

Development of Validated Stability Indicating HPTLC Method for Assay of Ozagrel and its Pharmaceutical Formulations

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ABSTRACT

The present paper describes stability indicating high-performance thin-layer chromatography (HPTLC) assay method for Ozagrel in bulk drugs. The method employed TLC aluminium plates precoated with silica gel 60F-254 as the stationary phase. The solvent system consisted of toluene: methanol: triethylamine (6.5: 4.0: 0.1 v/v/v). The system was found to give compact spot for Ozagrel (R_f value of 0.40 ± 0.010). Densitometric analysis of Ozagrel was carried out in the absorbance mode at 280 nm. The linear regression analysis data for the calibration plots showed good linear relationship with $r^2 = 0.999$ with respect to peak area in the concentration range 30 - 120 ng/spot. The developed HPTLC method was validated with respect to accuracy, precision, recovery and robustness. Also to determine related substance and assay determination of Ozagrel that can be used to evaluate the quality of regular production samples. The developed method can also be conveniently used for the assay determination of Ozagrel in pharmaceutical formulations. The limits of detection and quantitation were 4.069 and 12.332 ng/spot, respectively by height. Ozagrel was subjected to acid and alkali hydrolysis, oxidation, photochemical and thermal degradation. The drug undergoes degradation under acidic, basic, oxidation and heat conditions. This indicates that the drug is susceptible to acid, base hydrolysis, oxidation and heat. Statistical analysis proves that the method is repeatable, selective and accurate for the estimation of said drug. The proposed developed HPTLC method can be applied for identification and quantitative determination of Ozagrel in bulk drug and tablet formulation.

Keywords : Ozagrel, validation, HPTLC

I. INTRODUCTION

High Performance Thin Layer Chromatography (HPTLC) is the most powerful advanced form of Thin Layer Chromatography (TLC) and consists of chromatographic layers of utmost separation efficiency and the application of sophisticated instrumentation for all steps in the procedure include accurate sample application, standardized reproducible chromatogram development and software controlled evaluation. HPTLC is a concept that includes a widely standardized methodology

based on scientific facts as well as the use of validated methods for qualitative and quantitative analysis . HPTLC meets all quality requirements for today's analytical labs, to increase the resolution and to allow more accurate quantitative measurements.

HPTLC is the most advanced form of modern TLC. It uses HPTLC plates featuring small particles with a narrow size distribution which results in homogenous layers with a smooth surface to be obtained. HPTLC uses smaller plates (10 × 10 or 10 × 20 cm). HPTLC plates provide improved resolution,

higher detection sensitivity, and improved in situ quantification and are used for industrial pharmaceutical densitometric quantitative analysis. Normal phase adsorption TLC on silica gel with a less polar mobile phase, such as chloroform– methanol, has been used for more than 90% of reported analysis of pharmaceuticals and drugs.

The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte. The mobile-phase systems are used based on their diverse selectivity properties are diethyl ether, methylene chloride, and chloroform combined individually or together with hexane as the strength adjusting solvent for normal-phase TLC and methanol, acetonitrile, and tetrahydrofuran mixed with water for strength adjustment in reversed-phase TLC. Separations by ion pairing on C-18 layers are done with a mobile phase such as methanol–0.1 M acetate buffer (pH 3.5) containing 25 mM sodium pentanesulfonate (15.5:4.5).

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 methanol (conc.: 10.0 µg/ml).

❖ Optimization of chromatographic conditions

• Optimization of mobile phase

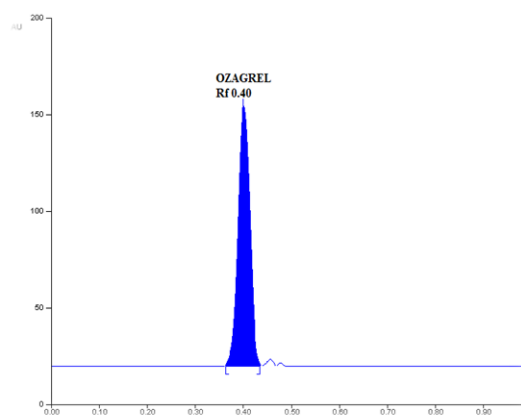
Aliquot portions of working standard solution (5 µl) were applied on TLC plates. Various pure solvents with varying polarity and their mixtures were tried for optimum movement of drug with sharp symmetrical peak. After trying several permutations and combinations, the mobile phase containing toluene: methanol: triethylamine (6.5: 4.0: 0.1 v/v/v) was found to be most satisfactory as it gave sharp

symmetrical peaks for the drug with R_F values 0.40 ± 0.010 .

