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ORIGINAL ARTICLE

Development and validation of stability indicating RP-HPLC and HPTLC for determination of Niclosamide in bulk and in synthetic mixture

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KEYWORDS

RP-HPLC;
HPTLC;
Niclosamide;
Stability indicating;
Validation;
Statistical comparisons

Abstract Two simple, specific, sensitive, accurate and precise stability indicating methods were described for quantitative determination of the anthelmintics drug Niclosamide. The first method was high performance liquid chromatographic with the use of a reversed phase hibar^R C-18 column (250 mm × 4.66 mm, 5 μm) and mobile phase of methanol: 1 mM ammonium phosphate buffer (85:15 v/v) at a flow rate of 1.2 mL/min. The retention time of drug was found to be 6.45 ± 0.02 min. Quantification of drug was achieved with diode array detection (DAD) at 332 nm. Linear calibration curve was obtained in concentration range 0.01–100 μg/mL with r^2 value of 0.999. The limit of detection and limit of quantification were found to be 0.048 μg/mL and 0.01 μg/ml respectively. The second method involved a high performance thin layer liquid chromatographic. Chromatographic separation was carried out with precoated silica gel G60 F254 aluminum sheets using toluene:ethyl acetate (7:3% v/v) as a mobile phase. Linearity of proposed method was found to be 200–700 ng/band at 332 nm with retention factor of 0.59 and r^2 value of 0.998. The limit of detection and limit of quantification were found to be 36.21 ng/band and 109.7 ng/band respectively. Both the developed methods were successfully validated as per International Conference on Harmonization guideline (ICH). Niclosamide was subjected to different stress conditions. The degraded product peaks were well resolved from the pure drug peak with significant difference in their retention time. Stress samples were successfully assayed by developed high

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performance liquid chromatographic and high performance thin layer liquid chromatographic method. Statistically analysis proves that there were no statistical significant differences between two developed methods.

1. Introduction

Niclosamide (NIC) chemically is 2', 5-dichloro-4' – nitrosalicylanilide and used as anthelmintics. It is used for treatment of tapeworm and intestinal fluke infection. NIC killing tapeworm by uncoupling of oxidative phosphorylation or stimulation of ATPase activity (Tripathi, 2010). The literature survey revealed several analytical methods for estimation of NIC from bulk and different pharmaceutical formulations. (Van Tonder et al., 1996; Cholifah et al., 2007; San-xia et al., 2013).

After extensive literature survey several methods have been found for determination of NIC including determination in pure form or in combination with other drugs. Determinations of NIC with thiabendazole (Onur and Tekin, 1994) and drotaverine hydrochloride (Daabees, 2000) by spectrophotometric method, and by spectrofluorimetric (Algarra et al., 2012) have been reported. Several chromatographic methods have been reported including HPLC (Schreier et al., 2000; Caldow et al., 2009), and GC (Churchill and Ku, 1980; John and Geoffrey, 1980). The electrochemical methods have been reported for the determination of Niclosamide based on square-wave voltammetry (Alemu et al., 2003), or by cyclic voltammetry at a glassy carbon electrode (Abreu et al., 2002), or modified electrode for electro-catalytic reduction of Niclosamide (Ghalkain and Shahrokhian, 2010).

As per reported literature no stability indicating HPLC and HPTLC methods were available for determination of NIC, so it was thought worthwhile to develop stability indicating HPLC and HPTLC methods for determination of NIC from bulk and synthetic mixture. Stability testing involves forced degradation or stress-studies indicating hydrolysis, oxidation, photolytic and thermal degradation, etc. Stability indicating methods are developed to supervise the stability of drug substance and pharmaceutical dosage forms for the duration of the early phase of medicine development, and once the medicine is entered to the marketed, for the continuing product stability studies which must be performed as per ICH or regulatory guidelines. The reason of stability studies testing is to give evidence on how the quality of drug differs with moment under the influence of a multiplicity of ecological factors such as humidity, temperature and light, enables suggested storage conditions, re-analysis intervals and shelf life to be recognized. Once the analytical method (Ivan et al., 2013) was developed it is to be validated according to regulatory guideline. In present study both the developed methods were validated as per ICH guideline and were used for estimation of NIC under stressed conditions. Both the developed methods successfully quantify the NIC in the presence of degradant product without any interference.

2. Experimental

2.1. Materials and methods

NIC was obtained from Prudence Pharma Chem., Ankleshwar as gift sample. All chemicals and reagents such as methanol, ammonium dihydrogen phosphate, hydrochloric acid, sodium hydroxide, and hydrogen peroxide solution used were of HPLC grade and were purchased from Merck Chemicals, India.

2.1.1. HPLC instrumentation and chromatographic conditions

The method was developed using Shimadzu HPLC-2010 instrument equipped with photodiode array detector. The hibar^{R} C-18 column (250 mm × 4.66 mm, 5 μm) was used as stationary phase. The mobile phase consisted of methanol:ammonium phosphate buffer (85:15 v/v) with pH 5.47 and was pumped at a flow rate of 1.2 mL/min. The mobile phase was filtered though a membrane filter of 0.22 μm. The elution was monitored at 332 nm and the injection volume was 20 μL.

2.1.2. HPTLC instrumentation and chromatographic conditions

The method was developed using Camag – HPTLC instrument using UV detector. The sample was spotted in the form of bands of width 6 mm with Camag microliter syringe on precoated silica gel aluminum plate G60 F254 purchased from Merck, Germany using Camag Linomat V (Switzerland). The space between two bands was 15 mm and the slit dimension was kept 5 mm × 0.45 mm micro. The mobile phase was consisted of toluene: ethyl acetate (7:3 v/v). Linear ascending development was carried out in the Camag twin though glass chamber saturated with mobile phase. The optimized saturation time for mobile phase was 25 min at room temperature (25 °C ± 2). TLC plates were dried in the current of air with help of an air dryer. Densitometric scanning was performed using Camag TLC scanner in the absorbance mode at 332 nm. The source of radiation utilized was deuterium lamp emitting continuous UV spectrum in the range of 190–400 nm.

