

Kinetic Approach to Determine 3-Amino Methyl Hexanoic Acid in Pharmaceutical Formulations

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ABSTRACT

A simple and sensitive colorimetric spectrophotometric method based on application of chemical kinetics was produced and developed for the quantitative determination of 3-amino methyl hexanoic acid (pregabalin) in bulk and pharmaceutical preparations. Ninhydrin, a chromogenic agent was reacted with pregabalin at 70 °C and the absorbance was measured at 575 nm and kinetic correlation was evaluated between absorbance and time. The initial-rate, rate-constant and fixed-time (80 min) methods were employed for the estimation of pregabalin in the range of 10–30 $\mu\text{g mL}^{-1}$ which showed good correlation >0.9990 in all three cases with LOQ values ranging from 1.2–1.5 $\mu\text{g mL}^{-1}$ and LOD values ranging from 0.76–0.95 $\mu\text{g mL}^{-1}$. Method validation was carried out using ICH recommendations and the variables were evaluated and optimized. The newly developed method has useful applications to determine pregabalin in pharmaceutical formulation with good recovery (98.38 to 102.12 %) and precision (RSD < 1.4 %). Standard free energy changes and association constant were found to be 1.212×10^3 and -4.747 , respectively, as determined by the Benesi–Hildebrand equation.

KEYWORDS

Colorimetric determination, pregabalin, ninhydrin, charge transfer complex.

1. Introduction

Pregabalin is chemically defined as 3-amino methyl hexanoic acid, having chemical formula $\text{C}_8\text{H}_{17}\text{NO}_2$ (Fig. 1). It is a structural and pharmacological analogue of the mammalian neurotransmitter gammaamino butyric acid (GABA). It is a white crystalline solid containing both acid and base function, i.e. zwitter ion. So it is soluble in water, both in basic and acidic range. Pregabalin is mainly used as an anticonvulsant drug, though its effects are much broader, as it is used for psychiatric diseases, treatment of partial seizures in adults, epilepsy, neuropathic pain and diabetic neuropathy. In addition, pregabalin has been prescribed in other physiological complications including inflammation, gastrointestinal damage, alcoholism and insomnia.^{1–4}

Pregabalin is an established α -2- δ potential ligand, therefore it binds to the α -2- δ subunit calcium channel receptors in the brain. Neurons are activated to decrease calcium influx at nerve

ending, and hence reducing the release of several neurotransmitters such as noradrenalin and dopamine,^{5–6} which subsequently results in the above-mentioned activities. However, the exact mechanism of action is unknown.

Ninhydrin (NIN) attained widespread utility in quantitative determination of amino acids and proteins. Thus its importance in pharmaceutical, biomedical, forensic, food, nutritional and plant studies cannot be ignored.^{7–11} NIN has special application in the pharmaceutical industry by visualization of amino and azo group-containing compounds on TLC plates and in kinetic studies.^{12–14}

A literature survey revealed that various analytical methods have been reported to quantify pregabalin, including spectrophotometry,^{15–19} spectrofluorimetry,^{20–21} high pressure liquid chromatography with UV detection,^{22–23} HPLC mass spectrometry,²⁴ HPLC with fluorescence detection²⁵ and HPLC with pre-column²⁶ and post-column²⁷ derivatization.

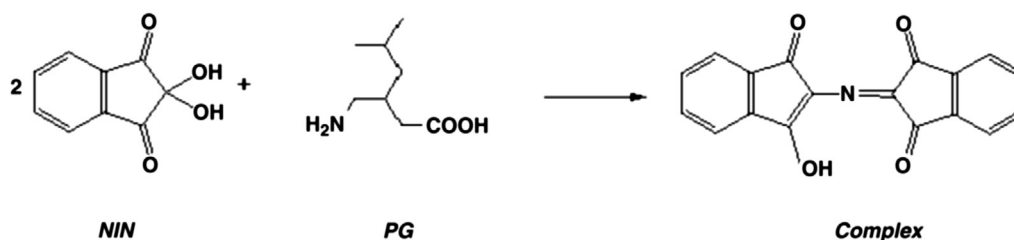


Figure 1 Structure of pregabalin and suggested reaction pathway between pregabalin and NIN.



