RESEARCH PAPER

Isolation of Azithromycin Using Liquid-Liquid Extraction in Azithromycin for Oral Suspension Formulation and Separation of Impurities by Using HPLC

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Received: 4th Sep, 2025; Revised: 24th Oct 2025; Accepted: 6th Nov, 2025; Available Online: 1st December, 2025

ABSTRACT
The isolation of Azithromycin in Azithromycin Powder for oral suspension using liquid-liquid extraction, development of stability-indicating method followed, an analytical method validation using HPLC for quantification of probable impurities of Azithromycin in Azithromycin for oral suspension. The ccritical separation between impurities after isolation from sample matrix of the drug product is achieved on X Bridge® C18, 5µ, 250 x 4.6mm column. pH 9.5 disodium Phosphate buffer (20mM) is phase A, acetonitrile and methanol in the ratio 75:25v/v is phase B in gradient elution. The flow is maintained at 1.0 mL/min, 210nm was fixed for quantification purpose at 60°C. Method is validated as per ICH recommended parameters. HPLC method is highly beneficial for determination of impurities of Azithromycin in Azithromycin for oral suspension. Liquid-liquid extraction eliminates the intervention of the complex placebo peaks which interferes with impurities related to Azithromycin.

Keywords: Azithromycin, Liquid-Liquid Extraction, Stability demonstrating and HPLC
How to cite this article: Padmakar GV, Vittal SP, Vasundhara D, Suryakala D; Isolation of Azithromycin Using Liquid-Liquid Extraction in Azithromycin for Oral Suspension Formulation and Separation of Impurities by Using HPLC. Int J Drug Deliv Technol. 2026;16(1): 472-480. DOI: 10.25258/ijddt.16.1.50
Source of support: Nil.
Conflict of interest: None

INTRODUCTION

Azithromycin (Azi), is an antibacterial drug which is used for sore throat, for some intestinal infections, number of sexually transmitted infections, pneumonia and also for treatment of malaria along with other medications. [1] It is"9-Deoxo-9a-aza-9a-methyl-9a-homoerythromycin A". Formula of Azi is $C_{38}H_{72}N_2O_{12}$ and molar mass is 748.98 $g.mol^{-1}$. The routes of administration of Azi is by mouth, intravenous and ophthalmic. Azi is derivative of erythromycin. The difference between erythromycin and Azi is, a methyl-substituted nitrogen atom is fused into the lactone ring. [2] The structure of Azi is illustrated in Figure 1.

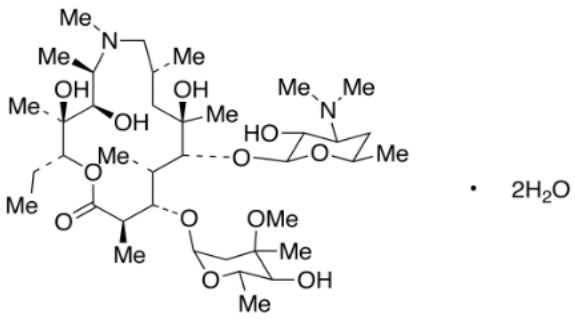

Figure 1. Azi chemical structure

It is marketed as ZITHROMAX. ZITHROMAX "Azi for oral suspension" contains Azi dihydrate equivalent to 1 g of Azi for adults. For pediatric use, ZITHROMAX 100 mg per 5 mL, 200 mg per 5 mL strengths. [3]

As per through literature assessment it is found that dissolution method for estimation of Azi in suspension is reported. [4] There are methods reported for determination of Azi by spectroscopic technique and determination of Azi in bulk and formulation by HPLC technique. [5-12] Few Methods are also reported for estimation of impurities in Azi using HPLC and UPLC techniques. [13-16] LCMS methods are also reported for quantification of Azi in plasma. [17-19]

This current paper describes about strategic extraction procedure used during sample preparation via liquid – liquid extraction to minimize the interference from flavors which are used in Azi oral solution sample viz., Cherry flavor and art ripe banana. Till now such method is not been published on liquid-liquid extraction to minimize the interference which is likely to come from sample matrix for Azi. Extracted sample was further assessed for identify of compound using identification procedure viz., Infrared spectroscopy. Also the developed analytical chromatographic method is capable enough to separate