2.1.3. Preparation standard stock solution

10 mg standard NIC was accurately weighed and transferred in 10 mL volumetric flask and dissolved in 10 mL methanol. The volumetric flask was sonicated for 10 min. From the above stock solution 1 mL of solution was taken and transferred to 10 mL volumetric flask and volume was made up to the mark with 10 mL methanol.

2.1.4. Preparation of test solution from synthetic mixture

Powder equivalent to 25 mg of NIC was weighed and transferred to 50 mL volumetric flask. Methanol was used as solvent and final volume was made with methanol to achieve concentration of 500 μg/mL. From the above stock solution 2 mL of solution was withdrawn and transferred into 10 mL volumetric flask and volume was made with methanol to achieve final concentration 100 μg/mL.

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2.2. Method validation

2.2.1. Precision

Repeatability, intra-day and inter-day precision studies were carried out by estimating a corresponding responses three times on the same day and three times on different days for one concentration of NIC (1 μg/mL for HPLC and 500 ng/band for HPTLC) and results are reported in terms of % relative standard deviation.

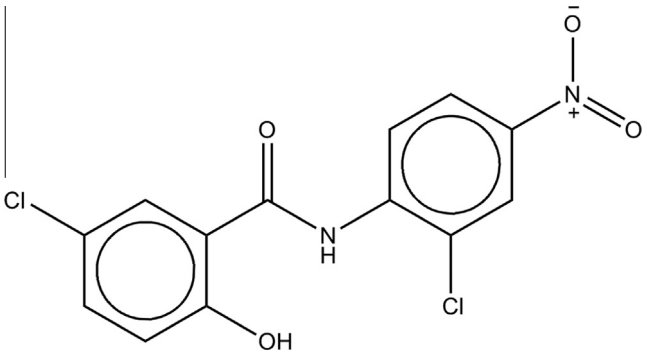

Figure 1 Structure of Niclosamide.

2.2.2. Accuracy

The accuracy of HPLC and HPTLC methods was determined by recovery study, carried out at three different concentrations (80%, 100%, and 120% test solution concentration). For each concentration three sets were prepared and % recovery was calculated.

2.2.3. Linearity

Calibration curve was constructed by plotting the peak area v/s concentration of NIC and regression equations were calculated for both the methods. The linearity of HPLC and HPTLC was determined by calibration curve, plotted over 5 and 6 different concentrations respectively. The linearity for HPLC was found to be 0.01–100 μg/mL whereas for HPTLC it was 200–700 ng/band.

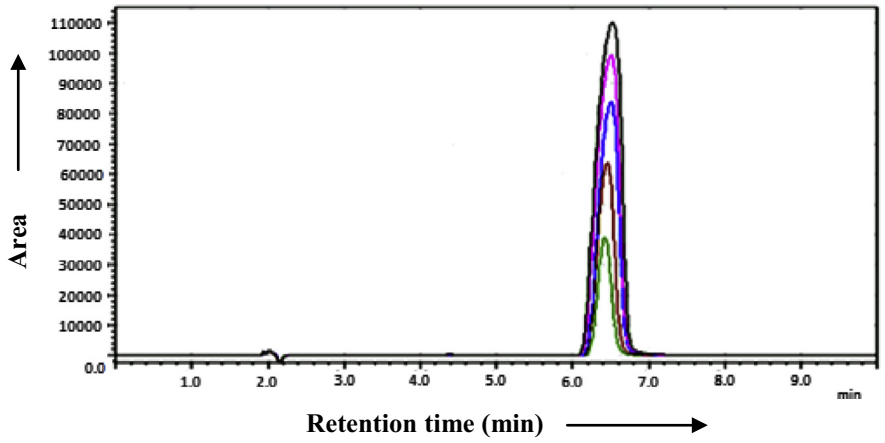

Figure 2 HPLC chromatogram for linearity of Niclosamide solution.

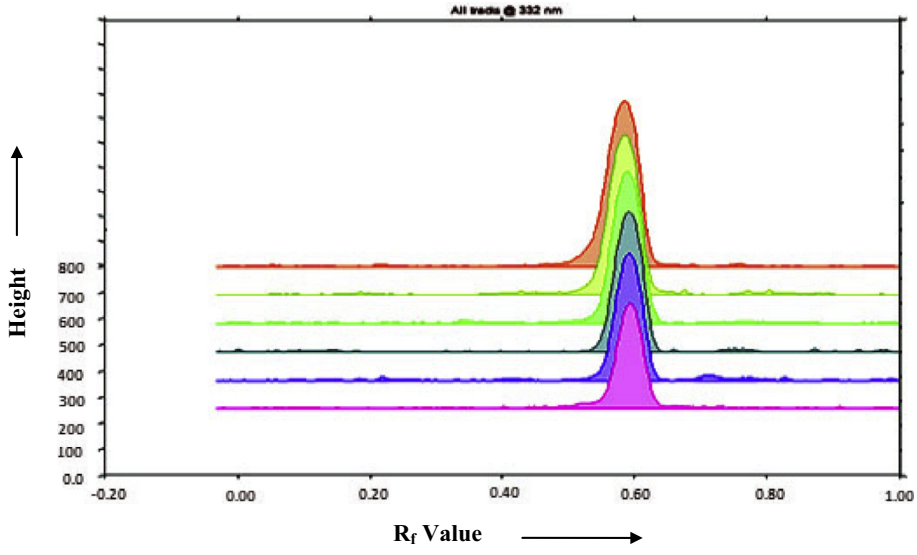

Figure 3 HPTLC chromatogram for linearity of Niclosamide solution.

