azithromycin and erythromycin in sample are presented in (Figure 2).

Figure 1. Representative Chromatogram of a Blank Plasma Sample for Azithromycin

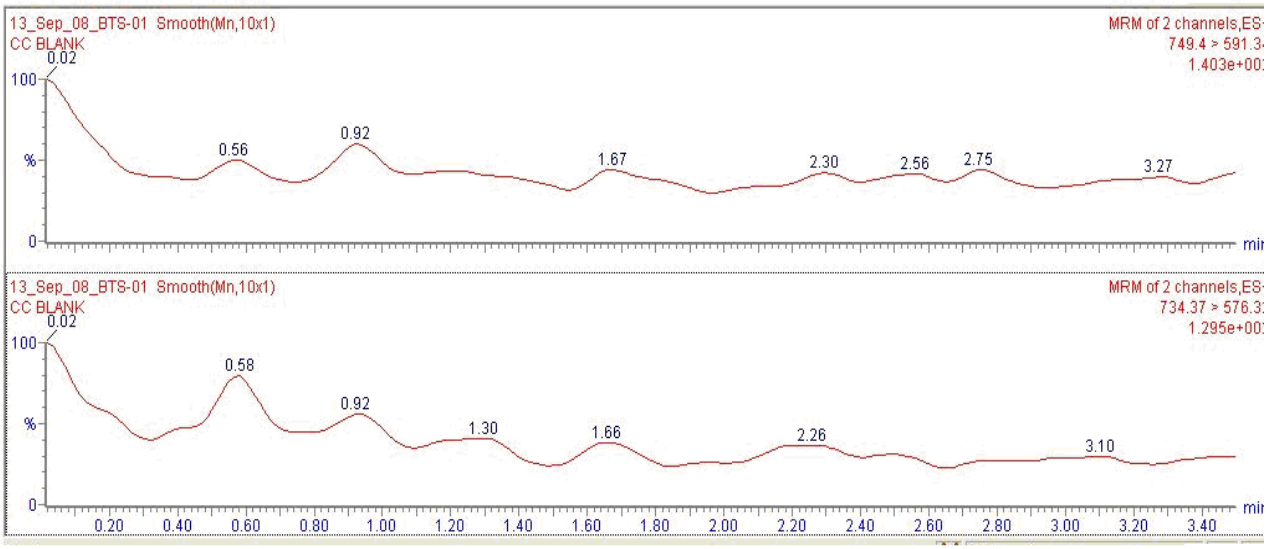
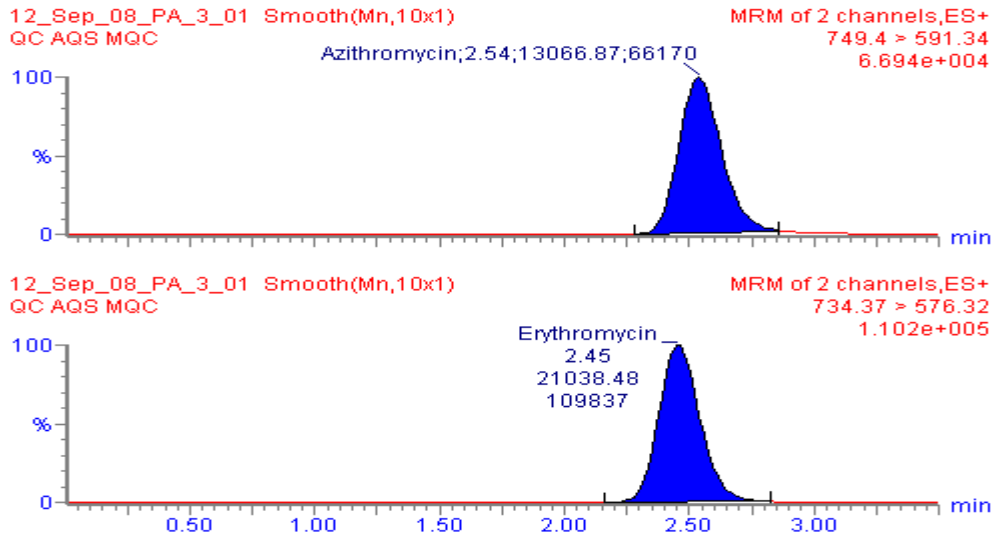


