Journal of Neonatal Surgery
ISSN(Online): 2226-0439
Vol. 14, Issue 6s (2025)
https://www.jneonatalsurg.com

OPEN ACCESS

Optimized Method development and validation By QBD-Based Liquid Chromatographic Method For Estimation Of Molnupiravir

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Cite this paper as: Meruva sathish Kumar,D.Swapna,Ravi Kumar Kota, Ch.Divyaja, S.Siva Prasad, S Marakatham, B.Ramu, (2025) Optimized Method development and validation By QBD-Based Liquid Chromatographic Method For Estimation Of Molnupiravir. *Journal of Neonatal Surgery*, 14 (6s), 259-269.

ABSTRACT

mplementation of quality by design (QbD) in developing greener analytical methods provides valuable knowledge about the use of greener chemicals and their impact on method performance.Green analytical chemistry(GAC) has mainly focussed on developing analytical methods that are safe for the environment and the analyst. The GAC works on 12 principles that mainly focus on reducing solvent usage, replacing toxic chemicals, reusing the generated waste, and avoiding unnecessary steps.The prime focus of the existing study was to develop analytical quality by design aided stability indicating green high performance liquid chromatography(HPLC) method for estimationof Molnupiravir in a capsule dosage form. The critical chromatographic factors were the % of ethanol inthe mobile phase, and flow rate, their overall effect on the responses like capacity factor, tailing factor and theoreticalplates were studied to optimize the method. A rotatable central composite design was employed, and the optimized conditions for chromatographic separation were made with a run time of 10 minutes using Zorbax C18 column (4.6× 150 mm, 5 µm) with 0.1%acetic acid and ethanol (70:30 v/v) as components of a mobile phase, flowing ata rate of 1.0 ml/minute. Photodiode array detection was carried out at 235 nm..The retention time was found to be 4.21 min. According to the ICH guidlines, the proposedmethod was validated and stress studies revealed that Molnupiravir is prone to acidic and basic stress conditions. An analytical eco-scale score evaluated the greenness profile, and a software-based evaluation Analytical Greenness metrics, which affirmed excellent greenness. The developed HPLC method is more eco-friendly and shall be adoptedin the routine quality control of Molnupiravir in a capsule dosage form

Keywords: *Green assessment, Molnupiravir, Method Validation, QBD, Stability testing,*

1. INTRODUCTION

Molnupiravir(MPV) is a prodrug of the synthetic nucleoside derivative N^4-hydroxycytidine and exerts its antiviral action by introducing copying errors during viral RNA replication.[1] Molnupiravir inhibits viral reproduction by promoting widespread mutations in the replication of viral RNA by RNA-directed RNA polymerase.[2] Molnupiravir is indicated for the treatment of mild-to-moderate coronavirus disease (COVID-19) in adults with positive results of direct SARS-CoV-2 viral testing who are at high risk for progression to severe COVID-19.The active drug incorporates into the genome of RNA viruses, leading to an accumulation of mutations known as viral error catastrophe.[3]The Chemical structure of MPV is shown in **Figure 1**.

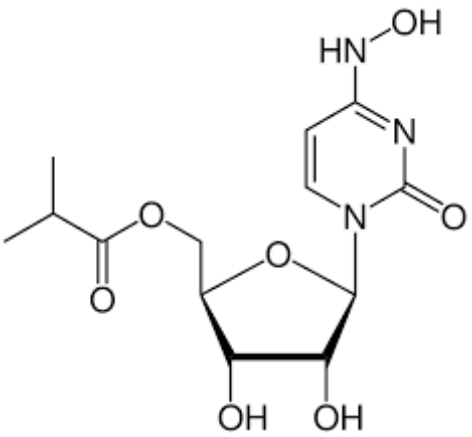

Figure 1: Chemical structure of Molnupiravir

Many more organic solvents are considered toxic. Thus the concept of green chemistry is introduced which eliminate or reduce the use of hazardous solvents/chemicals from analytical process for the eco-friendly environment and health without any compromise in Analytical performance.Organic solvents which are employed in wide range in the industrial process which have an vast impact on the economy as well as on the cost of environment. Organic solvents are noted to be combustible as well as poisonous/oncogenic also it may results into pollution. Thus the prime goal of the green chemistry is to reduce and/or eliminate the use of the chemicals which are more hazardous and to search the solvents that are comparatively safe. A literature survey for MPV revealed that several methods based on varied techniques like spectrophotometry[4,5], HPTLC[6,7],LC-MS[8] , HPLC[10-18] , UPLC[19] and colorimetric[20] are available for individual and combination drugs. Analytical quality by design (AQbD) has promoted the advancement of GAC principles in analytical procedures

