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METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR ESTIMATING DEGRADED PRODUCTS OF NEPAFENAC

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ABSTRACT

A simple accurate and reproducible stability indicating RP-HPLC method with UV-visible spectrophotometer was developed for the estimation of degraded products of Nepafenac. A novel isocratic RP-HPLC method has been developed using SHISEIDO CAP CELL PAK C18 column (4.6mm I.D× 250mm, 5µm) with the mobile phase composition of Acetonitrile: phosphate buffer (pH -4) [60:40] at flow rate 1ml/min, with column temperature 25^0 C and effluent was detected at 240nm. The method showed linearity over the range 0.1-4µg/ml with correlation coefficient of 0.9991. Nepafenac drug was subjected to acidic, alkaline, neutral, oxidative, thermal and photolytic stress conditions. Nepafenac was found to be degrading significantly in alkaline, acidic and oxidative stress conditions. Finally the drug was found stable in thermal, neutral and photolytic stress conditions. The developed method was validated according to ICH guidelines with respect to linearity, specificity, accurate, precise and robustness.

KEYWORDS

Nepafenac, Reverse phase high performance liquid chromatography (RP-HPLC), Stability indicating assay methods (SIAM's), Stress degradation, ICH Q1A (R_2) and Q_2R_1.

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INTRODUCTION[1-6]

Research entitled method development and validation of stability indicating RP-HPLC method for estimating degraded products of nepafenac includes the drug information as, molecular structure of Nepafenac is chemically designated as 2-(2-Amino-3-benzoylphenyl) acetamide or 2-Amino-3 benzoylbenzeneacetamide.

It is an yellow crystalline or powdery substance practically insoluble in water. An aqueous suspension at 0.1 % gives an average pH of 6.75. Nepafenac is an achiral substance and there are no

possible variations in the stereo chemical configuration.

It is a non-steroidal anti-inflammatory and analgesic prodrug. After topical ocular dosing, nepafenac penetrates the cornea and is converted by ocular tissue hydrolases to amfenac, a non-steroidal anti-inflammatory drug. Amfenac inhibits the action of prostaglandin H synthase (cyclooxygenase), an enzyme required for prostaglandin production. The aim of stability testing is to ensure the quality, safety, and efficacy of drug products up to their expiration date. Stress testing or forced degradation studies are nothing but testing of drug substances and drug products under conditions exceeding those used for accelerated testing. Forced degradation or stress testing is undertaken to demonstrate specificity when developing stability-indicating assay methods (SIAMs). Stress testing includes exposure to extremes of pH, light and oxidative conditions in addition to temperature, whereas accelerated stability studies include testing at high relative humidity and temperature (40 °C/75% RH) only and serves to generate the data which is useful in predicting the changes that might occur in the drug substance or product during normal storage conditions.

Literature review revealed that several methods have reported to determine nepafenac, but there are only one or two methods available to describe the forced degradation studies of nepafenac. The aim of the present work is to perform degradation studies of nepafenac by RP- HPLC, according to ICH requirements. And To perform stressed degradation studies of nepafenac by Hydrolytic Study (Acidic, Basic), Oxidative Study (Peroxide), Photolytic Study and Thermal Degradation Studies.

MATERIALS AND METHOD

Chemicals and Reagents[7]

Nepafenac was a gift sample from Mylan laboratories, Jedcherla, India. HPLC grade acetonitrile used in the present study was purchased from Merck (Mumbai, India) and used without further purification. HPLC grade Water from Merck (Mumbai, India). Analytical reagent grade disodium hydrogen phosphate ammonium acetate, potassium dihydrogen phosphate, hydrochloric acid, sodium hydroxide and hydrogen peroxide used in the present study were purchased from S.D Fine chemicals (Mumbai, India).HPLC instrument name is WATERS (M.F.G. By Waters Corp.), isocratic pump-515P, detector 2487-UV dual λ, column of SHISEIDO CAP CELL PAK C18 column (4.6mm I.D×250mm, Col.No:AKAD08222), loop used was Rheodyne (20µl) Hamilton syringe (25µl). Finally workstation of HPLC is YOUNGLINS Workstation (PC-pentium 4 processor).

Optimized Chromatographic Conditions

Mobile phase prepared with Acetonitrile: phosphate buffer (pH-4) in a ratio of 60:40, flow rate of 1ml/min, the retention time noted was 2.89min at a wavelength λ max of 240nm all these were chosen as optimal.

Preparation of Stock and Standard Solutions

Nepafenac of 1mg was weighed accurately and transferred to test tube containing 10ml of mobile phase which is labelled as primary standard (1mg/ml).

From the primary standard 0.1ml of drug solution was taken and transferred to an epperndorff vial(2ml) containing 0.9ml of mobile phase to get 100µg/ml of solution labelled as secondary standard. The stock solutions were stored in the freeze to avoid any kind of degradation. By serial dilution method aliquots of the stock solutions of Nepafenac were diluted with the respective solvent to yield standard solutions of 10, 30, 50, 70, 90 and 100ng/mL.

Preparation of phosphate buffer (p^h adjusted to 4)

Dissolve 5.04 g disodium hydrogen phosphate and 3.01 g of potassium dihydrogen phosphate in sufficient water to produce 1000 ml. Adjust the pH with glacial acetic acid.

Stress Studies

Stress studies were carried out under the conditions of dry heat, hydrolysis, oxidation and photolysis as mentioned in ICH Q1A (R2). A minimum of three samples were generated for every stress condition for each drug, viz., the blank solutions stored

under normal condition, zero time sample containing the drug (which was stored under normal conditions) and the drug solution subjected to stress treatment. For these studies 1N HCL, 0.1N HCL, 1N NaoH, 0.1N NaoH, 3%V/V H_2O_2 were prepared.

