Analysis of Azithromycin and Its Related Compounds by RP-HPLC with UV Detection

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Abstract

A simple, validated stability-indicating liquid chromatographic method is developed for the analysis of azithromycin in raw material and in pharmaceutical forms. Liquid chromatography with a UV detector at a wavelength of 210 nm using a reversed-phase C_{18} stationary phase has been employed in this study. Isocratic elution is employed using a mixture of phosphate buffer–methanol (20:80). This new method is validated in accordance with USP requirements for new methods for assay determination, which include accuracy, precision, specificity, linearity, and range. This method shows enough selectivity, sensitivity, accuracy, precision, and linearity range to satisfy Federal Drug Administration and International Conference of Harmonization regulatory requirements. The current method demonstrates good linearity over the range of 0.3–2.0 mg/mL of azithromycin. The accuracy of the method is 100.5% with a relative standard deviation of 0.2%. The precision of this method reflected by relative standard deviation of replicates is 0.2%. The method is sensitive with a detection limit of 0.0005 mg/mL for azithromycin. Impurities and degradation products of azithromycin can be selectively determined with a good resolution in both raw material and pharmaceutical forms.

Introduction

Azithromycin is a macrolide antibiotic related to erythromycin (Figure 1). It is used primarily to treat various bacterial infections caused by respiratory pathogens, such as aerobic Gram-positive and Gram-negative bacteria. Azithromycin prevents bacterial cells from manufacturing specific proteins necessary for their survival. Azithromycin is rapidly absorbed and is widely distributed to tissues and becomes concentrated in cells. Peak plasma concentrations are achieved within 2 to 3 h (1).

Because azithromycin is obtained from erythromycin, impurities present will undergo the same modifications and the azithromycin analogues of these impurities can be found in azithromycin bulk samples. In addition, degradation products of azithromycin as well as intermediate compounds of the semi-synthesis may be present (2); for instance, azithromycin is rapidly decomposed in acidic solution via intra-molecular dehydration to form erythromycin-6,6-hemiketal and then anhydroerythromycin (3). It is very difficult to determine small amounts of degradation products in a vast excess of parent drug and even more so when the compounds do not present a chromophore as this makes their detection more difficult (4).

Azithromycin has been analyzed by high-performance liquid chromatography1(HPLC) using electrochemical (5,6), fluorescence (7), mass spectrometry (8), and UV (9,10) for detection in bulk material and pharmaceutical forms. The USP method (11) describes the use of a high pH mobile phase (pH 11.0), which requires the use of a specific column, which is expensive and difficult to obtain commercially. Also, the USP method employs amperometric electrochemical detection, which is not available in many laboratories. A comprehensive, validated, and simple analysis method for azithromycin and its related substances, impurities, and degradation products is, therefore, crucial. HPLC with UV detector is a good selection as UV detector is available in most laboratories.

Liquid chromatography with UV detection has been already employed for the analysis of azithromycin in azithromycin tablets (4), in raw material, and in azithromycin tablets (9,10). In the current work, an HPLC method with UV detector will be developed for the determination of azithromycin and other related compounds, impurities, degradation products in raw material as well as in new pharmaceutical formulations: dry suspension and capsules. Validation of the method will be performed according to the requirements of USP for assay determination, which include accuracy, precision, specificity, linearity, and range. Additionally, in order to meet the regulatory guidance of the Federal Drug Administration\International Conference of Harmonization (ICH) (12), azithromycin will be forcibly degraded in acidic, basic, and strong oxidizing agent solutions. The method is able to detect and quantitatively estimate the degradation products generated.

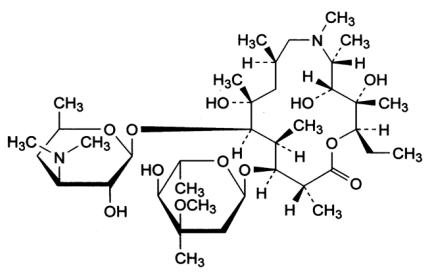

Figure 1. Structure of azithromycin.

