Advances in Bioresearch
Adv. Biores., Vol 14 (6) November 2023: 362-370
©2023 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
DOI: 10.15515/abr.0976-4585.14.6.362370

Advances in Bioresearch

ORIGINAL ARTICLE

Greenness Assessment and QbD based novel stability-indicating RP-HPLC for the Determination of Nimodipine in bulk and dosage form with characterization of degradation products and toxicity prediction by using in-silico approach

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ABSTRACT
Stability of Nimodipine in tablet and bulk form was developed and validated using chromatographic methods. The Nimodipine was exposed to the oxidizing agent like hydrogen peroxide, heat (dry and wet) and photolytic conditions, as well as acid, alkali, and water hydrolysis. However, in acid, alkali, oxidative, and wet heat thermal conditions, considerable degradation was observed. The drug's stability under photolytic and dry heat conditions was established. The drug and its degradation products were separated on Sapphirus C18 with specification of (250 mm × 4.6 mm, 5 µm) and mobile phase contain acetonitrile and water (80:20 % v/v). According ICH Q2 (R1) all method validation parameters are verified. Robustness of method was carried out by using QbD approach. Determination of the active pharmaceutical ingredient was not interfered by excipients or degradation products. In the 02–10 µg/mL range, the response was shown to be linear. LC-MS fragmentation experiments were used to characterize the degradation products. The findings may lead to the suggestion of a more thorough drug degradation mechanism. Method greenness was determine by using AGREE tool and comparison between reported method and developed method was carried out. The Toxtree software (Version 3.1.1) was then used to forecast the toxicity of the degradation products. Additional toxicity determination was carried out by using in-silico method.
Keywords: *Nimodipine, RP-HPLC, degradation products, Validation, in-silico toxicity, AGREE.*

Received 25.09.2023 Revised 08.10.2023 Accepted 26.11.2023

How to cite this article:
Kavita C, Sandeep S. Greenness assessment and QbD based novel stability-indicating RP-HPLC for the determination of Nimodipine in bulk and dosage form with characterization of degradation products and toxicity prediction by using in-silico approach. Adv. Biores., Vol 12 (6) November 2023: 362-370.

INTRODUCTION

A 1,4-dihydropyridine calcium channel blocker of second generation is nimodipine ($C_{21}H_{26}N_2O_7$). Through maintaining voltage-gated L-type calcium channels in their inactive conformation, it primarily affects vascular smooth muscle cells. Nimodipine reduces calcium-dependent smooth muscle contraction and subsequent vasoconstriction by blocking the influx of calcium into smooth muscle cells [1]. Nimodipine has larger impact on cerebral circulation than on peripheral circulation when compared to other calcium channel blockers [2].

Many reports are available in the literature on degradation behavior of Nimodipine (NMD). The drug is established to specifically sensitive to hydrolysis, oxidation and heat conditions. For this reason special handling precaution are employed to protect drug from hydrolysis, oxidation and heat conditions both in solution and solid state.

Alyne et al developed Stability-indicating methods for the enantioselective determination of dihydropyridines by high performance liquid chromatography and capillary electrophoresis [3]. Manoela K. Riekes et al and Sagar Suman Panda et al developed Stability-Indicating LC Method in the Presence of

its Degradation Products and Overall Kinetics study and Spiked Serum Using Systematized Design of Experiments (DoE) Approach [4, 5]. R. Swetha Sri et al developed a stability indicating study for quantitative estimation of nimodipine in bulk and pharmaceutical dosage form [6]. But there was lack of characterization and identification of degradation products with their toxicity prediction.
The current work's objective was to performed force degradation behavior of NMD at hydrolytic, oxidative, thermal (wet and dry heat) conditions and to characterize degradation products by using mass spectrometry. A greenness assessment for the analytical procedures by using AGREE tool was determined and additionally toxicity properties of DPs (degradation products) were compared to drug.

INSTRUMENTS AND EQUIPMENT

The HPLC analysis was carried out using dual pumps Waters Alliance e2695 separation module and Detector is a Photodiode array (model 2998) with wavelength range of 190-800 nm having rheodyne sample injection port with a 10 µl loop. The separation were made on a Sapphirus C18 with column specification (250 × 4.6 mm, 5 µm) and the analysis of data was carried out by using Waters Empower 3 Software. The other equipment used in analysis consists of digital weighing balance (AUX 220, Shimadzu Corporation)), electric hot air oven (MSI-66, Meta Lab Scientific industries, India), Ultrasonic bath (PCI Analytics Pvt. Ltd, Mumbai, India).

