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Original Article

DEVELOPMENT AND VALIDATION OF HPLC METHOD FOR QUANTITATIVE ESTIMATION OF NIMBOLIDE IN BULK AND PHARMACEUTICAL DOSAGE FORM

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ABSTRACT

Objective: The present study was aimed to a simple, new, rapid and highly sensitive Reverse Phase - High Performance Liquid Chromatographic (RP-HPLC) method has been developed and an assay was validated for the quantitative estimation of nimbolide in solid dosage form.

Methods: The chromatographic separation was achieved on an Agilent 1200 series HPLC system C18 (250 mm x 4.6 mm x 2.5 µ) column packing by using isocratic mobile phase consisting of acetonitrile: water (90:10 v/v), flow rate was adjusted to 1.0 ml/min at a fixed wave length of 207 nm.

Results: The nimbolide was eluted at 2.880 ± 0.05 min and established a dynamic range of linearity over the concentration range of 3.125-200 ppm/ml (r^2 = 0.9997 ± 0.005). The lower limit of detection and quantification was 0.007 ppm/ml and 0.021 ppm/ml respectively. The method was validated as per ICH guidelines. The accuracy of the method was determined by the recovery studies and the mean recovery was obtained 98.27%. Moreover the method was shown good reproducibility and recovery with percent relative standard deviation less than 2%.

Conclusion: Rapid, efficient and sensitive RP-HPLC method was developed for the estimation of nimbolide from the perspective of reducing the cost of analysis and time and thus by saving laboratory resources.

Keywords: Nimbolide, RP- HPLC, ICH guidelines.

INTRODUCTION

Azadirachta indica (Family-Meliaceae) is an important medicinal plant and aqueous extract of *A. indica* which is widely used as a tonic, stimulant and also against various ailments [1]. Biological activities of *A. indica* extract has been investigated intensively. In general, extracts of neem fruits, seeds, seed kernels, twigs, stem bark and root bark have been shown to possess anti-inflammatory and immune-stimulating activities [2-4]. Nimbolide, a chemical compound, is classified as a triterpenoid isolated from *Azadirachta indica* (neem tree) [5]. This compound has been shown to have some biological activities such as anti-feedent [6], anti-malarial [7] and antimicrobial activities [8, 9]. It has also been exhibited significant anti-cancer activity [10, 11].

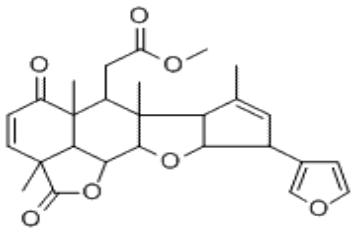

Fig. 1: Structure of Nimbolide

However, the toxicity of a compound has always become an issue in therapeutic use. It has been shown that nimbolide, when given through an intragastric route to experimental animals [12], did not show any toxicity. IUPAC name of Nimbolide is (4α,5α,6α,7α,15β,17α)-7,15:21,23-Diepoxy-6-hydroxy-4,8-dimethyl-1-oxo-18,24-dinor-11, 12-secochola-2,13,20,22-tetraene-4,11-dicarboxylic acid γ-lactone methyl ester (Mol. Formula: $C_{27}H_{30}O_7$; Mol. wt. 466.57 g/mole) in [Fig.1]. In the present study, HPLC method has been the development by using simple mobile phase which is sensitive and rapid for quantification of Nimbolide with the extract of *Azadirachta indica* as well as subsequent validation of developed method as per ICH guidelines [13-14].

MATERIALS AND METHODS

Chemicals and reagents

Nimbolide reference standard was purchased from Asthagiri Herbal research Foundation Centre, (Chennai, India). Acetonitrile, Methanol and water of HPLC grade were purchased from MERCK specialties Pvt Ltd. (Mumbai, India). Commercial capsules of nimbolide formulation were procured from local pharmacy. Neem capsule containing Nimbolide with labelled amount of 250 mg per capsule is manufactured by Himalaya Herbals, India.

