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METHOD DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF NIFEDIPINE AND LIGNOCAINE IN BULK FORM

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ABSTRACT

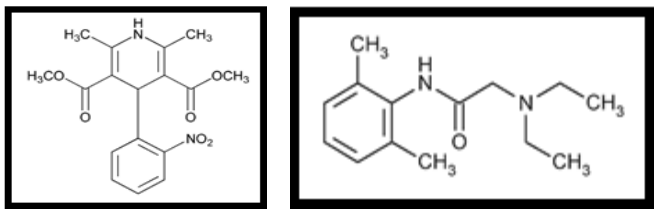
A simple, specific, precise and accurate stability indicating RP-HPLC method was developed and validated for simultaneous estimation of Nifedipine and Lignocaine in their bulk form that are used as Calcium Channel Blocker and Local Anesthetic respectively. The separation was achieved by using Inertsil C18, ODS column and mobile phase with a ratio of 75:25% v/v (Methanol :Acetonitrile) was fixed due to good symmetrical peaks and for good resolution at a flow rate of 1.0 ml/min and column oven temperature of 25oC. The instrument used was WATERS HPLC, Separation module 2690, photo diode array detector 996, Empower –software version-2. The retention times was found to be 3.305min for Nifedipine and 5.828min for Lignocaine. Analytical validation parameters such as specificity, linearity, system suitability, accuracy, precision, robustness, ruggedness, limit of detection and quantification (LOD & LOQ) were evaluated as per ICH guidelines. The bulk form of drug was subjected to degradation studies. The proposed method can be used for routine analysis.

KEYWORDS: *Nifedipine, Lignocaine, RP-HPLC, Method development and Validation, LOD & LOQ, Degradation Studies.*

INTRODUCTION

Nifedipine is a dihydropyridine calcium channel blocker with a chemical formula $C_{17}H_{18}N_2O_6$ and molecular weight 346.335 gm/mol. It is chemically 3,5-dimethyl-2,6-dimethyl-4(2-nitrophenly)1,4-dihydro pyrdine-3,5dicarboxylate, which is freely soluble in acetone and chloroform, sparingly soluble in ethanol and practically insoluble in water.

Lignocaine is a carboxylic acid derivative with a chemical formula $C_{14}H_{22}N_2O$ and molecular weight 234.34 gm/mol. It is chemically 2 (diethylamino)-N-(2,6-dimethylphenyl) acetamide, which is soluble in water, chloroform, ethanol and benzene [6].



(A) (B)

Fig. 1: Structure of Nifedipine (A) & Structure of Lignocaine (B)

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Mechanism of action of Nifedipine and Lignocaine:

Nifedipine is a Calcium Channel blocker and a vasodilator which is effective in reducing anal resting pressure and in healing chronic anal fissures. Lignocaine is a local anesthetic used to relive pain of anal fissures. Both drugs shows complementary action in reducing post symptomatic pain after haemorrhoidectomy [7-14].

MATERIALS AND METHODS

Instruments: HPLC WATERS 2690, Software: Empower-2, Detector: PDA 996, Inertsil ODS Column, Fast Clean Sonicator, Lab India pH Meter, Sartorious Electronic Balance.

Chemicals: Nifedipine, Lignocaine, HPLC Grade Methanol, Acetonirtile & Water

HPLC Method Development:

The RP-HPLC method development for the estimation of Nifedipine and Lignocaine was optimized by several trials for various parameters like different columns, flow rate and mobile phases; finally the following chromatographic conditions were set and the method was optimized.

Preparation of Standard Stock Solution:

Weight accurately 10 mg of Nifedipine and 10 mg of Lignocaine and transfer it separately into 10ml of volumetric flasks and dissolve in small amount of methanol as mobile phase and make up the mark with mobile phase.

Preparation of Working Standard Solution:

From the above stock solution pipette out 0.4ml of Nifedipine and 0.4ml of Lignocaine into 10ml of volumetric flask and makeup the mark with methanol as mobile phase. This solution is used for recording Chromatogram.

