Determination of Nimodipine Stability by UV-Spectroscopy along with Quantum Mechanics to Establish Method, Validation, and Force Degradation Study

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ABSTRACT: *Nimodipine is a Calcium Channel Blocker (CCB) used to treat high blood pressure. CCB worked as an antihypertensive drug. In this study, Nimodipine is used as a CCB to study spectral behavior by spectrophotometric method and further validated by computational methods to correlate with theoretical aspects. It is subjected to stress conditions by applying varying forced degradation parameters including temperature, photolytic, oxidative, acidic, and basic conditions to find the stability of the drug and predicts the chemical reactions that take place during degradation. The other parameters including order of reaction, shelf-life, half-life, and their energy of activation are determined. The method validation of a drug is performed according to the International Conference on Harmonization (ICH) guidelines by using parameters i.e. Linearity/ Range, Limit of Detection (LOD), Limit of Quantitation (LOQ), Stability, Accuracy, Precision, Robustness and Ruggedness. It is observed that the developed method using UV-spectroscopy can be considered a sensitive and fast reproducible method for the determination of Nimodipine. In the degradation study, it is observed that Nimodipine is thermally stable, and in the case of acidic, basic, and oxidative degradation, the reaction followed first-order kinetics whereas zeroth-order kinetics is observed in photolytic degradation. The recovery time of 10% and 50% drug degradation are also calculated. In the validation study, the range of linearity is observed between 2.5 to 40 µg/mL. The LOD and LOQ are calculated and it is found 0.1422 µg/mL and 0.4270 µg/mL in acidic medium and 0.1184 µg/mL and 0.3590 µg/mL in basic medium. Stability, inter-day precision, intra-day precision, robustness and ruggedness are calculated and results are within the acceptance criteria. This method can be employed to analyze any calcium channel blockers by slight modification. For a better understanding of CCB, the following perspective can be adopted in the future.*

KEYWORDS: *Nimodipine; Calcium Channel Blocker (CCB); UV-Vis spectrophotometry; quantum mechanics; forced degradation; robustness and ruggedness.*

INTRODUCTION

Nimodipine (NMD) is one of the known calcium channel blocker first developed for treating patients having high blood pressure. It was patented in 1971 and was approved as a medication in Germany in 1985 [1].

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A literature survey revealed that several chromatographic and spectroscopic methods have been reported for the estimation of Nimodipine. They include HPLC [2], GC [3], UPLC-MS/MS [4], LC-MS/MS [5], and UV-spectrophotometric techniques [6-8]. *Zhao et al* [9] have reported a spectrophotometric method for the determination of Nimodipine and also studied the oxidation of Nimodipine by cerium (IV) in an acid solution. A linear relationship was observed between absorbance and concentration of Nimodipine in the range of 1-16 μg/mL.

In 1995, *Chowdary et al.* [10] have developed a rapid spectrophotometric method for the estimation of Nimodipine in a commercial tablet. The method was based on the diazo-coupling reaction which reduced Nimodipine by *N*-(1-naphthyl) ethylene diamine dihydrochloride. The maximum absorbance of the product i.e. azo dye was observed at 550 nm and linear relation was observed in the range of 40 μg/mL.

In 1992, *Squella* [11] used Spectrophotometric and polarographic methods for the estimation of Nimodipine in tablets. For the estimation of Nimodipine polarographically, a buffer system i.e. phosphoric acid - acetic acid (pH-5.0) was used which contains 20% ethanol. The sharp polarographic peak of drug concentration against peak current was observed linearly in the range of 0.1 μM - 1 mM. The Nimodipine has a maximum absorbance at 360 nm in the UV region.

Lahoti et. al. in 2012, validated nimodipine in bulk and tablet formulation. They used the UV-spectrophotometric method and established a method for the estimation of Nimodipine in tablet formulation [12].

Riekes et. al. in 2013 worked on the degradation of Nimodipine under different conditions including photolysis, acidic and alkaline hydrolysis followed by a drug degradation kinetics study. Liquid Chromatography was used further for validation by using C18 column with UV-spectrophotometry. It observed that no degradation was observed for oxidative stress and thermal degradation [13].

In 2013, *Manoela K.* [13] studied Nimodipine by LC method for the determination of stress conditions along with kinetic study. The maximum absorbance of Nimodipine was observed at 235 nm in water. Linearity was found within the range of 5 μg/mL – 35 μg/mL.

Similarly, the literature also showed another approach for the study of drugs i.e. method validation. The method validation is the procedure of indicating that the analytical process is satisfactory for their deliberated use method and they also ensure the quality, purity, identity, and potency of the drug product [14]. The main advantage of method validation is that the degree of confidence is built, not only for the developer of the method but also for the user [15]. Different types of analytical tests could be used that includes Limit tests for the control of impurities, quantitative tests for impurities content, identification tests, and quantitative tests.

In this study, a combined approach is used to analyze and validate our methods. Initially, the spectrophotometric method was used to study the spectral behavior of Nimodipine and validation was followed by a computational approach to understanding the stability and transition of drug molecules in the UV region. The orbital energy of the Highest occupied molecular orbital (HOMO) is less than the lowest unoccupied molecular orbital (LUMO) showing a transition of an electron from π to π^* orbital, whereas experimental results showed a higher value of molar absorptivity also evidence of the transition between π to π^* orbital. In addition, Guidelines from the Food and Drug Administration (FDA) [14], United States Pharmacopoeia (USP) [16], and outlines provided by the International Conference on Harmonization (ICH) guidelines [17] for drug substance were used in force degradation study [16, 18]. The previous study showed that any drug can be easily degraded by the action of impurities, pH, light, temperature, or water. A drug can also react with an excipient or container which causes degradation. In addition, stress testing can be executed to provide data on decomposition mechanisms and decomposition products. The nature of the stability test depends on the type of drug product and individual drug product and some of the impurities may be formed due to degradation [19]. The degradation of drug substances may be due to solid-state stress, solution condition such as acid-base hydrolysis [20, 21], light exposure [17], oxidation [20, 21], temperature [22], and humidity. For thermal degradation temperature range varies from 40°C to 100°C [22]. Photolytic degradation is carried out by UV radiation sources such as UV lamps or sunlight. Light stress conditions can also persuade photo-oxidation by free radical mechanisms [17]. In this work, a forced degradation study is applied to Nimodipine to find the stability of the drug and predicts the chemical reactions that take place during degradation. The drug was subjected to stress conditions by varying temperature, pH, oxidation, and UV light and further

followed by a kinetic study to determine the order of reaction, shelf-life, half-life, and energy of activation by the Arrhenius equation [23]. For method validation of the drug, the International conference on harmonization (ICH) guidelines [17] were used. It includes linearity/ range, the Limit of Detection (LOD) and Limit of Quantitation (LOQ), stability, accuracy, precision, robustness and ruggedness. The results of LOD and LOQ were satisfactory. The accuracy and precision justify the proposed methods' repeatability and relative standard deviation (RSD) ensuring the robustness and ruggedness of the methods and suggesting that the system remains unaffected by a slight variation in the proposed methods and the analyst. The proposed validated study further can be used in pharmaceuticals to set dosage levels and the UV study is also helpful to find out interactions with other conditions.

EXPERIMENTAL SECTION

Chemicals and instrumentation

The reference standard of Nimodipine (1,4-dihydro-2,6dimethyl-4-(3nitrophenyl)-3,5-pyridinedicarboxylic acid 2) was kindly gifted by local Pharmaceuticals and was used as received. Nimoden contains 30 mg of Nimodipine per tablet and was purchased from the local market. The other chemicals include Methanol (Merck), Hydrochloric acid (Merck), Sodium Hydroxide (Merck), zinc dust (Merck), Hydrogen peroxide (Sigma Aldrich), Disodium hydrogen phosphate (Sigma Aldrich), Sodium dihydrogen phosphate (Sigma Aldrich), Double distilled water (0.06µs/cm) and deionized water were used throughout the experiment. Instruments used in the experiment include a thermal bath (BW-20H JEIO TECH, Korea), electrical weighing balance (DENVER TP-214), pH meter (JENWAY 3510), magnetic stirrer (LABTECH LMS-1008), UV-Visible spectrophotometer (Shimadzu UV-1600) and UV-lamp (UVGL-25 compact UV-lamp).