VALIDATION OF DEVELOPED METHOD[8-12]

The proposed isocratic RP-HPLC method was validated according to the International Conference on Harmonization guidelines all measurements were performed in triplicates.

Linearity, limit of detection and quantification

Under the experimental conditions, linear correlation between the peak area and applied concentration was found in the concentration range 0.1-4 μgmL^{-1}, as confirmed by the correlation coefficient of 0.9991. The peak area (y) is proportional to the concentration of nepafenac(x) following the regression equation Y=4119.x+1589. The experimentally derived LOD and LOQ for nepafenac were determined to be 1 and 10 $ngmL^{-1}$, respectively in Table No.1, Figure No.2-4.

Precision

Precision data on the intra- and inter-day variation for three different concentration levels are summarized in Table No.2. Both inter- and intra-day R.S.D were less than 2%, indicating a sufficient precision.

Precision of the assay for three different concentrations of nepafenac: intra-day and inter-day variations.

Accuracy

Nepafenac recovery from pharmaceutical dosage forms after spiking with 100, 200, and 400 $ngmL^{-1}$ of additional standard was 101.1% with R.S.D. below 2% for all analyzed concentrations Table No.3, confirming the accuracy of the method. Chromatograms shown in Figure No.5-7.

Specificity

The specificity was carried out through the comparison of the peak retention time of the formulation with nepafenac, standard drug sample and blank solution. No interference of the excipients was detected since no peak was detected in the same retention time of nepafenac. In the employed chromatographic conditions, nepafenac presented a retention time of approximately 2.8 minutes for formulations thus, allowing a rapid determination of the drug that is essential for routine analysis. Chromatograph for specificity was shown in Figure No.8.

Robustness

For the robustness test, peak area dependence on: the percentage of acetonitrile and pH of the mobile phase and the flow rate of the mobile phase; and the temperature of the column. Effects of the selected factors were evaluated over a range of conditions by determining the maximum response (quantification). At last it is concluded that chromatographic behavior of nepafeanc was mostly influenced by the mobile phase composition and pH. Highest response was obtained for concentrations within 45–65% and pH range 3.5–4.0. Among the studied factors, the flow rate had a minor influence on nepafenac peak area and was kept at the value of 0.8mL min^{-1}. The column temperature influences the retention time but has no significant impact on the peak area. It was therefore maintained at 25^0C throughout the study. Chromatographs with slight changes in mobile phase, temperature and flow rate were given in Figure No.9-11.

DEGRADATION STUDIES OF NEPAFENAC[13,14]

Hydrolytic Degradation

The hydrolytic degradation of Nepafeanc at 1mg/ml concentration in acidic and alkaline conditions can be studied by refluxing the drug in 0.1 N HCl/NaOH for 8 hrs and subsequently withdraw the samples at different time periods for each reaction condition and neutralize it. If reasonable degradation (5-20%) has seen, testing can be stopped at this point. However, in case no degradation has seen under these conditions, the drug should be refluxed in acid/alkali of higher strengths and for longer duration. Alternatively, if total degradation has seen after subjecting the drug to initial conditions, acid/alkali strength can be

decreased along with decrease in the reaction temperature. Once these peaks are separated in the chromatogram, peak purity is performed using PDA and/or mass spectrometry to determine if major degradation product(s) are co-eluting and to determine spectral homogeneity of the main component (API). If no degradation has observed, then it can be assumed that the compound is not prone to acid/base hydrolysis. Chromatogram during basic hydrolysis shown in Figure No.12, and for acidic hydrolysis Figure No.13.

Neutral Degradation

In a similar manner as in hydrolytic degradation, the degradation under neutral conditions can be started by refluxing Nepafeanc at 1mg/ml concentration in water for 8 hrs. Reflux time should be increased if no degradation has seen. If the drug is found to degrade completely, both time and temperature of study can be decreased.

Oxidative Degradation

The oxidative degradation may be carried out by placing Nepafeanc at 1mg/ml concentration in 3% H_2O_2 for 8 hrs at ambient temperature and subsequently withdrawing the samples at different time intervals during reaction condition. If reasonable degradation (i.e. 5-20%) has seen, testing can be stopped at this point. However, in case no degradation has seen under these conditions, the strength of H_2O_2 may be changed to 30% and also the duration of exposure set at higher conditions[3]. The ambient storage condition is sufficient for the oxidative degradation. Use of higher temperatures >30°C is not recommended, because the reaction rate in solution may actually be reduced at higher temp due to decrease in oxygen content of the solvent. But for some compounds, degradation may occur at higher temperature due to free radical reaction initiation. Chromatogram for oxidative stress was given in Figure No.14.

Thermolytic Degradation

Stress testing is likely to be carried out on single batch of Nepafeanc 1mg (API). Thermolytic degradation may lead to hydrolysis / dehydration / isomerization / epimerization / decarboxylation / rearrangements and some kinds of polymerization reactions. ICH guidelines suggest that thermolytic degradation study should be carried out at temperatures (in 10° increments e.g. 50°C, 60°C, etc.) above that for accelerated testing and withdraw the sample at different time intervals during reaction condition. If reasonable degradation (i.e. 5-20%) has seen, testing can be stopped at this point. However, in case no degradation has seen under these conditions, the study should be carried out with both elevated temperature and duration of exposure. Chromatogram was shown in Figure No.16.

Photolytic Degradation

Photo degradation is a surface-mediated phenomenon. The photolytic studies should be carried out by exposure to ultraviolet fluorescent lamps, using either a combination of short and long wavelength.

