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Development of a Validated Stability Indicating RP-HPLC Method for Assay of Ozagrel and Its Pharmaceutical Formulations

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ABSTRACT

A simple, precise, rapid, selective, and economic reversed phase high-performance liquid chromatography (RP-HPLC) method has been established for estimation analysis of DRO. A Brownlee ODS C-18 column (250×4.6 mm i.d) chromatographic column equilibrated with mobile phase methanol-0.02 M KH2PO4 (80:20, v/v) (Final pH adjusted to 4 using Orthophosphoric acid) was used. Mobile phase flow rate was maintained at 1 ml/min and effluents were monitored at 272 nm. The sample was injected using a 20 µl fixed loop, and the total run time was 9.946 min. Experimental conditions such as pH of mobile phase, column saturation time, selection of wavelength, etc. were critically studied and the optimum conditions were selected. In RP-HPLC linear range was found to be 1-10 µg/ml and, mean recovery was found to be 99.65 and Rt of Ozagrel was found to be 9.946 min. Degradation in acid, base, peroxide and thermal was found in range 5-20%. This stability indicating HPLC method is economic, sensitive, and less time consuming than other chromatographic procedures. It is a user-friendly and importance tool for analysis of Ozagrel in tablet dosage forms.

I. INTRODUCTION

Reversed phase HPLC is characterized by a situation in which the mobile phase used is MORE POLAR than the stationary phase. The name 'Reversed Phase' arises as this was the second, (chronologically), mode of chromatography after Normal (or 'Straight') phase in which a polar stationary phase is used in conjunction with a less polar mobile phase. Typical reversed phase stationary phases are hydrophobic and chemically bonded to the surface of a silica support particle. Other support materials and bonded phases are available.

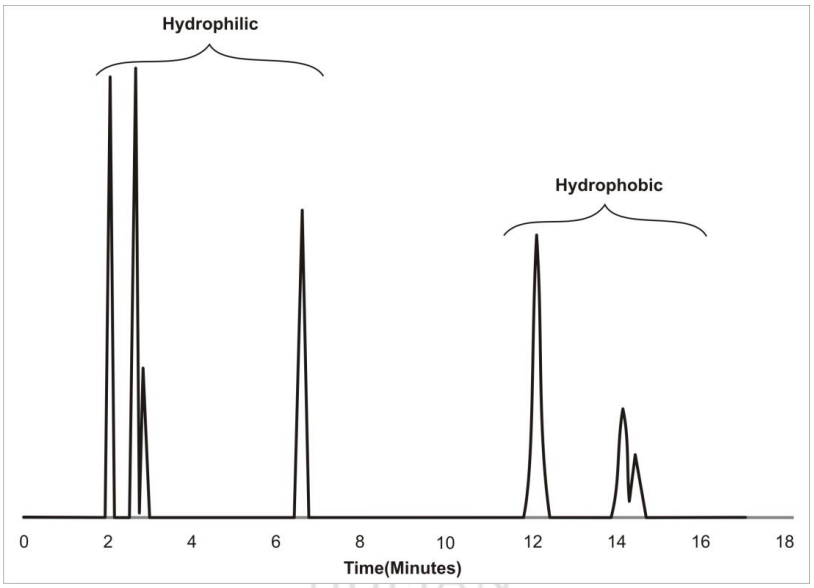


Figure: Representative reversed phase chromatogram detailing analyte retention order based on hydrophobicity or Hydrophilicity.

For neutral analytes, the mobile phase consists of water (the more polar component) and an organic modifier that is used to vary the retention of analytes by lowering the polarity of the mobile phase. The most common organic modifiers are shown. Increasing the water content will repel ('squeeze') hydrophobic (non-polar) analytes out of the mobile phase and onto the non-polar stationary phase where they will reside for a time until 'partitioning' out into the mobile phase again. Each 'on-off' partition is called a 'Theoretical Plate'. When ionizable (or ionic) analytes are present, other additives such as buffers or ion pairing reagents can be added to the mobile phase to control retention and reproducibility. The Chromatogram illustrates the general elution order of hydrophilic and hydrophobic analytes. When working with ionizable analytes the hydrophobicity and, hence, retention characteristics of the analyte will be affected

depending on its ionization state (ionized or non-ionized), this will be discussed later in the module.

II. EXPERIMENTAL WORK

❖ Standard solutions

- **Solution A (Stock standard solution)**

Accurately weighed quantity of Ozagrel (10.0 mg) was dissolved in methanol to make 10.0 ml solution. (conc.: 1.0 mg/ml)

- **Solution B (Working standard solution)**

Accurately measured 1.0 ml of solution A was diluted to 100.0 ml with mobile phase (conc.: 10.0 µg/ml).

- **Preparation of buffer**

10 mM Phosphate buffer was prepared by dissolving 13.609 g of Potassium dihydrogen phosphate in 100.0 ml of milli-Q water and 1.0 ml of resulting solution was diluted to 100.0 ml with milli-Q water and pH was adjusted to 6.5 with 0.1 M NaOH.

❖ Optimization of chromatographic conditions

The chromatographic studies were performed on C_8 analytical column. Initially, different mobile phases were tried in isocratic mode to get an adequate retention of Ozagrel. Mobile phase containing methanol: water, methanol: ammonium acetate was tried, but retention time of ozagrel was about 2.25 min. Then acetonitrile: water was tried, but retention time of ozagrel was about 3.56 min and peak was separated. Then acetonitrile: sodium hydrogen orthophosphate was tried, retention time of ozagrel was about 12.69 min but with excessive peak tailing and there was a large baseline noise. Further, acetonitrile: potassium dihydrogen phosphate (10 mM, pH 6.5 with 0.1 M NaOH) (10: 90 *v/v*) was tried which gave a retention time of 9.946 ± 0.039 min with flow rate of 1.0 ml/min. The same mobile phase when tried for forced degradation samples, Ozagrel was found to be well resolved from its degradation products.

