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Development and Validation of HPTLC Method for Estimation of Nepafenac in Ophthalmic Suspension

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Abstract— *A simple, accurate, precise and suitable High Performance Thin Layer Chromatography (HPTLC) method was developed and validated for estimation of Nepafenac in its marketed ophthalmic suspension formulation. Diclofenac sodium was used as Internal Standard (IS) in order to make the method more accurate and precise. Chromatographic separation of Nepafenac from suspension formulation and Diclofenac sodium was achieved on TLC Silica gel 60 F_{254} glass plates using the mobile phase comprising of Toluene: Ethyl acetate: Glacial acetic acid in the ratio of 65: 35: 0.2 % v/v/v. Densitometric detection and quantification was carried out at 280 nm. The Retention Factor (Rf) values of Diclofenac sodium and Nepafenac were found to be 0.453 and 0.268 respectively with good resolution and peak shapes. The method was validated in accordance with ICH guidelines for specificity, linearity, precision, recovery, sensitivity and robustness. The method was found to be linear in the concentration range of 50-500 ng/band with the Correlation coefficient value of 0.9991. Mean percent recovery of Nepafenac sample solutions was found to be 100.41 %. The Limit of Detection (LOD) and Limit of Quantitation (LOQ) values for Nepafenac were found to be 2.08 ng/band and 6.31 ng/band respectively. The proposed method was novel as no any HPTLC method was reported before for estimation of Nepafenac in eye drop and was applied successfully for the quantitative analysis of the same.*

Keywords— *High Performance Thin Layer Chromatography (HPTLC), Internal Standard (IS), Method development and Validation, Nepafenac, Ophthalmic suspension.*

I. INTRODUCTION

Cataract happens to be a leading cause of blindness worldwide and one of the main reason for decreased vision in elderly [1,2]. After cataract surgery which is the most common surgical procedures worldwide, one of the most common event that occurs is ocular inflammation. Activation of Cyclo-oxygenase-1 (COX-1) and Cyclo-oxygenase-2 (COX-2) during trauma causes production of Prostaglandins (PGs) which are the mediators for inflammation and increased production of PGs causes discomfort, pain and ocular inflammation. Nepafenac (CAS 78281-72-8) is a prodrug of Amfenac, a monocarboxylic acid amide having carboxylic acid group converted into the corresponding carboxamide [3]. The IUPAC name of Nepafenac is 2-(2-amino-3-benzoylphenyl)acetamide (Fig. 1) having molecular formula C15H14N2O2 [3]. Having a unique structure as prodrug, Nepafenac is converted to potent Cyclo-oxygenase inhibitor, Amfenac, by the action of intraocular hydrolases [4,5]. By permeating into the cornea, Nepafenac is metabolized by intraocular tissues [6] and gets converted to Amfenac resulting in optimal efficacy. Nepafenac is a target-specific NSAID, as its bioactivation to Amfenac is maximized in the iris, ciliary body, retina, choroid and happens to be lesser in cornea [7]. Nepafenac 0.1 % administered 3 times daily on the day before cataract surgery proves to be well tolerated and benefits by treating ocular inflammation and pain associated with surgery as shown by clinical trials [8],[9-11].

Few High Performance Liquid Chromatography (HPLC) and Ultra High Performance Liquid Chromatography (UHPLC) methods for estimation of Nepafenac in bulk and ophthalmic formulation are reported [12-15]. No any HPTLC method was found during the literature survey for analysis of Nepafenac in ophthalmic suspension. Hence, this work aims to develop simple, suitable, accurate, precise HPTLC method for estimation of Nepafenac in ophthalmic suspension.