UV-Visible spectrophotometry

At first, a buffer solution of pH 6.8 was prepared by taking a required volume of 0.1M disodium phosphate solution in a beaker, and then pH was adjusted to 6.8 by adding monosodium phosphate. This solution was used as a solvent in the method validation of NMD in a neutral condition.

Computational study

For the quantum mechanics study, the NMD structure was built by using gauss view 6.0 software. There are several algorithms available to determine the set of coordinates equivalent to the minimum energy. These are usually called optimization algorithms; it uses a basis set to attain accuracy in results. Initially, the geometry of NMD was optimized by using water as a solvation model with 6-31G (d, p) pople basis sets. It includes more sets of polarization functions and emphasizes the addition of extra sets of p and d functions to non-hydrogen atoms and an extra set of p functions to hydrogen atoms. The Density Function Theory (DFT) with B3LYP method was used for optimization. The optimized geometry was used to predict UV spectra by CIS methods. To visualize molecular orbitals HOMO/LUMO of NMD, the same level of theory was used to demonstrate the locality of electrons. All calculations have been done by using Gaussian 09 program [24, 25].

Preparation of standard solution of nimodipine at pH 3.3 and 6.8

For the preparation of standard solutions, a method used by Hosakere D. [8] was followed by adding HCl/Zn in preparation for the stock solution of NMD. The standard NMD of 100µg/mL solution was prepared by dissolving (10mg) NMD, (5mL) methanol, (5mL) 4M HCl, and (1g) zinc dust in a 100 mL of volumetric flask followed by shaking for 30 minutes to homogenized the mixture and then deionized water was added up to the mark. The pH of this stock solution was 3.3 and was used in forced degradation and method validation experiments at pH 3.3. Similarly, 100µg/mL solution of standard NMD at pH 6.8 was prepared by the same method followed by adding phosphate buffer of pH 6.8. This stock solution was also used in method validation.

Preparation of sample solution of nimodipine at pH 3.3 and 6.8

For sample preparation at pH 3.3 and pH 6.8, five tablets of Nimoden were weighted and an average weight of each table was calculated. The average weight of each Nimoden tablet was found to be 320 mg which was equivalent to the 30 mg of active ingredient (NMD) present in it. For the preparation of the sample solution of 100µg/mL, the powder form of Nimoden tablet was used which contains NMD (10mg), (5mL) methanol, (5mL) 4M HCl, and (1g) zinc dust in a 100 mL of a volumetric flask, followed by shaking for 30 minutes to the homogenized mixture and then deionized water was added up to the mark. The pH of this

stock solution was 3.3. Similarly, 100µg/mL sample solution of NMD at pH 6.8 was prepared by using phosphate buffer. The contents were filtered and used in the recovery study of method validation.

Forced degradation and kinetics study

The forced degradation of a standard solution of NMD was studied by various pathways i.e. thermal degradation, photodegradation, and oxidative degradation. It is also evaluated by applying acidic and basic conditions. After every degradation process with certain time intervals, the degraded samples were scanned between the UV range of 200-400 nm against a blank (deionized water), and absorbance was recorded at λ_{max} (238nm). Furthermore, the order of reaction was determined from the plots of zeroth-order kinetics, first-order kinetics, and second-order kinetics. The activation energy for acidic and basic degradation was calculated by increasing the temperature up to 10 degrees by using the Arrhenius equation.

$$\log\frac{k_2}{k_1}=\frac{E_a}{2.303R}\left(\frac{T_2-T_1}{T_1T_2}\right) \quad (1)$$

The validation further confirms by the Limit of Detection (LOD) and Limit of Quantification (LOQ) by using the following expressions.

$$LOD=3.3\ \frac{\sigma}{S} \quad (2)$$

$$LOQ=10\ \frac{\sigma}{S} \quad (3)$$

where, s = the slope of the calibration curve
σ = the standard deviation of the response

Validation and stability of nimodipine

The stability of the reagent, standard, and a sample is necessary for a sufficient time to produce reliable and reproducible results. To check stability 24 hours is preferred for reagents and solutions that need to be arranged for each analysis. The stability of the solution was evaluated by scanning of 20µg/mL of NMD and absorbance was recorded at 0 hours and then after 24 hours.

To perform recovery studies, a standard solution of NMD and sample (NMD) were prepared. The three different concentrations of standard i.e. 20 µg/mL, 25 µg/mL, and 30 µg/mL were prepared. It was assumed that 25 µg/mL is to be 100%, where 20 µg/mL and 30 µg/mL were 80% and 120% respectively. These three levels were studied versus 100% NMD standard (25 µg/mL) and pre-analyzed sample. For recovery studies, 2 mL of pre-analyze sample (25 µg/mL) + 2 mL of the standard were added.

To check the precession of the proposed method both intraday and inter-day precession were calculated. Robustness and ruggedness were determined by calculating the Relative Standard Deviation (RSD) of means at room temperature, 10℃ higher than the room temperature and 10℃ less than the room temperature. A 20µg/mL of dilute sample was drawn into three different 10 mL volumetric flasks. One sample was set at a room temperature of 30℃ , another sample was placed in a water bath by maintaining its temperature 40℃ and the third sample was placed in cold water to attain a sample temperature about of 20℃. The absorbance was noted for these three samples as triplicates and %RSD was calculated.

RESULTS AND DISCUSSIONS

UV Spectrum

The absorption spectrum of a standard solution of NMD at pH 3.3 and pH 6.8 was recorded on UV-800 spectrophotometer (Fig. 1a and Fig. 1b). The maximum absorbance (λ_{max}) was found at 238 nm and 230 nm at pH 3.3 and pH 6.8, respectively. The hypsochromic shift is observed due to the solvent effect of phosphate in the absorption spectrum of NMD at pH 6.8 [26]. The calculated value of the molar absorptivity coefficient of NMD was 1.74×10^4 L/mol.cm and 2.11×10^4 L/mol. cm at pH 3.3 and pH 6.8, respectively.

Computational results

Initially, geometry optimization is performed by using a basis set that is based on a semi-empirical method. Once the geometry is optimized to a lower level, it can be further used for the second optimization. In NMD, the Nitrogen atom of pyridine is sp^2 hybridized; it has a single bond and double bond with a lone pair of electrons on the top. It attributes a trigonal planar geometry during optimization to the lower level of theory. Geometry optimization is useful to describe the molecular geometry of a compound to acquire accurate results. For optimization, DFT calculation is performed on NMD. It is faster than Hartree-Fock and based on electron density to compute energy (Fig. 2).