Table No: 1 Optimized Chromatographic Conditions for Simultaneous Estimation of Nifedipine and Lignocaine by RP-HPLC

Mobile Phase	Methanol:Acetonitrile
Ratio	75:25
Column	Intersil C_{18} ODS
Flow Rate	1.0 ml/min
Column Temperature	Room Temperature (20-25°C)
Sample Temperature	Room Temperature (20-25°C)
Wavelength	210 nm
Injection Volume(µl)	20µl
Runtime (minutes)	10 min
Retention Time	3.305 min for Nifedipine and 5.828 min for Lignocaine

Method Validation:

System Suitability:

Standard solutions were prepared as per the test method and injected in to chromatographic system. The system suitability parameters like theoretical plates, resolution and asymmetric factor were evaluated.

Specificity:

It is the ability to measure accurately and specifically the analyte in the presence of components that may be expected to be present in the sample matrix. Solutions of standard and sample were prepared as per the test method and are injected into chromatographic system. The specificity was also performed by injecting blank.

Linearity:

A Series of solutions are prepared using Nifedipine and Lignocaine working standards at concentration levels from 20ppm to 80ppm of target concentration. Measure the peak area response of solution at Level 1 and Level 6 six times and Level 2 to Level 5 two times.

Accuracy:

Accuracy of the method was determined by Recovery studies. Drug Assay was performed in triplicate as per test method with equivalent amount of Nifedipine and Lignocaine into each volumetric flask for each spike level to get the concentration of Nifedipine and Lignocaine equivalent to 50%, 100%, and 150% of the labeled amount as per the test method. The average % recovery of Nifedipine and Lignocaine were calculated.

Precision:

1.Repeatability:

a. System precision: Standard solution were prepared as per the test method and injected five times.

b. Method precision: Prepare sample preparations of Nifedipine and Lignocaine as per the test method and inject six times in to the column.

2.Intermediate Precision (Analyst to Analyst Variability):

A study was conducted by two analyst as per the test method.

Ruggedness of test method:

System to system variability:

System to system variability study was conducted on different HPLC systems, under similar conditions at different times. Six samples were prepared and each was analyzed as per test method. Comparison of both the results obtained on two different HPLC systems, shows that the assay test method are rugged for System to system variability.

Robustness:

Effect of variation in flow rate:

A study was conducted to determine the effect of variation in flow rate. Standard solution were prepared as per the test method and was injected into the HPLC system at a flow rates, 1.0ml/min and 1.2ml/min. The system suitability parameters were evaluated and found within the limits for 1.0ml/min and 1.2ml/min flow.

Limit of Detection and Quantification (LOD & LOQ):

From the linearity data calculate the limit of detection and quantification, using the following formula:

$$LOD = \frac{3.3\,\sigma}{S}$$

$$LOQ = \frac{10\,\sigma}{S}$$

Where;

σ = Standard Deviation of the response

S = Slope of the calibration curve of the analyte

Stability Indicating RP-HPLC Method for Simultaneous Estimation of Nifedipine and Lignocaine: [1-5]

Degradation studies:

Oxidation: To 1 ml of stock solution of Nifedipine and Lignocaine, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60°C. For HPLC study, the resultant solution was diluted to obtain 40µg/ml & 40µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Acid Degradation: To 1 ml of stock solution Nifedipine and Lignocaine, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60°c .The resultant solution was diluted to obtain 40µg/ml&40µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample.

Alkali Degradation: To 1 ml of stock solution Nifedipine and Lignocaine, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60°c. The resultant solution was diluted to obtain 40µg/ml&40µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Dry Heat Degradation Studies: The standard drug solution was placed in oven at 105°c for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 40µg/ml&40µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

Photo Stability studies: The photochemical stability of the drug was also studied by exposing the 40µg/ml&40µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 40µg/ml&40µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample.

Neutral Degradation Studies: Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 40µg/ml&40µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample.