Hence, the mobile phase consisting of acetonitrile: potassium dihydrogen phosphate (10 mM, pH 6.5 with 0.1 M NaOH) with the ratio of 10: 90 *v/v* was selected as an optimum mobile phase which gave good resolution of Ozagrel from its degradation products along with good peak symmetry and retention of drug at about 9.946 ± 0.039 min.

The maximum absorption wavelength of reference drug and forced degradation samples was found to be 272 nm and hence selected as detection wavelength for analysis.

Following are the optimized chromatographic conditions for further study:

Column	**:**	**Enable C_8 column (250 x 4.6 mm, 5 µm)**
Mobile phase	**:**	**Acetonitrile: Potassium dihydrogen phosphate buffer, (10 mM, adjusted to pH 6.5 with 0.1 M NaOH), 10:90 *v/v***
Detection Wavelength	**:**	**272 nm**
Injection volume	**:**	**20 µl (Rheodyne injector)**
Flow rate	**:**	**1.0 ml/min**
Temperature	**:**	**25^0 C (Room Temperature)**

Procedure: The chromatographic conditions were set as per the given parameters and mobile phase was allowed to equilibrate with stationary phase as was indicated by the steady baseline. Working standard solution B was injected in the Rheodyne injector (20 µl) and the chromatograms were recorded for the drug. The mobile phase containing mixture of acetonitrile: potassium dihydrogen phosphate buffer, (10 mM, adjusted to pH 6.5 with 0.1 M NaOH), 10:90 *v/v* with a flow rate of 1.0 ml/min was found to yield satisfactory retention time of about 9.946 min with sharp symmetrical peak.

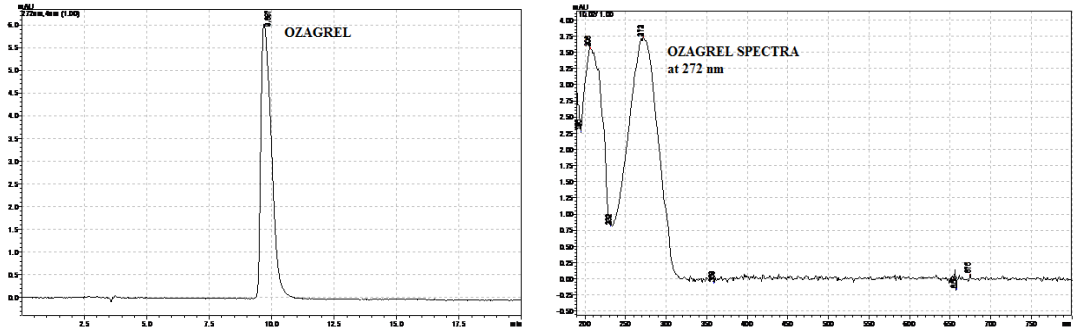
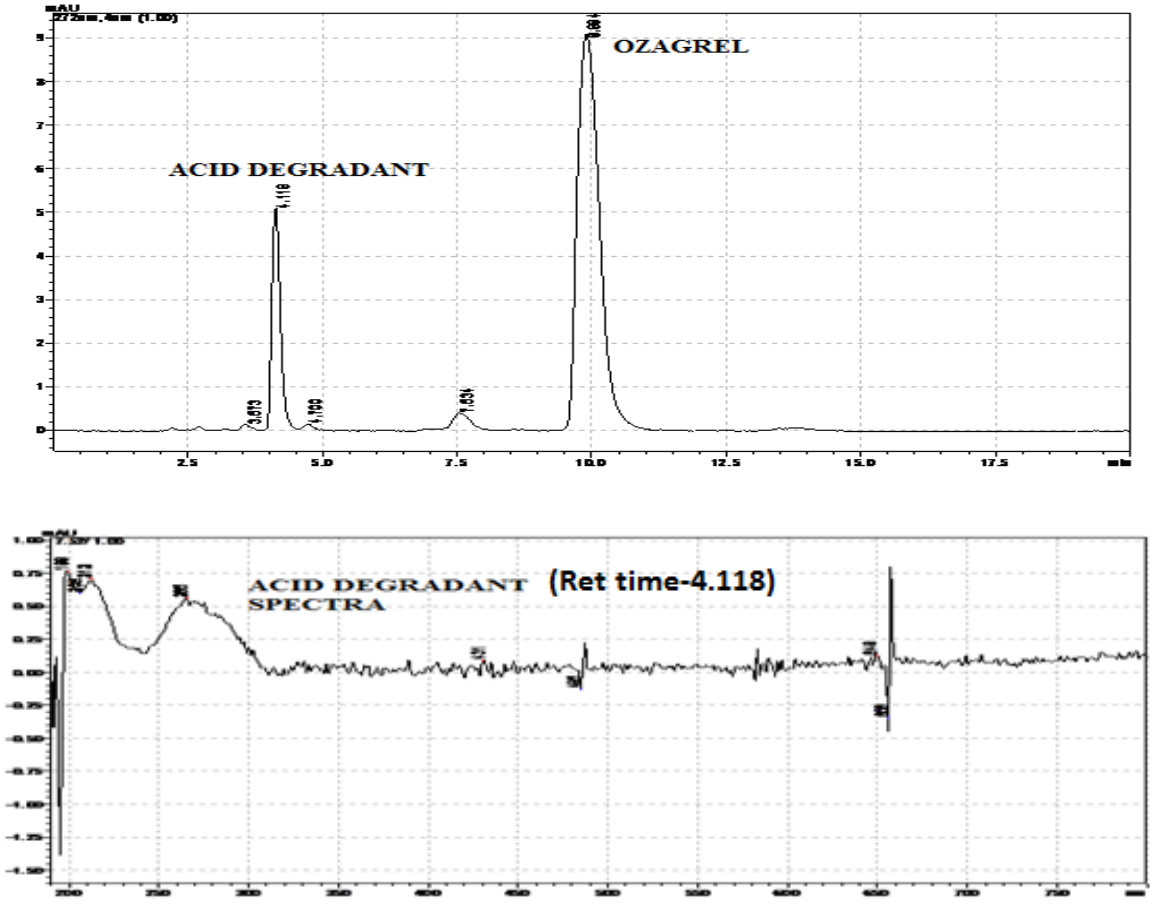
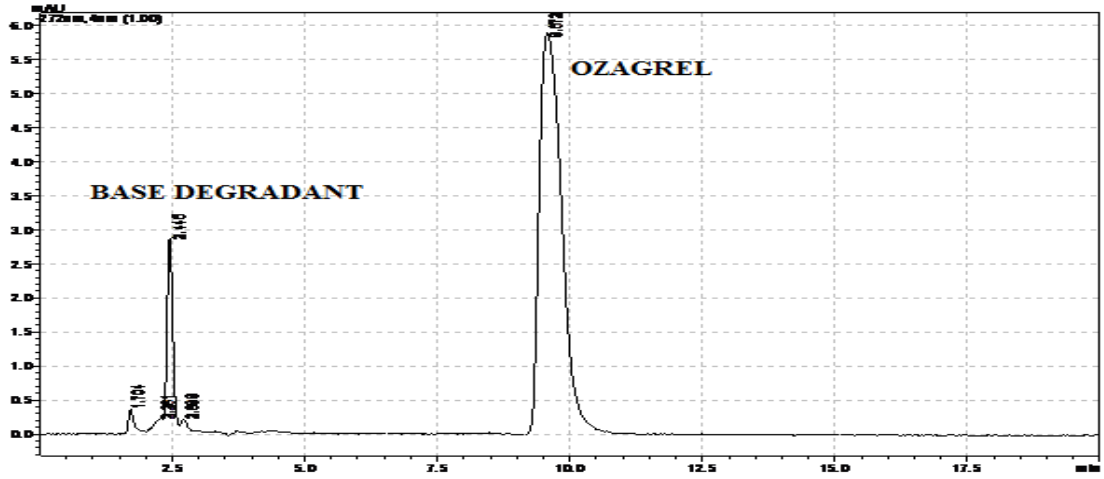


