

Development and validation of UV and RP-HPLC method for estimation of Nepafenac in bulk drug and ophthalmic formulation

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ABSTRACT

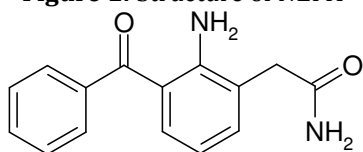
An ultraviolet (UV) and reverse phase high-performance liquid chromatographic (RP-HPLC) methods were developed and validated for the quantitative determination of Nepafenac in bulk and suspension formulation. UV method was performed with the λ_{\max} at 238.0 nm and 378.0 nm. RP-HPLC was carried out using C8 Olyster column with a mobile phase Acetonitrile: 10 mM Ammonium Formate buffer (pH 4.0 with formic acid): Methanol (27.5:45:27.5). The flow rate was 1.0 ml min⁻¹ and effluent was detected at 238 nm. The retention time of NEPA was 4.60 min. Both the methods showed good linearity, reproducibility and precision. No spectral or chromatographic interferences from the excipients were found in UV and RP-HPLC. Validation parameters such as linearity, precision, accuracy, and specificity were determined. The methods were successfully applied to commercial formulation.

Keywords: UV, RP-HPLC, Nepafenac, Q2 (R1)

Introduction

Nepafenac (NEPA), [2-(2-amino 3-benzoyl phenyl) acetamide] (Figure 1) is Antiinflammatory (NSAID's) which is indicated for the treatment of pain and inflammation associated with cataract surgery. NEPA is a prodrug. 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.

Figure 1: Structure of 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. These methods are based on the oxidative coupling reaction between

MBTH(3-Methyl-2-benzothiazolinone hydrazone) and NEPA resulting in the formation of pink colored chromogen (λ_{\max} 540 nm) and the complex formation between Potassium permanganate and NEPA resulting in the formation of blood red colored chromogen (λ_{\max} 425 nm). [2] One of the reported method utilizes phosphate buffer for development of UV method. [3] Determination of NEPA in plasma by RP-HPLC has also been reported. [4] The chemical purity of NEPA has been determined by HPLC-UV method. [5] Hence, the main aim of the present work is to develop a simple, rapid, accurate and precise UV and RP-HPLC method for determination of NEPA in suspension formulation. The proposed method was validated as per ICH guidelines. [6]

Experimental

Chemicals:

NEPA (Bulk drug) was kindly provided by Sun Pharma, Baroda. Nevanac [7] Ophthalmic Suspension 0.1%w/v (Alcon, USA) was purchased from local pharmacy. Analytical and HPLC grade Methanol (MeOH) and acetonitrile (ACN) were purchased from rankem, mumbai. HPLC grade Ammonium Formate and Formic acid were purchased from Loba Chemie Pvt Ltd, Mumbai.

Instrumentation and Analytical Conditions:

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Shimadzu UV-1700 double beam spectrophotometer connected to a computer loaded with Shimadzu UV Probe 2.10 software was used for all the spectrophotometric measurements. The absorbance spectra of the reference and test solutions were carried out in 1cm quartz cells over the range of 200-800 nm. 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 μ l. Data acquisition and integration was performed using Spinchrome software (Spincho biotech, Vadodara). The isocratic RP-HPLC was carried out at a flow rate of 1.0mLmin⁻¹ using C8 Olyster column with a mobile phase ACN: 10 mM Ammonium Formate buffer (pH 4.0 with formic acid): MeOH (27.5:45:27.5). Detection was carried out at 238.0 nm. The mobile phase was prepared daily, filtered through a 0.2 μ m membrane filter (Millipore) and sonicated for 5 min before use.

Preparation of Standard Solutions:

UV Method: NEPA stock solution (1mg mL⁻¹) was prepared by dissolving 25 mg of NEPA in 25 ml MeOH in 25ml 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. For preparation of calibration curve at 238.0 nm, aliquots ranging from 0.4 ml to 2 ml were taken, from standard stock solution, in 10 ml volumetric flask and diluted to 10 ml with MeOH to give final concentration of 4, 8, 12, 16, 20 μ g/ml of NEPA. For preparation of calibration curve at 378.0 nm, aliquots ranging from 2 ml to 6 ml were taken, from standard stock solution, in 10 ml volumetric flask and diluted to 10 ml with MeOH to give final concentration of 20, 30, 40, 50 60 μ g/ml of NEPA.