RESULTS

Optimized chromatographic conditions for estimation of Nifedipine and Lignocaine by RP-HPLC method:

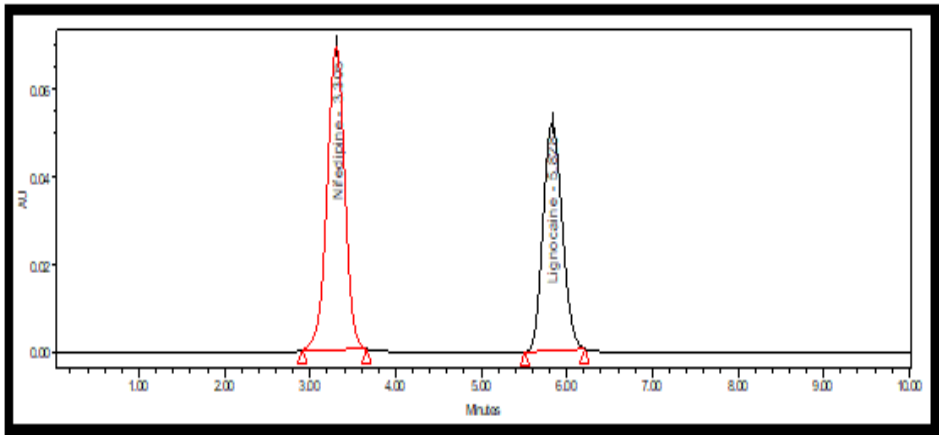


Fig. 2: Chromatogram showing optimized trial injection

Observation: Peak shape was good and the efficiency was more than 2000 with a resolution between two peaks >1.

Validation Parameters:

System Suitability:

Table No. 2: Data of System Suitability for Nifedipine

Injection	RT	Peak Area	USP Plate count	USP Tailing
1	3.300	3348846	9023.845712	1.14721
2	3.302	3348675	9010.547812	1.13384
3	3.302	3349834	9036.874214	1.18742
4	3.302	3348978	9027.254178	1.16547
5	3.301	3348226	9084.658952	1.17485
Mean	3.3014	3348801	9036.825471	1.1852313
SD	0.000894	591.3275	-------	-------
% RSD	0.027092	0.0176	-------	-------

Table No. 3: Data of System Suitability for Lignocaine

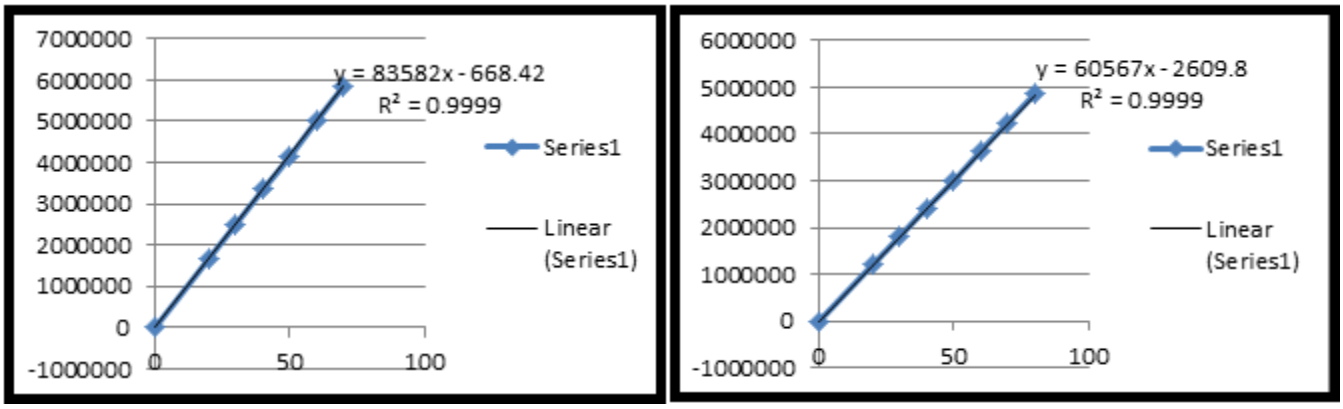
Injection	RT	Peak Area	USP Plate count	USP Tailing
1	5.821	2424582	8325.874512	1.284572
2	5.824	2424990	8384.547862	1.254872
3	5.821	2424001	8314.875424	1.278451
4	5.816	2423875	8372.784518	1.287451
5	5.818	2425012	8392.084512	1.298745
Mean	5.820	2424524	8358.8754210	1.255471
SD	0.003082	485.6573	-------	-------
% RSD	0.052959	0.020	-------	-------