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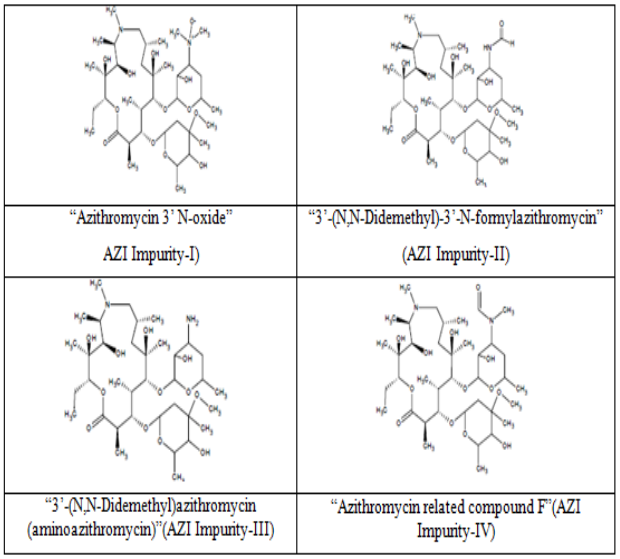
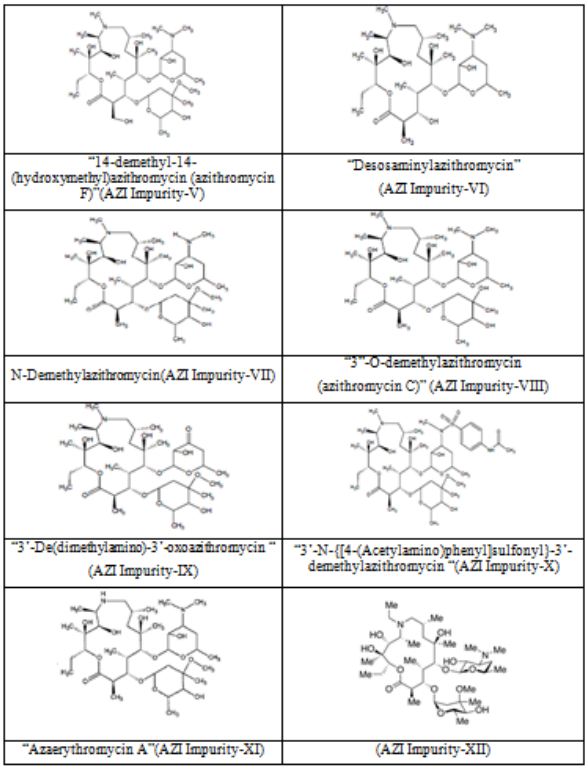
fourteen known impurities which are likely to come either by process or through degradation procedure. The existing reference cited monograph methods could not able to separate them. It has both advantages via separation of impurities and also completely removal of placebo peaks which are likely to interfere with probable impurities via liquid-liquid extraction procedure. Developed method was intended to validate as per ICH. [20-24]

Probable impurities from Azi are Azithromycin Ph. Eur. Impurity L (Azi impurity-I), Azithromycin Ph. Eur. Impurity M (Azi impurity-II), Azithromycin Ph. Eur. Impurity E (Azi impurity-III), Azithromycin Ph. Eur. Impurity F (Azi impurity-IV), Azithromycin Ph. Eur. Impurity D (Azi impurity-V), Azithromycin Ph. Eur. Impurity J (Azi impurity-VI), Azithromycin Ph. Eur. Impurity I (Azi impurity-VII), Azithromycin Ph. Eur. Impurity C (Azi impurity-VIII), Azithromycin Ph. Eur. Impurity N (Azi impurity-IX), Azithromycin Ph. Eur. Impurity H (Azi impurity-X), Azithromycin Ph. Eur. Impurity A (Azi impurity-XI), Azithromycin Ph. Eur. Impurity P (Azi impurity-XII), Azithromycin Ph. Eur. Impurity O (Azi impurity-XIII), Azithromycin Ph. Eur. Impurity G (Azi impurity-XIV), Azithromycin Ph. Eur. Impurity B (Azi impurity-XV). Figure.2





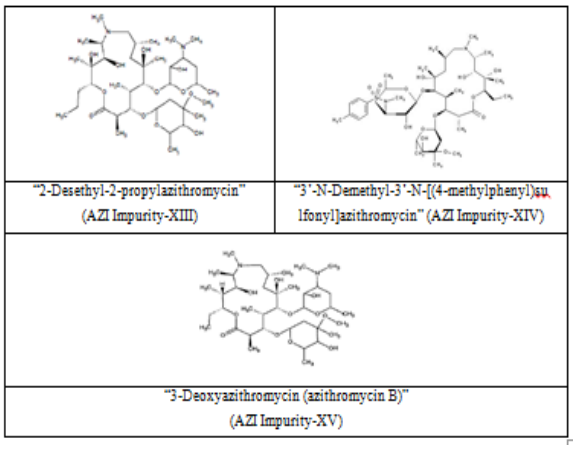


Figure 2. Azi Probable impurities

MATERIALS AND METHODS

Instruments

HPLC is equipped with gradient compatible quaternary pump; auto sampler with cooler chamber; temperature adjustable column oven compartment; Photo Diode Array (PDA) detector is employed. Empower 3 method validation software is used as an interface to monitor output signals which comes from detector. Waters X Bridge® C18, 5µ, 250 x 4.6mm column is used for testing purpose.