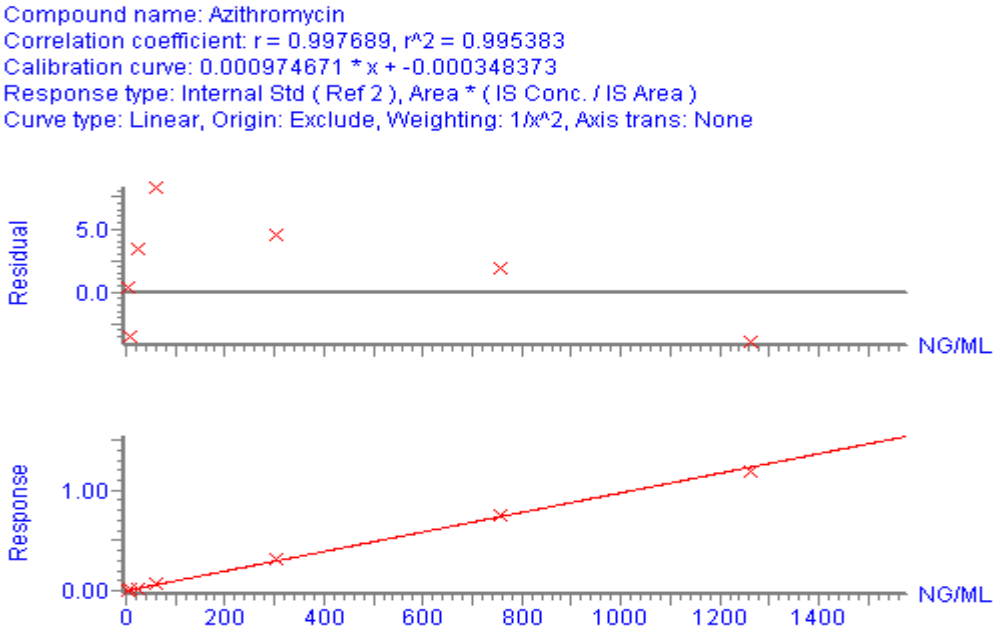
Figure 2. Representative Chromatogram of an Aqueous Standard Solution for Azithromycin



Linearity and lower limit of detection

The linear regression analysis of Azithromycin was constructed by plotting the peak–area ratio of Azithromycin to the internal standard (*y*) versus analyte concentration (ng/mL) in spiked plasma samples (*x*). The average regression equation of these curves and their correlation coefficients (*r*) were calculated. 1/*x*; showed good linear relationship between the peak areas and the concentrations (Figure 3). The lower limit of quantitation, defined as the lowest concentration analyzed with accuracy within ±15% and a precision ±15%.

Figure 3. Representative Regression Analysis of a Calibration Curve for Azithromycin



Precision

The intra-day precision (presented as relative standard deviation) is shown in Table 1. The precision for concentrations of Azithromycin were 5.20, 4.89, 2.61 and 3.94%, respectively. The accuracy, defined as (measured concentration/spiked concentration) ×100%, reached from 99.63 to 106.45 % throughout the four concentrations examined. The inter-day precision was studied, and the results are also given in Table 2.