Liquid chromatographic methods involve expansive equipment while spectrofluorimeters are not commonly available. Most of the available spectrometric methods in literatures are not highly sensitive or need comprehensive, complicated extractions procedures. Moreover, most of available methods are based on the absorbance in the near-UV region and thus specificity is questioned. Some of the methods in literature use reagents which do not produce linear response. Our endeavour was focused on development of an economical, rapid and sensitive analytical method for quantitative determination of pregabalin in pharmaceutical formulation and reference material involving kinetic approach.

Present study describes highly simple, sensitive and precise colorimetric spectrophotometric methods compared to the conventional existing spectrophotometric and expansive HPLC methods. The suggested methods involve reaction of NIN with amino group of PG developing purple colour complex called Ruhemann's purple (Fig. 1).¹⁴

The proposed method relies on simple and low-cost technique but it is sensitive enough to be reasonably compared to sophisticated and expensive techniques such as HPLC. ICH guideline was followed for various experiments to validate the methods.²⁸ The proposed spectrophotometrically monitoring kinetic approaches for determining pregabalin in pharmaceutical capsule and bulk material, using NIN as a reagent, is highly advantageous. It simply requires heating of reaction mixture and cooling. Conditions of reaction were optimized and applications of the proposed methods were established. Thermodynamic features like association constant (K_c) and the standard free energy changes (ΔG°) of the complex formed were investigated, in addition to stoichiometric composition of the complex.

2. Experimental

2.1. Instruments

UV-visible spectrophotometer of Shimadzu Corporation, Japan model number 1601 was used in this study. Spectrophotometer is double-beam-based with slit width of 2 nm while cell made of quartz with path length of 10 mm, controlled and run through UVPC version 3.9 software.

2.2. Chemicals and Reagents

Analytical grade reagents were used in this study. Pregabalin pure drug was kind gift of a national pharmaceutical company. Syngab[®] capsules 200 mg, 50 mg, 100 mg containing pregabalin were obtained from a local pharmacy. NIN was from Merck, Germany. Distilled and deionized water was used throughout this study.

2.3. General Procedure

2.3.1. Standard Stock Preparation

To prepare 100 $\mu\text{g mL}^{-1}$ solution of pregabalin in water, 100 mg of it was transferred to a 1 L volumetric flask, dissolved in water, diluted to the mark level and stored in a dark, cool (below 25 °C) place.

2.3.2. Preparation of Ninhydrin Solution

1 g of ninhydrin was taken in a 100 mL volumetric flask and sufficient methanol was added to dissolve; after having completely dissolved, the volume was adjusted up to the mark with methanol. NIN solution was prepared on fresh basis.

2.3.3. Reaction Procedure

Solutions of about 100 $\mu\text{g mL}^{-1}$ pregabalin were taken into heating tubes, to which 2 mL of 1 % NIN solution was added and

heated in a water bath for 80 min. Reaction mixture was allowed to cool down to room temperature. The mixture was shifted to a 50 mL volumetric flask and was added up to the mark level with water. Absorbance was measured at 575 nm as a function of time against a blank treated similarly (spectra 1). Absorbance time curve slope derived from different concentrations of the drug, was used to calculate the initial rate of reaction. The calibration curves were designed by plotting (i) logarithm of initial rate of reaction *versus* logarithm of molar concentration, (ii) rate-constant *versus* final concentration and (iii) absorbance measured at a fixed time *versus* final concentration of pregabalin.¹⁴

2.3.4. Sample Preparation of Pharmaceutical Formulations

A homogenous bulk sample was obtained by weighing twenty capsules and mixing their contents. The capsule powder equivalent to 10 mg of pregabalin was taken in to a 100 mL volumetric flask, sufficient amount of water was added and sonicated for 10 min as well as stirred for 30 min to completely dissolve the active content. Water was added up to volume and reaction procedure described above was followed.

