Overview:

The present chapter deals with the determination of azithromycin (AZI) in in-vitro study of solid oral dosage form using the developed and validated RP-HPLC method.
Determination of Azithromycine in *in-vitro* Study by RP-HPLC Technique

8.1 LITERATURE REVIEW

In-vitro study is used to guide development of new drug product and to assess lot-to-lot variability of drug product after its oral administration. Dissolution methods, as well as other analytical methods, are validated to ensure that they are suitable for their intended use and give accurate and reliable data. Guidance on validation characteristics and considerations has been published. Azithromycin (AZI) prevents bacteria from growing by interfering with their protein synthesis. AZI binds to the 50S sub-unit of the bacterial ribosome, and thus inhibits translation of mRNA. Nucleic acid synthesis is not affected. AZI is a macrolide antibiotic related to erythromycin. It is used primarily to treat various bacterial infection caused by respiratory pathogens, such as aerobic gram-positive microorganisms and aerobic gram-negative microorganisms. AZI prevents bacterial cells from manufacturing specific proteins necessary for their survival. AZI is rapidly absorbed and is widely distributed to tissues and concentrated in cells. Peak plasma concentration is achieved within 2 to 3h [1]. Available dosage forms are 250 mg capsule, 250 and 500 mg tablets and powder for oral administration as a suspension.

Desosaminylazithromycin and N-demethylazithromycin is major degradation product of AZI. AZI has been analyzed By HPLC using electrochemical detector [2, 3], fluorescence detector [4], mass spectrometry detector [5] and HPLC UV detector [6, 7] for the detection in bulk materials and pharmaceutical forms. The USP, and BP method describes the use of high pH (about 9-11) of mobile phase which required specific column which is expensive and difficult to obtain commercially. To the best of our knowledge, none of the currently available analytical method can separate AZI from its two major degradation products and accurately quantify AZI in *in-vitro* dissolution study of solid oral dosage form. IUPAC name and chemical structure of AZI is presented in Figure 8.1.
8.2 THE SCOPE AND OBJECTIVES OF PRESENT STUDY

AZI will be forcibly degraded in acidic, basic and strong oxidizing agent’s solution. There is no stability-indicating HPLC method reported in the literature that can adequately separate two degradation products from AZI and accurately quantify AZI during in-vitro study. It is, therefore, felt necessary to develop a new rapid, stability-indicating method for the determination of AZI. An attempt is made to determine whether HPLC can reduce analysis times without compromising the resolution and sensitivity.

Objectives of the present work are as follow:

- To separates AZI from its two major degradation product (Desosaminylazithromycin, N-Demethylazithromycin).
- Perform analytical method validation for the proposed method as per ICH guideline.

8.3 AZITHROMYCIN

The chemical (IUPAC) name of azithromycin is \((2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-2\text{-ethyl} 3,4,10\text{-trihydroxy}-3,5,6,8,10,12,14\text{-heptamethyl}-15\text{oxo}-11\{[3,4,6\text{-trideoxy}-3\text{- (dimethylamino)}-\beta\text{-Dxylo--hexopyranosyl]} \text{oxy}\}-1\text{-oxa-6-azacyclopentadec13-yl} 2,6\text{-dideoxy-3-C-methyl-3-O-methyl-a-L-ribohexopyranoside.}"

![Chemical structures and IUPAC name of AZI](image)
Determination of azithromycine in in-vitro study by RP-HPLC technique

Its molecular formula is $\text{C}_{38}\text{H}_{72}\text{N}_{2}\text{O}$, molecular weight is 748.98 g/mol. AZI is a semi-synthetic macrolide antibiotic of the azalide class. Like other macrolide antibiotics, AZI inhibits bacterial protein synthesis by binding to the 50S ribosomal subunit of the bacterial 70S ribosome. Binding inhibits peptidyl transferase activity and interferes with amino acid translocation during the process of translation. Its effects may be bacteriostatic or bactericidal depending of the organism and the drug concentration. Its long half life, which enables once daily dosing and shorter administration durations, is a property distinct from other macrolides.

