Research Article

A rapid, developed and validated RP-HPLC method for determination of azithromycin

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Abstract
Azithromycin (Azr) is a member of the macrolide antibiotic and it used on a wide scale in prescribed antibiotic drugs as anti-gram-positive and anti-gram-negative microorganisms. The present study aimed to develop an HPLC method of Azr analysis enjoyed highly linearity, repeatability, robust, rugged, selective and rapid to use. The chromatographic method uses a reversed phase column ODS-3 (250 mm × 4.6 mm x 5 µm). The mobile phase was prepared by mixing Methanol: Phosphate buffer (9:1, v/v) at flow rate 1.2 ml/min with PDA detector 210 nm, column oven adjusted to 40° C with injection volume 50 µL. The method revealed satisfied linearity regression R^2 (0.9996) with repeatability (0.66%), LOD and LOQ 28.7 µg/ml and 86.9 µg/ml respectively. The method showed a successful application for Azr determination in bulk and pharmaceutical formulations.

Keywords Azithromycin · HPLC · Determination · Validation · Pharmaceutical

Abbreviations

Azr	Azithromycin
HPLC	High- performance liquid chromatography
PDA	Photodiode array detector
UV	Ultraviolet
FT-IR	Fourier-transform infrared spectroscopy
USP	United States Pharmacopeia
LOD	Limit of detection
LOQ	Limit of quantitation
Conc	Concentration
P. A	Peak area
P. As	Peak areas
STDEV	Standard deviation
RSD	Relative standard deviation

1 Introduction

Azr is a member of the macrolide antibiotic, 15-membered azalactone ring, semi-synthetic and it is derived from erythromycin but, it differs from erythromycin in its chemical properties where the methyl-substituted nitrogen atom is incorporated into the lactone ring as shown in Fig. 1a, b.

It used for treating many and various bacterial infections, such as gram-positive and gram-negative microorganisms. The presence of the nitrogen atom into the ring introduce significant changes in the pharmacokinetics, microbiological and chemical properties of Azr [1]. It available in different oral dosage forms; powder for solution for infusion, powder for oral suspension and capsules.

Several analysis methods have been developed to determine of Azr in different pharmaceutical dosage forms. These methods include different analysis techniques as microbiological methods [2] and high-performance liquid chromatography (HPLC) [3].

Chromatographic separation technique is one of the most essential, easiest and powerful in most qualitative and quantitative analysis. HPLC is currently the most satisfied tool for an excellent and optimum separation [4].

Azr has been quantitatively analyzed in bulk material and different pharmaceutical dosage forms by

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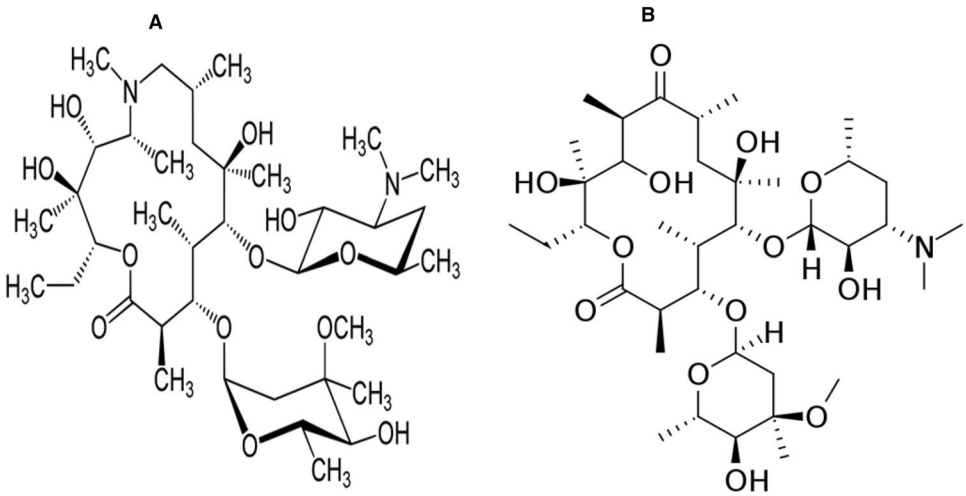

Fig. 1 Structure of azithromycin (**a**), Erythromycin (**b**)

Electrochemical using coulometric and amperometric detectors [5, 6], HPLC using fluorescence [7–9].