MATERIAL AND METHODS

Drug sample NMD was obtained as a gift sample from USV Pvt Ltd., Mumbai, India., certified to contain 99.74 %, w/w and used without further purification for analysis. The experiment called for HPLC-quality methanol and acetonitrile. Other chemical included analytical laboratory grade hydrogen peroxide, potassium dihydrogen orthophosphate, sodium hydroxide flakes, hydrochloric acid are procured from S D Fine-Chem Ltd. in Mumbai. Double distillation assembly used for preparation of double-distilled water. Nimodipine film-coated tablets were 30 mg (Nimodip 30 mg) purchase from medical store.

EXPERIMENTAL

Chromatographic condition

During analysis chromatographic conditions used consist of Sapphirus C18 column (250 mm × 4.6 mm, 5 µm) and mobile phase used as acetonitrile and water 80:20% v/v maintain at a flow rate of 1.0 mL/min. The eluents may be seen at 236 nm. Every analysis was performed at room temperature.

Forced degradation studies

Studies on forced degradation of NMD have been done. Every investigation was conducted with a target degradation rate of 5-20%. For acid and alkali hydrolysis carried out at room temperature in 0.1 N HCl or 0.1 N NaOH for 24 h. Additionally, an aqueous suspension of the medication was boiled under reflux for 4 hours at 70°C in order to perform the degradation under wet heat. At ambient temperature and in the dark for 48 hours, 30% v/v H_2O_2 was used to facilitate oxidative breakdown. Additionally, NMD was put through photolytic testing, which comprised exposing it to sunlight for 24 hours, as well as a dry heat study that involved placing the NMD in a hot air oven at 70°C for 4 hours. After being exposed, the drug samples were quenched. Lastly sample was to maintain a final concentration of drug 10 µg/mL, and analysis was performed on them. As a sign of degradation, it was thought that the NMD peak area had decreased and/or subsidiary peaks had begun to emerge. The errors were avoided each time by using the appropriate controls and blanks.

Characterization of Degradation Products:

Samples that had been stress under the various circumstances mentioned above were subjected to LC-MS analyses. The samples were examined using positive electrospray ionisation (ESI) in an MS spectrometer. A mobile phase compatible with MS (1 vol% formic acid: acetonitrile) and the HPLC technique were used to separate the samples. With the use of the LC-MS data, structure elucidation was carried out.

Validation of HPLC method

ICH Q2 (R1) guideline is commonly used for analytical method validation [7]. The standard NMD stock solution was added in the NMD tablet formulation stock solution at to achieve 80%, 100%, and 120% QC range along the calibration curve to evaluate the precision and accuracy. Every study was done in triplicate over the course of three days. Low percent RSD values were viewed as an indication of precision, and the percentage of recovery that reached the true added values was viewed as a sign of accuracy. The analytical data was evaluated for variance of analysis, and the intermediate precision was calculated by using F (theoretical) and F (observed) [9-12]. Following equation used for determination of detection and quantitation limit:
DL = (3.3σ / S) and QL = (10σ / S), σ where is the standard deviation (SD) of response (y-axis) and s is calibration curve slope. The percentage organic concentration, flow rate, and wavelength of the system

were slightly but purposefully changed under the ideal chromatographic circumstances mentioned previously, and the variations in the parameters were recorded. This was done to test the method's robustness by using QbD approach (design expert software). By completely separating NMD and the degradation products it produced from the excipients, the suggested method's specificity was demonstrated. Specificity was considered when there were no peaks in the blank runs during the retention time of NMD and its degradation products [13-18].

AGREE tool for greenness assessment for the analytical procedures

A greenness assessment for the analytical procedures is required because not all analytical techniques are green. The Green Analytical Procedure Index (GAPI), Analytical Method Greenness Score (AMGS), analytical eco scale, and AGREE metrics are used to assess how environmentally friendly the proposed technique is AGREE is a unique software tool that estimates the greenness profile [19-20]. The input requirements in AGREE represent the 12 major tenets of green analytical chemistry and are weighted differently to allow for confident flexibility. Each of the twelve principles of green analytical chemistry was used to create an aggregate scale ranging from 0 to 1. AGREE outcomes apply to all twelve principles of green analytical chemistry, and the net result is the product of each principle. A clockwise diagram is created, and the center of the chart presents the overall scores with a color that indicates the greenness profile. The red-yellow-green color scale on the clockwise diagram shows the presence of the procedure concerning the 12 principles, while, in contrast, the weight of each principle reflects the width of its corresponding segment. If the total score at the center of the clockwise pattern is close to one, the analytical technique is greener, and the color should be dark green. The color represents the procedure's performance for each of the section's assessment criteria, with the number corresponding to each criterion [8].