The main aim was to pursue a new outline for implementing GAC principles in tandem with AQbD philosophies. This combined framework was used to progress an eco-friendly and robust HPLC study of the drugs in bulk and marketed formulation.The current study focused on using National Environmental Method Index (NEMI),Green Analytical Procedure Index (GAPI) and Analytical Greenness(AGREE) to determine the greenness of the method.[21-23]

2. MATERIAL AND METHODS

2.1 Materials and reagents

MPV was procured from Sun Pharmaceuticals industries Ltd,India.The capsules purchased from local market; each capsule was labeled to contain 200 mg of MPV. Ethanol, HPLC grade was supplied by Sigma Aldrich, Germany. Water used throughout the procedure was HPLC grade

2.2 Equipment

Agilent technologies 1260 LC system with binary gradient pump , LC-10 AT VP solvent delivery system, Qualisial C18(250 cm × 4.6 mm) 5 µm column ,UV chamber (Camag), SPD M-10AVP photo diode array detector.

2.3 Preparation of standards and internal standards (IS)

In order to prepare stock solution, weighed accurately 10 mg MPV and transferred into 50 ml volumetric flask, added 35 ml of water and sonicated to dissolve the standard completely and diluted up to the mark with water (200 PPM). Further diluted 2 ml to 20 ml with water. (20 PPM)

2.4 Optimization of Developed RP-HPLC Method with Design Space and Control Strategy determination by optimization study:

All the computations for the current optimization study and statistical analysis were performed using Design Expert® software (Design Expert version 7.0.0; State-Ease Inc., Minneapolis, MN, USA).

2.5 Application of design of experiments for method optimization:

Design of experiments (DOE-1): 3^3 randomized response surface designs with a Box-Behnken design were used with 17 trial runs to study the impact of three factors on the three key response variables. In this design 3 factors were evaluated, each at 3 levels and experimental trials was performed at all 3 possible combinations. The mobile phase composition (X1), flow rate (X2) and column oven temperature (X3) were selected as independent variables and retention time (RT), asymmetry and theoretical plates were selected as dependent variables. The resulting data were fitted into Design Expert 7.0.0. software

and analysed statistically using analysis of variance (ANOVA). The data were also subjected to response surface methodology to determine the influence of mobile phase composition, flow rate and column oven temperature on dependent variables. The probable trial runs using 3^3 Box Behnken designs are as shown in **Table no 1.**

Table No. 1 Translation of coded levels in actual values

Level of Variable	Range of Factors		
	Ethanol (%v/v)	Flow Rate (ml/min)	Column oven temperature (°C)
Low Level (-1)	20	0.9	32
Medium Level (0)	30	1.0	35
High Level (1)	40	1.1	38

2.6 Sample preparation of Marketed sample:

Weighed 20 capsules and transferred the conent in mortar pestle and crushed to fine powder. Weighed the powder material equivalent to 100 mg of MPV and transferred to clean and dried 100 ml of volumetric flask. Added 70 ml of water, sonicated for 15 minutes with intermittent shaking after every five minutes. After 15 minutes allow to cool the solution to room temperature and made volume up to the mark with mobile phase. Filtered the solution through suitable

0.45 µ syringe filter discarding 3-5 ml of initial filtrate. Further diluted 0.5 ml of filtered stock solution to 50 ml with water (10 µg of MPV), injected the resultant solution and chromatograms were recorded (**Figure 1)** and results are recorded.