**Strengths**

AZI tablets are available in two strengths 250mg and 500mg of azithromycin as an active ingredient for oral administration.

**Innovator**

Immediate release tablets, Pfizer.

**Pharmacology**

**Indication**

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.

**Pharmacodynamics**

AZI, a semisynthetic antibiotic belonging to the macrolide subgroup of azalides, is used to treat STDs due to chlamydia and gonorrhea, community-acquired pneumonia, pelvic inflammatory disease, pediatric otitis media and pharyngitis, and Mycobacterium avium complex (MAC) in patients with advanced HIV disease similar in structure to erythromycin. AZI reaches higher intracellular concentrations than erythromycin, increasing its efficacy and duration of action.
Mechanism of Action

Azithromycin binds to the 50S subunit of the 70S bacterial ribosomes, and therefore inhibits RNA-dependent protein synthesis in bacterial cells.

Pharmacokinetics

Absorption

Bioavailability is 37% following oral administration. Absorption is not affected by food. Azithromycin is extensively distributed in tissues with tissue concentrations reaching up to 50 times greater than plasma concentrations. Drug becomes concentrated within macrophages and polymorphonucleocytes giving it good activity against chlamydia trachomatis.

Volume of distribution

31.1 lit/kg

Protein binding

Serum protein binding is variable in the concentration range approximating human exposure, decreasing from 51% at 0.02 µg/mL to 7% at 2 µg/mL.

Metabolism

Hepatic. In vitro and in vivo studies to assess the metabolism of AZI have not been performed.

Route of elimination

Biliary excretion of AZI, predominantly as unchanged drug, is a major route of elimination.

Half life

68 hours

Clearance

Apparent plasma cl=630 mL/min [following single 500 mg oral and i.v. doses]
Toxicity

Potentially serious side effects of angioedema and cholestatic jaundice were reported.

8.4 EXPERIMENTAL

8.4.1 Materials and reagents

AZI tablets, placebo, AZI working standard, Desosaminylazithromycin working standard and N-Demethylazithromycin working standard are provided by Cadila Pharmaceutical Ltd. Dholka, Ahmedabad, India. HPLC grade methanol is obtained from J.T. Baker (NJ., USA). GR grade potassium dihydrogen phosphate and sodium hydroxide are obtained from Merck Ltd (Mumbai, India). PVDF membrane filters (0.45 μ) and PVDF syringe filters are purchased from Millipore, India. High purity water is generated with Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

8.4.2 Equipments

Acquity HPLC™ system (Waters, Milford, USA), consisting of a quaternary solvent manager, sample manager and PDA (photo diode array) detector. System control, data collection and data processing are accomplished using waters empower chromatography data software.

8.4.3 Preparation of mobile phase

Buffer Preparation:

Dissolved 4.55 g of potassium dihydrogen orthophosphate in 1000 mL of Milli-Q water, adjusted the pH 7.5 with diluted sodium hydroxide solution.

Mobile Phase:

The mobile phase consisted of methanol: buffer (65:35 % v/v) mixture. Mobile phase is filtered through 0.45 μ PVDF filter and degassed under vacuum prior to use.
8.4.4 Diluent preparation

Accurately weigh and transfer 6.8 g of potassium dihydrogen phosphate in to 1000 ml plastic beaker add about 800 ml of purified water, dissolve, and adjust pH-6.0 with diluted sodium hydroxide and makeup volume 1000 with measuring cylinder.

8.4.5 Standard solution preparation

Standard solution is prepared by dissolving standard substance in diluent to obtain solution containing 555 µg/mL of AZI.