Instrumentation and chromatographic conditions

The HPLC analysis was performed on Agilent 1200 HPLC series Liquid Chromatography comprising a C18 Column - 250 mm X 4.6 mm X 2.5 micron particle size and a Prominence UV-Vis detector. A manually operating Rheodyne injector with 10 µL sample loop was equipped with the HPLC system. The HPLC system was equipped with "Spinchrom" data acquisition software. The mobile phase was consisting of Acetonitrile and water in the ratio of 90:10 v/v. These were filtered through 0.45µm membrane filter and degassed by sonication before use. The mobile phase was pumped isocratically in a flow rate 1.0 ml/min during analysis at ambient temperature. The total run time was set at 5.0 min, eluent was monitored at 207 nm and the base line was continuously monitored during the process.

Preparation of stock and working standard solution of nimbolide

About 25 mg of Nimbolide were weighed accurately and dissolved in 100 ml of diluent by means of sonicated for 15 min and filter through Whatman filter paper. Pipette out 1.0 ml from the stock solution and diluted up to10 ml with the same solvent (25 ppm/ml).

Preparation of sample solution

Twenty capsules (Neem) were weighed accurately and powder equivalent to 25 mg containing Nimbolide and transfer into 100 ml volumetric flask. By adding 70 ml of diluent, it was placed in an ultra sonication bath until dissolution was complete. Same solvent was added to bring up the volume to 100 ml. Pipette out 1.0 ml of the

sample solution into a 10 ml volumetric flask and dilute up to the mark and mix well. The resulting solution was filtered using 0.2 µm filter and degassed by sonication. The resulting solution will give a concentration of approximately 25 ppm/ml.

Selection of wavelength

The UV spectrum of diluted solutions of various concentrations of nimbolide in mobile phase was recorded using UV spectrophotometer.

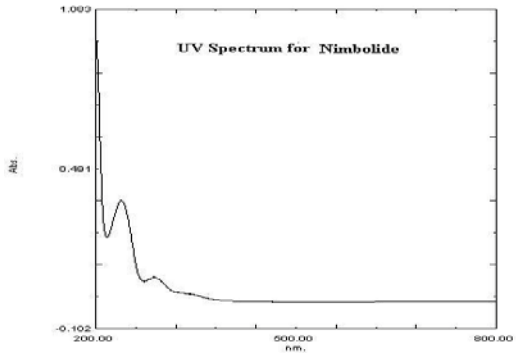

Fig. 2: Absorption spectra of Nimbolide (25 ppm/ml)

The wavelength of maximum absorbance was observed at 207 nm. This wavelength was used for detection of nimbolide.

Calibration curve for nimbolide

Seven different concentrations of Nimbolide were analyzed and their calibration curve was constructed in the specified concentration range (3.125-200 ppm/ml). The calibration plots were made by replicate analysis (n = 6) at all concentration levels. Ten micro lot quantity of the dilution were injected each time into the column at a flow rate of 1.0 ml/min. The nimbolide in the elutes was monitored at 207 nm.

The results were presented in Table 1 and the corresponding standard chromatograms of nimbolide have been depicted between Fig. 4 and Fig. 10. From these chromatograms the mean peak areas were calculated and a plot of concentration over the peak area was constructed. The regression of the plot was computed by least squares regression method and is presented in Fig. 3. The data of regression analysis are presented in Table 2.

Assay of nimbolide in neem capsule

The proposed method was applied for the assay of Nimboide pharmaceutical dosage form. The drug content was calculated as an average of six determination and results of the assay are shown in Table 6. The results were close to the labelled value of commercial capsule. The percentage assay of nimbolide was found to be 98.972%. The sample chromatogram of nimbolide is shown in Fig. 12.

Table 1: Calibration data of the proposed HPLC method for estimation of Nimbolide

S. No.	Concentration in ppm	Retention time	Peak Area	% RSD
1	3.125	2.880	103465.3	0.8796
2	6.25	2.887	200726	1.3639
3	12.5	2.880	419504	0.4569
4	25	2.880	797974	0.1245
5	50	2.880	1646982	0. 2266
6	100	2.880	3459291	0.3473
7	200	2.880	7068030	0.1678
Slope 35,426.1822, Intercept - 51,970.2950, Correlation Coefficient 0.9997				

Table 2: Linear regression data of the proposed HPLC method of Nimbolide

Parameter	Method
Detection of wavelength	UV at 207 nm
Linearity range	3.125- 200 ppm/ml
Regression equation (Y= a - bX)	Y= 51,970.29 + 35,426.18
Slope(b)	35,426.18
Intercept(a)	-51,970.29
Regression Coefficient	0.9997