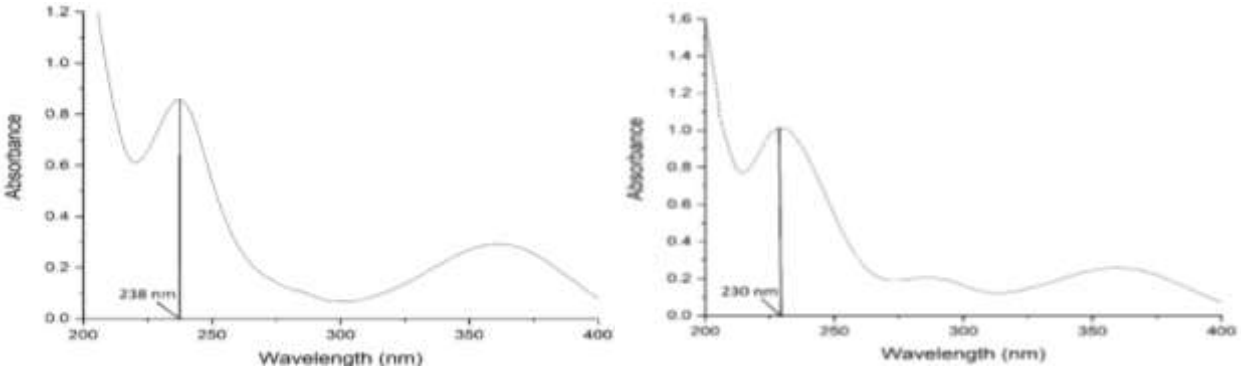

Fig. 1: (a) UV absorption spectrum of nimodipine at pH 3.3 and 1(b) UV Spectrum of nimodipine at pH 6.8

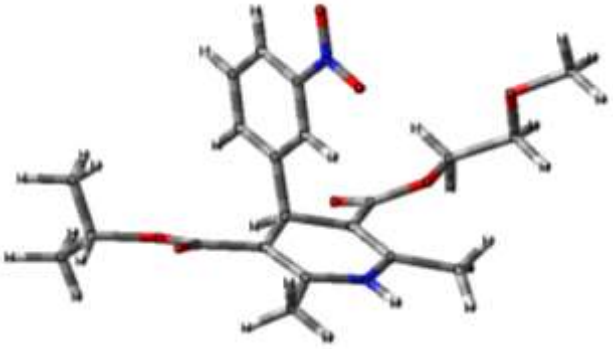

Fig. 2: Optimized geometry of nimodipine model

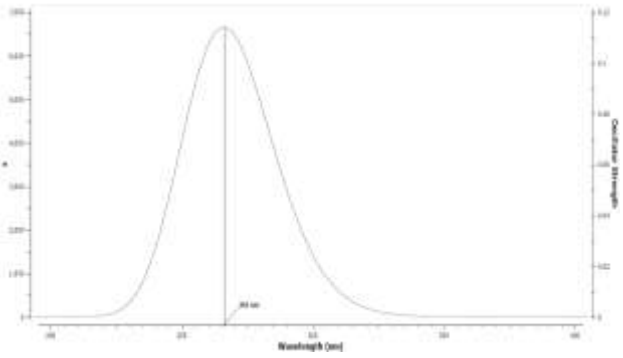

Fig. 3: Computational UV-visible spectrum using water as solvent

Absorption spectra

The calculated electronic absorption spectrum of NMD between molar absorptivity versus wavelength by CIS method is shown in Fig. 3, having absorption bands with the λ_{max} at 262 nm in water which is similar to the electronic absorption spectrum of pyridine [27] whereas the experimental spectrum of NMD has absorption bands with the λ_{max} at 238 nm in water as solvent. The absorption band of the experimental spectrum of NMD with two acetate groups as auxochrome attached with pyridine which arise a certain degree of hypsochromic shift. The same results are reported by *Yi Li* and *Yuan-Yuan Liu* [28] who discussed and compare experimental data of 1,4-Dihydropyridines with calculated data and explain hypsochromic shift due to the Cl_2 and $–OCH_3$ as auxochrome besides pyridine.

The molar absorptivity ε for π–π* transitions is typically in between 1000 to 100,000, whereas for n–π* transitions molar absorptivity is less than 1000. Experimental and computational spectra exhibit an intense band at 220–300 nm and molar absorptivity between 1000 to 100,000 which confirm the π–π* transitions in the pyridine ring. The spectra of methylpyridines measured in 1, 2-dichloroethane [29]. The π–π* bands go along with a shoulder on the low energy side assigned as n–π* transitions of the C=N chromophore [29]. Transfer of strong charge for π–π* and n–π* transitions are slightly overlapped and a single band (220–290 nm) is visualized instead of two separated bands [30]. The π–π* and n–π* bands for un-substituted pyridine show hypsochromic shift of a few nanometers in the visible region differentiating to the spectrum of 2-or 3-substituted pyridines. This effect noticed for all examined pyridines specifies that the energy of π–π* and n–π* transitions are heavily dependent on the substituent's position, which is in acceptance with the results reported by H. C. Brown and J. H. Rush [31, 32].

According to the computational results and literature survey, we can say that π–π* and n–π* transitions can be the favorable transition that occurs in NMD.

Electronic properties

The HOMO and LUMO energy gap reflects the stability of structures and it is seen that the Orbital HOMO is mainly located at the Pyridine and partly related to both

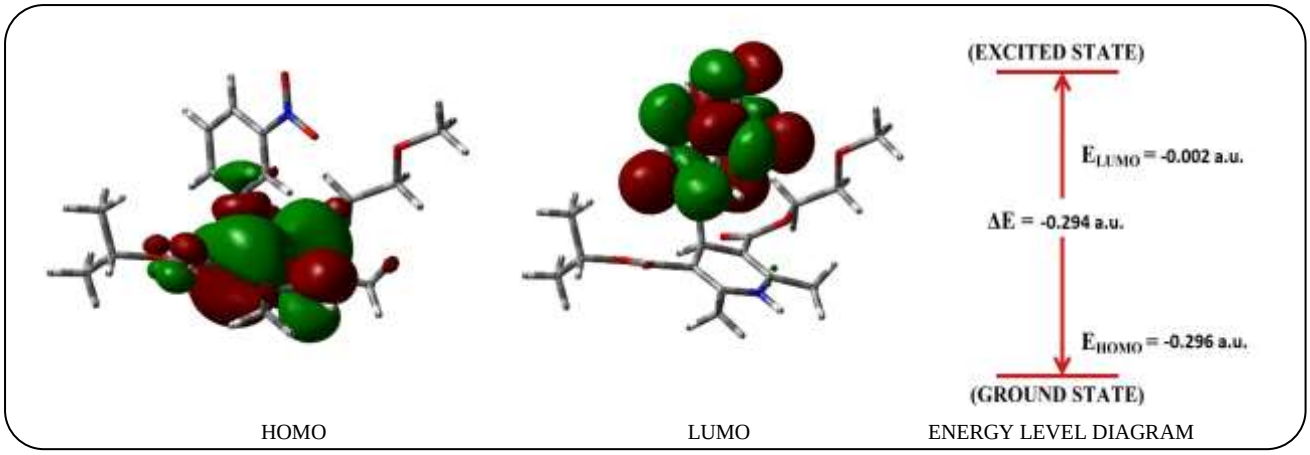

Fig. 4: (a) HOMO and LUMO orbitals with electrostatic contour maps. 4(b) Energy level diagram of HOMO and LUMO orbital by using water as solvent

acetate groups. In the case of LUMO, it is mostly located at nitro benzene. The shapes of these orbitals are visualized in Fig. 4(a). The energy level diagram between HOMO and LUMO is shown in Fig. 4(b). These results indicate that the Pyridine to nitro-benzene charge transfer takes place occurring with higher wavelength (i.e. lower energy) in acceptance with the reported data.

Forced degradation and kinetic study

Thermal degradation

Thermal degradation was studied by taking 25 mL of 20 µg/mL sample solution and kept in a water bath and then absorbance was noted after every hour by using deionized water as a blank. Thermal degradation was carried out at 60℃ and 70℃ by maintaining the constant temperature of the water bath. The absorbance data was used to calculate the Percent recovery (%) by following Equation (4).

$$\text{Percent recovery (\%)} = \frac{\text{absorbance at time (t)}}{\text{initial absorbance}} \times 100 \qquad (4)$$

It has been observed that increase in temperature also increases the percent recovery (%) in thermal stress because some un-dissolved particles starts dissolving in aqueous solution when temperature was increased or a small part of solvent evaporates by increasing the temperature as a result it concentrate the solution (Table1). Hence in aqueous medium Nimodipine showed unpredicted behaviors, where degradation was mainly observed in terms of loss of assay (Fig. 5a and Fig. 5b). Therefore, it can be concluded that Nimodipine is not susceptible to degrade in thermal conditions.