8.4.6 Dissolution parameters and sample solution preparation

Dissolution parameter

Carry out the dissolution test using USP Apparatus II dissolution apparatus. Use paddle-stirring assembly and maintain the parameters as follows:

<table>
<thead>
<tr>
<th>Medium</th>
<th>Phosphate buffer (pH-6.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume</td>
<td>900 mL</td>
</tr>
<tr>
<td>RPM</td>
<td>75</td>
</tr>
<tr>
<td>Temperature</td>
<td>37°C ± 0.5°C.</td>
</tr>
<tr>
<td>Time</td>
<td>45 minutes</td>
</tr>
</tbody>
</table>

Sample preparation:

Instrument is set as mentioned in the test method and degassed the medium prior to use. Transferred one tablet in each vessel used 900 ml of medium and operated the apparatus for exactly 45 minutes. At the end of 45 minutes, withdraw 10 ml of the test solution from zone midway between the surface of medium and top of the rotating paddle not less than 1 cm from the vessel wall. Filtered the solution through 0.45 µm PVDF filter and discarded initial 5 ml of the filtrate and use the collected filtrate as a sample solution.
**8.4.7 Placebo solution preparation**

Instrument is set as mentioned in the test method and degassed the medium prior to use. Transferred one placebo tablet in each vessel used 900 ml of medium and operated the apparatus for exactly 45 minutes. At the end of 45 minutes, withdraw 10 ml of the test solution from zone midway between the surface of medium and top of the rotating paddle not less than 1 cm from the vessel wall. Filtered the solution through 0.45 µm PVDF filter and discarded initial 5 ml of the filtrate and use the collected filtrate as a placebo solution.

**8.4.8 Chromatographic conditions**

The chromatographic condition is optimized using a Cosmosil RP-18 (50 x 4.6mm, 5µ) column. The mobile phase consisted of methanol: buffer (65:35, v/v) mixture. The mobile phase was filtered through a 0.45µ PVDF membrane filter and degassed under vacuum prior to use. The flow rate is 2.0 mL/min. The monitoring wavelength is 210 nm and the injection volume is 20 µL with maintaining column oven temperature at 50ºC. The phosphate buffer is used as diluent.

**8.5 METHOD VALIDATION**

The RP-HPLC method described herein has been validated for determination of AZI in in-vitro study of solid oral dosage form.

**8.5.1 Specificity**

Specificity of the method is demonstrated by prepared the solutions like mobile phase, diluent, standard, sample, placebo solution, placebo spiked with API and placebo spiked with impurities. Injected each solution on to the chromatography equipped with PDA detector and recorded the chromatograms.

**8.5.2 System Suitability**

System suitability parameters are measured so as to verify the system, method and column performance. System precision is determined on five replicate injections of standard preparation.
All important characteristics including % RSD, tailing factor and theoretical plate number for AZI are measured.

### 8.5.3 Precision

The precision is the parameter that expresses the closeness of agreement between a series of measurements obtained from multiple analysis of the same sample under the prescribed conditions. In our study the repeatability was evaluated as follows.

**Method precision:**

Prepared six consecutive sample preparations and injected into chromatography system and chromatograms are recorded. Calculate the % drug release of AZI with respected to the standard solutions. The % RSD of six drug released value of sample preparation should not more than 6.0.

**Intermediate precision:**

The aim of the study consists of establishing the effect of the random events on the analytical method and the intermediate precision is evaluated by analyzing a sample by different analysts with different columns on different day. Difference between precision and intermediate precision mean value should not more than 5%.

### 8.5.4 Accuracy

Accuracy of a method is defined as the closeness of the measured value to the true value for the sample. The recovery of method is studied at concentration levels 25%, 100% and 150% of the claimed content. Prepared three sets for each concentration level and injected them in HPLC system. The recovery is calculated with respect to the standard solution.

### 8.5.5 Linearity

The linearity study verifies that the sample solutions are in a concentration range where an analyte response is linearly proportional to the concentration. This study is performed by evaluating the system and method linearity. For the system linearity, standard solutions of AZI at
five concentration levels (50%, 75%, 100%, 125% and 150%) are prepared and each standard solution is injected. The experimental results are plotted to obtain the calibration curve and carrying out the necessary statistical study.