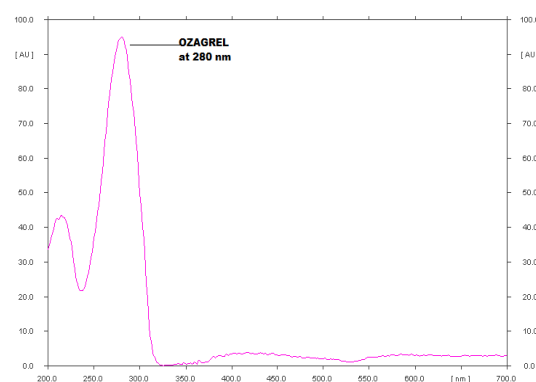
• Optimization of wavelength for densitometric evaluation

Aliquot portion of working standard solution B (5 µl) was applied on TLC plate. The plate was developed using optimized mobile phase. After development, the plate was removed and dried with the help of hair dryer. The migrated band was scanned over the wavelength range 200– 400 nm in an absorbance/reflectance mode and an *in situ* UV-absorption spectrum of drug was obtained.

A 280.0 nm was selected as scanning wavelength as it gave maximum absorption for the drug.



(a)



(b)

Figure 1 : (a) HPTLC chromatogram and (b) *In situ* UV Spectrum of Ozagrel

• Chamber saturation time

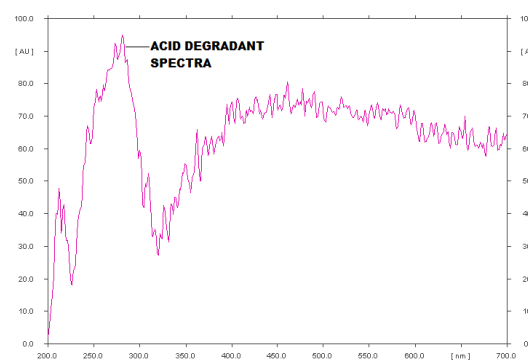
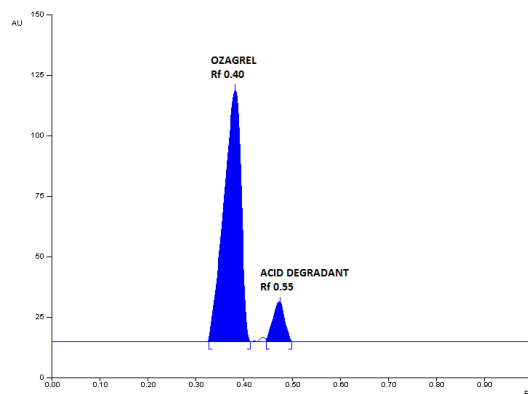
The chamber saturation time was optimized by allowing the spotted plates to equilibrate for varying

time with vapours of mobile phase in twin trough chamber and then developing them using optimized mobile phase. The optimum saturation time was found to be 15 min, which resulted in to dense and compact spot.

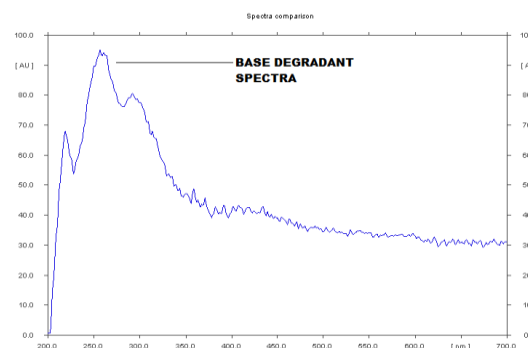
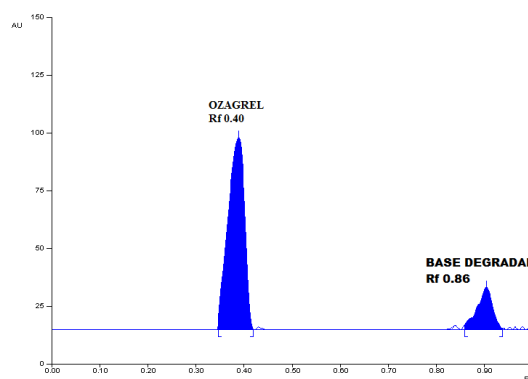
On the basis of exhaustive experimentation the chromatographic conditions optimised are as follows:

Stationary Phase	Pre-coated Silica Gel 60 F254 TLC Plate (10 x10 cm)
Thickness	200 mm
Mobile Phase	Toluene Methanol
Mode of Application	Band
Band Width	5mm
Sample Volume	5 ml
Separation Technique	Ascending
Development Chamber	Twin trough glass chamber , 10 x 10 cm
Chamber Saturation Time	15 min
Migration Distance	90 mm
Detection	UV Densitometric Scanning
Scanning Mode	Absorbance/ Reflectance
Scanning Wavelength	280.0 nm
Scanning Speed	20 mm/s
Slit Dimension	4.0 x 0.45 mm
Temperature	25 ± 3 °C

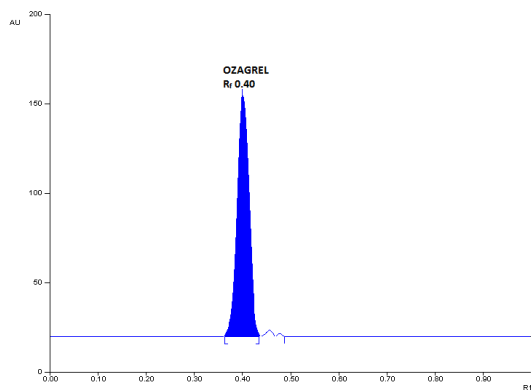
The samples of stress studies were spotted (5 µl bands) on TLC plates and developed and scanned under optimized conditions. The study has shown adequate resolution of parent drug and its degradation products from one another under optimized chromatographic conditions by normal phase mode. Hence these chromatographic conditions were finalized for further experimentation.



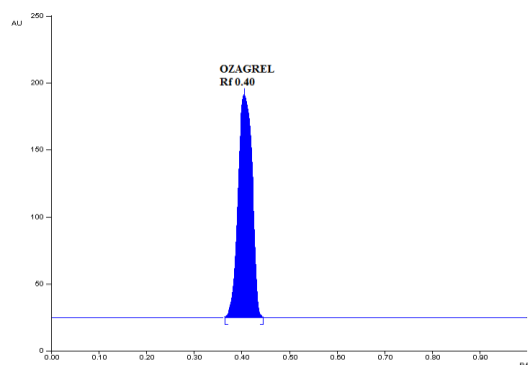
A) Acid (1M HCL, 24 h reflux) and Spectra of degradant



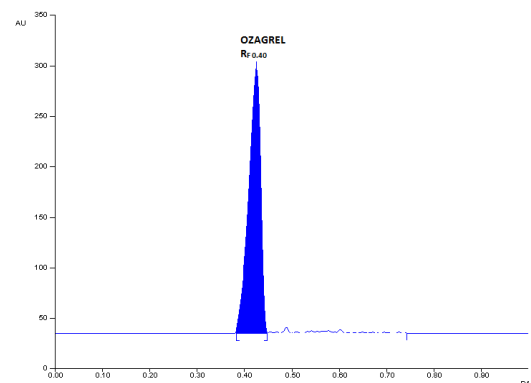
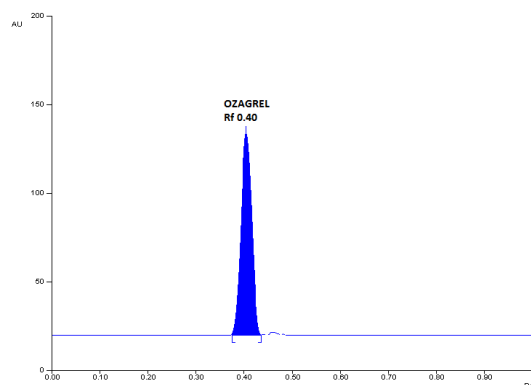
B) Base (1M NaOH, 24 h reflux) and Spectra of degradant



C) Neutral stress, 24h reflux



D) Oxidative stress (3% H₂O₂, 48 h at R.T.)



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

Thermal Stress (dry heat, 15 days at 700c

Figure 2 : HPTLC chromatograms of forced degraded samples (A-F).