2.3.5. Stoichiometric Study

To evaluate the stoichiometric composition of the complex, Job's continuous variation method²⁹ was used. Equimolar solution of both drug and reagent were prepared individually. Different complementary proportions of both solutions were reacted together in a series of 5 mL flasks. These complementary proportions were 0:5, 1:4, 2:3...4:1 mL. complex was measured through absorbance at 575 nm after a due time against a reagent blank treated similarly.

Excipients Interference: Common excipients such as hydroxy-propyl methyl cellulose, magnesium stearate, talcum powder, pyrrolidone, glucose, and lactose were spiked with 50 mg of pregabalin and were determined as given under General Procedure.

3. Results and Discussion

Pregabalin does not possess prominent chromophore, therefore it is a very low UV absorbing molecule.¹⁷ Thus use of conventional UV spectrophotometric methods was out of question due to its poor sensitivity. For high sensitivity and specificity HPLC is usually used but due to low specific absorption, a high concentration has to be injected onto the column to get viable peak which would cause column deterioration. In literature, derivatization techniques (post and pre-column) are found but they are always laborious tasks. There is a critical need to develop a visible range spectrophotometric analytical method that can accurately quantitate pregabalin in pharmaceutical formulations, therefore the present work was designed.

3.1. Reaction with Ninhydrin (NIN)

It has been found that introduction of an aromatic ring to molecule increases its UV response showing momentous increase in its absorbance; moreover, UV absorbance can be further increased by introduction of a nitrogen atom bearing a lone pair. Thus to achieve the increase in absorbance, pregabalin was reacted with NIN. NIN reagent is a recommendation for study of aliphatic primary amine or amino acids. Methanolic medium was provided for the interaction of pregabalin and NIN. The reaction takes place through an oxidation process of deamination of the amine which later on condenses with reduced NIN. A purple coloured complex results from this reaction which absorbs at 575 nm (λ_{max} at 575 nm) as shown in Figs. 1 & 2.

Taking a hypothetical approach that various factors might interfere with the reaction, many factors were studied and it was found that temperature played a key role as the optimum colour was obtained on heating the reaction mixture at a temperature of $70 \pm 5^\circ\text{C}$ for 80 min. Extended heating or heating at higher degrees resulted in decreased chromogenic intensity. In addition, the choice of best extracting solvent was tested by trying different solvents such as water, isopropanol, ethanol, methanol, dioxane, acetone and acetonitrile. Best results were found with methanol so it was used in subsequent analysis.

3.2. Kinetic Approach

3.2.1. Initial Rate Method

The kinetic parameters of proposed reactions were studied. For this purpose, a time curve graph of the slope of the initial tangent to the absorbance was used to determine the initial rate of reaction. In order to establish the order of reaction with respect to pregabalin, different concentrations of pregabalin were tried but the concentration of NIN was kept constant. A plot of $A_{\text{inf}}/A_{\text{inf}} - A_t$ versus time was obtained. A straight line was obtained that showed that the reaction is first order.

The order of reaction was also established with respect to NIN. Now the concentration of pregabalin was kept constant and that of NIN varied. The plot of $\log A_{\text{inf}}/A_{\text{inf}} - A_t$ against time was again a straight line which confirmed that it also involved first-order kinetics. For initial rate of reaction, the following equation applies:

$$\text{Rate} = dA/dt = k'C^n \quad (1)$$

where, k' is the rate constant, C is the concentration of pregabalin, and n represents the order of the reaction. Logarithmic form of the above equation is as follows:

$$\log \text{rate} = \log k' + n \log C \quad (2)$$

$$\log \text{rate} = \log k' + 0.01011 \log C \quad (3)$$

The regression equation gave rise to first-order reaction with respect to pregabalin. The concentration range was between 10 and $30 \mu\text{g mL}^{-1}$. A linear plot was obtained when absorbance of different concentrations were plotted against time. Statistical features like slope, intercept and correlation coefficient were

calculated from the linear regression equation. Results are shown in Table 1.