8.5.6 Robustness

The robustness is a measure of the capacity of a method to remain unaffected by small but deliberate changes, change in column oven temperature (± 5°C), changes in flow rate (± 0.2 mL/min), change in wavelength detector (± 2 nm) and change in pH of phosphate buffer solution (± 0.2 units).

8.5.7 Solution stability

The stability of the sample solution is established by storage of the sample solution at ambient temperature for 24h. The sample solution is re-analyzed after 2, 8, 16 and 24h, and % area difference are calculated. The stability of standard solution is established by the storage of the standard solution at ambient temperature for 24h. The standard solution is re-injected after 2, 8, 16 and 24h, and % area difference are calculated.

8.6 RESULTS AND DISCUSSION

8.6.1 Method Development and Optimization

The introduction of new HPLC methods for a routine quality control of pharmaceutical preparations begins with a series of preliminary investigations, which enables in establishing the optimal experimental conditions and provide maximum relevant information by analyzing the experimental data [8]. As a part of optimization various mobile phases and stationary phases are studied. Based on development trial final optimized chromatography condition are; Cosmosil RP-18 (50 x 4.6mm, 5µ) column, mobile phase consisted of methanol:buffer (65:35, v/v) mixture, flow rate is 2.0 mL/min, monitoring wavelength is 210 nm, and injection volume is 20 µL and column oven temperature at 50°C. The phosphate buffer is used as diluent.
8.6.2 Analytical Parameters and Validation

After satisfactory development of method it is subjected to method validation as per ICH guideline [9]. The method is validated to demonstrate that it is suitable for its intended purpose by the standard procedure to evaluate adequate validation characteristics (specificity, system suitability, precision, accuracy, linearity, robustness and solution stability).

8.6.2.1 Specificity

From the study, it is observed that the drug eluted at a retention time of 2.3. No interferences with the analyte peaks due to presence of placebo, blank and impurities have been observed. On the basis of that, the method results specific for the qualitative analysis of AZI in-vitro study. The peak purity angle is less than peak purity threshold for AZI peak, indicating that peak is pure. The purity factor for the drug assures that there is no co-elution of other peaks. Therefore, the method is specific and suitable for routine work. Specimen chromatograms of specificity study are presented in Figure 8.2 to 8.6.

![Specimen chromatogram of blank](image-url)
Determination of azithromycine in *in-vitro* study by RP-HPLC technique

Figure 8.3 Specimen chromatogram of placebo

Figure 8.4 Specimen chromatogram of spike impurities into sample solution

Figure 8.5 Specimen chromatogram of desosaminyl azithromycin impurity
8.6.2.2 System suitability

System suitability parameters are measured so as to verify the system, method and column performance. The % RSD (relative standard deviation) of AZI area count of five replicate injections (standard preparation) is below 1.0 %. Low values of % RSD of replicate injections indicated that the system is precise. Results of other system suitability parameters such as theoretical plates and tailing factor are summarized in Table 8.1. As seen from this data, the acceptable system suitability parameters would be: theoretical plates for AZI is not less than 800, tailing factor for AZI is not more than 2.0 and % RSD of replicate injections is not more than 2.0. Results of system suitability parameters from precision and robustness study are also summarized in Table 8.1.

Table 8.1 System suitability results (system suitability, precision, intermediate precision and robustness study)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>% RSD* of AZI peak</th>
<th>Theoretical plates* for AZI</th>
<th>Tailing factor* for AZI</th>
</tr>
</thead>
<tbody>
<tr>
<td>System suitability</td>
<td>0.71</td>
<td>1105</td>
<td>1.0</td>
</tr>
<tr>
<td>Precision</td>
<td>0.63</td>
<td>1102</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Determination of azithromycine in *in-vitro* study by RP-HPLC technique