Degradation products toxicity determine by using in-silico approach.

TOXTREE (Toxic Hazard Estimation), version 3.1.1 was used to determine toxicity of NMD degradation products. The results of the toxicity were produced [9].

RESULTS AND DISCUSSION

Chromatographic conditions for analysis:

To properly retain NMD from the degradation products it produced, various mobile phases were used. The retention of NMD was adequate at 5.508 min with acceptable system suitability when stationary phase Sapphirus C18 column used with mobile phase of acetonitrile: water (80:20, v/v) at flow rate of 1.0 mL/min. The excipients and degradation products that had formed could be easily separated from the peak of NMD. At the ideal wavelength of 236 nm, all eluents were detected. Figure 1 displays the nimodipine chromatogram with optimized chromatographic conditions.

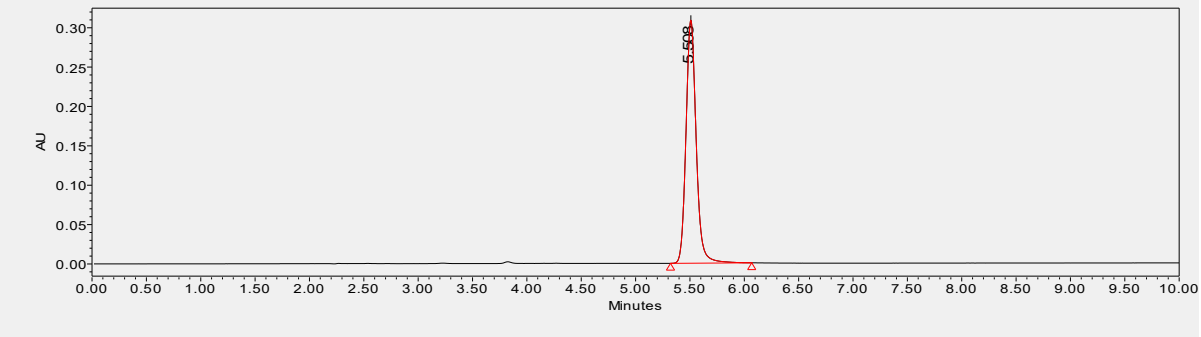


Figure 1: The chromatogram of Nimodipine with optimized chromatographic condition

Forced degradation studies

NMD degradation was observed when exposed to conditions that contained acid, alkali, peroxide-mediated oxidative degradation, and heat. It was discovered that the peak area of NMD reduced in conjunction with the generation of DPs under hydrolytic, oxidative, and thermal. However, under photolytic and humidity circumstances, the peak area of NMD decreased without producing any degradation products. Table 1 and Figures 2, 3, 4, and 5 show an overview of NMD's degradation behavior under various forced degradation studies.

Table 1. Summary of forced degradation behaviour of NMD.

Sr. no	Stress conditions	Retention time	% degradation
1	**Acid degradation - 0.1N HCl at room temperature for 24hr.**	**2.116, 5.261**	**50.56**
2	**Alkali degradation - 0.1N NaOH at room temperature for 24hr.**	**2.132, 3.515, 5.291**	**41.23**
3	**Oxidative degradation -30 % H2O2 at room temperature for 24 hr.**	**1.966, 3.511, 3.697, 5.299**	**55.09**
4	**Photolytic degradation exposed to Sunlight for 24 hr.**	**2.440, 3.079, 5.306**	**30.37**
5	**Dry heat- 70° C for 4 hr**	**2.452, 3.735, 5.361**	**30.45**
6	**Wet heat degradation to 70°C for 4 hr.**	**2.162, 2.458, 5.361**	**41.98**

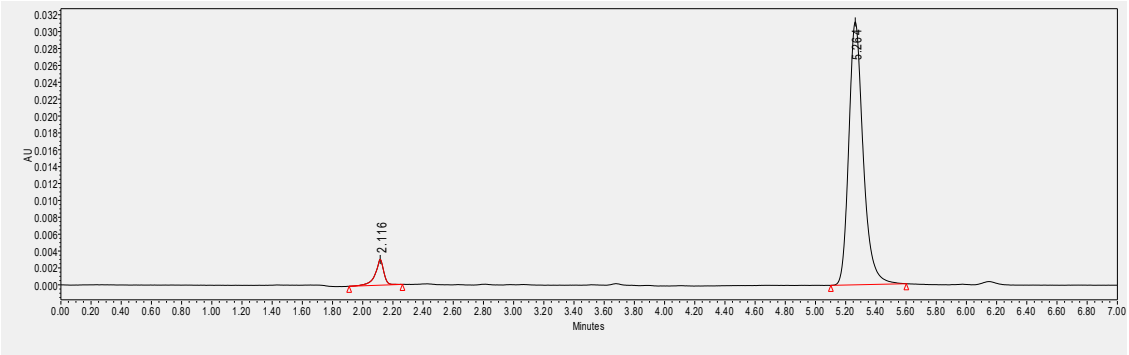


Figure 2: Chromatogram of Acid degradation of NMD.

