

## **Analysis of azithromycin and its related compounds by reversed-phase high-performance liquid chromatography with UV detection**

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### **Abstract**

A simple, validated stability-indicating liquid chromatographic method was developed for the analysis of azithromycin raw material and in pharmaceutical forms. The chromatograph is equipped with a UV detector at a wavelength of 210 nm on a reversed phase C18 column. Isocratic elution was employed using a mixture of phosphate buffer and methanol (20:80). This new method has been 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/International Conference of Harmonization regulatory requirements. The current method demonstrated 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 was 0.2%. The method is sensitive with a detection limit of 0.0005mg/mL for azithromycin. Impurities and degradation products of azithromycin can be selectively determined with a good resolution in both raw material and pharmaceutical forms.

Key words: azithromycin, assay, accuracy, precision, linearity, specificity, pharmaceutical dosage forms.

## 1. 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 microorganisms and Aerobic gram-negative microorganisms. 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 hours [1].

Since 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 intramolecular 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 analysed by 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 specific column which is expensive and difficult to obtain commercially. Also 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.

The objective of the present work is the development and validation of a HPLC method with UV detector for the determination of azithromycin and other related compounds, impurities, degradation products in raw material and pharmaceutical formulations (dry suspension and capsules) to be employed in routine and stability tests. 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.

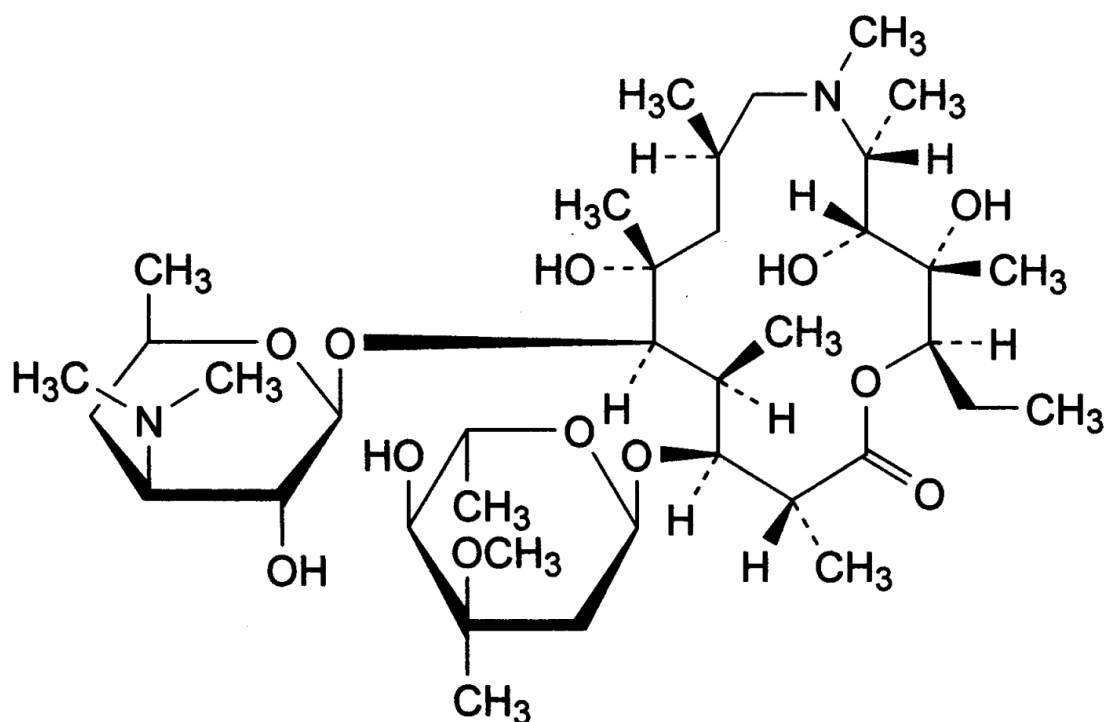


Figure 1: Structure of azithromycin.