<table>
<thead>
<tr>
<th>Intermediate Precision</th>
<th>0.54</th>
<th>1020</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column temperature (45˚C)</td>
<td>0.06</td>
<td>971</td>
<td>1.0</td>
</tr>
<tr>
<td>Column temperature (55˚C)</td>
<td>0.11</td>
<td>1163</td>
<td>1.0</td>
</tr>
<tr>
<td>Flow rate (1.8 ml/min)</td>
<td>0.07</td>
<td>1166</td>
<td>1.0</td>
</tr>
<tr>
<td>Flow rate (2.2 ml/min)</td>
<td>0.05</td>
<td>986</td>
<td>1.0</td>
</tr>
<tr>
<td>Wavelength (208 nm)</td>
<td>0.12</td>
<td>1075</td>
<td>1.0</td>
</tr>
<tr>
<td>Wavelength (212 nm)</td>
<td>0.15</td>
<td>1066</td>
<td>1.0</td>
</tr>
<tr>
<td>Buffer pH (7.3 units)</td>
<td>0.09</td>
<td>936</td>
<td>1.0</td>
</tr>
<tr>
<td>Buffer pH (7.7 units)</td>
<td>0.34</td>
<td>1065</td>
<td>1.0</td>
</tr>
</tbody>
</table>

* ... Determined on five values

### 8.6.2.3 Precision

The precision of the analytical method is verified by repeatability and by intermediate precision. Precision is investigated using sample preparation procedure for six real samples of tablets and analyzing by proposed method. Intermediate precision is studied using different column, and performing the analysis on different day. The average % drug release (n=6) of AZI is 97.1 with RSD of 2.4 % for precision. Results are summarized in Table 8.2 along with intermediate precision data. Low values of % RSD and % difference between precision and intermediate precision indicates that the method is precise. Overlaid specimen chromatograms of precision study are presented in Figure 8.7.

**Table 8.2** Precision and intermediate precision results (n=6 unit)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precision at 100 %</th>
<th>Intermediate precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Drug Release #</td>
<td>% RSD*</td>
</tr>
<tr>
<td>AZI</td>
<td>97.1</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Difference between precision and intermediate precision is 1.7%

# … Average of six determinations;  * … Determined on six values
8.6.2.4 Accuracy

To confirm the accuracy of the developed method, recovery experiments are carried out by standard addition technique. Three different levels (25 %, 100 % and 150 %) of standards are added to pre-analyzed placebo samples in triplicate. The percentage recoveries of AZI at each level and each replicate are determined. The mean of percentage recoveries (n=9) and the % RSD is calculated. The amount recovered is within ± 3 % of amount added, which indicates that the method is accurate and also there is no interference due to excipients present in tablets. The results of recoveries for % drug release are shown in Table 8.3. Overlaid specimen chromatograms are presented in Figure 8.8.

Table 8.3  Accuracy results

<table>
<thead>
<tr>
<th>Azithromycin</th>
<th>At 25 %</th>
<th>At 100 %</th>
<th>At 150 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Recovery #</td>
<td>97.6</td>
<td>99.6</td>
<td>99.5</td>
</tr>
<tr>
<td>% R.S.D.*</td>
<td>0.20</td>
<td>0.04</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* … Determined on three values;  # … Mean of three determinations
8.6.2.5 Linearity

Linearity is demonstrated from 50% to 150% of standard concentration using minimum five calibration levels (50%, 75%, 100%, 125% and 150%) for the AZI compound, which gives us a good confidence on analytical method with respect to linear range. The response is found linear for AZI from 50% to 150% of standard concentration and correlation coefficient is also found greater than 0.997. Y-intercept bias is also found within ± 2. The result of Correlation coefficients, Y-intercept bias and linearity equations for AZI are summarized in Table 8.4. Overlaid specimen chromatograms of linearity study are presented in Figure 8.9. Linearity curve is presented in Figure 8.10.

