ejpmr, 2017,4(06), 449-454

SJIF Impact Factor 4.161

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

FIRST ORDER DERIVATIVE METHOD DEVELOPMENT AND VALIDATION FOR SIMULTANEOUS ESTIMATION OF AZITHROMYCIN AND DEXAMETHASONE FROM EYE DROPS

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Article Received on 27/03/2017 **Article Revised on 17/04/2017** **Article Accepted on 07/05/2017**

ABSTRACT

Simple, precise, accurate, and sensitive first order derivative spectroscopic method have been developed for the estimation of Azithromycin and Dexamethasone in market formulation. The wavelength maxima for Azithromycin and Dexamethasone are 215.16 nm and 239.07 nm respectively. For the first order derivative spectroscopic method, the zero crossing points (ZCPs) for Azithromycin and Dexamethasone were obtained at 256.63 nm and 214.35 nm respectively in 0.1N NaOH using Shimadzu 1800 UV- visible double beam spectrophotometer. The proposed method were successfully utilised for the determination of Azithromycin and Dexamethasone in eye drops, with good linearity, high percentage of recovery and acceptable precision. Different analytical validation parameters like linearity, accuracy, precision, limit of detection and limit of quantification were determined according to International Conference on Harmonization ICH Q2 (R1).

KEYWORDS: Azithromycin, Dexamethasone, First order derivative method, UV-visible spectrophotometer.

INTRODUCTION

Azithromycin (AZI)[1] is a novel macrolide antibiotic and a semisynthetic- erythromycin derivative. It has a methyl-substituted nitrogen at position 9a in the lactone ring to create a 15-membered-ring macrolide. AZI produces an enhanced spectrum and potency against bacteria compared with other macrolides and superior stability in acid environment. Its mechanism is similar to erythromycin, appearing to bind to the same receptor, 50s ribosomal subunits of susceptible bacteria and suppresses protein synthesis. Azithromycin[2] has the chemical name (2R,3S,4R,5R,8R,10R,11R,12S,13S,14R)-L-ribo-hexopyranosyl)α13-[(2,6-dideoxy-3-C-methyl-3-O-methyl-D-xylo-hexopyranosyl]oxy]-1-βoxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)- oxa-6-azacyclopentadecan-15-one. Molecular formula is $C_{38}H7_2N_2O_{12}$. Chemical structure of Azithromycin is shown in figure1.

Dexamethasone (DEX)[3] is a corticosteroid hormone (glucocorticoid). It has anti inflammatory and immunosuppressant effect. Dexamethasone's chemical name (9α-fluoro-11β, 17, 21-trihydroxy- 16α-methylpregna- 1, 4- diene-3, 20-dione). Dexamethasone[4] is very potent and highly selective glucocorticoid and also used topically. Chemical structure of Dexamethasone is shown in figure 2.

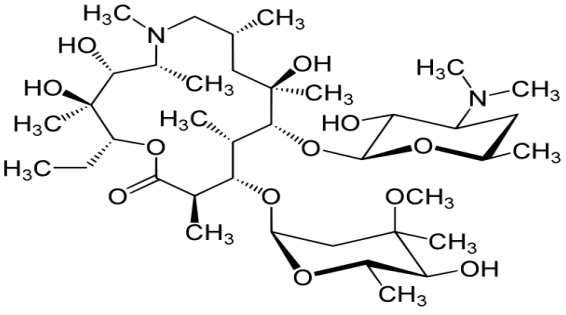

Fig. 1 Chemical structure of Azithromycin

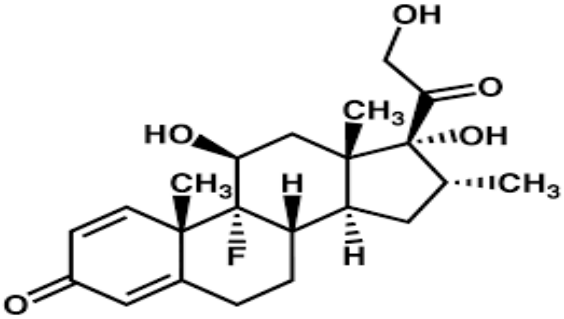

Fig.2 Chemical structure of Dexamethasone

For AZI the individually methods reported are UV[5]HPLC[6-13] TLC-densiometry[14] Stability indicating HPLC method with TLC [15-16] LC-MS/MS[17] For DEX the individually methods reported are UV[18-19] HPLC[20-24]The review of literature revealed that no isocratic analytical method for AZI and DEX as in individual dosage or combined dosage form was not available. Therefore the present study aims to develop and validate analytical method for simultaneous estimation of Azithromycin and Dexamethsone in Eye drops. This proposed method can be successfully employed for quality control during manufacture.