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Experimental

Chemicals

HPLC-grade methanol was from J.T. Baker (Phillipsburg, NJ). Potassium dihydrogen phosphate, hydrochloric acid, sodium hydroxide, and hydrogen peroxide are from Merck (Darmstadt, Germany). Azithromycin RS, azaerythromycin RS, desosaminyl-azithromycin RS, and *N*-demethylazithromycin RS are from USP (Rockville, MD).

Apparatus

HPLC system (Merck Hitachi Lachrome Elite HPLC system, Tokyo, Japan) with an L-2130 pump, an L-2200 autosampler, L-2300 column oven, and L-2490 UV detector was employed. The Ezochrom Elite software was employed. The chromatographic analysis was performed on HX749288, LiChroCart, HPLC-cartrage Purospher STAR RP-18 endcapped (5 µm), (150 mm length, 4.6 mm inner diameter) (Waters, Milford, MA), as well as on RP-8 endcapped (5 µm), (150 mm length, 4.6 mm inner diameter) (Waters). The column is stored at 50°C.

Standard solutions and HPLC conditions

Phosphate buffer was prepared by dissolving 4.55 g of potassium dihydrogen phosphate in 1000 mL of water (0.3 M), adjusted to different pHs (6.0, 6.5, 7.0, and 7.5) with 10% sodium hydroxide solution. Different volume fractions of methanol (50, 60, 70, 80, and 90) have been used for the mobile phase. A filtered and degassed mixture of phosphate buffer (different pH), and methanol (different volume fractions) has been tested as a mobile phase for the separation of azithromycin and other related compounds. The best combination was 80% methanol and 20% buffer with pH 7.5. For this combination, different buffer concentrations have been tested (0.020, 0.030, and 0.050 M). The optimum concentration for the mobile phase was found to be 0.03 M. Different column temperatures (40, 50, and 60°C), as well as different flow rates (1.0, 1.5, and 2.0 mL/min) have been tested. UV detection was performed at 210 nm, and injection volume was 20 µL.

Stock standard solution was prepared by dissolving a quantity of azithromycin dihydrate equivalent to 500 mg of azithromycin base in 50 mL of mobile phase to obtain a solution having a known concentration of 10 mg/mL azithromycin.

Nominal standard solution was prepared by diluting 5 mL of stock standard solution to 50 mL mobile phase to obtain a solution having a known concentration of 1.0 mg/mL azithromycin.

Nominal solutions of the formulated azithromycin capsules (Zitrocin 250 mg/capsule) were prepared by dissolving a quantity equivalent to 250 mg of azithromycin (~ 480 mg of the capsules powder which is the average weight of the capsules) in a 250 mL the mobile phase.

The nominal solutions of the formulated azithromycin dry suspension (Zitrocin dry suspension contains 200 mg of azithromycin in each 5 mL after reconstitution) is prepared by reconstituting the dry suspension bottle with the quantity of water specified by the label on the bottle and taking a volume of 5 mL, which is equivalent to 200 mg of azithromycin and dissolving it with a quantity of the mobile phase sufficient to give 200 mL of the solution.

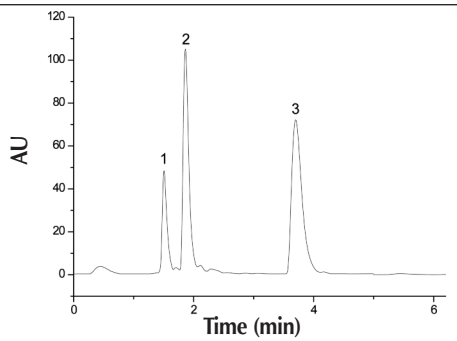

Figure 2. Chromatogram of azithromycin and its related compounds. Analytes: 1, Desosaminylazithromycin; 2, N-demethylazithromycin; and 3, Azithromycin. Mobile phase: methanol–phosphate buffer, pH 7.5 (80:20, v/v), flow rate 2.0 mL/min, injection volume 20 mL. Column: reversed phase C18, 5 mm, 25 cm length, 4.6 mm inner diameter, column temperature: 50°C. UV detection: 210 nm.