Complicated procedures for sample pretreatment were required in detection using Fluorescence involving pre-column derivatization of the analyte. Electrochemical detection is often required a much more time in the analysis so, both in the sample preparation steps classified as a time-consuming technique and not desired in assay determination. In many HPLC assay methods of analysis, a high pH mobile phase about 11.0 was required. Moreover, a specific column also wanted to get the optimum separation as in the United States Pharmacopeia [10]. Mass spectrometric methods may have the highest sensitivity, but the determination process is complicated to use and very expensive [11]. A spectrophotometric UV–Vis method has been reported by Suhagia et al. for determination of Azr in pharmaceutical dosage forms. Although the method is easy and simple to perform, it suffers from the lack of separation where unknown excipients that might interfere which makes fluctuation in UV absorption reading for Azr [12]. FT-IR transmission spectroscopy for determination of Azr was reported by Mallah et al. It is easy to execute but it is not being suitable for formulations with the unknown composition of excipients [13].

In the present study, an HPLC method with a photodiode array detector (PDA) was developed for the determination of a lower concentration of Azr in different pharmaceutical dosage forms. The proposed analytical method of Azr was found to be precise, repeatable, linear, accurate, rugged, robust, specific and selective.

2 Materials and methods

2.1 Chemicals and reagents

Azr standard (98.4%) was supplied by Century pharmaceuticals LTD. (India) as a gift sample from Smart pharma company (Assuit, Egypt). HPLC-grade methanol, HPLC-grade acetonitrile, potassium dihydrogen phosphate, Hydrochloric acid, Sodium hydroxide and Hydrogen peroxide (Scharlau, Spain). Deionized water used in the analysis. Phosphate buffer pH 8.0 was prepared by mixing of 100 ml of 0.2 M potassium dihydrogen phosphate and 93.6 ml of Sodium hydroxide 0.2 M, then diluted to 1000 ml by deionized water.

2.2 Instrument and chromatographic system

Azr was measured using LC-20A HPLC instrument with the PDA (Shimadzu, Japan). The method was performed on reversed phase column ODS-3 (250 mm × 4.6 mm × 5 µm) (Thermo Scientific, USA). The mobile phase was prepared by mixing Methanol: Phosphate buffer (9:1, v/v) at flow rate 1.2 ml/min with PDA detector 210 nm, column oven adjusted to 40° C with injection volume 50 µL.

2.3 Preparation of standard solution

An accurately weighed quantity of Azr (100 mg) was transferred to a 100 ml volumetric flask, approximately 50 ml of the mobile phase was added and dissolved in the ultrasonic bath. The solution was completed to the marked volume by the mobile phase and mixed to obtain a final

concentration of 1.0 mg/ml. The prepared stock solution was stored at 4 °C in a glass vial.

2.4 Method validation parameters

2.4.1 System suitability

Firstly, we assured from the system suitability and the instrument performance. The sample was prepared by dissolving accurately 100 mg of standard in 100 ml volumetric flask and dilute with mobile phase.

2.5 Linearity and range

Linearity is defined by the correlation coefficient, which should be ≥ 0.999 [14], using peak area (P. A) responses, where the range included the concentrations between the minimum and the maximum concentration in linearity test including the target concentration (1 mg/ml).

Regression linearity equation:

$$P.\ A = a\ (Conc.) + b \quad (1)$$

where (P. A) presents Peak area, (Conc.) presents the concentration (%), a represents the slope and b is the intercept.

Linearity was performed by preparing 5 different concentrations of (50, 80, 100, 120 and 150%) of Azr standard. The sample was prepared by weighing accurately 1000 mg of Azr standard and dissolved in 100 ml of mobile phase in 100 ml volumetric flask (stock solution). Subsequently, serial dilutions were prepared by taking (5 ml, 8 ml, 10 ml, 12 ml and 15 ml) from the stock solution (10.0 mg/1 ml) and completed to 100 ml with mobile phase, then injected in triplicates for each concentration.

2.5.1 Limit of detection (LOD)

It was defined as the smallest concentration of an analyte in the sample which can be detected by the detector and it is not significant to undergo the linearity and precision test (it is not to be quantified) [15–17].

2.5.2 Limit of quantitation (LOQ)

It was defined as the smallest concentration of an analyte in the sample which can be detected by the detector and it can be determined quantitatively with appropriate precision and accuracy [15, 17, 18].

LOD and LOQ were calculated according to the linearity of the calibration curve and its standard error according to the following equations:

$$LOD = 3.3\sigma/S \quad (2)$$

$$LOQ = 10\sigma/S \quad (3)$$

where σ: is standard error and S: is a slope of the linearity calibration curve.

