Spectrophotometric and High Performance Liquid Chromatographic Determination of Ofloxacin and Azithromycin in Pharmaceutical Tablets

Madhuri A. Hinge, Nikita A. Desai, Rajvi J. Mahida and Hitesh P. Dalvadi

ROFEL Shri G.M. Bilakhia College of Pharmacy, Vapi, Gujarat-396191, India.

ABSTRACT

Absorbance ratio spectrophotometric method and high performance liquid chromatographic method were developed for the determination of Ofloxacin and Azithromycin in tablet dosage form. In UV-Spectrophotometric method, estimation of Ofloxacin and Azithromycin was carried out at 230.60 nm (isoabsorptive point) and 288.60 nm (λ<sub>max</sub> of Ofloxacin) for absorbance ratio method. Calibration curves were linear in the range of 4–20 μg/ml for Ofloxacin and 5-25 μg/ml Azithromycin for absorbance ratio method. Correlation coefficient found to be close to 0.9995 for both the drugs. Accuracy for both the drugs was in the range of 98-101%. A simple liquid chromatographic assay has been developed for the determination of Ofloxacin and Azithromycin. A C<sub>18</sub> (250 mm × 4.6 mm, 5 μ) column was used with a mobile phase consisting of Phosphate buffer: Methanol ( pH adjusted to 5.0 with ortho phosphoric acid) at a flow rate of 1.0 ml min<sup>-1</sup>. Quantitation was achieved with UV detection at 215 nm based on the peak height ratios. Beer’s law was obeyed in a concentration range of 12-28 µg ml<sup>-1</sup> for Ofloxacin and 15-35 µg ml<sup>-1</sup> for Azithromycin and the regression line equation was derived with a correlation coefficient of 0.9970 and 0.9980 for Ofloxacin and Azithromycin respectively. The validity of the methods was further confirmed using the standard addition method. The proposed procedures were successfully applied to the determination of Ofloxacin and Azithromycin in bulk and tablet form, with high percentage of recovery, good accuracy and precision.

Key words: Absorbance ratio method, Azithromycin, HPLC, Ofloxacin, Tablets.

INTRODUCTION

Ofloxacin (OFO), 7-fluro-2-methyl-6-(4-methylpiperazin-1-yl)-10-oxo-1-aztricyclo[7.3.10{5,13}]trideca-5(13),6,8,11-tetraene-11-carboxylic acid (Figure 1), a synthetic fluoroquinolone (fluoroquinolones) anti-bacterial agent that inhibits the supercoiling activity of bacterial DNA gyrase, halting DNA replication. It is used for the treatment of (infection of kidney, skin, soft tissue) Urethra and survival gonorrhoea.

Azithromycin (AZI), (2R, 3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S, 14R)-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-15-oxo-11-{{[3,4,6-trideoxy-3-(dimethylamino)-β-D-xylo-]oxy}-1-oxa-6-azacyclopentadec-13-yl 2,6-dideoxy-3-C-methyl-3-O-methyl-α-L-ribopyranoside (Figure 2), a semi-synthetic macrolide antibiotic of Azide class. It is indicated for the treatment of patients with mild to moderate infections caused by susceptible strains of the designated microorganisms in the specific conditions: \( H.\ influenzae\), \( M.\ catarrhalis\), \( S.\ pneumoniae\), \( C.\ pneumoniae\), \( M.\ pneumoniae\), \( S.\ pyogenes\), \( S.\ aureus\), \( S.\ Agal\).

Ofloxacin is official in Indian Pharmacopoeia 2010 and USP32-NF27 and describes Liquid Chromatographic method for its estimation and a literature survey reveals that RP-UPLC, HPTLC, HPLC and Spectrophotometric methods has been developed for its estimation in alone or in combination with other drugs. Azithromycin is official in Indian Pharmacopoeia 2010 and USP32-NF27 British Pharmacopoeia 2012, USP-30 NF-25. Liquid Chromatographic method is given for the estimation of Azithromycin in IP, BP and USP. Various HPLC and Spectrophotometric methods have been reported for the determination of Azithromycin alone or in combination with other active pharmaceutical agents in
dosage forms or in biological fluids. However, simultaneous determination of Ofloxacin and azithromycin has not been described previously. The purpose of present study was to develop and validate spectrophotometric and HPLC method for simultaneous determination of Ofloxacin and azithromycin in tablet dosage form.