Photo degradation

In a previous study, *Rao* [33] worked on a degradation mechanism and it has similar results for photodegradation. For photodegradation of Nimodipine, 25mL sample solution was carried out from 20 µg/mL stock sample solution and kept under the UV lamp. The absorbance was noted at room temperature (25°C) before and after every hour for up to four hours by using deionized water as a blank (Fig. 6).

At room temperature, the initial absorbance of NMD was 0.626 at zero time. The decrease in absorbance was observed when UV radiation was introduced into the sample solution. The absorbance was noted for 4 h with the same stress condition and after 240 minutes absorbance of NMD was found to be 0.563 which indicates that NMD was not steady in photolytic stress (Fig. 6). The percent recovery after photodegradation for time is shown in Table 1.

Kinetic study of photodegradation

To find the order of reaction for photochemical degradation, graphs were plotted. For zero order kinetics, the graph between absorbance versus time, for first order reaction Ln of absorbance versus time, and for second-order reaction reciprocal of absorbance versus time was plotted. It was observed that photodegradation of NMD follows zeroth order kinetics in an aqueous medium [34-37] (Fig. 7).

Table 1: The percen recovery after force degradation by thermally, photo, oxidative and in acidic and basic conditions

Forced Degradation	Time (Minutes)			
	60	120	180	240
Thermal degradation at 60°C	100.34	100.53	100.87	100.39
Thermal degradation at 70°C	100.52	100.05	101.57	101.92
Photo degradation	98.40	97.28	95.20	89.93
Oxidative degradation	97.54	94.40	92.50	89.71
Acidic Condition at 60°C	63.04	39.61	38.33	20.64
Acidic Condition at 70°C	53.37	34.55	30.24	16.63
Basic Condition at 60°C	70.90	34.54	33.43	21.81
Basic Condition at 70°C	65.84	40.07	35.57	16.75

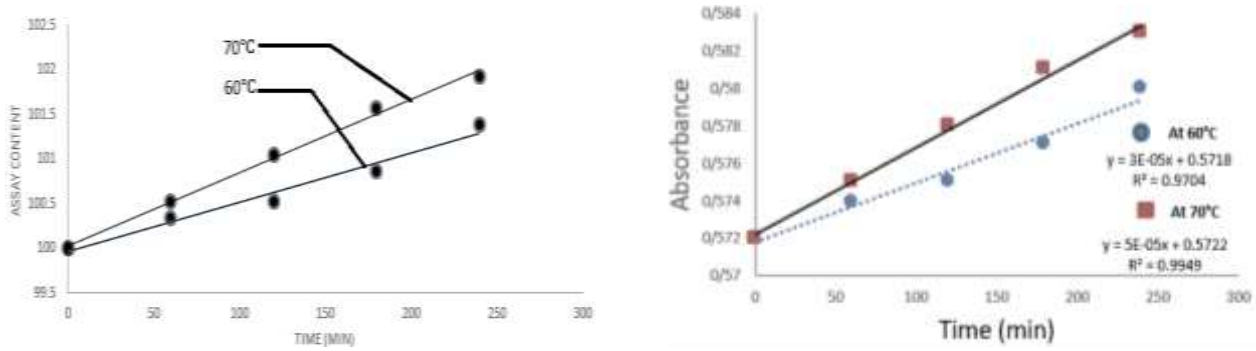

Fig. 5: (a) Assay content vs time (5b) Absorbance vs time of thermal degradation at 60°C and 70°C

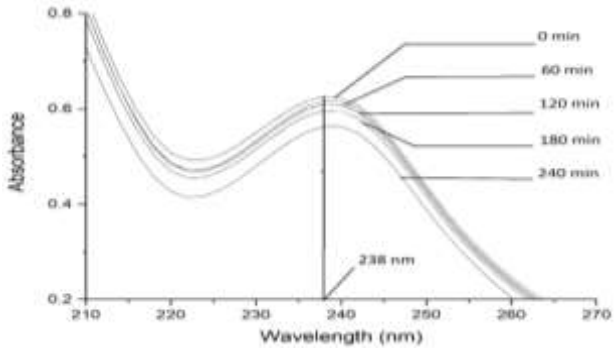

Fig.6: Photo degradation spectra at different time intervals

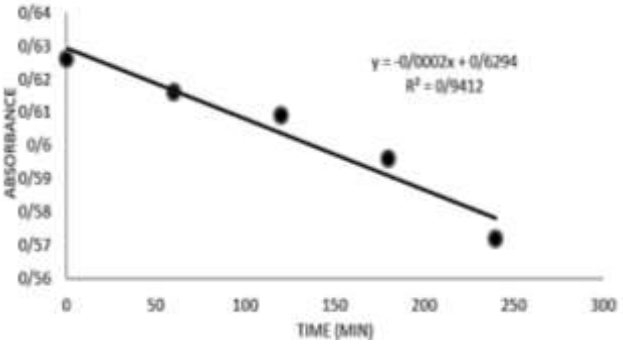

Fig. 7: Zeroth order plot for photo degradation

Time ($t_{50\%}$ and $t_{90\%}$) for Photo degradation

Time of photo degradation was calculated by the help of the following equations for zeroth order kinetics

$$t_{50\%}=\frac{A\square}{2k} \quad (5a)$$

and

$$t_{90\%}=0.1\ \frac{A\square}{k} \quad (5b)$$

Where A_o absorbance at zero time and k is the rate constant. It was observed that photo degradation was time dependent. 10.0% of degradation was noticed in 5 hours and 13 minutes whereas 50.0% of degradation was noticed in 26 hours and 5 minutes at room temperature.

Oxidative degradation

From the 20µg/mL NMD sample solution, 25mL sample was subjected to oxidation by adding 25 mL of 0.3% hydrogen peroxide and absorbance was noted from 0-240 m after regular interval of time. Oxidative degradation was carried out at room temperature (25°C) and the plot of absorbance versus wavelength was predicted (Fig. 8a).

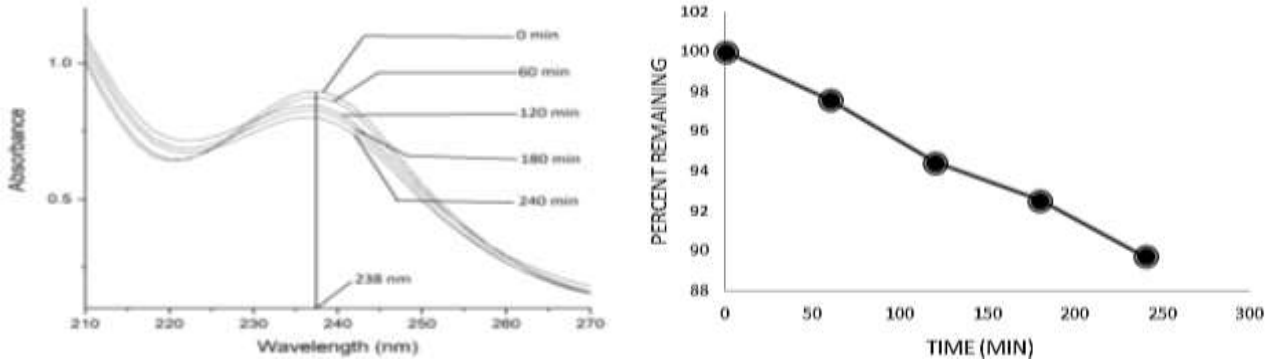

Fig. 8: (a) Oxidative degradation spectra at different time interval; 8(b) percent recovery Vs time

Absorbance was start decreasing once the oxidizing agent was added to the solution, which indicates that NMD was not steady in oxidative stress as shown in Fig. 8a and 8b. The percent recovery after oxidative degradation for time is shown in Table 1 (Fig. 8b).