## 2. Experimental

### 2.1 Chemicals

Methanol HPLC grade is from J.T Baker (NJ, USA). Potassium dihydrogen phosphate, hydrochloric acid, sodium hydroxide, and hydrogen peroxide are from Merck (Darmstadt, Germany). Azithromycin RS, azaerythromycin RS, desosaminylazithromycin RS, and N-demethylazithromycin RS are from USP (Rockville, MD, USA).

### 2.2 Apparatus

HPLC system (Merck Hitachi Lachrome Elite HPLC system, Japan) with a 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-cartridge Purospher STAR RP-18 endcapped , and RP-8 endcapped (5  $\mu$ m), (150 mm length, 4.6 mm inner diameter) (Waters Corporation, Milford, Massachusetts, USA). The column is kept at 50 °C.

### **2.3 Standard solutions and HPLC conditions**

Phosphate buffer was prepared by dissolving 4.55 g of potassium dihydrogen phosphate in 1000 mL of water, adjusted to pH 7.5 with 10% sodium hydroxide solution. A filtered and degassed mixture of phosphate buffer pH 7.5, and methanol (20:80) has been used as a mobile phase. UV detection was performed at 210nm, flow rate was 2.0 mL/min, and injection volume was 20  $\mu$ L.

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 per 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 per 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 [ about 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 which 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 .

## **3. Results and discussion**

### **3.1 Method development**

We have started method development by testing two reversed-phase stationary phases: C8 and C18. Azithromycin has retention on both C8 and C18 stationary phases, however, C18 shows better resolution and separation of related compounds

and impurities from azithromycin compared to C8. Therefore, C18 reversed-phase column, 5  $\mu\text{m}$ , 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 pharmaceutical industry, since these compounds interact strongly with 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 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 optimum percentage. Temperature was increased to facilitate mass exchange with the corresponding decrease of peak broadening and increase in sensibility. Fifty degree Celsius 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 (see Figure 1). Isocratic elution (80:20, methanol/buffer) with flow rate of 2.0 ml/min was found to be good for the elution of azithromycin and its related substances.

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). A good separation with adequate resolution has been obtained, see Figures 2-3.

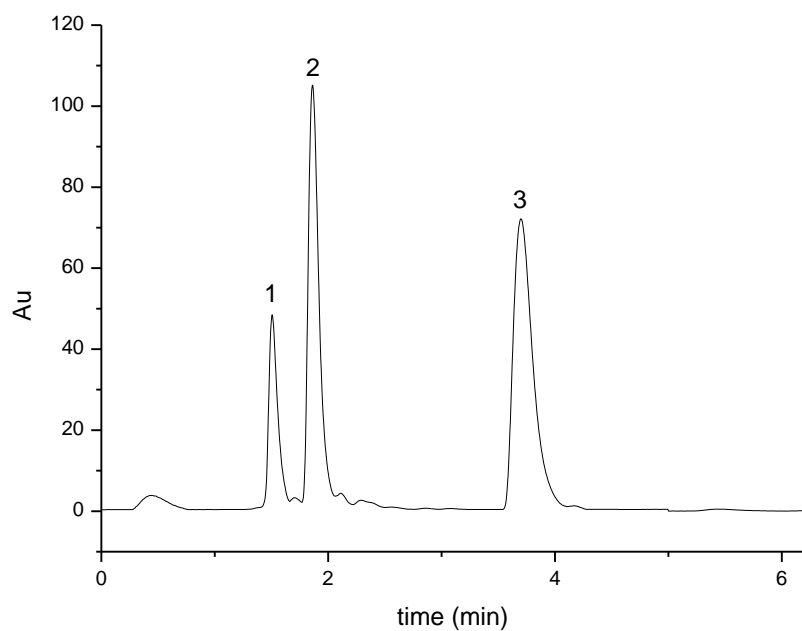


Figure 2: Chromatogram of azithromycin and its related compounds. Analytes: (1) Desosaminylazithromycin, (2) N-Demethylazithromycin, and (3) Azithromycin. Mobile phase: methanol/phosphate buffer, pH 7.5 (80:20, v/v), flow rate 2.0 mL/min, injection volume 20  $\mu$ L. Column: reversed phase C18, 5  $\mu$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