Chemicals

Azi pure compound, impurities of Azi, Azi for oral suspension (Zithromax) are from Aurobindo Pharma limited. Gradient grade methanol, acetonitrile, disodium hydrogen phosphate anhydrous, ammonium dihydrogen phosphate, sodium hydroxide and Ammonia solution

(≈30%) are from Merck life science private limited. The water used for analysis is taken from Evoqua water purification system.

Optimization and development of method

Aim of this study is to exclude placebo/flavor peaks which are likely to come from sample matrix and propose a method which is capable to resolve Azi probable impurities eluting from sample matrix and impurities eluting during stress conditions in compliance with regulatory requirement.

Azi drug substance and Azi Tablets is cited in USP monograph. Azi drug substance USP monograph covers about fourteen impurities. The related substances method contains disodium hydrogen phosphate buffer pH 8.9 as Elution phase A, acetonitrile along with methanol in the proportion of 75:25 as Elution phase B with flow of 1.0mL/m in gradient elution mode, detection at 210nm and column temperature 60°C using Water's X terra MS C18, 5µ, 250 x 4.6mm column. There is no official monograph for Azi for oral suspension. The related substances method of drug substance was verified for Azi for oral suspension. Due to presence of flavours (cherry flavor and art ripe banana flavor) in suspension, placebo peaks which are having very high area interfered with probable impurities. The other draw back of the related substances method of drug substance is that Azi impurity IX which is a potential impurity is co eluting with Azi impurity X. Azi impurity X is having high RRF value i.e. 10 and hence the above method cannot be used to quantify the probable impurities of suspension.

Initially to separate probable impurities in chromatography, trials were initiated using the above mentioned chromatographic conditions with modification of gradient, but placebo peaks interfered with one or another probable impurities. Trials were initiated with disodium hydrogen phosphate buffer pH 8.9 as Elution phase, acetonitrile along with methanol in the proportion 75:25 as Elution phase B with flow rate 1.0 $mL.min^{-1}$ in gradient elution mode, detection at 210nm and column temperature 60°C using X Bridge® C18, 5µ, 250 x 4.6mm column. Above column is opted as it can withstand high pH. Separation between potential impurities could not be achieved with the above mentioned chromatographic conditions.

Several logical gradients were carried out by changing Elution phase, pH and column temperature to attain the separation between probable impurities and placebo peaks. From the above trials it was understood that high pH Elution phase and column temperature 60°C is must for separation of impurities. By taking several trials, separation between impurities were achieved, but placebo peaks separation from impurities could not be achieved.

In order to have a specific method it has become necessity for removal of placebo from sample matrix. In this regard trials were initiated for separation of placebo using liquid-liquid extraction technique using various organic solvents. A conclusion could not be established. Literature was searched thoroughly and found that Azi is insoluble in water. Placebo was checked for solubility in water and found soluble. Suitable trials were initiated for removal of placebo from suspension. Entire suspension from the bottle was transferred into a separating flask and sufficient water was added and shaked for some time and left aside. The process of separation was monitored after 20 minutes and it was found that insoluble matter settled at the bottom. The insoluble matter was transferred into a Buchner funnel which is already pre-saturated with water. The Buchner funnel is connected to vacuum pump and sediment is washed cautiously using 200 to 250 mL of water for complete flushing of placebo if any. The leftover sample is collected along with whatman filter paper and heated in a preheated air circulation oven at temperature about 60°C . In random time intervals the sample was checked physically for moisture presence .Finally sample heating for 30 minutes at temperature 60°C was finalized. The obtained sample was cross checked with pure drug substance for purity by using Infrared spectroscopy (Figure 3a & 3b) and the correlation between pure drug substance and extracted sample is more than 0.98. The extracted sample from suspension was prepared at concentration of 8000µg/mL with diluent. The diluent adopted is ammonium dihydrogen phosphate buffer pH10: methanol: acetonitrile in the ratio 35:35:30 as given in the USP drug substance monograph.