Results and Discussion

Method development

We have started method development by testing two reversed-phase stationary phases: C_8 and C_{18}. Azithromycin has retention on both C_8 and C_{18} stationary phases; however, C_{18} shows better resolution and separation of related compounds and impurities from azithromycin compared to C_8. Therefore, C_{18} reversed-phase column, 5 mm, 25, or 15 cm length and 4.6 mm inner diameter has been used for this method. Problems when analyzing basic drugs, such as azithromycin, are known in the pharmaceutical industry, as these compounds interact strongly with the polar ends of HPLC column packing materials, causing peak asymmetry and low separation efficiencies. However, we have not encountered these problems using this reversed-phase stationary phase.

Regarding the mobile phase, a mixture of phosphate buffer and methanol was used. In order to improve the separation and peak symmetry, the chromatographic variables: buffer concentration, buffer pH, temperature, and methanol percentage have been investigated. Phosphate buffer with high pH (7.5) was used to avoid problems with silica dissolution. Moreover, the stability of azithromycin and related compounds is low in acidic media. Phosphate buffer concentration has been varied until optimum concentration (0.03M) has been obtained. Additionally, different methanol percentages in the mobile phase have been tested to get optimum separation and resolution of azithromycin and its related compounds. 80% of methanol was found to be the

Table I. Chromatographic Parameters for the Separated Peaks in Figure 2

Parameter	Desosaminylazithromycin	*N*-Demethylazithromycin	Azithromycin
Resolution	–	2.1	5.3
Capacity factor	2.8	3.3	7.7
Asymmetry	1.09	1.18	1.32
Selectivity	–	1.18	2.33

optimum percentage. Temperature was increased to facilitate mass exchange with the corresponding decrease of peak broadening and increase in sensitivity; 50°C was a good selection. We have selected low wavelength (210 nm) to be used for UV detection due to the lack of chromophores other than the ester group (Figure 1). In the current study, the elution is simplified by using isocratic elution (80:20, methanol–buffer) with a flow rate of 2.0 mL/min, compared with gradient elution employed by Miguel et. al for the separation of azithromycin and its related substances (4).

After this optimization, this method has been used for the separation of azithromycin from its related compounds (e.g., desosaminylazithromycin and *N*-demethylazithromycin) (Figure 2) as well as separation from azaerythromycin A (Figure 3). Good separation with adequate resolution has been obtained (Figures 2–3). Chromatographic parameters of the separated peaks (desosaminylazithromycin, *N*-demethylazithromycin, and azithromycin) (Table I).

Method validation

After method development, the validation of the current test method for azithromycin has been performed in accordance with USP requirements for assay determination (Category I: Analytical methods for quantitation of active ingredients in finished pharmaceutical products), which include accuracy, precision, specificity, linearity, and range (13).

Linearity and range

Linearity is the ability of a method to elicit test results that are directly proportional to analyte concentration within a given range. Linearity is generally reported as the variance of the slope of the regression line. Range is the interval between the upper and lower levels of the analyte that have been demonstrated to be determined with precision, accuracy, and linearity using the method as written. The range is normally expressed in the same units as the test results obtained by the method. A minimum of five concentration levels along with certain minimum specified ranges are required. For assay, the minimum specified range is from 80–120% of the target concentration. For content uniformity testing, the minimum range is from 70–130% of the test or target concentration (14).

Acceptance criteria for linearity are that the correlation coefficient (R^2) is not less than 0.990 for the least squares method of analysis of the line. Additionally, the relative standard deviation (RSD) will not be greater than 5.0% at all standard concentrations (14).