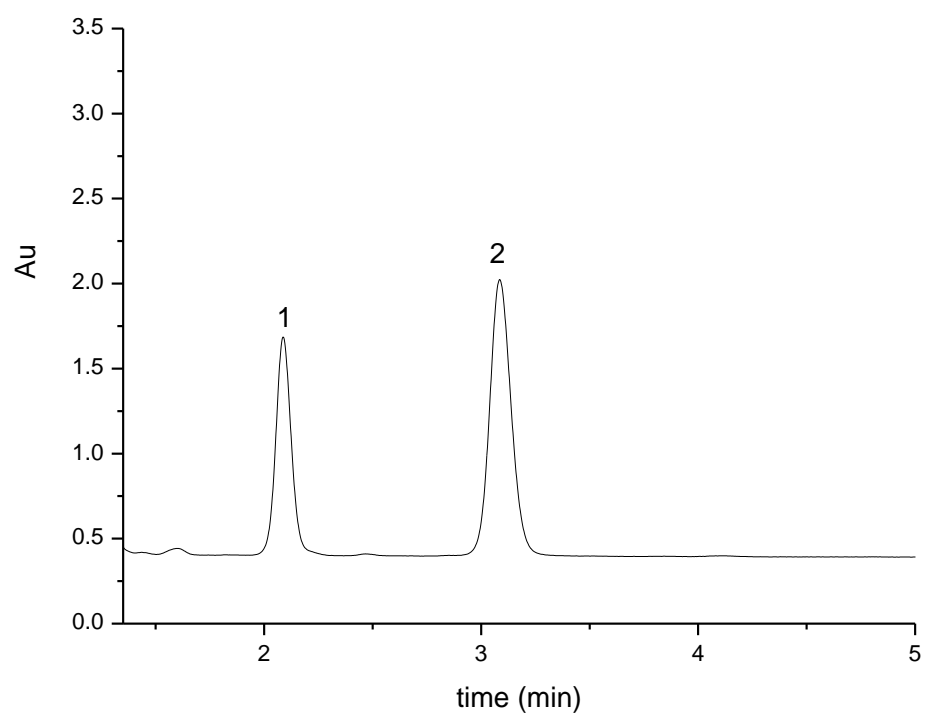


Figure 3: Chromatogram of azithromycin (1) and azaerythromycin (2). Mobile phase: methanol/phosphate buffer, pH 7.5 (80:20, v/v), flow rate 2.0 mL/min, injection

volume 20  $\mu$ L. Column: reversed phase C18, 5  $\mu$ m, 25 cm length, 4.6 mm inner diameter, column temperature: 50  $^{\circ}$ C. UV detection: 210 nm.

### **3.2 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].

#### **3.2.1 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 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 (1.0mg/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 has 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  $3 \times 10^6$  has also been obtained. Standard deviation of the slope and y-intercept is 145921 and 15761,

respectively. Standard error was 23798. These findings demonstrate linearity of this method over the specified range.

### 3.2.2 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, by 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  $100 \pm 2.0\%$  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 1.2mg/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 is within  $100 \pm 2.0\%$  at each concentration, and the RSD is lower than 1.0%, see Table 1.

Table 1: Accuracy of azithromycin in drug formulation (capsules) at three concentration levels.

<b>Concentration</b>	<b>Concentration of azithroycin (mg/ml)</b>	<b>% accuracy (recovery)</b>	<b>RSD for 3 replicates</b>
80	0.8	99.9	0.8%
100	1.0	100.0	0.3%
120	1.20	101.3	0.9%

### 3.2.3 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, i.e. 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 for the recovery data analyzed in accuracy study (see section 3.2.2) 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. The RSD 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.

### 3.2.4. 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 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, 10mL of 2M sodium hydroxide, and 10mL 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, see 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.