Acceptance criteria:

1. The % RSD for the retention times of Nifedipine and Lignocaine Peaks from 5 replicate injections of each Standard solution should be not more than 2.0 %
2. The number of theoretical plates (N) for the Nifedipine and Lignocaine peaks should not be less than 3000.
3. The Tailing factor (T) for the Nifedipine and Lignocaine peak is not more than 2.0.

Observation: The % RSD for the retention times and peak area of Nifedipine and Lignocaine were found to be less than 2%. The plate count and tailing factor results were found to be satisfactory and are found to be within the limit.

Linearity:



(A) **(B)**

Fig. 3: Linearity Plot (Concentration v/s Response) of Nifedipine (A) & Lignocaine (B)

Table No. 4: Data of Linearity of Nifedipine

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	83582
20	1674311	y-Intercept	-668.4
30	2511466	Correlation Coefficient	0.999
40	3348621		
50	4145002		
60	5022932		
70	5860087		
80	6697242		

Table No. 5: Data of Linearity of Lignocaine

Concentration (ppm)	Average Area	Statistical Analysis	
0	0	Slope	60567
20	1212548	y-Intercept	-2609
30	1818358	Correlation Coefficient	0.999
40	2424865		
50	2993456		
60	3636458		
70	4242689		
80	4849487		

Acceptance criteria:

The relationship between the concentration of Nifedipine and Lignocaine and area of Nifedipine and Lignocaine should be linear in the specified range and the correlation coefficient should not be less than 0.999.

Observation:

The correlation coefficient for linear curve obtained between Concentration vs Area for Standard preparations of Nifedipine and Lignocaine is 0.999. The relationship between the Concentration of Nifedipine and Lignocaine and Area of Nifedipine and Lignocaine is linear in the range examined since all points lie in a straight line and the correlation coefficient is well within limits.

Accuracy:

Table No. 6: Data of Accuracy for Nifedipine

Concentration % of spiked level	Area	Amount added(ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50%	1674301	20	20.04	100.20	**MEAN**	100.19
	1674008	20	20.03	100.18		
	1674233	20	20.03	100.19	**%RSD**	0.009
100 %	3348571	40	40.07	100.17	**MEAN**	100.17
	3347789	40	40.06	100.15		
	3348518	40	40.07	100.17	**%RSD**	0.013
150%	5022964	60	60.10	100.17	**MEAN**	100.17
	5022835	60	60.10	100.17		
	5022856	60	60.10	100.17	**%RSD**	0.0013

Table No. 7: Data of Accuracy for Lignocaine

Concentration % of spiked level	Area	Amount added(ppm)	Amount found (ppm)	% Recovery	Statistical Analysis of % Recovery	
50%	1211548	20	20.04	100.23	**MEAN**	100.28
	1212002	20	20.05	100.27		
	1212984	20	20.07	100.35	**%RSD**	0.060
100 %	2421005	40	40.01	100.03	**MEAN**	100.15
	2422203	40	40.03	100.08		
	2428052	40	40.13	100.32	**%RSD**	0.155
150%	3636215	60	60.07	100.13	**MEAN**	100.12
	3634215	60	60.04	100.07		
	3637021	60	60.09	100.15	**%RSD**	0.039

Acceptance criteria: The % Recovery of Nifedipine and Lignocaine should lie between 98 % and 102%.

