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Stability indicating HPLC method development and validation for the simultaneous determination of Azithromycin & Ofloxacin in bulk and its dosage forms

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Abstract

A simple, precise, sensitive and reproducible stability indicating Reverse Phase High Performance Liquid Chromatographic (RP-HPLC) method for determination of Azithromycin and Ofloxacin in tablet dosage form was developed. Chromatographic separation was achieved on Hypersil-Keystone RP C_{18} ($_{250}$ × 4.6 mm, 5µm) column maintained at 30 ºC eluted with mobile phase at flow rate of 1.2 ml/min. The mobile phase consists of Buffer (0.02 M potassium dihydrogenphosphate): methanol: acetonitrile in the ratio 65:25:10 v/v at pH maintained at 3.2 with OPA was used and the determination was carried out at 285 nm. The retention time for Azithromycin and Ofloxacin were 9.7 min and 5.01 respectively. The linearity was found in the range of 5–50 µg/ml for Azithromycin and 4–40 µg/ml for Ofloxacin. In stability studies the drugs were well separated from degradation products. The degradation was studied in the individual standard drugs, their mixture and formulation which gave the idea about the orgin of the degradant products. The analytical method was validated as per ICH guideline for linearity, accuracy, precision, and specificity, limit of detection, limit of quantification, stability in analytical solution etc. and method can be extended to the analysis of Azithromycin and Ofloxacin in tablet formulations.

Keywords: Azithromycin, Ofloxacin, HPLC, Stability Indicating, Validation

1. Introduction

Azithromycin (AZI) is a *(2R,3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S,14S)*-13-[(2,6-Dideoxy-3-*C*-methyl-3-*O*-methyl-α-L- ribo-hexopyranosyl)oxy] -2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,-14 heptamethyl-11-[[3,4,6- trideoxy-3-(dimethylamino) β-D-xylohexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15- one is a semi-synthetic macrolide antibiotic related to Erythromycin. It differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring. Macrolide antibiotics are a bacteriostatic agent that inhibits protein synthesis by binding reversibly to 50S ribosomal subunits of sensitive microorganisms, at or very near the sites that binds chloramphenicol (Figure 1). It is used in the treatment of S. pneumonia, Community-acquired pneumonia due to C. pneumoniae, Pharyngitis or tonsillitis, skin and skin structure infections. Ofloxacin (OFL) is a broad spectrum fluorinated quinolones antibacterial with chemical name 9-Fluoro-2,3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H- pyridol[1,2,3-de]-1,4 benzoxazine -6-carboxylic acid (Figure 2). It is used in the treatment of respiratory tract infections, pharyngitis, community-acquired pneumonia, mild to moderate bacterial exacerbation, sexually transmitted diseases, acute and uncomplicated urethral and cervical gonorrhea, urethritis, complicated urinary tract infections, prostatitis[1-2].

Figure 1: Structure of Azithromycin

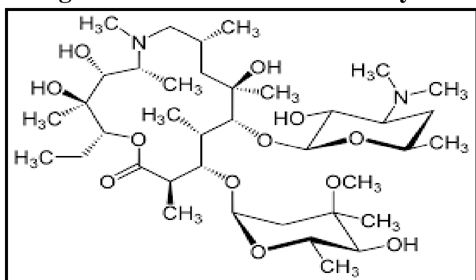
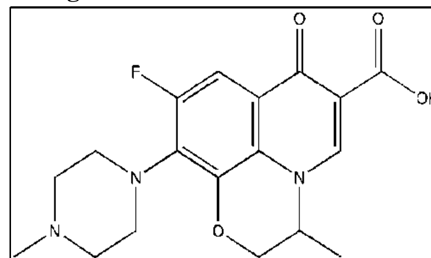


Figure 2: Structure of Ofloxacin



Literature survey reveals that there are few reported methods to determine AZI[3-8] and OFL[9-12] alone in dosage forms by spectrometry and HPLC. Few methods are also available in combination with other drugs[13-22] in dosage forms by spectrometry and HPLC, but no HPLC method is available for the simultaneous estimation of AZI and OFL in its dosage form. Therefore the purpose of this study is to develop stability indicating HPLC method for the simultaneous estimation of AZI and OFL in tablet dosage form.

2.0 Materials and Methods

2.1 Chemicals and Reagents

AZI and OFL standards were obtained as a gift samples from Alkem Laboratories, Mumbai. Methanol and acetonitrile of HPLC grade (E. Merck) and potassium dihydrogenphosphate of AR grade (S.D. Fine Chemicals Ltd.) was purchased from local supplier. Milli Q water was used. All the other reagents were of analytical grade. Marketed formulation Zithium-O (Alkem Laboratories Limited, Mumbai, India) was purchased from local pharmacy.