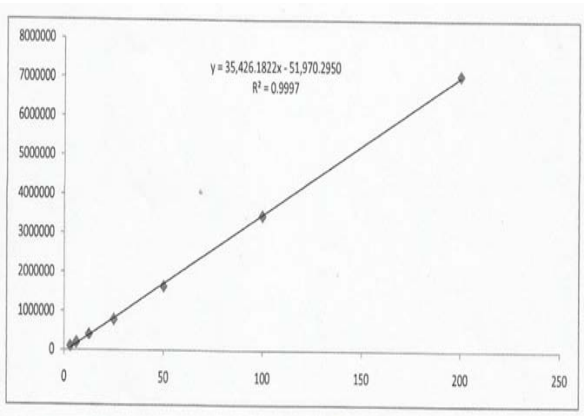

Fig. 3: Calibration plot of Nimbolide

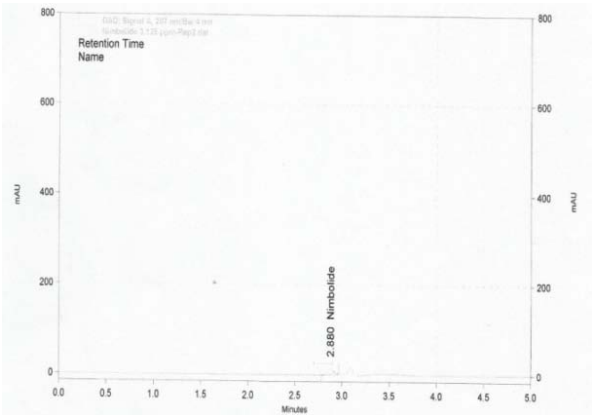

Fig. 4: Standard Chromatogram of Nimbolide 3.125 ppm/ml

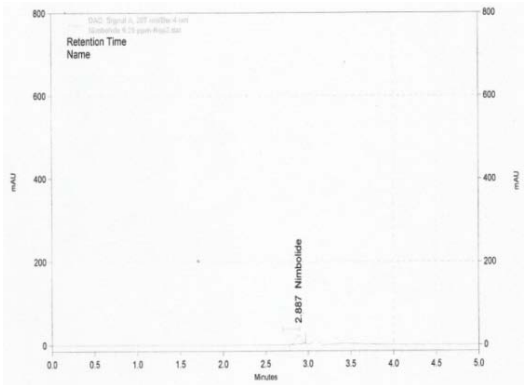

Fig. 5: Standard Chromatogram of Nimbolide 6.25 ppm/ml

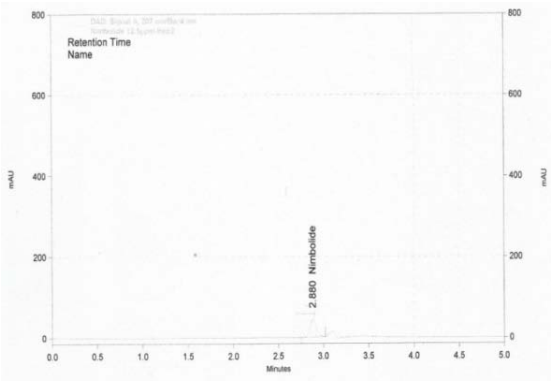

Fig. 6: Standard Chromatogram of Nimbolide 12.5 ppm/ml

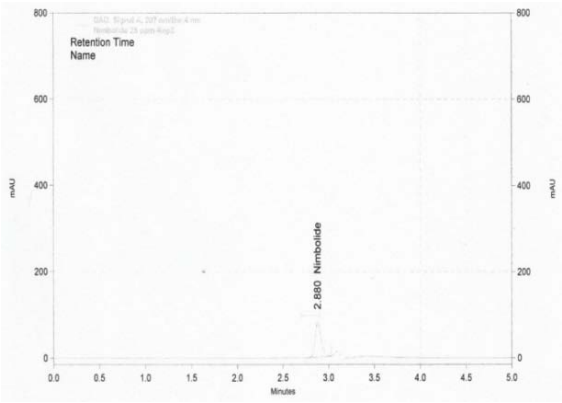

Fig. 7: Standard Chromatogram of Nimbolide 25 ppm/ml

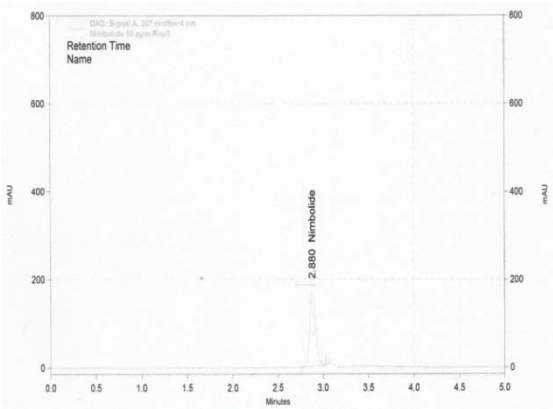

Fig. 8: Standard Chromatogram of Nimbolide 50 ppm/ml

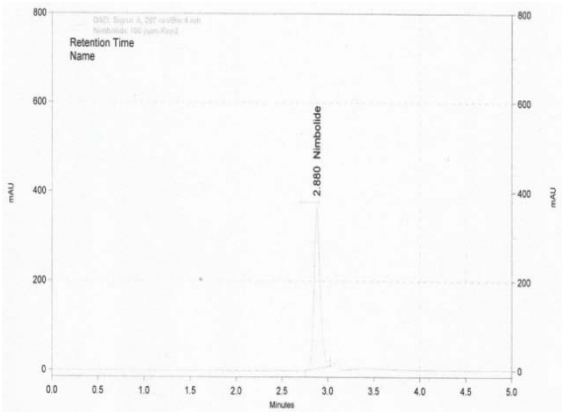