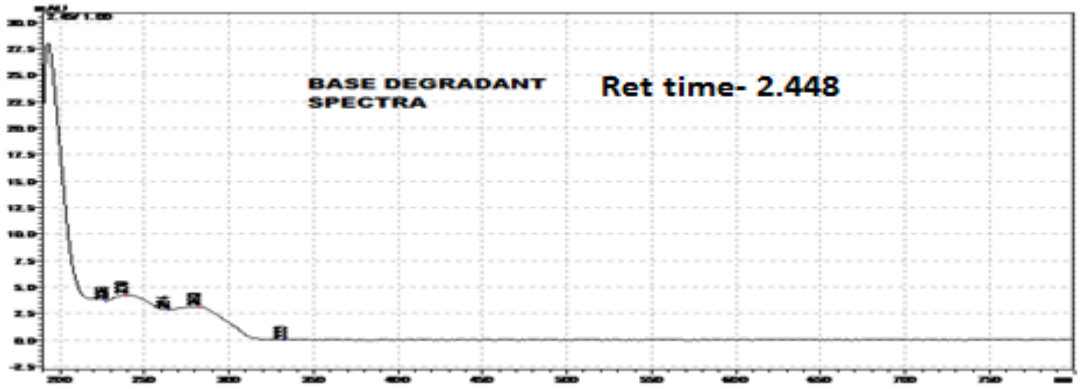
Figure: (a) HPLC chromatogram and (b) *in situ* spectrum of Ozagrel

All the stability samples were analyzed to study the extent of degradation and if the reasonable degradation (5-20%) with respect to parent drug has seen, the stress testing was stopped at that point.