All the stressed samples (hydrolytic, oxidative, thermal and photolytic stress) are diluted with mobile phase to 10 times. All these prepared samples are filtered through 0.22 μm membrane filter before HPLC analysis. Chromatogram was shown in Figure No.15.

SUMMARY

A new RP-HPLC method is developed and validated for Nepafenac, the following parameters were validated according to ICH guidelines:

Mobile phase-Acetonitrile: Phosphate Buffer (adjusted with glacial acetic acid to pH-4) 60:40.

Flow rate 1ml/min

Retention Time 2.89min

Uv λ_{max}= 240nm

Limit of detection=1ng.

Limit of quantification=10ng.

Linearity-100ng-4000ng, a linear regression square of 0.9991 and Y=4119.x+1589 were obtained.

Precision, accuracy along with their mean, standard deviation and relative standard deviations were calculated.

Specificity, Recovery and Robustness were performed and found to within limits of ICH guidelines.

Stressed degradation studies were performed for Nepeafenac by the following methods:

Alkaline hydrolytic studies show that a degraded product at Rt3.37min.
Acidic hydrolytic studies show that a degraded product at Rt3.15min.
Oxidative (peroxide) hydrolytic studies show that a degraded product at Rt3.37min.
For thermal and photolytic studies no degraded products were obtained.

RESULTS AND DISCUSSION

Linearity

Table No.1: Standard graph data

S.No	X-axis- Concentrations (ng/ml)	Y-Axis- Peak area (AU)
1	100.	8117.
2	200.	10967.
3	400.	19930.
4	800.	42214.
5	1000.	59268.
6	2000.	115870.
7	4000.	236877.

Precision

Table No.2: Precision studies

S.No	Different concentration	Intraday			Interday		
1	Concn.ng/ml	200	400	800	200	400	800
2	Mean concn.ng/ml	200.60	400.04	800.08	199.8	398.9	795.3
3	S.D	0.081	0.050	0.0129	0.69	0.23	5.18
4	R.S.D%	0.04	0.012	0.0016	0.34	0.057	0.65

Table No.3: Accuracy studies

S.No	Concentration ($ngmL^{-1}$)	Mean ±S.D.	Recovery (μgmL^{-1})	Accuracy (%)	R.S.D. (%)
1	100	97±1.6	97	97	1.64
2	200	199.8±0.69	199	99	0.34
3	400	398.9+0.23	398	99	0.57