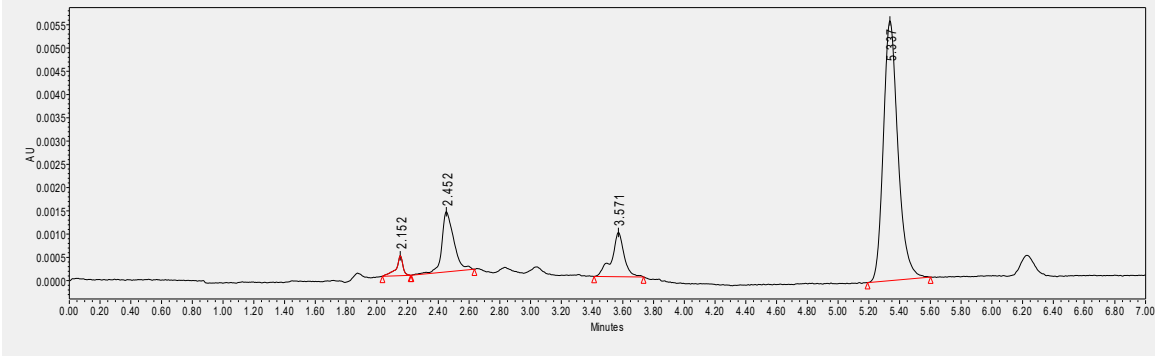


Figure 3: Chromatogram of Alkali degradation of NMD.

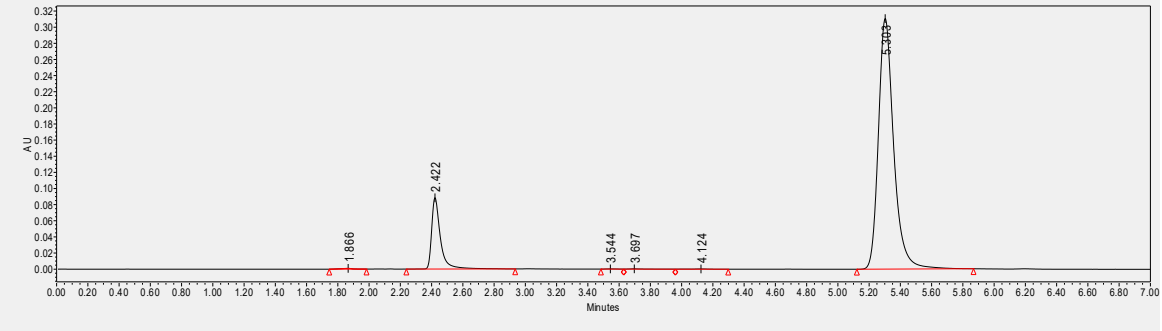


Figure 4: Chromatogram of Oxidative degradation of NMD.

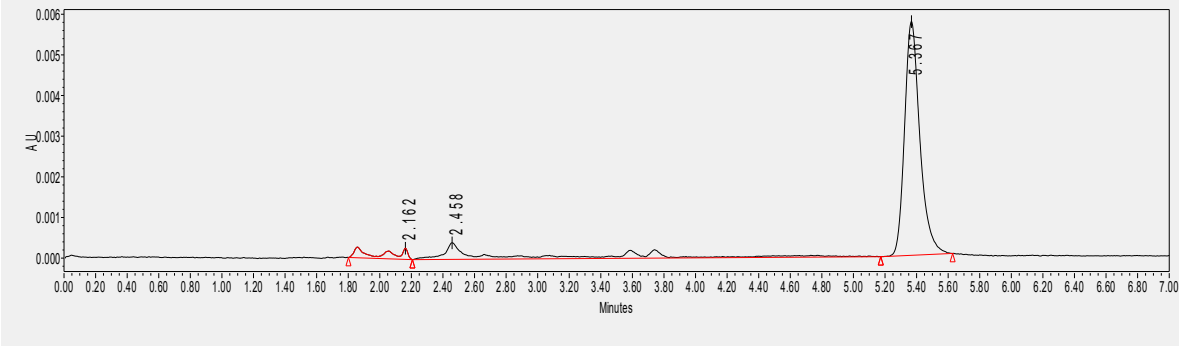


Figure 5: Chromatogram of Wet heat degradation of NMD.