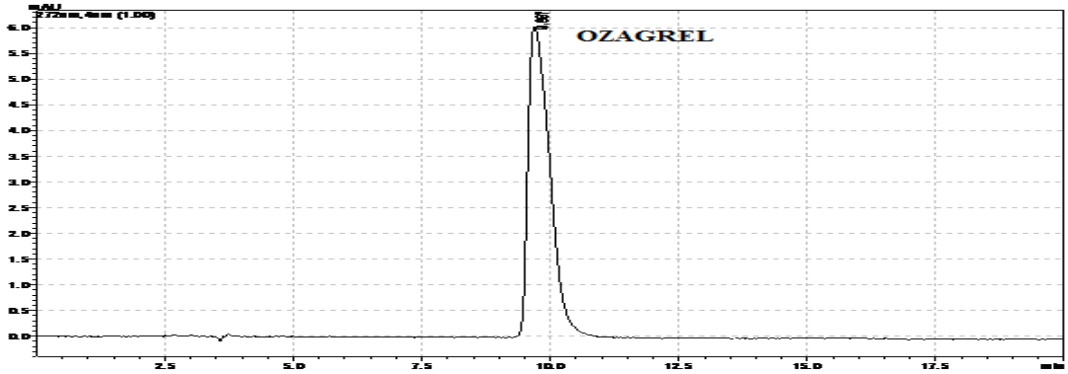


A) Acid (1M HCL, 24 h reflux) and Spectra of degradant

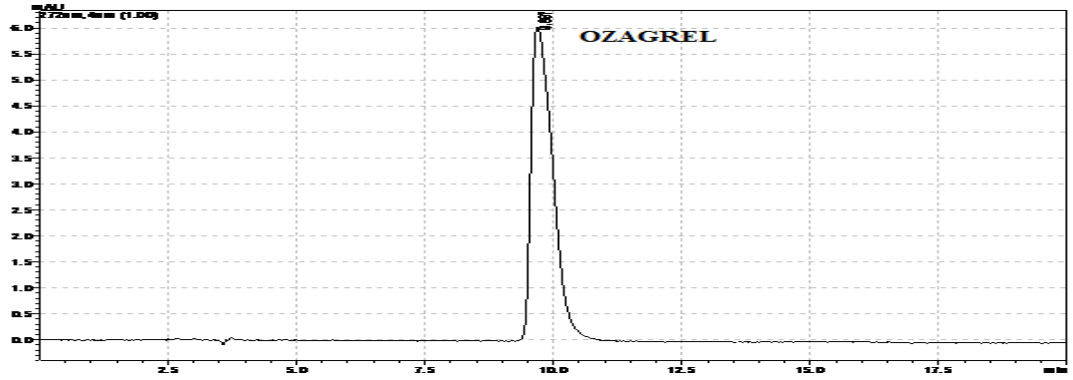




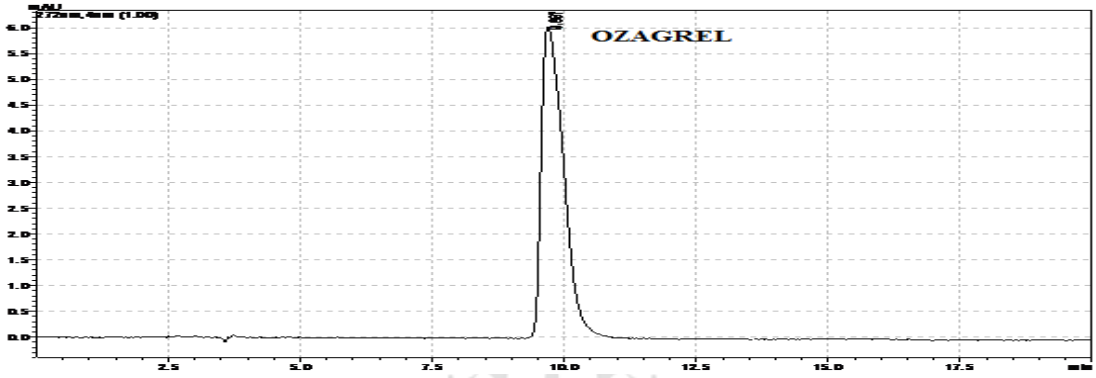
B) Base (1M NaOH, 24 h reflux) and Spectra of degradant



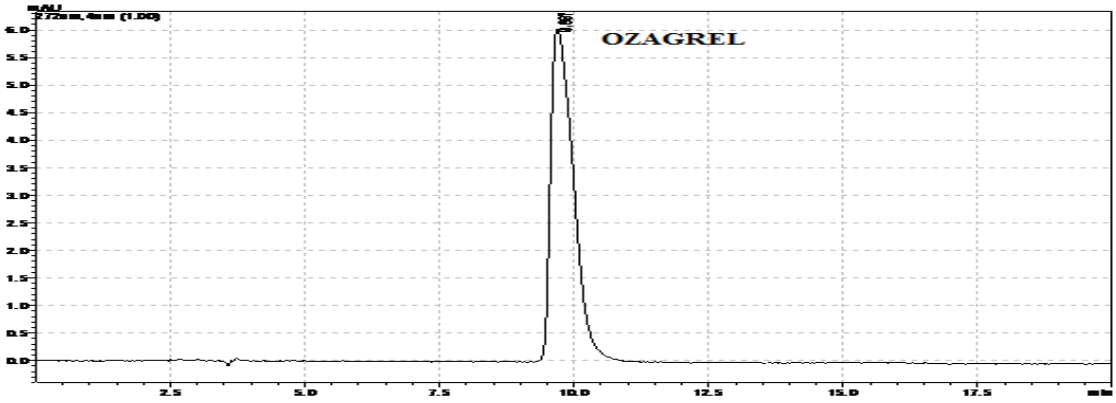
C) Neutral Stress, 24h reflux



D) Oxidative Stress (3% H_2O_2, 48 h at R.T.)



E) Photolytic Stress, (15 days, 4 lac lux h)



F) Thermal Stress, (dry heat, 15 days at 70^0C)

Figure: HPLC chromatograms of forced degraded samples (A-F)

Chromatogram of mixed degradation products

Chromatogram was also obtained under similar chromatographic conditions for mixed degradation products.

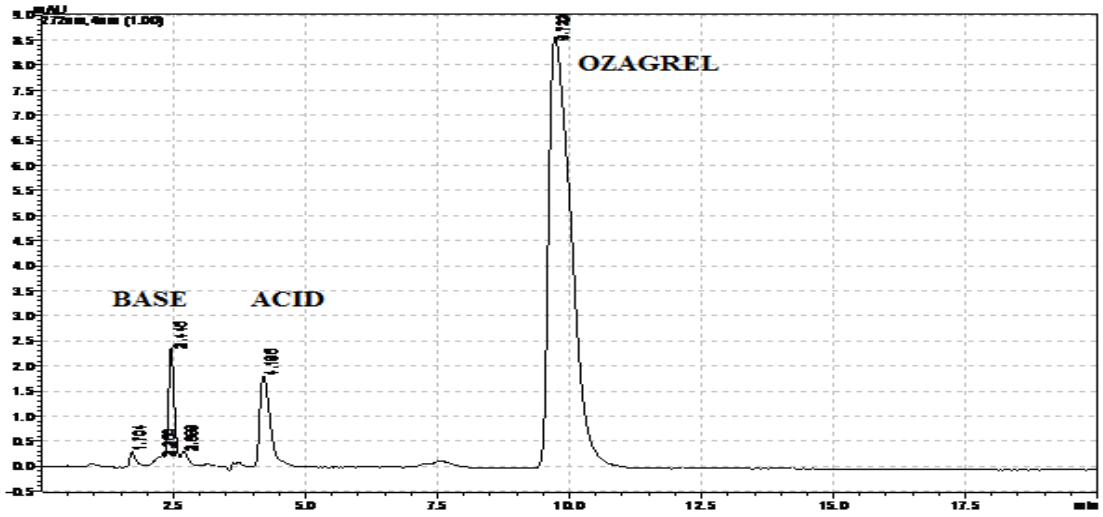


Figure: Chromatogram of mixed degradation products

❖ **Study of system suitability parameters**

The chromatographic conditions were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase to get steady baseline. Five replicate injections of working standard solution B were made separately and the chromatograms were recorded.