Standard solutions covering the range between 30–120% of the nominal standard concentration (1.0 mg/mL azithromycin) have been prepared by diluting specific volume of the stock standard to get several concentrations (0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.20, 1.60, and 2.0 mg/mL). Then, these standards have been chromatographed using UV detector at 210 nm. Three runs have been performed for every concentration. The peak responses (e.g., peak area) have been recorded and plotted versus standard concentrations. Results have shown that the method is linear over the specified range with R^2 of 0.9999, insignificant y-intercept (6977), and a slope of 3×100^6 has also been obtained. Standard

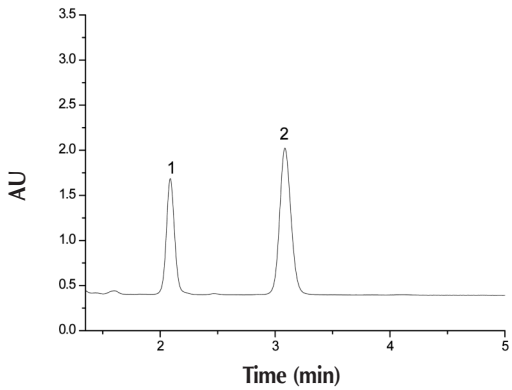

Figure 3. Chromatogram of 1, azaerythromycin (capacity factor and peak asymmetry for azaerythromycin peak are 2.85 and 1.06, respectively); and 2, azithromycin (resolution, capacity factor, peak asymmetry, and selectivity for azithromycin peak are 6.4, 4.70, 1.09, and 1.64, respectively). Other experimental conditions are the same as in Figure 2 except for the length of the column, which was 15 cm instead of 25 cm.

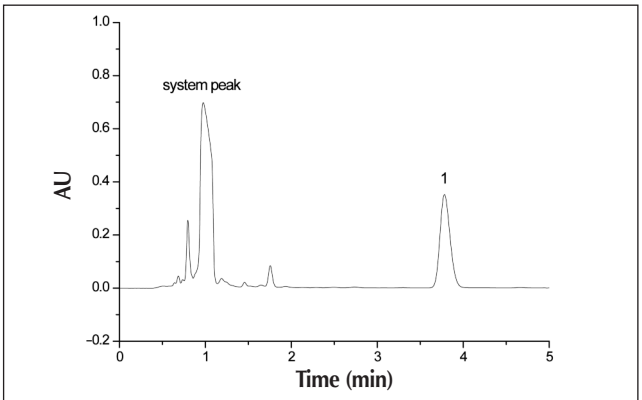

Figure 4. Chromatogram of 1, azithromycin (1.0 mg/mL azithromycin, added to it 10% of 2M sodium hydroxide). Other experimental conditions are the same as in Figure 2. Resolution, capacity factor, and peak asymmetry for azithromycin peak are 5.3, 4.45, and 1.19, respectively.

Table II. Accuracy (recovery) of Azithromycin in Capsules and Dry Suspension Formulations at Three Concentration Levels as well as in Raw Material at the Nominal Conc.

Azithromycin	% Accuracy (recovery)			RSD for 3 replicates		
Conc. (mg/mL)	Raw material	Capsules	Dry suspension	Raw material	Capsules	Dry suspension
0.8	–	99.9	99.5	–	0.8%	0.3%
1.0	99.6	100.0	100.0	0.8%	0.3%	0.8%
1.20	–	101.3	99.9	–	0.9%	0.5%

Table III. Precision of Azithromycin Analysis in Capsules and Dry Suspension Formulations at Three Concentration Levels, as well as in Raw Material at the Nominal Conc.

Azithromycin conc. (mg/mL)	RSD		
	Raw material	Capsules	Dry suspension
0.8	–	0.8%	0.3%
1.0	0.2%	0.3%	0.8%
1.20	–	0.9%	0.5%

deviation of the slope and y-intercept is 145,921 and 15,761, respectively. Standard error was 23,798. These findings demonstrate linearity of this method over the specified range.

