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Original Article

DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD OF NEPAFENAC AND ITS DEGRADATION PRODUCTS: APPLICATION TO DEGRADATION KINETIC

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ABSTRACT

Objective: The objective of present work was to develop and validate simple, precise, accurate and specific stability indicating method for determination of nepafenac in presence of its degradation products and application of the method to study degradation kinetics.

Methods: A novel isocratic RP-HPLC method has been developed using C_8 Olyster column, (250X4.6 mm i. d, 5µ particle size) with the mobile phase composition of ACN: 10 mM Ammonium formate buffer (pH 4.0): Methanol (27.5:45:27.5). The flow rate was at 1.0 ml min^{-1} and effluent was detected at 238 nm. Nepafeac was subjected to stress degradation under acid, base, neutral hydrolysis, oxidation, dry heat, humidity and photolysis, conditions. Kinetic study was also performed.

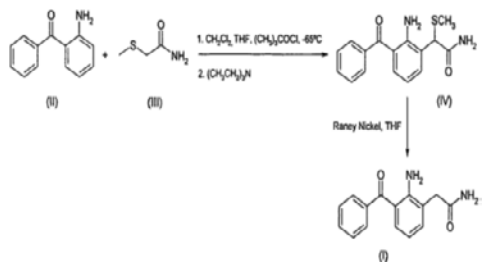
Results: The degradants peaks were well resolved from Nepafenac peak. Significant degradation was observed in acid, base and oxidative degradation. The drug is relatively stable towards photolysis and dry heat.

Conclusion: Stability of Nepafenac was determined by stability indicating assay method. Three degradation related impurities was identified by LC-MS. The hydrolytic degradation pathway of nepafenac was postulated. The developed stability indicating method was applied to determine acid, base and oxidative degradation kinetic. Acid hydrolysis and oxidative degradation followed zero order while base hydrolysis followed first order kinetic. Degradation rate constants and half-life were determined.

Keywords: Nepafenac, Reverse Phase High Performance Liquid Chromatography (RP-HPLC), Stability Indicating Assay Methods (SIAM'S), Stress Degradation, ICH Q1A (R2), Q2 R1.

INTRODUCTION

Nepafenac (NEPA), [2-(2-amino 3-benzoyl phenyl) acetamide] (II, Fig. 1) is antiinflammatory (NSAID's) 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 bioactivation to amfenac, which is a potent NSAID that uniformly inhibits the COX1 and COX2 activity [1]. Amfenac is thought to inhibit the action of prostaglandin H synthase (cyclooxygenase), an enzyme required for prostaglandin production. Preparation of NEPA is disclosed in US patent 4313947 and the scheme is depicted in Fig. 1[2].



I=NEPA, II= 2-amino-benzophenone,III=2-Methylsulfanyl-acetamide, IV= 2-amino-3-benzoyl-α-(methylthio)-benzeneacetamide

Fig. 1: Synthetic Scheme of NEPA

There is always need for significant SIM'S in modern analytical labs. Environmental factors, such as the temperature, pH,buffer species, ionic strength, light, oxygen, moisture, additives and excipients, can play an important role in the stability of drug substances. Stress testing can help in identifying degradationproducts and provide important information about the intrinsic stability of drug substances [3]. Stability indicating assay is defined as a validated quantitativeanalytical procedure that can detect the changes with time in the pertinent properties (e. g., active ingredient, preservative level) of the drug substance and drug product. A stability-indicating assayaccuratelymeasures the active ingredients without interferencesfrom degradation products, process impurities, excipients, or other potential impurities [4]. No official USP (United States Pharmacopeia Convention) andEP (European Pharmacopoeia) monographs currently exist for NEPA. The literature survey reveals that. Two simple and sensitive visible spectrophotometric methods have been developed for the estimation of NEPA in pure and pharmaceutical dosage forms [5,6]. Determination of NEPA in plasma by RP-HPLC has also been reported [7]. Method for determining chemical purity of NEPA by High performance liquid chromatography with ultraviolet visible detector(HPLC-UV)was disclosed in patent document [8]. The method utilizes gradient elution using mobile phase Ammonium formate buffer (PH 4.25 with formic acid) and acetonitrile (ACN). The separation was carried out on C-18 column. The chromatographic run time was at least 65 min. HPLC-UV studies of the chemical purity or assay of the NEPA was also reported by ElzbietaLipiec-Abramska et al.[9]As in patent document, the method also utilizes gradient elution with mobile phase ammonium formate (pH 4.10 ± 0.02 adjusted with formic acid) and ACN using C18 column at a column temperature of 30^0C. But the chromatographic run time is 29 min. Hence, the main objective of our work was to develop a simple, accurate and precise, stability-indicating HPLC-UV method for determination of NEPA and its degradation products within a short run time. In contrast to the above reported HPLC-UV methods, the chromatographic run time of the present method is 12 min with isocratic elution. Stress degradation was also performed to determine the most potential degradation related impurities. The validated method was applied to study the rate of degradation of NEPA under acid, base hydrolysis and oxidative degradation.