Table 1 Intra-Batch Precision and Accuracy of Azithromycin

	LOQQC	**LQC**	**MQC**	**HQC**
Actual Concentration (ng/mL)	**4.9469**	**13.0180**	**650.9022**	**1183.4585**
	4.9117	13.9384	635.5910	1102.5028
	5.1421	13.2497	612.8674	1079.6424
	4.7558	14.1104	623.5893	1105.8255
	5.2785	14.9289	642.4505	1129.6742
	4.9597	12.8241	639.4090	1095.2098
	5.3950	13.7780	629.3510	1116.2869
Mean	**5.07380**	**13.80492**	**630.54303**	**1104.85693**
SD	**0.240897**	**0.727388**	**11.027950**	**17.215463**
%CV	**4.75**	**5.27**	**1.75**	**1.56**
%Nominal	**102.57**	**106.04**	**96.87**	**93.36**
	5.2968	13.7527	640.4680	1050.6787
	5.5247	14.8527	655.3918	1177.9657
	5.1128	15.0391	643.7426	1279.5804
	5.2020	14.3736	677.1835	1272.5243
	5.7029	13.8763	682.9706	1218.9732
	5.4215	14.7190	607.0989	1223.2866
Mean	**5.37678**	**14.43557**	**651.14257**	**1203.83482**
SD	**0.217653**	**0.529436**	**27.632446**	**83.891821**
%CV	**4.05**	**3.67**	**4.24**	**6.97**
%Nominal	**108.69**	**110.89**	**100.04**	**101.72**
	5.4831	12.3233	684.5666	1213.2573
	5.7980	12.6268	701.0277	1274.4391
	5.6753	13.8682	711.0484	1190.9572
	5.1237	12.5554	680.3789	1254.9060
	5.1498	12.8370	679.3554	1260.6098
	4.8542	11.6598	695.6507	1176.8123
Mean	**5.34735**	**12.64508**	**692.00462**	**1228.49695**
SD	**0.363678**	**0.723264**	**12.711095**	**40.378551**
%CV	**6.80**	**5.72**	**1.84**	**3.29**
%Nominal	**108.09**	**97.14**	**106.31**	**103.81**

Table 2 Inter-batch or Total Precision and Accuracy of Azithromycin

QC ID	LOQQC	LQC	MQC	HQC
Actual Concentration ng/mL	**4.9469**	**13.0180**	**650.9022**	**1183.4585**
	4.9117	13.9384	635.5910	1102.5028
	5.1421	13.2497	612.8674	1079.6424
	4.7558	14.1104	623.5893	1105.8255
	5.2785	14.9289	642.4505	1129.6742
	4.9597	12.8241	639.4090	1095.2098
	5.3950	13.7780	629.3510	1116.2869
	5.2968	13.7527	640.4680	1050.6787
	5.5247	14.8527	655.3918	1177.9657
	5.1128	15.0391	643.7426	1279.5804
	5.2020	14.3736	677.1835	1272.5243
	5.7029	13.8763	682.9706	1218.9732
	5.4215	14.7190	607.0989	1223.2866
	5.4831	12.3233	684.5666	1213.2573
	5.7980	12.6268	701.0277	1274.4391
	5.6753	13.8682	711.0484	1190.9572
	5.1237	12.5554	680.3789	1254.9060
	5.1498	12.8370	679.3554	1260.6098
	4.8542	11.6598	695.6507	1176.8123
Mean	**5.26598**	**13.62852**	**657.89674**	**1179.06290**
SD	**0.299344**	**0.986976**	**31.601235**	**75.227724**
%CV	**5.68**	**7.24**	**4.80**	**6.38**
%Nominal	**106.45**	**104.69**	**101.07**	**99.63**

Recovery and stability

The absolute recoveries of Azithromycin at concentrations of QC samples were 92.88, 77.84 and 96.98%**,** respectively. Stability of Azithromycin during sample handling (freeze–thaw and short-term temperature) and the stability of processed samples were evaluated and Azithromycin was stable for at least 6 h at room temperature in plasma samples, for 24 h in autosampler.

Conclusion

It was shown that LLE improves the sample clean-up to remove internal substances from plasma and thereby decrease the amount of matrix injected onto the column, thus the ion suppression effect was minimized. The results indicated that there was no significant difference between the signals of analytes extracted from human plasma and the mobile phase, which proves that there were no matrix effects. The developed method is applicable to pharmacokinetic studies.

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