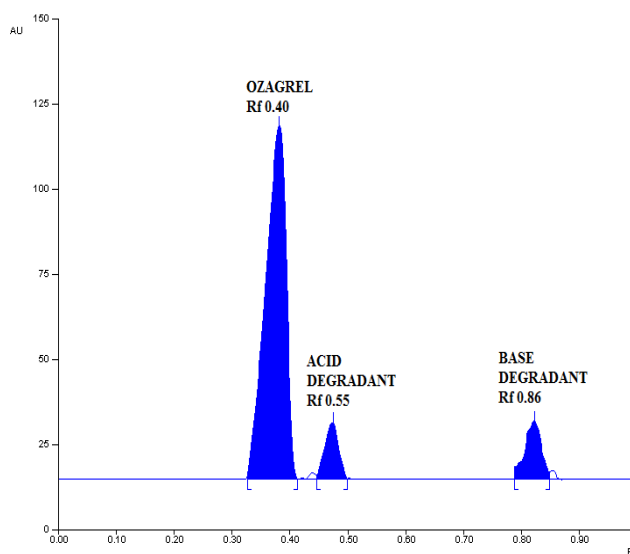
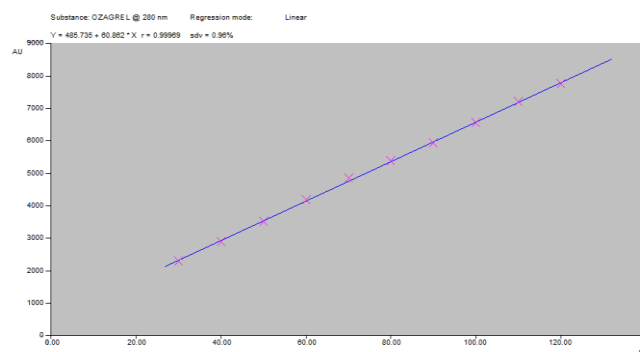


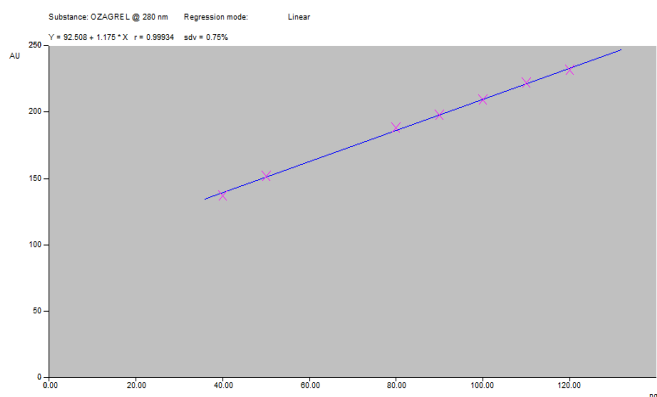
Figure 3 : Chromatogram of mixed degradation sample

❖ **Study of Linearity of response**

Aliquots portions of standard solution B (3-12 µl) were applied on TLC plate and chromatograms were developed under optimized chromatographic conditions. The linear regression curves are depicted along with correlation coefficient, slope and y-intercept. The curves were found to be linear between concentration ranges 30-120 ng/spot.



(a)



(b)

Figure 4 : Linearity (a) by area (b) by height

Concentration range	30- 120 ng/spot	
Parameter	Height	Area
Slope	1.175	60.862
Y-intercept	92.508	485.735
Correlation coefficient	0.999	0.999

Table 1 : Results of Linearity studies

❖ Estimation of Ozagrel in Tablet by Proposed Method

- **Standard solution:** Working standard solution 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 methanol to get concentration of 10.0 µg/ml (on labelled claim basis). Replicate sample solutions were prepared in similar manner.
- **Procedure:** Two bands of standard solution and six bands of sample solution of equal volume (5 µl) were applied on TLC plate and the plate was developed and scanned as per optimized chromatographic conditions.
- **Calculation:** The instrument directly gives the weight of constituent in volume of sample solution applied by comparison with concentration of standard. This value was subsequently converted to percent of labelled claim using following formula.

Pulmoza tablet (Avg. wt: 359.82 mg., Labelled claim: 200 mg per tablet)					
Sr. No.	Wt. of tablet powder taken (mg)	Amt. of Ozagrel estimated in applied 5 µL vol. (ng)		% of labelled claim	
		By Height	By Area	By Height*	By Area*
1.	14.70	41.08	40.96	100.55	100.26
2.	16.00	44.27	44.15	99.55	99.28
3.	18.30	50.82	50.94	99.92	100.15
4.	21.20	58.87	59.14	99.91	100.37
5.	22.70	62.99	62.75	99.84	99.46
* Each value is mean of five observations		Mean		99.95	99.90
		±S.D.		0.365	0.497
		% RSD		0.365	0.498

Table 2 : Results of estimation of Ozagrel in tablet

VALIDATION

❖ Validation of the proposed method

Validation of proposed method was ascertained on the basis of accuracy, precision, linearity & range, limit of detection, limit of quantitation, specificity, ruggedness and robustness.

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

- **Standard solution:** Working standard solution 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 (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 methanol.