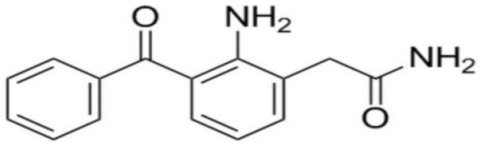

Fig. 1. Chemical structure of Nepafenac

II. EXPERIMENTAL

2.1 Chemicals, Reagents and Materials

Nepafenac reference standard having potency 99.4 % and Diclofenac sodium reference standard having potency 99.6 % were obtained from Central Drugs Testing Laboratory (CDTL), Mumbai. Nepafenac ophthalmic suspension with the brand name Nepaflam® ophthalmic suspension having strength 0.1 % w/v was procured from the local market. Analytical Reagent (AR) grade Toluene, Glacial acetic acid, HPLC grade Ethyl acetate and Methanol were obtained from Finar Chemicals, Gujarat, India. TLC Silica gel 60 F_{254} glass plates were procured

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from Sigma-Aldrich, India. Nylon membrane filter (0.45 µm) was obtained from Axiva Sichem Pvt. Ltd.

2.2 Instrumentation and Chromatographic conditions

CAMAG Linomat 5 sample applicator (CAMAG, Muttenz, Switzerland) and CAMAG microsyringe (100 µl) were used for applying bands on TLC Silica gel 60 F_{254} glass plates (20 cm × 10 cm, 250 µm thick; Sigma-Aldrich). CAMAG twin trough glass chamber was saturated with mobile phase comprising of Toluene: Ethyl acetate: Glacial acetic acid (65: 35: 0.2 % v/v/v) for 30 min with the lid closed. Activation of plates was done at 110 °C for 10 min on CAMAG TLC Plate Heater III. Sample spotting was done in the form of narrow bands having length 8 mm at a constant rate of 15 nl/s using a nitrogen aspirator. In order to avoid edge effect, the application positions X and Y were kept at the distance of 8 mm and 20 mm respectively. Distance between two bands was kept 20 mm. Chromatogram was developed in a linear ascending manner upto the run distance of 80 mm. Drying of plates was carried out in hot air stream using an air dryer in a wooden chamber having adequate ventilation. Plates were scanned at 280 nm for spectro densitometric quantification of the separated components using CAMAG TLC Scanner 4 equipped with deuterium lamp by keeping the sensitivity at auto mode, during which the slit dimension was 6.0 mm × 0.3 mm and scanning speed was 100 nm/s. Evaluation of peak areas was carried out using CAMAG visionCATS software version 3.0. Sartorius Analytical Balance was used for all weighings.

2.3 Selection of wavelength of maximum absorbance

20 ug/ml solution of Nepafenac solution was scanned in the range of 200.0 to 400.0 nm using CAMAG TLC Scanner 4. Nepafenac showed maximum absorbance at 280.0 nm as shown in Fig. 2. Hence the same wavelength was selected for the analysis of Nepafenac.

2.4 Preparation of standard solution

A mix standard solution was prepared containing 20 µg/ml of each of Nepafenac and Diclofenac sodium (IS).

2.5 Analysis of Marketed Formulation

1.0 ml of Nepafenac ophthalmic suspension was transferred to 50.0 ml of volumetric flask. Further, 2.0 ml of Diclofenac sodium (IS) from the previously prepared stock solution (500 µg/ml) was added in the same flask and was dissolved in sufficient quantity of methanol. The contents were sonicated for 10 min using an ultrasonicator and made upto the mark with methanol so as to prepare the sample solution containing 20 µg/ml of each of Nepafenac and Diclofenac sodium (IS). The assay was repeated six times by injecting 10 µl volumes of standard and sample solutions for analysis of Nepafenac in ophthalmic suspension. Mean, Standard Deviation (SD) and Percent Relative Standard Deviation (% RSD) of sample peak areas and % estimation were calculated and reported. The results are shown in Table 6.