Kinetic study of oxidative degradation

The order of reaction for oxidative degradation was determined by plotting a graph between absorbance versus time for zeroth order kinetics, Ln of Absorbance versus time for first-order kinetics, and reciprocal of absorbance versus time for second order kinetics to determine the order of a reaction. The oxidative degradation of NMD was followed 1st order kinetics in an aqueous medium (Fig. 9). The rate constant was found $5.0 \times 10^{-4} s^{-1}$ and it was determined from the linear equation of first-order, where *Dhara R. Patel* [38] also has similar results for oxidative degradation.

Time ($t_{50\%}$ and $t_{90\%}$) for oxidative degradation

For first order reactions ($t_{50\%}$ and $t_{90\%}$) are calculated by the given equations:

$$t_{50\%} = \frac{0.693}{k} \quad (6a)$$

and

$$t_{90\%} = \frac{0.106}{k} \quad (6b)$$

It was observed that oxidative degradation was time-dependent. 10.0% of degradation was noticed in 3 hours and 32 minutes whereas 50.0% of degradation was noticed in 23 hours and 6 minutes at room temperature.

Degradation in Acidic condition

Acidic degradation was carried out by taking 30mL of 20 µg/mL standard NMD solution in 100 mL of volumetric flask and add 30mL of 5M HCl. 10 mL of solution was drawn in four different vials and after every 60 minutes, neutralized by adding 5 mL of 5M NaOH. The absorbance of these four samples was noted by using deionized water as blank (Fig. 10a). Degradation was carried at 60℃ and 70℃ (Fig. 10b) by maintaining the constant temperature of the water bath, where kinetic study and percent recovery was calculated.

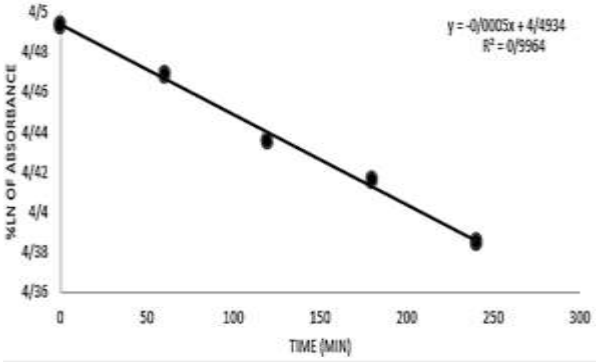

Fig. 9. First order plot for oxidative degradation

At 60℃, the initial (zero time) absorbance of NMD was 1.323. Absorbance was start decreasing once the acid was added to the solution, which indicates that NMD was not steady in acidic stress at 60℃. Analysis was performed for four hours with the same stress condition. The final absorbance of NMD was found 0.273. Acidic degradation spectra at 60℃ is shown in Fig. 10(a) and the percent recovery after acidic degradation with time is shown in Table 1 (Fig.11(a) and Fig. 11(b)).

NMD in an aqueous medium was found to be unstable under the acidic condition at 60℃. Similarly acid degradation was also carried out at 70℃. NMD in an aqueous medium was also unstable under the acidic condition at 70℃ (Fig. 10b).

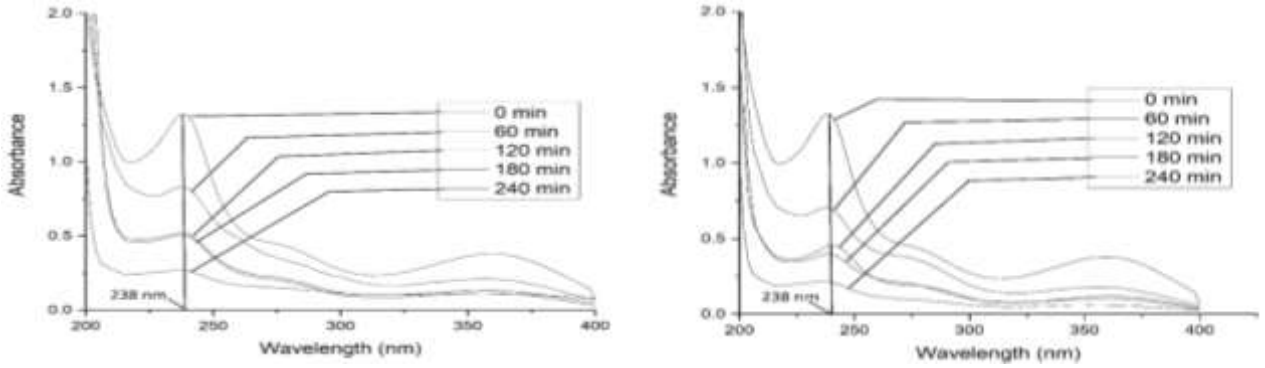

***Fig. 10: (a) Acidic degradation spectra at different time interval at 60*°C *and 10(b) acidic degradation spectra at different time interval at 70*°C**

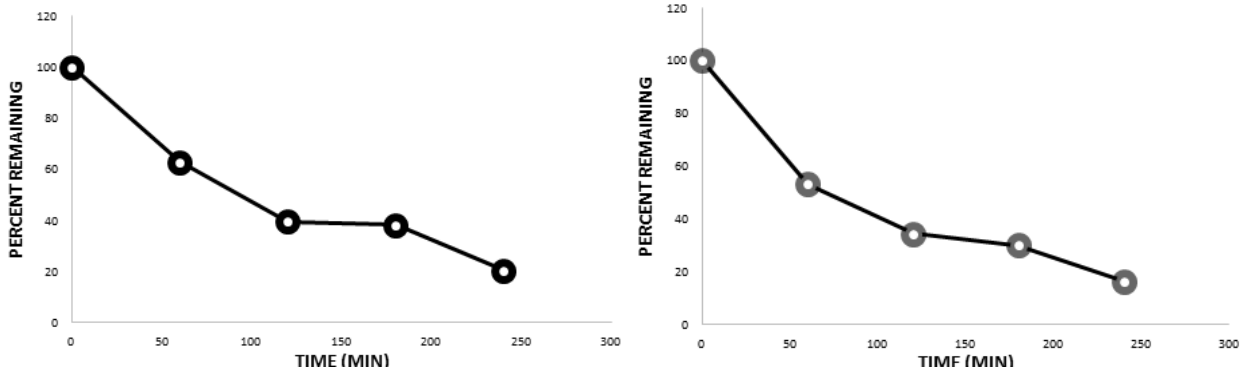

***Fig. 11: (a) Assay content Vs Time under Acidic degradation at 60*°C *and 11(b) percent recovery Vs Time under Acidic degradation at 70*°C**

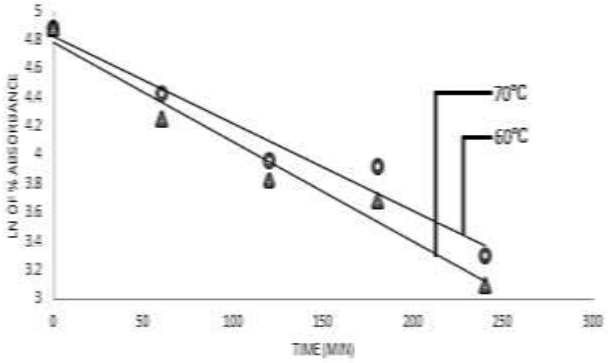

***Fig. 12: First-order plot of acidic hydrolysis at temperature 60*°C *and 70*°C**

Kinetic study of acidic degradation at 60°C *and 70°C*

A kinetic study was carried out by plotting the graph between Ln of absorbance versus time and it indicates that acidic hydrolysis in water followed the first-order reaction at 60°C and 70°C. The rate constant was determined from the linear equation of first-order which was $6.1 \times 10^{-3} s^{-1}$ for 60°C and $6.9 \times 10^{-3} s^{-1}$ for 70°C as shown in Fig.12. The activation energy was found to be 11.7 k joules / mole or 2.79 k calories / moles. The activation energy was calculated by the help of equation (1)

Time ($t_{50\%}$ and $t_{90\%}$) for degradation in acidic condition

For first order reactions ($t_{50\%}$ and $t_{90\%}$) are calculated by the given equations:

$$t_{50\%} = \frac{0.693}{k} \quad (6a)$$

and

$$t_{90\%} = \frac{0.106}{k} \quad (6b)$$

It was observed that acidic degradation was time dependent. 10.0% of degradation was noticed in 17.37min whereas 50.0% of degradation was noticed in 1 hours and 53.6 minutes at 60 °C temperature. At 70°C 10.0% of degradation was observed in 15.36 minutes, whereas 50.0% of degradation was noticed in 1 hour and 40.43 minutes.