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2.2.4. Limit of detection and limit of quantification

LOD and LOQ for both the developed methods were calculated using following Eq. (1) as per ICH guideline

$$\text{LOD} = 3.3\,\sigma/s \quad \text{and} \quad \text{LOQ} = 10\,\sigma/s \tag{1}$$

where the σ the standard deviation of the precision and s is the slope of the calibration curve.

2.2.5. Robustness

The robustness of HPLC method was studied by changing mobile phase composition (±2%), pH of mobile phase (±1), flow rate (±0.2 mL/min) and working wavelength (±2 nm). The robustness of HPTLC was studied by changing mobile phase composition (±1%), saturation time (±5 min) and wavelength (±2 nm). Robustness of both the developed methods was calculated in terms of % RSD.

2.2.6. Specificity

Specificity of the developed methods was checked by recording chromatogram of placebo and was compared with chromatogram of NIC. Specificity of both the developed methods was further studied by conducting the force degradation study, including acid hydrolysis, alkaline hydrolysis, photodegradation and thermal degradation. In all tested conditions, interference of degradation product was determined.

2.2.7. System suitability test

Analytical system performance before and/or during the analysis was evaluated by system suitability test. System suitability tests are an integral part of method development and are performed to evaluate the behavior of the chromatographic system such as capacity factor (k'), plate number (N) and tailing factor (T).

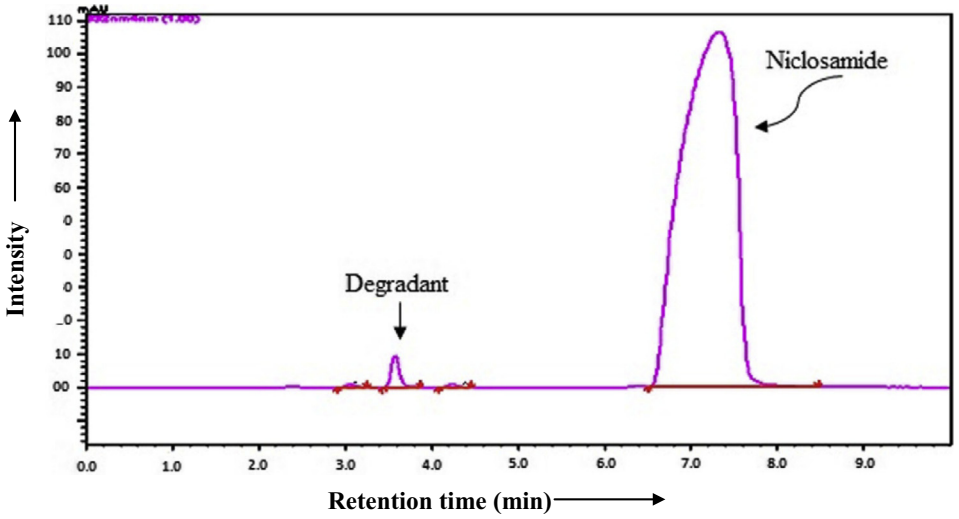

Figure 4 HPLC chromatogram of acid hydrolysis.

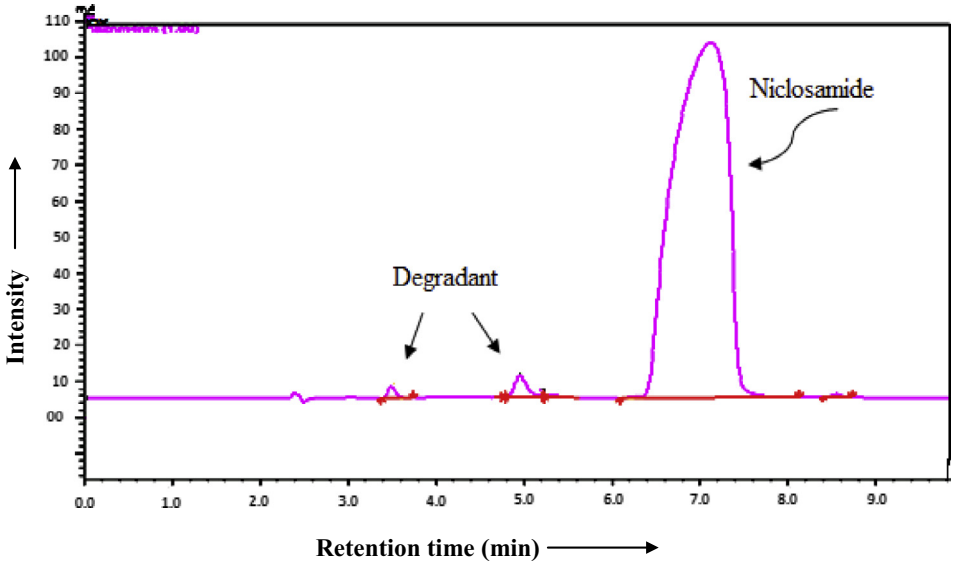

Figure 5 HPLC chromatogram of alkaline hydrolysis.

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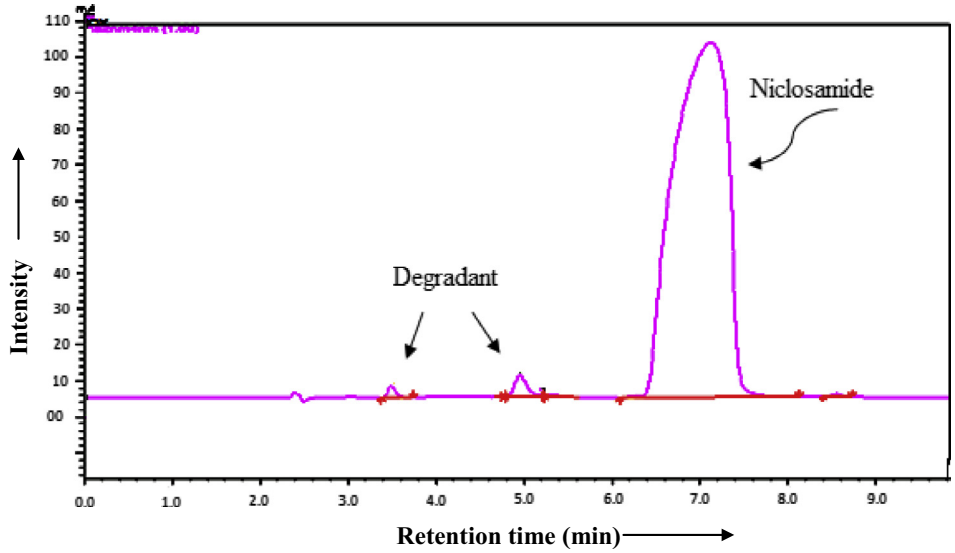