3.2.2. Rate-constant Method

The rate constant method was used to determine different nominal concentrations of pregabalin by plotting the slopes of $\log A_{\text{inf}}/A_{\text{inf}} - A_t$ against time. Rate constant values were plotted against concentration to create a calibration curve and a linear response was obtained in the concentration range $10\text{--}30 \mu\text{g mL}^{-1}$. Table 2 gives details of the data.

3.2.3. Fixed Time Method

Fixed time assay method was carried out for pregabalin at pre-selected time. A plot of absorbance vs concentration was constructed at fixed times of 20, 40, 60 and 80 min. Regression equations were developed and related analytical parameters were calculated. These parameters were in accordance with acceptable limits. Experimental results detailed in Table 3 suggest that any of the outlined fixed time methods can be used for the assay of pregabalin.

Evaluating the regression equations, good linearity was exhibited by obtaining correlation coefficients of more than 0.999. Excellent sensitivity of the method is obvious from low limit of detection and quantification values and the low value of the standard deviation indicated good precision. Pharmaceutical samples were assessed with these projected methods over a range of concentrations and results obtained in Table 4 suggest excellent accuracy of the method.

3.3. Stoichiometry of the Reaction

Job's method of continuous variation was employed to find

Table 1 Statistical and regression data of the proposed method.

	Initial rate	Rate constant	Fixed time
Intercept	0.0019	-0.1184	-0.0122
Slope	0.01218	0.01356	0.01425
r^2	0.9995	0.9993	0.9996
Linearity range/ $\mu\text{g mL}^{-1}$	10–30	10–30	10–30
LOD/ $\mu\text{g mL}^{-1}$	0.76	0.95	0.82
LOQ/ $\mu\text{g mL}^{-1}$	1.2	1.4	1.5

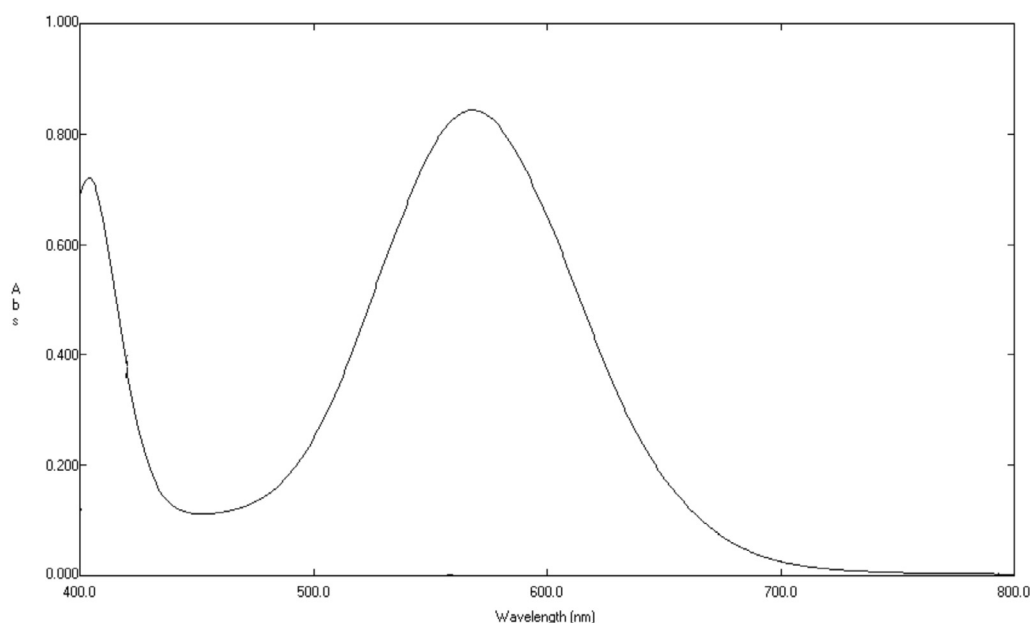


Figure 2 Absorption spectra of the reaction products of pregabalin with NIN.

