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DEVELOPMENT AND VALIDATION OF A NEW HPLC METHOD FOR THE ESTIMATION OF AZITHROMYCIN IN BULK AND TABLET DOSAGE FORM

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ABSTRACT

A simple, reproducible and efficient reversed phase high performance liquid chromatographic (RP-HPLC) method has been developed for quantitative determination of azithromycin in drug substance. The separations were carried out on a Xterra C₁₈ column (150 × 4.6 mm; 5μ) with UV detection at 215 nm. The mobile phase consisting of acetonitrile and phosphate buffer (pH adjusted to 7.5) in a ratio of 50:50 v/v. The injection volume was 50 μl and flow rate was 1.0 mL/min. The linear dynamic response was found to be in the concentration range of 300μg-700 μg/mL and coefficient of correlation was found to be 0.998. The %RSD value was below 2.0 for intraday and interday precision indicated that the method was highly precise. The percentage recovery value was higher than 100 %, indicating the accuracy of the method and absence of interference of the excipients present in the tablet formulation. The proposed method was simple, economic, accurate, precise and reproducible and hence can be applied for routine quality control analysis of azithromycin in bulk and dosage form.

INTRODUCTION: Azithromycin is a macrolite antibiotic that acts by interfering protein synthesis in bacteria. Due to the differences in the way proteins are made in bacteria and humans, the macrolite antibiotics do not interfere with humans' ability to synthesis proteins. It binds to the 50s rRNA subunit of the 70s bacterial ribosome's, therefore inhibits RNA-dependent protein synthesis.

It inhibits the translation of mRNA in bacterial cells at the chain elongation step; result in the blockage of transpeptidation. Azithromycin is rapidly absorbed and is widely distributed to tissues and becomes concentrated in cells. Peak plasma concentrations are achieved within 2 to 3 hours¹.

Chemically azithromycin is (2R, 3S, 4R, 5R, 8R, 10R, 11R, 12S, 13S, 14R)-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-xyllo-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one shown in **figure 1**.

It is derived from erythromycin; however, it differs chemically from erythromycin in that a methyl-substituted nitrogen atom is incorporated into the lactone ring².

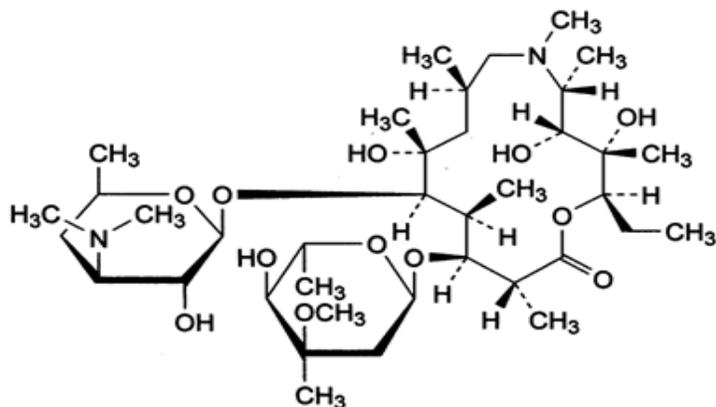


FIGURE 1: STRUCTURE OF AZITHROMYCIN

Azithromycin has been analyzed by HPLC using electrochemical^{3,4}, fluorescence⁵, mass spectrometry⁶, and UV^{7,8} for detection in bulk material and pharmaceutical forms. The USP method⁹ describes the use of a high pH mobile phase (pH 11.0) which requires the use of specific column which is expensive. Also USP method employs amperometric electrochemical detection which is not available in many laboratories. So development and validation of a simple method for the analysis of azithromycin is still required. In this study a new HPLC method has been developed for determination of azithromycin in bulk and tablet dosage forms which is very much simple, economic and accurate.

MATERIALS AND METHODS:

Apparatus and chromatographic condition: The chromatographic separation was performed on a Prominence Shimadzu high performance liquid chromatographic instrument equipped with a Xterra C₁₈ column (150 x 4.6mm; 5 μ m) integrated with UV detection at 215nm. The mobile phase consisting of acetonitrile and phosphate buffer (pH adjusted to 7.5) in a ratio of 50:50 v/v and was prepared freshly, filtered and sonicated before use and delivered at a flow rate of 1 mL/min. The volume of each injection was 50 μ L. Column temperature was maintained 40 $^{\circ}$ C for the analysis.

Chemicals and reagents: Azithromycin was obtained as a gift sample from Renata Pharmaceuticals Ltd. Methanol and water used were of HPLC grade and phosphate buffer used were of analytical grade. The commercially available Azithromycin tablets claimed to contain 500 mg of active ingredients were procured from local market of Bangladesh.