Table: Study of system suitability parameters

Sr. No	Retention Time (min)	Asymmetry	No. of Theoretical plates	Capacity Factor	Peak Area	Peak height
1	9.894	1.269	6597.560	1.769	126335	6391
2	9.932	1.254	6653.906	1.810	125598	6387
3	9.969	1.234	6674.371	1.804	126089	6288
4	9.939	1.231	6616.350	1.854	126370	6404
5	9.996	1.230	6622.484	1.826	126119	6396
Mean	**9.946**	**1.244**	**6632.934**	**1.813**	**126102.20**	**6373.20**
± SD	**0.039**	**0.017**	**30.791**	**0.031**	**308.459**	**48.049**
%RSD	**0.389**	**1.386**	**0.464**	**1.717**	**0.245**	**0.754**

❖ **Linearity of response**

Aliquot portions of standard solution B (1.0, 2.0, 3.0, 4.0, 5.0, 6.0 & 10.0 ml) were diluted to 100.0 ml with mobile phase to get concentration 1-6.0 µg/ml. The chromatographic conditions

were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase to get the steady baseline. Prepared standard solutions of different concentration were injected separately and the chromatograms were recorded.

A graph was plotted as peak area vs. concentration of drug (µg/ml)

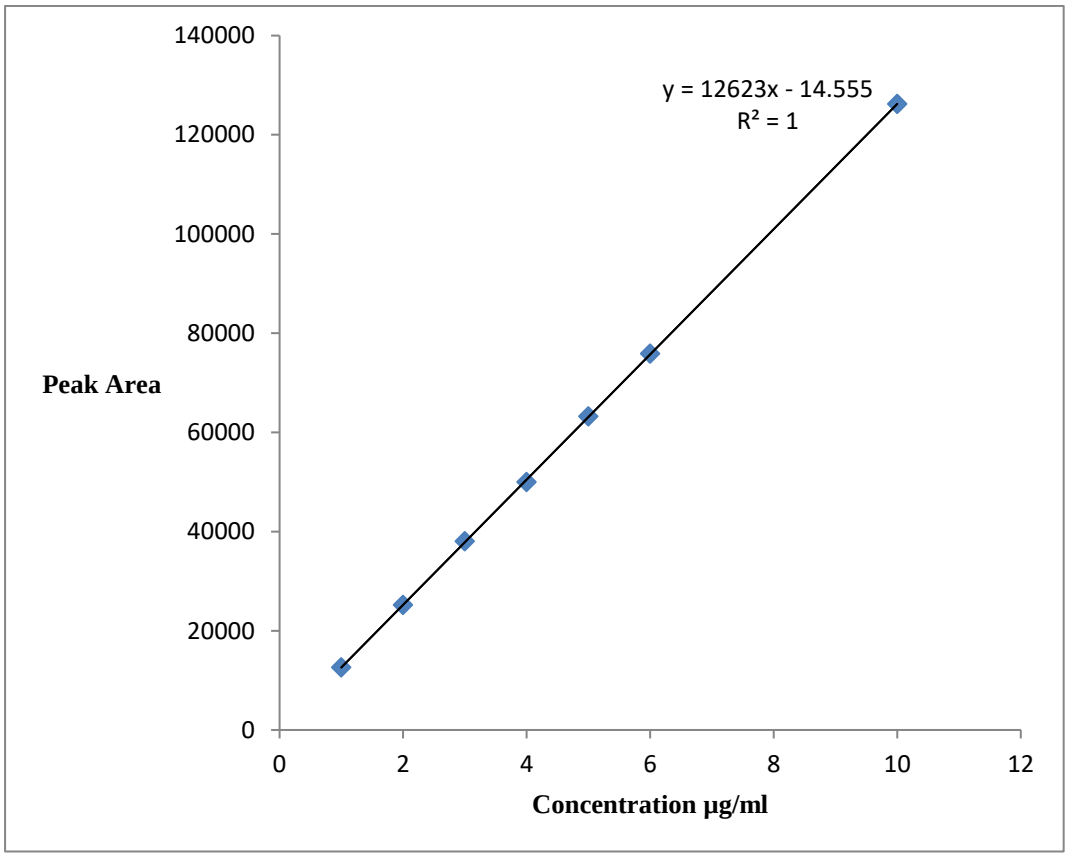


Figure: Calibration graph of Linearity studies

Table: Results of Linearity studies

Concentration range	1.0- 10.0 µg/ml
Equation for straight line	Y= 12623X-14.55
Slope	12623
Y-intercept	(-) 14.55
Correlation coefficient	1

❖ **Estimation of Ozagrel in Tablet by proposed HPLC method**

- **Standard solution:** Working standard solution B was prepared (10.0 µg/ml) as described under preparation of standard solution.

- **Sample solution:** Twenty tablets were weighed and average weight was calculated. Tablets were crushed to a fine powder. An accurately weighed quantity of tablet powder equivalent to about 10.0 mg of Ozagrel was shaken with about 8.0 ml of methanol, sonicated for 15 minutes, the volume was made up to 10.0 ml with methanol, and solution was filtered through Whatman Grade I filter paper.One ml of the filtrate was diluted to 100.0 ml with mobile phase to get concentration of 10.0 µg/ml (on labelled claim basis). Replicate sample solutions were prepared in similar manner.

Procedure: After equilibration of column with mobile phase, replicate injection of standard and each of five sample solutions were made separately and chromatograms were recorded.