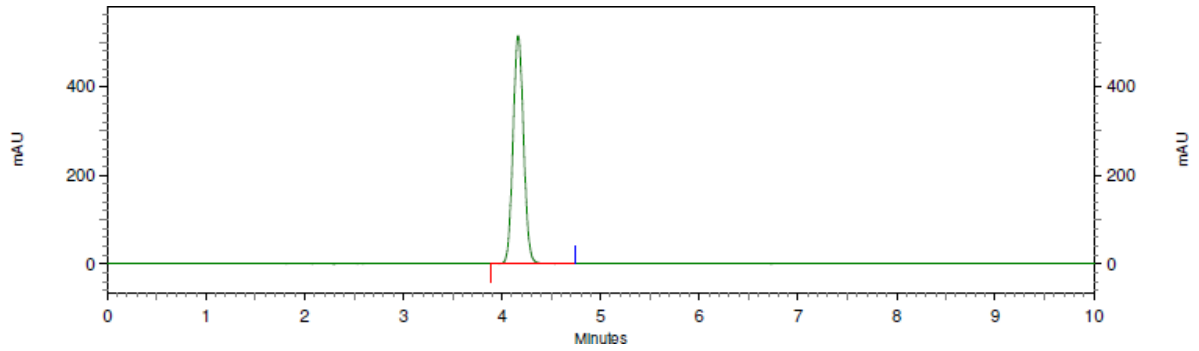

Figure 2: Chromatogram of MPV

2.7 Validation

The developed HPLC method was validated for system suitability, linearity, accuracy, precision, robustness, Limit of Detection (LOD), Limit of Quantification (LOQ) in accordance with ICH guidelines.

2.8 Linearity

From stock solution, aliquots of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 ml were transferred into 10 ml volumetric flasks and diluted up to the mark with mobile phase such that the final concentration of The range 2 – 12 µg/ml Volume of 10 ml of each sample was injected with the help of syringe. All measurements were repeated five times for each concentration and calibration curve was constructed by plotting the peak area versus the drug concentration. Regression analysis, which was performed using the least square regression approach, was used to determine linearity.

2.8.1 Accuracy

Accuracy is measured as a percentage of recovery. For both methods, a known amount of MPV B standard drug powder corresponding to 50, 100, and 150 percent of label claim was added, mixed, and analyzed by running chromatograms in optimized mobile phase.

2.8.2 Precision

Precision of the method was assessed by repeatability, intra-day and Inter-day. The precision measures the similarity of measurements obtained from multiple samplings of the same homogeneous sample under the specified conditions. The intra-

day precision was determined by analysing standard drug solutions three times on the same day within the calibration range of individual drugs. Inter-day precision was determined by analyzing drug solutions over a week on three different days within the calibration range.

2.8.3 Ruggedness

Different analysts used aliquots from homogenous lots and operational and environmental circumstances to assess an analytical method's robustness. The assay was conducted utilizing the parameters, such as in various settings, by various analysts, and on various dates.

2.8.4 Robustness

Robustness was studied by comparing the results obtained for deliberate changes in chromatographic conditions.

2.8.5 Limit of Detection (LOD) and Limit of quantitation (LOQ)

Concentrations in the calibration curve's lower linear range were used to determine the detection and quantification limits. The amount of drugs used versus the average response (peak area) was plotted, and the regression equation was determined. Response standard deviations (S.D.) were computed. The average of standard deviations was calculated from this data (A.S.D.). LOD was calculated using the formula (3.3 xA.S.D.)/b, and LOQ was calculated using the formula (10 xA.S.D.)/b, where "b" corresponds to the slope obtained in the method's linearity study.

2.8.6 Specificity

Specificity of the method was determined by means of entire separation of standard drugs in the presence of other excipients normally present in the dosage forms.

2.8.7 System suitability

The suitability of the system was evaluated in order to ensure the chromatographic system's quality performance. Six replicates of MPV working standards samples were injected, and parameters such as capacity factor (K), injection repeatability tailing factor (T), theoretical plate number (N), and resolution (Rs) for the main peak and its degradation product were tested. The system suitability parameters were revealed to be within acceptable limits.

