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QUANTIFICATION DETERMINATION OF NEPAFENAC IN OPHTHALMIC SUSPENSION USING HIGH PERFORMNACE THIN LAYER CHROMATOGHRAPHY

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ABSTRACT

Nepafenac is a pro drug with low inherent cyclooxygenase-inhibiting activity. The active non steriodial anti-inflammatory drug compound of nepafenac is called amfenac (2-amino-3-benzoylbenzeneacetic acid) which is an non steriodial anti-inflammatory drug with an arylacetic acid structure exhibiting potent antipyretic and analgesic properties. Nepafenac is designated chemically as 2-amino-3-benzoylbenzeneacetamide with an empirical formula of $C_{15}H_{14}N_2O_2$ and molecular weight of 254.28 g/mol. Nepafenac is a member of the new class of non steriodial anti-inflammatory pro drugs for ophthalmic use, providing a novel drug delivery mechanism. The analgesic and anti-inflammatory effect of nepafenac is the result of its fast penetration through the cornea in addition to conversion to amfenac.Present paper describes a high performance thin layer chromatographic for determination of nepafenac from Ophthalmic suspension. Separation was performed on aluminium-backed silica gel 60 F_{254} HPTLC plates with Toluene : Acetone : Glacial acetic acid (6 : 4 : 0.25) as mobile phase. After development, plates were observed under UV and detection and quantification were performed by densitometry at $\lambda = 254$ nm. Detection and quantitation limits for nepafenac is 0.8 µg/mL and 2 µg/mL. Nepafenac response was linear over the range 10 µg/mL to 30 µg/mL. The developed method was validated to determine its accuracy, precision and stability by carrying out linearity and stability experiments. The method developed is simple, fast, accurate and precise and hence can applied for routine quality control analysis from Pharmaceutical preparations / Ophthalmic suspensions.

Keywords: Nepafenac, HPTLC, Silica Gel, Densitometery, Ophthalmic suspension

INTRODUCTION

Ophthalmic drug forms have been one of the most important and widely developed areas of pharmaceutical technology for dozens of years. The main reason of continuously strong interest of scientists in these drug forms is the problem of a low bio availability of medicinal substance after the application to the eyeball[1]. Ophthalmic suspensions may be used to increase the corneal contact time of a drug substance and thus provide a more sustained action[2]. Ophthalmic solutions are formulated to achieve long shelf-life, effective anti-microbial action, comfort to the patient, penetration and action of the active agent(s), and minimal side effects. Ocular disorders are mainly treated by using drug formulations in the form of eye drops. One such drug is nepafenac[3]. Chemically nepafenac is (NEPA) { [2-(2-amino 3-benzoyl phenyl) acetamide] with an empirical formula of $C_{15}H_{14}N_2O_2$ and molecular weight of 254.28 g/mol} is non steriodial anti-inflammatory drug (NSAIDs) which is indicated for the treatment of pain and inflammation associated with cataract surgery. NEPA is a pro drug. After penetrating the cornea, NEPA undergoes rapid bio activation to amfenac, which is a potent NSAID that uniformly inhibits the COX1 and COX2 activity. Amfenac is thought to inhibit the action of prostaglandin H synthase (cyclooxygenase), an enzyme required for prostaglandin production[4]. Non-steroidal Anti-Inflammatory Drugs include aspirin and agents such as aceclofenac, bromfenac, dexibuprofen, dexketoprofen, diclofenac, etodolac, ketorolac, mefenamic acid, meloxicam, nabumetone, naproxen, nepafenac, parecoxib, piroxicam, sulindac, tenoxicamtiaprofenic acid tolfenamic acid[5,9]. One of the more recent products commercialized for topical ophthalmic use is nepafenac, approved for use in the US for the treatment of postoperative inflammation after cataract surgery. Nepafenac ophthalmic suspension 0.1% was recently made available for ophthalmic use and supplied as a sterile drug with a pH of approximately 7.4. Nepafenac is indicated for relief of eye pain and inflammation after cataract surgery[6,7]. It is unique as a topical NSAID for two main reasons First, it is delivered in the form of a prodrug, which penetrates the cornea and is converted to amfenac by tissue hydrolases. Amfenac is thought to inhibit prostaglandin-H synthase (cyclooxygenase), an enzyme that plays a role in prostaglandin production[8].

EXPERIMENTAL

Reagents and Chemicals:

The formulation were procured from Sion Healthcare Gujarat - India and standards were procured from Suyaash Pharmaceuticals, Gujarat - India. Toluene, acetone, glacial acetic acid were from Merck. All dilutions were performed in standard volumetric flasks. Standard stock solution was prepared by weighing 99.94 % pure nepafenac (25 mg) into a 25 mL volumetric flask, dissolving in methanol, and diluting to volume with methanol.