2.5.3 Accuracy and recovery

Accuracy and recovery, each of them is a face for the same coin. The accuracy of a measurement is defined as the closeness of the measured value (actual conc) to the true value (Theoretical conc) [19] where recovery it is defined as how much was recovered from the initial concentration using the purposed method [19].

Accuracy and recovery were evaluated by addition of 3 sets of Azr standard to the formula placebo to get concentration at 80% [0.8 mg/ml], 100% [1.0 mg/ml] and 120% [1.2 mg/ml]. Then injected in triplicate for each concentration. The average P. As for each concentration was calculated. The actual concentration for each average P. As from the linearity equation was calculated, then the recovery was calculated according to the following equation:

$$\text{Recovery } \% = \text{ActualConc.}\%/\text{TheoreticalConc.}\% \times 100 \quad (4)$$

2.5.4 Precision and repeatability

Repeatability expresses "The precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision" [20].

Repeatability was conducted using 6 different preparations [15] of the 100% test concentration [1.0 mg/ml] of Azr.

2.5.5 Robustness

Robustness was determined by observing how a method stands up to slight variations [14, 21] in normal operating parameters. This could be a slight variation in mobile phase composition, temperature, flow rate and etc.

The analytical method validation was performed by deliberated changes of the target method parameters. Changes included a different organic solvent (Methanol) ratio at (± 10%), different temperature ± 5°C and different flow rate ± 0.2 ml. where the other method parameters were kept constant in each study.

The robustness of method can be evaluated by calculation of the pooled RSD % of the total number of replicates that have been made in each parameter change.

2.5.6 Ruggedness

Ruggedness of an analytical method is the degree of reproducibility [18, 21, 22] of the test results obtained by the analysis of the same samples under a variety of conditions. The major change such as, different analysts, HPLC columns of different serial number or different supplier, different days and etc.

(A) Day to Day: Single sample freshly preparation (1.0 mg/ml) were used to determine a single day precision then on the second day and the third day were used to provide information concerning day-to-day precision. This acquired by the same analyst.
(B) Analyst to Analyst: Person-to-person precision was performed to provide information about ruggedness values between different analysts. A single sample preparation (1.0 mg/ml) was used to determine a person precision then another person prepared another sample using the same method parameters.
(C) Column to Column: The same analytical method was performed on HPLC columns of the same packing materials but of a different serial number.

The ruggedness of method can be evaluated by calculation of the pooled RSD % of the total number of replicates that have been made in each parameter change.

2.5.7 Auto-sampler stability (solution stability)

This test was performed by injection the test at a target concentration of 100% [1.0 mg/ml]. It was injected at different time intervals to assess the solution stability at analysis conditions.

2.5.8 Selectivity and specificity

Selectivity means the ability to measure accurately an analyte in the presence of interference [23]. It was performed by separate injection of inactive of formulation placebo, Azr standard and formula solutions with Azr at the same test target concentration 1 mg/1 ml. Also, specificity it was performed using forced degradation to provide an indication of the stability–indicating properties of the procedure and indicating on the absence of other interference with a good separation of analyte principle peak [14, 24].

(A) Heating degradation of Azr: It was performed by weighing accurately 100 mg in 100 ml volumetric flask and completed with mobile phase then boiled under reflux for 5 min, allowed to coll. Then it was injected onto HPLC.
(B) Acid hydrolysis: It was performed by weighing accurately 100 mg of in 100 ml volumetric flask and dissolved in 80 ml of mobile phase then a 20 ml of HCl 0.1 N was added and left for 15 min, then neutralized and injected onto HPLC.
(C) Base hydrolysis: It was performed by weighing accurately 100 mg of in 100 ml volumetric flask and dissolved in 80 ml of mobile phase then a 20 ml of NaOH 0.1 N was added and left for 15 min, then neutralized and injected onto HPLC.
(D) Oxidation hydrolysis: It was performed by weighing accurately 100 mg of in 100 ml volumetric flask and dissolved in 80 ml of mobile phase then add 20 ml of H_2O_2 3% and left for 15 min, then neutralized and injected onto HPLC.

2.6 Application of the validated test method for Azr analysis in the different commercial dosage forms in the Egyptian local market

The drug finished products have undergone the analysis, Zithromax 500 mg powder for solution for infusion, Zithromax 250 mg capsules and Zithromax 900 mg/22.5 ml powder for oral suspension. The tested samples were prepared by dissolving an accurate quantity of the drug product in mobile phase to obtain the final concentration 1 mg/ml of Azr, then sonicated for 5 min. Subsequently, the samples were filtered through *Whatman* No.1 filter paper and injected onto HPLC under the prescribed validated method parameters.

