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Validated Stability Indicating HPTLC of Clopidogrel and its Pharmaceutical Formulations

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ABSTRACT

The present paper describes stability indicating high-performance thin-layer chromatography (HPTLC) assay method for clopidogrel 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 Clopidogrel ($R_f$value of 0.40 ± 0.010). Densitometric analysis of Clopidogrel was carried out in the absorbance mode at 254 nm. The linear regression analysis data for the calibration plots showed good linear relationship with $r2$ = 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 Clopidogrel 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 Clopidogrel .The limits of detection and quantitation were 4.062and 12.322ng/spot, respectively by height.

Keywords : Clopidogrel, Validation, HPTLC

I. INTRODUCTION

Estimation of Clopidogrel 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 Clopidogrel 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 clopidogrel estimated in applied 5 µL vol. (ng)		% of labelled claim	
		By Height	By Area	By Height*	By Area*
1.	14.60	41.09	40.96	100.55	100.28
2.	16.00	44.27	44.15	99.55	99.26
3.	18.30	50.84	50.94	99.92	100.15
4.	21.20	58.85	59.14	99.91	100.37
5.	22.60	62.97	62.79	99.85	99.48
* Each value is mean of five observations			**Mean**	**99.94**	**99.90**
			±S.D.	**0.366**	**0.497**
			% RSD	**0.365**	**0.498**

Table 2: Results of estimation of Clopidogrel in tablet

II. 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 Clopidogrel 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 Clopidogrel 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.
- ✓ **Calculation:** Amount of Clopidogrel (ng/5µl) obtained from instrument was converted to total Drug

Estimated by using following formula:

$$T = \frac{Ew \times 1000}{Vs}$$

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 Clopidogrel 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 Clopidogrel in tablet

❖ **Precision**

✓ **Repeatability**

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

✓ **Intermediate precision**

The samples were analyzed 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	ByHeight	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**

Table 4 : Result of precision studies

*** Each value is mean of three observations**

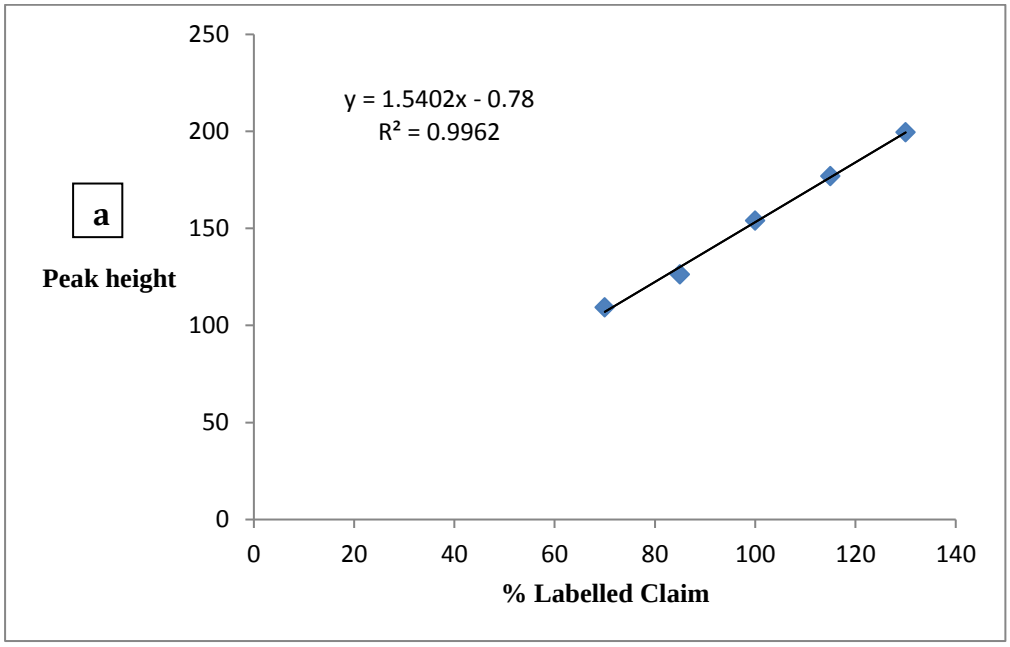
❖ **Linearity and Range**

- **Linearity of response**

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

- **Range of the method**

Sample weights of pre- analyzed 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.



