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Method Development and Validation of An RP-HPLC Method for Simultaneous Determination of Pregabalin and Nortriptyline Hydrochloride in Pharmaceutical Dosage Form

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Abstract:
The assay of Pregabalin and Nortriptyline hydrochloride was performed with tablets and the % assay was found to be 99.46 & 99.50% which shows that the method is useful for routine analysis. The linearity of Pregabalin and Nortriptyline hydrochloride was found to be linear with a correlation coefficient of 0.999 and 0.999, which shows that the method is capable of producing good sensitivity. The acceptance criteria of precision is RSD should be not more than 2.0% and the method show precision 0.37 and 0.18 for Pregabalin and Nortriptyline hydrochloride which shows that the method is precise. Which shows that the method is repeatable when performed in different days also. The total recovery was found to be 99.39-100.45 % and 99.32-99.37%, for Pregabalin and Nortriptyline hydrochloride. The validation of developed method shows that the accuracy is well within the limit, which shows that the method is capable of showing good accuracy and reproducibility.

Keywords: Pregabalin, Nortriptyline, RSD, accuracy

Introduction:

Chemically known as 3-(10, 11-dihydro-5H-dibenzo [a, d] cyclohepten-5-ylidene)-N-methyl-1-propanamine hydrochloride, nortriptyline hydrochloride is a member of the broad class of tricyclic antidepressant medications. The chemical formula for pregabalin is (S)-3-(amino methyl)-5-methylhexanoic acid. It belongs to the anticonvulsant class. [1-5].

Clinical studies have demonstrated that the combination of Pregabalin and the antidepressant medication Nortriptyline hydrochloride is superior to either medication alone in controlling pain from diabetic polyneuropathy or postherpetic neuralgia. Combination treatment should be taken into consideration when monotherapy is unable to provide sufficient pain management. Pregabalin and nortriptyline hydrochloride work well together as an antidepressant, an anticonvulsant, and to treat neuropathic pain. [6].

Additionally, nortriptyline binds to cholinergic, histaminergic, and alpha-adrenergic receptors. Because nortriptyline increases the activation of adrenergic receptors, long-term use of the drug results in a down-regulation of these receptors. Official in IP, BP, and USP; insoluble in the majority of organic solv

ents; sparingly soluble in methanol.(7-9)

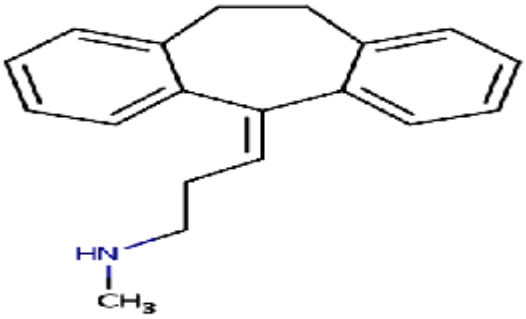

Figure No.:-1 Structure of Pregabalin

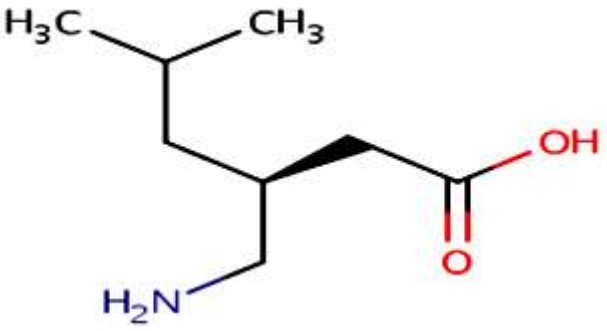

Figure No. 2: Structure of Pregabalin

The alpha 2-delta site, an auxiliary subunit of voltage-gated calcium channels, is where pregabalin, a (3S)-3-(aminomethyl)-5 methyl hexanoic acid, binds most strongly in tissues of the central nervous system. Pregabalin exact mode of action is unknown, but research using genetically altered mice and substances structurally similar to pregabalin (like gabapentin) indicates that binding to the alpha2-delta subunit may play a role in the antinociceptive and antiseizure effects of pregabalin in animal models. (10,11)

MATERIAL AND METHODS

Instrumentation Chemicals and reagents:

The HPLC system consisted of Agilent connected with PDA detector. Pregabalin and Nortriptyline hydrochloride standard gift samples were provided by Zydus Cadila healthcare ltd., Ahmedabad, India. Methanol, acetonitrile and water (HPLC grade) were purchased from Merck Chemical Company. Sodium hexane Sulphonic acid, glacial acetic acid (Analytical grade) and 0.22 µm pump Nylon filter were purchased from S.D. Fine Chemicals Ltd., Mumbai. The Whatman filter paper No. 41 was obtained from Modern Science Lab. All other reagents used were analytical grade. All the glassware used were borosilicate glass. Tablet formulations (PREGABID NT) were obtained from the local market.