2.6 Method Optimization

TLC Silica gel 60 F_{254} glass plate was used for the separation of Nepafenac by considering its chemical nature and polarity. Diclofenac sodium (IS) was added along with Nepafenac in the solution so as to make the method more precise and accurate and to increase the reproducibility of method which can be lost due to loss of sample during preparation steps in case of suspension formulation. Initial trials were carried out using mobile phase containing Toluene: Ethyl acetate: Glacial acetic acid in the ratio 68: 32: 0.2 % v/v/v. Good resolution was obtained between the peaks of Nepafenac and Diclofenac sodium (IS), but peak shapes were poor. Hence further trial was carried out using the same mobile phase in the ratio of 65: 35: 0.3 % v/v/v. This led to better resolution, but splitting peaks were observed. Finally better resolution along with symmetrical and sharp peak shapes and improved Rf values of both molecules was observed by using mobile phase containing Toluene: Ethyl acetate: Glacial acetic acid in the ratio of 65: 35: 0.2 % v/v/v and was used throughout the analysis. HPTLC densitogram of Nepafenac and Diclofenac sodium (IS) under optimized conditions is shown in Fig. 3. Image of HPTLC plate taken at 254 nm is shown in Fig. 4.

2.7 Method Validation

Validation of the developed HPTLC method was done by checking the parameters such as specificity, linearity, precision, accuracy, Limit of Detection (LOD), Limit of Quantitation (LOQ) and robustness as per ICH Q2 (R1) guidelines [16].

2.7.1 Specificity

Standard and sample solutions of Nepafenac were analysed for demonstrating the specificity of the method. By comparing the Rf value and spectrum of the band with that of standard, the band for Nepafenac was confirmed. The spectrum was compared at three different regions of the band viz. peak start (S), peak apex (M) and peak end (E) for determining the peak purity of Nepafenac. Overlain peak purity spectra of Nepafenac is depicted in Fig. 7.

2.7.2 Linearity

Linearity studies on Nepafenac were performed in the concentration range of 50-500 ng/band by applying seven different concentrations of mix standard solution of Nepafenac and Diclofenac sodium (IS) twice. The linearity graph of peak areas verses concentrations was plotted to assess the linearity of Nepafenac. The three-dimensional densitogram for Nepafenac linearity is shown in Fig. 5. The plot of peak areas verses respective concentrations is shown in Fig. 6 and the results of Nepafenac linearity are shown in table Table 1.

2.7.3 Precision

Precision of the proposed method was determined in terms of repeatability and intermediate precision. % RSD for repeatability was determined by applying 200 ng/band of mix standard solution of Nepafenac and Diclofenac sodium (IS) six times. % RSD for intraday precision was determined by analysing 150, 200 and 250 ng/band Nepafenac standard solution each applied thrice on the plate. Interday precision was determined by analysing 150, 200 and 250 ng/band Nepafenac standard solution each applied thrice on the plate on different

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days over a period of one week. The results of repeatability and intermediate precision studies of Nepafenac are depicted in Table 2 and 3 respectively.

2.7.4 Accuracy

Accuracy of the method was established by using standard addition method. Known amount of standard was added to preanalyzed formulation at three different levels (110, 120 and 130 %). Recovery studies were conducted by performing three determinations at each level and mean % recovery was calculated and reported. The results of accuracy studies of Nepafenac are exhibited in Table 4.

2.7.5 Sensitivity

Sensitivity of measurement of Nepafenac by the proposed method was determined in terms of Limit of Detection (LOD) and Limit of Quantitation (LOQ) by using the formulae:

$LOD = 3.3 \times \propto/s$

$LOQ = 10 \times \propto/s$

Where $\propto$ is the standard deviation of regression line and s denotes the slope obtained from calibration curve.

2.7.6 Robustness

Robustness of the proposed method was evaluated by changing the volume of mobile phase in the range of ±5 ml, saturation time in the range of ±5 min and distance travelled by the solvent front in the range of ± mm. The results of Robustness of the proposed method for analysis of Nepafenac are displayed in Table 5.

III. Results and Discussion

A simple, suitable, precise and accurate HPTLC method was developed for estimation of Nepafenac in ophthalmic suspension using Diclofenac sodium as internal standard. Internal standard was added along with the drug so as to increase the accuracy and precision of the method by compensating the losses occurred during stepwise sample preparations.

The spectra of Nepafenac was compared at peak start (S), peak apex (M) and peak end (E) positions to determine its peak purity. Correlation (r^2) values were found to be more than 0.999 for Nepafenac. It was confirmed that there is no any interference in quantitation of Nepafenac in sample solution and the method is specific as the correlation values and peak purity were found to be within limits.