The obtained R^2 value for the current method (0.9999) is comparable to the value obtained by Zubata et. al (9) for the LC method for azithromycin analysis in raw material and in azithromycin tablets (0.9994), and better than the value obtained by Miguel et. al (4) for the LC method for azithromycin analysis in azithromcin tablets (0.996).

Accuracy

The accuracy of an analytical procedure measures the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value and value found (i.e., accuracy is a measure of exactness of an analytical method). Accuracy is measured as the percent of analyte recovered by assay after spiking samples in a blind study (15). For the assay determination of azithromycin in drug formulations (capsules and dry suspension), accuracy is evaluated by analyzing synthetic mixtures spiked with known quantities of azithromycin.

To document accuracy, a minimum of nine determinations over a minimum of three concentration levels covering the specified range (for example, three concentrations, three replicates for each) were collected. It is performed at 80, 100, and 120% levels of label claim. At each level studied, replicate samples are evaluated. The RSD of the replicates provides the analysis variation and gives an indication of the precision of the test method. Moreover, the mean of the replicates, expressed as % of label claim, indicates the accuracy of the test method. The mean recovery of the assay should be within 100 ± 2.0% at each concentration over the range of 80–120% of nominal concentration (15).

To prepare accuracy standard solutions, placebo of the drug formulation (e.g., capsule or drug suspension) has to be prepared according to the formulation procedure. To the required quantity of placebo, a known quantity of azithromycin with the same proportion as in the drug formulation has been added to get three concentrations [0.8, 1.0 (nominal concentration), and 1.2 mg/mL of azithromycin]. These standards, then, have been chromatographed. Three runs have been performed for every concentration, and then peak area has been recorded. The average recovery and the RSD for each level have been calculated. Results have shown that the mean recovery of the assay for both drug suspension and capsules as well as for azithromycin raw material is within 100 ± 2.0% at each concentration, and the RSD is lower than 1.0% (Table II). Furthermore, results have shown that recovery data obtained was within the 99.9–101.3% range for capsule formulation (mean = 100.4%) and 99.5–100.0% range for dry suspension (mean = 99.8%), and the mean recovery for raw material at the nominal concentration (1.0 mg/mL) is 99.6% (Table II). Zubata et al. has obtained comparable recovery data for azithromycin in raw material and in azithromycin tablets (99.8–100.0% with a mean of 99.4%) (9).

Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the RSD for a statistically significant number of samples. Precision is performed at one level (repeatability). Repeatability is the result of the method operating over a short time interval under the same conditions (injection precision or instrument precision). It is determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each), or from a minimum of six determinations, at 100% of the test or target concentration. RSD for replicate injections should not be greater than 1.5% (16).

The RSD of the peak areas for the recovery data analyzed in accuracy study (see the Method validation section) for each level (80%, 100%, and 120% of the nominal concentration) has been calculated, and it has been found to be less than 1.0% for each level (Table III). The RSD of the peak areas of six replicate injections for the nominal standard concentration (100%) has also been calculated to be 0.2%. These results show that the current method for azithromycin analysis is repeatable.

Specificity (stability-indicating evaluation)

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present, such as impurities, degradation products, and matrix components (17). It is a measure of the degree of interferences from such components, ensuring that a peak response is due to a single component only. Specificity is measured and documented in a separation by the resolution, plate count (efficiency), and tailing

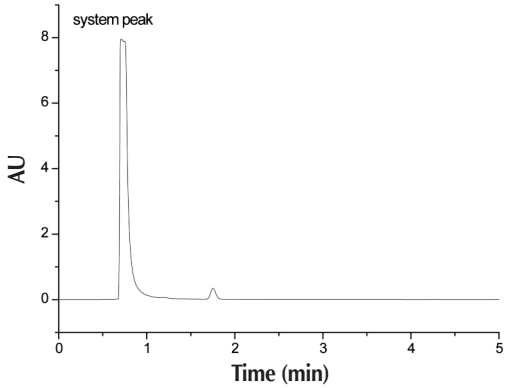

Figure 5. Chromatogram of 1, azithromycin (1.0 mg/mL azithromycin, added to it 10% of 2M hydrochloric acid). Other experimental conditions are the same as in Figure 2. 1: azithromycin is no more present as it is completely degraded.