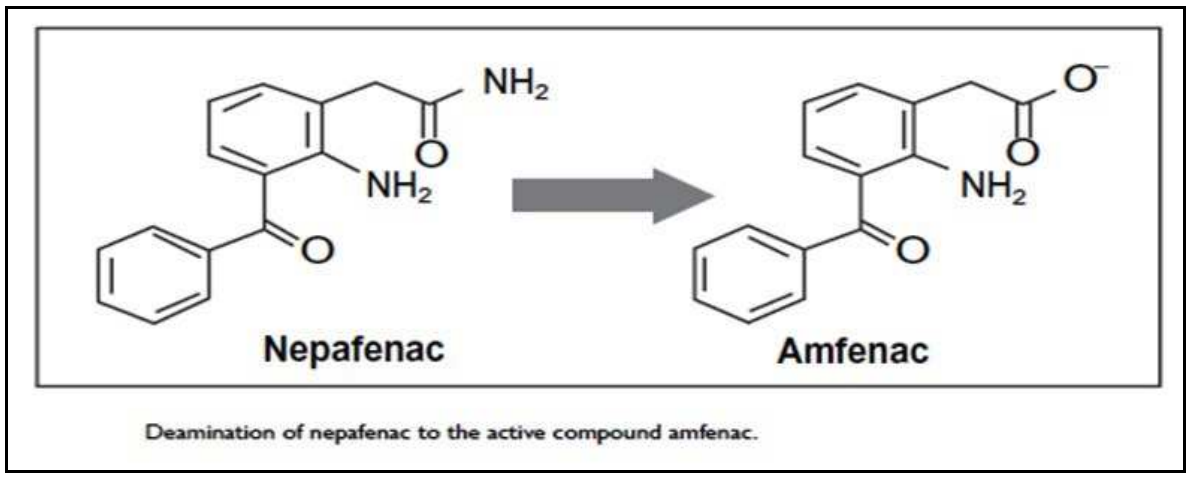

Figure No.1: Conversion of nepafenac to amfenac

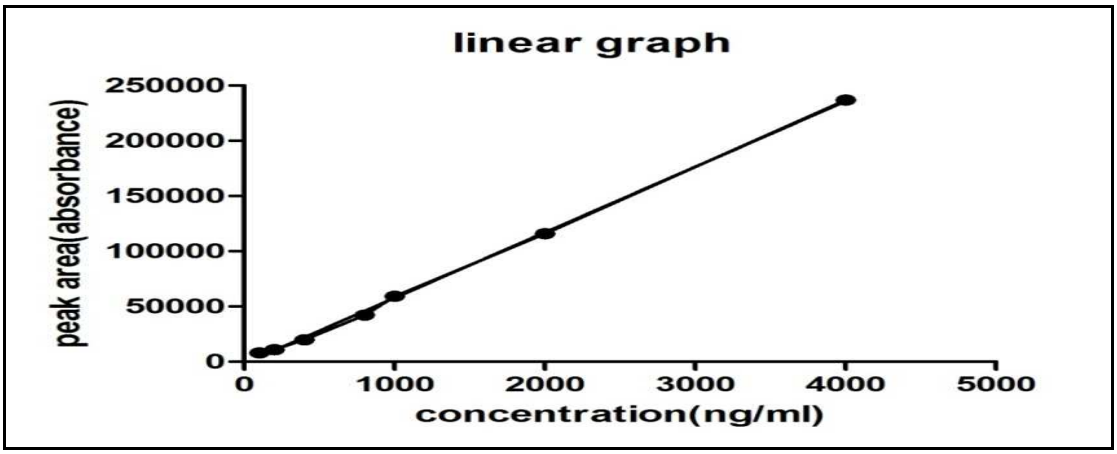

Figure No.2: Standard linear graph of Nepafenac

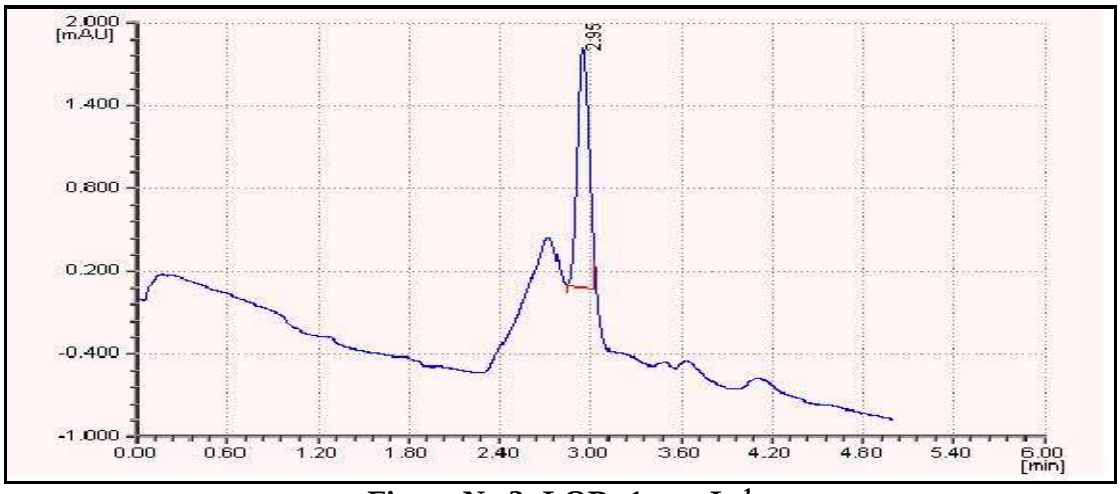

Figure No.3: LOD: 1 ngmL^{-1}

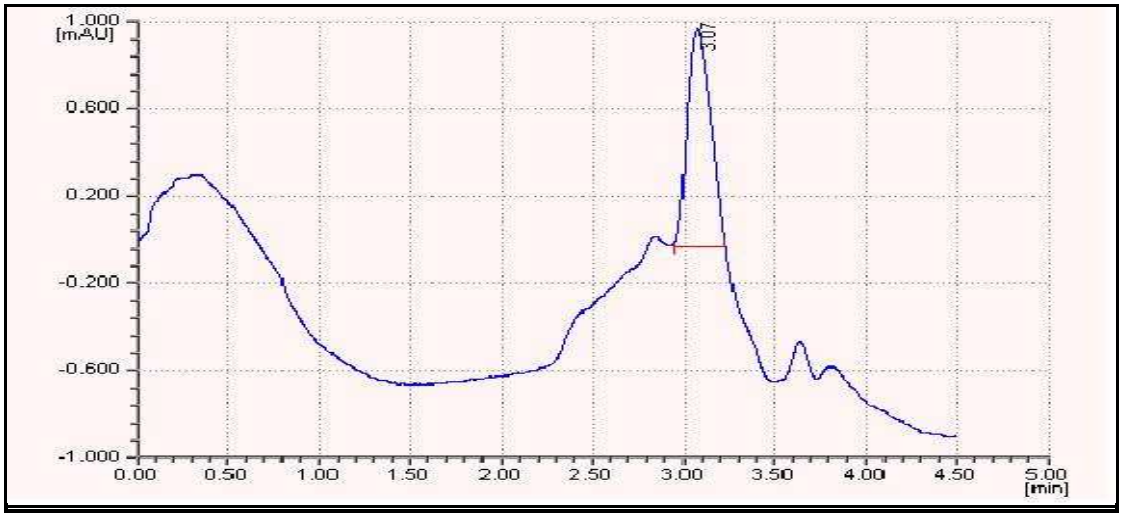

Figure No.4: LOQ: 10 ngmL^{-1}

Accuracy results

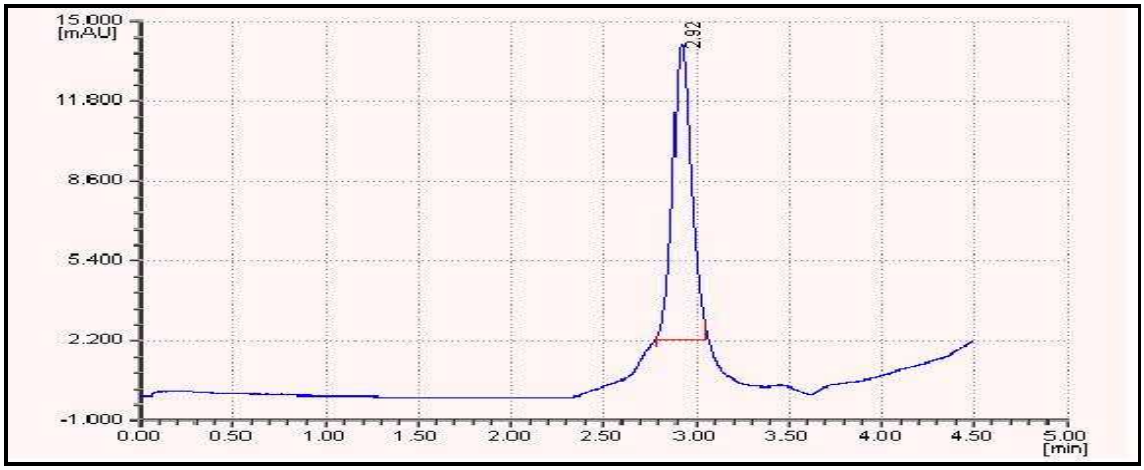

Figure No.5: Chromatogram of 100ng/ml

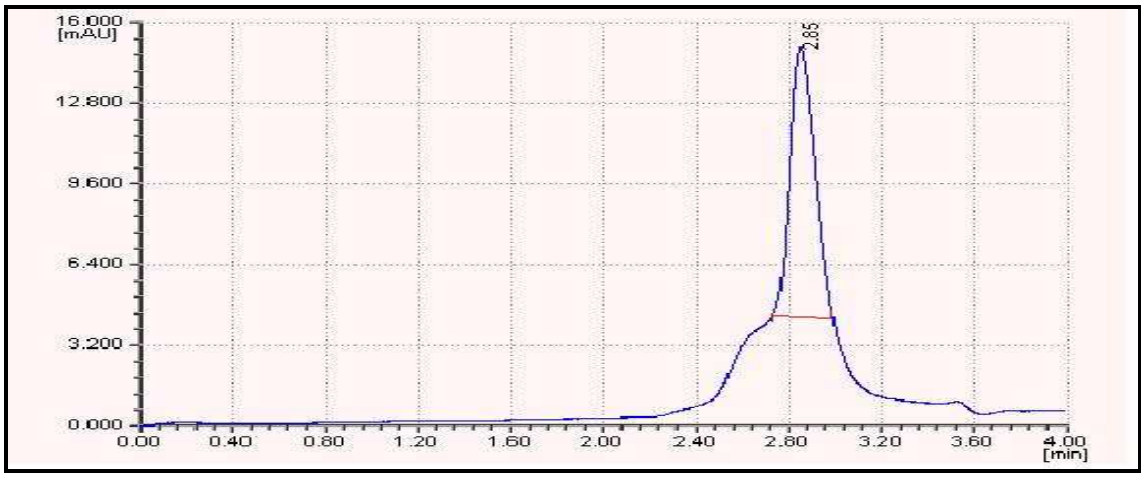

Figure No.6: Chromatogram of 200ng/ml

