Development and Validation Of Stability Indicating HPTLC Method for Determination of Nepafenac as Bulk Drug and in Pharmaceutical Dosage Form

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Abstract-*A new simple, accurate, precise and selective stability indicating high performance thin layer chromatographic method has been developed and validated for estimation of Nevanac ophthalmic suspension. The mobile phase selected was Toluene: Methanol(7:3v/v) with UV detection at 234nm.The retension factor for Nepafenac was found to be 0.40±0.006.The method was validated with respect to linearity, accuracy, precision and robustness as per the ICH guidelines. The drugs were subjected to stress condition of hydrolysis(acid, base), oxidation, photolysis and thermal degradation. Results found to be linear in concentration range of 500-4000ng/band. the thermal method has been successfully applied for the analysis of drug in pharmaceutical formulation. The % assay (Mean ± S.D) was found to be 100.54±1.48.The developed method can be used for checking the stability of Nepafenac in bulk drug and pharmaceutical dosage form.*

Keywords-Forced degradation, High performance thin layer chromatography (HPTLC), Nepafenac, stability-indicating method, Validation

I. INTRODUCTION

Nepafenac is a member of the nonsteroidal anti-inflammatory drug (NSAID) class. Nepafeanc chemically designated as 2-Amino-3- benzoylbenzeneacetamide.It is a yellow crystalline powder with a molecular weight of 254.28.It is very soluble in methanol and DMSO and poorly soluble in water. The drug is formulated as a suspension applied by the topical ocular route, and is intended for the prevention and treatment of pain and inflammation associated with cataract surgery. Nepafenac (amfenac amide) is a prodrug which is converted to amfenac by intraocular hydrolases. Amfenac inhibits both cyclooxygenase COX-1 and COX-2 activity.The literature survey revealed few methods like UV-spectroscopy , RP-HPLC and HPLC for the determination of nepafenac.

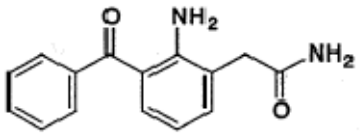


Figure 1: Chemical structure of Nepafenac

The above survey of literature shows no report of stability indicating HPTLC method for estimation of Nepafenac. The aim of the study was to develop a simple, accurate, precise and specific stability indicating HPTLC method for the determination of Nepafenac . The present work involve stress degradation as per ICH Q1A(R2) and Q1B . The proposed method was validated for linearity, accuracy, precision, robustness, LOD and LOQ according to ICH guidelines.

II. MATERIALS AND METHODS

Chemicals and Reagents

Nepafenac was provided as a gift sample by Sun pharmaceutical Ltd., Mumbai. Ethyl Acetate, Methanol and Toluene of AR grade purchased from Merck Pvt. Ltd. Mumbai, India. Hydrochloric acid, hydrogen peroxide and sodium hydroxide were purchased from LOBA CHEMIE PVT. LTD. Mumbai.

Selection of detection wavelength

The standard solution of Nepafenac in methanol was scanned over the range of 200 – 400 nm by using UV-Visible spectrophoto-meter. Wavelength 234 nm was selected for analysis where Nepafenac showed higher absorbance.

Instrumentations and Chromatographic conditions

Chromatographic separation of drug was performed on Aluminum plates pre-coated with silica gel 60 F_{254}, (10 cm × 10 cm with 250 µm layer thickness). Samples were applied on the plate as a band of 4 mm width using Camag 100 µL sample syringe (Hamilton, Switzerland) with a Linomat 5

applicator (Camag, Switzerland). The mobile phase was composed of Toluene:Methanol(7:3v/v). CAMAG twin through glass chamber 10 cm × 10 cm was used for linear ascending development of TLC plate under 15 min saturation conditions and 10 mL of mobile phase was used per run, migration distance was 90 mm. Densitometric scanning was performed using Camag TLC scanner 3 in the range of 400-200 nm, operated by winCATS software, slit dimensions were 3.00 x 0.45 mm and Deuterium lamp was used as a radiation source.