- **Procedure:** Same as described under estimation of Ozagrel in tablet. (p.no: 59)
- **Calculation:** Amount of Ozagrel (ng/5µl) obtained from instrument was converted to total drug estimated by using following formula:

$$T = \frac{E_w \times 1000}{V_s}$$

The percent recovery was then calculated using the formula:

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

where,

T	=	total drug estimated (mg)
E _w	=	Wt. (µg) of drug calculated by instrument in V _s
V _s	=	Volume (µl) of sample solution applied
B	=	amount of drug contributed by pre-analyzed tablet powder (mg)
C	=	weight of pure drug added (mg)

Pulmoza tablet (Avg. Wt: 359.82 mg., Labelled claim: 200 mg per tablet)					
Flask No.	Wt. of tablet powder taken (mg) + Amt of pure drug added (mg) (% of labelled claim)	Amt. of Ozagrel estimated in applied 5µL vol. (ng)		% Recovery	
		By Height	By Area	By Height*	By Area*
1.	12.80 + 0 (70 %)	35.7	35.8	100.49	100.89
2.	12.60 + 1.5 (85 %)	42.4	42.5	99.88	100.05
3.	12.90 + 3.0 (100 %)	50.9	50.6	100.11	99.64
4.	12.70 + 4.5 (115 %)	56.8	57.1	98.98	98.88
5.	12.50 + 6.0 (130 %)	65.1	65.3	100.55	100.95

* Each value is mean of five observations	Mean	100.00	100.08
	±S.D.	0.634	0.872
	%RSD	0.634	0.872

Table 3 : Results of recovery studies of Ozagrel in tablet

❖ 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), and by different analysts.

Sr. No.	Observations	% of labelled claim					
		Intra-day		Inter-day		Different Analysts	
		By Height	By Area	By Height	By Area	By Height	By Area
1.	I	99.78	99.57	100.03	99.46	100.23	100.33
2.	II	99.96	99.36	99.79	99.25	99.52	99.92
3.	III	100.06	99.86	98.94	99.12	100.76	100.25
Mean*		99.93	99.60	99.59	99.28	100.17	100.17
±S.D.		0.142	0.251	0.573	0.172	0.622	0.217
% R.S.D.		0.142	0.252	0.575	0.173	0.621	0.217

* Each value is mean of three observations

Table 4 : Result of precision studies

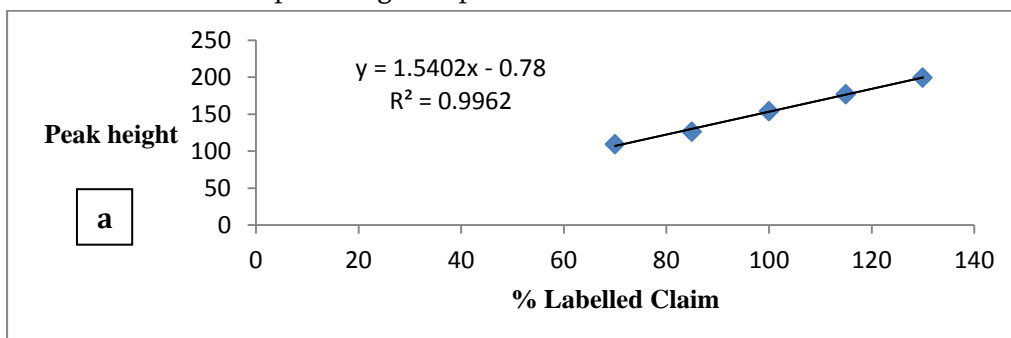
❖ Linearity and Range

- Linearity of response

Chromatographic response (peak height / 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 samples were processed as discussed under accuracy studies. The graph plotted as percent labelled claim vs. peak height or peak area.



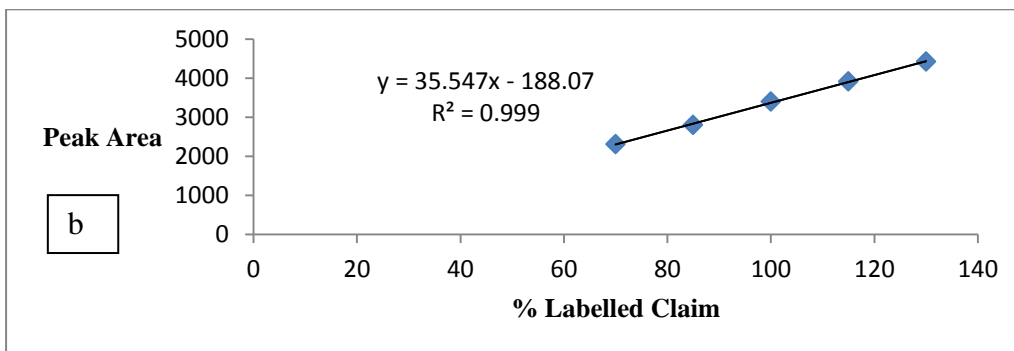


Figure 5 :Calibration curve of range of method (a) by height (b) by area

Concentration range	70- 130% of labelled claim	
Parameter	Height	Area
Regression equation	Y=1.540X-0.78	Y=35.54-188.0
Slope	1.540	35.34
Y-intercept	(-) 0.78	(-) 188.0
Correlation coefficient	0.996	0.999

Table 5 : Results of range of method

❖ **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 term of peak height or peak area of standard solution of conc. 30.0 ng/spot for five times and σ was calculated) = 1.455201, 48.71276 by height and area resp.