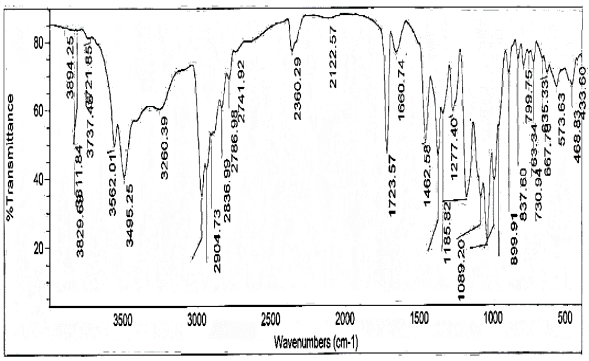


Figure 3a. IR Spectrum of pure drug substance

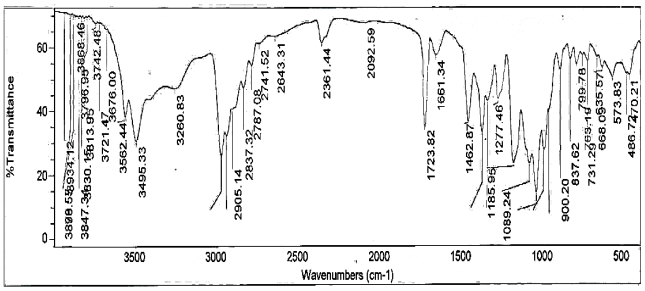


Figure 3b. IR Spectrum of Extracted sample

The adopted chromatographic conditions are summarized in Table 1.

Solutions of impurity mixture were prepared at concentrations as given in Table 3.The sample concentration of Azi is finalized at 8000 µg/mL using ammonium dihydrogen phosphate buffer pH10: methanol: acetonitrile in the ratio 35:35:30 as a diluent. 50µL sample solution loaded into photodiode array detector. In the drug substance USP monograph 210nm is given for detection of impurities. Individual impurity of Azi shows maxima in between 200-210nm wavelength. Based on the spectral data and USP monograph for drug substance, 210 nm was opted for quantification purpose (Figure 4). From the spiked sample chromatogram it is clear that the resolution between the impurities is found acceptable from column to column and found adequate area counts. The established method is capable to separate Azi impurity IX and Azi impurity X with resolution of more than 2.0.

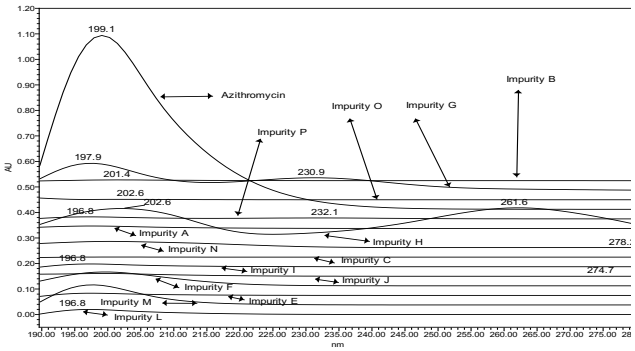


Figure 4. Spectra of Azi and probable impurities

Finalized method parameters:

For finalized chromatographic conditions, Refer Table 1.

Table 1. Finalized Chromatographic conditions

Column	Waters X Bridge® C18, 5µ, 250 x 4.6mm.
Flow & temperature	1.0 mL/min.,60°C
Inj. volume	50 µL.
Wavelength (nm)	210
Elution phase~A	2.84g/L of disodium hydrogen phosphate anhydrous pH 9.5 with 0.1M sodium hydroxide.
Elution phase~B	Acetonitrile along with methanol in the proportion of 75:25.
Diluent	Ammonium phosphate buffer pH10: methanol: acetonitrile in the proportion of 35:35:30.

Gradient programme	Time interval (in min)	% Elution phase~A	% Elution phase~B
	0.0	53	47
	35.0	53	47
	80.0	35	65
	105.0	35	65
	106.0	53	47
	120.0	53	47

The retention times of Azi impuritiesare given in the Table 2 along with the system suitability criteria.