The amount of drug estimated in sample weight was calculated using formula:

$$Ew = \frac{Au \times Cs}{As}$$

where, E_w = drug estimated in sample weight (mg)

Cs = concentration of standard (µg/ml)

Au = peak area of unknown sample

As = peak area of standard

Amount of drug present in average weight of tablet (as % of labelled claim) was calculated using following formula:

$$\%\ \text{of labelled claim} = \frac{Ew \times W_{AV}}{Lc \times Ws} \times 100$$

where, E_w= amount estimated in sample weight (mg)

W_{AV}= average weight of tablet (mg)

W_s= sample weight (mg)

L_c= labelled claim (mg/tablet)

Table: Result of estimation of Ozagrel in tablet

Pulmoza tablet (Avg. wt: 359.82 mg., Labelled claim: 200 mg per tablet)				
Sr. No.	**Wt. of tablet powder (mg)**	**Detector response* (Peak area)**		**% of labelled claim**
		Standard (Conc:µg/ml)	**Sample**	
1	12.70	126196	88649	99.51
2	15.20		106085	99.49
3	17.90		126182	100.49
4	20.60		143769	99.49
5	23.60		163402	98.70
*Mean of five observations			**Mean**	**99.54**
			±SD	**0.635**
			%RSD	**0.638**

III. VALIDATION

❖ **Accuracy:** Accuracy of the proposed method was ascertained on the basis of recovery studies performed by standard addition method.

- **Standard solution:** Working standard solution B was prepared (10.0 µg/ml) as described under preparation of standard solution.

- **Sample solution:** Accurately weighed quantities of pre-analyzed tablet powder equivalent to about 7.0 mg of Ozagrel were transferred to five different 10.0 ml volumetric flasks and 1.5 mg, 3.0 mg, 4.5 mg and 6.0 mg of standard Ozagrel were added to 2nd, 3rd, 4th & 5th flask respectively (flasks representing 70- 130% of labelled claim). This was followed by addition of methanol to make volume to about 8.0 ml in each flask, and the contents were shaken and sonicated for 15 minutes. Sufficient methanol was added to each flask to adjust the volume to 10.0 ml mark and filtered. One ml of each of the filtrate was diluted to 100.0 ml with mobile phase.

Procedure: Same as described under estimation of Ozagrel in tablet.

The percent recovery was then calculated by using formula:

$$\% \text{ Recovery } = \frac{Ew - B}{C} \times 100$$

where,

E_w = total drug estimated (mg)

B= amount of drug contributed by preanalyzed tablet powder (mg)

C= weight of pure drug added (mg)

Table: Result of recovery studies

Pulmoza tablet (Avg. wt: 359.82 mg., Labelled claim: 200 mg per tablet)				
Flask No.	**Wt. of tablet powder (mg) + wt. of standard added (mg)**	**Detector response* (Peak area)**		**% Recovery**
		Standard	**Sample**	
1	12.40 + 0 (70 %)	126253	87603	100.67
2	12.30 + 1.5 (85 %)		100692	99.46
3	12.50 + 3.0 (100 %)		123995	98.72
4	12.90 + 4.5 (115 %)		146592	99.49
5	12.60 + 6.0 (130 %)		164022	99.90
* Mean of five observations			**Mean**	**99.65**
			±SD	**0.712**
			%RSD	**0.715**

❖ **Precision**

- **Repeatability**

Precision of proposed method was ascertained by replicate analysis of homogeneous samples of tablet powder.

- **Intermediate precision**

The samples were analysed by proposed method on different days (intra-day & inter-day), by different analysts and on two different HPLC.

S. No	Observations	% of labelled claim				
		Intra-day	Inter-day	Different Analysts	HPLC	
					I	II
1.	I	100.78	100.93	100.30	100.15	99.63
2.	II	100.60	99.71	100.86	99.62	99.25
3.	III	99.24	100.14	100.76	99.35	100.21
Mean*		**100.21**	**100.26**	**100.64**	**99.61**	**99.70**
±S.D.		**0.842**	**0.619**	**0.299**	**0.471**	**0.483**
%R.S.D.		**0.840**	**0.617**	**0.297**	**0.473**	**0.485**

*** mean of three observations**

Table: Result of precision studies

❖ **Linearity and Range**

- **Linearity of response**

Chromatographic response (peak area) as a function of concentration was studied.

- **Range of the method**

Sample weights of pre- analysed tablet powder were fortified by addition of standard drugs to have the range 70- 130 % of labelled claim and the sample were processed as discussed under accuracy studies. The graph was plotted percent labelled claim vs. peak area using the data under accuracy studies.

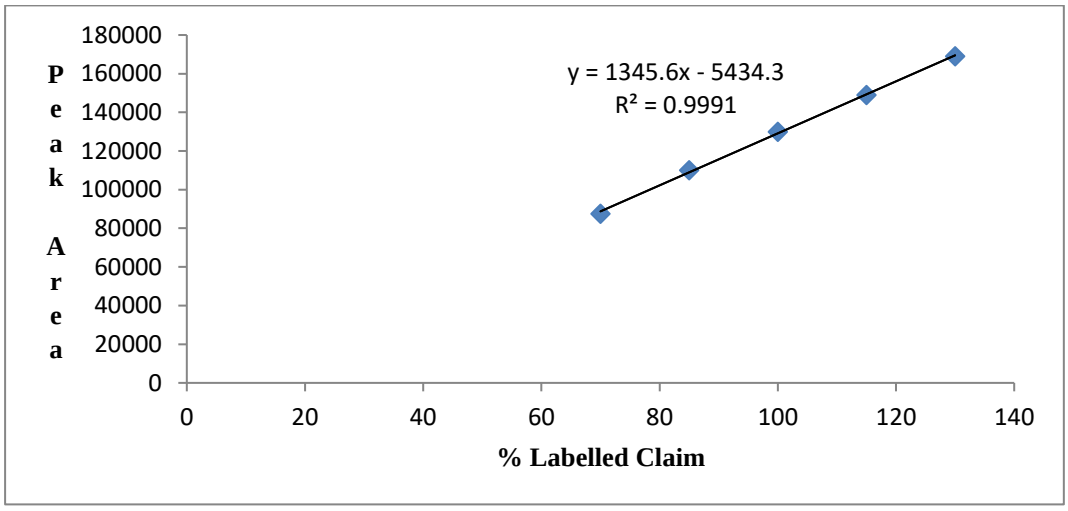


Fig: Calibration curve of range of method

Table: Results of range of method by area

Parameters	Results
Range	70- 130% of labelled claim
Slope	1345
Y-intercept	(-) 5434
Correlation coefficient	0.999
Equation for straight line	Y=1345.X-5434