Figure 6 HPLC chromatogram of 10% H_2O_2.

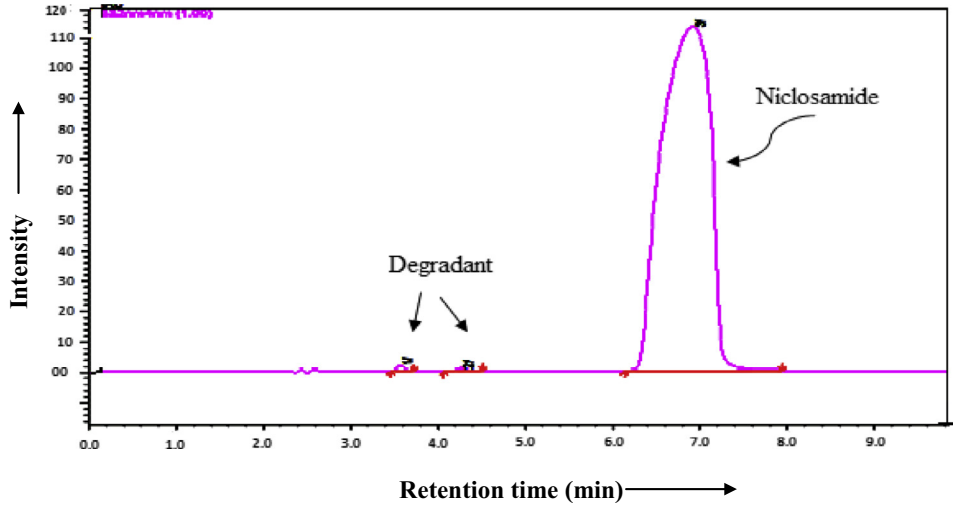

Figure 7 HPLC chromatogram of thermal degradation at 24 h.

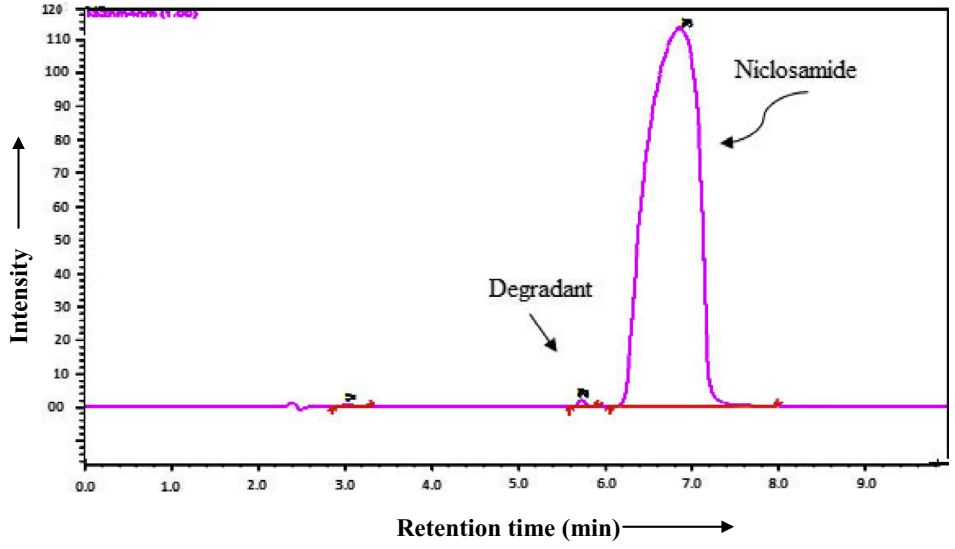

Figure 8 HPLC chromatogram of photodegradation at 254 nm for 24 h.

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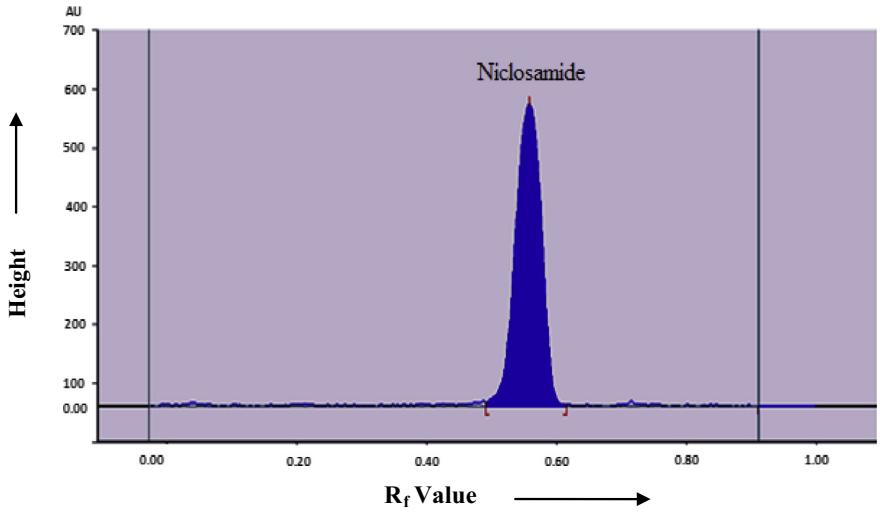

Figure 9 HTPLC chromatogram of 500 ng/band Niclosamide.