S = Slope of calibration curve (obtained from calibration curve) = 1.18, 60.86 by height and area resp.

S. No	Parameters	By Height	By Area
1.	LOD (ng/spot)	4.069	2.641
2.	LOQ (ng/spot)	12.332	8.004

Table 6 : Results of LOD and LOQ studies

❖ **Solution State Stability and stability on plate**

The chromatograms of the same standard were obtained periodically over a period of 24 h.

Time (h)	Solution state stability		Stability on plate	
	Peak height*	Peak area*	Peak height*	Peak area*
1	151.96	3498.52	151.85	3498.63
3	152.14	3498.96	151.90	3498.22
7	152.36	3491.25	151.93	3495.55
24	151.99	3496.39	152.25	3495.96
Mean	152.11	3496.82	151.98	3497.09
± SD	0.183	3.536	0.181	1.560
% RSD	0.120	0.101	0.119	0.045

*mean of three observations

Table 7 : Results of Solution State Stability and stability on plate

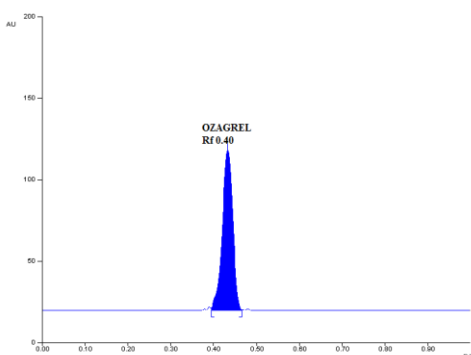
❖ **Specificity**

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

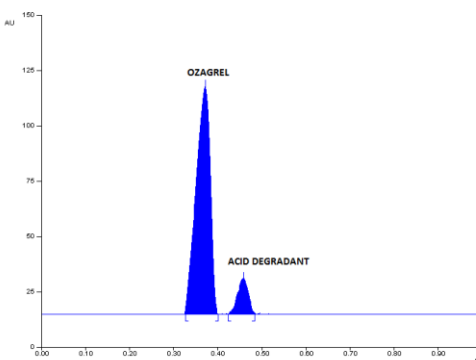
- **Sample solution:** Accurately weighed quantities of tablet powdered equivalent to about 10.0 mg of Ozagrel were transferred to six different 10.0 ml volumetric flasks. The samples were then exposed to stress conditions as follows:

- 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 % H₂O₂ up to 10.0 ml mark for 48 h.
- 5) At 80° C (dry heat) for 24 h (after 24 h; methanol was added to make volume to 10.0 ml mark).
- 6) Sunlight for 24 h (after 24 h; methanol was added to make volume to 10.0 ml mark).

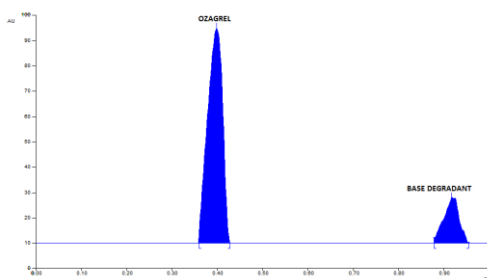
After stipulated time of each stress conditions flasks were sonicated for 15 minutes and filtered. One ml each of filtrates was further diluted to 100.0 ml with methanol and analyzed in similar manner as described under estimation of Ozagrel in tablets.