Degradation in Basic Condition

Base degradation was carried out by taking 30mL

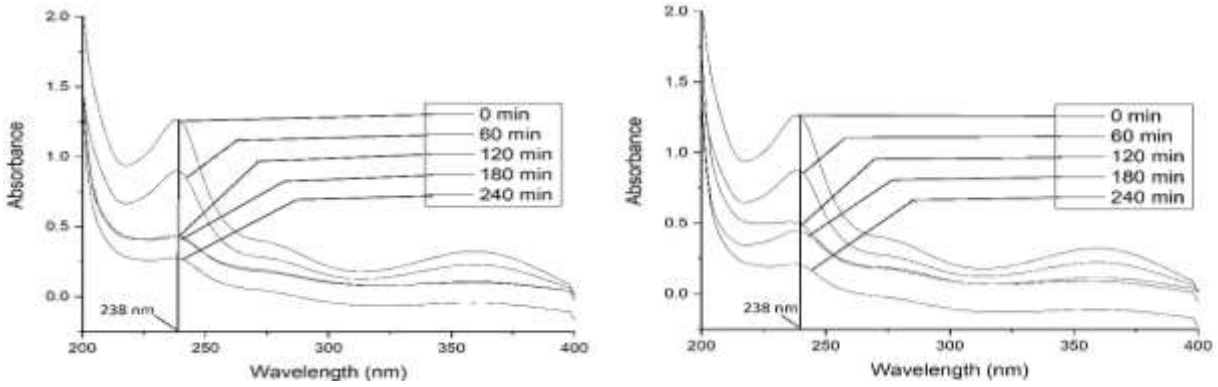

***Fig. 13(a) Basic degradation spectra at different time interval at 60*℃ *and 13(b) Basic degradation spectra at different time interval at 70*℃**

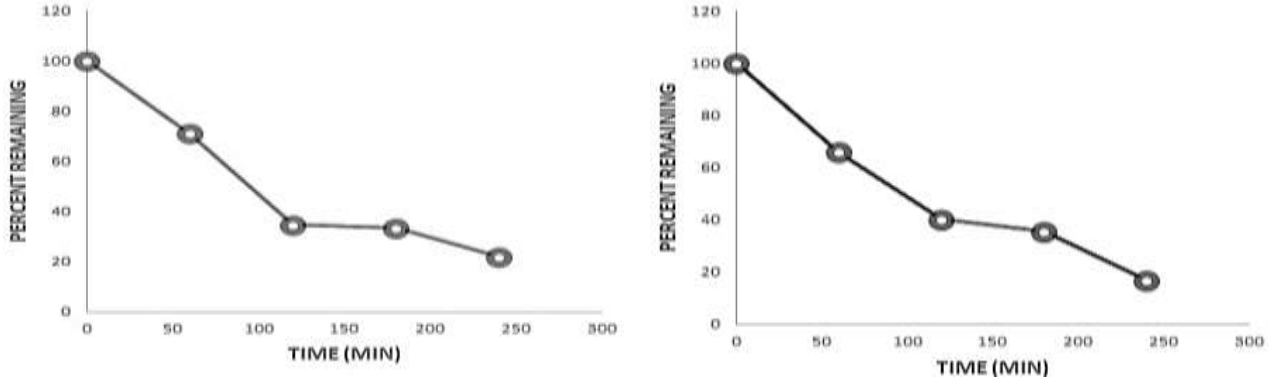

Fig. 14: (a) Assay content vs time under basic condition at 60°C; (b) Assay content vs time under basic condition at 70°C

of 20 µg/mL solution in 100 mL of volumetric flask and add 30 mL of 0.1 M of NaOH then 10 mL of this solution was drawn in four different vials after every 60 minutes, each sample was neutralized by adding 5 mL of 0.1 M HCl. The degradation was carried at 60℃ and 70℃ by maintaining the constant temperature of water bath. The absorbance of these four samples was noted by using deionized water as a blank and used to study the kinetic and percent recovery. The spectra is shown in Fig. 13a and 13b, it showed that NMD was not steady in basic stress at 60°C and 70°C and the percent recovery after basic degradation with time is shown in Table 1 (Fig. 14(a) and Fig. (b)).

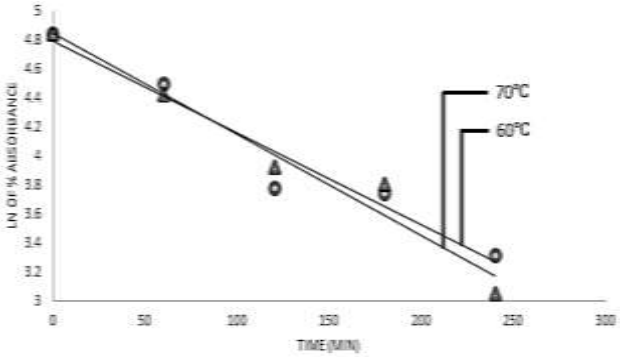

***Fig. 15: First-order plot of basic hydrolysis at temperature 60*℃ *and 70*℃**

Kinetic study of basic degradation at 60℃ *and 70°C*

Kinetic study was carried out by plotting the graph between Ln of absorbance versus time and it indicates that basic degradation in water followed a first-order reaction at 60℃ and 70°C (Fig. 15). The rate constant at 60℃ and 70°C were determined from the linear equation of first-order which was $6.3 \times 10^{-3} s^{-1}$ and $7.0 \times 10^{-3} s^{-1}$ respectively. It was observed that basic degradation was time dependent and Manoela K. [13] also has similar results for photolytic, acidic and basic degradation. The activation energy was calculated by the help of equation (1) and it was found to be 10.00 k joules / mole or 2.39 k calories / moles.

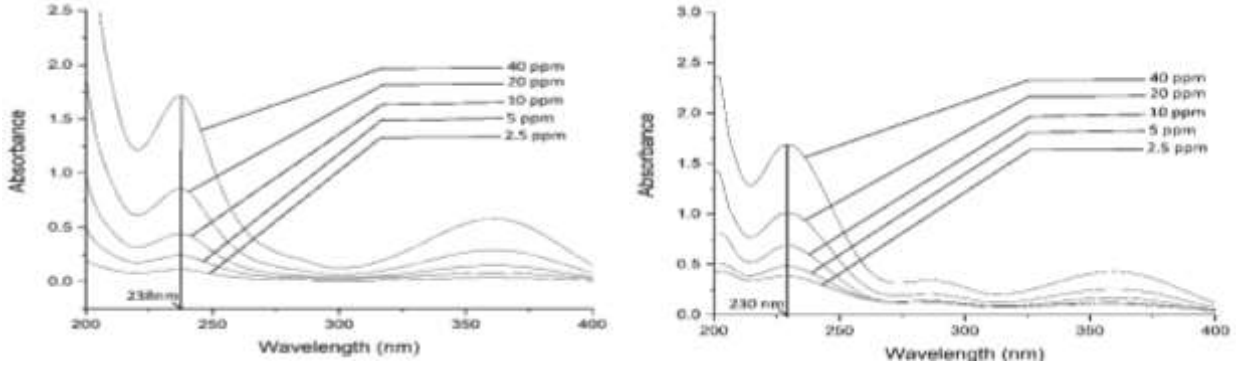

Fig. 16: (a) Overlay scan of nimodipine at pH 3.3 concentration range from 2.5 µg/mL to 40 µg/mL and 16(b) Overlay scan of nimodipine at pH 6.8 concentration range from 2.5 µg/mL to 40 µg/mL

Time ($t_{50\%}$ and $t_{90\%}$) for degradation in acidic condition

For first order reactions ($t_{50\%}$ and $t_{90\%}$) are calculated by the given equations:

$$t_{50\%}=\frac{0.693}{k} \quad (6a)$$

and

$$t_{90\%}=\frac{0.106}{k} \quad (6b)$$

It was observed that basic degradation was time dependent. 10.0% of degradation was noticed in 16.82 min whereas 50.0% of degradation was noticed in 1 hours and 50 minutes at 60 °C temperature. At 70°C 10.0% of degradation was observed in 15.14 minutes, whereas 50.0% of degradation was noticed in 1 hour and 39 minutes.