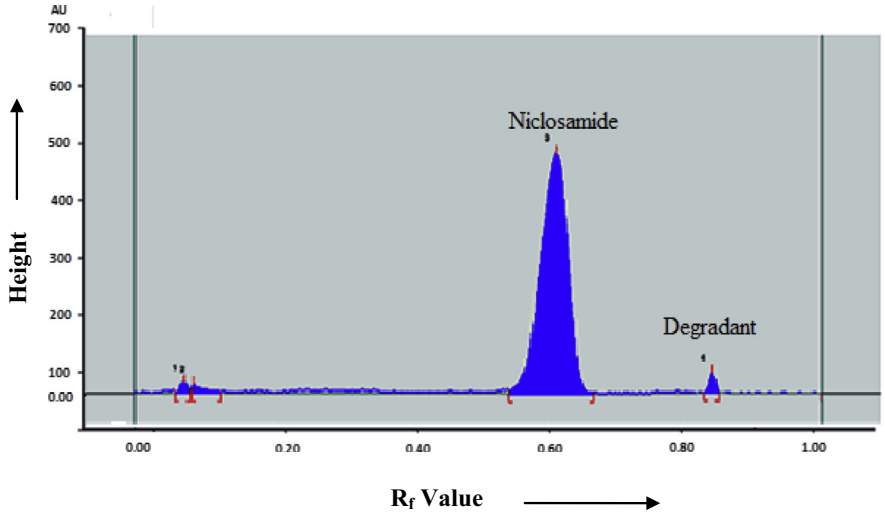

Figure 10 HPTLC chromatogram of acid hydrolysis.

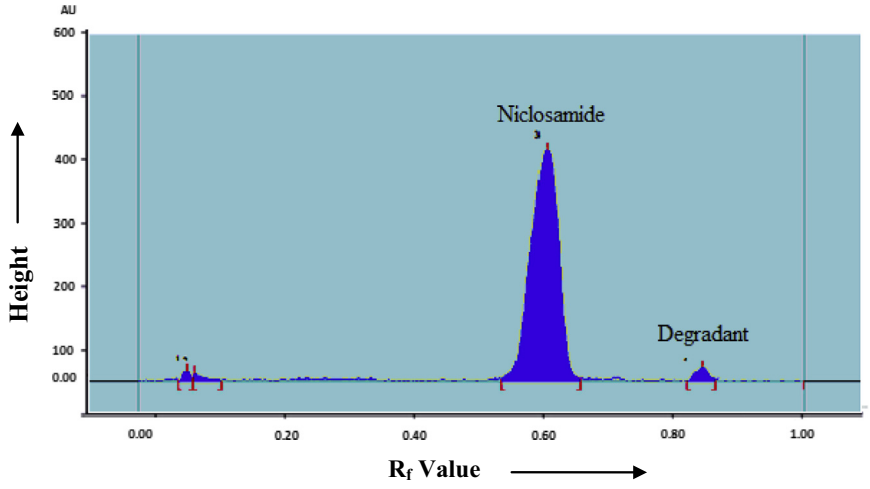

Figure 11 HPTLC chromatogram of alkaline hydrolysis.

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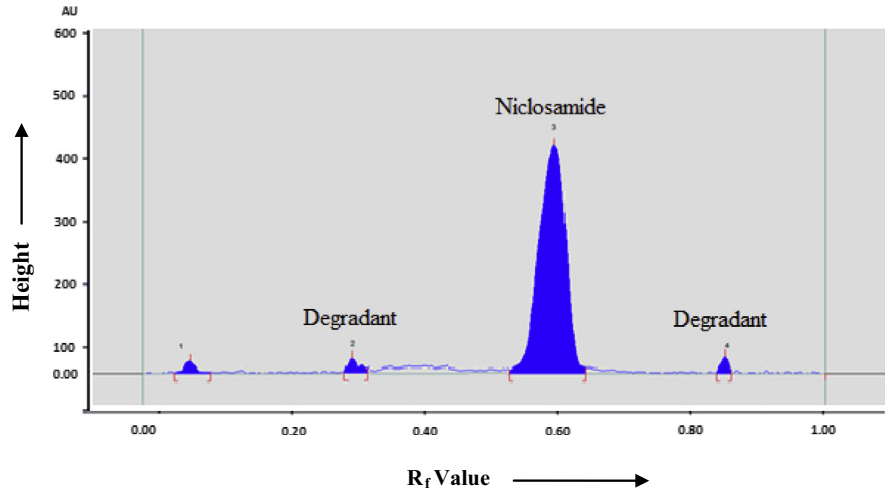

Figure 12 HPTLC chromatogram of 10% H_2O_2.

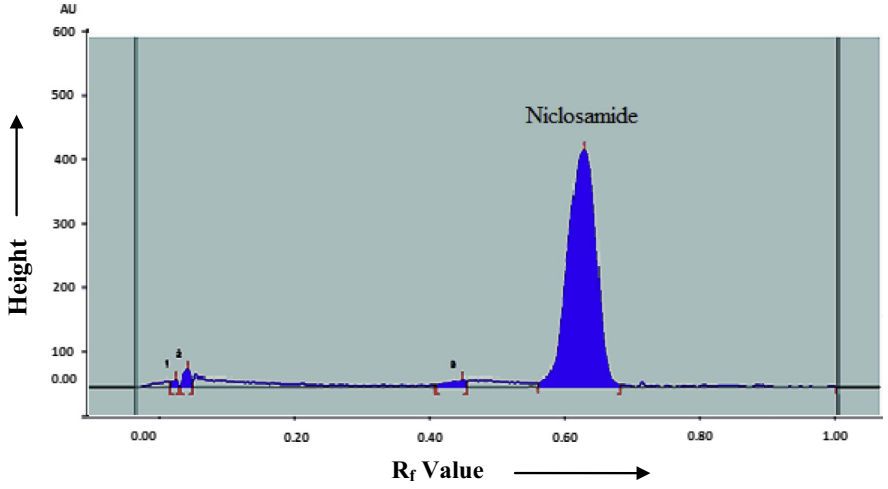

Figure 13 HPTLC chromatogram of thermal degradation at 24 h.