HPLC Method

NEPA stock solution (1mg mL⁻¹) was prepared by dissolving 25 mg of NEPA in 25 ml MeOH in 25ml 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 μ g mL⁻¹ of NEPA. 20 μ l 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.

Preparation of Sample Solutions

For both methods 5ml of 0.1% aqueous suspension of NEPA was transferred to 50 ml of volumetric flask and sufficient MeOH was added to get the working sample (100 μ g mL⁻¹). The solution was filtered through 0.2 μ whatman filter paper. Different volumes of above prepared solution were taken and diluted with MeOH to get different concentrations of NEPA. The above prepared solutions were analyzed by UV and HPLC for the content of NEPA.

Results and Discussion

Validation of Methods

Linearity and range

The proposed spectrophotometric method showed good linearity in the concentration range of 4 to 20 μ g mL⁻¹ for NEPA (Figure 2 (a and b)). For chromatographic method the linearity was observed over the concentration range of 1-200 μ g mL⁻¹ (Figure 3). Linear relationships between the the peak area signal of NEPA versus the corresponding drug concentration were observed, as shown by the results presented in Table 1. The standard deviations of the slope and intercept were low. The determination coefficient (r^2) exceeded 0.99.

Figure 2 (a): Calibration Curve for NEPA at 238nm (UV)

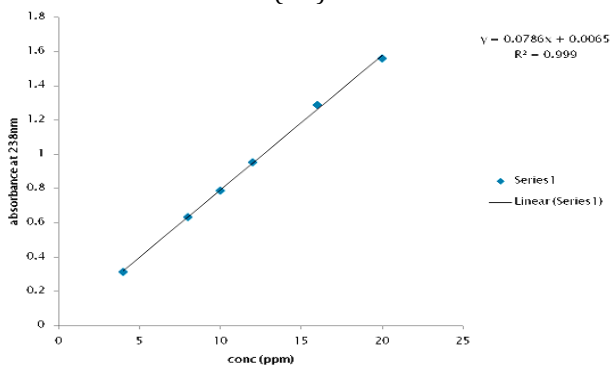


Figure 2 (b): Calibration Curve for NEPA at 378nm (UV)

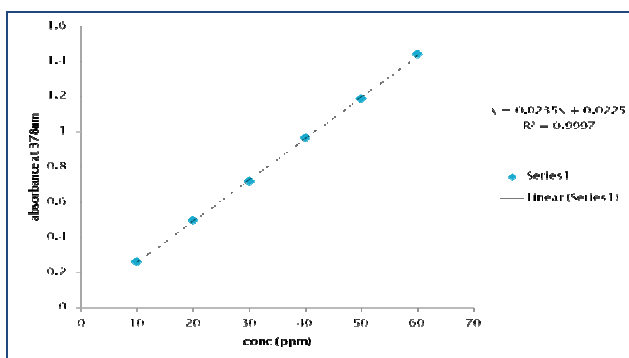


Figure 3: Calibration curve of NEPA (RP-HPLC)

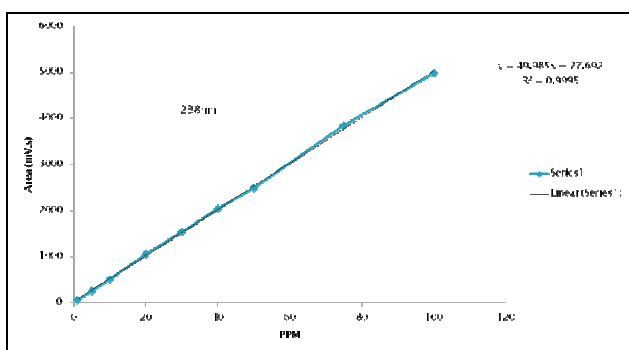


Table 1: Results of Linearity data for the quantitative determination of NEPA

Statistical parameters	UV (238)	UV (378)	HPLC
Concentration range ($\mu\text{g mL}^{-1}$)	4- 20	4- 20	1-200
Regression equation	0.0786X+0.0065	0.0235X+0.0225	49.985X+27.692
Correlation coefficient (r)	0.999	0.9997	0.9995

Precision

Inter-day and intra-day precision for both methods were measured in terms of % RSD. The experiment

was repeated 3 times in a day (Intraday precision) for 3 different concentrations and the average % RSD values of the results were calculated. Similarly the experiment was repeated on 3 different days (Inter day precision) and the average % RSD value for peak area of NEPA was calculated. The low value of SD obtained confirms the precision of the method (Table 2).