❖ **Limit of Detection (LOD) and Limit of Quantitation (LOQ)**

LOD and LOQ were determined by the method based on standard deviation of the response and the slope of calibration curve as per ICH guidelines and are as follows:

$$LOD = \frac{3.3\sigma}{S} \text{ and } LOQ = \frac{10\sigma}{S}$$

Signal to noise ratio (k) = 3.3 and 10 for LOD and LOQ respectively

σ = Standard deviation of response (Estimated by measuring the response in terms of peak area of standard solution of conc. 1.0 µg/ml for five times and σ was calculated) = 1251.393

S = Slope of calibration curve (obtained from calibration curve) = 12639

Table: Results of LOD and LOQ studies

S. No	Parameters	Values
1.	LOD (µg/ml)	0.327
2.	LOQ (µg/ml)	0.990

❖ **Specificity**

- **Standard solution:** Working standard solution was prepared (10.0 µg/ml) as described under preparation of standard solution.

- **Sample solution:** Accurately weighed quantities of tablet powdered equivalent to about 10.0 mg of Ozagrel were transferred to six different 10.0 ml volumetric flasks. The samples were then exposed to stress conditions as follows:

1) Normal (control) for 24 h

2) Reflux for 24 h after addition of 1M HCL up to 10.0 ml mark.

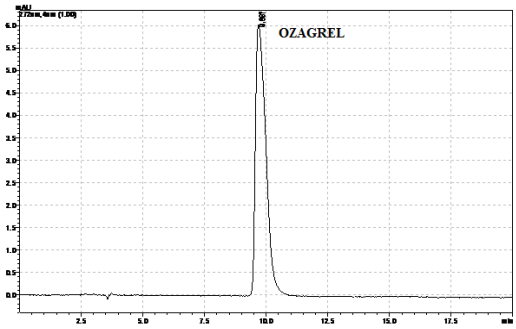
3) Reflux for 24 h after addition of 1M NaOH up to 10.0 ml mark.

4) At room temperature in dark after addition of 3 % H_2O_2 up to 10.0 ml mark for 48 h.

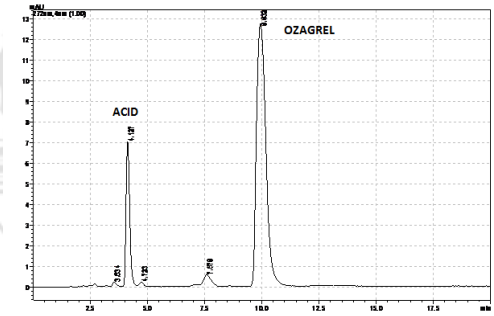
5) At 80^0 C (dry heat) for 24 h (after 24 h; mobile phase was added to make volume to 10.0 ml mark).

6) Sunlight for 24 h (after 24 h; mobile phase was added to make volume to 10.0 ml mark).

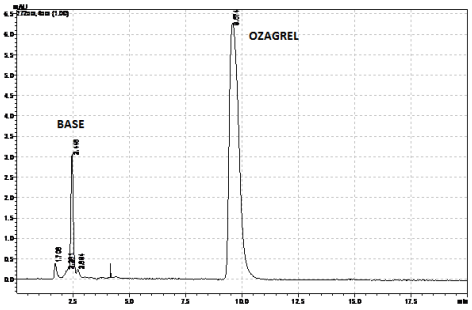
After stipulated time of each stress conditions flasks were sonicated for 15 minutes and filtered. One ml each of filtrates was further diluted to 100.0 ml with mobile phase and analyzed in similar manner as described under estimation of Ozagrel in tablets.



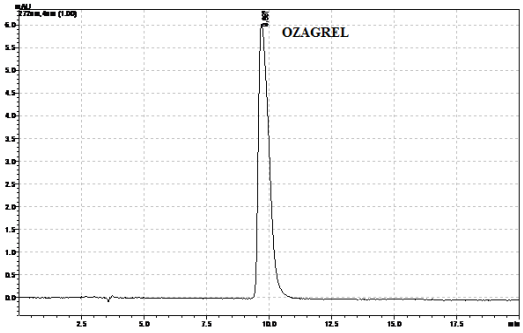
A) Normal, 24 h



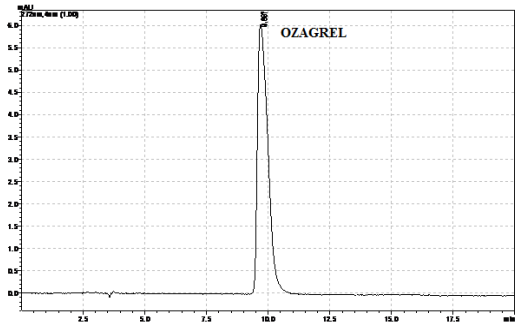
B) 1M HCL, 24 h reflux

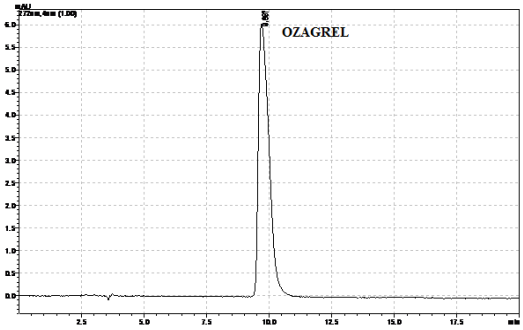


C) 1 M NaOH, 24 h reflux



D) 3% H_2O_2, 48 h





E) Thermal, 24 h F) Sunlight, 24 h

Figure: HPLC chromatograms of specificity studies

Table: Results of Specificity studies

S. NO	Sample	% of Labelled claim* ± S.D.
1	Normal	99.96 ± 0.237
2	Acid	85.54 ± 0.505
3	Alkali	83.93 ± 0.596
4	Oxide	99.52 ± 0.225
5	Heat	100.13 ± 1.420
6	Sunlight	99.26 ± 0.535

***mean of three observations**

❖ **Robustness:** The studies were carried out for different parameters by changing the wavelength of detection.