2.9 Forced degradation studies

Test solutions (10 µg/ml) of MPV was exposed todifferent conditions like 0.1M HCl, 0.1M NaOH, 3 % H2O2, dry heat and photo light, and the extentof degradation was analyzed to the time of exposure.

3. RESULT AND DISCUSSION

The aim to the paper is to apply a trio combination of AQbD,HPLC, and GAC concepts in a single system to progress longstanding viability and robustness. The five steps in the AQbD framework help understand method variables and their interactions, recognize factors that significantly impact process performance, and assign the acceptable limits of their variance. This chapter provides a detailed descriptionof AQbD based on GAC principles for applying analytical methods. Instead of using traditional approaches, a combined trio technique was used on a drug combination that enlightens its applicability in other drug analyses.

3.1 Method Optimization using rotatable Central Composite Design

The risk assessment method identified an optimal organic phase concentration (30%), a flow rate of 1 min/ml as the most promising with C18 (RP18, ODS, Octadecyl) (150 × 4.6 mm i.d × 5 µm) column; and mobile phase composition of 0.1 % Acetic acid and ethanol (70: 30). Reduced run time resultsin reduced mobile phase usage, improved laboratory time management, and waste management in adherence to GAC principles, enabling higher analysis efficiency. Following that, DoE principles were included in all relevant CMPs and tracked their outcome solely on CMAs to analyze the required method model equations.A rotatable central composite design with a quadratic design model was used in the study. The following parameters were chosen for the DoE analysis built on the initial method of risk assessment; percentage of ethanol and flow rate (0.8 –1.2 ml/min). The following parameters were chosen: Asymmetry; theoretical plates and retention time of the drug, Results are shown in **Table 2.**

Table No. 2 : Optimization method parameters for central composite design

	Factor1	Factor 2	Factor 3	Response 1	Response 2	Response 3	Response 4

Runs	A: % Ethanol	B: Flow rate	C: COT (°C)	Retention time (RT)	Asymmetry	TP	Resolution
1	28	0.85	35	7.30	1.11	10721	6.18
2	27	0.9	32	6.73	1.12	8695	6.82
3	30	1.0	35	4.27	1.17	8772	4.75
4	33	1.1	32	4.85	1.15	9110	3.23
5	32	1.0	35	4.27	1.17	8781	3.63
6	32	0.85	32	3.30	1.13	6682	3.83
7	30	1.14	32	3.95	1.15	7791	4.73
8	30	1.0	38	6.47	1.11	10768	4.64
9	30	1.0	35	4.27	1.18	8750	4.77
10	30	1.1	38	3.83	1.14	9324	4.78
11	**30**	**1.0**	**38**	**4.70**	**1.12**	**10718**	4.81
12	28	1.0	35	4.28	1.18	8758	6.19
13	30	0.9	35	3.65	1.14	7534	4.72
14	30	1.0	38	3.24	1.13	8900	4.56
15	30	1.0	35	4.27	1.17	8786	4.59
16	28	1.1	35	5.93	1.11	9179	6.06
17	30	1.14	35	2.98	1.13	6652	489

Derringer's desirability statistical model was used to find the best set of conditions found on the importance and constraints of each response. The desirability method illustrated the attainment of specific objectives within limits imposed, and a precise experimental region explored for configurations whereby constraints set was reached to the limit(Table 3)

Table 3:Goals selected for the optimized method

Name	Goal	Lower	Upper	Importance
A: EtOH	is in range	27.87	32.12	3
B: Flow Rate	is target = 1.00	0.85	1.14	3
Asymmetry Factor	is in range	1.11	1.18	3
RT	minimize	4.23	9.43	3
Theoretical plates of peak	is in range	6652	10768	3
Rs	is in range	3.23	6.82	3