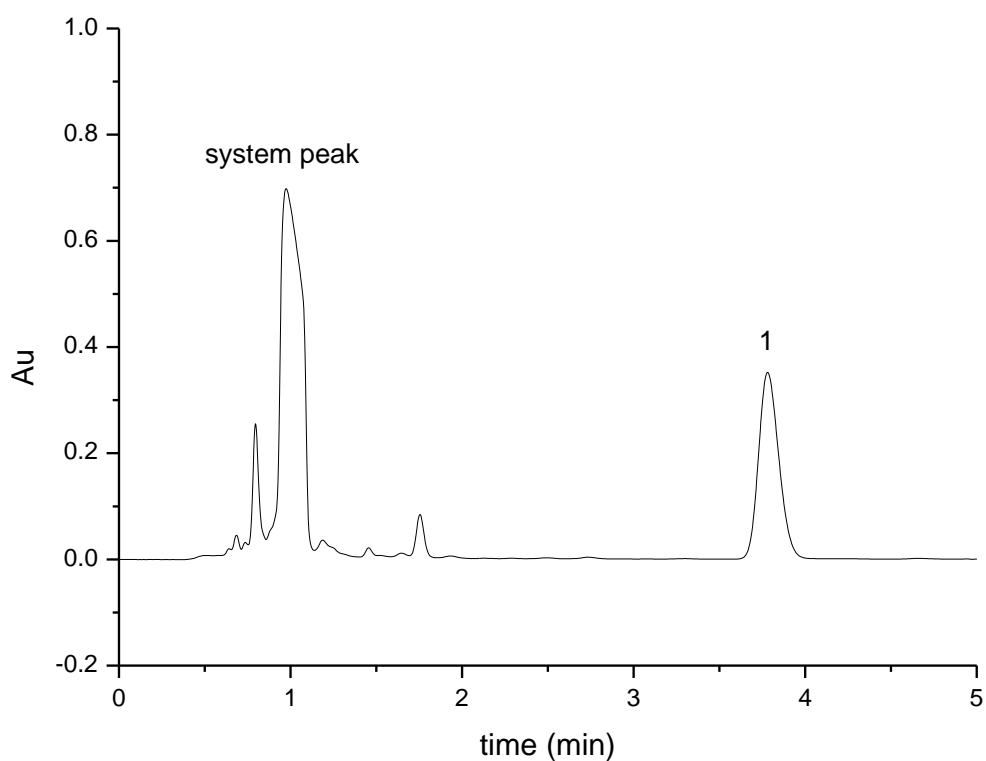


Figure 4: Chromatogram of azithromycin (1) (1.0mg/ml azithromycin, added to it 10% of 2M sodium hydroxide). Mobile phase: methanol/phosphate buffer, pH 7.5 (80:20, v/v), flow rate 2.0 mL/min, injection volume 20  $\mu$ L. Column: reversed phase

C18, 5  $\mu\text{m}$ , 25 cm length, 4.6 mm inner diameter, column temperature: 50  $^{\circ}\text{C}$ . UV detection: 210 nm.