$$\%\text{Recovery} = \frac{Amount\ found}{Amount\ added} \times 100$$

Observation: The percentage mean recovery of Nifedipine and Lignocaine was found to be between 98 % to 102 % respectively

Precision:

1. Repeatability:

(a) System precision:

Table No. 8: Data of Repeatability (System precision) for Nifedipine

	Injection	Peak Areas of Nifedipine	%Assay
Concentration 40ppm	1	3348798	100.18
	2	3349568	100.20
	3	3348271	100.16
	4	3348683	100.18
	5	3348711	100.18
	6	3348123	100.16
Statistical Analysis	**Mean**	3348962	100.18
	SD	505.97	601.10
	% RSD	0.015	0.015

Table No. 9: Data of Repeatability (System precision) for Lignocaine

	Injection	Peak Areas of Lignocaine	%Assay
Concentration 40ppm	1	2424333	100.17
	2	2424789	100.19
	3	2423789	100.15
	4	2425554	100.22
	5	2424220	100.17
	6	2424515	100.18
Statistical Analysis	**Mean**	2424533	100.18
	SD	599.9676	0.024
	% RSD	0.024	0.024

Acceptance criteria: The % Relative Standard Deviation of assay preparations of Nifedipine and Lignocaine should be not more than 2.0%.

Observation: Test results for Nifedipine and Lignocaine are showing that the %RSD of Assay results are within limits.

(b) Method precision:

Table No. 10: Data of Repeatability (Method precision) for Nifedipine

	Injection	Peak Areas of Nifedipine	%Assay
Concentration 40ppm	1	3348900	100.18
	2	3348201	100.16
	3	3348578	100.17
	4	3348907	100.18
	5	3348577	100.17
	6	3348008	100.16

Statistical Analysis	**Mean**	3348528	100.17
	SD	364.48	0.0109
	% RSD	0.0108	0.0108

Table No. 11: Data of Repeatability (Method precision) for Lignocaine

	Injection	**Peak Areas of Lignocaine**	**%Assay**
Concentration 40ppm	1	2422124	100.08
	2	2428654	100.35
	3	2426902	100.28
	4	2421845	100.07
	5	2421109	100.04
	6	2425858	100.23
Statistical Analysis	**Mean**	2424415	100.17
	SD	3131.126	0.129
	% RSD	0.129	0.129

Acceptance criteria: The % Relative Standard Deviation of assay preparations of Nifedipine and Lignocaine should be not more than 2.0%.

Observation: Test results for Nifedipine and Lignocaine are showing that the %RSD of Assay results are within limits

Intermediate precision:

1. Analyst 1:

Table No. 12: Data of Intermediate Precision (Analyst 1) for Nifedipine

	Injection	**Peak Areas of Nifedipine**	**%Assay**
Concentration 40ppm	1	3348900	100.18
	2	3348201	100.16
	3	3348578	100.17
	4	3348907	100.18
	5	3348577	100.17
	6	3348008	100.16
Statistical Analysis	**Mean**	3348528	100.17
	SD	364.48	0.0109
	% RSD	0.0108	0.0108

Table No. 13: Data of Intermediate Precision (Analyst 1) for Lignocaine

	Injection	**Peak Areas of Lignocaine**	**%Assay**
Concentration 40ppm	1	2422124	100.08
	2	2428654	100.35
	3	2426902	100.28
	4	2421845	100.07
	5	2421109	100.04
	6	2425858	100.23
Statistical Analysis	**Mean**	2424415	100.17
	SD	3131.126	0.129
	% RSD	0.129	0.129

2. Analyst 2:

Table No. 14: Data of Intermediate Precision (Analyst 2) for Nifedipine

	Injection	**Peak Areas of Nifedipine**	**%Assay**
Concentration 40ppm	1	3347897	100.15
	2	3348915	100.18
	3	3347684	100.15
	4	3348555	100.17
	5	3349564	100.18
	6	3348652	100.20

Statistical Analysis	**Mean**	3348544	100.17
	SD	685.27	0.0204
	% RSD	0.0204	0.0204