3 Results and discussion

3.1 System suitability

The retention time of Azr. peak appeared about at 5.8 min as in Fig. 2, also the RSD %, tailing and plates evaluated as in Table 1. The RSD % for each parameter of system suitability was found to be < 2.0% for 6 replicates according to the requirements in (European Pharmacopoeia [25].

3.2 Linearity and range

It's clear from the output results that, the method linear in the range 50–150% from the target conc 1 mg/ml. Calibration curve of Azr showed also, a good regression coefficient R^2 as in Table 2 which shows the linear proportional between the response of P. As and the corresponding concentrations. So, the method was found to be linear as the R^2 is 0.9996 ≥ 0.999 and the curve follows the linear equation: P. A = 1138.1(Conc)+9.0828.

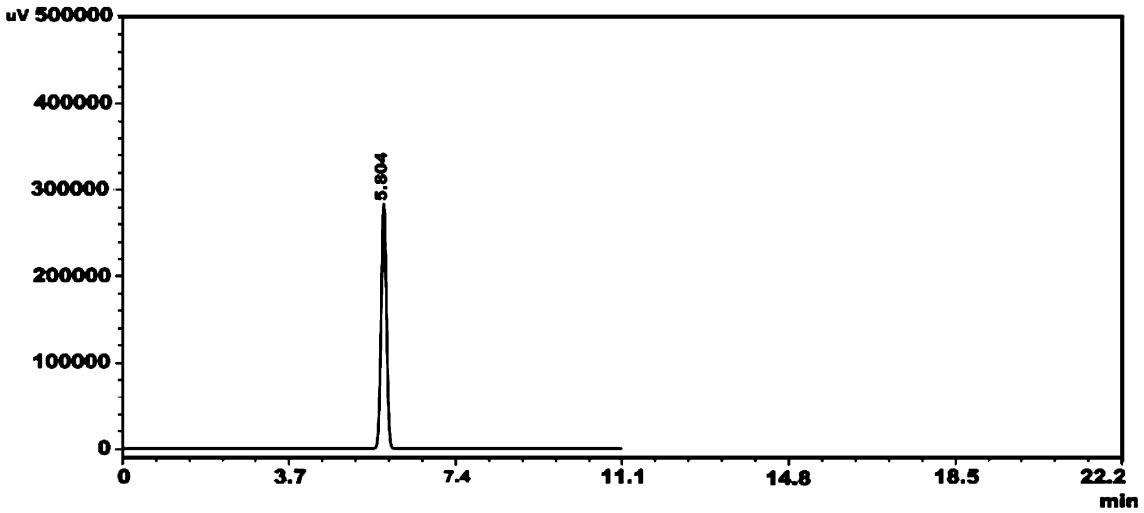

Fig. 2 Azr chromatogram

Table 1 System suitability results

#	P. A	Tailing	Plates
1	113,965	1.015	18,547
2	113,629	1.015	18,704
3	113,741	1.015	18,390
4	112,509	1.015	18,613
5	112,173	1.016	18,183
6	113,741	1.016	18,390
RSD %	0.12%	0.05%	1.02%

Table 2 Linearity data and parameters of Azr calibration curve

Conc %	Average P. As	Statistical data	
50	57,935	R^2	0.9996
80	90,220	Slope	1138.1
100	113,013	Intercept	9.0828
120	136,478	Standard error	988.8
150	171,452		

Table 3 Accuracy and recovery of Azr results

Theoretical conc %	Average P. As	Actual conc %	Recovery %
80	90,171	79.2	99.0
100	113,890	100.1	100.1
120	136,325	119.8	99.8

Table 4 Repeatability of Azr

#	P. A	Statistical data	
1	113,965	Average P. As	113,293
2	113,629	STDEV	752.987
3	113,741	RSD %	0.66%
4	112,509		
5	112,173		
6	113,741		

3.3 LOD and LOQ

LOD and LOQ values were calculated from the linearity calibration curve data of Azr and they found to be 2.87% and 8.69% from the target conc 100%. That is mean LOD equal 28.7 µg/ml where LOQ equal 86.9 µg/ml. These values have been indicated on the method sensitivity at lower concentrations.

3.4 Accuracy and recovery

The results revealed that the method was found to be accurate within the range from 80% (0.8 mg/ml) to 120% (1.2 mg/ml) of the standard conc with an addition of formula placebo as shown in Table 3.