3.2 Statistical method validation and design space

The process model equations were estimated and statistically validated using ANOVA based on the DoE results with evaluated each model coefficient. The model coefficients used in this portion were statistically significant (P values< 0.0001). Additionally, the F-ratios indicate the importance of each coefficient in the model. Higher R^2 and LOF values show a well-

fitting model, whereas higher F-ratios indicate statistical significance for the analytical model equation. The 3D contour plots for the A and B interactions are depicted in **Figure 3** . ANOVA results, LOF non-significance, reasonable standards of R^2, and adjusted and predicted R^2 were then used to ensure thestatistical viability of the regression models for all the three responses. These interlinked responses showed a need for a sensible method that does not alter the other responses.The overlay plots for the optimized plots indicating that the yellow region is the range that does not require any revalidation further with each interaction are depicted in **Figure 4.**

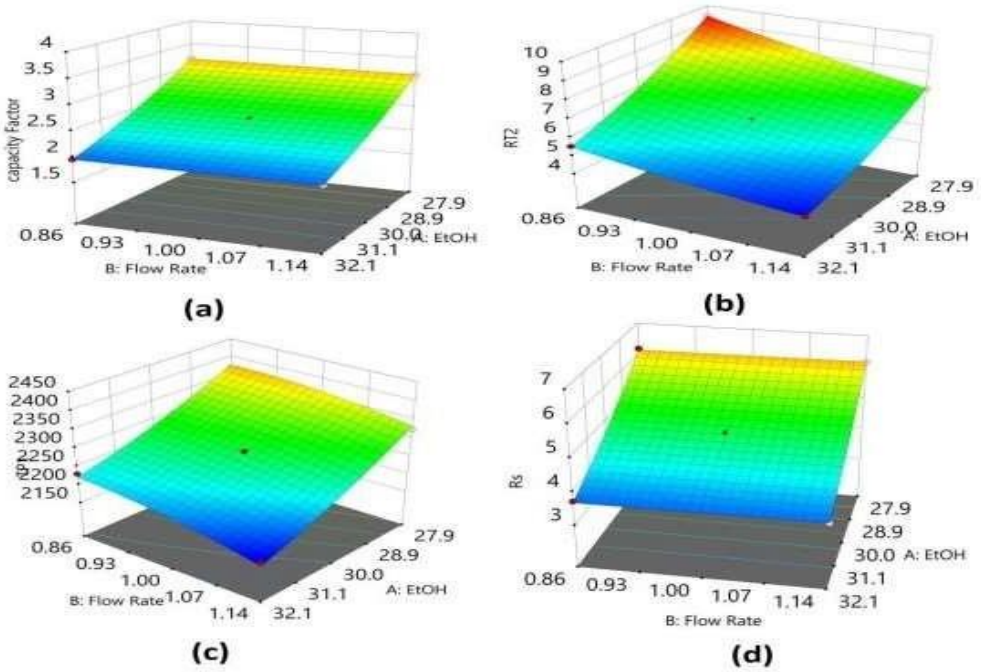

Figure 3. 3D Contour plots for optimized method with interaction of twofactors on (a) R 1, (b) R 2, (c) R 3 and (d) R 4.

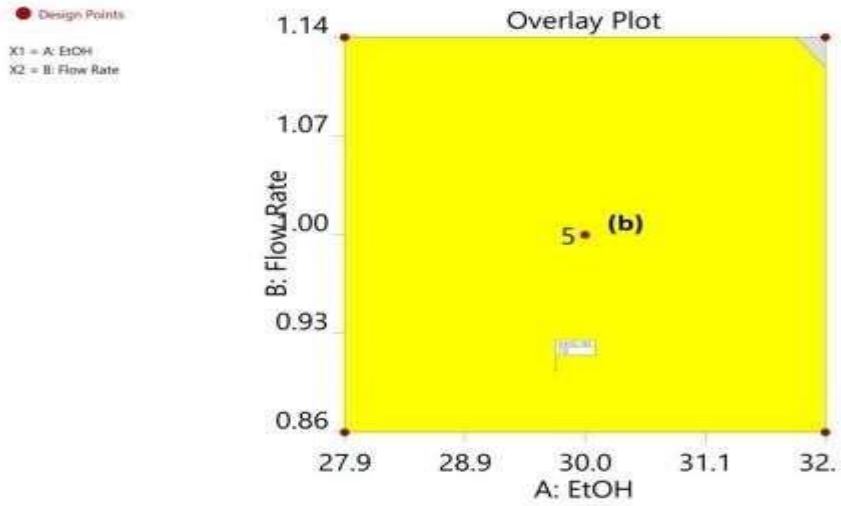

Figure 4: Overlay plots for the variables and responses with the interaction oftwo factors on (a) R 1, (b) R 2,

(c) R 3 and (d) R 4.