Chromatographic conditions:

Chromatographic Conditions:

a. Oven Temp: 30˚C
b. Flow rate: 1 ml/min.
c. Mobile Phase: 0.1% Acetic acid: Acetonitrile(55: 45, % v/v)
   Preparation of 0.1% Acetic acid: In 1000 ml HPLC water, 1 ml of Glacial Acetic acid was added and mixed well and filtered through 0.45-micron membrane filter and sonicated to degas for 10 minutes.
d. Runtime: 6 minutes
e. Injection Volume: 20µl
f. Wavelength: 232nm
g. Diluents: 0.1% Acetic acid: Acetonitrile (50: 50, % v/v)
h. Column: Agilent Zorbax Bonus RP (250 x 4.6 mm, 5µ)

Standard Preparation:

Pregabalin Standard Stock solution-I (SSS-I): Prepare a Standard Stock Solution (SSS-I) of by addi-

ng7.5 mg of Pregabalin in 10 ml volumetric flask & add 5 ml diluent, sonicate for 5 minutes and make the volume to 10 ml with diluent.(Conc. Of Pregabalin in SSS-I = 750 µg/ml).

Nortriptyline Standard Stock solution-II (SSS-II): Prepare a Standard Stock Solution (SSS-II) of by adding 10mg of Nortriptyline in 100 ml volumetric flask & add 50 ml diluent, sonicate for 5 minutes and make the volume to 100 ml with diluent. (Conc. of Nortriptyline in SSS-II = 100 µg/ml).

Then add 1 ml of SSS-I and 1 ml of SSS-II in 10 ml volumetric flask and add 5 ml diluent and vortex and make up the volume with diluent. (Conc. of Pregabalin=75 µg/ml and Conc. of Nortriptyline = 10 µg/ml).

Preparation of Drug Product sample solution: The drug product sample solution was prepared by taking 10 tablets and crushing them using mortar and pestle and powder equivalent to 7.5 mg of Pregabalin and 1 mg of Nortriptyline weighed accurately in 100 ml volumetric flask and 50-70diluent was added to it and sonicated for 5 minutes and made up to the mark with diluent.

Selection of Wavelength: The sample was scanned from 190-400 nm with DAD detector. The Wavelength selected for analysis chosen was 232 nm on the basis of isobestic point.

Method Validation:

i) Specificity & Assay:

Individual sample of Blank, Pregabalin working standard (75 µg/ml), Nortriptyline working standard (10 µg/ml), Mixture working standard and Drug product of was prepared and peak was for identified from Retention Time.

1. % Assay was calculated as follows:

$$\%\mathbf{Assay} = \frac{\mathbf{Samplearea}}{\mathbf{Standardarea}} \times \mathbf{100}$$

ii) Repeatability& System Suitability:

2. A single working standard was prepared as described in section 2 and 6 injections were made from same solution and checked for system suitability.
3. System suitability parameters are as below:

1. Retention Time,
2. Theoretical plates,
3. Asymmetry (Tailing factor),
4. Resolution.

iii) Linearity & Range:

Samples of varying concentrations ranging from 80-120% were prepared. The concentrations are given below.

Table: 1 Concentration for linearity Study of HPLC

% Level	Pregabalin Conc. (µg/ml)	Nortriptyline Conc. (µg/ml)
80	60	8
90	67.5	9
100	75	10
110	82.5	11
120	90	12

4. The sample preparations are given as below;

5. X ml of Pregabalin and Y ml of Nortriptyline standard solution was added to 10 ml diluent to make up the concentrations given above:

X ml of SSS-I	X ml of SSS-II	Diluted to
0.8	0.8	10 ml
0.9	0.9	10 ml
1.0	1.0	10 ml
1.1	1.1	10 ml
1.2	1.2	10 ml

iv) Accuracy:

Samples were prepared of 80%, 100% and 120% concentration by spiking the same amount of concentration given in table for Linearity. Samples were injected in triplicate to calculate % RSD. % Recovery was also calculated.

v) LOD/ LOQ:

Limit of detection and quantification was calculated by using ANOVA technique.

Formula:

$$\text{LOD} = \frac{3.3 \times \text{Std. Error of Intercept}}{\text{Coefficients of X Variable 1}}$$

$$\text{LOQ} = \frac{10 \times \text{Std. Error of Intercept}}{\text{Coefficients of X Variable 1}}$$

vi) Robustness:

The Robustness was performed by changing the column temperature and Wavelength by ± 2˚C and ± 2 nm. Each Sample was injected and % RSD of peak area was calculated at each condition.

Condition	Increased	Normal	Decreased
Column Oven Temperature	32˚C	30˚C	28˚C
Wavelength	234 nm	232 nm	230 nm

Vii) Intra & Inter-day Precision:

Single mixture working standard and drug product was prepared and injected twice in a day at different time intervals to evaluate intra-day precision. Same mixture working standard was analysed on second day to evaluate the inter-day precision. % RSD of peak was calculated at each interval and stability of solutions were estimated.