**MATERIALS AND METHODS**

**Instrumentation**
A double beam, UV-visible spectrophotometer (Simadzu-1800, Software –UV Probe, Version 2.42) with 1 cm matched quartz cells was used. The spectral band width was 2 nm and the wavelength scanning speed was medium. The HPLC (Agilent SHIMADZU) instrument was equipped with a C18 (250 mm×4.6 mm, 5 μ) column, an auto injector, UV-Detector and Chem 32 software.

**Materials**
Pharmaceutical grade of Ofloxacin and Azithromycin used as reference standard were supplied by Vapi care, Vapi. Combined tablet formulation (Zithium O BD) manufactured by Synochem Pharmaceutical Ltd. and marketed by Alkem Laboratory Ltd., was procured from local market. All other reagents used were AR grade and HPLC grade. AR grade methanol acquired from S.D. Fine Chemicals. Distilled water (HPLC grade- Merk, Renkem), Acetonitrile (HPLC grade-Merk, Renkem), Methanol (HPLC grade-Merk, Renkem), Ortho phosphate (Tri Ethyl Amine), Potassium dihydrogen phosphate (Merk, Renkem) were used for analysis.

**Chromatographic conditions**
The mobile phase was prepared by mixing phosphate buffer and methanol in the ratio of (60:40 v/v) and pH 5.0 adjusted with ortho phosphoric acid. It was filtered through 0.45 μm membrane filter. All determinations were performed at ambient temperature (20°C) using C18, 250 mm×4.6 mm,5 μ, reverse phase column (Agilent SHIMADZU). The column effluent was monitored at 215 nm, which represents the wavelength of maximum absorbance of OFO and AZI. The injection volume was 20 μl with a flow rate of 1 ml min⁻¹.

**Standard solutions and calibration graphs for spectrophotometric measurements**
**Absorbance ratio Method**
A stock solution was prepared by dissolving OFO and AZI in methanol and dilution was made by 0.2 N NaOH to obtain a concentration of 100 µg ml⁻¹. The standard solutions were prepared by dilution of the stock solution in 0.2 N NaOH to reach concentration ranges of 4–20 µg ml⁻¹ and 5-25 µg ml⁻¹ for OFO and AZI respectively for absorbance ratio method. Each solution was scanned between 200-400 nm. Wavelengths were selected from the overlay spectra of OFO and AZI. The absorbance of the solutions was measured at 288.60 nm and 230.60 nm against 0.2 N NaOH as a reagent blank. The concentrations versus their Absorbance were plotted in order to obtain the calibration graphs.

**Standard solutions and calibration graphs for chromatographic procedure (HPLC)**
Standard solutions of OFO and AZI containing concentration range of 12-28 µg ml⁻¹ for OFO and 15-35 µg ml⁻¹ for AZI were prepared in the mobile phase. Triplicate 20 µl injections were made for each concentration and the peak height ratio of each concentration were plotted against the corresponding concentrations to obtain the calibration graph.
Sample preparation
A total of 20 tablets containing OFO and AZI as the active ingredients were weighed and finely powdered. The powder equivalent to 20 mg of OFO and 25 mg of AZI was taken in 100 ml volumetric flask and dissolved in mobile phase. The volume was made up to mark and the solution was filtered through 0.45 micro membrane filter. The appropriately diluted solution was analyzed under optimized chromatographic conditions. The areas of resulting peak were measured at 215 nm. The peak-height ratios were used for the determination of OFO and AZI in each sample.

For Absorbance ratio spectrophotometric method, a 1.2 ml of this solution was diluted to 10 ml with methanol and 0.2N NaOH and in another flask 1.5 ml of this solution was further diluted to 10 ml with methanol and 0.2N NaOH. Absorbance of the resulting solution was measured at 288.60 nm and 230.60 nm against methanol. The concentration of AZI and OFO can be obtained as,

\[ C_x = \frac{(Q_m - Q_y)}{(Q_x - Q_y)} \times A_1/ a_x1 \]
\[ C_y = \frac{(Q_m - Q_x)}{(Q_y - Q_x)} \times A_1/ a_y1 \]

Where, Qx and Qy are value of AZI and OFO respectively, ax1 and ay1 are absorptivity value at isobestic point for OFO and AZI.