Fig. 9: Standard Chromatogram of Nimbolide 100 ppm/ml

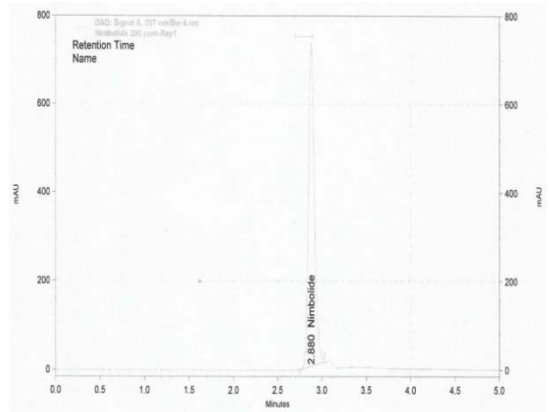

Fig. 10: Standard Chromatogram of Nimbolide 200 ppm/ml

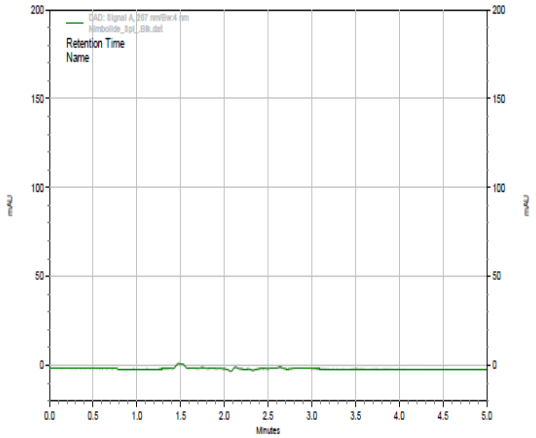

Fig. 11: Chromatogram for placebo

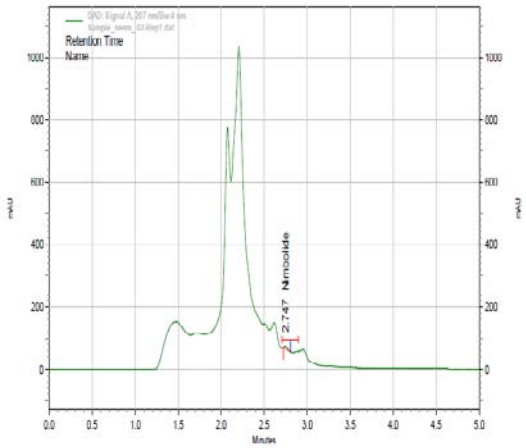

Fig. 12: Chromatogram of Market Formulation

Method validation: The method was validated as per the parameters like accuracy, precision, robustness, LOD, LOQ and system suitability by the following procedures:

Precision: The precision was examined by performing the intra-day and inter-day assays of six replicate injections.