Result and Discussion:

i) Selection of analytical wavelength:

The sample was scanned from 200-400 nm with DAD detector. The Wavelength selected for analysis chosen was 232 nm on basis of appropriate intensity of both the peaks.

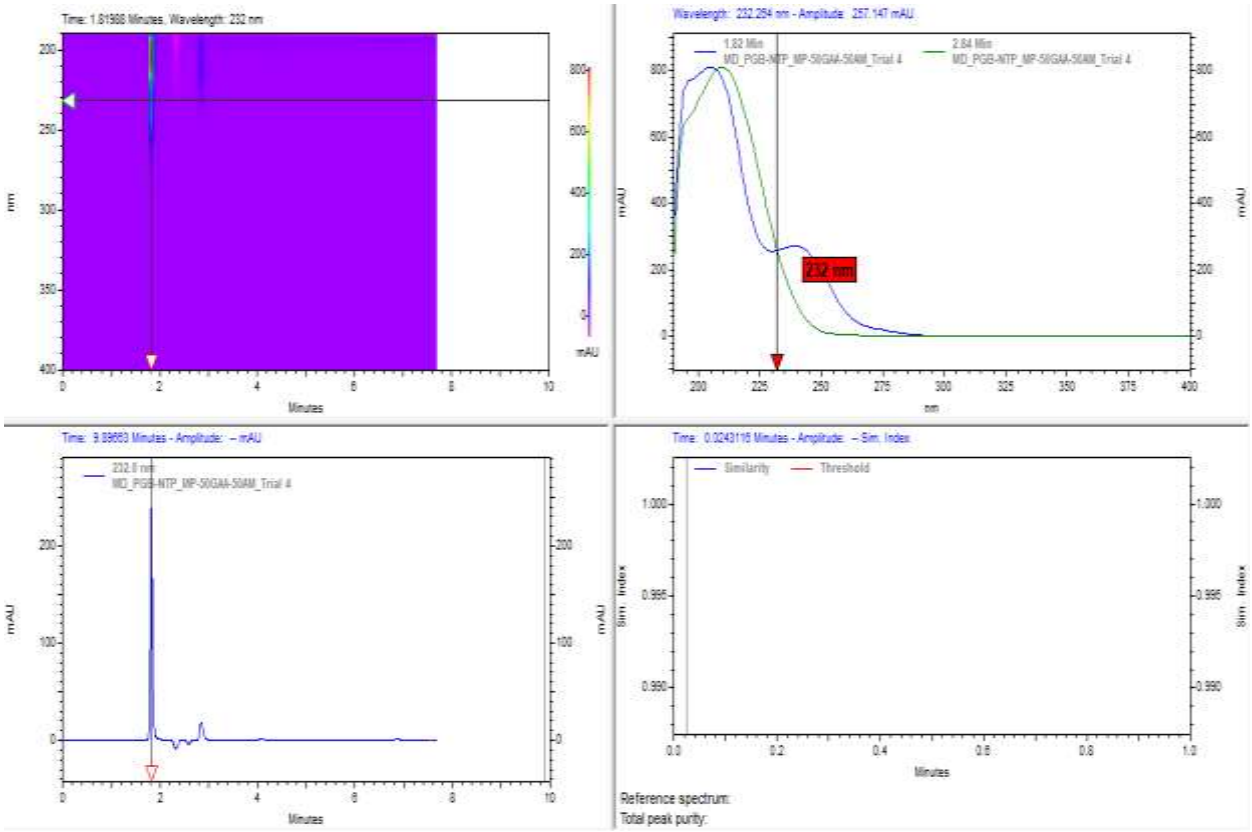

Figure 1: Spectrum of Pregabalin and Nortriptyline hydrochloride between 200-400nm in mobile phase.

Pregabalin RT 1.87 min and Nortriptyline hydrochloride RT 2.85 min show the maximum absorbance at 232 nm. Hence, HPLC analysis was carried out at 232 nm. (Figure.1)

