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Eco-Compatible RP-HPLC Method for Selective Determination of Molnupiravir and Its Critical Impurity C: A Mechanistic and Sustainability-Oriented Analytical Approach

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ABSTRACT

A green RP-HPLC analytical method was established for the selective determination of Molnupiravir in the presence of related Impurity C. Separation of analytes was carried out on an Inertsil ODS-3 C18 column employing isocratic chromatographic conditions with a mobile phase of formic acid (0.1%) and ethanol in a 48:52 (v/v) ratio. Detection was carried out at 236 nm using a photodiode array detector with a flow rate maintained at 0.8 mL/min. Under optimized experimental conditions, Molnupiravir and Impurity C exhibited retention at approximately 2.10 min and 9.45 min, respectively, within a total analysis duration of 14 min. Validation findings demonstrated satisfactory analytical behavior in terms of linearity, accuracy, precision, robustness, and sensitivity according to ICH recommendations. Replacement of conventional hazardous solvents with ethanol significantly improved the environmental acceptability of the analytical procedure. Eco-scale, AGREE, and MoGAPI assessments collectively confirmed the favorable greenness profile of the developed method. The proposed analytical approach is therefore appropriate for routine impurity monitoring and pharmaceutical quality evaluation of Molnupiravir formulations.

Keywords: Molnupiravir; Impurity C; RP-HPLC; Green Analytical Chemistry; Method Validation; Ethanol-Based Mobile Phase

Introduction

Molnupiravir is an orally active antiviral nucleoside analogue extensively investigated for therapeutic management of viral infections [1,2] Ensuring the pharmaceutical quality and safety of Molnupiravir formulations requires reliable analytical methodologies capable of monitoring process- and degradation-related impurities at low concentration levels [3,4]. Among the reported impurities, Impurity C possesses analytical significance because variations in its polarity and chromatographic behaviour may influence selective quantification during routine analysis [5,6]. Several reported chromatographic procedures for Molnupiravir analysis employ solvent systems containing acetonitrile or other environmentally unfavourable organic modifiers [7-10]. Increasing regulatory and scientific attention toward sustainable analytical practices has encouraged the development of greener chromatographic methodologies with reduced ecological burden [11,12]. Ethanol has emerged as a promising alternative organic solvent because of its comparatively lower toxicity, renewable origin, and improved biodegradability [13,14]. Nevertheless, implementation of ethanol within reversed-phase chromatographic systems necessitates careful adjustment of experimental variables to maintain satisfactory separation efficiency and acceptable peak characteristics [15]. Apart from solvent sustainability, understanding analyte retention characteristics remains essential for rational RP-HPLC method optimization [16]. Variations in molecular polarity and hydrophobic interaction with the stationary phase markedly affect analyte migration and selectivity. Accordingly, the present investigation describes

the establishment of a sustainability-oriented RP-HPLC procedure for selective quantitative analysis of Molnupiravir and Impurity C using an ethanol-based mobile phase. In addition, analyte retention characteristics and sustainability characteristics of the developed method were critically evaluated. The chemical structures of Molnupiravir and Impurity C are presented in (Figure 1).