Validation Procedure
System suitability
The typical values for evaluating system suitability of a chromatographic procedure include the RSD <1%, tailing factor <2 and theoretical plates >2000. The determination of system suitability of analytical method was accomplished by assaying six samples of Ofloxacin and Azithromycin. The sample concentration of Ofloxacin and Azithromycin used in this analysis was 12-28 µg/ml and 15-35. µg/ml, respectively. The retention time, peak area, theoretical plates and tailing factor were evaluated for system suitability.

Sensitivity
The limit of detection (LOD) and quantification limit (LOQ) were determined by gradually diluting the sample and analysing by the proposed method. The signal/noise ratio (S/N) was determined for each tested strength. The typical S/N ratio recommended by the International Conference on Harmonisation (ICH) is 3/1 and 10/1 for LOD and LOQ, respectively.

Calibration curve
The above-mentioned calibration standards were analysed for determining linearity. The sample strengths ranged from 12-28 µg/ml and 15-35 µg/ml for Ofloxacin and Azithromycin, respectively for HPLC method and 4-20 µg/ml and 5-35 µg/ml of Ofloxacin and Azithromycin for Absorbance ratio method. The regression analysis was accomplished by slope, intercept and correlation coefficient (r²).

Accuracy and precision
The accuracy was determined by percent recovery method. Furthermore, precision (inter-day variance and intra-day

Figure 3: Overlay UV-spectra of OFO and AZI
variance) were determined by assaying samples over a period of 1 day and 3 days, respectively. The standard concentrations used for these study were 12, 20, and 24 µg/ml for Ofloxacin and 15, 25, and 35 µg/ml for Azithromycin for HPLC method and for Absorbance ratio method the concentrations used were 4, 12, 20 μg/ml and 5, 15 and 25 μg/ml for Ofloxacin and Azithromycin.

Robustness

The influence of slight deliberate changes in chromatographic conditions such as column temperature, flow rate of mobile phase and pH of mobile phase on the retention time and peak area were observed one by one. The test was performed in triplicate for each set of conditions. The standard concentrations of Ofloxacin and Azithromycin used in this analysis were 20 µg/ml and 25 µg/ml, respectively.

RESULTS AND DISCUSSION

Absorbance ratio method

Tablet powder was dissolved in methanol and 0.2 N NaOH. Figure 3. shows the absorption (zero-order) UV spectra of (a) OFO and AZI standard solution. However, the application of the absorbance ratio spectrophotometric technique allowed complete elimination of the back-ground absorption due to the excipients.

<table>
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<th>Parameters</th>
<th>Data obtained</th>
<th>OFO</th>
<th>AZI</th>
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<tr>
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<td>Resolution</td>
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Figure 4a: Calibration curve for Ofloxacin at 288.60 nm

Figure 4b: Calibration curve of Ofloxacin at 230.60 nm

Chromatographic procedure (HPLC)

A reversed phase HPLC method was developed to provide a specific procedure suitable for rapid quality control of OFO and AZI tablet dosage form. A mobile phase consisting of phosphate buffer and methanol in the ratio of (60:40 v/v) and pH 5.0 adjusted with ortho phosphoric acid, was chosen after several trials with acetonitrile: water and methanol: water. The apparent pH of the aqueous phase was adjusted to 5 using orthophosphoric acid. The above described chromatographic system allowed an adequate resolution (R, 7.04) between OFO (t, 4.060) and AZI (t, 6.227) in a reasonable time (R, resolution; t, retention time). The applied analytical conditions produced the peaks with suitable peak symmetry (<2).

The typical conditions for system suitability of an analytical method encompass the relative standard deviation (RSD) < 1%, peak symmetry <2 and theoretical plates >2000. The results of system suitability of present chromatographic method are described in Table 1. The peak area, retention time, tailing factor and theoretical plates were within the recommended limits. Therefore, the method was considered as suitable for quantitative determinations a linear calibration graph (Y=150.61x-609.61, r²=0.9972; n=3, for OFO and Y=140.85x-755.24, r²=0.9989; n=3) was obtained over the working concentration range of 12-28 µg ml⁻¹ for OFO and 15-35 µg ml⁻¹ for AZI.

The specificity and selectivity of the HPLC system were ascertained by a separate chromatographic analysis of either the excipient mixtures or sample; no interfering peaks at the retention times of OFO and AZI peaks were observed.