Linearity of Nepafenac was found to be linear having Correlation coefficient (r^2) value of 0.9991. The regression equation obtained was y=8.4804x+24.89 with the slope 8.4804 and y-intercept 24.89.

% RSD values for repeatability and intermediate precision of Nepafenac were found to be 1.66 % and 0.67 % respectively and were within acceptance limits (<2 %). Hence the method was found to be precise.

The mean % recovery of Nepafenac sample solutions was found to be 100.41 % which is within the acceptance limit of 98 %-102 % proving the method to be accurate and suitable for the routine analysis of Nepafenac in ophthalmic suspension.

The LOD and LOQ values for Nepafenac were found to be 2.08 ng/band and 6.31 ng/band respectively which indicate the method to be sensitive.

The method was found to be robust as reproducible results were obtained in the form of precise Rf values and low % RSD values. The results remained unaffected by deliberate changes in parameters.

The % Nepafenac in sample solutions was found to be 101.14 % which indicates no any interference by excipients in analysis of Nepafenac in ophthalmic suspension. The Rf value of Nepafenac and Diclofenac sodium (IS) was found to be 0.268 and 0.453 respectively and the densitogram of Nepafenac from the dosage form was observed to be identical to the reference standard of Nepafenac. Hence it indicates that the proposed method can be implemented for the routine analysis of Nepafenac present in ophthalmic formulation.

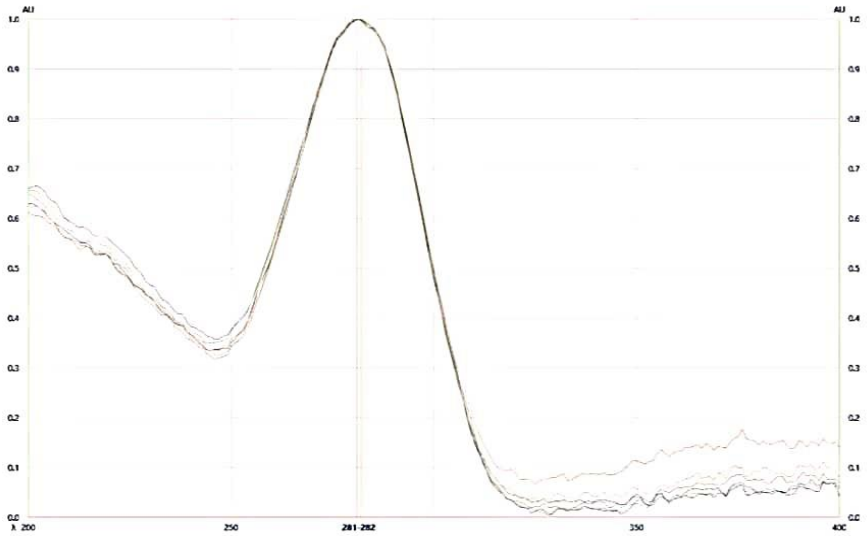

Fig. 2. UV Spectra of Nepafenac

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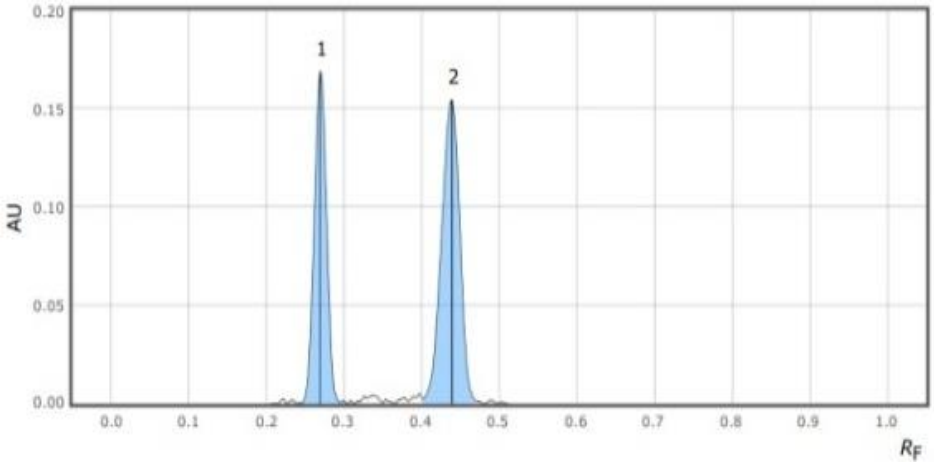