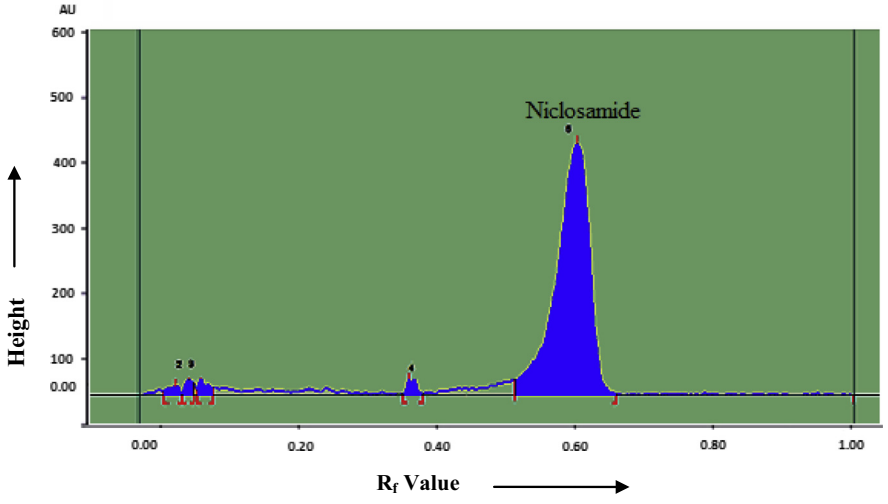

Figure 14 HPTLC chromatogram of photodegradation at 254 nm for 24 h.

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2.2.8. Solution stability

Stability of sample solution was studied at ambient temperature for 48 h.

2.3. Forced degradation studies

2.3.1. Acid degradation studies

10 mg of NIC was separately dissolved in 10 ml of 0.1 N HCl and kept for 24 h at room temperature in dark. From the above solution 1 mL was withdrawn and neutralized with 0.1 N NaOH and diluted to 10 mL methanol. Solution was analyzed by proposed HPLC and HPTLC method.

2.3.2. Alkali degradation studies

10 mg of NIC was separately dissolved in 10 ml of 0.1 N NaOH and kept for 24 h at room temperature in dark. From the above solution 1 mL was withdrawn and neutralized with 0.1 N HCl and diluted to 10 mL methanol. The prepared solution was analyzed by HPLC and HPTLC method.

2.3.3. Oxidative degradation studies

10 mg of drug was dissolved in 10 ml of 10% H_2O_2 in 10 ml volumetric flask. This solution was kept for 24 h at room temperature in dark. From the above solution 1 ml was withdrawn and make up with methanol up to 10 ml. The prepared solution was analyzed by HPLC and HPTLC method.

2.3.4. Thermal degradation studies

For thermal decomposition drug powder was kept at 70 °C for 24 h. From that powder solution having concentration of 100 µg/ml was prepared and analyzed for thermal degradation study. The prepared solutions were analyzed by HPLC and HPTLC method.

2.3.5. Photodegradation studies

A sample of drug was exposed to a near ultraviolet lamp in a UV Chamber. Drug was kept in petri dish for 24 h and solution having concentration of 100 µg/ml was prepared and analyzed for photolytic (UV Light) degradation study. The prepared solution was analyzed by HPLC and HPTLC method.

3. Result and discussion

3.1. Method development and optimization

Prime objective of development and validation of HPLC and HPTLC method for determination of NIC in single run and should be accurate, precise, reproducible, robust and stability indicating. All degradation products from stress conditions should be well separated from each other and method should be simple to useful for routine analytical work. Stability-indicating methods demonstrate the capability of the method for

Table 1 Degradation Niclosamide at different stress conditions at 24 h by HPLC and HPTLC.

Stress condition	Standard Niclosamide concentration		% Area	
	For HPLC (µg/mL)	For HPTLC (ng/band)	For HPLC	For HPTLC
0.1 N HCl	100	500	14.6	14.2
0.1 N NaOH	100	500	13.16	12.25
10% H_2O_2	100	500	16.41	15.92
Thermal 70 °C	100	500	10.58	10.6
UV(254 nm)	100	500	9.14	9.57

Table 2 Linearity data of NIC by HPLC and HPTLC.

Sr. no.	Concentration for HPLC (µg/mL)	Area for HPLC	Concentration for HPTLC (ng/band)	Area for HPTLC
1	0.01	2733	200	9880.94
2	0.1	9711	300	13005.93
3	1	61312	400	15561.88
4	10	557,410	500	17813.49
5	100	5,442,709	600	20395.40
6			700	23218.53

Table 4 Data of % recovery study of NIC by HPLC.

Recovery level (%)	Conc. of test sol. (µg/mL)	Conc. of std. sol. (µg/mL)	% Recovery	SD	% RSD
80	1	0.8	100.47	1.106	1.101
100	1	1	101.56	1.464	1.441
120	1	1.2	100.00	1.587	1.587

Table 5 Data of % recovery study of NIC by HPTLC.

Recovery level (%)	Conc. of test sol. (ng/band)	Conc. of std. sol. (ng/band)	% Recovery	SD	% RSD
80	300	240	99.36	0.481	0.484
100	300	240	100.41	1.039	1.035
120	300	240	99.80	0.654	0.655

Table 3 Data of precision of Niclosamide by HPLC and HPTLC.