MATERIALS AND METHODS

Chemicals, Reagents and Solutions

NEPA (Bulk drug) and impurities were kindly provided by Sun Pharmaceuticals. Baroda and were used as received. Nevanac

Ophthalmic suspension 0.1%w/v (Alcon, USA) was purchased from local pharmacy. HPLC grade Methanol (MeOH) and ACN were purchased from Rankem Ltd, Mumbai. HPLC grade Ammonium Formate and formic acid were purchased from LobaChemie Pvt Ltd, Mumbai. Analytical grade Hydrochloricacid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide (H_2O_2)were purchased from S D FineChem. Ltd. Mumbai, India. A 10 mM Ammonium Formate buffer was prepared by dissolving 0.6306 g of Ammonium Formate in sufficient water to produce 1000 ml, and then the pH 4.0 was adjusted with Formic acid.

Instrumentation and Chromatographic Conditions

HPLC-UV

Analytical Chromatography was performed on Shimadzu (Shimadzu Corporation, Kyoto, Japan) chromatographic system equipped with Shimadzu LC-20AT pump and Shimadzu SPD-20AV absorbance detector. Samples were injected through a Rheodyne 7725 injector valve with fixed loop at 20 µl. Data acquisition and integration was performed using Spinchrome software (Spincho biotech, Vadodara). Chromatographic separation of NEPA was achieved at ambient temperature using C-8 Olyster column (250 X 4.6 mm i. d., 5 µ particle size) with mobile phase composition of ACN: 10 mM Ammonium Formate buffer (pH 4.0 adjusted with formic acid): MeOH (27.5:45:27.5). The flow rate was at 1.0 ml min^{-1}at ambient column temperature and effluent was detected at 238 nm. Before use, the mobile phase was filtered through a 0.2 µ nylon membrane filter and sonicated for 5 min.

Construction of the calibration curve

NEPA stock solution (1mg mL^{-1}) was prepared by dissolving 25 mg of NEPA in 25 ml MeOH in 25 ml volumetric flask. NEPA working solution (0.1 mg mL^{-1}) was prepared by transferring 2.5 ml from stock solution to 25 ml volumetric flask and diluted up to the mark with MeOH. Aliquots ranging from 0.01 ml to 2 ml were taken, from standard stock solution, in 10 ml volumetric flask and diluted to 10 ml with Mobile phase to give final concentration of 1, 5, 10, 25, 50, 75, 100, 150 & 200 µg mL^{-1} of NEPA. 20 µl of each concentration were injected in the chromatographic system and chromatogram was recorded. Calibration graph was constructed by plotting peak area versus concentration of each drug and the regression equation was calculated.

LC-MS (Liquid Chromatography –Mass Spectroscopy)

An LC–MS system (Thermo Scientific, Sunnyvale, CA, USA) comprised of a LCQ Fleet Ion Trap Mass Spectrometer. The analysis was performed in positive electro-spray/positive ionization mode with an ion source voltage of 5000 V and a source temperature of 450^{0}C. The curtain gas flow was 20 psi. The data was collected and processed using Surveyor plus HPLC System with Qual browser software. Column, mobile phase, flow rate and column temperature were same as in HPLC-UV.

Forced Degradation Study

Hydrolytic conditions

Acid, alkali and water induced degradation

10 mg of API & ophthalmic suspension (equivalent to 10 mg NEPA)were taken in 10 ml volumetric flasks separately, 4 ml of MeOHwas added to dissolved the API and sonicated for 3 min. 0.01N HCL,1N NaOH and Waterwere added separately to make up the volume and subjected to the conditions specified in table1. Degradation was performed in the dark in order to exclude possible degradation effect of light.

Oxidizing conditions

hydrogen peroxide-induced degradation

10 mg of API & ophthalmic suspension (equivalent to 10 mg NEPA) were taken 10 ml volumetric flask, 5 ml of MeOH added & sonicated then volume was made with 30 % H_2O_2. The solution was transferred to RBF and subjected to the conditions specified in table1.

Thermal conditions

dry heat

API and ophthalmic suspension (equivalent to 10 mg NEPA) were placed in an oven at 80ºC for 7 days under dry heat condition in the dark and then cooled to room temperature.

Photolytic degradation

For the photochemical stability API and ophthalmic suspension (equivalent to 10 mg NEPA) was spread in 1 mm thickness on a petridish and exposed to 5382 LUX and 144UW/cm^2 for 8 days.

Thermal & Humidity

API and ophthalmic suspension (equivalent to 10 mg NEPA) was placed in Stability Chamber at 40ºC±2º C and 75±5 % RH for 15 days.

Sample collection, storage and preparation

The sample from acid and base induced degradation was neutralized by adding appropriate strength of NaOH/formic acid. All samples were stored at 2–8 ^{0}C in the refrigerator. On theday of analysis samples were diluted with the mobile phaseto make the final concentrationof 100 µg/ml in all conditions, filtered with a 0.22 µm membrane syringe filterand injected three times for each sample into HPLC.