Method validation at pH 3.3 and at pH 6.8

i- Linearity and Range

To check linearity 100 µg/mL stock solution was diluted into 2.5 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL and 40 µg/mL in 10 mL of volumetric flask and spectra was recorded (Fig. 16a) at pH 3.3. The calibration curve showed a correlation coefficient i.e. R^2 =0.9998, it confirms linearity over the range of concentration (2.5 µg/mL - 40 µg/mL) as shown in Fig. 17. Similar analysis was performed at pH 6.8. For this 50:50(v/v) phosphate buffer and double distilled water were used as blank. The overlay spectrum was recorded (Fig. 16b). The calibration curve shows Correlation Coefficient (R^2 = 0.9992) near 1 and it confirms linearity over a range of concentration from 2.5 µg/mL to 40 µg/mL at pH 6.8 (Fig. 18).

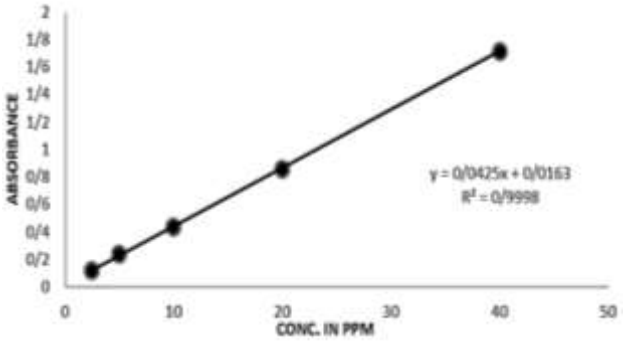

Fig. 17: Calibration curve at pH 3.3

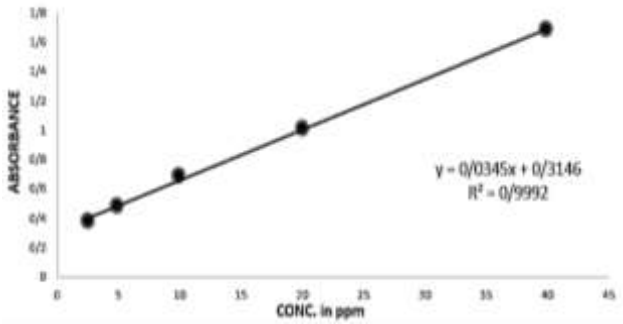

Fig. 18: Calibration curve at pH 6.8

ii- LOD and LOQ

To calculate limit of detection (LOD) and limit of quantification (LOQ), standard deviation was calculated by five replicates i.e. 1.815×10^{-3} at pH 3.3. The slope was calculated from the calibration curve i.e. 0.0425 (Fig. 17). LOD and LOQ was calculated and found to be 0.1422 µg/mL and 0.4270 µg/mL in aqueous medium using equation (2) and (3) respectively. At pH 6.8 the LOD was found to be 0.1184 µg/mL and LOQ was found to be 0.3590 µg/mL in aqueous medium.

iii- Stability tests

For stability testing stock solution (100 µg/mL) was diluted into 20 µg/mL by adding deionized water and 4 M HCl as a catalyst, pH of solution was found to be 3.3. At 0 hour and 24 hours absorbance was recorded in triplicate to check stability. Relative Standard Deviation (RSD) at 0 hours was found to be 0.68 and it is under the range of 2 (Table 2). Similarly, RSD after 24 hours just lies above the range. Similarly, for pH 6.8 absorbance was recorded at 0 hour and then after 24h. Relative standard deviation (RSD) at pH 6.8 was 1.54 as per ICH guideline [17] (Table 2).

iv- Accuracy

The percentage recovery of NMD from the analyzed solution of formulation was calculated in the recovery range from 80% to 120%. The individual percent recovery of NMD was found to be 102.07%, 102.63% and 97.55% for 100%, 80% and 120% accuracy level respectively at pH 3.3. The average % recovery for 80%, 100% and 120% accuracy level of NMD was 100.75% at pH 3.3. Results of recovery studies for NMD are shown in Table 3. The overlay spectrum is shown in Fig. 19. Similarly, the percentage recovery of NMD from the analyzed solution of formulation was calculated in the recovery range from 80% to 120% for pH 6.8. The individual percent recovery NMD was found to be 101.4414%, 99.14237% and 98.33333% for 100%, 80% and 120% accuracy level respectively. The average % recovery for 80%, 100% and 120% accuracy level of NMD was 99.64% at pH 6.8. The overlay spectrum is shown in Fig. 20 and results of recovery studies for NMD at pH are shown in Table 3. The Statistical analysis was also carried out through student's t-test for comparison of two means of pH 3.3 and 6.8., in which significant results showed a probability greater than 5% ($p > 0.05$ with a 95% confidence level). For statistical analysis MS-Excel software was used.

v. Precession

Intraday precession or repeatability

To evaluate intraday precession, precision was calculated on same day but different time interval for 20µg/mL NMD solution. The analysis were performed in triplicate and the absorbance was taken five times after every hour and RSD was calculated for pH 3.3 and pH 6.8 (Table 4).

Table 2: Absorbance of 20 µg/mL before 0 hour and after 24 hours at pH 3.3 and pH 6.8

pH	Time (hours)	Absorbance at 20 µg/mL drug	RSD
pH = 3.3	0	0.727 0.727 0.726	0.68
	24	0.733 0.734 0.735	
pH = 6.8	0	0.974 0.974 0.975	1.54
	24	0.953 0.951 0.955	

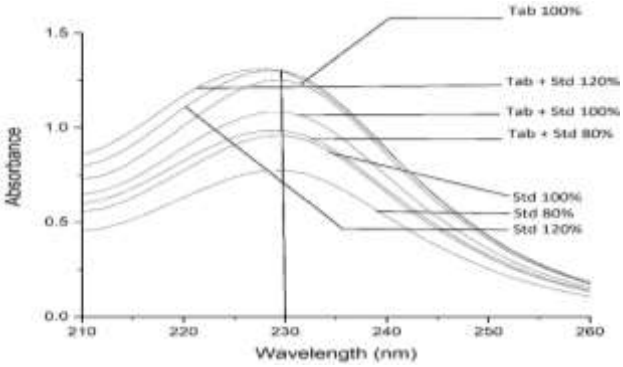

Fig. 19: Overlay Spectra of sample and standard drug at different concentration level at pH 3.3.