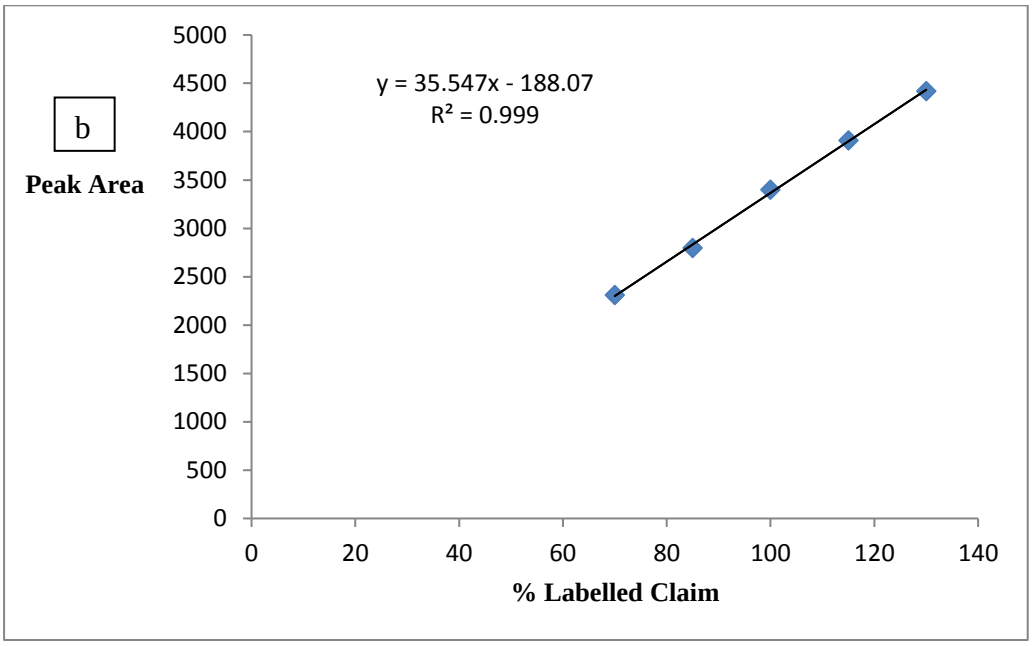


Figure 5: Calibration curve of range of method (a) by height (b) by area

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:

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

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 respectively

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**

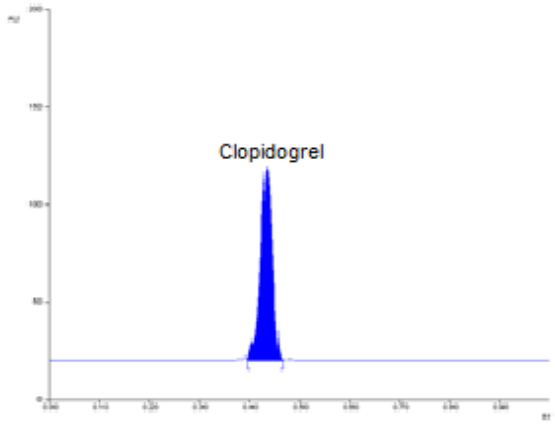
Table 7 : Results of Solution State Stability and stability on plate

❖ **Specificity**

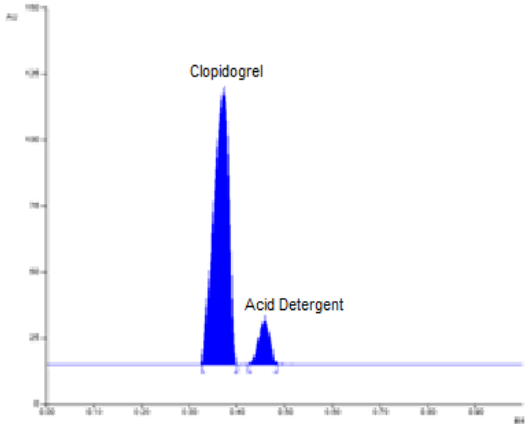
- **Standard solution:** Working standard solution was prepared (10.0 µg/ml) as described under preparation of standard solution.
- **Sample solution:** Accurately weighed quantities of tablet powdered equivalent to about 10.0 mg of Clopidogrel were transferred to six different 10.0 ml volumetric flasks. 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; 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).