Table 2: Intra and Inter-day precision data for estimation of NEPA

Precision	RSD (%)		
	UV (238)	UV (378)	HPLC
Intra day	0.44521	0.404324	0.511283
Inter day	1.289294	1.181257	1.167331

LOD and LOQ:

Calibration curve was repeated for 9 times and the standard deviation (SD) of the intercepts was calculated. Then LOD and LOQ were measured as follows. The results obtained are shown in Table 3.

LOD=3.3 * SD/slope of calibration curve

LOQ=10 * SD/slope of calibration curve

SD = Standard deviation of intercepts

Table 3: LOD and LOQ of NEPA

Parameter	UV ($\mu\text{g ml}^{-1}$)		HPLC ($\mu\text{g ml}^{-1}$)
	238	378	
LOD	0.034339	0.028325	0.309582
LOQ	0.107179	0.088407	0.938128

Accuracy

Accuracy of the method was confirmed by recovery study from marketed formulation at 3 level of standard addition (80%, 100%, and 120%) of label claim. Recovery greater than 98 % with low SD justified the accuracy of the method. The result of accuracy study is shown in table 4.

Table 4: Recovery from Marketed formulation

Excess drug added to analyte (%)	Theoretical Content ($\mu\text{g/ml}$)	UV (238)		UV (378)		HPLC	
		*Amount Found ($\mu\text{g/ml}$)	Recovery (%) \pm S.D.	*Amount Found ($\mu\text{g/ml}$)	Recovery (%) \pm S.D.	*Amount Found ($\mu\text{g/ml}$)	Recovery (%) \pm S.D.
0	10.0	9.953	99.253 \pm 0.0498	10.1	101.0 \pm 0.0233	9.984	99.849 \pm 0.0454
80	18.0	17.91	99.500 \pm 0.423	17.84	99.166 \pm 0.421	17.980	99.895 \pm 0.043
100	20.0	19.852	99.261 \pm 0.627	20.138	100.69 \pm 0.641	20.041	100.242 \pm 0.127
120	22.0	21.82	99.217 \pm 0.599	22.04	100.21 \pm 0.523	22.971	99.872 \pm 0.043

*Average of three experiments

Robustness

The robustness of spectroscopic method was determined by using MeOH of 3 different manufacturers for the preparation of stock solution of standard drugs. The average value of % RSD for determination of NEPA less than 2 % revealed the robustness of the method (Table 5).

Table 5: Robustness study using MeOH from three different manufacturers

MeOH	NEPA (mg) Taken	*NEPA Found	% Recovery NEPA
1	1	0.992	99.2 \pm 0.701
2	1	0.982	98.2 \pm 0.441
3	1	0.99	99.0 \pm 0.143

To evaluate robustness of the chromatographic method few parameters were deliberately varied. The parameters included variation in 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. Each factor selected to examine were changed at 3 levels (-1, 0, 1). One factor at the time was changed to estimate the effect. Thus replicate injections (n=3) of standard solution at 3 concentration levels were performed under small changes of 3 chromatographic parameters (factors). Results, presented in Table 6 indicate that the selected chromatographic parameter s remained unaffected by small variation in these factors, which demonstrates that the developed method is robust.

Table 6: Robustness of HPLC method

Factor	Level	Chromatographic Parameter (RT)
A. Flow Rate		
0.9	-0.1	5.183
1.0	0	4.65
1.0	0.1	4.260
Mean \pm S.D.		4.7143 \pm 0.4616
B. pH		
3.8	-0.2	4.56
4.0	0	4.650
4.2	0.2	4.79
Mean \pm S.D.		4.6667 \pm 0.1159
C. Detection wavelength (nm)		
235	-3.0	4.58
238	0.0	4.69
241	0.3	4.65
Mean \pm S.D.		4.65 \pm 0.0608
D. Mobile Phase Ratio [Buffer:ACN: MeOH (v/v/v)]		
45:30:25		3.503
45:27.5:27.5		4.65
45:25:30		4.42
Mean \pm S.D.		4.46 \pm 0.1734

Stability in sample solutions:

Solutions containing different concentrations of NEPA were prepared from standard stock solution and stored at room temperature for 24hrs. They were then injected in to LC system. No additional peaks found in chromatogram & area under the peak comes within 2.0 % of RSD indicate the stability of NEPA in the sample solution.