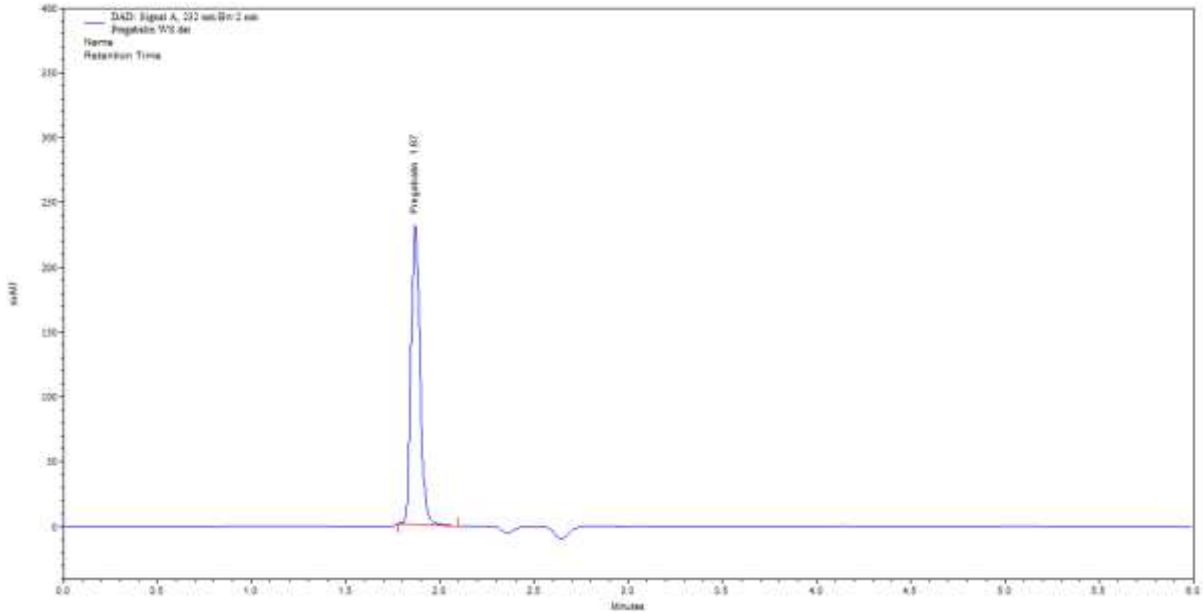

Figure 2: Chromatogram of Standard Pregabalin.

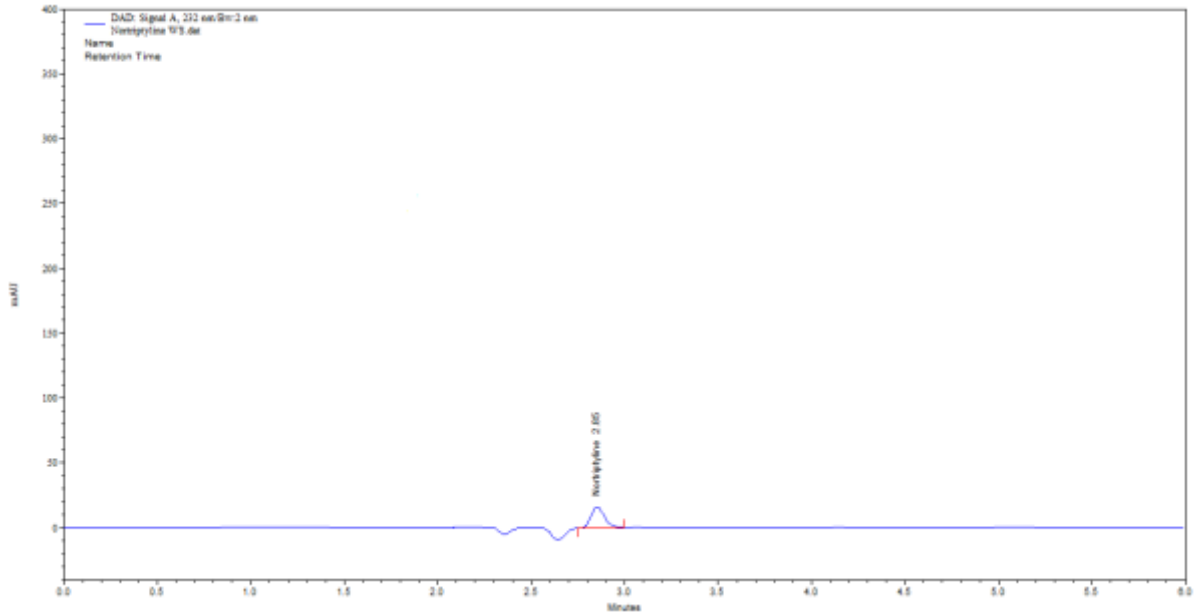

Figure 3: Chromatogram of Standard Nortriptyline hydrochloride.