Fig. 3. HPTLC densitogram under optimized conditions showing Rf value of 0.26 for Nepafenac (200 ng/band) and 0.45 for Diclofenac sodium IS (200 ng/band)

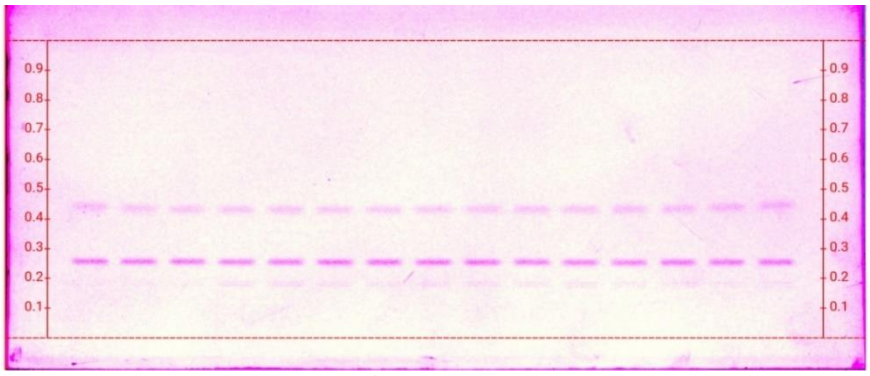

Fig. 4. Image of HPTLC plate taken at 254 nm

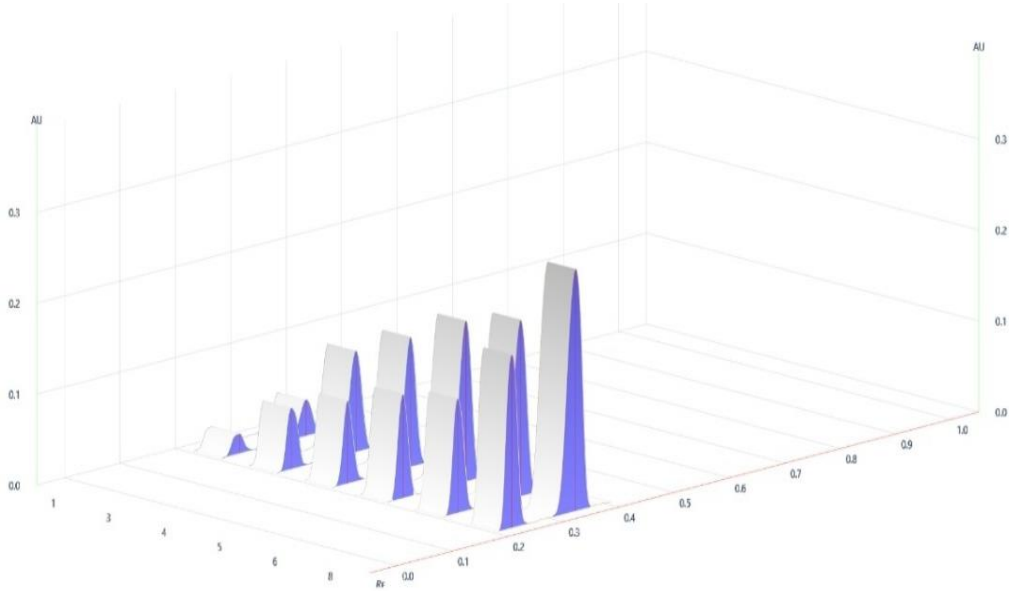

Fig. 5. Three-dimensional densitogram for the linearity of Nepafenac at 280 nm