MATERIAL AND METHODS

Chemical Reagents

AZI sample were obtained from Indiana Ophthalmics, Surendranagar. DEX was obtained from Balaji Enterprise, Surat and Market formulation having brand name AYZEECON-D [Azithromycin dehydrate (10mg) and Dexamethasone Sodium Phosphate (1mg)] was procured from Indiana Ophthalmics, Surendranagar. Methanol and NaOH of AR grade were obtained from Merck.

Intruments

- Double beam UV-visible spectrophotometer (Shimadzu UV-1800) with matching pair of 1cm quartz cuvettes
- Electronic Analytical balance (Shimadzu AUX 220)
- Soltec-Sonica Ultrasonic Cleaner, Sonicator (Spincotech Pvt. Ltd.)
- Whatman filter paper no. 41
- Glass wares (Borosil) - Volumetric flasks (10, 25, 50 and 100ml), Pipettes (1, 2, 5 and 10 ml), Beakers (50, 100 and 250 ml)
- All instruments and glass wares were calibrated.

Spectrophotometric Conditions

- Mode: Spectrum
- Scan speed: Medium
- Wavelength range: 400-200 nm
- Derivative order: 1st order

The derivative spectra were recorded by using digital differentiation (Convolution method) with a derivative wavelength difference (Δλ) of 1 nm in the range of 200-400 nm.

Selection of Solvent

AZI was very soluble in methanol. DEX was sparingly soluble in methanol but became completely soluble when ultrasonicated for 2 minutes. So, methanol was selected as a solvent. Derivatization of both drugs was done with 0.1N NaOH.

Preparation of Standard Solutions

Standard stock and working standard solution of Azithromycin (AZI)

10mg of AZI was transferred to 10 ml volumetric flask, dissolved and diluted up to mark with methanol to give a standard stock solution (AS_1=1000 µg/ml). An aliquot (5ml) of the solution was transferred to a 50 ml volumetric flask and diluted to the mark with 0.1N NaOH to obtain a working standard solution (AS_2=100 µg/ml) of AZI.

Standard stock and working standard solution of Dexamethasone (DEX)

10mg of DEX was transferred into 10ml volumetric flask, dissolved and diluted up to mark with methanol to give a standard stock solution (DS_1=1000 µg/ml). An aliquot (5ml) of the solution was transferred to a 50 ml volumetric flask and diluted to the mark with 0.1N NaOH to obtain a working standard solution (DS_2=100 µg/ml) of DEX.

Preparation of sample solution

One ml of sample solution (eye drops) containing 10mg AZI and 1mg DEX was transferred to 100ml volumetric flask. 4mg of DEX standard was transferred to 100ml volumetric flask and diluted with (10ml) Methanol. It is then kept in ultrasonicator for 10 min to get optimum dissolution of the active ingredients and diluted up to mark with 0.1N NaOH (100µg/ml of AZI and 50µg/ml of DEX). An aliquot (4ml) of this solution was transferred to a 10 ml volumetric flask and diluted to the mark with 0.1N NaOH (40µg/ml of AZI and 20µg/ml of DEX).

Selection of Wavelength for Estimation

Standard solution of 40µg/ml of AZI and 20µg/ml for DEX were prepared for selection of wavelength and each solution was scanned between 200-400 nm against 0.1N NaOH as a blank. The first order derivative spectrum of each solution was obtained and checked for zero crossing points and it was selected as wavelength for estimation.