Table No. 15: Data of Intermediate Precision (Analyst 2) for Lignocaine

	Injection	**Peak Areas of Lignocaine**	**%Assay**
Concentration 40ppm	1	2421796	100.07
	2	2429938	100.40
	3	2420157	100.00
	4	2423315	100.13
	5	2424210	100.17
	6	2426598	100.26
Statistical Analysis	**Mean**	2424335	100.17
	SD	3506.366	0.144
	% RSD	0.144	0.144

Acceptance criteria: The individual assays of Nifedipine and Lignocaine should be not less than 98% and not more than 102% and %RSD of assays should be NMT 2.0% by both analysts.

Observation: Individual Assays and %RSD of Assay are within limit and passes the intermediate precision.

Ruggedness:
System to System variability:
For System 1:

Table No. 16: Data of system to system variability (Nifedipine)

	Injection	**Peak Areas of Nifedipine**	**%Assay**
Concentration 40ppm	1	3348900	100.18
	2	3348201	100.16
	3	3348578	100.17
	4	3348907	100.18
	5	3348577	100.17
	6	3348008	100.16
Statistical Analysis	**Mean**	3348528	100.17
	SD	364.48	0.0109
	% RSD	0.0108	0.0108

Table No. 17: Data of system to system variability (Lignocaine)

	Injection	**Peak Areas of Lignocaine**	**%Assay**
Concentration 40ppm	1	2422124	100.08
	2	2428654	100.35
	3	2426902	100.28
	4	2421845	100.07
	5	2421109	100.04
	6	2425858	100.23
Statistical Analysis	**Mean**	2424415	100.17
	SD	3131.126	0.129
	% RSD	0.129	0.129

For System-2:

Table No. 18: Data of system to system variability (Nifedipine)

S. No.	**Peak area**	**Assay % of Nifedipine**
1	3348655	100.18
2	3348863	100.18
3	3348279	100.17
4	3348878	100.18
5	3348256	100.16
6	3348965	100.19
Mean	3348649	100.18
%RSD	0.0093	0.0093

Table No. 19: Data of system to system variability (Lignocaine)

S. No.	Peak area	Assay % of Lignocaine
1	2405987	99.45
2	2404550	99.31
3	2406663	99.44
4	2401245	99.22
5	2406987	99.45
6	2402543	99.27
Mean	2404662	99.36
%RSD	0.097	0.0971

Acceptance criteria: The % Relative Standard Deviation of Nifedipine and Lignocaine from the six sample preparations should be not more than 2.0%. The % assay of Nifedipine and Lignocaine should be between 98.0%-102.0%.

Observation: The % RSD was found within the limit

Robustness:

Table No. 20: Data for Effect of Variation in Flow rate (Nifedipine)

	Std Area	Tailing factor		Std Area	Tailing factor		Std Area	Tailing factor
Flow 0.8 ml	3310845	1.175	**Flow 1.0 ml**	3349687	1.142	**Flow 1.2 ml**	3381258	1.136
	3314284	1.178		3346456	1.145		3381992	1.126
	3314254	1.168		3347398	1.126		3382879	1.152
	3313846	1.176		3348562	1.152		3382225	1.143
	3319635	1.169		3349322	1.148		3382987	1.135
	3318975	1.170		3348544	1.135		3382798	1.138
Avg	3315306	1.172	**Avg**	3348328	1.141	**Avg**	3382356	1.138
SD	3358.128	0.0041	**SD**	1207.769	0.0094	**SD**	666.680	0.0086
%RSD	0.101	0.356	**%RSD**	0.036	0.8287	**%RSD**	0.0197	0.7631

Table No. 21: Data for Effect of Variation in Flow rate (Lignocaine)