Table 1: Stress conditions for forced degradation study of Nepafenac

Stress condition	Solvent	Temperature (^{0}C)	Time (Hrs)
Hydrolytic			
Acid	MeOH, 0.01N HCl	RT	0.5
Base	MeOH, 1N NaOH	RT	6
Neutral	MeOH, Water	80	4
Oxidative	30% H_2O_2	40	6
Thermal			
Dry Heat	--	80	168
Thermal & Humidity		40ºC±2 ºC & 75% RH	336
Photolytic	--	5382 LUX and 144UW/cm^2	192

RESULTS AND DISCUSSION

Method Development and Optimization

A detection wavelength 238 nm was selected from the full range UV spectral data due to its high sensitivity for all degradation products and minimal difference in response factors. NEPA having pKa (SA) 15.82 and pKa (SB) 1.83, pH 4.0 buffer was selected to enable better column performance. Isocratic run was accessed using mobile phase 10 mM Ammonium formate buffer pH 4.0: ACN: MeOH (45:27.5:27.5) (v/v/v) on C8 and C18 columns. The C8 column provided the highest number of peaks and better resolution. Thus, further experiments were carried out using anOlyster C8 column.

Forced Degradation Study

The results from the stress testing studies indicate the method is highly specific for NEPA. Degradation products were completely

distinguishable from the parent compound. The drug undergoes significant degradation under acid, alkaline and oxidative condition. Acidic degradation was faster than basic & oxidative degradation. Acid hydrolysis resulted in complete degradation of NEPA. Little degradation was observed in photolysis. The drug was relatively stable in thermal & relative humidity, dry heat and wet heat degradation. Three degradation products were obtained at R_t of 3.3, 4.0 & 5.3. And some additional peaks are at 6.3 & 9.1. During experiments, it was found that NEPA Drug has inherent impurity at RT 5.29(Fig.2) that increases in stressed conditions and is common in all degradation condition. The typical chromatograms from assay of stressed samples are shown in Fig.3.

In acidic hydrolysis degradants peaks were obtained at RT of 3.3, 3.4 & 5.3 min on heating. In basic hydrolysis & oxidative additional degradants peaks were obtained at RT of 4.0 & 5.3 min apart from drug peak. Overall NEPA degradants peaks were observed at RT of 3.3, 3.4, 4.0, 4.9 5.3, 6.3, 9.1. The MS data shows that bifurcated peak at 3.3 & 3.4 have same m/z & fragment at 268 & 222.08 respectively. This indicates the presence of enantiomer. Results of forced degradation study are summarized in table 2.

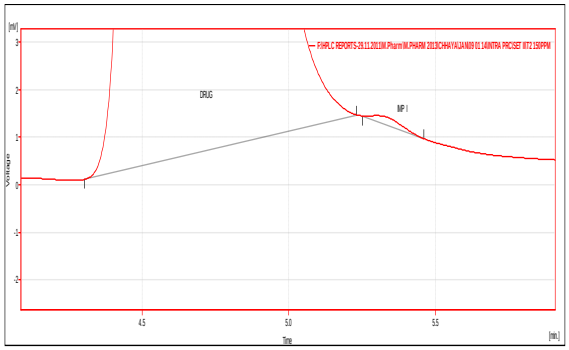
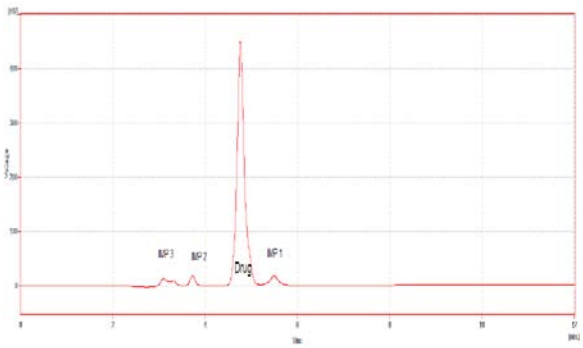
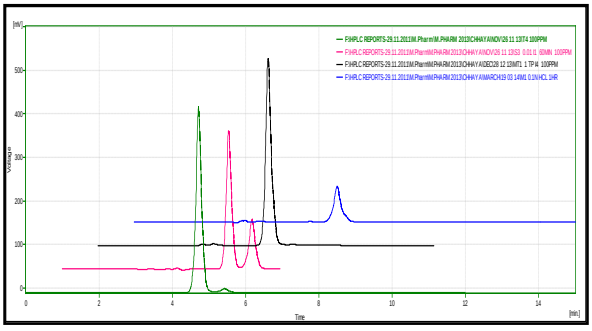


Fig. 2: Chromatogram of NEPA showing inherent impurity.

a)



(b)