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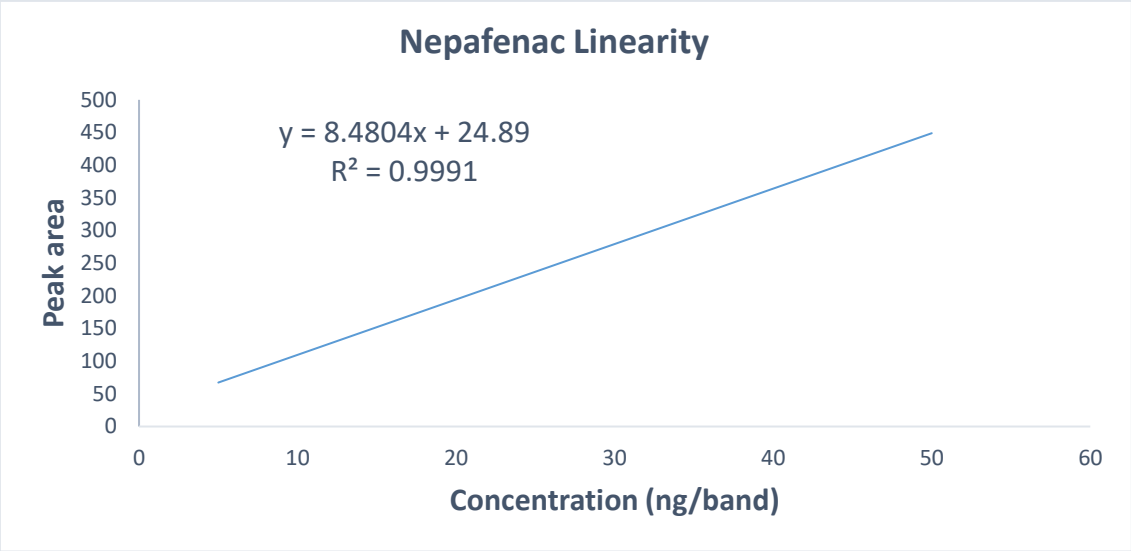

Fig. 6. Linearity graph of Nepafenac

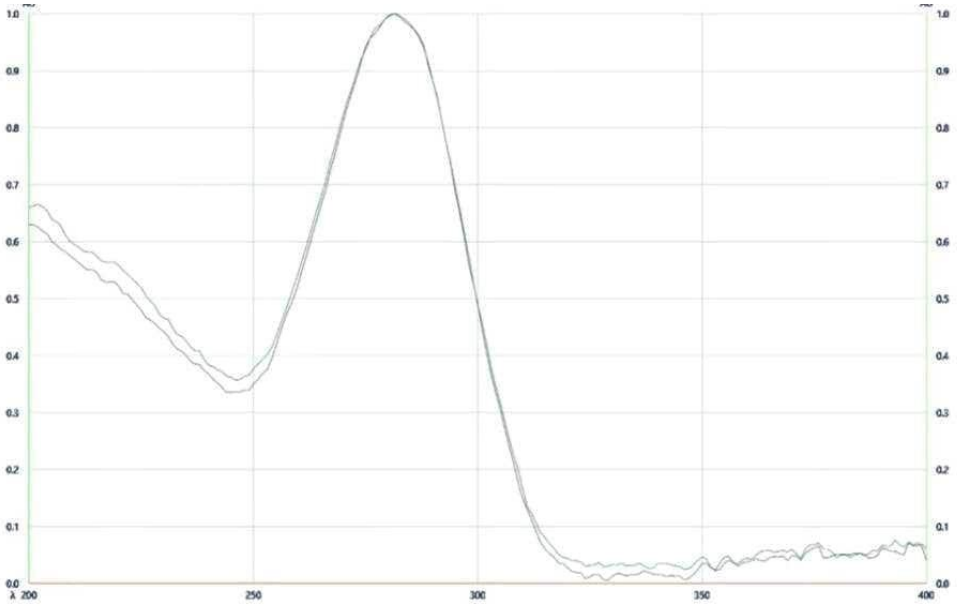

Fig. 7. Overlain peak purity spectra of Nepafenac

TABLE 1. Linearity data of Nepafenac

Concentration (ng/band)	Peak Area
50	0.00063
150	0.00151
200	0.00196
250	0.00243
300	0.00281
500	0.00445