2.2 HPLC instruments and analytical conditions

The separation was carried out on HPLC (Waters 2998 with PDA detector), Waters 600 controller (water's 486 UV- visible tunable absorbance detector) were used to develop and validate the method. The chromatographic separation was carried out by using (Hypersil-keystone RP C_{18} 250 × 4.6 mm, 5µm) column, maintained at 30ºC. Data acquisition was done by using Data ace software and Empower 2 software.

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2.3 Preparation of Mobile phase and Diluent

The mobile phase consisting of buffer (0.02 M potassium dihydrogenphosphate): methanol: acetonitrile (pH 3.2 adjusted with orthophosphoric acid) was filtered through 0.45µ membrane filter before use, degassed and was pumped from the solvent reservoir in the ratio of 65:25:10 v/v at a flow rate of 1.2 ml/min. The detection was monitored at 285 nm and the run time was 25 min. The volume of injection loop was 10µl.

2.4 Preparation of standard and sample (Azithromycin 500 mg + Ofloxacin 400 mg) solutions

Accurately weighed about 500.0 mg of AZI and 400.0 mg of OFL working standards and transferred into separate 100 ml volumetric flasks containing 50 ml of diluent and dissolved by sonication. The volume was made up to 100 ml with diluent (5000 µg/ml and 4000 µg/ml of AZI and OFL respectively- stock A). Solutions were further appropriately diluted to get concentration of 10 µg/ml of AZI and 8 µg/ml of OFL in separate volumetric flask. Ten tablets of Zithium-O were accurately weighed and crushed to fine powder. Accurately weighed powder sample (1231mg) equivalent to 500 mg of AZI (400 mg of OFL) was transferred into 100 ml volumetric flask and dissolved by sonication for 25 min with intermittent shaking. The filtered solution was further diluted to get the concentration 10 µg/ ml of AZI (8 µg/ml of OFL).

2.5 Method Development and Validation[23-24]

During mobile phase optimization Buffer: methanol: acetonitrile in the ratio of 65:25:10 at λ_{max}285 nm was found to be satisfactory for both drugs. After mobile phase selection, effect of pH and flow rate was studied on resolution of peaks of both the drugs from each other. It was found that pH 3.2 (adjusted with OPA) and flow rate1.2 ml/min was suitable for both drugs. The method was validated for the parameters such as system suitability, selectivity, linearity and range, precision (interday and intraday), accuracy, robustness, ruggedness limit of quantitation (LOQ) and limit of detection (LOD).

For the system suitability study, the standard solutions containing 10 µg/ml of AZI and 8 µg/ml of OFL were prepared and the column efficiency, resolution and peak asymmetry was calculated. The method specificity was assessed by comparing the chromatograms obtained from blank (mobile phase), chromatograms of single drug with the chromatograms of sample. The precision of the method was established by carrying out method precision, system precision, intraday and interday analysis and relative standard deviation (%RSD) was calculated. Method precision and system precision was determined by injecting the sample and standard solution containing the mixture of 10 µg/ml of AZI and 8 µg/ml of OFL (six replicates) into HPLC system. The intraday precision was studied by performing analysis at regular interval in a day at three different concentration levels (10, 20, 30 µg/ml for AZI and 8, 16, 24 µg/ml for OFL), while interday precision was performed on three different days (day 1, 2, and 3). The accuracy of the method was determined by performing the recovery study by addition of standard drug to the preanalyzed sample at three different levels 80 %, 100 % and 120 % and average percentage recovery was determined. To determine the linearity of the method different concentration levels (5–50 µg/ml for AZI and 4–40 µg/ml for OFL) were prepared from standards stock solutions and each solution was injected into the HPLC system. The peak area of the chromatogram obtained was noted and calibration curve was constructed by plotting response factor against concentration of drugs. Effect of small changes in the HPLC conditions such as change of pH 3.0 & 3.4), change in temperature (25°C-35°C) & change in flow rate (1 & 1.3 ml) was studied to evaluate robustness of the method while ruggedness was studied by performing the assay by the different analyst. The LOD and LOQ were determined based on the standard deviation of the response and the slope of the calibration curve.