Table 2. System suitability data

Component	Retention Time	Relative Retention Time	Purity Angle	Purity Threshold
Azi impurity-I	15.070	0.21	0.384	1.389
Azi impurity-II	21.389	0.30	0.230	0.893
Azi impurity-III	24.693	0.35	1.127	3.416
Azi impurity-IV	32.707	0.46	0.221	0.567
Azi impurity-V&VI	35.160	0.49	0.634	1.598
Azi impurity-VII	38.186	0.54	1.450	2.771
Azi impurity-VIII	52.518	0.74	11.973	38.519
Azi impurity-IX	55.148	0.77	0.085	0.464
Azi impurity-X	56.905	0.80	0.090	0.370
Azi impurity-XI	60.253	0.85	0.563	2.134
Azi impurity-XII	66.665	0.94	0.422	1.715
Azi impurity-XIII	83.715	1.18	1.830	7.540
Azi impurity-XIV	86.700	1.22	0.069	0.320
Azi impurity-XV	88.958	1.25	1.377	4.297

Solution Preparations

Standard solution preparation

43mg of Azi dihydrate is weighed into a 50mL clean and dry volumetric flask and dissolved with 40mL of diluent with aid of sonication, and diluted 5mL of the above solution to 25mL and mixed thoroughly with diluent. The above solution contains 160 µg/mL of Azi.

System suitability solution preparation

Stock solution of Azi impurity-IV: 0.95mg of pure substance is taken in 10mL and sonicated to dissolve by adding 5mL of diluent and diluted to volume with diluent.

Stock solution Azi impurity-VI: 1.25mg of pure substance is taken in 10mL and sonicated to dissolve by adding 5mL of diluent and diluted to volume with diluent.

System Suitability Solution: Dilute 4mL of Stock solution of Azi impurity-IV and 5mL of Stock solution Azi impurity-VI into 20mL with diluent to get of 16.5µg/mL and 27µg/mL respectively.

Sample solution preparation

Reconstituted the bottle containing Azithromycin powder for oral suspension as per the label with purified water. Transferred the entire quantity into a 500mL separating funnel and added 250mL of water. The separating funnel was shaken for about 5 minutes and left a side for about 30 minutes. The clear visible sediment was transferred into a Buchner funnel which is already pre-saturated with water. The Buchner funnel is connected to vacuum pump and sediment is washed cautiously using 200 to 250 mL of water for complete flushing of placebo. The leftover sample is collected along with whatman filter paper and heated at temperature about 60°C in an air circulated preheated oven for about 60 minutes.

80mg of dried sample collected from the above procedure is weighed into a 10mL clean and dry volumetric flask. 8 mL of diluent is added and sample solution is filtered through suitable 0.45µ membrane. The above solution contains 8000µg/mL of Azi.

METHOD VALIDATION

ZITHROMAX is available in 100mg/mL and 200mg/mL strength. Validation is performed on 100mg/mL strength.

Note: Azi system suitability mixture is available in Ph.Eur. Which contains all fourteen potential impurities in a mixture. This mixture is used for chromatographic separations. However for validation purpose four process related impurities are not considered due to unavailability in market. Remaining ten probable are used in validation.

Specificity and Stress degradation

Table 3. Limit of impurities

Component	Specification Limit in %	Specification Limit in µg/mL
Azi impurity-I	1.0	80
Azi impurity-II	1.0	80
Azi impurity-III	0.5	40
Azi impurity-IV	1.0	80
Azi impurity-V&VI	0.5	40
Azi impurity-VII	0.7	56
Azi impurity-VIII	0.5	40
Azi impurity-IX	0.5	40
Azi impurity-XIV	0.5	40

Analytical method was evaluated by injecting the diluent and test solutions spiked with known impurities are outlined in Table 3.

Samples were prepared and subjected to various forced degradation conditions to assess the method. For acid degradation, the sample was treated with 1 M hydrochloric acid and heated at 85 °C for approximately 5 minutes. Base degradation was carried out by exposing the sample to 1 M sodium hydroxide and heating at 85 °C for about 15 minutes. Samples were neutralized prior to HPLC injection. Degradation was performed by treating the sample with 0.5% hydrogen peroxide and allowing it to react for 30 minutes at room temperature. Thermal stress studies involved exposing the sample to 80 °C for 72 hours, followed by preparation of the test solution was exposed to 90% relative humidity at 25 °C for 120 hours before analysis. Photolytic degradation was conducted by exposing the sample to light intensity of 10 K lux for 120 hours and UV exposure of 200 watt-hours/m². All stressed samples were subsequently prepared and analyzed by HPLC.

Precision

Method precision was evaluated by preparing six individual test solutions from extracted sample powder, each is injecting them into the HPLC system. The results were expressed as % w/w, and the %RSD was calculated for every impurity. Intermediate precision (ruggedness) was assessed by repeating the analysis on a different day using a different instrument and column.

Sensitivity (LOD and LOQ)

The limits of detection (LOD) quantification (LOQ) were established by injecting a series of impurity solutions covering concentrations from 1% to 150% of the proposed specification limits. Precision at the LOQ level was evaluated using predicted concentrations obtained from the linearity plots.