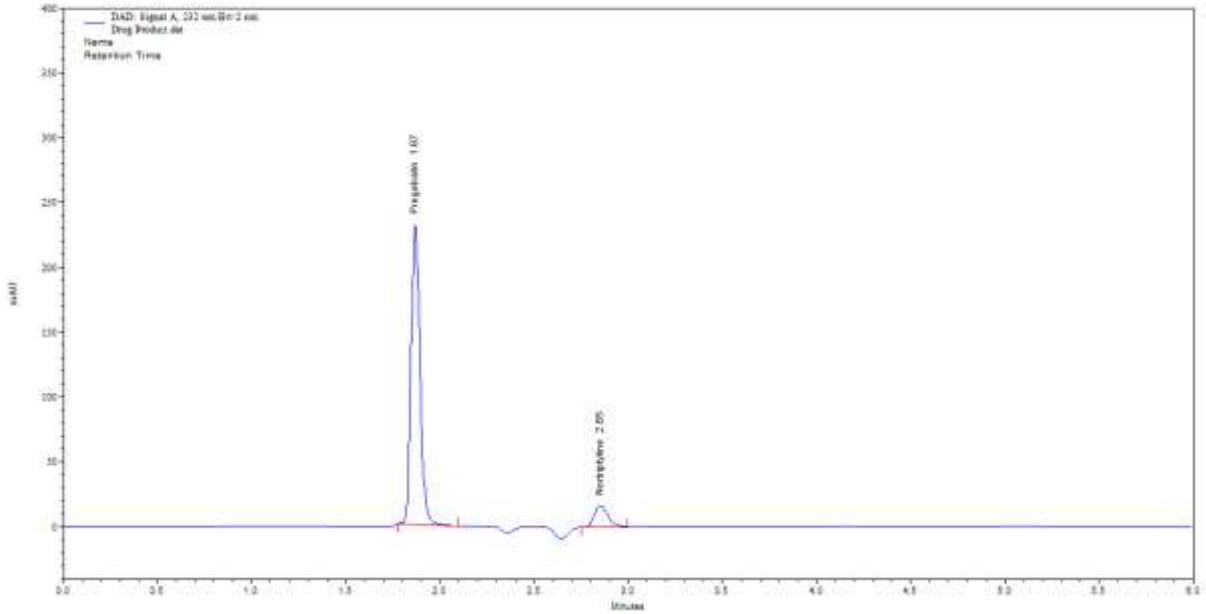

Figure 4: Chromatogram of Standard Mixture of Pregabalin and Nortriptyline hydrochloridein optimized chromatographic conditions.

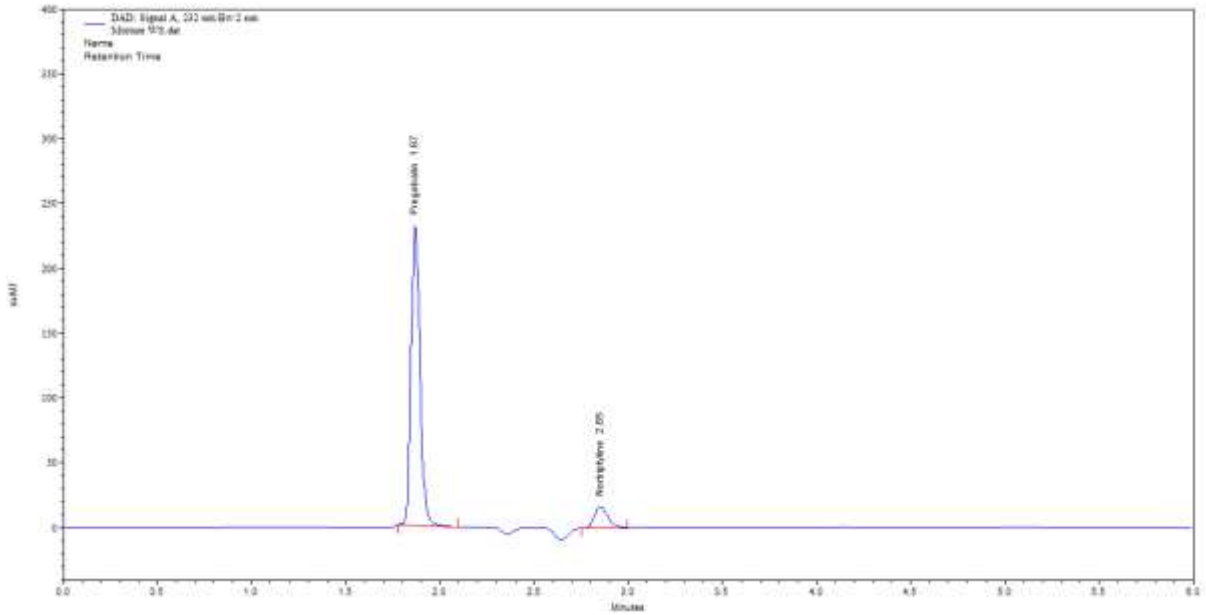

Figure 5: Chromatogram of Sample of Pregabalin and Nortriptyline hydrochloride in optimized chromatographic conditions.

iii) Analysis of tablet formulation:-

Table no. 2 Analysis of Marketed formulation.