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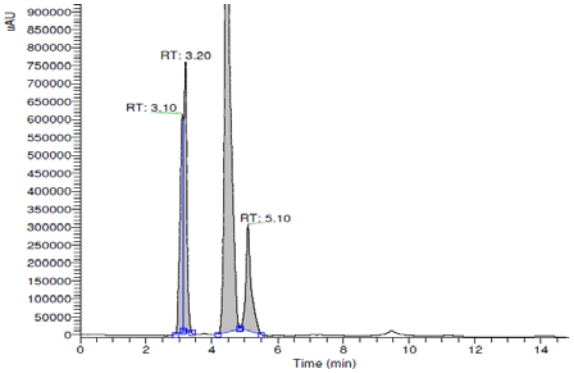
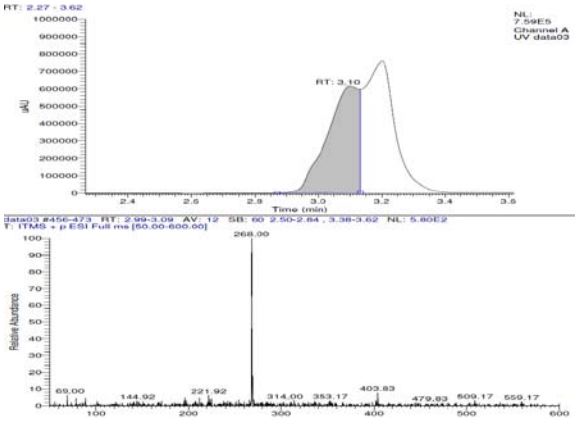
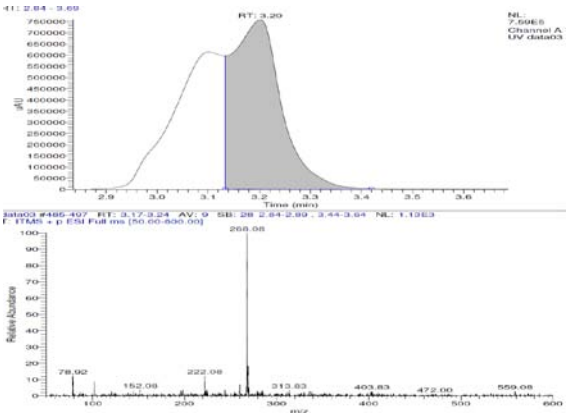


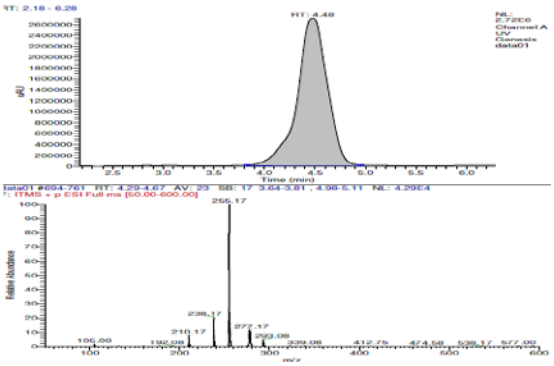
(d)



(e)



(f)



(g)

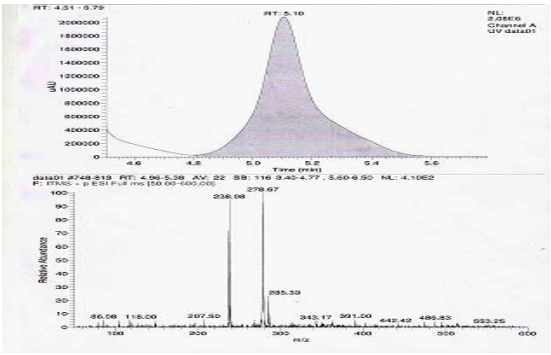
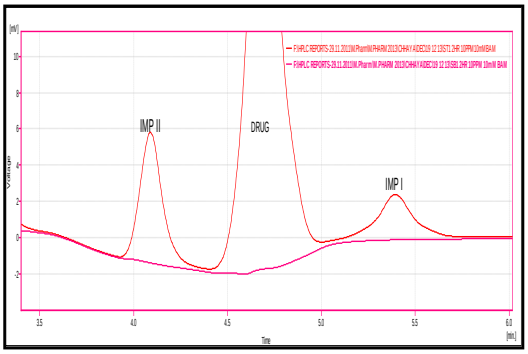


Fig. 3: (a) Separation of all degradants in stressed samples in optimized condition. (b) Overlain Chromatograms of acid degradation (0.01 N HCl, at room temperature for 30 min, Green – API, Black- Market Formulation, Pink- API treated with Acid Stressor, Blue- Formulation treated with Acid Stressor (c) LC-MS spectra (d) MS Spectra of NEPA degradation product in HCl at RT 3.10 (e) MS Spectra of NEPA degradation product in HCl at RT 3.2 (f) MS Spectra of NEPA in HCl at RT 4.4 (g) MS Spectra of NEPA degradation product in HCl at RT 5.1

(a)