Characterization of degradation Product by LC-MS

Alkali hydrolysis

Degradation products (DPs) produced during the alkali degradation experiment showed how sensitive NMD is to alkali treatment. The protonated molecular ion peak of alkali degradant was detected at m/z 360 with specific fragment ions at m/z 272, 228, 151, 123, 107 and 61 in MS2. Mass spectra of alkali degradation product was shown in figure 6A.

Oxidative degradation:

Degradation products (DPs) produced during the Oxidative degradation experiment showed how sensitive NMD is to oxidative treatment. The protonated molecular ion peak of Oxidative degradant was detected at m/z 432 with specific fragment ions at m/z 330, 244, 151, 122 and 75 in MS2. Mass spectra of oxidative degradation product was shown in figure 6B.

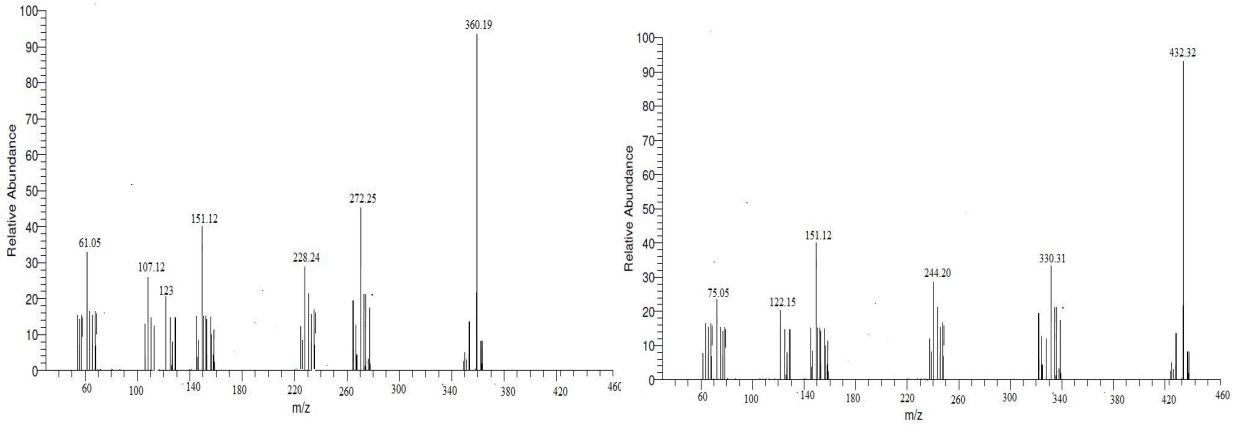


Figure 6: Mass spectra of A) alkali and B) oxidative degradation.

Method Validation

Table 2 provides a summary of the findings from the precision and accuracy experiments. The accuracy of the suggested procedure is demonstrated by the fact that the mean amount found was determined to be relatively near the sum that was contributed. Low readings of % RSD demonstrate that the technique is accurate at each QC level on each day. The F values at each levels observed will less than the F (theoretical) [F (2,6) = 5.143 at α = 0.05] when the data obtain from precision were used to calculate the analysis of variance and value prove that intermediate precision are within given calibrated range. The DL and QL were found to be, respectively, 0.651 µg/mL and 1.973 µg/mL, when they were calculated using the response of SD and calibration curve slope. Because the chromatographic parameters were optimized for the % of organic content, flow rate, and detection wavelength, the NMD peak exhibited appropriate system suitability, indicating the technique's versatility. Robustness of method were check by change in mobile phase composition and flow rate on the effect of retention time, tailing and theoretical plate was used to design response surface Central composite design (CCD) was used to study in depth effect these independent factors. It is shown in figure 7-9. When the NMD were compared before and after being subjected to the specified forced degradation circumstances, there was no interference peak to be seen at the retention time of NMD. Furthermore, specificity of method depends on separation of NMD degradation products.