3.3 Validation as per ICH guidelines

3.3.1 Accuracy (recovery):

The accuracy of an analytical method is the closeness of test results obtained by that method to the true value. The accuracy

of an analytical method is determined by applying the method to analyzed samples to which known amounts of analyte have been added.The results are shown in **Table 4.**

Table 4 : Result and statistical data of Accuracy

Level (%)	Area	Recovered conc (µg/ml)	Added conc (µg/ml)	% Recovery	Mean Recovery	% RSD
50	3849637	5.03	5.02	100.20	99.47	0.7061
	3811921	4.98	5.01	99.40		
	3786175	4.95	5.01	98.80		
100	7760187	10.15	10.02	101.30	100.53	0.7466
	7721087	10.09	10.04	100.50		
	7641804	9.99	10.01	99.80		
150	11284198	14.75	15.04	98.07	99.42	1.4863
	11386304	14.89	15.01	99.20		
	11596386	15.16	15.01	101.00		

3.3.2 Precision

Repeatability of sample application was assessed by multiple injections of a homogenous sample of 10 µg/ml of MPV.The % R.S.D. of MPV was found to be 0.79 .Intra-day variation of the method was performed by analyzing, the three different concentrations 6 µg/ml, 8 µg/ml and 10 µg/ml of MPV, for three times in the same day. Inter- day variations of the method were performed by analyzing the same concentrations for the period of the three consecutive days over a period of week. Results are shown in **Table 5.**

Drug	Conc. [µg/ml]	Intra- day Amount found [µg/ml]		Inter- day Amount found [µg/ml]	
		Mean ± SD [n= 3]	% RSD	Mean ± SD [n= 3]	% RSD
MPV	6	5.98 ± 0.23	0.34	5.97 ± 0.16	1.23
	8	7.94 ± 0.14	0.63	8.09 ± 0.42	0.23
	10	10.09 ± 0.06	0.39	10.03 ± 0.24	0.67

3.3.3 Ruggedness

The ruggedness of the proposed method was evaluated by two different analysts. The results for MPV was found to be 99.65%, 99.91% respectively.

3.3.4 Robustness

The standard deviation of peak areas were calculated for each parameter and % R.S.D. was found to be less than 2%. The low % R.S.D. values as shown in **Table 6** indicated robustness of the method.

Table 6: Robustness Studies

Sr. No.	Parameter	± SD of Peak Area	% RSD
	Mobile phase composition		
1.	a) 0.1% Acetic acid: ethanol (65:35 v/v)	5163.12	0.87
	b) 0.1% Acetic acid: ethanol (75:25 v/v)	3820.70	0.65
	Change in Flow Rate		
2.	a) 0.9	4234.25	0.69
	b) 1.0	5673.34	0.88
Change in Column Temperature			
3.	a) 35 ^{0}C	3245.32	0.78
b) 40 ^{0}C		2567.34	1.21

3.3.5 System Suitability

Chromatographic condition such as Retention Time (Rt), Capacity Factor (K0), Theoretical Plate (N), Tailing Factor (T) were tested and results are given in **Table 7**.

Table7: System Suitability Test

System Suitability Parameters	Proposed Method
Retention time (T_R)	4.7 min
Capacity factor (K ')	1.21
Theoretical plate (N)	10718
Tailing factor (T)	1.24

3.4 Forced Degradation studies

Stability of MPV was carried out by forced degradation study. The chromatograms of samples degraded with acid, base, hydrogen peroxide and light showed well separated spots of pure MPV as well as some additional peaks at different Rt values.The number of degradation products with their retention time values, content of LPV and RTV remained, and percentage recovery were calculated and listed in **Table 8**.