Chromatographic conditions:

Chromatography was performed on silica gel $60F_{254}$ HPTLC plates (Merck #5548). The mobile phase was Toluene : Acetone : Glacial acetic acid (6 : 4 : 0.25). The RF was found to be at 0.42 **Fig. 1**. Before use the plates were washed with methanol and dried in an oven at 105 °C for 2 h, because adsorbents with a large surface are absorb water vapor and other impurities from the atmosphere and other volatile substances often condense, particularly after the packing has been opened. Samples (20 µL) were spotted 16 mm from the edge of the plates by means of a Linomat apparatus V sample applicator (Camag, Muttenz, Switzerland). Plates were developed to a distance of 80 mm in a Camag twin-trough chamber previously equilibrated with mobile phase. The chromatographic conditions had been optimized to achieve the best resolution and peak shape **Fig. 2 and Fig. 3**. Plates were evaluated by densitometry at λ = 269 nm with a Camag TLC Scanner IV, with CATS software for quantitation. The wavelength used for densitometry was selected after acquiring the in-situ UV spectra of the drugs **Fig. 4**.

Figure 1

Chromplate of nepafenac Standard and Sample RF value 0.42

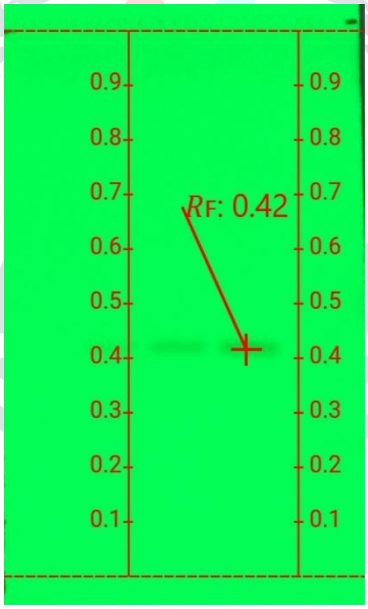


Figure 2

Chromatogram of standard nepafenac band (0.02 mg/mL) using Toluene : Acetone : Glacial acetic acid (6 : 4 : 0.25) as mobile phase. RF value 0.42

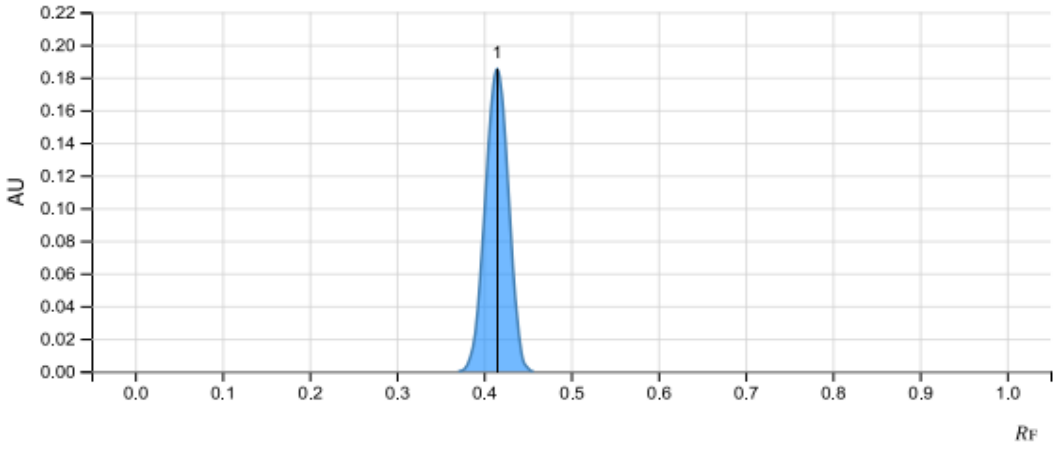


Figure 3

Chromatogram of Sample nepafenac Ophthalmic Suspension band (0.02 mg/mL) using Toluene : Acetone : Glacial acetic acid (6 : 4 : 0.25) as mobile phase. RF value 0.42