Preparation of Standard stock solution

A standard stock solution of Nepafenac was prepared by dissolving 10 mg of drug in10 ml of methanol to get concentration of 1000µg/ml. From the standard stock solution, working standard solution was prepared to contain 100µg/ml of Nepafenac.

Preparation of sample solution

5ml of 0.1% aqueous suspension of Nepafenac was transferred to 50 ml of volumetric flask and sufficient methanol was added to get the working sample (100µg/ml). The solution was filtered through 0.2 µ whatman filter paper. Different volumes of above prepared solution were taken and diluted with methanol to get different concentrations of Nepafenac. The above prepared solutions were analyzed by HPTLC for the content of Nepafenac.

III. STRESS DEGRADATION STUDY OF BULK DRUG

Stress degradation studies were carried under condition of acid/ base/ neutral hydrolysis, oxidation, dry heat. For each study, samples were prepared as follows

1. The blank subjected to stress in the same manner as the drug solution.
2. Working standard solution of Nepafenac subjected to stress condition.

Dry heat was carried out in solid state. 2.5µL of the resultant solution was then applied at TLC plate and densitogram was developed.

Stress test conditions like strength of reagent and exposure time were optimized to get 10-30% degradation. The optimized conditions are as follows:

Degradation under alkali catalyzed hydrolytic condition

To 1 mL of 1000 µg/ml solution of Nepafenac, 1mL of 1N methanolic NaOH was added. The volume was made upto 10 mL with methanol. The above solution was kept for 4 hrs at room temperature in dark place.

Degradation under acid catalyzed hydrolytic condition

To 1 mL of 1000 µg/ml solution of Nepafenac, 1 mL of 0.1N methanolic HCl was added. The volume was made upto 10 mL with methanol. The above solution was kept for 15 mins at room temperature in dark place.

Degradation under neutral hydrolytic condition

To 1 mL of 1000 µg/ml solution of Nepafenac, 1mL of distilled water was added. The volume was made upto 10 mL with methanol. The above solution was kept for 4 hrs at room temperature in dark place.

Degradation under oxidative condition

To 1 mL of 1000 µg/ml solution of Nepafenac, 1 mL of 30% H2O2 was added. The volume was made upto 10 mL with methanol. The above solution was kept for 24 hrs at room temperature in dark place.

Degradation under dry heat

Dry heat studies were performed by keeping drug sample in oven (60^0 C) for a period of 7 days. Sample was withdrawn, dissolved in methanol and diluted to get 100 µg/ml.

Multiple wavelength scanning was done for each stress condition to locate the peak of degradation product; but no peak of degradation product was obtained even upon spotting ten times higher concentration. The drug peak area reduced under stress conditions.

IV. METHOD VALIDATION

The method was validated according to the ICH Q2 (R1) guidelines for the following parameters.

Specificity

The specificity of the method was ascertained by peak purity profiling studies in winCATS software. It involves comparison of UV spectra at peak start, middle and end. The peak purity values were found to be more than 0.998, indicating the noninterference of any other peak of degradation product or impurity.

Linearity and Range

From the standard stock solution (1000µg/ml) of Nepafenac, solution was prepared containing 100µg/ml of Nepafenac. This solution was used for spotting. Six replicates per concentration were spotted. The linearity was determined by analyzing eight concentrations over the concentration range of 500-4000 ng/band for Nepafenac. The peak areas were plotted against the corresponding concentrations to obtain the calibration graph. The LOD and LOQ were calculated based on the equation:

LOD = 3.3 × σ/ S and LOQ = 10 × σ/ S.

Where, σ is standard deviation of the lowest response of linearity equation and S is slope of the calibration curve of the analyte.

Accuracy

To check accuracy of the method, recovery studies were carried out by adding standard drug to sample at three different levels 50, 100 and 150%. Basic concentration of sample chosen was 1000 ng/band. % recovery was determined from linearity equation.