2. 6 Forced Degradation study

Forced degradation studies were performed to evaluate the stability indicating properties (specificity) of the proposed method. Samples were subjected to stress conditions such as acid hydrolysis, base hydrolysis, heat or thermal, photolytic, oxidation and reduction. Individual drug solutions, standard mixture and sample solution were subjected to the same stress conditions to ensure the effective separation of degradation peaks and main peaks. Standard stock and sample stock solution (stock B) was further diluted to get the concentration 10 µg/ml of AZI and 8 µg/ml of OFL in individual solutions, their mixture and sample solution and same solutions were also kept as control sample. Acid degradation was carried for individual drug solutions, standard mixture and sample solution using 10 ml of 1 N hydrochloric acid; alkali degradation was carried out in 10 ml of 0.1N sodium hydroxide. The stressed solutions were neutralized and then diluted with diluent. Oxidation degradation was performed by adding 10 ml of 30% H_2O_2 and then diluted with diluent. 10 ml of 10% solution of sodium bisulphate was added in to each flask to carry out the reduction study. For the thermal degradation study solid samples (equivalent to 500 mg of AZI & 400 mg of OFL) and working standards were kept in oven at 60ºC for 24 hrs which were further diluted appropriately and chromatographed. Photolytic degradation was carried out in by exposing to UV radiations (1.2 million lux hr). All these solutions were filtered through the 0.45 µ membrane filter before injecting.

3.0 Results and Discussion

3.1 The Finalized Chromatographic Conditions

Based upon system suitability parameters the finalized chromatographic conditions was as follows

Column	: Hypersil keystone RP C18
Wave length	: 285 nm
Column Temp	: 30ºC
Injection Volume	: 10 µl
Run Time	: 25 min
Flow Rate	: 1.2 ml / min
Pump Mode	: Isocratic
Retention time	: About 5.0 to 5.5 min (For Ofloxacin) About 9.5 to 9.8 min (For Azithromycin)
Mobile Phase	: Buffer: methanol: acetonitrile (pH 3.2 OPA) (0.02 M potassium dihydrogenphosphate)
Diluent	: Mobile Phase

3.2 Method Validation

In system suitability the values obtained demonstrated the suitability of the system for the analysis of these drugs combination. The values for resolution, capacity factor, theoretical plate, HETP and asymmetric factor were given in Table 1. The values were found well within the acceptable limit. The method was found linear over the concentration range of 5–50 µg/ml for AZI and 4–40 µg/ml for OFL. The correlation coefficients (r^2) were found to be 0.998 & 0.997 for AZI and OFL respectively. The result showed that an excellent correlation exists between responses and concentration of drugs (Table 2). The calibration curves for AZI and OFL are shown in Figure 3 & 4. In method precision and system precision studies the % RSD of the assay for both the drugs was found in the range of 0.26-0.85%. Intraday and interday analysis was performed for precision study. Repeatability or intraday precision was investigated by injecting six replicate sample solutions on the same day. Interday precision was assessed in triplicate over three consecutive days. The relative standard deviation (%RSD) was found to be well within the acceptable limit. The % RSD for interday and intraday study was found in the range of 0.1 and 1.02 for AZI and OFL. The results implied that the method developed was precise for the determination. The recovery study at three different levels 80, 100 and 120 % was performed to assess the accuracy of the method. The % recovery of AZI and OFL was found in the range of 99.73 –.99.83% and the results of the recovery study are shown in table 3. The LOD and LOQ were found to be 0.00062 µg/ml and 0.00185µg/ml for AZI and 0.006 µg/ml and 0.00210 µg/ml for OFL. The % RSD in robustness and ruggedness was found well within the acceptable limit (in the range of 0.0008% - 0.63%). The proposed method was applied for the determination of AZI and OFL in tablet dosage form. The % assay for AZI and OFL was found to be 99.74% & 99.02% respectively. Assay determination was carried in five replicates and % RSD was found less than 2% (Table 4) and the representative chromatogram of formulation is shown in figure 5.

3.3 Forced Degradation

Forced degradation studies were performed on standard drug alone, mixture of standard drugs and their formulation. From the degradation of these solutions under the same stress condition gives us an idea about the origin of degradant products. Peak purity study was performed by using purity angle and purity threshold parameters. Degradants did not show any interference with the elution of drug peaks as the peak purity of AZI and OFL was well within acceptance criteria for stressed samples. Hence, the method is stability indicating.