The inter-day precision was assayed on two consecutive days by two different analysts. The percent relative standard deviation (% RSD) was calculated which is within the acceptable criteria of not more than 2.0. The intra-day and inter-day precision results are shown in Table 3 and Table 4 respectively.

Table 3: System Precision

S. No.	Injection	Retention Time	Peak Area	Average	SD	%RSD
1	Injection 1	2.887	6859292	68896545	51657.6504	0.75
2	Injection 2	2.887	6866262			
3	Injection 3	2.880	6874227			
4	Injection 4	2.880	6865277			
5	Injection 5	2.887	6878709			
6	Injection 6	2.880	6994160			

Table 4: Intermediate Precision

S. No.	Analyst	Retention Time	Area 1	Area 2	Average Area	SD	%RSD*
1	1	2.82	7155900	7149312	7152606	131501.71	1.81
2	2	2.81	7332256.00	7344899	7338578		

*Acceptance criteria < 2

Table 5: LOD LOQ

S. No.	Sample	Retention Time	Peak Area	Average	%RSD	LOD	LOQ
1	Injection 1	2.887	219219	219474	0.336	0.007	0.021
2	Injection 2	2.887	219269				
3	Injection 3	2.880	220144				
4	Injection 4	2.887	218253				
5	Injection 5	2.887	220276				
6	Injection 6	2.887	219683				

Table 6: Assay results of Nimbolide Formulation

Formulation	Labeled Amount	Amount found	% Assay
Neem capsule (Himalaya Herbals)	250mg/cap	247.43 mg/cap	98.972

Table 7: Optimized chromatographic conditions and system suitability parameters of Proposed-HPLC method for Nimbolide

Parameters	Chromatographic Conditions
Instrument	Agilent 1200 series LC system
Column	250 mm X 4.6 mm X 2.5 µ
Detector	UV detector
Mobile phase	90:10 (Acetonitrile-water)
Flow rate	1 ml/min
Run time	5 min
Temperature	Ambient temperature
Volume of Injection loop	10 µl
Retention time	2.880 min
Theoretical Plates	9098
Trailing Factor	1.46

Limit of detection and limit of quantification

The LOD and LOQ were determined by kD/S where k is constant (3.3 for LOD and 10 for LOQ), SD is the standard deviation of the analytical signal and s is the slope of the concentration/response graph. The LOD and LOQ values are presented in Table 5. The results of LOD and LOQ supported the sensitivity of the developed method.

Specificity:: The specificity of the method was determined by observing interference of any encountered ingredients present in the formulations. The test results obtained were compared with the results of those obtained for standard drug. It was shown that those ingredients were not interfering with the developed method. Furthermore the well-shaped peaks also indicate the specificity of the method. The results for specificity are tabulated in Table 8. The chromatogram for placebo indicating the specificity of developed method is presented in Fig. 11.

Robustness: The robustness test aimed to examine the sources potentially subject to variations through evaluation of one or a set of responses inherent to the method. In this sense, the analyst, the wavelength (± 2) the column temperature (15 and 20°C) and the flow rate (0.9 and 1.1 ml/ min) were deliberately altered. Mean and SD were calculated for each peak and % RSDs was calculated for each component during each change. The results for robustness study in Table 9 indicated that the small change in the conditions did not significantly affect the determination of Nimbolide

Accuracy

The accuracy of the method was determined by calculating the recoveries of Nimbolide by the method of standard addition. A known amount of standard (50%, 100% and 150%) was added to pre analyzed sample solution and the amount of the standard was estimated by measuring the peak areas and by fitting these values to the straight-line equation of calibration curve. The recovery results for accuracy study of Nimbolide are presented in Table 10.

System suitability

The chromatographic systems used for analysis must pass the system suitability limits before sample analysis. Set up the chromatographic system; allow the HPLC system to stabilize for 30 min. Inject blank preparation (single injection) and standard preparation (at least six replicates) and record the chromatograms to evaluate the system suitability parameters like tailing factor [NMT 1.5], theoretical plate count [NLT 3000] and % RSD for peak area of six replicate injections of Nimbolide standard [%RSD NMT 2.0]. The system suitability data is reported in Table 11.