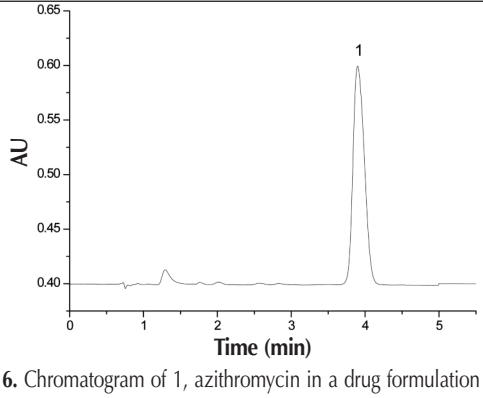

Figure 6. Chromatogram of 1, azithromycin in a drug formulation product (capsule). Other experimental conditions are the same as in Figure 2. Resolution, capacity factor, and peak asymmetry for azithromycin peak are 4.8, 4.4, and 1.34, respectively.

factor. Resolution between the active ingredient (e.g. azithromycin) and all the components have to be at least 1.5. Efficiency of the column is not less than 1500 theoretical plates for azithromycin peak, and the tailing factor is not more than 1.5.

Specificity may be demonstrated by enhancing degradation of the azithromycin under stress conditions (acid and base hydrolysis, and oxidation). Accordingly, 10 mL of 2M hydrochloric acid, 10 mL of 2M sodium hydroxide, and 10 mL of 35% hydrogen peroxide has been added to 100 mL of assay solution. These solutions have been chromatographed, and all the peaks in the chromatograms are recorded. Results showed that the resolution between azithromycin and all other degradation products or components is higher than 1.5, which indicates that the method is specific for determination of azithromycin and can separate and detect possible degradation products (Figure 4–5).

We have also found that azithromycin is stable in basic solutions (e.g., sodium hydroxide solution) as it gives no degradation product when it is exposed to 10% of 2M sodium hydroxide (Figure 4). On the other hand, azithromycin has degraded completely in both acidic (10% of 2M hydrochloric acid) and in hydrogen peroxide (10% of 35% hydrogen peroxide) solutions (Figure 5). Zubata et al. has observed that azithromycin showed degradation products following both alkaline and acid hydrolysis (9) while Miguel et al. has observed that azithromycin was slightly degraded in basic media and it was completely degraded in acidic media (4).

Detection limit and quantitation limit

Detection limit is the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions (16). Detection limit can be determined by preparing a solution that is expected to produce a response that is ~ 3 to 10 times baseline noise. The solution is injected three times, and the signal and the noise for each injection are recorded. Each signal-to-noise ratio (S/N) is then calculated, and averaged. The concentration of the solution is used for the determination of the detection limit if the average S/N ratio is between 3 and 10. If it is not between 3 and 10, the solution concentration is modified as necessary and the experiment is repeated. The limit of detection (LOD) may be expressed as:

$$LOD = 3.3\ \sigma/S$$

where σ is the standard deviation of the response, and S is the slope of the calibration curve.

Limit of quantitation can be determined in the same manner by using the formula:

$$10\ \sigma/S$$

Results showed that the detection and quantitation limits for azithromycin using this method are 0.0005 and 0.0008 mg/mL, respectively.

After successful development and validation of this method, we have employed it for the analysis of azithromycin in two drug formulations (capsules and drug suspension) as well as in raw material (Figure 6).

Conclusion

A new, specific, and validated method for the analysis of azithromycin by using HPLC equipped with UV detection at 210 nm has been developed. This method is accurate, precise, specific, sensitive, and linear. This method can be employed for the analysis of azithromycin in different drug formulations as well as raw material. Related compounds (e.g., impurities, degradation products, and matrix components) can be also separated with good resolution using this validated method.

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