Table 2. Data of Accuracy and precision

Amount Added	Amount Found (µg/mL)			Within mean square	Between mean square	F value
	Day 1	Day 2	Day 3	0.000613	0.0000823	0.13441
80% (7.2 µg/mL)	7.139	7.185	7.184			
	7.142	7.162	7.175			
	7.199	7.144	7.152			
Mean	7.160	7.163	7.170			
Recovery (%)	98.75	99.07	99.25			
SD	0.03380	0.02055	0.01650			
%RSD	0.472	0.287	0.23			
100% (8 µg/mL)	7.914	7.912	7.914	2.555	1.333	0.521
	7.911	7.911	7.915			
	7.914	7.914	7.912			
Mean	7.913	7.912	7.913			
Recovery (%)	97.82	97.8	97.82			
SD	0.001732	0.001528	0.001528			
%RSD	0.022	0.019	0.019			
120% (8.8 µg/mL)	8.727	8.741	8.744	0.0000243	0.0000581	2.388
	8.738	8.733	8.744			
	8.733	8.733	8.736			
Mean	8.732	8.735	8.741			
Recovery (%)	98.3	98.37	98.52			
SD	0.005508	0.004619	0.004619			
%RSD	0.063	0.053	0.053			

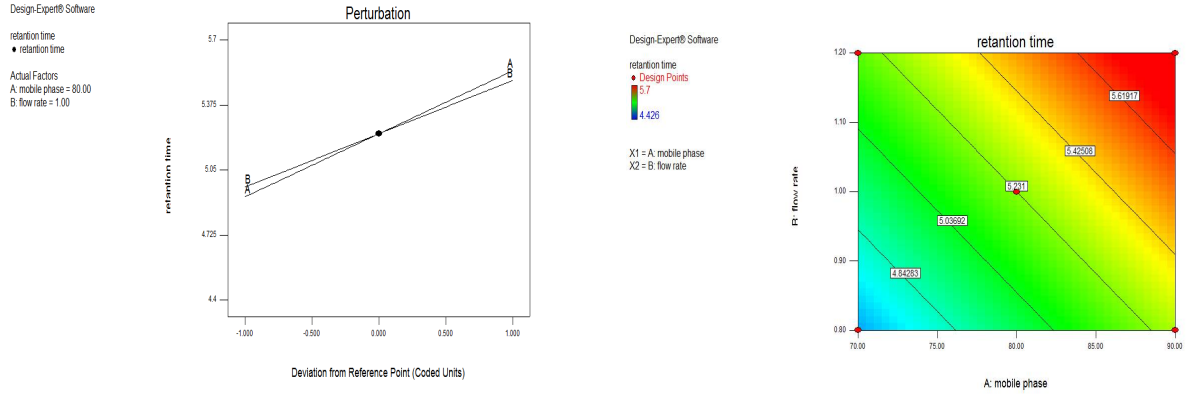


Figure 7: Perturbation plot and counter plot of Retention time.

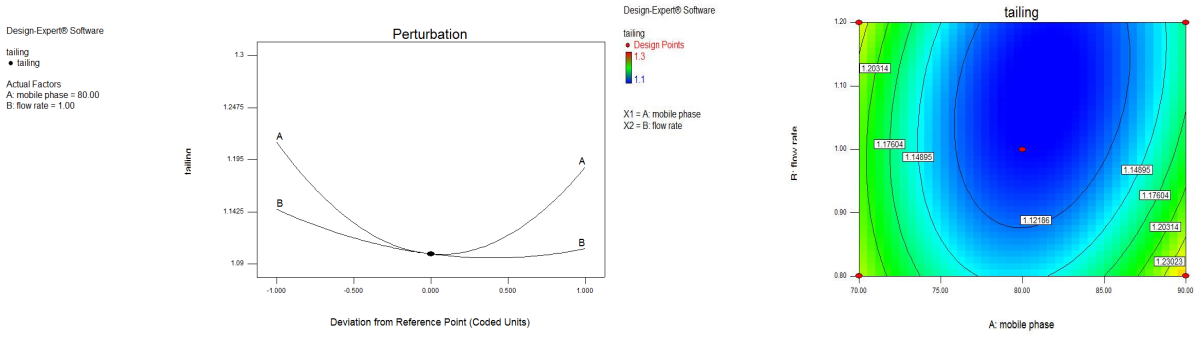


Figure 8: Pertubation plot and counter plot of tailing.