Sample ID	Pregabalin			Nortriptyline		
	RT	Area	% Assay	RT	Area	% Assay
PGB WS	1.87	1612055	-	-	-	-
NTP WS	-	-	-	2.85	163714	-
MIX WS	1.87	1610548	-	2.85	165959	-
Drug Product	1.87	1601905	99.46	2.85	165125	99.50

Amount of drug present in the marketed formulation was calculated using RP-HPLC. Amount of Pregabalin and Nortriptyline hydrochloridewas found to be 99.46 & 99.50% respectively. This method can be employed for routine analysis of Pregabalin and Nortriptyline hydrochloride. The result of assay of marketed formulation is given in Table 2.

VALIDATION OF RP-HPLC METHODE: (12-13)

I. Linearity:

Different concentration of solution prepared for Linearity of both Pregabalin and Nortriptyline hydrochloride are shown in (Table -3 and Table -4) calibration curves are shown in Figure 6 & 7 respectively.

Table No. 3 Linearity dilutions for Pregabalin.

Pregabalin		
% Level	Conc. (µg/ml)	Area
80	60	1275044

90	**67.5**	**1439094**
100	**75**	**1610548**
110	**82.5**	**1774840**
120	**90**	**1938560**

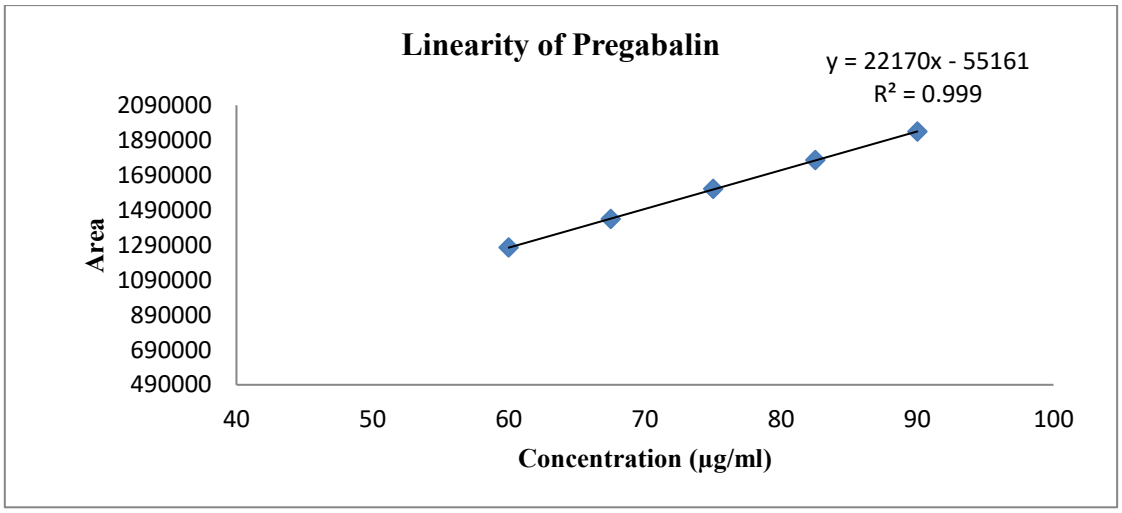

Figure No. 6 Calibration curve of Pregabalin.

Table No. 4 Linearity dilutions for Nortriptyline hydrochloride.

Nortriptyline hydrochloride.		
% Level	**Conc. (µg/ml)**	**Area**
80	**8**	**131850**
90	**9**	**148706**
100	**10**	**165959**
110	**11**	**182395**
120	**12**	**197314**

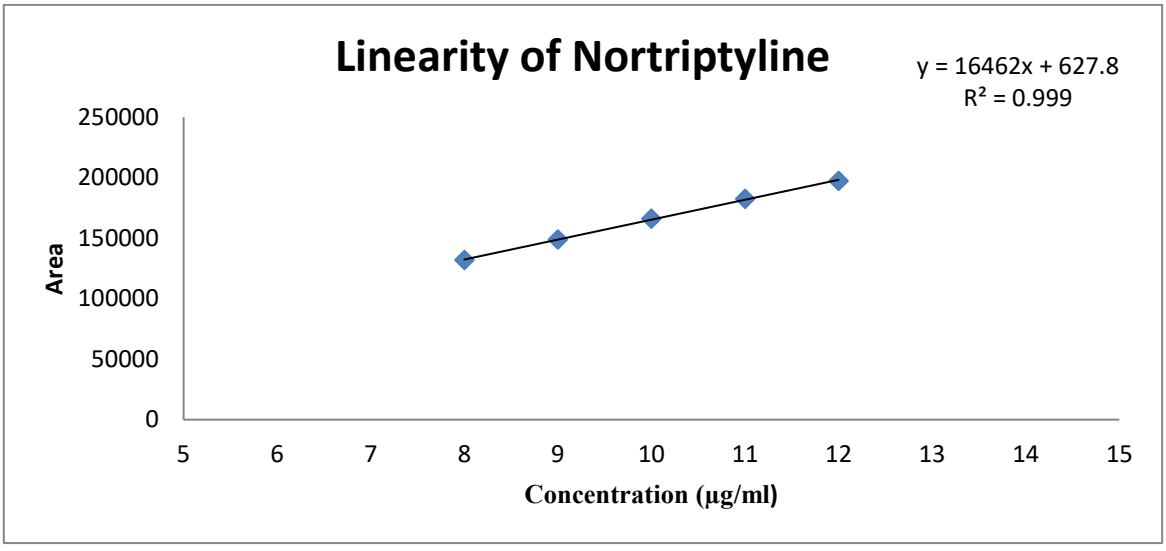

Figure No. 7 Calibration curve of Nortriptyline hydrochloride.