Precision	Concentration		SD[a]		% RSD[b]	
	For HPLC (µg/mL)	For HPTLC (ng/band)	For HPLC	For HPTLC	For HPLC	For HPTLC
Repeatability	1	500	80.031	285.685	0.130	1.635
Intermediate	1	500	654.154	180.654	1.055	1.023
Reproducibility	1	500	665.116	185.784	1.075	1.062

[a] SD = standard deviation.
[b] RSD = relative standard deviation.

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the accurate determination of active ingredients without interference from possible degradation products, process impurities, excipients or other potential impurities (see Fig. 1).

For both the developed methods mobile phase selected with a view to best sensitivity and selectivity along with short elution time. In HPLC method the combination of methanol:ammonium phosphate buffer (85:15 v/v) resulted in high sensitivity, short analysis time and good peak symmetry (about 1.026) (Fig. 2). Among the different columns hibar^{R} 250-4.6 C-18 columns (250 mm × 4.6 mm i.d. with particle size of 5 μm) analytical column was selected, as it provided the best chromatographic separation and good peak characteristics. The detection was carried out with PDA detector at 332 nm, indicated good resolution of NIC from its degradant. Whereas for HPTLC method, precoated silica Gel G60 F254 aluminum sheets (10 × 10 cm) was used as stationary phase and toluene:

Table 6 Robustness parameter for NIC by HPLC method.

Sr. no.	Parameter	Normal condition	Variable 1	Variable 2
1	Wavelength	332 nm	330 nm	334 nm
	Area	492,457	496,829	499,832
		496,725	495,682	499,856
		497,832	496,542	498,562
	Average	495671.3	496,351	499416.7
	SD	2838.189	596.877	740.260
	% RSD	0.572	0.120	0.148
2	Flow rate	1.2 ml/min	1.0 ml/min	1.4 ml/min
	Area	499,535	692,959	559,086
		495,632	693,567	557,524
		496,853	695,824	554,852
	Average	497,340	694116.7	557,154
	SD	1996.554	1509.22	2141.113
	% RSD	0.401	0.217	0.384
3	pH	5.47	4.47	6.47
	Area	495,263	510,814	450,112
		498,526	512,856	452,145
		493,591	510,442	455,214
	Average	495793.3	511370.7	452490.3
	SD	2509.88	1299.714	2568.471
	% RSD	0.506	0.254	0.567

Table 7 Robustness parameter for NIC by HPTLC method.

Sr. no	Parameter	Normal condition	Variable 1	Variable 2
1	Wavelength	332 nm	330 nm	334 nm
	Area	17453.1	17516.8	17460.9
		17540.9	17548.2	17,567
		17428.2	17356.8	17397.7
	Average	17474.07	17475.2	17473.93
	SD	59.203	85.551	102.648
	% RSD	0.338	0.489	0.587
2	Mobile phase composition (v/v)	Toluene:ethyl acetate (7:3 v/v)	Toluene:ethyl acetate (6:4 v/v)	Toluene:ethyl acetate (8:2 v/v)
	Area	17580.3	17666.4	17321.07
		17421.78	17842.31	17560.74
		17456.81	17576.9	17592.48
	Average	17486.3	17695.2	17491.43
	SD	83.272	135.029	148.387
	% RSD	0.476	0.763	0.848
3	Saturation time	25 min	20 min	30 min
	Area	17657.6	17416.4	17149.8
		17444.23	17,633	17027.3
		17563.2	17,275	17176.6
	Average	17555.01	17441.47	17117.9
	SD	106.920	180.311	79.597
	% RSD	0.609	1.033	0.464

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ethyl acetate (7:3 v/v) as mobile phase with 25 min saturation time. Detection was carried out at 332 nm. Linearity of developed HPTLC method was mentioned in Figs. 3 and 9 shows a typical chromatogram obtained by the proposed HPTLC method. The retention factor was observed 0.59. Both the developed methods were validated according to ICH guideline for the analysis of NIC in bulk and in synthetic mixture.

3.2. Method validation

Stability indicating properties of HPLC and HPTLC methods was performed by forced degradations study. The results of stress testing indicated that the developed methods were highly specific in nature. The study data revealed that selected drug was unstable in acidic, basic and oxidative medium. In HPLC method, acidic stress led to 14.6% degradation with three unknown degradation peaks at 3.0, 3.8 and 4.3 min, respectively, whereas a prominent peak of NIC was stable at 6.45 min (Fig. 4). Alkaline stress led to 13.16% degradation with two unknown degradation peaks at 3.5 and 5.0 min, respectively, whereas a prominent peak of NIC was stable at 6.45 min (Fig. 5). Peroxide stress led to 16.41% degradation with three unknown degradation peak at 4.1, 5.1 and 9.3 min, whereas a prominent peak of NIC was stable at 6.45 min (Fig. 6). The force degradation studies by thermal and UV conditions of NIC resulted in a significant decrease of the peak area, 10.58% and 9.14% respectively, with detectable degradation product at 3.4 and 5.9 min (Figs. 7 and 8). In HPTLC method, acidic stress led to 14.2% degradation with one unknown degradation peaks at 0.82, whereas a prominent peak of NIC was stable at 0.59 (Fig. 10). Alkaline stress led to 12.25% degradation with one unknown degradation peaks at 0.83, whereas a prominent peak of NIC was stable at 0.59 (Fig. 11). Peroxide stress led to 15.92% degradation with one unknown degradation peak at 0.82, whereas a prominent peak of NIC was stable at 0.59 (Fig. 12). The force degradation studies by thermal and UV conditions of NIC resulted in a significant decrease of the peak area, 10.6% and 9.57% respectively, without detectable degradation product (Figs. 13 and 14). The results of degradation study of NIC at each stress condition were shown in Table 1.