Table: Results of Robustness Studies

% Estimation	Change in wavelength (± 2 nm)		
	270.0*	**272.0***	**274.0***
Mean	100.25	100.98	99.80
± SD	1.362	0.993	0.589
% RSD	1.359	0.983	0.590

***mean of three observations**

IV. DISCUSSION AND SUMMARY

❖ HPLC Method Development

The chromatographic separation of Ozagrel and its degradation products was done on Enable C_8 column (150 x 4.6 mm, 5 µm). The mobile phase containing mixture of acetonitrile: phosphate buffer (10 mM, pH 6.5 with 0.1 M NaOH) (10: 90 *v/v*) was found to be most satisfactory as it gave good resolution of drug from its degradation products with reasonably symmetrical sharp peaks. A detection wavelength 272 nm was optimized as Ozagrel has substantially high absorbance at this wavelength. A flow rate of 1.0 ml/ min at room temp was found to be optimum. The retention time under optimized chromatographic conditions was found to be 9.946 ± 0.039 min with sharp symmetrical peak (asymmetry of peaks 1.244 ± 0.017). The capacity factor was 1.813 ± 0.031 with mean theoretical plates 6632.934 ± 0.791. The degradation studies on Ozagrel have established that the drug degrades under acidic and alkaline stress conditions. However, no degradation was observed under neutral, thermal, oxidative and photolytic stress conditions.

Table: Summary of forced degradation studies by HPLC

Stress conditions	Duration of exposure (API)	Retention Time of degradation products (min.)
Acid (1M HCl)	24 h, reflux	3.573, 4.198, 4.700, 7.534
Alkali(1M NaOH)	24 h, reflux	1.704, 2.261, 2.445, 2.699
Neutral (Water)	24 h, reflux	No degradation
Oxidative (3% H_2O_2)	48h	No degradation
Thermal (80 ^{0}C, 120 ^{0}C)	15 days	No degradation
Photo (Sunlight)	15 days	No degradation

The detector response was found to be linear over the concentration range 1.0-10.0 µg/ml with correlation coefficient value 0.999 by area and height both. The system suitability tests have indicated the reproducibility of detector response with respect to retention time, tailing factor, theoretical plates, capacity factor, peak area and peak height for the drug..

A standard solution of Ozagrel (10.0 µg/ml) was prepared in mobile phase. Accurately weighed quantities of tablet powder of Ozagrel was extracted with methanol and diluted with mobile phase to adjust the concentration to about 10.0 µg/ml (on the basis of labelled claim). Chromatograms of

standard and sample solutions were recorded under optimized conditions and the drug contents were calculated.

Table: Summary of result of estimation of Ozagrel in tablet

Statistical parameters	% of labelled claim
Mean*	99.54
±SD	0.635
%RSD	0.638

*** mean of five observations**

❖ **Validation of Proposed HPLC method**

The proposed HPLC method was validated for accuracy, precision, linearity & range, limit of detection, limit of quantitation, specificity and robustness.

a) Accuracy: Accuracy was ascertained by carrying out recovery studies on marketed formulations with standard addition method over the range of 70 to 130 % of labelled claim. Recoveries of the drug were observed to be very close to 100% representing the accuracy of the method and also non interference of excipients.

Table: Summary of results of accuracy studies

Statistical parameters	% Recovery
Mean*	99.65
±S.D.	0.712
% RSD	0.715

*** mean of five observations**

b) Precision: Replicate estimation of Ozagrel in tablet by proposed HPLC method have yielded quite concurrent results with a small value of S. D., which speak about repeatability of proposed HPLC method.

Parameters	% of labelled claim			
	Intra-day	Inter-day	Different Analyst	Jasco HPLC
Mean*	100.21	100.26	100.64	99.70
±S.D.	0.842	0.619	0.299	0.407
%R.S.D.	0.840	0.617	0.297	0.408

*** Mean of three observations**

Table: Summary of results of precision studies

c) Linearity and Range, LOD and LOQ

Chromatographic response (peak area) as a function of concentration was studied

Table: Summary of results of Linearity, Range, LOD and LOQ

Concentration range	1.0- 10.0 µg/ml
Slope	12623
Y-intercept	(-) 14.55
Correlation coefficient	1
LOD	0.327
LOQ	0.990

d) Specificity: These studies were carried out to ascertain how accurately and specifically, the analyte of interest are estimated in presence of other components (e.g. impurities, degradation products, and excipients) by exposing the tablet powder samples to different stress conditions such as light, heat, oxidation, acids and alkali and then analyzing them by proposed method. They are indicative of specificity and selectivity of the method. The proposed HPLC method is capable of estimating intact drug contents free of interference from its degradation products.

e) Robustness: Robustness of the proposed method was ascertained by deliberate change in the chromatographic parameters like change in wavelength by ± 2nm. There was no significant difference in results obtained by change of detection wavelength by ±2 nm

The results of assay of Ozagrel tablet obtained by proposed HPLC method are quite concurrent and reproducible. The recoveries of the drug from tablet matrix were about 100% indicating

accuracy and reliability of method and non-interference of excipients. At the same time method is simple, precise, accurate, rapid, reasonably specific and rugged. Hence, it may be adopted for routine assay of Ozagrel free of interferences from its degradation products in tablet formulation. The proposed HPLC method in true sense can be said to be selective Stability Indicating Assay Method for Ozagrel, due to its capacity to estimate the drug content unequivocally free of interference from its degradation products. The degradants may be estimated if they are identified and their standards are available.

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