Preparation of Lambert-Beer's Curve

From working standard solution (AS2), aliquots (2, 3, 4, 5 and 6 ml) were transferred into series 10 ml volumetric flasks and volume were adjusted upto the mark with 0.1N NaOH to get 20, 30, 40, 50 and 60 µg/ml solutions of AZI. Similarly, from working standard solution (DS2), aliquots (1, 1.5, 2, 2.5 and 3 ml) were transferred into series 10 ml volumetric flasks and were adjusted upto the mark with 0.1N NaOH to get 10, 15, 20, 25 and 30 µg/ml solutions of DEX. Zero order overlain spectra were scanned between 200-400nm and showed absorption maxima at 215.16nm for AZI and at 239.07nm for DEX. All the spectra were converted to first order derivative spectra using inbuilt software UV-Probe and ZCP of AZI and DEX were obtained. Absorbance of each solution was measured at ZCP of AZI and DEX which were 256.63 nm and 214.35nm respectively. The graph of absorbance v/s concentration was plotted and straight line equation was obtained.

Method Validation

As per ICH Q2R1[25] guidelines, the method validation parameters studied were linearity, accuracy, precision, limit of detection and limit of quantification.

Linearity and Range

The linearity response was determined by analyzing 5 independent levels of different standard solution in the range of 20-60 µg/ml for AZI and 10-30 µg/ml for DEX. Response of each solution was measured at 214.35 nm and 256.63 nm for AZI and DEX, respectively using first order derivative method. The graph of response obtained versus respective concentration was plotted and the regression equations were calculated. Each response was average of five determinations.

Accuracy (% Recovery)

It was determined by calculating the recovery of AZI and DEX from eye drops formulation by standard addition method. To a fixed amount of test 50%, 100% and 150% amount of standard was added and the amount of standard added was calculated using regression equation. Known amount of standard solutions of AZI (0,10, 20 and 30µg/ml) and DEX (0, 5, 10 and 15µg/ml) were added to a pre-quantified sample solution of AZI and DEX (20.0 and 10.0 µg/ml, respectively). Each solution was scanned in triplicate and the percentage recovery was calculated by measuring the responses and fitting these values into the regression equations of the respective calibration curves.

Precision

The repeatability of the proposed method was determined by measuring the corresponding responses 6 times for 100% test concentration of AZI (40µg/ml) and DEX (20µg/ml), each.

The intra-day and inter-day precisions of the proposed method was determined by measuring the corresponding responses 3 times on the same day and on 3 different days over a period of 1 week for 3 different concentration of AZI (20, 40 and 60 µg/ml) and DEX (10, 20 and 30 µg/ml), each. The results were reported in terms of relative standard deviation.

Limit of detection (LOD) and Limit of quantitation (LOQ)

The LOD and LOQ were separately determined as per the ICH Q2 (R1) using 5 calibration curves. The residual standard deviation of y- intercept of regression lines was used to calculate LOD and LOQ.

LOD=3.3*SD/S and LOQ=10*SD/S

Where, SD is the standard deviation of the Y- intercepts of the 5 calibration curves and S is the slope of the 5 calibration curves.

Estimation of Azithromycin and Dexamethasone in the Marketed Formulation

The absorbance of the Marketed Sample (eye drops) solution was measured using first order derivative spectrophotometry at selected wavelengths for determination of AZI and DEX. The concentration of each drug and assay result was calculated using equation of straight line.

RESULT AND DISCUSSION

Selection of Wavelength for Estimation

In preliminary studies it was found that λmax of AZI and DEX were nearer and zero order spectra overlapped for both drugs show that precise quantitation could not be done using simultaneous equation method due to interfering of one drug at estimation of the other. Hence, Derivative spectroscopic method was performed to get better resolution and precise quantitation of AZI and DEX in combination.

From the zero order overlain spectra of both drugs, λmax of AZI was found to be at 214.15 nm (Fig. 3) and λmax of DEX was found to be at 239.07 nm (Fig.4).