Table 2 Determination of rate constant by the proposed method.

Concentration / $\mu\text{g mL}^{-1}$	Calculated value of rate constant (slope) / min^{-1}
10	0.0145
14	0.0143
18	0.0144
20	0.0144
30	0.0143

Table 3 Regression characteristics of pregabalin concentrations at different time intervals.

	Fixed time			
	20	40	60	80
Intercept	−0.0061	−0.0032	−0.0034	−0.0035
Slope	0.0115	0.0125	0.0155	0.0217
Correlation coefficient/r	0.9995	0.9997	0.9994	0.9998
Linearity range/ $\mu\text{g mL}^{-1}$	10–30	10–30	10–30	10–30
LOD/ $\mu\text{g mL}^{-1}$	0.92	0.94	0.88	0.82
LOQ/ $\mu\text{g mL}^{-1}$	1.7	1.8	1.5	1.5

out the molar ratio of pregabalin with NIN^{17,29} was used. The ratio found through method was 1:2 for NIN.

3.4. Determination of Association Constants

Benesi–Hildebrand equation³⁰ was used to know about the association constants of the complex produced as a result of the interaction of pregabalin with NIN using 1.212×10^3 .

$$\frac{Ca}{A} = \frac{1}{\epsilon} + \frac{1}{K_c \cdot \epsilon} \cdot \frac{1}{Cb} \quad (4)$$

where K_c is the association constant, ϵ is the molar absorptivity and A is the absorbance of the complex. Ca and Cb are the concentrations of the acceptor and donor, respectively.

3.5. Determination of Standard Free Energy Changes

A plot of Ca versus A in Eq. 4 produced a straight line. The

following equation was used to determine free energy changes ($\Delta G^\circ = -4.747$) associated with the complex which shows that the complex is fairly stable.

$$\Delta G^\circ = -2.303 R T \log K_c \quad (5)$$

where ΔG° is the standard free energy change of the complex (kJ mol^{-1}), K_c is the association constant of drug-acceptor complexes (mol^{-1}), T the temperature in Kelvin ($273 + ^\circ\text{C}$), and R the gas constant ($1.987 \text{ cal mol}^{-1} \text{ deg}^{-1}$).

3.6. Method Validation

The developed colorimetric analytical methods were validated for linearity, accuracy and precision. Calibration curves were obtained by plotting absorbance against concentration and Beer's law was obeyed in the given range. Beer's law plots were found to be linear for all the studied cases and the intercepts found for them were very small (Tables 1, 2 & 3). The values of slopes ranged from 0.01218–0.01425 while the correlation coefficient was always more than 0.9990. The limit of detection LOD and limit of quantification LOQ values for pregabalin with NIN were calculated. The cross-check was performed from theoretically calculated LOD and LOQ values by experimentally verifying these concentrations using newly developed methods. LOQ values ranging from 1.2–1.5 $\mu\text{g mL}^{-1}$ and LOD values ranging from 0.76–0.95 $\mu\text{g mL}^{-1}$ were calculated for the proposed methods.

In order to evaluate accuracy and precision, pregabalin was analyzed at five concentration levels; each was analyzed in replicate. Excellent accuracy is shown in Table 4 while low mean relative standard deviation (RSD) and the mean standard deviation (SD) values indicate satisfactory precision.