Linearity and Range

Linearity and range were determined by analyzing impurity concentrations from the established LOQ level up to 150% . The slope, y-intercept, and correlation coefficient for each impurity were calculated from the corresponding calibration curves.

Accuracy

Accuracy was demonstrated by spiking impurity stock solutions into control samples at LOQ, 50%, 100%, and 150% of the proposed specification levels. The percentage recovery (% w/w) was calculated at each level. Each concentration level was prepared and analyzed in triplicate.

Solution stability

Sample solutions was assessed by storing the solutions at refrigerated conditions (~4 °C) and injecting them at predefined time intervals. The results obtained at each time point were compared against those from freshly prepared solutions.

Robustness

Method robustness was evaluated by introducing deliberate variations in chromatographic conditions. The parameters varied included flow rate (±10%; 0.9 and 1.1 mL/min), column temperature (±5 °C; 55 °C and 65 °C), gradient composition (±1 absolute), pH (±1 unit; 9.4 and 9.6), and detection wavelength (±5 nm; 205 nm and 215 nm). In each study, only one parameter was altered while keeping all other conditions unchanged.

RESULTS AND DISCUSSION

Specificity and Stress studies

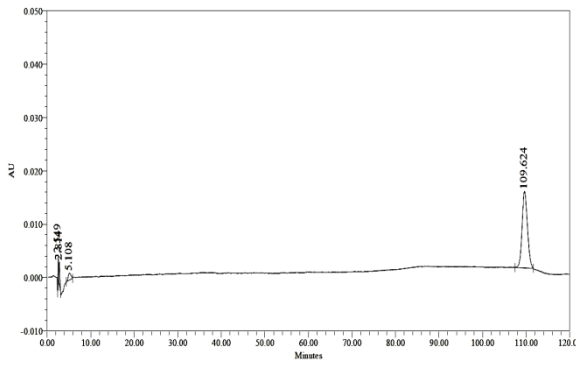
For comprehensive degradation data refer Table 4 and Figure 5.

Table 4. Comprehensive degradation data

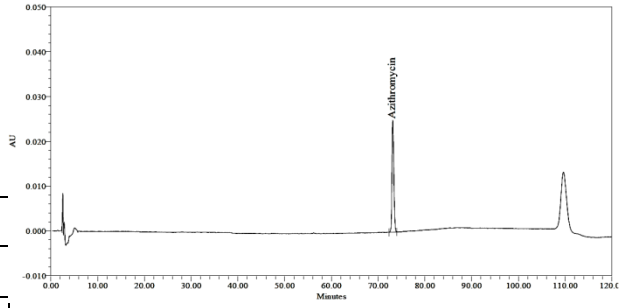
	% impurities+ %Degradation products		
Stress condition	Time	Azithromycin	Major Appeared impurities
Acid (1M HCl / 85°C)	5 minutes	25	Azi Impurity-VI significantly formed & Major unknown at RRT 0.41 is seen.
Base (1M NaOH / 85°C)	15 minutes	8.9	Major unknown at RRT 0.05, 0.07, 0.41 & 0.65 were seen.
Oxidation (0.5% H2O2 / RT)	30 minutes	11.4	Azi Impurity-I significantly formed & Major unknown is not seen.
Thermal (Heated at 80°C)	72 hours	0.1	No significant known and unknown impurities were seen.
Humidity (90%RH / 25°C)	120 hours	1.0	No significant known and unknown impurities were seen.
Photolytic (10K Lux along with UV 200 watt hours/m2).	7 days	0.3	No significant known and unknown impurities were seen.