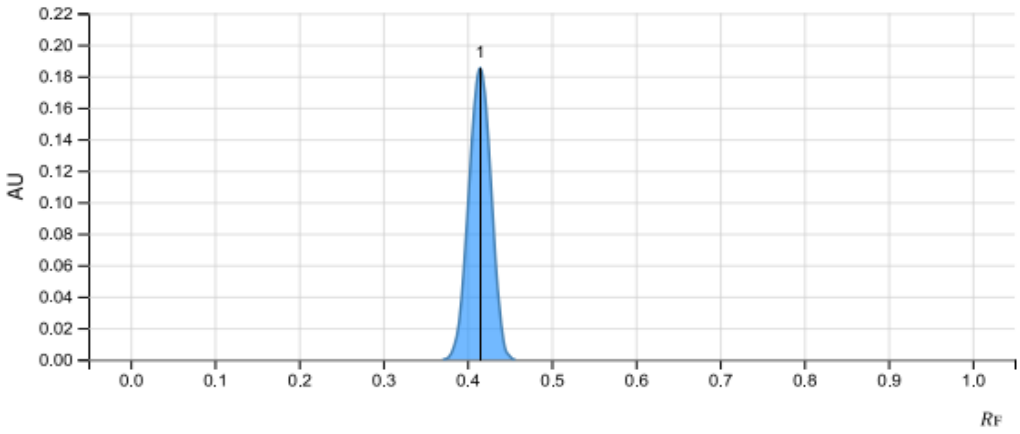
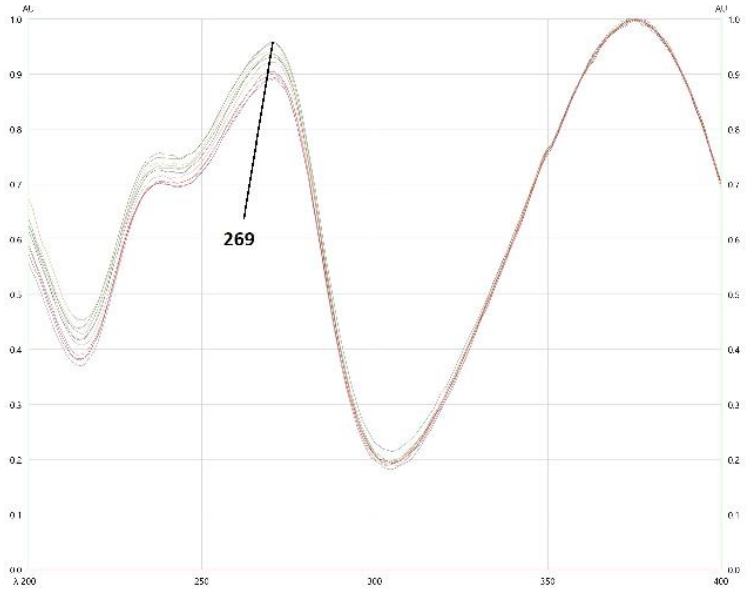


Figure 4

Overlay Spectra of Standard and Sample in Precision Experiment



Linearity of Detector Response

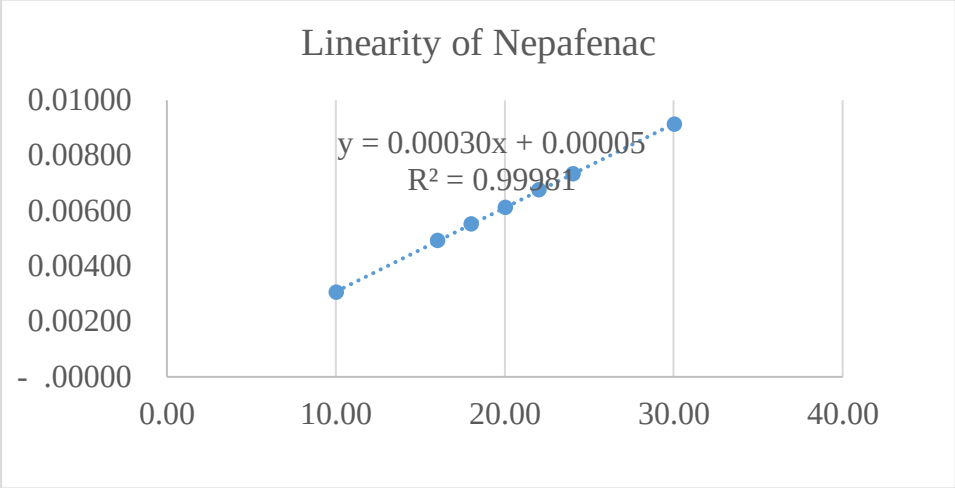
Solutions containing nepafenac at seven different concentrations were prepared in methanol Each of these solutions (20 µL) was applied to a plate, the plate was developed, and the detector response to the different concentrations was measured. The peak-area was calculated for each concentration and a graph was plotted of drug concentration against this peak area. The plotted of drug concentration against this ratio. The plot was linear for nepafenac in the range 10 to 30 µg/mL. This experiment was performed thrice and the mean was used for calculations. The data were analyzed by linear regression least-squares fitting. The statistical data obtained are given in **Table 1**.