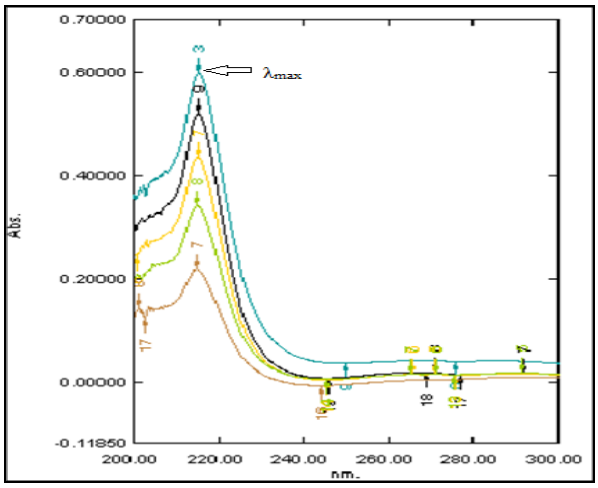

Fig.3: Zero order overlain spectra of AZI (214.15nm) (20-60 µg/ml)

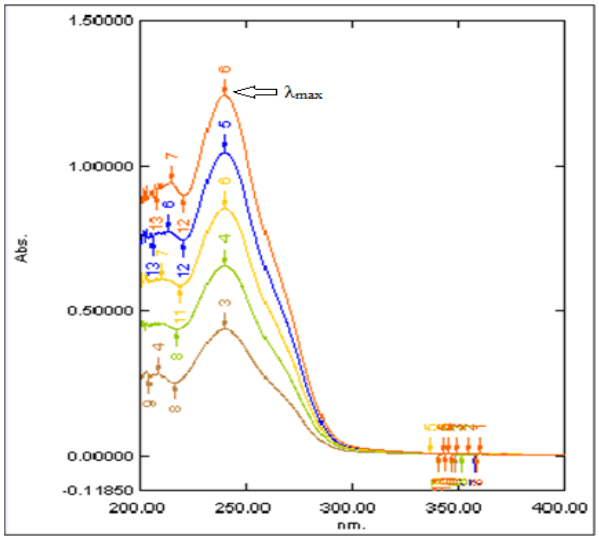

Fig.4: Zero order overlain spectra of DEX (239.07nm) (10-30µg/ml)

All spectra were converted to first order derivative spectra. The wavelength selected for estimation of AZI was 214.35 nm (ZCP of DEX) shown in (Fig.5) while wavelength selected for estimation of DEX was 256.63 nm (ZCP of AZI) (Fig.6).

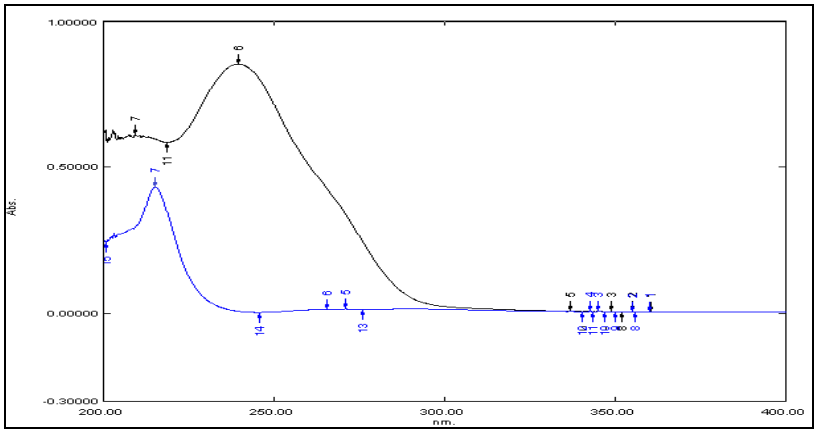

Fig.5: Zero order overlain spectra of AZI (40µg/ml)and DEX (20µg/ml)

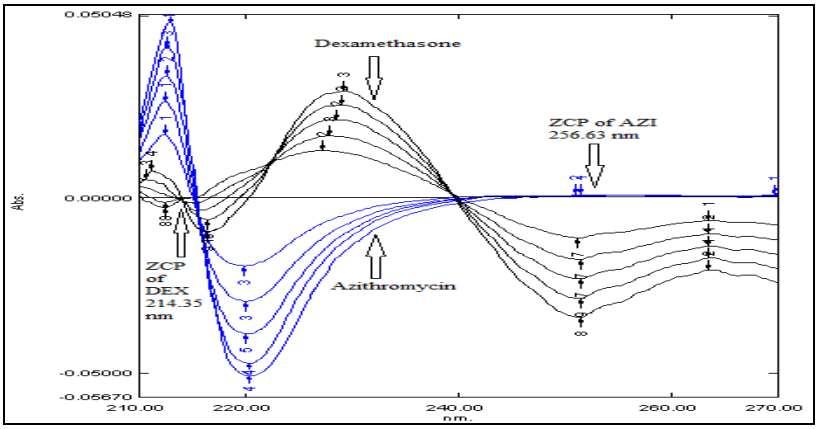

Fig. 6: First order overlain spectra of AZI (256.63nm) (20-60 µg/ml) and DEX (214.35nm) (10-30µg/ml)