	Std Area	Tailing factor		Std Area	Tailing factor		Std Area	Tailing factor
Flow 0.8 ml	2420132	1.086	**Flow 1.0 ml**	2424658	1.054	**Flow 1.2 ml**	2428956	1.075
	2420654	1.074		2423956	1.087		2428684	1.079
	2420580	1.078		2424110	1.084		2428658	1.075
	2420003	1.078		2424198	1.087		2428368	1.078
	2420981	1.077		2424689	1.087		2428789	1.072
	2420777	1.078		2424741	1.087		2428184	1.080
Avg	2420521	1.077	**Avg**	2424392	1.081	**Avg**	2428606	1.078
SD	378.9329	0.0039	**SD**	342.935	0.013	**SD**	282.667	0.0030
%RSD	0.0156	0.369	**%RSD**	0.014	1.228	**%RSD**	0.011	0.280

Acceptance criteria: The Tailing Factor of Nifedipine and Lignocaine standards should be NMT 2.0 for Variation in Flow.

Observation: The Tailing Factor for Nifedipine and Lignocaine was found to be within the limits

Limit of Detection and Limit of Quantification (LOD and LOQ):

Nifedipine: From the linearity plot the LOD and LOQ were calculated

$$LOD = \frac{3.3\,\sigma}{S}$$

$$= \frac{3.3\times 591.3275}{83582} = 0.023\ \mu g/ml$$

$$LOQ = \frac{10\,\sigma}{S}$$

$$= \frac{10\times 591.3275}{83582} = 0.070\ \mu g/ml$$

Lignocaine:

$$LOD = \frac{3.3\,\sigma}{S}$$

$$= \frac{3.3\times485.6573}{60567} = 0.026\ \mu g/ml$$

$$LOQ = \frac{10\,\sigma}{S}$$

$$= \frac{10\times485.6573}{60567} = 0.080\ \mu g/ml$$

Observation: The LOD for this method was found to be and 0.023µg/ml for Nifedipine and 0.026µg/ml for Lignocaine**.** The LOQ for this method was found to be and 0.070µg/ml for Nifedipine and 0.080µg/ml for Lignocaine

Force Degradation Studies:
Data for Degradation Studies:

Table No. 22: Nifedipine % Degradation

Parameter	Standard Area	Sample Area	% Assay	% Degradation
Acid Degradation	3351637	3058015	91.15	8.85
Alkali Degradation	3351637	3233569	96.38	3.62
Oxidation	3351637	3218482	95.93	4.07
Dry Heat Degradation	3351637	3154742	94.03	5.97
Photo Stability	3351637	3236891	96.48	3.52
Neutral Degradation	3351637	3323448	99.06	0.94

Table No. 23: Lignocaine % Degradation

Parameter	Standard Area	Sample Area	% Assay	% Degradation
Acid Degradation	2447065	2338602	95.47	4.53
Alkali Degradation	2447065	2361236	96.40	3.60
Oxidation	2447065	2335804	95.36	4.64
Dry Heat Degradation	2447065	2357470	96.24	3.76
Photo Stability	2447065	2382586	97.27	2.73
Neutral Degradation	2447065	2440052	99.61	0.39

CONCLUSION

HPLC is at present the highly developed tool used for routine analysis. The estimation of Nifedipine and Lignocaine was done by RP-HPLC. The method was optimized using Methanol:Acetonitrile (75:25 v/v) as mobile phase using Inertsil C_{18} ODS Column as stationary phase, at a flow rate of 1.0ml/min and Rt was found to be 3.305min for Nifedipine and 5.828min for Lignocaine The detection was carried out using PDA detector at 210nm. From System Suitability data it was found that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done.Specificity data confirms that the diluents or excipients peaks are not interfering with the Nifedipine and Lignocaine peaks.The correlation coefficient for linear curve obtained between Concentration vs Area for Standard preparations of Nifedipine and Lignocaine was 0.999.The accuracy of the methods was assessed by recovery studies at three different levels and the percentage mean recovery of Nifedipine and Lignocaine was found to be between 98 % to 102 % . The method was found to be precise as indicated by the repeatability analysis, showing %RSD less than 2. Degradation Studies were performed and results of degradation was found within limits. All statistical data proves validity of the methods and can be used for routine analysis.

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