RESULT AND DISCUSSION

A simple HPLC method was adopted for the determination of Nimbolide in herbal formulation. To optimize the proposed HPLC method, all of the experimental conditions were investigated. To optimize the mobile phase, different systems were tried for chromatographic separation of the components. The best resolution was achieved using a mobile phase consisting of acetonitrile - water in the ratio of 90:10 v/v, which gave satisfactory result with sharp well defined and resolved peak with minimum tailing. The absorption spectrum of Nimbolide is shown in Fig. 2. The wavelength 207 nm was used for quantification of sample. The calibration curves (n=3) constructed for the Nimbolide were linear over the concentration range 3.125-200 ppm/ml of Nimbolide. Peak areas of the Nimbolide were plotted versus the concentration and linear regression analysis performed on the resultant curve.

The coefficients of determination 0.9997 for Nimbolide with % RSD values ranging from 0.1245 to 2% across the concentration range studied were obtained following linear regression analysis Table 2.

Table 8: Specificity Study for Nimbolide

Name of the solution	Retention time
Mobile phase	No Peak
Placebo	No Peak
Nimbolide 25 ppm/ml	2.880 min.

Table 9: Robustness

S. No.	Conditions Varied	Changed Conditions	Retention Time	Peak Area	Theoretical Plates*	Tailing Factor	SD	%RSD
1	Wavelength (nm)	205	2.88	7035815	9319	1.37	9169.39	0.14
		207	2.88	7245592	9326	1.30	131501.7	0.18
		209	2.88	7128595	9349	1.44	9051.05	0.13
2	Temperature (˚C)	15	2.92	7828836.67	8329	1.26	104813.05	1.26
		20	2.88	7245592	9326	1.30	131501.7	0.18
		25	2.89	7485205	8959	1.45	8762.33	1.37
3	Flow Rate (ml/min)	0.9	3.2	7841481	9954	1.34	6189.99	0.08
		1.0	2.88	7245592	9326	1.30	131501.7	0.18
		1.1	2.62	6653009	8825	1.30	619.06	0.01

* Plate count > 3000

Table 10: Recovery Study of Nimbolide added to preanalysed sample using the Proposed HPLC method (n=3)

% Concentration (at specification level)	Area	Amount added (mg)	Amount found (mg)	% recovery	Mean% recovery	%RSD
50	1647387	1.0	0.98	98.00		0.23
100	3467234	2.0	1.97	98.50	98.27	0.34
150	5015587	3.0	2.95	98.33		0.19

Table 11: System Suitability

Parameters	Nimbolide
Linearity range (ppm)	3.125 to 200
Slope	35426.1822
Intercept	-51970.295
Correlation coefficient (r^2)	0.9997
Theoretical plate	9098.0
Retention time (min)	2.88
Tailing factor	1.46
% RSD	0.75

The precision result of the solution at medium concentration is presented in Table 4, and it was shown that the RSD values of retention time were less than 1%, while the RSD values of peak area were less than 2 % both for intra-day assay and inter-day assay precision. The LOD and LOQ were found to be 0.007 ppm/ml and 0.021 ppm/ml respectively for Nimbolide.

The robustness study indicated that selected factor remained unaffected by small variation of these parameters. To ensure the insensitivity of the developed HPLC method to minor changes in the experimental conditions, it is important to demonstrate its robustness. None of the alterations caused a significant change in, retention time and theoretical plates.

A system suitability test was performed to evaluate the chromatographic parameter (retention time, number of theoretical plate and tailing factor) before the validations run (Table 11). The accuracy was studied by the standard addition technique. Three different levels of standard were added to the previously analyzed samples, each level being repeated thrice. The percentage recovery of Nimbolide was 98.27 in herbal formulation as shown in Table 10.

CONCLUSION

The developed HPLC method has various advantages like less retention time, good peak symmetry and phenomenal linearity, highly sensitive, simple, precise and robust for the analysis of Nimbolide. Since the proposed mobile phase effectively resolves nimbolide, the method can be used for qualitative as well as quantitative analysis of nimbolide in herbal formulations. The drug solutions employed in the study were stable up to 48 hours. These attribute the high quality of the method. The RSD values for all parameters were found to be less than 2, which indicates the validity of method and results obtained by this method is with fair agreement. The proposed method can be used for the routine analysis of Nimbolide in bulk preparations of the drug and in pharmaceutical dosage forms for routine application in quality control laboratories.

CONFLICT OF INTERESTS

Declared None

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