Precision

The precision of the method was demonstrated by Intra-day and Inter-day variation studies. In the Intra-day studies 3 replicates of 3 concentrations were analyzed on the same day, for the inter day variation studies, 3 replicates of 3 concentrations were analyzed on 3 consecutive days and % RSD was calculated.

Robustness

Robustness of the method was determined by introducing small deliberate changes in mobile phase ratio, chamber saturation time, time from spotting to development and development to scanning. For all changes in conditions, the samples were analyzed in triplicate and the effects on the peak area and R_f value was noted. It was found that results did not vary by more than 2%

V. RESULTS AND DISCUSSION

Optimization of mobile phase

The analytical method was developed by studying different parameters. First of all, maximum absorbance was found to be at 234 nm. The stationary phase used for study of Nepafenac was Aluminum plates pre-coated with silica gel 60 F_{254}, (10 cm × 10 cm) with 250 µm layer thickness. Different ratios of mobile phase constituents were studied, mobile phase with Toluene: Methanol in the ratio of 7:3 v/v was chosen due to good symmetrical peak. Retention Factor was 0.40 ± 0.006. The analytical method was found linear over the range of 500-4000ng/band.

Forced degradation studies

Forced degradation studies were conducted to evaluate the stability and specificity of the method. No degradation product of Nepafenac was observed when the drug was subjected to acidic, basic and neutral treatment, exposure to heat and Nepafenac was degraded in oxidative condition with two peak degradation at Retention Factor 0.28 and 0.32 . The drug peaks obtained from all the stressed samples were found to be homogenous and pure. Hence the method is found to be specific. The results are given in (**Table 1**).

Table 1: Summary of stress degradation of Nepafenac

Stress degradation conditions	**Percent recovery (%)**	**Percent degraded (%)**	**Peak purity data**	
			r(s,m)	**r(m,e)**
Initial	100	-	0.999	0.999
Base (0.1 N NaOH, kept for 2hrs)	86.86	13.13	0.998	0.997
Acid (0.01 N HCl, Kept for 15 min)	85.90	14.09	0.999	0.997
H_2O_2 6% v/v (kept for 24 hrs)	77.67	22.32	0.997	0.998
Neutral (kept for 4 hrs)	100.17	12.32	0.998	0.998
Heat dry (60^0 C, 7 days)	100.37	10.47	0.997	0.998

Linearity and Range

The linear calibration range was found to be 500 to 4000 ng/band. The calibration curve obtained by the least square regression analysis between average peak area and concentration showed linear relationship with a correlation coefficient of 0.994, the equation of the calibration curve found for Nepafenac was y = 1.3525x – 507.99 (**Fig. 3**).

Accuracy and Precision

Recovery of standard drug was found to be 101.15-100.27 % with less than 2% of RSD values, indicating that the proposed method was accurate (**Table 2**). (**Table 3**) shows the precision study results. The RSD values for intraday and interday-precision were not more than 2 %, indicating the repeatability and reproducibility of the method.

Limit of detection and quantification (LOD and LOQ)

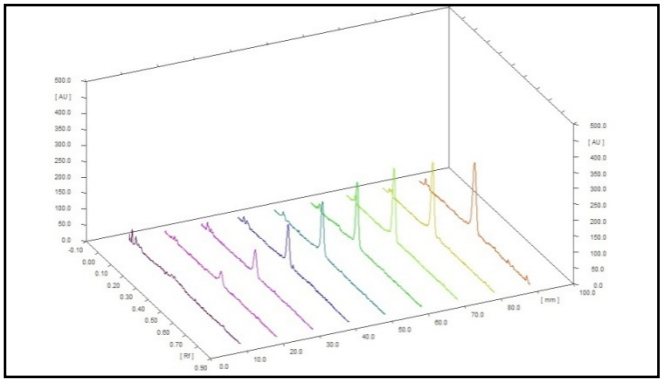
The LOD (Limit of Detection) and LOQ (Limit of quantitation) were estimated from the standard deviation of the lowest response and the slope of the calibration curve. LOD and LOQ were found to be 69.20 ng/ band and 209.72ng/band respectively.