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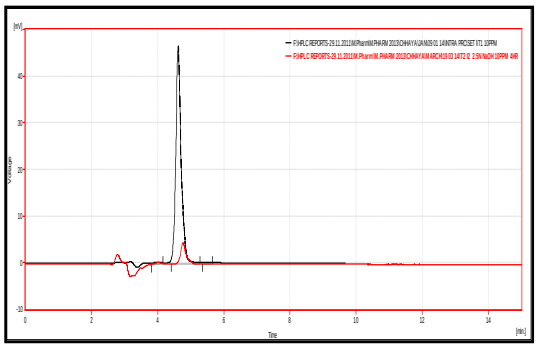

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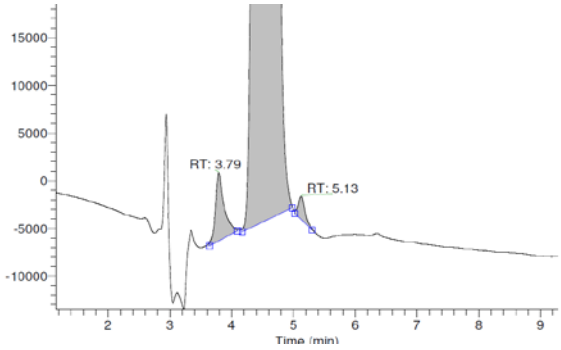


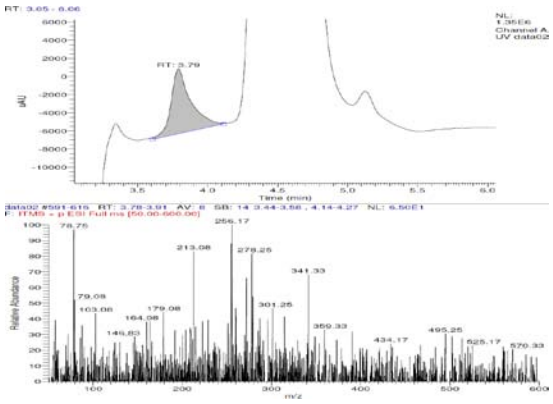


Fig. 4: Chromatogram of base degradation (1 N NaOH, at room temprature for 1 hrs.): (a) Overlain of API & blank: (RT= 4.1, 4.60 and 5.3 min.), (b) overlain Chromatograph of Ophthalmic suspension: Black without treatment & red after treated with Base Stressor: degradant (RT= 4.05 and 5.398 min.) (c) LC-MS & MS spectra of NEPA degradation product in NaOH at RT 3.79

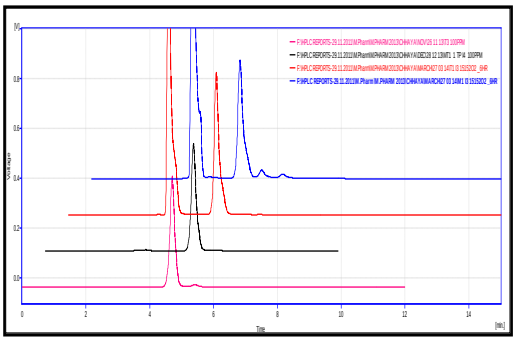


Fig. 5: Overlain Chromatograms of degradation in hydrogen peroxide (30 % H_2O_2, refluxed at 40ºC for 6 hrs.): Pink – API, Black- Ophthalmic suspension, Red- API treated with H_2O_2 stressor, blue- formulation treated with H_2O_2 stressor (API: 4.6, degradant 5.31 & 6.3)

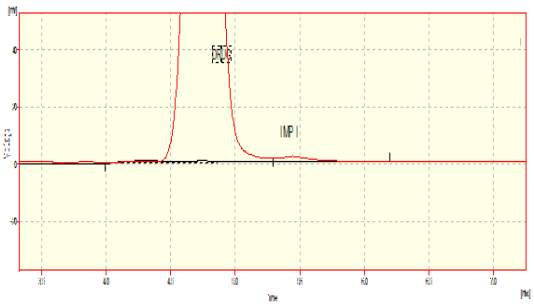


Fig. 6: Chromatogram of photochemical degradation (8 days)

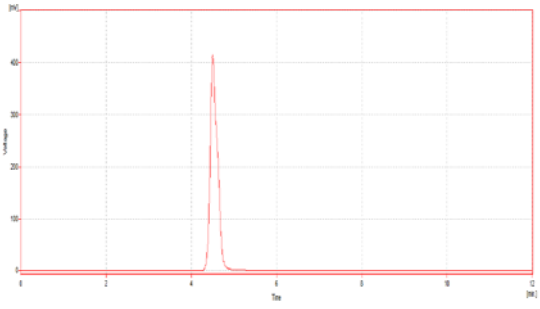


Fig. 7: Chromatograms of dry heat (80°C for 7 days)