TABLE 2. Precision data of Nepafenac as Repeatability

Concentration (ng/band)	Peak Area
200	0.00313
200	0.00319
200	0.00324
200	0.00323
200	0.00322
200	0.00329
Average (n=6)	0.003216667
SD	5.3541261
% RSD	1.6644951

TABLE 3. Intermediate Precision data of Nepafenac

Concentration (ng/band)	Intraday precision		Interday precision	
	Peak area ± SD (n=3)	% RSD	Peak area ± SD (n=3)	% RSD
150	0.0015533 ± 0.00001	0.4991946	0.00179 ± 0.00002	1.0362109
200	0.0022189 ± 0.00001	0.4413282	0.00233 ± 0.00001	0.5302848
250	0.0025811 ± 0.00001	0.2998984	0.00271 ± 0.00001	0.4476737

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TABLE 4. Accuracy studies of Nepafenac

% level	Amount spiked (ng/band)	Amount recovered (ng/band)	% recovery	Mean % recovery	% RSD
100	200	201.68			
100	200	201.68	101.14		0.5317
100	200	203.52			
110	220	200.08			
110	220	199.24	99.95		0.2956
110	220	200.38		100.41	
120	240	200.58			
120	240	200.30	100.38		0.2766
120	240	201.36			
130	260	200.06			
130	260	200.46	100.17		0.1167
130	260	200.46			

TABLE 5. Robustness studies of Nepafenac

Change in mobile phase composition (65: 35: 0.2 % v/v ± 0.2 in toluene content)			
Ratio (% v/v)	**Rf**	**Peak area ± SD (ng/band)**	**% RSD**
65.2: 35: 0.2	0.25 ± 0.02	0.00262 ± 0.00001	0.5615
64.8: 35: 0.2	0.25 ± 0.02	0.00265 ± 0.00001	0.3085
Change in chamber saturation time (30 min ± 5)			
Saturation time (min)	**Rf**	**Peak area ± SD (ng/band)**	**% RSD**
35	0.25 ± 0.02	0.00262 ± 0.00001	0.5615
25	0.25 ± 0.02	0.00266 ± 0.00003	0.8833
Change in mobile phase volume (20 ml ± 5)			
Volume (ml)	**Rf**	**Peak area ± SD (ng/band)**	**% RSD**
25	0.25 ± 0.02	0.00266 ± 0.00003	1.0006
15	0.26 ± 0.02	0.00260 ± 0.00001	0.4041
Change in distance travelled by solvent front (80 mm ± 5)			
Distance travelled (mm)	**Rf**	**Peak area ± SD (ng/band)**	**% RSD**
85	0.26 ± 0.02	0.00265 ± 0.00001	0.3085
75	0.26 ± 0.02	0.00269 ± 0.00001	0.4497

TABLE 6. Analysis of Marketed Formulation

Label claim (mg/ml)	Amount found (mg)	% estimation	% RSD
1	1.01	101.14	1.10

IV. CONCLUSION

The proposed HPTLC method was validated successfully with respect to ICH guidelines and was found to be simple, accurate and precise for the quantification of Nepafenac in ophthalmic suspension without interference of excipients. All validation parameters were found to be within their acceptance limits. The method offers better resolution between drug and excipients and higher sensitivity. No HPTLC method was reported earlier for analysis of Nepafenac in ophthalmic suspension; hence this method is worthwhile. Using this method can be highly beneficial due to easy sample preparation, method's high capacity (15 bands per plate) and method's flexibility to run qualitative and quantitative assays at a time. Hence the method can be routinely used for analysis of Nepafenac in ophthalmic suspension.

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