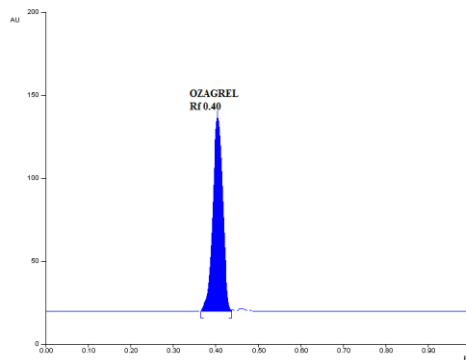
A) Normal, 24 h



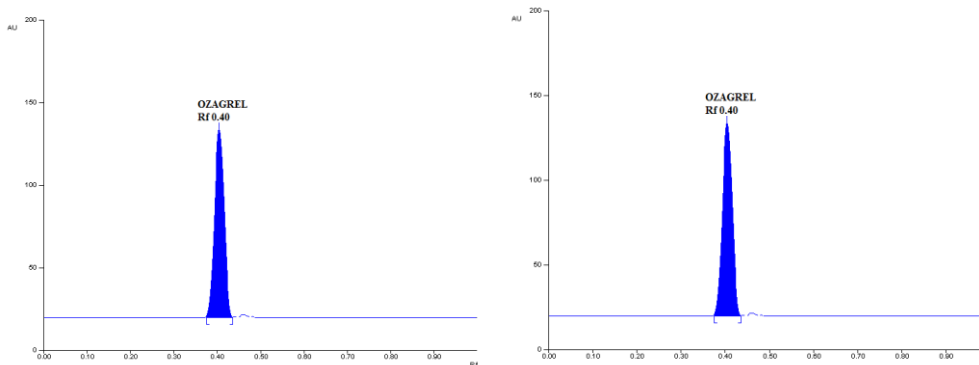
B) 1M HCL, 24 h reflux



C) 1 M NaOH, 24 h reflux



D) 3% H₂O₂, 48 h



E) Thermal, 24 h

F) Sunlight, 24 h

Figure 6 : HPTLC chromatogram of specificity studies

Sr. No.	Sample	% of labelled Claim* ± S.D.	
		By Height	By Area
1	Normal	99.21 ± 1.837	99.84 ± 1.276
2	Acid	88.04 ± 1.905	87.10 ± 0.560
3	Alkali	87.27 ± 0.445	87.80 ± 1.286
4	Oxide	99.80 ± 0.571	99.30 ± 0.582
5	Heat	99.40 ± 1.877	99.03 ± 1.212
6	Sunlight	99.10 ± 0.456	99.62 ± 1.934

*mean of three observations

Table 8 : Results of specificity studies

❖ **Robustness (Change in Scanning Wavelength)**

The tablet sample of Ozagrel was analyzed using proposed method after a deliberate change in detection wavelength for estimation by ±2 nm.

% Estimation	Change in wavelength (± 2 nm)					
	278.0		280.0		282.0	
	By Height	By Area	By Height	By Area	By Height	By Area
Mean*	99.48	100.42	100.17	100.17	99.19	100.53
± SD	0.069	0.035	0.622	0.217	0.618	0.720
%RSD	0.069	0.034	0.621	0.217	0.631	0.710

*mean of three observations

Table 9 : Results of robustness studies

III. DISCUSSION AND SUMMARY

❖ **HPTLC Method Development**

HPTLC technique is in wide use for qualitative and quantitative analysis of drugs as well as finds applications in the development of stability indicating assay methods for the drugs. HPTLC has been employed as an alternative to HPLC for development of SIAM for selective estimation of Ozagrel in tablet formulation. Normal phase separation mode was used to resolve the intact drug from their degradation products.

The standard solution of Ozagrel (10.0 µg/ml) was prepared in methanol. The stationary phase used was pre-coated silica gel 60 F₂₅₄ TLC plates. Several mobile phase combinations of varying polarity were tried for resolution of degradation products from the parent drug. The mobile phase having composition toluene: methanol: triethylamine (6.5: 4: 0.1 v/v/v) was found to be satisfactory for the resolution of degradation products from the intact drug. The intact drug and its degradation products were adequately resolved on TLC plates under optimized chromatographic conditions. The R_F value of intact drug was 0.40 ± 0.010 with sharp symmetrical peak. The λ_{max} of Ozagrel, 280 nm from its *in situ* UV spectrum was found to be sensitive enough for densitometric evaluation of the degradation products also. The optimized chromatographic conditions for proposed HPTLC method are as follows:

Stationary Phase	Silica Gel 60 F ₂₅₄ TLC Plate 5 x 10 cm and 10 x 10 cm
Mobile Phase	Toluene: Methanol: Triethylamine 6.5: 4.0: 0.1 v/v/v
Saturation Time	15 min.
Detection wavelength	280 nm in absorption/reflectance mode
Sample volume applied	5 µl in band of 5 mm width
Slit Dimension	4.0 x 0.45 mm
Temperature	25 ± 3 °C

Table 10 : Summary of optimized chromatographic conditions

The degradants formed under various stress conditions were well resolved from the intact drug.