The effect of common adjunct and excipients were experimentally considered on the projected methods before applying these methods to the analysis of pharmaceutical preparations. Interference from these additives or degradation product of the parent molecules is possible which in turn depends upon donor acceptor affinities. Amine functionality in pregabalin render it highly effective for complexation with NIN compared to other additives and hence the method was found to be specific and selective for pregabalin in presence of excipients (Table 5).

Table 4 Accuracy and precision of proposed method in pharmaceutical analyses.

Concentration / $\mu\text{g mL}^{-1}$	Initial rate		Rate constant		Fixed time	
	Found	% Recovery	Found	% Recovery	Found	% Recovery
10	10.16	101.6	9.92	99.2	9.94	99.4
14	14.09	100.64	14.15	101.07	13.89	99.21
18	18.06	100.33	17.96	99.78	18.14	100.78
20	19.93	99.65	19.91	99.55	20.07	100.35
30	30.27	100.9	30.09	100.3	29.98	99.933
Mean	18.5	100.62	18.406	99.97	18.404	99.93
STD	7.579	0.718	7.57	0.729	7.56	0.6496
%RSD	40.96	0.713	41.12	0.729	41.1	0.65

STD = Standard deviation, RSD = relative standard deviation.

Table 5 Recovery of pregabalin in presence of different excipients.

	Pyrro	Lactose	Talc	Mag St	Starch	HPMC
Initial rate	99.86	99.99	101.26	99.97	101.26	98.92
Rate constant	98.99	100.66	101.33	99.85	101.67	99.11
Fixed time	100.68	99.78	100.27	98.99	99.67	100.14

Pyrro: pyrrolidone, Mag St: magnesium stearate, HPMC: hydroxypropyl methyl cellulose.

3.7. Determination of PG in Dosage Forms

The newly developed methods were applied to determine pregabalin in pharmaceutical commercial products along with the reference method.²³ Comparisons of the proposed methods were made to the reference method by employing *t*- and *F*-tests. There was no considerable difference between the developed method and the reference method at 95 % level of confidence. Data in Table 6 indicate that these procedures can be applied for quantitative determination of pregabalin in dosage form without interference. Percentage recoveries were found to be in the range of 98.38–102.12 %.

3.8. Spectroscopic Studies

3.8.1. Infrared Spectra

It was found from the IR spectra of pregabalin that a multiplet of peak is found in the region 2500–3000 cm⁻¹ and the usual amine and carboxyl peaks at 3200–3400 cm⁻¹ and 1710 cm⁻¹, respectively, were absent. This can be attributed to the existence

of zwitterions. It is further confirmed by the presence of asymmetric and symmetric peaks of carboxylate ion at 1600 and 1400 cm⁻¹ as observed in amino acids³¹ along with NH₃⁺ bending at 1550 cm⁻¹. NIN spectra showed the presence of two OH groups by exhibiting two broad peaks at 3300 and 3250 cm⁻¹ along with a stretching frequency at 1061 cm⁻¹ owing to secondary alcohol C-O, carbonyl peaks in the region 1660–1760 cm⁻¹ and aromatic frequency at 750 cm⁻¹.

It is reported that NIN forms a complex called Ruhemann's purple with primary amines.²⁰ Complex formation with pregabalin was confirmed by comparing the spectral data of NIN with complex. Doublet of the carbonyl changed into a singlet at 1680 cm⁻¹ and the O-H stretching shifted to 3400 cm⁻¹ which confirms the formation of complex between pregabalin and NIN.

3.8.2. Nuclear Magnetic Resonance Spectra

¹H NMR spectra of pregabalin confirmed three –CH₂ peaks at δ 2.443, δ 2.553 and δ 2.873 ppm and a multiplet for tertiary

Table 6 Determination of pregabalin in pharmaceuticals formulations by proposed.