3.5 Precision and repeatability

Precision was evaluated by repeatability term. Repeatability of the standard was expressed using RSD % of P. As shown in Table 4. The obtained RSD % of the 6 samples preparations was found to be 0.66% ≤ 2.0% according to repeatability requirements in European Pharmacopoeia [25, 26]. This revealed that the method was precise and repeatable.

Table 5 Change in organic ratio results

#	1	2	3	Statistical data	
Organic P. A at 900 ml	114,861	114,749	115,197	Average P. As	114,139
Organic at +10% P. A	112,173	111,949	111,837	STDEV	1641.4
Organic at – 10% P. A	115,645	115,533	115,309	RSD %	1.44%

Table 6 Change in temperature results

#	1	2	3	Statistical data	
Temp. P. A at 40° C	114,861	114,749	115,197	Average P. As	113,965
Temp. at 40° C +5° C P. A	111,725	111,277	111,389	STDEV	1900.7
Temp. at 40° C – 5° C P. A	115,645	115,533	115,309	RSD %	1.67%

Table 7 Change in flow rate results

#	1	2	3	Statistical data	
Flow rate at 1.2 ml P. A	114,861	114,749	115,197	Average P. As	114,394
Flow rate at 1.0 ml P. A	116,429	116,317	116,261	STDEV	1967.2
Flow rate at 1.4 ml P. A	112,173	111,949	111613	RSD %	1.72%

Table 8 Day-to-day precision results

#	1	2	3	Statistical data	
First Day P.A	114,861	114,749	115,197	Average P. As	114,077
Second Day P.A	114,413	114,301	114,637	STDEV	965.1
Third Day P.A	112,845	112,621	113,069	RSD %	0.85%

Table 9 Analyst-to-Analyst precision results

#	1	2	3	Statistical data	
First Analyst P. A	114,861	114,749	115,197	Average P. As	114,674
Second Analyst P. A	114,637	114,189	114,413	STDEV	351.8
				RSD %	0.31%

3.6 Robustness

The results of deliberated changes included organic (± 10%), temperature ± 5°C and flow rate ± 0.2 ml were evaluated by RDS % calculations. The observed RSD % results were 1.44% for change in organic ratio, 1.67% for change in temperature and 1.72% for the flow rate change.

In all case studies, the RSD % was < 2% as shown in Tables 5, 6 and 7. So, the obtained results indicating that the method is a robust.

3.7 Ruggedness

According to the obtained results after major changes application on the analysis method including day-to-day, analyst-to-analyst and column-to-column precisions. The method was found to be rugged as revealed results in Tables 8, 9 and 10. The ruggedness of method was evaluated using RSD % and it was 0.85, 0.31 and 1.69% for day-to-day, analyst-to-analyst and column-to-column precisions respectively and also as in Robustness challenge all the RSD % < 2.0%.

3.8 Auto-sampler stability (solution stability)

The test solution was found to be stable in the auto-sampler within about 12 h at room temperature with RSD % equal 0.67% as in Table 11.

3.9 Selectivity and specificity

The peak of Azr was appeared well resolved from other degradation peaks and any adjacent peak with resolution at least 3.6 as in Fig. 3a, b. The results also were revealed that good assay reading after forced degradation within 5% from the initial assay P. As of Azr. So, the method was found to be specific and selective for Azr determination.

3.10 Analysis of different commercial dosage forms "Finished products"

The Azr average assay results of Zithromax 500 mg powder for solution for infusion, Zithromax 250 mg capsules and Zithromax 900 mg/22.5 ml powder for oral suspension revealed good results; 101.4%, 102.2% and 99.8 respectively.

Table 10 Column-to-Column precision results

#	1	2	3	Statistical data	
First Column P. A	114,861	114,749	115,197	Average P. As	113,200
Second Column P. A	111,613	111,277	111,501	STDEV	1910.5
				RSD %	1.69%

Table 11 Auto-sampler stability solution

#	0 h	3 h	6 h	12 h	Statistical data	
Test P. A	113,405	112,845	112,397	111,613	Average P. As	112,565
					STDEV	756.9
					RSD %	0.67%