AZI was completely degraded in 1N HCl and four degradants products were appeared at 0.87 min (68.88%), 1.91 min (5.32%), 2.4 min (6.78%) and 3.7 min (18.90%), whereas AZI did not show any degradation when exposed to the stress conditions such as 0.1 N NaOH, 30% hydrogen peroxide solution, 10% sodium bisulphate and photolytic condition, temperature. This indicates that AZI is sensitive to the acid hydrolysis. On the other hand, OFL showed 50.01 % degradation in acidic condition where degradant product appeared at around 4.34 min. In alkaline degradation OFL showed one degradation product peak at 4.23 min and % degradation was found to be 25.01 %. OFL showed 19.82 % and 16.4% degradation in peroxide & photolytic degradation respectively, but the peak of degradant product was not detected by the detector. The degradation can be confirmed from the reduced % assay (80.41% in peroxide & 83.81% w/w in photolytic degradation). OFL did not show any degradation in sodium bisulphate. In thermal degradation study OFL showed 29.13 % degradation which appeared at around 4.19 min. The percentage assay, percentage degradation at each condition along with their purity angle and purity threshold for AZI and OFL are tabulated in the table 5.

Table 1: System Suitability Parameters

Sr. No	Name of drug	Retention Time*(±SD)	Area*(±SD)	USP Resolution* (±SD)	USP Tailing*(±SD)	USP Plate Count*(±SD)
1	**Azithromycin**	9.735 (±0.024)	418968.3 (±1234.1)	12.54 (±0.035)	1.12 (±0.008)	214568 (±1413.7)
2	**Ofloxacin**	5.03 (±0.060)	50010.1 ±(466.8)	——	1.083 (±0.004)	125631 (±223.6)

**Average of six determination*

Table 2: Linearity and Range

Sr. No.	Conc.(µg/ml) of Azithromycin	Area*(± SD)	Conc.(µg/ml) of Ofloxacin	Area*(± SD)
1	5	208437.31 (±833.1)	4	24392.1(±502.4)
2	10	421488.02(±488.64)	8	50010.40(±627.42)
3	20	835972.13(±256.72)	16	98003.73 (± 452.88)
4	30	1186500.74(±783.82)	24	150761.91 (±829.15)
5	40	1682947.06(±535.76)	32	212616.01 (± 681.04)
6	50	2115445.87(±425.41)	40	250004.46 (± 374.82)
	Equation of line	**y = 42093X–12268**		**y = 6419X – 1703**
	Slope	**42093**		**6419**
	y-intercept	**– 12268**		**– 1703**
	r^2	**0.998**		**0.997**

**Average of six determinations*

Table 3: Recovery Study

Recovery Studies level	80%		100%		120%	
Recovery (%)	**AZI**	**OFLO**	**AZI**	**OFLO**	**AZI**	**OFLO**
Amount of Std. Added (mg)	399.2	320.5	498.1	400	601.1	480.6
	398.7	319.7	500.7	399.5	600.4	478.9
	401.4	319.4	500.9	400.8	600.2	479.6
Amount Recovered (mg)	398.1	318.8	497.8	398.52	600.66	479.98
	398.5	319.5	499.6	398.26	599.89	478.84
	400.5	318.8	500.74	400.32	598.69	478.26
% Recovery	99.72	99.46	99.93	99.63	99.92	99.87
	99.94	99.93	99.78	99.68	99.91	99.98
	99.77	99.81	99.96	99.88	99.74	99.72
Mean Recovery	**99.81**	**99.73**	**99.89**	**99.73**	**99.86**	**99.85**
SD	**0.1181**	**0.2421**	**0.1012**	**0.130**	**0.0997**	**0.1337**
%RSD	**0.1183**	**0.2428**	**0.1013**	**0.131**	**0.099**	**0.1339**

Table 4: Assay of marketed formulation

Brand Name	AZITHROMYCIN		OFLOXACIN	
	Label Claim (mg)	**% Assay**	**Label Claim (mg)**	**% Assay**
Zithium-O (AZI 500 mg+ OFL 400 mg)	500	99.7	400	99.5
	500	99.8	400	98.9
	500	99.8	400	99.2
	500	99.6	400	98.1
	500	99.8	400	99.4
Mean		**99.74**		**99.02**
SD		**0.0894**		**0.5630**
%RSD		**0.0896**		**0.5685**

Table 5: Forced Degradation Study

Sr. No	Condition	% Assay of Azithromycin	% degradation w.r.t. Control	% Assay Of Ofloxacin	% degradation w.r.t. Control	Peak Purity for Azithromycin peak: Purity Angle	Peak Purity for Azithromycin peak: Purity Threshold	Peak Purity for Ofloxacin: Purity Angle	Peak Purity for Ofloxacin: Purity Threshold
1	Control Sample	100.15	---	100.23	---	0.176	1.163	0.185	1.077
2	Acid degradation	0.00	100.00	50.22	50.01	0.34	1.463	0.191	1.082
3	Base degradation	99.93	0.22	75.22	25.01	0.317	1.288	0.156	1.079
4	Peroxide degradation	99.83	0.32	80.41	19.82	0.346	1.219	0.207	1.103
5	Reduction degradation	99.68	0.47	100.23	0.00	0.314	1.239	0.184	1.075
6	Thermal degradation	99.80	0.35	71.10	29.13	0.315	1.261	0.166	1.072
7	Photolytic degradation	99.78	0.37	83.81	16.42	0.385	1.233	0.173	1.081