Figure 5. Typical chromatogra

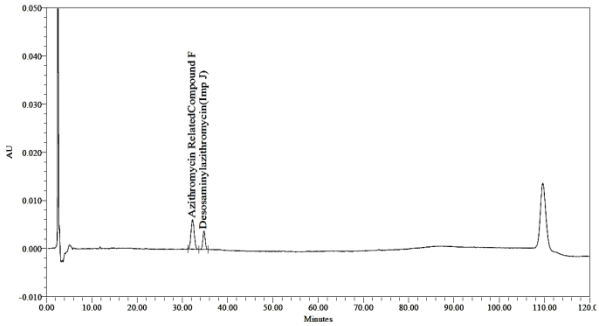
(a) Diluent



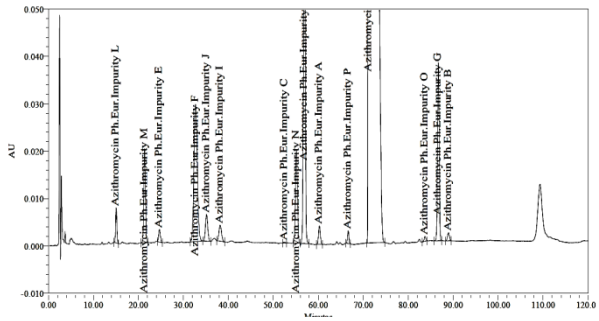
(b) Diluted standard



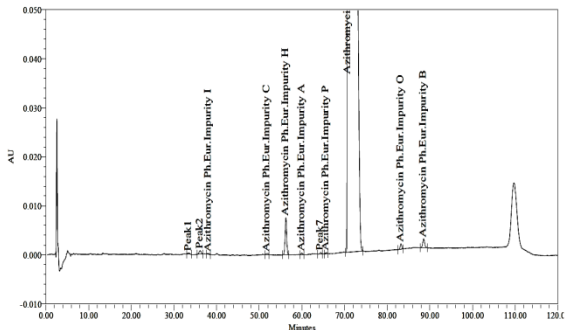
(c) System suitability



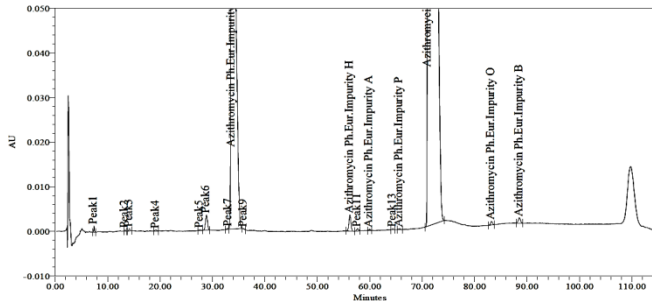
(d) Impurities Spiked Sample solution

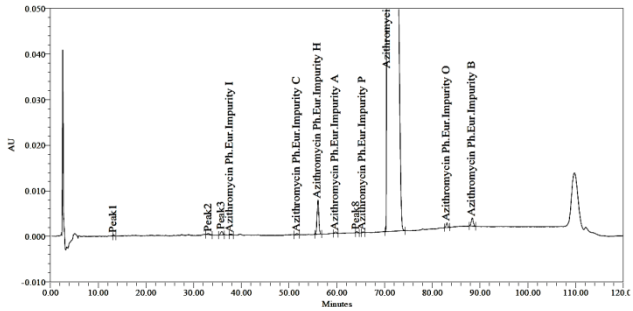


(e) Control Sample

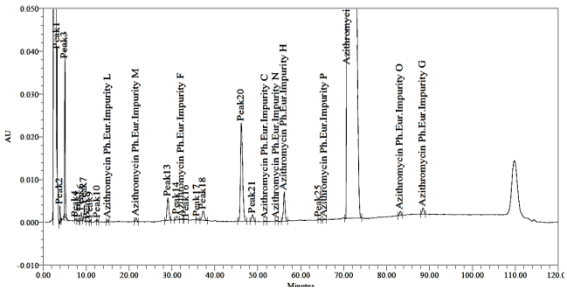


(f) Acid Stressed Sample

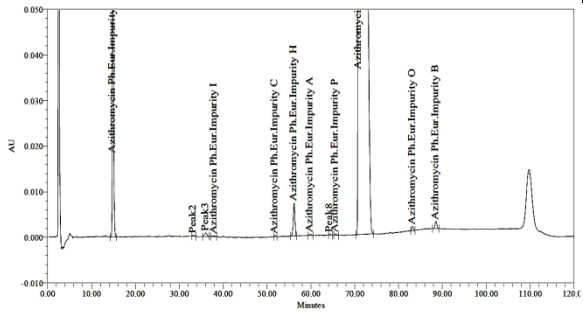




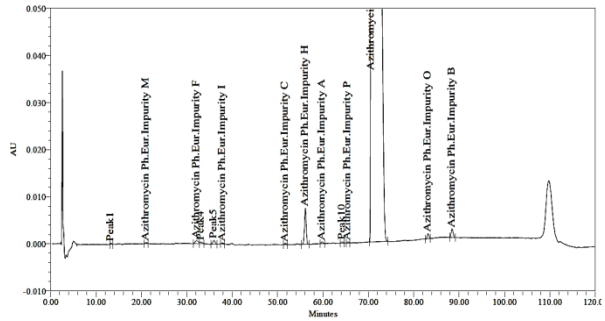
(g) Base Stressed Sample



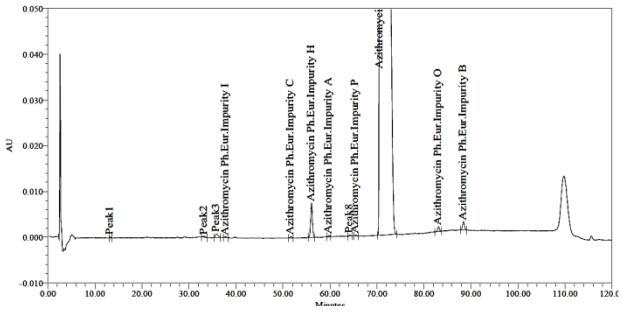
(h) Oxidation Stressed Sample



(i) Thermal Stressed Sample



(j) Photolytic Stressed Sample



(k) Humidity Stressed Sample

Method Validation

The validation results of AZI and impurities are elaborated in Table 5a and 5b.

Table 5a Results for AZI and impurities

Parameter	IMP-I	IMP-II	IMP-III	IMP-IV	IMP-VI	IMP-VII
Precision (%RSD)(n=6)	1.5	2.6	1.7	2.4	2.2	1.9
Ruggedness (%RSD) (n=6)	1.2	0.5	2.5	0.3	2.1	4.2
Accuracy@LOQ (n=3)	113.2	112.3	106.3	102.5	103.0	103.8
Accuracy@50% (n=3)	103.7	103.5	104.9	104.2	101.8	103.2
Accuracy@100% (n=3)	104.1	105.3	104.4	103.3	101.5	99.5
Accuracy@150% (n=3)	107.1	104.4	106.3	104.2	102.8	103.2
LOD (%w/w)	0.042	0.013	0.033	0.006	0.017	0.041
LOQ (%w/w)	0.126	0.039	0.101	0.020	0.051	0.125
Correlation coefficient	0.9993	0.9998	0.9984	0.9999	0.9996	0.9987
slope	1697	6152	1669	18292	4829	2435
Intercept	751	-14	1050	777	1538	-638

Table 5b Results for AZI and impurities

Parameter	IMP-IX	IMP-X	IMP-XI	IMP-XIV	AZI
Precision (%RSD)(n=6)	1.3	3.6	2.9	2.3	0.7
Ruggedness (%RSD) (n=6)	1.0	0.4	2.1	0.4	0.6
Accuracy@LOQ (n=3)	102.3	108.0	92.0	109.9	105.1
Accuracy@50% (n=3)	105.9	106.8	98.3	103.6	106.4
Accuracy@100% (n=3)	106.0	106.3	104.2	104.1	102.8
Accuracy@150% (n=3)	105.3	106.3	104.4	104.5	104.1
LOD (%w/w)	0.011	0.011	0.002	0.004	0.021
LOQ (%w/w)	0.032	0.005	0.101	0.014	0.063
Correlation coefficient	0.9999	0.9999	0.9973	0.9999	0.9999
slope	7499	53194	2405	24490	4195
Intercept	3826	4106	3206	2731	-289