Preparation of Stock Solution: Stock solution of azithromycin was prepared by dissolving 100 mg of azithromycin in a 100 mL of volumetric flask containing 50 mL of mobile phase and the solution was sonicated for 5 min and then volume was made up to the mark with mobile phase to get a concentration of 1.0 mg/mL. Subsequent dilutions of this solution were made with mobile phase to get concentrations of 300 μ g/ml to 700 μ g/ml and the chromatogram was recorded by maintaining the chromatographic condition shown in figure 3.

Preparation of Sample Solution: A quantity equivalent to equivalent to 100 mg of azithromycin drug substance was accurately weighed and transferred into a 100 ml volumetric flask containing 50 mL of mobile phase and the solution was sonicated for 5 min and then made up to the mark with mobile phase to get a concentration of 1 mg/mL. Then this solution was filtered through 0.45 μ membrane filter and diluted suitably using mobile phase to obtain a solution 500 μ g/mL.

Development and validation of HPLC method: Present study was conducted to obtain a new, affordable, cost-effective and convenient method for HPLC determination of azithromycin in bulk and tablet dosage form. The experiment was carried out according to the official specifications of USP-30, ICH-1996, Global Quality Guidelines-2002. The method was validated for the parameters like system suitability, selectivity, linearity, accuracy, precision, and robustness.

The linearity of an analytical method is its ability to elicit that test results are proportional to the concentration of analyte in samples within a given range. This was determined by means of calibration graph using increasing amounts of standard solutions (300- 700 μ g/mL). These standards were tested six times in agreement to the International Conference on Harmonization (ICH).

Calibration curves were constructed and the proposed method was evaluated by its correlation coefficient and intercept value, calculated in the corresponding statistical study (ANOVA) ($p < 0.05$).

The precision is a measure of the ability of the method to generate reproducible results. The precision of the assay was determined by repeatability (intraday) and intermediate precision (inter-day) and reported as %RSD. For this, 500 µg/mL of the solution was measured three times in a day and the same was repeated in next three days.

Accuracy indicates the deviation between the mean value found and the true value. Accuracy was determined by means of recovery experiments, by the addition of active drugs to placebo formulations. The accuracy was calculated from the test results as the percentage of the analyte recovered by the assay.

The system suitability tests were carried out on freshly prepared standard stock solution of azithromycin to evaluate the suitability of the system. The system was evaluated by analyzing the repeatability of retention time, tailing factor, theoretical plates (Tangent) of the column. The robustness of the method was assessed by altering the some experimental conditions such as, by changing the flow rate, amount of acetonitrile and the temperature of the column.

RESULTS AND DISCUSSION: All of the analytical validation parameters for the proposed method were determined according to Conference on Harmonization (ICH) guidelines¹⁰.

Linearity: The linearity of this method was determined at ten concentration levels ranging from 300-700 µg/mL (**Table 1**). The plot of peak area of each sample against respective concentration of drug was found to be linear shown in figure 2 in the range of 300–700 µg/mL. The regression equation was found to be $Y = 2338.6x - 217080$ and the correlation coefficient (r) of the standard curve were found to be 0.998 shown in **table 2**.

TABLE 1: DATA FOR CALIBRATION CURVE

Concentration µg/ml	Response of peak area
300	465924
400	740912
500	951350
600	1194520
700	1408426

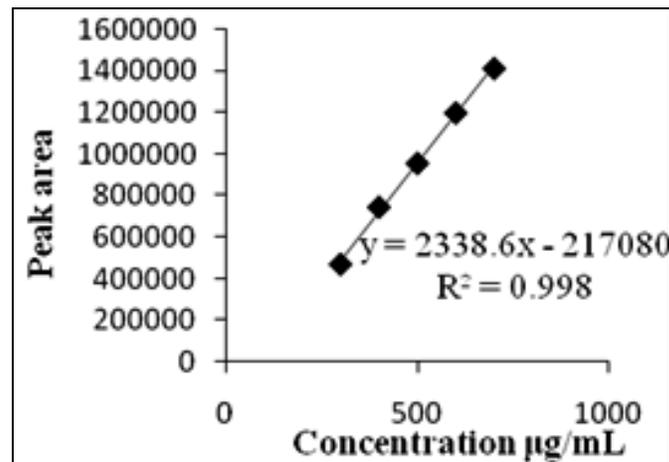


FIGURE 2: CALIBRATION CURVE OF AZITHROMYCIN

TABLE 2: VALIDATION PARAMETERS

Parameters	Results		
Linearity range (µg/ml)	300 to 700		
Standard Regression equation	$Y = 2338.6x - 217080$		
Correlation coefficient	0.998		
Precision	Concentration	Intraday (%RSD)	Interday (%RSD)
	500(µg/ml)	0.354	0.265

Precision: The precision of the method was investigated with respect to repeatability (intra-day), intermediate precision (inter-day variation). The precision (measurements of intraday and inter day) results showed good reproducibility with percent relative standard deviation (% RSD) was below 2.0% shown in **table 2**. This indicated that method was highly precise.