Table 8 : Forced Degradation studies

Sample exposure condition	Number of degradation products [Rt values]	MPV remained [10ug/ml]	SD	Recovery [%]
0.1 M HCl, 8h,RT[a]	2 (2.2,3.1)	8.32	4.72	89.07
0.1M NaOH,8h, RT[a]	2 (1.2,2.0)	8.313	10.12	83.13
3%H2O2,8h,RT[a]	No degradation	9.97	1.31	99.87
Dry Heat	No degradation	9.956	12.23	99.56
Photo, 8 h	No degradation	9.92	1.26	99.28

[a]RT = Room Temperature

3.5 Green evaluation of the proposed HPLC method

The greenness of the current method was assessed by the tools like tools like NEMI and an advanced tool, GAPI, were used to express the method greenness in the pictogram . AGREE is a modern tool that evaluates all 12 green principles using appropriate software.. It considers sample preparation, sample handling as well as the chemicals consumed and the instrumentation. Every variable was colored from green to yellow to red indicating low, medium and high negative environmental impact respectively. The applied HPLC method had seven green zones and one red zone. Finally, AGREE tool was used, checking environmental friendliness profile of the analytical methods as a numerical value. The obtained value was 0.82 and confirmed the superior green characters of the developed HPLC method.. The developed method was assessed using green assessment tools, and the results were depicted in **Figure 5,** The results show that the new method is completely eco-friendly and can be used as a long-term method.

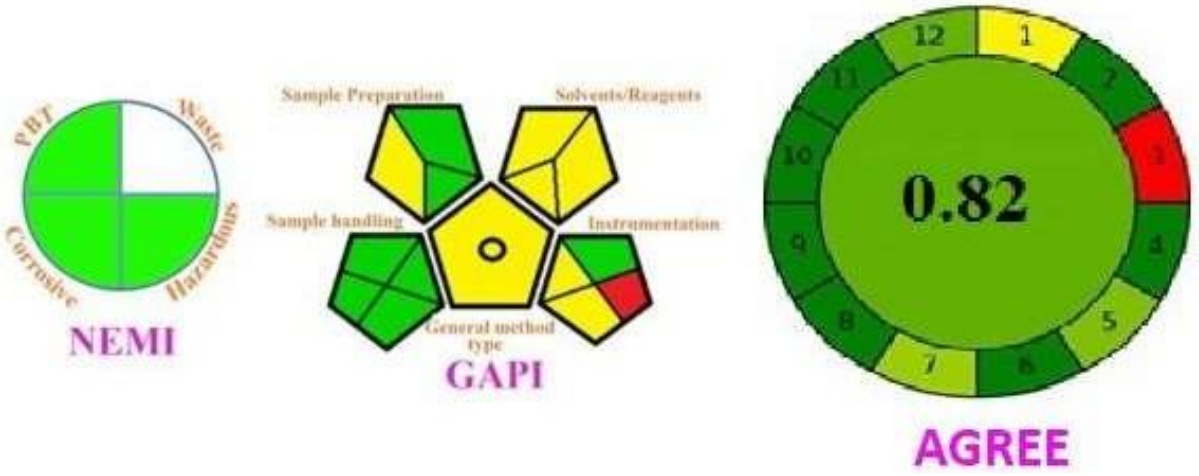

Figure 5: Greenness assessment for the proposed method by using (a) NEMI, (b)GAPI,(c) AGREE metrics.

4. CONCLUSION

The proposed green RP-HPLC method is accurate, precise, robust, sensitive and stability-indicating. QbD and green chemistry combination was demonstrated through the development of a new analytical method for the analysis of degradation products of an MPV in bulk and a pharmaceutical product.Different tools (such as NEMI, GAPI, and AGREE) used for the evaluation of the greenness of the HPLC methods unambiguously demonstrate that eco-friendly methods have advantages over the conventional methods in terms of ecological impact, operator's safety, and energy consumption. Along with ecological and economic benefits, the eco-friendly methods provide better method performances.The implementation of the green strategies in the pharma analysis will provide lower method cost and lower waste disposal costs. This technique will also help commercial and industrial lab research and testing departments adopt and evaluate the various combination in bulk and dosage forms

Conflict of interest

The authors declare no conflict of interest.

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