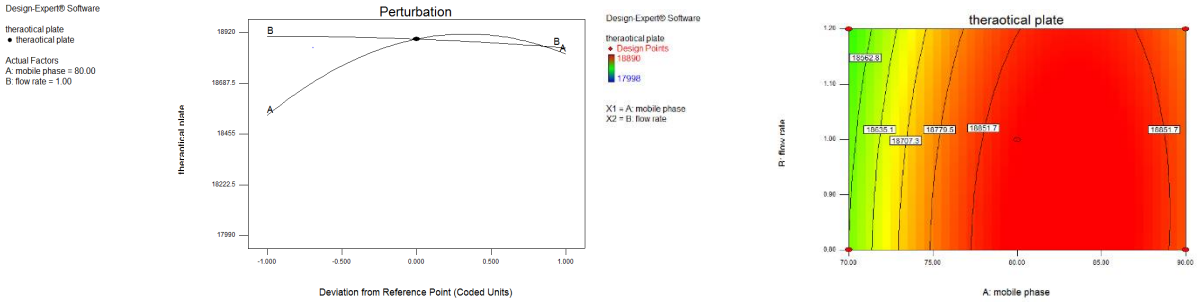
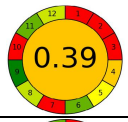
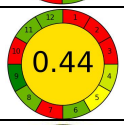


Figure 9: Pertubation plot and counter plot of theoretical plate.

Assessment of greenness of the proposed method

The AGREE software score for the proposed and reported HPLC procedures in table 3 shows that the suggested approach is more environmentally friendly.

Table 3: Evaluation of reported and proposed method:

Mobile Phase	**Greenness Profile**
	AGREE Score
ACN and Potassium Phosphate Monobasic buffer 25mM (50:50, v/v)	
methanol: water (pH 3.5 maintained by o-phosphoric acid), 80:20, v/v	
Acetonitrile: methanol: water (55:11:34, v/v/v)	
ACN : water (80:20 v/v)	

Degradation products toxicity determine by using in-silico approach.

Toxicity prediction was determined for Cramer rules, Carcinogenicity, in-vitro mutagenicity (Ames test) and biodegradability. Table 4 and 5 shows toxicity results of alkali and oxidative degradation product.

Table 4: Toxicity prediction of Alkali degradation product:

Method	Results
Cramer rules	High class (Class III)
Carcinogenicity	Structure alert for genotoxic and non-genotoxic carcinogenicity
In-vitro mutagenicity (Ames test)	Structure alert for S. typhimurium mutagenicity
Biodegradability	Class II (persistent chemical)

Table 5: Toxicity prediction of Oxidative degradation product:

Method	Results
Cramer rules	High class (Class III)
Carcinogenicity	Structure alert for genotoxic and negative for non-genotoxic carcinogenicity
In-vitro mutagenicity (Ames test)	Structure alert for S. typhimurium mutagenicity
Biodegradability	Class II (persistent chemical)

CONCLUSIONS

The estimate of NMD in bulk formulation with its produced degradation products has been developed and validated. Drug and its degradation products was separated on Sapphirus C18 column used with mobile

phase of acetonitrile: water (80:20, v/v) at flow rate of 1.0 mL/min. Each eluent was distinguished at a wavelength of 236 nm. However, it was found that the NMD remained stable in the presence of dry heat and photolysis while degrading in the presence of hydrolysis, wet heat, and peroxide-treated oxidative. Under the mentioned chromatographic conditions, the drug was determined to be liner at 02 to 10 µg/mL. By using QbD, method was found to be robust. Degradation product formed in FDS was characterized by using mass spectrometry. The detected alkali and oxidative degradation product was found to be very harmful, according to the in-silico toxicity prediction. Method greenness was determined by using AGREE tool which show developed method was greener than reported method.

ACKNOWLEDGEMENT
The authors are grateful to the principle, Dr. S.J. Kshirsagar, the management and trustees of Mumbai Educational Trust in Nashik, as well as USV Laboratories Ltd. in Mumbai, who gave a gift sample of NMD.

CONFLICT OF INTERESTS
No conflicts of interest

AUTHOR CONTRIBUTION
Stability indicating methods, mass spectra interpretation and in-silico toxicity study were performed by the kavita chandramore under the supervision of Sandeep sonawane.

FUNDING
Received funding from Dr. Babasaheb Ambedkar research and training institute (Barti) pune. Award letter no BARTI/Fellowship/BANRF-2020/21-22/850.

ETHICS STATEMENTS
Not applicable to study performed.