	Initial rate		Rate constant		Fixed time		Reference method	
Syngab 100 mg								
Taken con.	Found	% Rec	Found	% Rec	Found	% Rec	Found	% Rec
100	100.09	100.09	99.76	99.76	100.79	100.79	99.22	99.22
100	100.17	100.17	98.91	98.91	100.19	100.19	101.24	101.24
100	99.89	99.89	99.59	99.59	99.56	99.56	101.66	101.66
100	100.12	100.12	100.34	100.34	98.76	98.76	98.38	98.38
100	99.37	99.37	100.27	100.27	100.39	100.39	100.73	100.73
Mean	99.928	99.928	99.774	99.774	99.938	99.938	100.246	100.246
STD	0.3295755	0.329575	0.580284413	0.580284	0.794399	0.794399	1.392509	1.392509
%RSD	0.3298129	0.329813	0.581598826	0.581599	0.794892	0.794892	1.389091	1.389091
t-test	0.79		0.82		0.64			
F-test	1.39		1.47		1.25			
Syngab 200 mg								
Taken con.	Found	% Rec	Found	% Rec	Found	% Rec	Found	% Rec
200	198.96	99.48	199.67	99.835	201.24	100.62	201.22	100.61
200	200.47	100.235	200.68	100.34	198.96	99.48	199.34	99.67
200	199.79	99.895	200.41	100.205	200.29	100.145	199.07	99.535
200	201.22	100.61	198.37	99.185	201.57	100.785	201.22	100.61
200	199.47	99.735	199.27	99.635	199.58	99.79	201.09	100.545
Mean	199.982	99.991	199.68	99.84	200.328	100.164	200.388	100.194
STD	0.8823661	0.441183	0.924283506	0.462142	1.096207	0.548104	1.085435	0.542718
%RSD	0.4412228	0.441223	0.462882365	0.462882	0.547206	0.547206	0.541667	0.541667
t-test	0.86		0.66		0.73			
F-test	1.41		1.29		1.3			
Syngab 50 mg								
Taken con.	Found	% Rec	Found	% Rec	Found	% Rec	Found	% Rec
50	50.97	101.94	50.09	100.18	49.74	99.48	50.77	101.54
50	49.92	99.84	50.22	100.44	49.61	99.22	49.82	99.64
50	49.34	98.68	50.12	100.24	50.71	101.42	51.06	102.12
50	50.11	100.22	49.63	99.26	50.33	100.66	50.22	100.44
50	50.49	100.98	49.72	99.44	49.92	99.84	49.51	99.02
Mean	50.166	100.332	49.956	99.912	50.062	100.124	50.276	100.552
STD	0.6118251	1.22365	0.262925845	0.525852	0.452736	0.905472	0.643529	1.287059
%RSD	1.2196012	1.219601	0.526314847	0.526315	0.904351	0.904351	1.279993	1.279993
t-test	0.81		0.75		0.73			
F-test	1.17		1.22		1.34			

hydrogen at δ 1.365 ppm. Zwitterion was confirmed from the absence of NH_2 peak near δ 2 ppm and that of carboxylic OH near δ 11; instead a peak at 4.849 δ ppm attributing to NH_3^+ was observed. NIN exhibited two singlets at δ 7.240 and δ 7.448 ppm due to four aromatic protons along with a singlet at δ 1.52 ppm for two OH protons. ^1H -NMR of the complex indicated a singlet at δ 4.803 ppm representing enolic proton and amine protons (NH_2) completely diminished. The experimental results coincide with the reported ones. The ^1H -NMR spectra in association with UV-VIS and IR confirmed the structure of the complex.

4. Conclusion

This endeavor produced simple, low cost, sensitive and highly effective procedures for quantitative analyses of pregabalin in pharmaceutical bulk and dosage form. Low cost, rapidity and simplicity are the advantages of this method. The proposed methods are effective alternatives for other analytical methods with respect to either cost or simplicity and speed. Quality control laboratories in the pharmaceutical industry may use these methods for routine analysis of pregabalin in its raw bulk and dosage forms.

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