Recovery studies (Accuracy): Recovery studies were performed to judge the accuracy of the method. The studies were carried out by adding a known quantity of pure drug to the pre-analyzed formulation and the proposed method was followed. From the amount of drug found, the percent recovery was calculated. Recovery study was carried out at three levels 80%, 100% and 120% for the formulation concentration of 500 µg/mL shown in **table 3**.

The percentage recovery value which was higher than 100 %, indicated that the accuracy of the method and absence of interference of the excipients present in the tablet formulation.

TABLE 3: RECOVERY STUDY

Level of Addition (%)	Addition of pure drug ($\mu\text{g/mL}$)	% Recovery of pure drug	Recovery (%) \pm S.D.
80	400	100.45	100.52 \pm 0.39
100	500	100.89	
120	600	100.23	

System Suitability Test: The parameters of system suitability study were presented in **table 4**. From the typical chromatogram of azithromycin as shown in Figure 3, it was found that the average retention time \pm standard deviation for azithromycin was found to be 7.33 \pm 0.004 min for five replicate injections. The asymmetry factor was found to be 1.12, which indicated asymmetric nature of the peak. The number of theoretical plates was found to be 3639, which suggested an efficient performance of the column. The absence of additional peaks in the chromatogram

indicated non-interference by the common excipients used in the tablet formulation. To optimize the chromatographic conditions, various combinations of acetonitrile and buffer were tested and the ratio of 50:50 v/v afforded peak with good shape and resolution.

TABLE 4: SYSTEM SUITABILITY PARAMETER

Retention time (min) \pm S.D.	7.33 \pm 0.004
No. of theoretical plates	3629
Asymmetric factor	1.12

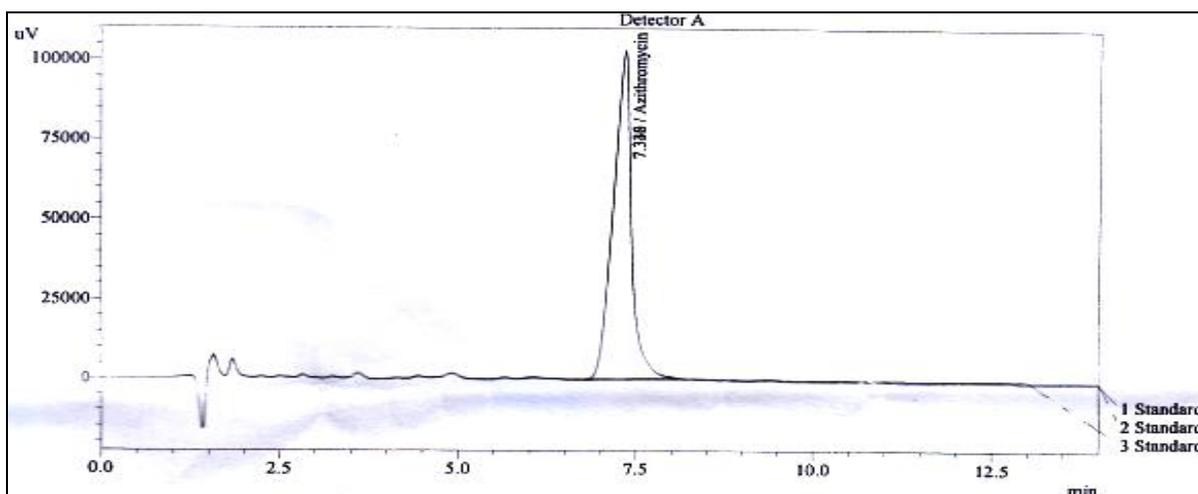


FIGURE 3: TYPICAL CHROMATOGRAM OF AZITHROMYCIN

Robustness: Robustness was performed by small but deliberate variation in the chromatographic conditions and was found to be unaffected by small variations like $\pm 2\%$ in volume of mobile phase composition, ± 0.1 mL/min in flow rate of mobile phase and $\pm 1\%$ change in column temperature. It was observed that there were no marked changes in the chromatograms, which demonstrated that the proposed method was robust.

CONCLUSION: From the above discussion it is clear that the proposed method was simple, sensitive and reliable with good precision and accuracy. Hence, this method can be used for the routine analysis of azithromycin in pure drug substance.

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