Stress conditions	Duration of exposure (API)	R_F of degradation products
Acid (1M HCl)	Reflux, 24 h	0.55
Alkali (1M NaOH)	Reflux, 24 h	0.86
Neutral (Water)	Reflux, 24 h	No degradation
Oxidative (3% H ₂ O ₂)	48 h	No degradation
Thermal (80 °C, 120 °C)	15 days	No degradation
Sunlight	15 days	No degradation

Table 11 : Summary of forced degradation studies by HPTLC

Linear relationships between concentration of Ozagrel and corresponding peak area or peak height was seen over the range of 30.0-120.0 ng/spot with correlation coefficient 0.999 by height and area both. The optimized chromatographic conditions were kept constant for further experimentation and used for estimation of Ozagrel in tablet formulation. The standard solution of Ozagrel was prepared in methanol (conc.:10.0 µg/ml). The sample solutions were prepared by shaking accurately weighed quantities of tablet powder with methanol, filtering the solutions and diluting aliquot portions of the filtrate to obtain concentration in close proximity to standard solution. Two bands of standard and six bands of sample solutions of equal volume (5 µl) were applied on TLC plate and the plate was developed and scanned as per optimized chromatographic conditions. The concentration of

sample solution applied was displayed directly by the instrument by comparison with concentration of standard.

Statistical parameters	% of labelled claim	
	Area	Height
Mean*	99.95	99.90
±S.D.	0.365	0.497
% RSD	0.365	0.498

*mean of five observations

Table 12 : Summary of result of estimation of Ozagrel in tablet

❖ Validation of proposed HPTLC Method

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

1. **Accuracy:** The accuracy of the method was ascertained on the basis of recovery studies performed by standard addition method and the recovery was found to be very close to 100% by area and height over the range of 70-130% of labelled claim representing the accuracy of the method and non interference of the sample matrix.

Statistical parameters	% Recovery	
	Area	Height
Mean*	100.08	100.00
±SD	0.634	0.872
% RSD	0.634	0.872

*mean of five observations

Table 13 : Summary of result of accuracy studies

2. **Precision:** The precision was ascertained by replicate estimations of the drugs in tablet formulation by proposed method. A small value of R.S.D. well below 2.0 is indicative of repeatability of the proposed method.

Parameters	% of labelled claim					
	Intra-day		Inter-day		Different Analyst	
	Height	Area	Height	Area	Height	Area
Mean*	99.93	99.60	99.59	99.28	100.17	100.17
±S.D.	0.142	0.251	0.573	0.172	0.622	0.217
%R.S.D.	0.142	0.252	0.575	0.173	0.621	0.217

* Mean of three observations

Table 14 : Summary of results of precision studies

3. Linearity and Range, LOD and LOQ

Chromatographic response (peak height/ peak area) as a function of concentration was studied. A linear response was seen over conc. range studied. The LOD and LOQ values down to few ng per spot are indicative of sensitivity of the method with respect to detection and quantitation of Ozagrel.

Concentration range	30- 120 ng/ spot	
Parameter	Height	Area
Slope	1.175	60.862
Y-intercept	92.508	485.735
Correlation coefficient	0.999	0.999
LOD (ng/ spot)	4.069	2.641
LOQ (ng/ spot)	12.332	8.004

Table 15 : Summary of results of Linearity, Range, LOD and LOQ

4. Solution State Stability and stability on plate

The chromatograms of the same standard were obtained periodically over a period of 24 h.

Results indicate that the Ozagrel in methanolic solution and on silica gel TLC plate is quite stable over a long period of about 24 h.

5. Specificity: The chromatograms of control and sample solutions showed no interfering peak at the retention time of the drug, so the concentration of Ozagrel can be accurately measured, indicating specificity of the developed method. Moreover, the peaks for degradation products are also well resolved which may enable their estimation if they are identified and their standards are generated.

6. Robustness: A deliberate change in the chromatographic parameters i.e. changes in wavelength by ± 2 nm of λ_{\max} did not have any effect on result indicate the robustness of method with respect to detection parameters.

The results of the assay of Ozagrel tablet obtained by proposed HPTLC methods are quite concurrent and reproducible. The recovery of the drug from the tablet matrix was about 100% indicating accuracy and reliability of method and non interference of excipients. At the same time the method is simple, precise, accurate, rapid, reasonably specific, selective and rugged. Hence, it may be adopted for routine assay of Ozagrel free of interferences from its degradation products in tablets formulation. The

proposed HPTLC method in true sense can be said to be Stability Indicating Assay Method for Ozagrel due to its capacity to estimate the intact drug content unequivocally free of interference from its degradation products. It may also be possible to determine degradation products if they are identified and their standards are generated.

IV. REFERENCES

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