Table 1: Analysis Performance Data of nepafenac

Linear working range	10 µg/mL to 30 µg/mL
Slope	0.00030
Intercept	0.000048
Correlation Coefficient	0.999906

Figure 5

Plot of concentration of standard nepafenac versus Mean area



Assay from the Pharmaceutical Preparation

Sample preparation

Twenty vials of the suspension were shaken emptied in the beaker. Accurately weighed and transferred 5 ml of sample (equivalent to 5 mg nepafenac) to 50 mL volumetric flask add 30 mL of methanol sonicated for 20 minutes and make up to volume with diluent. The solution was filtered through Whatman filter paper no 41. Further diluted 2 mL of the sample and diluted to 10 mL methanol. This procedure was repeated six times, Standard and sample solutions (20 µL) were spotted on the plate and the plate was developed and evaluated as described above. The densitometric responses from the standard and sample were used to calculate the amounts of drug in the ophthalmic suspension .

Limit of Detection and Limit of Quantitation:

The LOD was found to be 0.8 µg/mL for nepafenac was established at a signal to noise ratio of 30 and Limit of Quantitation (LOQ) was established at a signal to noise ratio of 10. The LOQ nepafenac were determined experimentally by analysis of six injection of each drug at LOQ concentration. The LOQ of nepafenac were found to be 2.0 µg/mL

Spotting repeatability / Instrument precision

This is carried out by spotting seven bands of the standard nepafenac (20 µg/cm^3) on to the chromatographic plate. Twenty microlitre of this solution was spotted seven times at a distance of 15 mm from the edge of the chromatographic plate as bands of 7 mm width with the help of Linomat apparatus V sample applicator (Camag, Muttenz, Switzerland). The mobile phase was prepared and poured on one side of the twin trough chamber. The chamber was allowed to saturate with mobile phase vapours for 20 minutes. The plate was

kept on the other side of the chamber and the plate surface was allowed to saturate for 10 minutes. The plate was then kept in the mobile phase and allowed to develop until 90 mm above the position of sample application. After plate development, the plate was removed from the chamber and dried under current of air at room temperature. The UV response of the band was monitored using a scanning densitometer set at a wavelength of 269 nm. The densitograms were recorded and the peak areas were recorded. The mean, standard deviation and coefficient of variation (%) of all these parameters were calculated. The results of spotting repeatability/ instrument precision are given in **Table 2**.

Table 2: Results of Spotting repeatability / Instrument precision

Sample No.	Concentration in (µg/mL)	Sample wt (in g)	Test Area	% Assay
1	20	5.50100	0.00609	99.6
2	20	5.50300	0.00614	100.4
3	20	5.50400	0.00615	100.6
4	20	5.52000	0.00609	99.3
5	20	5.50615	0.00613	100.2
6	20	5.52140	0.00619	100.9
			Mean	**100.0**
			SD	**0.6001**
			% RSD	**0.6**

Intra-Assay /Within day Precision

This experiment is carried out in one laboratory, on one day at three different concentration levels, and with three repetitions each. A sets of experiment was carried out, six different preparations were made of 20 µg/mL. Each of the levels were independently prepared (separate weighing) according to the method procedure. The contents were dissolved in a minimum quantity of methanol and diluted up to the mark with methanol. Twenty microliter of each of these solutions were spotted at a distance of 15 mm from the edge of the chromatographic plate as bands of 7 mm width at a distance of 8 mm from each other with the help of Linomat apparatus V sample applicator (Camag, Muttenz, Switzerland). The mobile phase was prepared and poured on one side of the twin trough chamber. The chamber was allowed to saturate with mobile phase vapours for 30 minutes. The plate was kept on the other side of the chamber and the plate surface was allowed to saturate for 10 minutes. The plate was then kept in the mobile phase and allowed to develop until 90 mm above the position of sample application. After plate development, the plate was removed from the chamber. The plate was dried under current of air at room temperature and the plate was scanned as 269 nm This experiment was carried out thrice and the mean, standard deviation, coefficient of variation and response

factors for each level were calculated. The results are tabulated in **Table 3**. The mean, standard deviation and coefficient of variation of the peak area ratios and the amount found were calculated.