Figure 3: Calibration Curve of Azithromycin

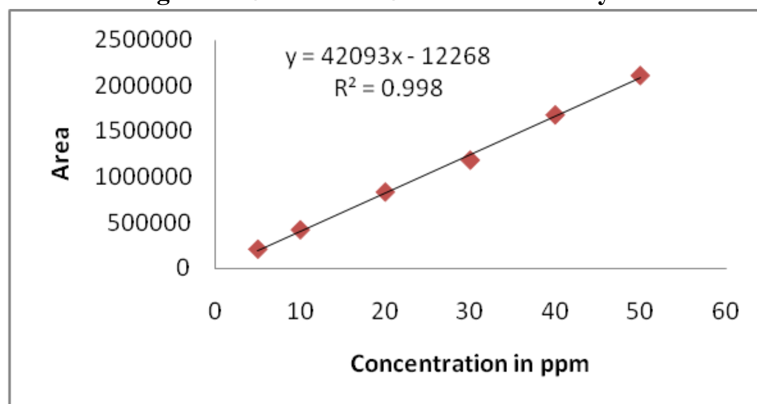


Figure 4: Calibration Curve of Ofloxacin

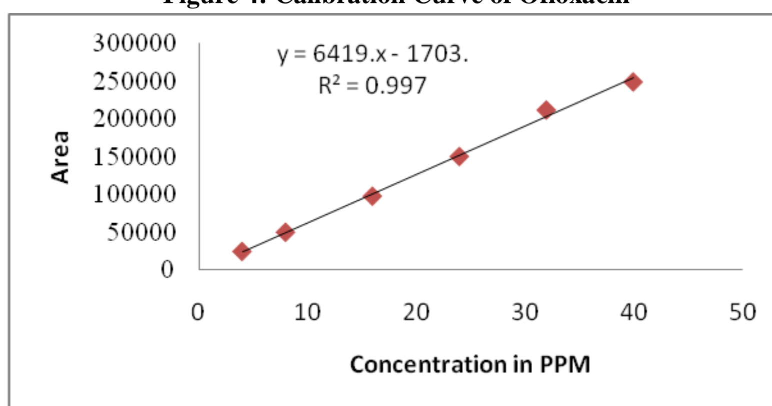
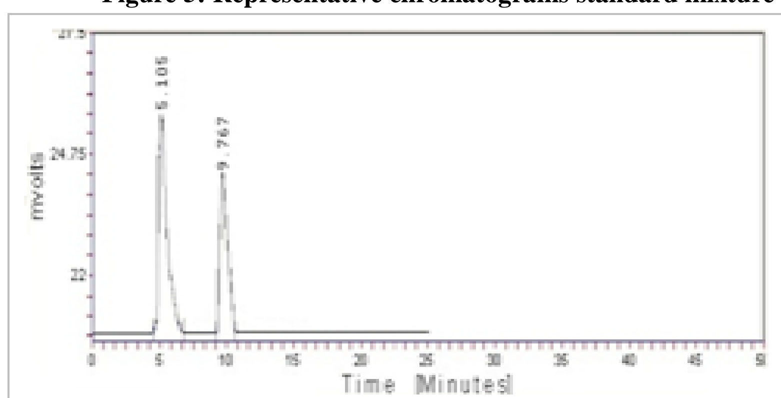


Figure 5: Representative chromatograms standard mixture



4.0 Conclusion

A simple and precise stability indicating HPLC method has been developed for estimation of AZI and OFL in tablet dosage form. The %RSD values in precision, recovery studies, robustness and ruggedness studies was found less than 2.0% which indicates that the method is precise, accurate and robust. The LOD and LOQ were found to be 0.00062 µg/ml and 0.00185µg/ml for AZI and 0.006 µg/ml and 0.00210 µg/ml for OFL indicates that the method is sensitive for the determination of lower concentration of the both the drugs. The % assay was found to be well within the acceptable limit. In degradation studies it was found that AZI was more sensitive to acidic degradation, while OFL showed degradation in acidic, alkali, peroxide, photolytic thermal conditions. The peaks of the degradants in each condition were well separated from main peaks. Purity plot confirmed that there is no interference of any degradants at the retention time of the main peaks indicates that the developed method is stability indicating. The proposed method can be used as an alternative method for the analysis of AZI and OFL in its dosage forms.

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