Specificity of the analytical method is its ability to measure accurately and specifically the analyte of interest in the presence of sample matrix. In the present study, the ability of the methods to separate the drug from its degradation products without interference of other sample components indicated the specificity of the developed methods. Values of peak purity index were found to be higher than 0.9999 indicated that the proposed methods are specific.

The linearity of a method reveals the linear relationship of response against the selected concentration of the analyte. For HPLC method, linear correlation was obtained between peak areas and concentrations of NIC in the range of 0.01–l00 μg/mL. The following regression equation was found by plotting the peak area (y) versus the NIC concentration (x) expressed in μg/mL: $y = 54368x + 6605$. The correlation coefficient (r^2: 1) obtained for the regression line demonstrates the excellent relationship between peak area and concentration of NIC. For HPTLC method, linear correlation was obtained between peak areas and concentrations of NIC in the range of 200–700 ng/band. The following regression equation was found by plotting the peak area (y) versus the NIC concentration (x) expressed in μg/mL: $y = 26.03x + 4932$. The correlation coefficient (r^2: 0.998) demonstrated excellent relationship between peak area and concentration of NIC. Data of regression analysis are summarized in (Table 2). The precision, for both the developed methods was evaluated as repeatability and calculating the % RSD. Six determinations of the l μg/mL and 500 ng/band sample of NIC were performed on the same day and under the same experimental conditions for HPLC and HPTLC respectively and % RSD was found to be 0.130% and 1.635% respectively. The RSD values of repeatability study were found to be $<2\%$, indicated that the proposed methods are repeatable. The intermediate precision of HPLC and HPTLC methods was determined by analyzing one sample for six times on three different days (interday) and % RSD was found to be 1.055% and 1.023% respectively. The between-analysts precisions of developed methods were determined by calculating the % RSD for the analysis of one sample of the NIC by two different analysts. % RSD was found to be 1.075% for HPLC and 1.062% for HPTLC. The % RSD of intermediate precision for both the developed methods was found to be $<2\%$, which indicated that the proposed methods are reproducible (Table 3).

The accuracy of both HPLC and HPTLC methods was assessed by the standard addition method. Three replicate determinations were performed at three different levels. The recoveries were obtained in a range of 100.00–101.56% and 99.36–100.41% for HPLC and HPTLC respectively. (Tables 4 and 5) The high values indicated that the proposed HPLC and HPTLC methods are accurate. The LOD and LOQ were determined from slopes of linear regression curves. LOD and LOQ for HPLC method were found to be 0.0048 and 0.01 μg/mL, respectively, whereas for HPTLC, were found to be 36.217 ng/band and 109.7 ng/band, respectively. Both the

Table 8 System suitability parameters by HPLC.

System suitability parameters	Niclosamide	Standard value
Tailing factor	1.026	<2
Theoretical plates	3706.83	>2000
Capacity factor	2.24	2–10

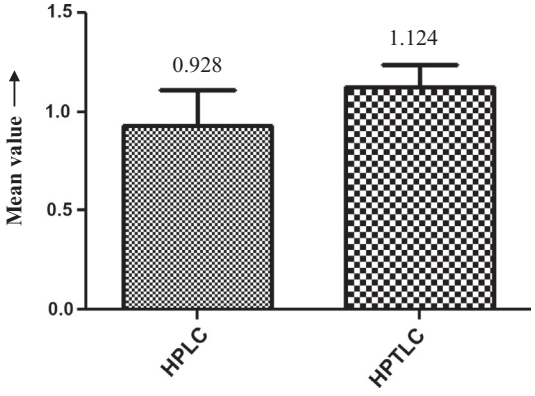

Figure 15 Comparison of unpaired t-test for HPLC and HPTLC method.

Table 9 Summary of results for stability indicating RP-HPLC methods.

Parameters	HPLC	HPTLC
λmax	332 nm	332 nm
Linearity	0.01–100 μg/mL	200–700 ng/band
Linearity equation	$y = 54368.16x + 6605.768$	$y = 26.03x + 4932$
Correlation coefficient (r^2)	0.999	0.999
Accuracy (n[a] = 3) (% RSD)	1.376	0.724
Precision (% RSD) (n = 6)		
Repeatability (% RSD)	1.300	1.635
Intraday precision (% RSD)	1.055	1.023
Interday precision (% RSD)	1.075	1.062
% Recovery	100.67	99.85
LOD[b]	0.0048 (μg/mL)	36.12 (ng/band)
LOQ[c]	0.01 (μg/mL)	109.7 (ng/band)

[a] n = number of determination.
[b] LOD = limit of detection.
[c] OQ = limit of quantification.

Table 10 Data of unpaired *t*-test (two tailed).

Parameter	Column 1	Column 2
Mean	0.928	1.124
Observation	7	7
Df	12	
T Stat	0.909	
P (*T* <= *t*) two-tail	0.381	

developed methods were evaluated for robustness (Tables 6 and 7). There were no significant changes were observed in the chromatographic pattern when the modifications were made in the experimental conditions, indicated that method was robust. The stability of sample solutions was tested at intervals of 12 h, 24 h, and up to 48 h. The methods were found to be rugged as there was no change in the peak area was found for NIC in both the developed methods. The system suitability test results were within the acceptable range as shown in (Table 8), indicated that the system is suitable for the intended analysis. Both the developed methods were statistically evaluated by unpaired *t*-test, demonstrated that the *P*-value = 0.3811 > 0.05 indicated that there were no statistical significant differences in between two methods for analysis of NIC (Fig. 15) (see Tables 9 and 10).

4. Conclusion

This study presents simple and validated stability indicating RP-HPLC and HPTLC methods for estimation of NIC in the presence of degradation products. The developed methods are sensitive, specific, rapid, robust, precise and accurate. All the degradation products were well separated from the analyte peak demonstrating that the developed methods were specific and stability indicating. Statistically analysis proves that there were no statistical significant differences in between two developed methods. Developed methods can be used as quality-control tool for routine quantitative analysis of NIC.

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