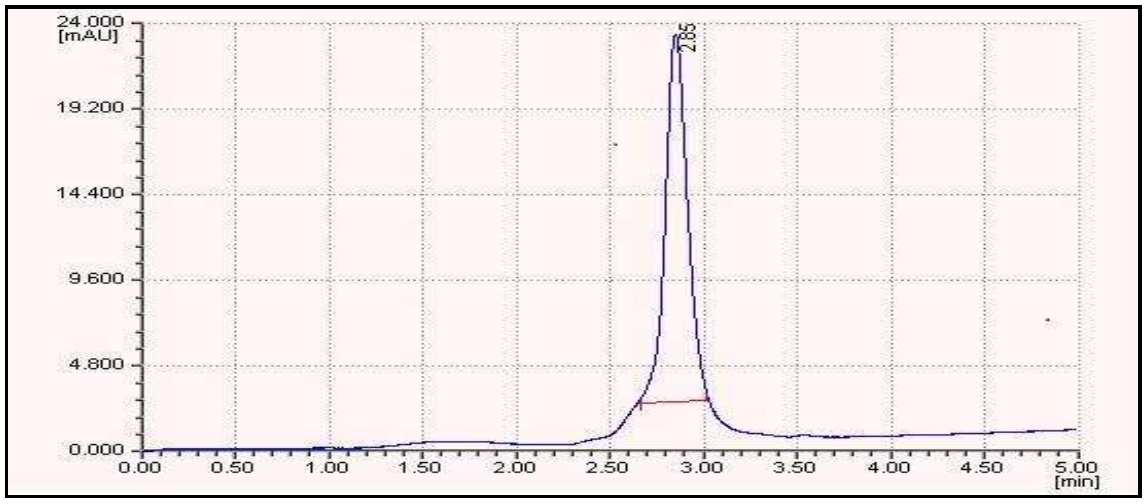

Figure No.7: Chromatogram of 400ng/ml

Specificity

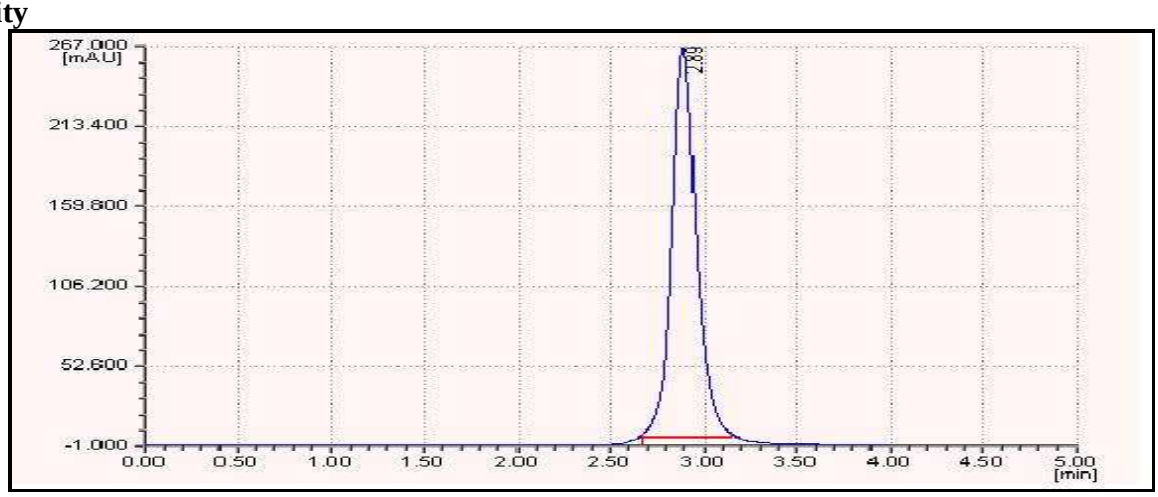

Figure No.8: Chromatogram of specificity

Robustness

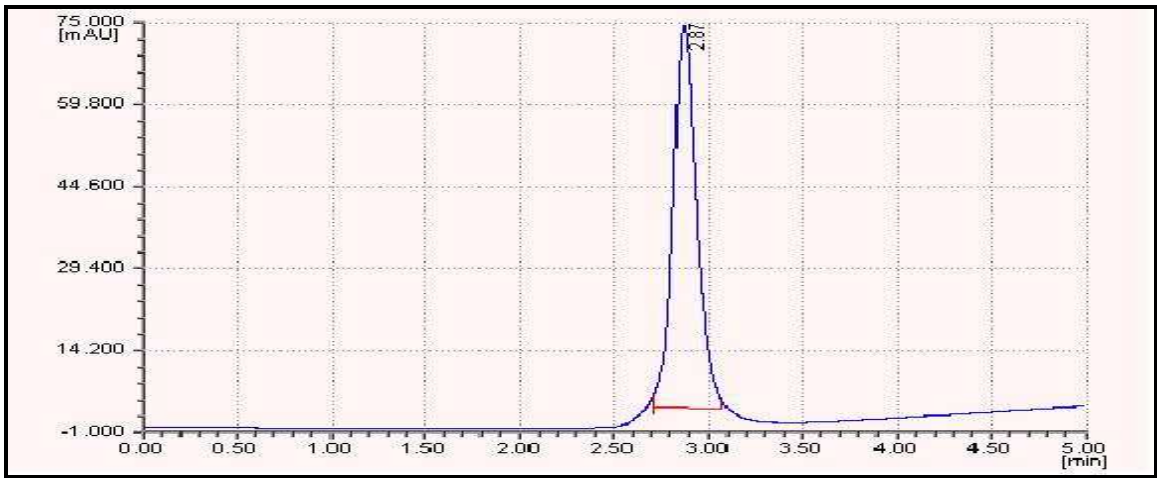

Figure No.9: Chromatogram of Slight Change in mobile phase

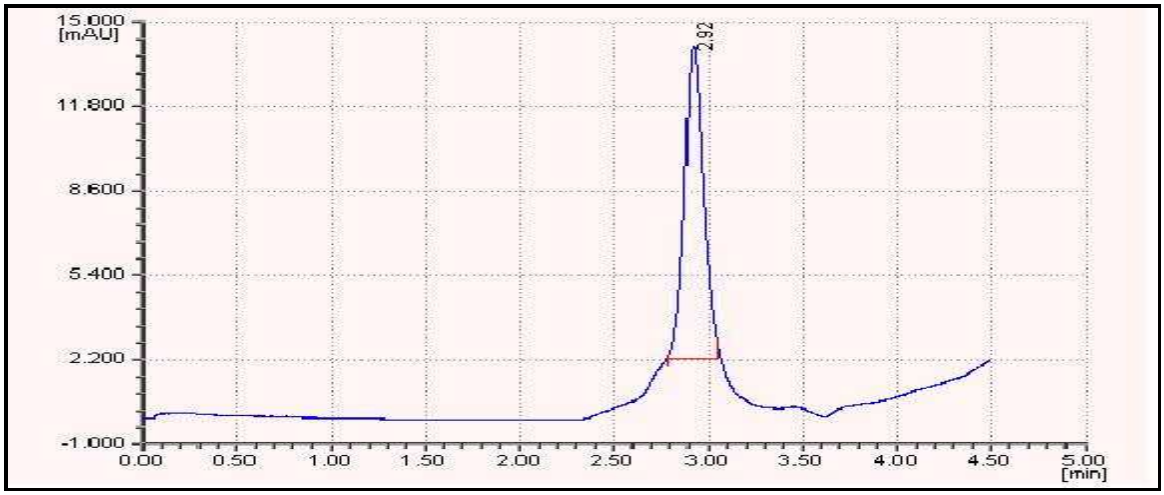

Figure No.10: Chromatogram of Slight change in flow rate

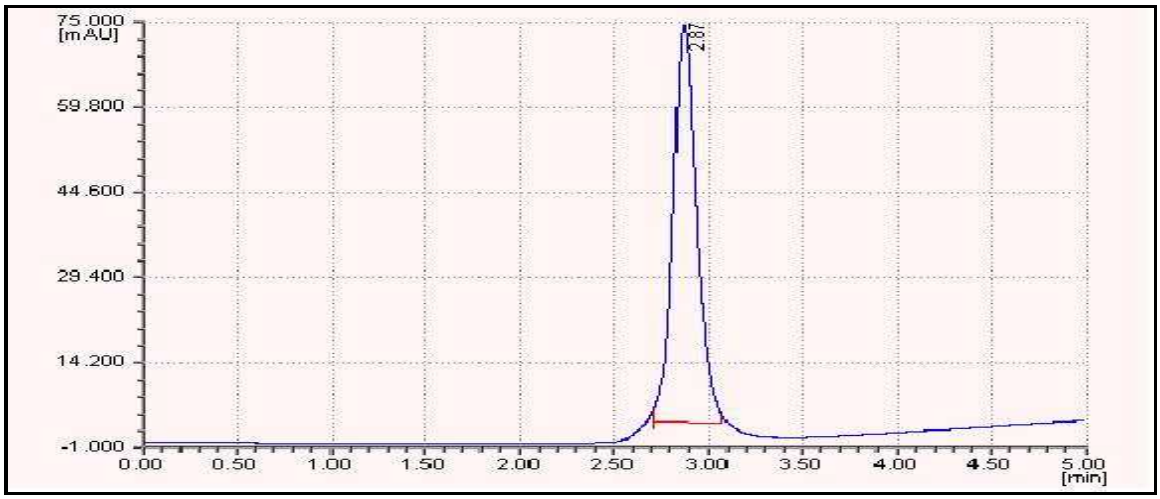

Figure No.11: Chromatogram of Slight change in temperature

DEGRADATION STUDIES