According to ICH guideline linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration of an analyte Linearity was studied by plotting a graph of area v/s concentration. A series standard solution of Pregabalin and Nortriptyline hydrochloridewere prepared in the concentration range of 60 µg/ml to 90 µg/ml and 8 µg/ml to 12 µg/ml respectively with linearity range 80-120% for both the drug and is shown in Table 3 and 4.

II. Precision:

The Precision study of Pregabalin and Nortriptyline hydrochlorideare shown Table 5 respectively.

Table No. 5 Precision of Pregabalin and Nortriptyline hydrochloride.

Pregabalin				
Condition	**Sample ID**	**RT**	**Area**	**% Assay**
Morning	**WS**	**1.87**	**1610548**	**-**
	DP	**1.87**	**1601905**	**99.46**
Evening	**WS**	**1.87**	**1608252**	**-**
	DP	**1.87**	**1597054**	**99.30**
% RSD				**0.11**
Day 2	**WS**	**1.87**	**1590175**	**-**
	DP	**1.87**	**1570543**	**98.77**
% RSD				**0.37**

Nortriptyline				
Condition	**Sample ID**	**RT**	**Area**	**% Assay**
Morning	**WS**	**2.85**	**165959**	**-**
	DP	**2.85**	**165125**	**99.50**
Evening	**WS**	**2.85**	**163357**	**-**
	DP	**2.85**	**162301**	**99.35**
% RSD				**0.10**
Day 2	**WS**	**2.85**	**161704**	**-**
	DP	**2.85**	**160325**	**99.15**
% RSD				**0.18**

The accuracy of an analytical process used to determine intra-day and inter-day variation. The percentage relative standard deviation (RSD) for Intraday and Inter-day precision was 0.011 and 0.37 % for Pregabalin and Intraday and Inter-day precision 0.10 and 0.18 % for Nortriptyline. The obtained findings are less than 2% suggests a high level of precision.

III. Accuracy:

The accuracy study of Pregabalin and Nortriptyline hydrochlorideare shown in Table 6 and 7 respectively.

Table No. 6. Accuracy Study of Pregabalin.

% Level	Reps	Spiked Conc. (µg/ml)	Area	Amount Recovered (µg/ml)	% Recovery	AVG	STDEV	RSD
80%	Rep 1	59.94	1275044	59.44	99.17	99.39	0.215647	0.22
	Rep 2	59.94	1280587	59.70	99.60			
	Rep 3	59.94	1277958	59.58	99.39			
100%	Rep 1	74.93	1610548	75.08	100.21	99.93	0.274167	0.27
	Rep 2	74.93	1601745	74.67	99.66			
	Rep 3	74.93	1605785	74.86	99.91			
120%	Rep 1	89.91	1938560	90.37	100.51	100.45	0.069873	0.07
	Rep 2	89.91	1937450	90.32	100.46			
	Rep 3	89.91	1935878	90.25	100.37			

Table No. 7. Accuracy Study of Nortriptyline hydrochloride.

% Level	Reps	Spiked Conc. (µg/ml)	Area	Amount Recovered (µg/ml)	% Recovery	AVG	STDEV	RSD
80%	Rep 1	7.99	131850	7.95	99.47	99.32	0.193624	0.19
	Rep 2	7.99	131367	7.92	99.10			
	Rep 3	7.99	131759	7.94	99.40			
100%	Rep 1	9.99	165959	10.01	100.16	99.97	0.442318	0.44
	Rep 2	9.99	166155	10.02	100.28			
	Rep 3	9.99	164799	9.94	99.46			
120%	Rep 1	11.99	197314	11.90	99.24	99.38	0.165295	0.17
	Rep 2	11.99	197522	11.91	99.34			
	Rep 3	11.99	197958	11.94	99.56			

The method's accuracy defines how close the method's results are to the true value. The results of the accuracy testing revealed that the technique is accurate within acceptable ranges. When the % RSD for Pregabalin and Nortriptyline hydrochlorideis calculated, all of the results are within acceptable bounds. A maximum RSD of 2.0% indicated acceptable accuracy within the range. The results are shown in Table 6 and 7.

According to the Accuracy research, the percent recovery of Pregabalinis 99.39-100.45 % and Nortriptyline hydrochlorideis 99.32-99.37 %, both of which are within the ICH standards.