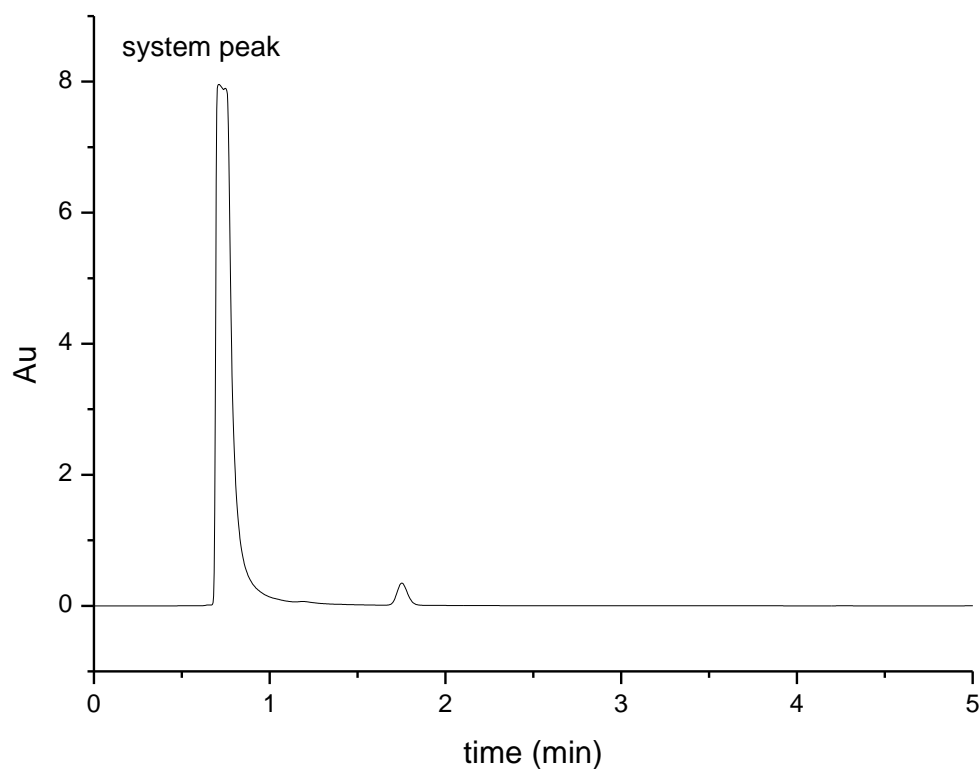


Figure 5: Chromatogram of azithromycin <sup>(1)</sup> (1.0mg/ml azithromycin, added to it 10% of 2M hydrochloric acid). Mobile phase: methanol/phosphate buffer, pH 7.5 (80:20, v/v), flow rate 2.0 mL/min, injection volume 20  $\mu\text{L}$ . Column: reversed phase C18, 5  $\mu\text{m}$ , 25 cm length, 4.6 mm inner diameter, column temperature: 50  $^{\circ}\text{C}$ . UV detection: 210 nm. <sup>(1)</sup>: azithromcin is no more present as it is completely degraded.

### 3.2.5 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 approximately 3 to 10 times base line 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 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 (DL) may be expressed as:

$$DL = 3.3 \sigma/S$$

Where  $\sigma$  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 but using the formula

$$10 \sigma/S.$$

Results showed that detection limit and quantitation limit 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, see Figure 6.

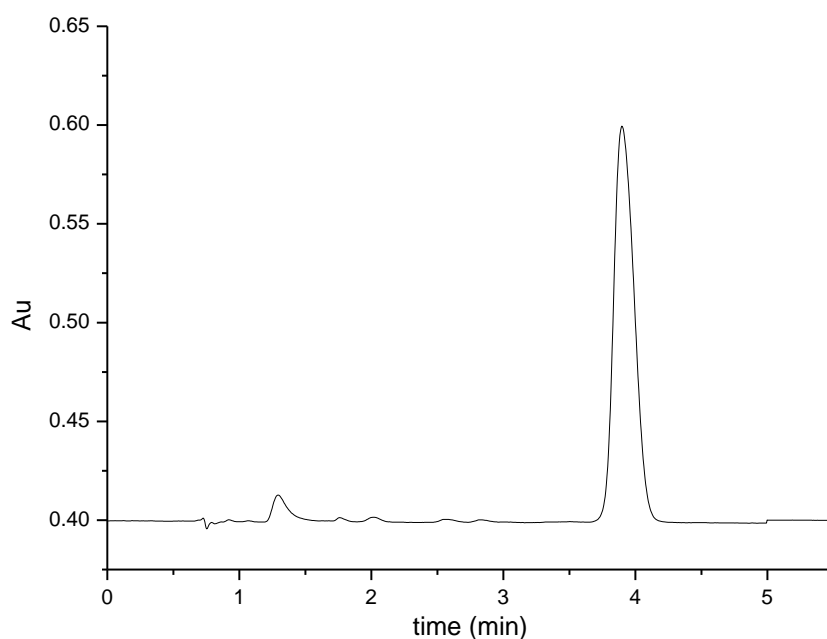


Figure 6: Chromatogram of azithromycin in a drug formulation product (capsule). Mobile phase: methanol/phosphate buffer, pH 7.5 (80:20, v/v), flow rate 2.0 mL/min, injection volume 20  $\mu$ L. Column: reversed phase C18, 5  $\mu$ m, 25 cm length, 4.6 mm inner diameter, column temperature: 50  $^{\circ}$ C. UV detection: 210 nm.

#### 4. Conclusion

A new specific, validated method for the analysis of azithromycin by using HPLC equipped with UV detection at 210nm 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, matrix components can be also separated with good resolution using this validated method.

## 5. References

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