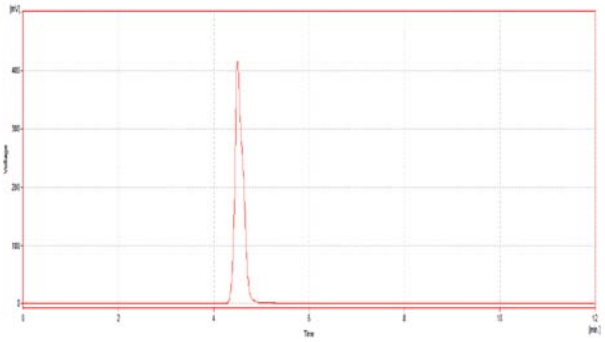


Fig. 8: Chromatograms of Wet heat (80°C for 4 hrs)

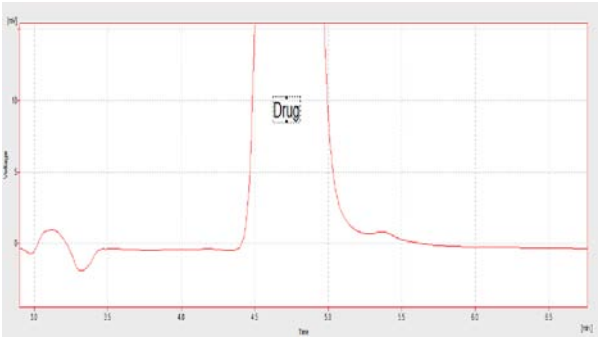
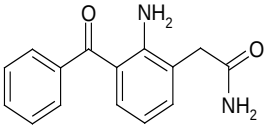
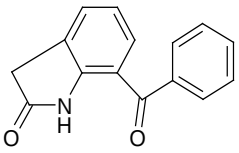
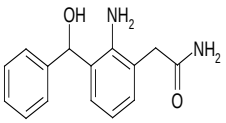
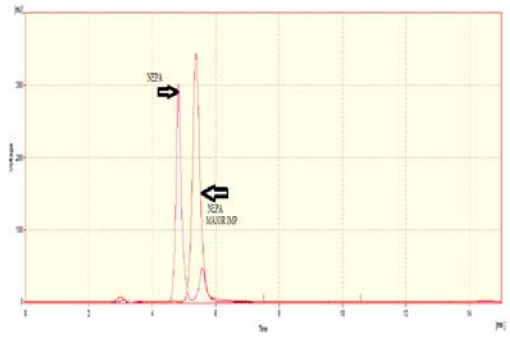


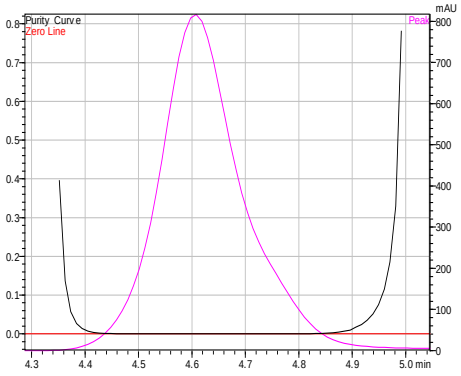
Fig. 9: Chromatogram of Thermal- Humidity (40°C±2 °C & 75% RH for 2 week

Table 2: Summary of forced degradation studies and structure of possible degradation products

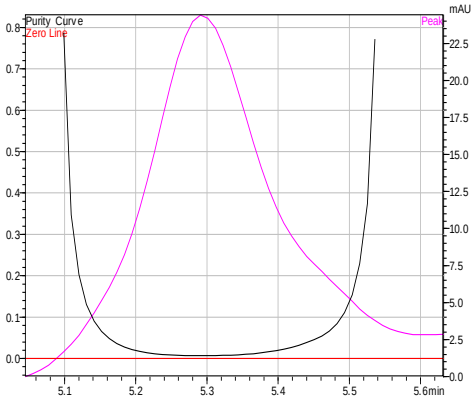
Compound	RT	m/z	Fragments	Structure	Systematic Name
NEPA	4.48	255.13	238.17, 277.17		[2-(2-amino 3-benzoyl phenyl) acetamide]
IMP-1[1]	5.10, (Major Degradant)	238.08, 278.67	178.07, 210.03; 209.94		7-benzoyl-1,3- dihydro-indol-2-one
IMP-2[2]	3.79, (Base, Oxidative Degradation)	256.17	213.08,278.25		2-{2-amino-3-hydroxy(phenyl)methyl¨phenyl} acetamide



(a)



(b)



(c)

Fig. 10: (a) Overlain Chromatogram of NEPA & its Degradation Product. (b) Peak purity for NEPA Drug Peak (c) Peak purity for major degradation Peak in HCl

Method Validation

Linearity and range

The linearity of proposed HPLC method was evaluated by analyzing a series of different concentrations of NEPA (n=11). The linear regression equation was generated from calibration data. Under the optimized conditions described above, the measured peak areas were found to be proportional to concentrations of the analyte (Table 3).