METHOD VALIDATION

Linearity and Range (n=5)

The calibration curves for AZI and DEX were prepared and regression line equations were computed. Lambert-Beer's law was followed in the ranges of 20-60µg/ml and 10-30µg/ml for AZI and DEX depicted in table 1and fig.7 and fig.8 respectively.

Table 1: Data of absorbances of AZI at 214.35nm and DEX at 256.63nm

AZI			DEX		
Conc. (µg/ml)	**Mean response ± SD (n=5)**	**% CV**	**Conc. (µg/ml)**	**Mean response± SD (n=5)**	**% CV**
20	0.0058±0.00010	1.72	10	0.0105±0.00010	0.95
30	0.0107±0.00015	1.42	15	0.0149±0.00010	0.67
40	0.0158±0.00020	1.27	20	0.0193±0.00021	1.08
50	0.0207±0.00017	0.84	25	0.0236±0.00025	1.06
60	0.0255±0.00025	0.99	30	0.0281±0.00032	1.14

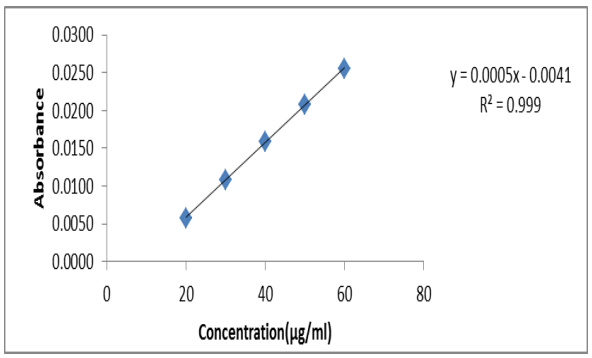

Fig.7: ***Calibration curve for AZI (20,30,40,50,60 µg/ml) at 214.35nm***

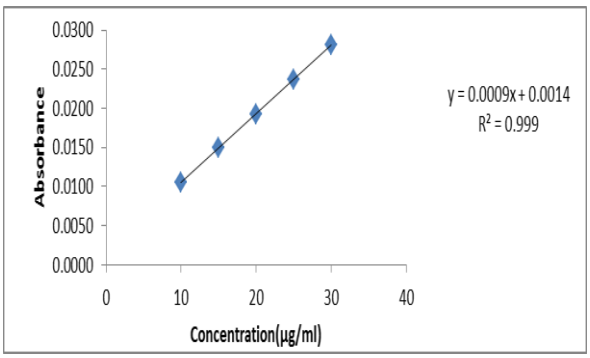

Fig.8: ***Calibration curve for DEX (10,15,20,25,30 µg/ml) at 256.63nm***

Acceptance criteria: The r value should be more than 0.995 over the working range[25]. The r value for each component is well within the limit of acceptance criteria that means the areas obtained are directly proportional to the concentration of analyte in the sample. The method can therefore be considered to be linear in the range specified.

Accuracy (n=3)

The data of accuracy study for AZI and DEX are shown in table 2. Percentage recoveries for AZI and DEX were found to be in the range of 99.33-100.67 % and 99.51-100.74 % respectively.

Acceptance Criteria: % Recovery (individual) at each level should be 98% to 102 %.Individual recovery at each level meets the established acceptance criteria. Hence, the method is accurate in the considered range.

Precision

- **Repeatability (n=6)** was carried out and %CV was found to be 1.19 for AZI and 0.99 for DEX as shown in table 3.
- **Intraday precision (n=3)** was carried out and % CV was found to be 0.6-1.02% for AZI and 0.72-0.97% for DEX as shown in table 4.
- **Interday precision (n=3)** was carried out and % CV was found to be 0.83-1.33% for AZI and 1.03-1.08% for DEX as shown in table 4.