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

Table No. 8. The LOD and LOQ of Pregabalin and Nortriptyline hydrochloride.

Sr.No	Name of drug	LOD (µg/mL)	LOQ(µg/mL)
1.	Pregabalin	1.14	3.44
2.	Nortriptyline hydrochloride	0.52	1.56

iv. System suitability:

System suitability data of Pregabalin and Nortriptyline hydrochloridegiven in below Table 9 and 10.

Table No. 9 System suitability parameter of Pregabalin.

Pregabalin					
Sample ID	Area	RT	TP	Asymmetry	Resolution
100% Rep 1	1610548	1.87	7379	1.23	0.00
100% Rep 2	1601745	1.87	7205	1.21	0.00
100% Rep 3	1605785	1.87	7456	1.19	0.00
100% Rep 4	1609112	1.87	7433	1.22	0.00
100% Rep 5	1608204	1.87	7312	1.24	0.00
100% Rep 6	1607870	1.87	7259	1.24	0.00
AVG	1607211	1.87			
STDEV	3100.978	2.4316			
% RSD	0.19	0.00			

Table No. 10 System suitability parameter of Nortriptyline hydrochloride.

Nortriptyline					
Sample ID	Area	RT	TP	Asymmetry	Resolution
100% Rep 1	165959	2.85	7635	1.22	9.07
100% Rep 2	166155	2.85	7528	1.17	9.07
100% Rep 3	164799	2.85	7689	1.23	9.07
100% Rep 4	165725	2.85	7721	1.20	9.07
100% Rep 5	164895	2.85	7520	1.22	9.07
100% Rep 6	166622	2.85	7563	1.21	9.07
AVG	165693	2.85			
STDEV	718.9302	0			
% RSD	0.43	0.00			

The system, method, and column performance were validated by testing system suitability features. Six times, a standard solution of Pregabalin and Nortriptyline hydrochloridewas injected into the system, and the system's suitable features were evaluated. Results are shown in Table 9 and 10.

V. Robustness: Robustness data of Pregabalin and Nortriptyline hydrochloridegiven in below

Table No. 11 Robustness parameter of Pregabalin and Nortriptyline hydrochloride.

Variation in Column temperature (Pregabalin)							
Condition	**Sample ID**	**RT**	**Area**	**% Assay**	**Average**	**STDEV**	**% RSD**
28˚C	WS	1.87	1607250	-	99.61	0.127694	0.13
	DP	1.87	1602045	99.68			
30˚C	WS	1.87	1610548	-			
	DP	1.87	1601905	99.46			
32˚C	WS	1.87	1605057	-			
	DP	1.87	1600114	99.69			

Variation in wavelength (Pregabalin)							
Condition	**Sample ID**	**RT**	**Area**	**% Assay**	**Average**	**STDEV**	**% RSD**
230 nm	WS	1.87	1605778	-	99.40	0.134993	0.14
	DP	1.87	1593725	99.25			
232 nm	WS	1.87	1610548	-			
	DP	1.87	1601905	99.46			
234 nm	WS	1.87	1678987	-			
	DP	1.87	1670575	99.50			

Variation in Column temperature (Nortriptyline)							
Condition	**Sample ID**	**RT**	**Area**	**% Assay**	**Average**	**STDEV**	**% RSD**
28C	WS	2.85	163154	-	99.40	0.085817	0.09
	DP	2.85	162058	99.33			
30C	WS	2.85	165959	-			
	DP	2.85	165125	99.50			
32C	WS	2.85	164225	-			
	DP	2.85	163220	99.39			

Variation in Column temperature (Nortriptyline)							
Condition	**Sample ID**	**RT**	**Area**	**% Assay**	**Average**	**STDEV**	**% RSD**
230 nm	**WS**	**2.85**	**171054**	**-**	**99.42**	**0.078905**	**0.08**
	DP	**2.85**	**169925**	**99.34**			
232 nm	**WS**	**2.85**	**165959**	**-**			
	DP	**2.85**	**165125**	**99.50**			
234 nm	**WS**	**2.85**	**162214**	**-**			
	DP	**2.85**	**161257**	**99.41**			

Robustness was investigated using various deliberate alterations in chromatographic settings, such as changes in column Condition like 28˚C, 30˚C and 32˚C. RSD was shown to be less than 2% in the Pregabalin and Nortriptyline hydrochloriderobustness studies. As a result, it is strong and adheres to ICH criteria. Results are shown in Table 11.

Conclusion:

The developed and verified RP-HPLC method simplifies and accelerates the quantitative determination of Pregabalin and Nortriptyline hydrochloride from their formulations. According to ICH criteria, all validation parameters were determined to be within the permitted ranges. Regardless of the excipients used, it was discovered that the proposed technique was easy, precise, accurate, robust, and specific to the drugs of interest. It can be used to conduct routine analyses of commercial formulations.

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