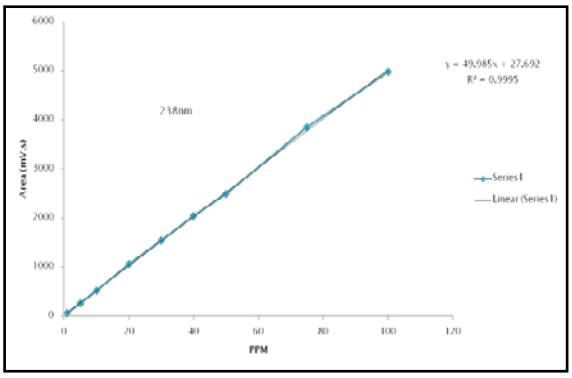


Fig. 11: Calibration curve of NEPA

Table 3: Analytical Validation Parameters for the determination of NEPA using HPLC method

S. No.	Parameters	NEPA
1.	Detection wavelength (nm)	238 nm
2.	Linearity range (µg mL^{-1})	1-200
3.	Regression Equation-	y=49.985x+27.692
	Intercept	27.692
	Slope	49.985
	Correction coefficient	0.9995
4.	Accuracy	99.914±0.060
5.	Intraday Precision(%RSD)	0.5112
6.	Interday Precision(%RSD)	1.1673
7.	LOD (µg mL^{-1})	0.3095
8.	LOQ(µg mL^{-1})	0.9381
9.	Reproducibility (%RSD)	1.4372

Detection and quantification limits

According to the pharmaceutical recommendations, limit of detection (LOD) and limit of quantification (LOQ) were determined, at signal to noise ratios of 3:1 and 10:1, respectively (Table 3).

Precision and Accuracy

The within-day (intra-day) precision and accuracy for the proposed method were studied at three concentration levels of NEPA using three replicate determinations for each concentration within one day. Similarly, the between-day (inter-day) precision and accuracy were tested by analyzing the same three concentrations using three replicate determinations repeated on three days. Recoveries were calculated using the regression equation and they were satisfactory. The percentage of relative standard deviation (%RSD) were less than 1.5% providing the high repeatability, intermediate precision and accuracy of the developed method for the estimation of NEPA in bulk form (table 3).

Robustness

The robustness was examined by evaluating the influence of small variations in different conditions such as flow rate, change in pH of buffer, detection wavelengths and organic ratio. The average value of % RSD for determination of NEPA less than 2 % revealed the robustness of the method.

Ruggedness

To evaluate ruggedness of the method few parameters were deliberately varied. The parameters included different instruments. The average value of % RSD for determination of NEPA less than 2 % revealed the ruggedness of the method.

Stability of solutions

Solutions containing different concentrations of NEPA were prepared from standard stock solution and stored at room temperature for 24 hrs. They were then injected in to LC system.

No additional peaks were found in chromatogram & area under the peak comes within 2.0 % of RSD indicate the stability of NEPA in the sample solution.

Degradation Kinetic Study

For relatively unstable drug, systematic kinetic studies of the degradation of drugs using stability testing techniques are essential for the quality control. In this work, we report a kinetic investigation of NEPA in acid, alkaline and oxidative condition. Calculations have been based on the measurement of the concentration of the Drug remaining using the previously described HPLC-UV method. At the selected temperatures and time interval (Table 4), the acid and oxidative degradation followed zero order kinetic while alkaline degradation followed first order kinetics (Fig. 12). A regular decrease in the percentage of drug remaining with increasing time intervals was observed. From the slopes of the straight lines, it was possible to calculate the degradation rate constant (Kobs) and half-life ($t_{1/2}$) of NEPA (Table 4).