Table 2: Accuracy study of AZI and DEX

Drug	Amt. of taken (µg)	% Amt. of std added	Total amt spiked. (µg)	Total Amt. found (µg)	Mean % Recovery± SD (n=3)	% CV
AZI	20	0 %	20	19.93	99.67± 0.00006	0.98
	20	50 %	30	30.00	100.67±0.00010	0.92
	20	100 %	40	39.80	99.33± 0.00020	1.27
	20	150 %	50	50.07	100.44±0.00015	0.73
DEX	10	0 %	10	10.04	100.37±0.00015	1.46
	10	50 %	15	15.07	100.74±0.00021	1.39
	10	100 %	20	20.00	99.63±0.00010	0.52
	10	150 %	25	24.96	99.51± 0.00015	0.640

Table 3: Repeatability data of AZI and DEX

AZI		DEX	
Conc. (µg/ml)	**Response (nm)**	**Conc. (µg/ml)**	**Response (nm)**
40	0.0158	20	0.0191
	0.0156		0.0189
	0.0160		0.0187
	0.0159		0.0188
	0.0157		0.0192
	0.0155		0.0190
Mean response	0.0158	**Mean response**	0.0190
SD	0.00019	**SD**	0.00019
% CV	1.19	**% CV**	0.99

Table 4: Intraday and Interday precision data of AZI and DEX (n=3)

Drug	Conc. (µg/ml)	Intraday precision		Interday precision	
		Mean response± SD (n=3)	% CV	Mean response± SD (n=3)	% CV
AZI	20	0.0057±0.00006	1.02	0.0043±0.00006	1.33
	40	0.0154±0.00015	0.99	0.0155±0.00020	1.29
	60	0.0257±0.00015	0.60	0.0252±0.00021	0.83
DEX	10	0.0103±0.00010	0.97	0.0097±0.00010	1.03
	20	0.0190±0.00015	0.80	0.0188±0.00020	1.06
	30	0.0278±0.00020	0.72	0.0283±0.00031	1.08

Acceptance Criteria: RSD of assay values sample preparations should not be more than 2.0%.[25] The results obtained lie well within the limit of acceptance criteria. Hence the method can be termed as precise.

LOD and LOQ

LOD for AZI and DEX were found to be 1.19(µg/ml) and 0.73(µg/ml) respectively. Similarly LOQ for AZI and DEX were found to be 3.6(µg/ml) and 2.22(µg/ml) respectively.

Applicability of The Proposed Method for The Analysis of Pharmaceutical Dosage Form by The Developed First Order Derivative Method

The assay results obtained were summarized in table 5.

Table 5: Assay data for AZI and DEX (n=5)

AZI			DEX		
Label Claim (mg)	**Amt. found (mg)**	**% Assay**	**Label Claim (mg)**	**Amt. found (mg)**	**% Assay**
10	9.85	98.50	1	0.983	98.33
	10.05	100.50		0.994	99.44
	9.9	99.00		0.989	98.89
	10.15	101.50		0.992	99.17
	9.95	99.50		1.011	101.11
Mean	**9.98**	**99.80**	**Mean**	**0.994**	**99.39**
SD	**120.42**	**1.20**	**SD**	**10.468**	**1.047**
% CV	**1.21**	**1.21**	**% CV**	**1.05**	**1.05**

CONCLUSION

First order Derivative Spectrophotometry was developed. Method were statistically validated in terms of accuracy, precision, linearity and reproducibility as per ICH guidelines.

In First order derivative method ZCP for AZI and DEX were found to be 256.63 nm and 214.35 nm respectively. The linearity range was found to be of correlation coefficient 0.999 and 0.999 for AZI for DEX, respectively. The recovery was in the range of 99.33-100.67 % for AZI and 99.51-100.74 % for DEX. Precision study showed RSD values less than 2 for both AZI and DEX respectively in all selected concentrations. Marketed preparation was analyzed by the proposed method and the amount of AZI and DEX was found to be 99.80% and 99.39% of the labeled amount, respectively.

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