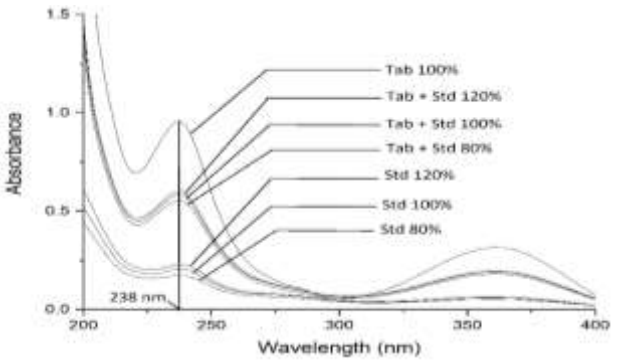

Fig. 20: Overlay spectra of sample and standard drug at different concentration level at pH 6.8

Inter-day or intermediate precision

To assess the intermediate precision (also known as the ruggedness) of the purposed method, precision was performed on different days. Inter-day precision of 20 µg/mL NMD was determined by taking absorbance three times after every hour on three consecutive days and %RSD was calculated. The %RSD for inter-day precision is 1.70 which is less

Table 3: Percent recovery at different concentration level of nimodipine at pH 3.3

pH	Conc. Level of studies (%)	Absorbance of sample 10 µg/mL	Absorbance of spiked standard	Absorbance of sample + standard	Recovery (%)	Mean Recovery (%)
pH=3.3	100	1.252	0.959	1.083	102.07	
	80	1.252	0.774	0.984	102.63	100.75
	120	1.252	1.304	1.310	97.55	
pH=6.8	100	0.951	0.205	0.583	99.14237	
	80	0.951	0.175	0.555	101.4414	99.64
	120	0.951	0.229	0.6	98.33333	

Table 4. Absorbance at different time interval within a day (Intra day) at pH 3.3 and 6.8

pH	Time(min)	Absorbance	RSD
pH = 3.3	0	0.749	0.60
	60	0.759	
	120	0.751	
	180	0.759	
	240	0.754	
pH = 6.8	0	0.974	1.59
	60	0.956	
	120	0.979	
	180	0.994	
	240	0.993	

Table 5: Absorbance at different time interval in three consecutive days at pH 3.3 and 6.8

pH	Time (min)	Day 1	Day 2	Day 3	%RSD
pH = 3.3	0	0.727	0.734	0.756	1.70
	60	0.725	0.721	0.754	
	120	0.726	0.732	0.737	
	Mean	0.726	0.729	0.749	
pH = 6.8	0	0.974	0.953	0.945	1.98
	60	0.956	0.929	0.926	
	120	0.979	0.943	0.933	
	Mean	0.969	0.941	0.934	

than 2 at pH 3.3 (Table 5). It justifies the proposed method repeatability and intermediate precision. In case of pH 6.8 all values of RSD fulfill the ICH guideline [17] (Table 5).

vi. Robustness and Ruggedness

The mean % RSD of absorbance was calculated to check the robustness measured in triplicates. The result showed that % RSD is within limits for variation at temperature (±10℃). The allowable temperature should be between 20℃ to 40℃. It can be concluded that the temperature variation cannot affect the proposed method. Robustness was determined by calculating %RSD of means at room temperature, 10℃ higher than room temperature, and 10℃ less than room temperature, same as reported for NMD at pH 3.3. % RSD of absorbance was within limits for variation in temperature (±10℃) that is 0.29 at pH 6.8 (Table 6). Hence it shows that the method is robust even with slight temperature changes and has the capacity to remain unaffected.

Similarly ruggedness of the method was evaluated by repeating the analysis with same experimental conditions by other analyst and RSD of mean exhibit the ruggedness of the proposed method as shown in Table 7.

vii. Statistical analysis

The statistical analysis of the data was performed through one way of analysis of variance (ANOVA), in which significant results showed the probability greater than 5% ($p > 0.05$ with 95% confidence interval). For this statistical evaluation MS Excel software was used.

CONCLUSION

It is concluded that the NMD sample was prepared at two different pH and validation was performed to check the proposed method following International Conference on Harmonization (ICH) guidelines. The parameters such as linearity/range, the Limit of Quantification (LOQ), Limit of Detection (LOD), stability, accuracy, precision, robustness and ruggedness were used to determine the stability of NMD, and results were expressed in Relative Standard Deviation (RSD) with acceptance criteria of less than 2. The NMD sample was scanned on UV-Spectrophotometer between 200-400 nm against blank. NMD shows maximum absorbance at 238 nm and 230 nm at pH 3.3 and 6.8 respectively. The experimental results were also compared

Table 6: Robustness of proposed method analyzed at different temperature and at pH 3.3 and pH 6.8

Replicates	30℃ (Room temperature)	40℃	20℃	RSD of mean
1	0.727	0.729	0.730	0.29
2	0.725	0.730	0.729	
3	0.726	0.731	0.729	
Mean	0.726	0.730	0.729	
1	0.946	0.913	0.933	1.67
2	0.948	0.915	0.931	
3	0.946	0.917	0.932	
Mean	0.946	0.915	0.932	

Table 7: Ruggedness of proposed method analyzed at different temperature and at pH 3.3 and pH 6.8

Replicates	30℃ (Room temperature)	40℃	20℃	RSD of mean
1	0.728	0.731	0.726	0.52
2	0.724	0.732	0.725	
3	0.726	0.733	0.724	
Mean	0.726	0.732	0.725	
1	0.947	0.953	0.936	1.04
2	0.948	0.952	0.934	
3	0.949	0.954	0.932	
Mean	0.948	0.953	0.934	

with the theoretical data, the geometry of the NMD structure was optimized by using the density functional theory method, and then ground state calculation was examined with the help of the CIS method. Hypsochromic shift was observed in the experimental absorption band when compared with the calculated absorption band which is due to the acetate group as auxochrome neighboring with pyridine. HOMO and LUMO were also calculated which demonstrates the electron transfer from pyridine to nitro-benzene. The molecular electronic transition was also predicted. Therefore, with the help of the molar absorptivity coefficient, absorption bands difference, and molecular orbitals it can be concluded that the absorption spectrum of NMD is dominated by π–π^* and n–π^* transitions. The energy gap between a ground state and exited state was also calculated which was -0.294 a.u. which indicates high polarizability as well as high chemical reactivity of NMD. Hence, it can be concluded that the developed methods using UV-spectroscopy can be considered sensitive and fast reproducible methods for the determination of NMD.

Furthermore, thermal, oxidative, photolytic, acidic, and basic degradation was performed in water, and the order of reaction was calculated along with associated assay content. No degradation was observed in thermal degradation. First-order kinetics was observed in acidic, basic, and oxidative degradation whereas zeroth-order kinetics was observed in photolytic degradation. NMD was observed highly unstable in a basic medium, during the forced degradation study it was noticed that 0.1 M of NaOH having pH 12.5 is enough to form an unstable medium for a drug. In our results, the accuracy of the system showed that average % recovery for 80%, 100%, and 120% accuracy levels of NMD, and it was found 100.75% and 99.64% at pH 3.3 and pH 6.8 respectively. According to the student' s t-test for the comparison of two means there is no significant difference between the two means ($p > 0.05$) at pH 3.3 and 6.8 and the values were within acceptable ranges of official method (100±2.0%) (13). For precession, both intraday/repeatability as well as inter-day/intermediate systems were studied. The %RSD for intraday precision and inter-day precision was calculated was found 0.60 and 1.70 respectively which were less than 2. It justifies the proposed method's repeatability and intermediate precision.

Robustness was determined by calculating the RSD of means at room temperature, 10℃ higher than room temperature and 10℃ less than room temperature. At pH 3.3 % RSD of absorbance was within limits for variation in temperature (±10℃) that is 0.29. Ruggedness was also determined by repeating the developed method at both pH under same experimental conditions with other analyst and then calculates RSD and its value was found to be 0.52 for pH 3.3 and 1.04 for pH6.8 which showed the ruggedness of method. Our results validated the reliability and robustness of our analytical methods and suggested that the system remains unaffected by a slight variation in proposed method parameters. It is suggested that this method can be employed for routine quality control in drug testing laboratories and can be used for process assessment in pharmaceutical manufacturing firms.

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