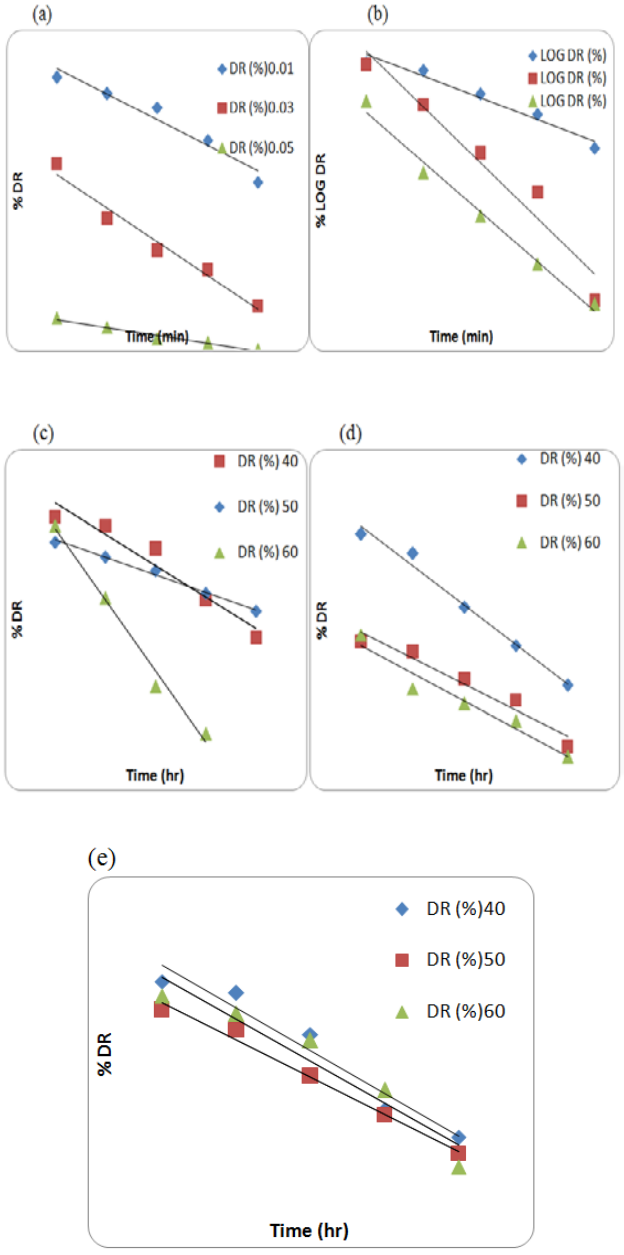


Fig. 12: (a) Zero order Kinetics Plot of NEPA in 0.01, 0.03 & 0.05 N HCl, (b) First order Kinetic plot of NEPA in 0.5, 1.0 & 2.0 N NaOH (c): Zero order Kinetic plot of NEPA in 30% H_2O_2 (d): Zero order Kinetic plot of NEPA in 15% H_2O_2 (e): Zero order Kinetic plot of NEPA in 6% H_2O_2.

Table 4: Kinetic Data - Degradation rate constants & half-lives for acid, base and oxidative degradation

Stressor	Temperature (⁰C)	Concentration (N/%)	Regression equation	R^2	$t_{1/2}$
HCl	RT	0.01	Y=-0.7961x + 95.651	0.9442	62.86
		0.03	Y=-1.0517x + 65.394	0.9717	47.54
		0.05	Y=-0.2473x + 12.081	0.9709	20.02
NaOH	RT	0.5	Y=-0.0134x + 2.2692	0.9611	51.71
		1.0	Y=-0.0348x + 2.6227	0.9506	22.35
		2.0	Y=-0.031x + 1.9935	0.9873	19.91
H_2O_2	40	30%	Y=-12.99x + 110.95	0.9365	3.85
	50		Y=-7.23x + 89.95	0.9915	3.85
	60		Y=-29.36x + 116.5	0.9876	1.70
	40	15	Y=-6.043x + 104.32	0.9858	8.27
	50		Y=-3.969x + 86.047	0.9488	12.62
	60		Y=-4.231x + 84.209	0.9488	11.82
	40	6.0	Y=-4.84x + 104.74	0.9475	10.33
	50		Y=-4.22x + 99.96	0.9891	11.84
	60		Y=-4.74x + 103.26	0.9245	9.2

CONCLUSION

The proposed LC method presented the ability to separate Nepafenac from all its degradation products and therefore can be applied in stability testing of the commercially available formulation. Nepafenac was rapidly degraded in acidic & basic medium and in the presence of hydrogen peroxide too, while it was more stable in UV radiation and thermal conditions. The structure of degradation related impurities were determined. Kinetic studies shows the acid Hydrolysis & Oxidation showed zero-order kinetics & Basic Hydrolysis Showed first order kinetics.

CONFLICT OF INTERESTS

Declared None

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REFERENCES

1. Gamache DA, Graff G, Brady MT, Spellman JM, Yanni JM. Nepafenac. A unique nonsteroidal prodrug with potential utility in the treatment of trauma-induced ocular inflammation: I. Assessment of Anti-Inflammatory Efficacy. Inflammation 2000;24(4):357-70.
2. James R. Shanklin Jr, Dwight A, Shamblee, David A. Walsh US 4313949, Method of producing an inhibitory effect on blood platelet aggregation 1982;2.
3. Saranjit S, Monika B. Guidance on conduct of stress tests to determine inherent stability of drugs. Pharm Tech 2000;24:1–14.
4. FDA Guidance for Industry, Analytical Procedures and Method Validation: CDER, August; 2000.
5. Anupama B, Madhavi D, Amareswari S, Hari Prasad CH: new visible spectrophotometric methods for determination of nepafenac. AJRC 2011;4:611.
6. Yadav SJ, Doshi, MN Panchori HP, Bhalero SS. A simple & rapid spectrophotometric methods for determination of nepafenac in pharmaceuticals. JPR 2012;5(8):4292.
7. Phani Kumar V, Sunandamma Y. A Novel RP-HPLC Method for quantification of Nepafenac in formulation, Plasma (*In Vitro*). Int J Pharm Bui Sci 2012;3(4):847-54.
8. Ana GE. WO 2010/115906 A12-{2-amino-3-[hydroxy(phenyl) methyl] phenyl}acetamide. 2010;10;14.
9. Elzbieta LA, Łukasz J, Adam F, Jacek R, Marcin C, Maria P, Joanna Z. Development and validation of high performance liquid chromatography with a spectrophotometric detection method for the chemical purity and assay of Nepafenac. J Pharm Biomed Anal 2014;91:1-6.