Hydrolytic Studies

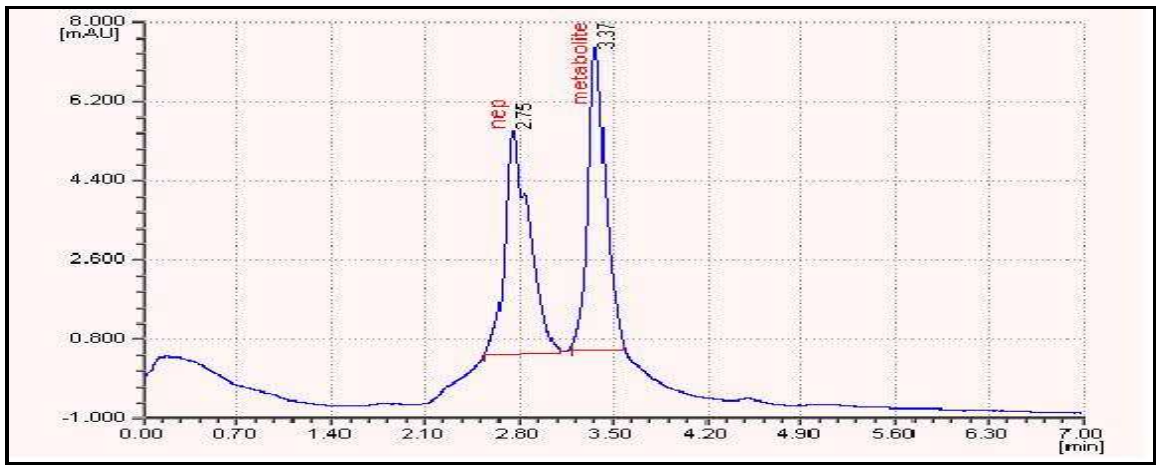

Figure No.12: Chromatogram of basic hydrolysis

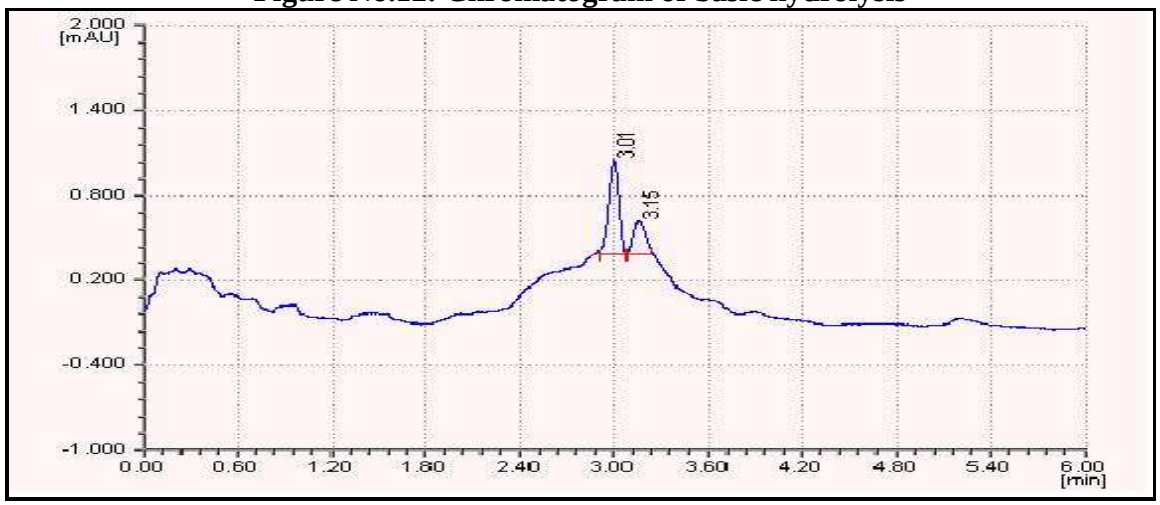

Figure No.13: Chromatogram of Acidic hydrolysis

Oxidative Studies

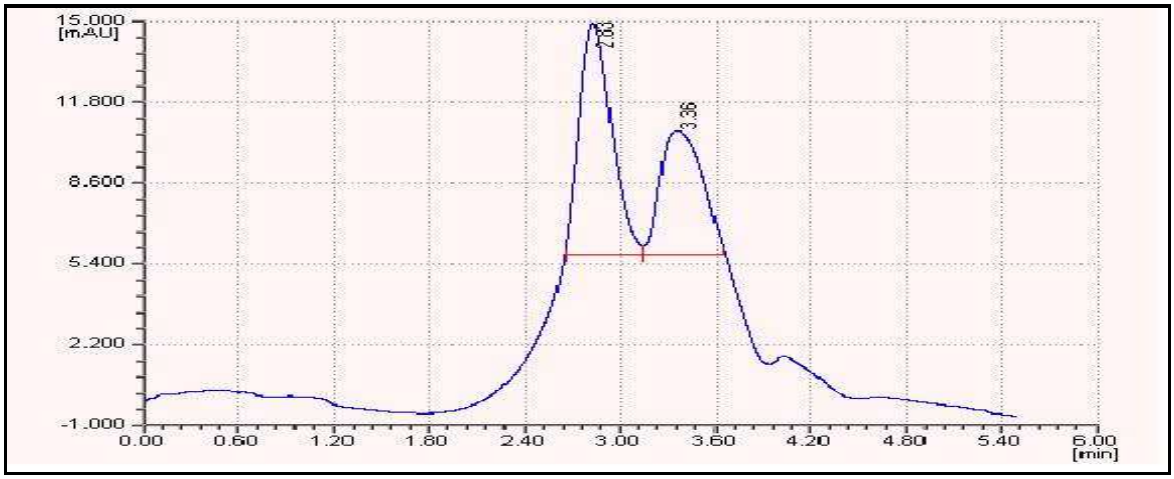

Figure No.14: Chromatogram of Peroxide hydrolysis

Photolytic Studies

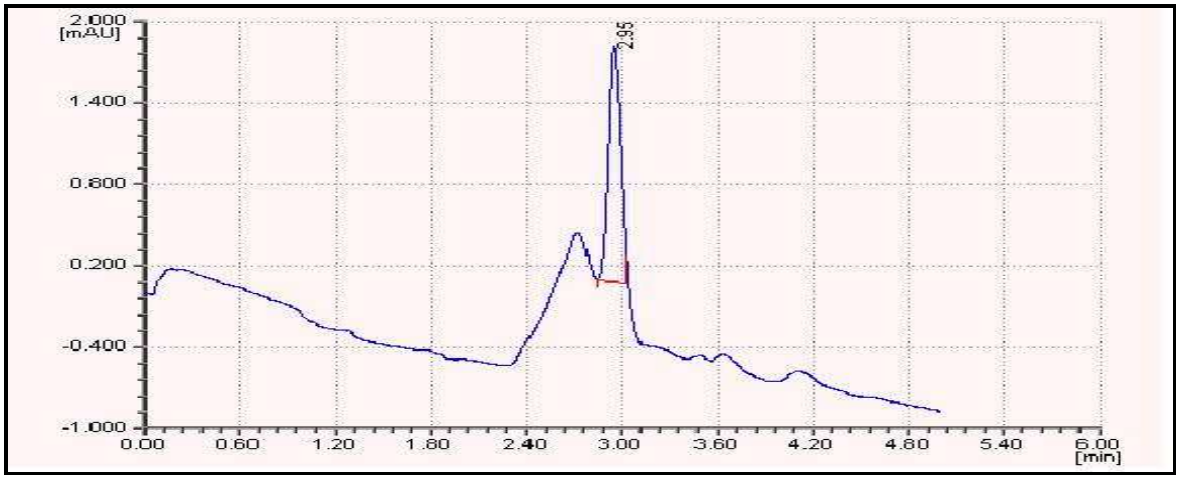

Figure No.15: Chromatogram of Photolytic Studies

Thermal Studies

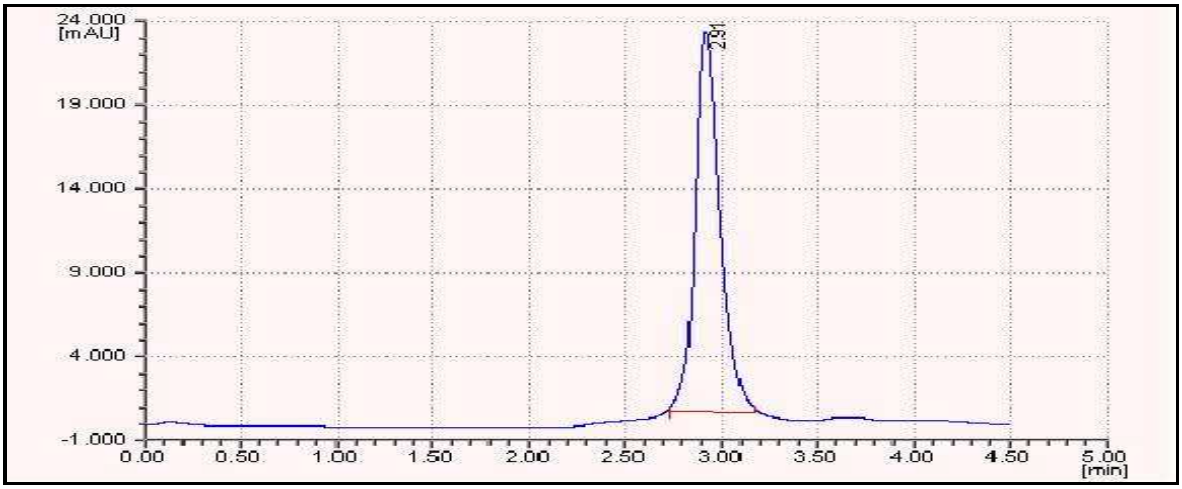

Figure No.16: Chromatogram of thermal studies

CONCLUSION

Reducing expenses and improving efficiency have been a focus for many pharmaceutical companies. A simple, harmonized approach to HPLC method screening can reduce cycle time for method development. So, this developed method is useful for determination of nepafenac even at low concentration (i.e. at ng level), and the solvents used are relatively cost effective when compared to other solvents which will be economical for both research laboratories and pharmaceutical industries for qualitative and quantitative determination. Conditions can therefore be fairly harsh, and simple procedures can be used. LC–UV will often give fully satisfactory (quantitative) results; nevertheless, LC–single-stage MS is making headway because it provides more selective and, consequently, more reliable data.

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CONFILICT OF INTEREST

We declare that we have no conflict of interest.

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