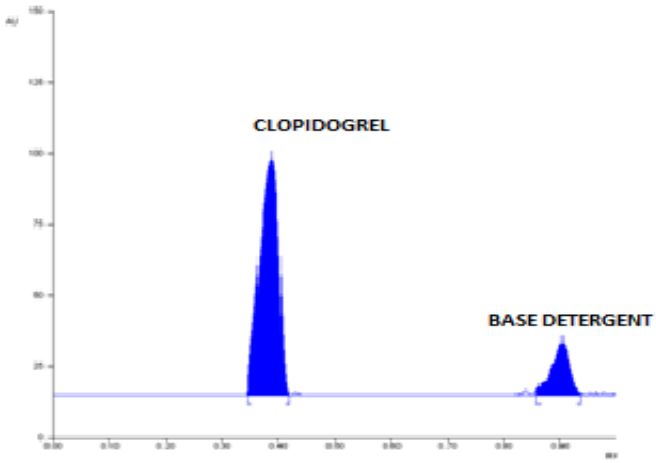
After stipulated time of each stress conditions flasks were sonicated for 15 minutes and filtered. One ml each of filtrates was further diluted to 100.0 ml with methanol and analyzed in similar manner as described under estimation of Clopidogrel in tablets.



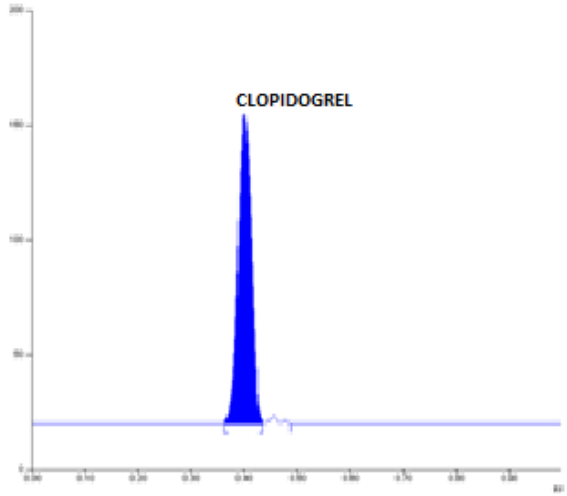
A) Normal, 24 h



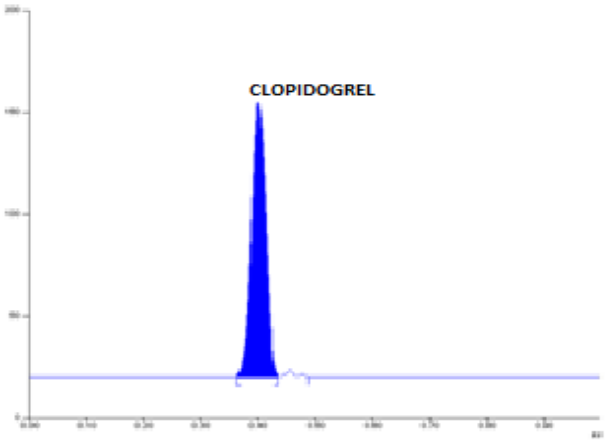
B) 1M HCL, 24 h reflux



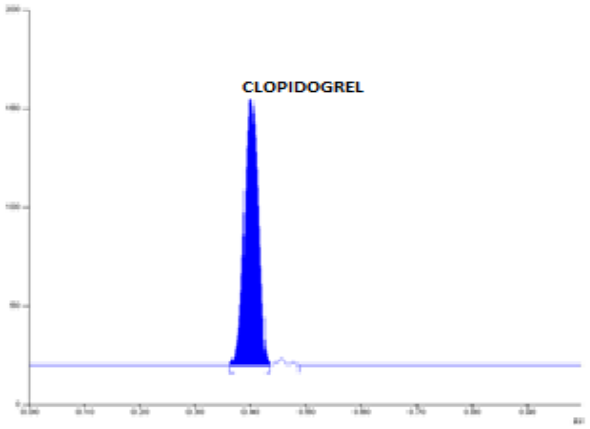
C) 1 M NaOH, 24 h reflux



D) 3% H_2O_2, 48 h



E) Thermal, 24 h



F) Sunlight, 24 h

Figure 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**

Table 8: Results of specificity studies

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

The tablet sample of Clopidogrel 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 Clopidogrel in tablet formulation. Normal phase separation mode was used to resolve the intact drug from their degradation products.

The standard solution of Clopidogrel (10.0 µg/ml) was prepared in methanol. The stationary phase used was pre-coated silica gel 60 F_{254} 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 Clopidogrel, 254 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 degradant 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_2O_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 Clopidogrel 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 Clopidogrel in tablet formulation. The standard solution of Clopidogrel 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 Clopidogrel 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 Clopidogrel.

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)	4.062	2.641
LOQ (ng/ spot)	12.322	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 Clopidogrel 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 Clopidogrel 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 Clopidogrel 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 Clopidogrel 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 Clopidogrel 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.

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