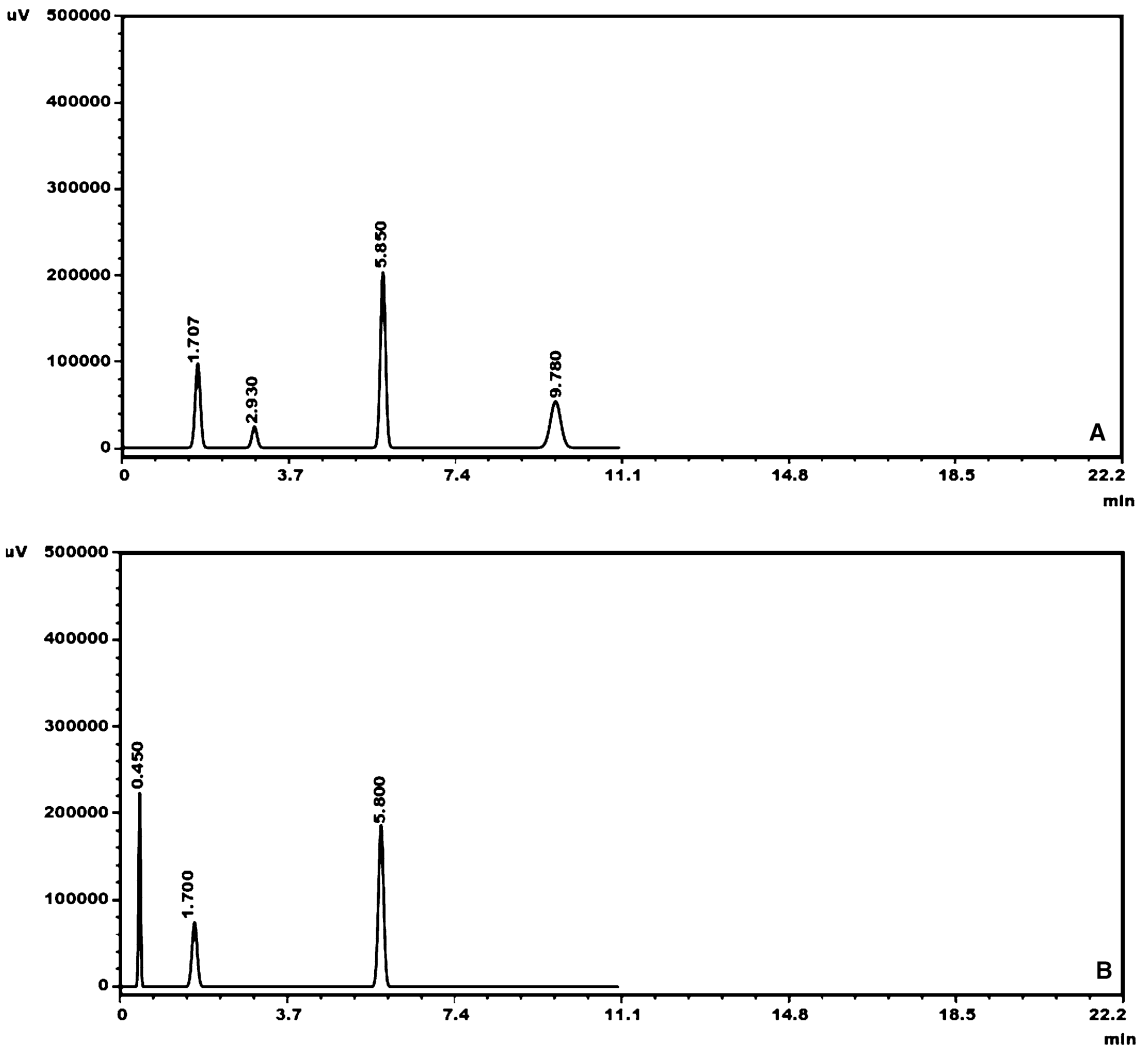

Fig. 3 Effect of heating degradation (**a**) and Hydrogen peroxide (**b**) in specificity test

4 Conclusion

The present study introduces a rapid, easy, cheap and accurate method of Azr analysis. The analysis run time takes about 8 min. The method revealed a good behaviour as linear, precise (repeatable), robust, rugged, selective and specific as the resolution factor between Azr peak and any adjacent peak at least anyway > 1.5. LOD and LOQ also, evaluated and showed an appreciated and satisfied value as 28.7 µg/ml and 86.9 µg/ml respectively. So, the analysis method is valid to use for Azr determination at the minimum level of concentrations with convenient tools of analysis. The validated method gave satisfying results for practical application of Zithromax assay determination for three different dosage forms as revealed the results.

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Compliance with ethical standards

Conflict of interest The corresponding author states that there is no conflict of interest.

Human and animal rights Research is not involving Human Participants and/or Animals.

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