System suitability

Following parameters were calculated for system suitability of HPLC method (Table 7)

Table 7: Results of system suitability parameters

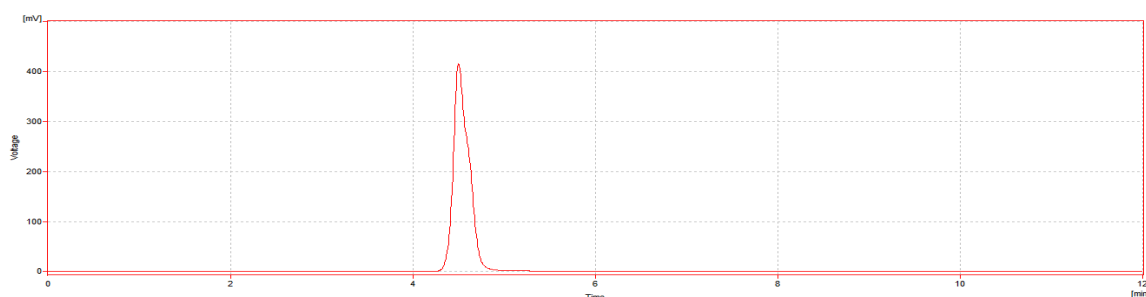
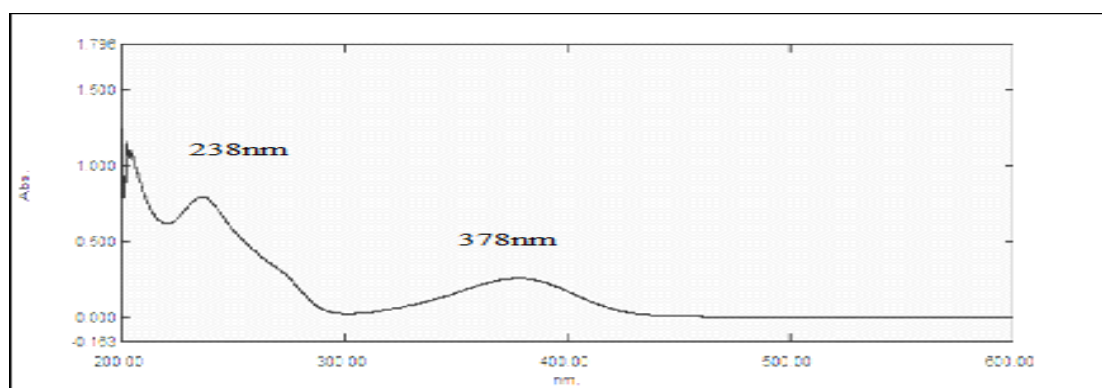
Parameters	Data obtained
Theoretical Plates per meter \pm RSD	102397 \pm 0.835
Theoretical Plates per column \pm RSD	5120 \pm 0.556
Capacity Factor	0.45
Symmetry Factor \pm RSD	1.143 \pm 0.106
Resolution \pm RSD	2.3
% RSD of 5 injected solution	1.4372 \pm 0.204

Assay:

Applicability of the proposed method was tested by analyzing the commercially available ophthalmic suspension formulation containing 0.1% w/v of NEPA. Each sample was analyzed in Triplicate. Figure 4 and 5 shows an HPLC chromatogram and spectrogram of NEPA in suspension formulation. None of the tablets ingredients interfered with the analyte peak. The results presented in Table 8 are in good agreement with the labelled content.

Table 8: Analysis of marketed formulation

Formulation	Drug	λ max	Labeled (0. and 1%w/v)	UV		HPLC	
				Amt*. Found	% Assay	Amt*. Found	% Assay
NEVANAC	NEPA	238nm	0.1	0.0982	98.2 \pm 0.441	0.0996	99.62 \pm 0.234
		378nm	0.1	0.0997	99.7 \pm 0.564	---	---

Figure 4: HPLC chromatogram of NEPA in suspension formulation**Figure 5:** Spectrogram of NEPA in suspension formulation**Conclusion**

A validated UV and isocratic HPLC methods have been developed as per ICH guideline for the determination of NEPA in suspension formulation. The proposed methods are simple, rapid, accurate, precise, and specific. Its chromatographic run time of 4.6 min allows the analysis of a large number of samples in a

short period of time. Therefore, it is suitable for the routine analysis of NEPA in pharmaceutical dosage form.

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