Robustness

The %RSD values of all robustness parameters were examined and found to be within the limit of 2%, showed that the proposed method was robust (**Table 4**).

Solution Stability

Freshly prepared solution was kept in a freeze (cool condition) after use. UV absorbance of freeze solution was compared with absorbance of fresh solution. It was observed that drug solution have stability of 3 days.



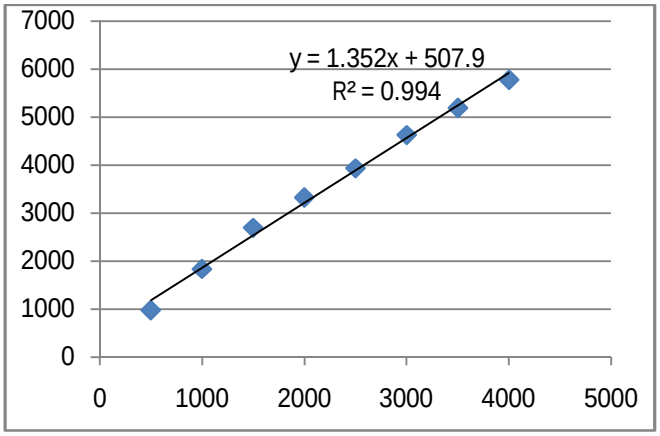


Figure 3: I) Densitogram of linearity of Nepafenac (500-4000 ng/band), II) Calibration curve for Nepafenac

Table 2: Accuracy of the proposed method

Conc. ng/band Level (%)	Theoretical Conc. (ng/band)	Avg. of Area	Recovered Conc. (ng/band)	% Recovery
50	1500	2559.36	1517.35	101.15
100	2000	3217.83	2004.38	100.21
150	2500	3897.06	2506.78	100.27

Table 3: Precision study

Concentration (ng/band)	Mean area*	SD	% RSD
Inter day			
1000	1860.42	0.6214	0.621
1500	2529.86	0.1835	0.184
2000	3229.63	0.5934	0.589
Intra day			
1000	1868.82	0.2591	0.257
1500	2534.47	0.2236	0.223
2000	3230.79	0.7051	0.702

Table 4: Results of robustness study

Sr. No.	Parameters	Robust condition	% RSD
1.	Saturation time(15min) ± 2 min.	13min	0.02
		17min	0.05
2.	Mobile phase composition Toluene: Methanol (7:3 v/v)±0.2 toluene	Toluene: Methanol (6.8: 3.2 v/v)	0.05
		Toluene: Methanol (7.3: 2.7 v/v)	0.11
3	Time from spotting to development (immediate)	After 30min.	0.43
		After 1hr	0.54
4	Time from development to scanning (immediate)	After 30min.	1.45
		After 1hr	1.78

Table 5: Summary of validation study

Sr. No.	Validation parameters	Nepafenac
1.	Linearity Range	$y = 1.3525x + 507.99$ $R^2 = 0.9944$ 500- 4000 ng/band
2.	Precision (% RSD) Interday Intraday	 0.464 0.394
3.	Accuracy	% Recovery
	50	101.15
	100	100.21
	150	100.27
4.	Limit of Detection	69.20ng/band
5.	Limit of Quantitation	209.72ng/band
6.	Specificity	Specific
7.	Robustness	Robust

VI. CONCLUSION

The developed method was found to be simple, sensitive, selective, cost-effective and time saving for analysis of Nepafenac in Ophthalmic suspension without any interference from the excipients. The results indicated the suitability of the method to study stability of Nepafenac under various forced degradation conditions as prescribed by ICH Q1A(R2) guidelines.

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