The LOD and LOQ in accordance with the ICH guidelines is 3/1 and 10/1, respectively. LOD and LOQ values for
ofloxacin and azithromycin for Absorbance ratio method and HPLC method are given in Table 2.

Each calibration curve was constructed with five standard strengths (Figure 3). For Absorbance ratio method, the calibration curve of ofloxacin was made with 4, 8, 12, 16 and 20 µg/ml concentrations (Figure 4a). Similarly, the concentrations used in the formation of calibration curve of azithromycin were 5, 10, 15, 20 and 25 µg/ml (Figure 4b). Figure 5 shows typical chromatogram for Ofloxacin and Azithromycin. For HPLC method, the calibration curve of ofloxacin was made with 12, 16, 20, 24 and 28 µg /ml concentrations (Figure 6a). Similarly, the concentrations used in the formation of calibration curve of azithromycin were 15,20,25,30 and 35 µg/ml (Figure 6b). The regression analysis is displayed in Table 2. The correlation coefficient ($r^2$) was close to 0.9999 for both ofloxacin and azithromycin.

The results of accuracy and precision (inter-day variance and intra-day variance) are shown in Table 3 and 4. For accuracy, all the recovery values were within ±5%. By Absorbance ratio method, the mean recovery value of ofloxacin and azithromycin was 98.9-101.10% and 100.30-
101.41% at 288.60 nm and 100.55-102.50% and 98.33-101.44% at 230.60 nm. In HPLC method the recovery values of ofloxacin and azithromycin was 99.77% and 99.72%. For inter-day and intra-day variance assessment % RSD was calculated. All the samples exhibited RSD values <1% confirming that the analytical method was precise. Robustness study was carried out by making minor changes in conditions like composition of mobile phase, flow rate of mobile phase and pH of mobile phase. No substantial variances were observed in the retention time and peak area of each component when the chromatographic conditions were slightly changed one by one. Moreover, the RSD for

<table>
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<th>Drug</th>
<th>HPLC method</th>
<th>Absorbance ratio method</th>
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<td></td>
<td>Amt. (μg/ml)</td>
<td>% RSD Intraday</td>
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<td></td>
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<tr>
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each value was <1%. Thus, the proposed method was considered as robust.

Statistical evaluation of the developed procedures
The HPLC method was chosen as the analytical reference method. Absorbance ratio spectrophotometric procedures were compared with HPLC. The slopes, intercepts and linearity of each calibration graph were calculated and summarized in Table 2. The order of linearity for the calibration graphs in the ranges stated in Table 2 for the different analytical method was: Absorbance ratio /HPLC. The concentration ranges, detection limits and quantitation limits are summarized in Table 2. The lowest detection limit calculated was obtained for absorbance ratio method indicating the highest sensitivity. Relative sensitivities, based on detection limits, were calculated with respect to the chromatographic method. The order of sensitivity for this method was: Absorbance ratio/HPLC. Commercially available tablets were analyzed using the HPLC and the Absorbance ratio spectrophotometric methods. The results obtained were summarized in Table 5. No significant differences were found between the results obtained by the HPLC and the spectrophotometric procedures, for the same batch at the 95% confidence level. Statistical comparison was done on assay results obtained from UV and HPLC methods for marketed formulation (Zithium O BD) by using student’s t-test. Calculated values for t-test were-0.121 and 0.609 for OFO and AZI respectively which is less than t_critical value (12.706) indicating that there was no significant difference between the HPLC method and UV method.

CONCLUSION

The HPLC method and the spectrophotometric (Absorbance ratio) method were found to be reproducible and accurate in the analysis of Ofloxacin and Azithromycin dihydrate in pharmaceutical tablets. Under the experimental conditions, mentioned above, the Absorbance ratio method was the most sensitive method; however, better selectivity
was obtained with the HPLC method. All the proposed methods were linear with good reproducibility and sensitivity. In general, all the proposed methods can be used for the routine analysis of Ofloxacin and Azithromycin dihydrate in bulk and tablet dosage form.

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REFERENCES


ABBREVIATIONS

HPLC: High Performance Liquid Chromatography
UV: Ultra violet
OFO: Ofloxacin
AZI: Azithromycin
ICH: International Conference of Harmonization
S/N: Signal to noise
RSD: Relative standard deviation
Madhuri Hinge, et al.: UV spectrophotometric and HPLC method for simultaneous estimation of Ofloxacin and Azithromycin