Solution Stability:

Standard, system suitability and sample solutions were injected for about 48 hours on periodical intervals at refrigerated temperature (~4°C). From the above observations it can be stated that standard and sample solutions are found stable at refrigerated condition (~4°C) up to 48 hours.

Robustness

Robustness data revealed that closely eluting peaks Azi Impurity-IV and Azi Impurity-VI resolution decreases with decrease in temperature. For remaining other probable impurities from different robustness conditions, significant variation in relative retention times are not observed when compared to test procedure conditions. The

response is also found to be satisfactory for each impurity at every robustness condition adopted. From the above conclusions the optimized method can be assessed as robust.

CONCLUSIONS

Liquid-liquid extraction optimized ensures the complete removal of placebo from suspension matrix. The optimized chromatographic method is suitable for quantification of Azi probable impurities present in Azi for oral suspension in a single injection. The analytical method was validated in accordance with ICH guidelines and relevant compendial recommendations. Forced degradation studies adequately demonstrated the stability behavior of the drug product and confirmed the specificity of the method. All known and potential impurities were well separated from the main analyte, meeting the acceptance criteria of USP/Ph.Eur. The selected detection wavelength of 210 nm was found to be appropriate for impurity quantification, with no interference observed from placebo components. Linearity was established over the specified range with correlation coefficients greater than 0.99. Accuracy results confirmed satisfactory recovery using the proposed diluent. Therefore, the method is considered selective, specific, precise, stability-indicating, suitable for the quantitative determination of impurities.

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