Table 3: Results of Intra-assay / Within day precision for nepafenac

Sample No.	Concentration in (µg/mL)	Sample wt (in g)	Test Area	% Assay
1	20	5.4950	0.00610	100.7
2	20	5.5200	0.00615	101.1
3	20	5.5150	0.00610	100.4
4	20	5.5320	0.00614	100.7
5	20	5.4980	0.00610	100.7
6	20	5.5200	0.00604	99.3
			Mean	**100.7**
			SD	**0.6276**
			% RSD	**0.6**

RESULTS AND DISCUSSION

Use of pre-coated silica gel HPTLC plates with Toluene : Acetone : Glacial acetic acid (6:4:0.25 V/V) resulted in good separation of the drug and the internal standard. **Fig. 5** shows a typical densitogram obtained from nepafenac.

Regression analysis of the calibration data for nepafenac showed that the dependent variable (peak area) and the independent variable (concentration) were represented by the equations y = 0.00030x+ 0.00005 for nepafenac. The correlation of coefficient obtained was 0.99981 for nepafenac The system suitability experiment was carried out before the determination of nepafenac in unknown samples. The coefficient of variation was less than 2 % for replicate measurements of the same sample. This shows that the method and the system both are suitable for the determination of unknown samples.

The precision studies including the instrument precision, intra-assay precision and intermediate precision was carried out to evaluate the precision of the method. The intermediate precision included analysis on a different day and by a different analyst. The values of standard deviation and coefficient of variation were calculated. The standard deviations for intra assay precision was 0.6276, for Inter assay precision for

nepafenac 0.6001. The coefficient of variation for nepafenac was 0.6 and for interday nepafenac was 0.6. The low values of standard deviation and coefficient of variation indicate high precision of the method.

The accuracy of the experiment was established by spiking Placebo with known amounts of the drugs at three different concentration levels i.e. 50, 100 and 150 % of the drug in the Ophthalmic suspension (the external standard addition technique). The spiked samples were then analyzed. The results from recovery analysis are given in **Table 4**. The mean recovery is within acceptable limits, indicating the method is accurate.

Table 4: Results from recovery analysis for nepafenac

Level	Wt of Placebo (in g)	Amt of std stock solution added (in mL)	Area	% Recovery	Mean	SD	%RSD
50% 1	5.5123	1.0	0.00306	99.80	100.50	0.6523	0.6
50% 2	5.5142	1.0	0.00308	100.45			
50% 3	5.5145	1.0	0.00310	101.11			
100% 1	5.5001	2.0	0.00612	99.80	100.10	0.2491	0.2
100% 2	5.5136	2.0	0.00614	100.13			
100% 3	5.5012	2.0	0.00615	100.29			
150% 1	5.1450	3.0	0.00924	100.45	100.10	0.3075	0.3
150% 2	5.5123	3.0	0.00919	99.91			
150% 3	5.5145	3.0	0.00923	100.35			

Solution Stability

The stability of standard and sample solutions was determined by monitoring the peak- area of solutions of nepafenac in nepafenac Ophthalmic suspension over a period of 2 days. The results showed that the retention factor and peak area nepafenac were almost unchanged (RSD % < 2.0) and that no significant degradation is observed within the given period, indicating the solutions are stable for at least 2 days.

The robustness of the method was studied, during method development, by determining the effects of small variation, of mobile phase composition (±2 %), duration of plate pre-washing, chamber saturation period, development distance and scanning time (10 % variation of each). The mobile phase comprised three components, toluene, acetone, glacial acetic acid in the ratio 6 : 4 : 0.25(% V/V). Removal of glacial acetic acid from the mobile phase resulted in broad peaks volume ratios of two of the other three components were varied simultaneously while keeping the third constant to determine the effect both on RF and on the response

to nepafenac no significant change of RF or response to nepafenac was observed, indicating the robustness of the method. All the validation parameters are studied on the basis of ICH Q2R1 guidelines[10].

Method Application

The validated HPTLC method was used for nepafenac in the dosage form. The mean assay results, expressed as a percentage in the label claim, are shown in **Table 3**. The results indicated that the amount of each drug in the ophthalmic suspension is within the requirements of 80 to 110 % of the label claim.

Conclusion

The proposed method is highly accurate, selective, and precise and can therefore be used for a routine quality-control analysis and quantitative simultaneous determination of nepafenac in pharmaceutical preparations The method is also fast, and requires approximately 45 min for analysis

Nepatop Ophthalmic suspension were used as sample manufactured by Sion healthcare Gujarat- India with a label claim of 0.1% w/v nepafenac and 0.01% v/v benzalkonium chloride solution IP and sterile aqueous vehicle q.s. (Batch no. SH2104173; Mfg. Date- 04/2021; Exp. Date- 09/2022).

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