<table>
<thead>
<tr>
<th>Linearity range (µg/mL)</th>
<th>Correlation Coefficient ($r^2$)</th>
<th>Linearity (Equation)</th>
<th>Y- intercept bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>280 to 840</td>
<td>0.998</td>
<td>$y = 255.7(x) - 871.1$</td>
<td>0.6%</td>
</tr>
</tbody>
</table>
The robustness is a measure of method capacity to remain unaffected by small, but deliberate changes in chromatographic conditions are studied by testing influence of small change in column oven temperature (± 5°C), changes in flow rate (± 0.2 mL/min), change in wavelength (± 2 nm) and change in buffer pH (± 0.2 units). No significant effect is observed on system suitability parameters such as theoretical plates, tailing factor and % RSD of AZI, when small but deliberate changes are made to chromatographic conditions. The results are summarized in Table 8.1 along with system suitability parameters of precision study. Thus, the method is found to be robust with respect to variability in above conditions.
8.6.2.7 Solution stability

Drug stability in pharmaceutical formulations is a function of storage conditions and chemical properties of the drug, preservative and its impurities. Condition used in stability experiments should reflect situations likely to be encountered during actual sample handling and analysis. Stability data is required to show that the concentration of analyte in the sample at the time of analysis corresponds to the concentration of analyte at the time of sampling. Stability of sample solution is established by storage of sample solution at ambient temperature for 24h. AZI sample solution is re-analyzed after 2, 8, 16 and 24h time intervals and % drug release is determined and compared against fresh sample. Sample solution does not show any appreciable change in area of AZI, when stored at ambient temperature up to 24h, which are summarized in Table 8.5. The results from solution stability experiments confirmed that sample solution is stable for up to 24h during drug release determination.

Standard solution is re-injected after 2, 8, 16 and 24h time intervals and % change in area of AZI of all injected standard injections are calculated. Standard solution did not show any appreciable change in % area (for AZI) value when stored at ambient temperature up to 24h [Table 8.5].

<table>
<thead>
<tr>
<th>Time point</th>
<th>% Difference in Area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Standard</td>
</tr>
<tr>
<td>2 Hr</td>
<td>1.6%</td>
</tr>
<tr>
<td>8 Hr</td>
<td>0.3%</td>
</tr>
<tr>
<td>16 Hr</td>
<td>0.4%</td>
</tr>
<tr>
<td>24 Hr</td>
<td>1.4%</td>
</tr>
</tbody>
</table>
8.7 CALCULATION FORMULA

8.7.1 % Drug release (% w/w)

Calculated the quantity, in % drug release, of AZI in the portion of solid oral pharmaceutical formulation using the following formula:

\[
\text{Drug release (\%)} = \frac{C_{\text{std}} \times R_s \times 100}{C_s \times R_{\text{std}}}
\]

Where,

\( C_{\text{std}} \) = Concentration of standard solution in mg/mL

\( C_s \) = Concentration of sample solution in mg/mL

\( R_s \) = Compound peak response obtained from the sample preparation

\( R_{\text{std}} \) = Compound peak response (mean peak area) obtained from the standard preparation

8.7.2 Relative standard deviation (% RSD)

It is expressed by the following formula and calculated using Microsoft excel program in a computer.

\[
\text{Related Standard Deviation (\%)} = \frac{SD \times 100}{\bar{X}}
\]

Where,

\( SD \) = Standard deviation of measurements

\( \bar{X} \) = Mean value of measurements

8.7.3 Accuracy (% Recovery)

It is calculated using the following equation:

\[
\% \text{ Recovery} = \frac{\text{Amount of substance found (mg)} \times 100}{\text{Amount of substance added (mg)}}
\]
8.8 CONCLUSION

A dissolution method with HPLC analysis for AZI tablets has been developed to meet global regulatory requirements. The methodology is evaluated for specificity, linearity, precision, accuracy and range in order to establish the suitability of the developed analytical method. Stability of analytical solution is also observed for the suitability of the method. In addition, intermediate precision as per regulatory requirements is reformed and showed that there are no significant differences among the different “intermediate conditions” evaluated. The total run time is 3.0 min, within which the drug and their degradation products are eventually separated. Developed method can be applied for the routine analysis, formulation stability study and pre formulation study, where sample load is higher and high throughput is essential for faster delivery of results.
8.9 REFERENCES