Informed consent
Not applicable

REFERENCES
1. Tomassoni, D., Lanari, A., Silvestrelli, G., Traini, E. and Amenta, F., (2008). Nimodipine and its use in cerebrovascular disease: evidence from recent preclinical and controlled clinical studies. *Clinical and experimental hypertension*, *30*(8), pp.744-766.
2. Carlson, A.P., Hänggi, D., Macdonald, R.L. and Shuttleworth, C.W., (2020). Nimodipine reappraised: an old drug with a future. *Current neuropharmacology*, *18*(1), pp.65-82.
3. Galvão, A.F., Ajimura, T.O., Aguiar, F.A., Borges, K.B. and de Gaitani, C.M., (2012). Stability-indicating methods for the enantioselective determination of dihydropyridines by high performance liquid chromatography and capillary electrophoresis. *Analytical Methods*, *4*(9), pp.2953-2961.
4. Riekes, M.K., Rauber, G.S., Kuminek, G., Tagliari, M.P., Cardoso, S.G. and Stulzer, H.K., (2013). Determination of nimodipine in the presence of its degradation products and overall kinetics through a stability-indicating LC method. *Journal of chromatographic science*, *51*(6), pp.511-516.
5. Panda, S.S., Ravi Kumar, V.V.B. and Mahapatra, S., (2020). Stability-Indicating LC Method for Estimation of Nimodipine in Dosage Form and Spiked Serum Using Systematized Design of Experiments (DoE) Approach. *Analytical Chemistry Letters*, *10*(4), pp.459-476.
6. Sri, R.S., Sri, K.B., Afreen, A. and Sumakanth, M., (2021). A stability indicating study for quantitative estimation of nimodipine in bulk and pharmaceutical dosage form. World journal of pharmaceutical research, 10 (13), pp.2318-2325.
7. Guideline, ICH. (2005). Validation of analytical procedures: text and methodology Q2 (R1).
8. Youssef, Y.M., Mahrouse, M.A. and Mostafa, E.A., (2023). Assessment of environmental impact of a novel stability-indicating RP-HPLC method and reported methods for the determination of selexipag in bulk and dosage form: A comparative study using different greenness assessment tools. *Microchemical Journal*, *185*, p.108256.
9. Bhatia S, Schultz T, Roberts D, Shen J, Kromidas L, Api AM. (2015). Comparison of cramer classification between toxtree, the OECD QSAR Toolbox and expert judgment. Regulatory Toxicology and Pharmacology.1;71(1):52-62.
10. Barmpalexis, P., Kanaze, F.I. and Georgarakis, E., (2009). Developing and optimizing a validated isocratic reversed-phase high-performance liquid chromatography separation of nimodipine and impurities in tablets using experimental design methodology. *Journal of Pharmaceutical and Biomedical Analysis*, *49*(5), pp.1192-1202.
11. Shang, X., Ma, S. and Li, Z., (2013). Development and validation of a RP-HPLC method for determination of nimodipine in sustained release tablets. *Journal of Chemistry*, *2013*.

12. Rahman, Z., Siddiqui, A. and Khan, M.A., (2013). Assessing the impact of nimodipine devitrification in the ternary cosolvent system through quality by design approach. *International journal of pharmaceutics*, *455*(1-2), pp.113-123.
13. Patel, M.M., Doshi, D.B. and Ghosh, C., (2017). Development and Validation of HPLC method for estimation of Nimodipine in soft gelatin Capsule. *Pharma Science Monitor-An International Journal of Pharmaceutical Sciences*, *8*(2), pp.200-209.
14. Rahman, Z., Siddiqui, A. and Khan, M.A., (2013). Assessing the impact of nimodipine devitrification in the ternary cosolvent system through quality by design approach. *International journal of pharmaceutics*, *455*(1-2), pp.113-123.
15. Shang, X., Ma, S. and Li, Z., (2013). Development and validation of a RP-HPLC method for determination of nimodipine in sustained release tablets. *Journal of Chemistry*, *2013*.
16. Raghunath, C.H., Hemamalini, K., Harika, V., Prasanna, A.L., Bhavani, K., Sree, M.R. and Sagar, B., Method Development and Validation of UV-Spectrophotometric Method for Quantitative Estimation of Nimodipine in Pharmaceutical Dosage Form.
17. Nirogi, R.V.S., Kandikere, V.N., Maurya, S., Mudigonda, K. and Boosi, R., (2006). Liquid chromatographic–electrospray tandem mass spectrometric method for the quantification of nimodipine in human plasma. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, *61*(10), pp.828-834.
18. Qiu, F., Chen, X., Li, X. and Zhong, D., (2004). Determination of nimodipine in human plasma by a sensitive and selective liquid chromatography–tandem mass spectrometry method. *Journal of Chromatography B*, *802*(2), pp.291-297.
19. Scriabine, A., Schuurman, T. and Traber, J., (1989). Pharmacological basis for the use of nimodipine in central nervous system disorders. *The FASEB journal*, *3*(7), pp.1799-1806.
20. Tomassoni, D., Lanari, A., Silvestrelli, G., Traini, E. and Amenta, F., (2008). Nimodipine and its use in cerebrovascular disease: evidence from recent preclinical and controlled clinical studies. *Clinical and experimental hypertension*, *30*(8), pp.744-766.