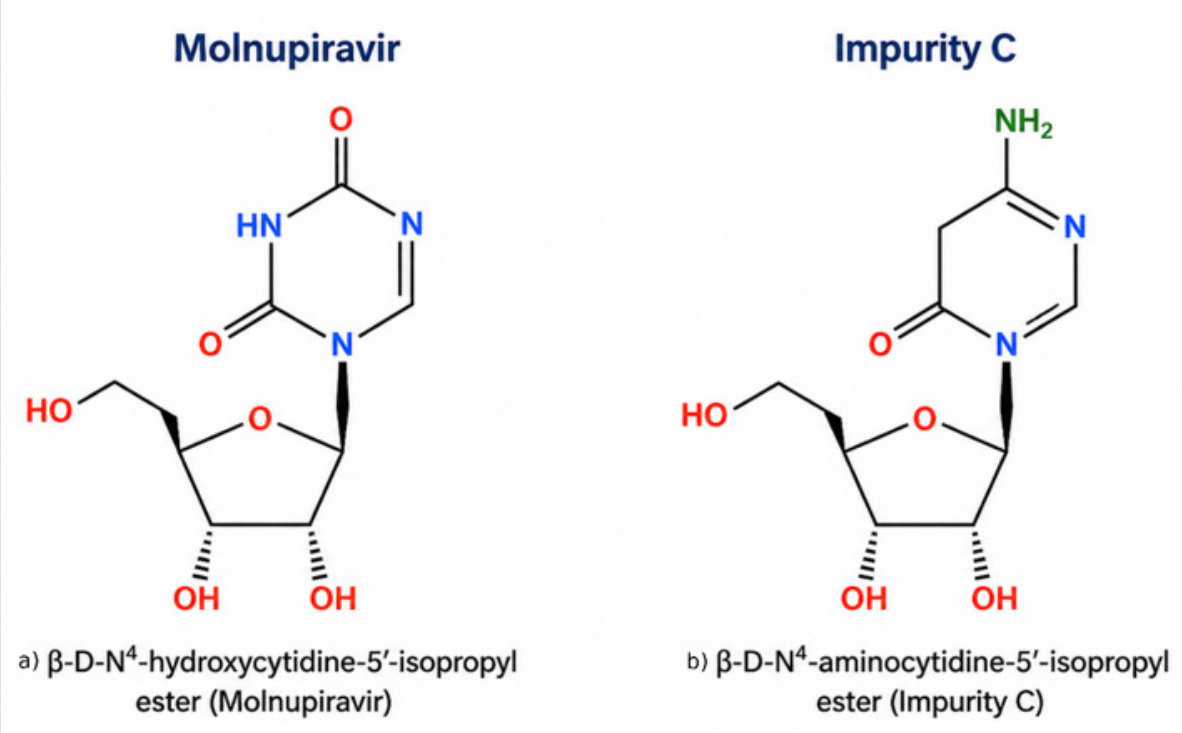

Figure 1: Chemical structures of Molnupiravir and its related Impurity C.

Materials and Methods

Chemicals and Reagents

Certified reference materials of Molnupiravir and Impurity C were procured from authenticated commercial sources. Ethanol (HPLC grade) and formic acid were used for preparation of the mobile phase. Ultrapure water was generated using a laboratory purification system. Analytical-grade reagents were utilized directly throughout the study without additional treatment.

Instrumentation

The experimental analysis employed a Shimadzu UFLC platform incorporating a binary solvent delivery module, autosampler, and photodiode array detector. Data acquisition and processing were carried out using LabSolutions software.

Chromatographic Conditions

Separation of analytes was achieved on an Inertsil ODS-3 C18 stationary phase (150 × 4.6 mm, 5 µm). A mixture of 0.1% aqueous formic acid and ethanol (48:52, v/v) served as the mobile phase and was delivered at 0.8 mL/min. The chromatographic system was operated at 30 °C, while analyte detection was achieved at 236 nm using a PDA detector. Samples of 10 µL volume were injected throughout the study, and complete separation was achieved within 14 min. The optimized chromatographic separation is illustrated in (Figure 2).

Preparation of Standard Solutions

Stock solutions of Molnupiravir and Impurity C were prepared at a concentration of 1000 µg/mL in diluent (water:ethanol, 80:20 v/v). Working standard solutions were prepared by appropriate dilution to obtain concentrations in the range of 10–60 µg/mL for Molnupiravir and 1–10 µg/mL for Impurity C.

Calibration Curve Preparation

A series of standard solutions were prepared and injected in triplicate. Chromatographic responses were measured and calibration plots were generated using analyte concentration versus detector response. Regression statistics including slope, intercept, and correlation coefficient were subsequently calculated. The calibration curves obtained are presented in (Figure 3).

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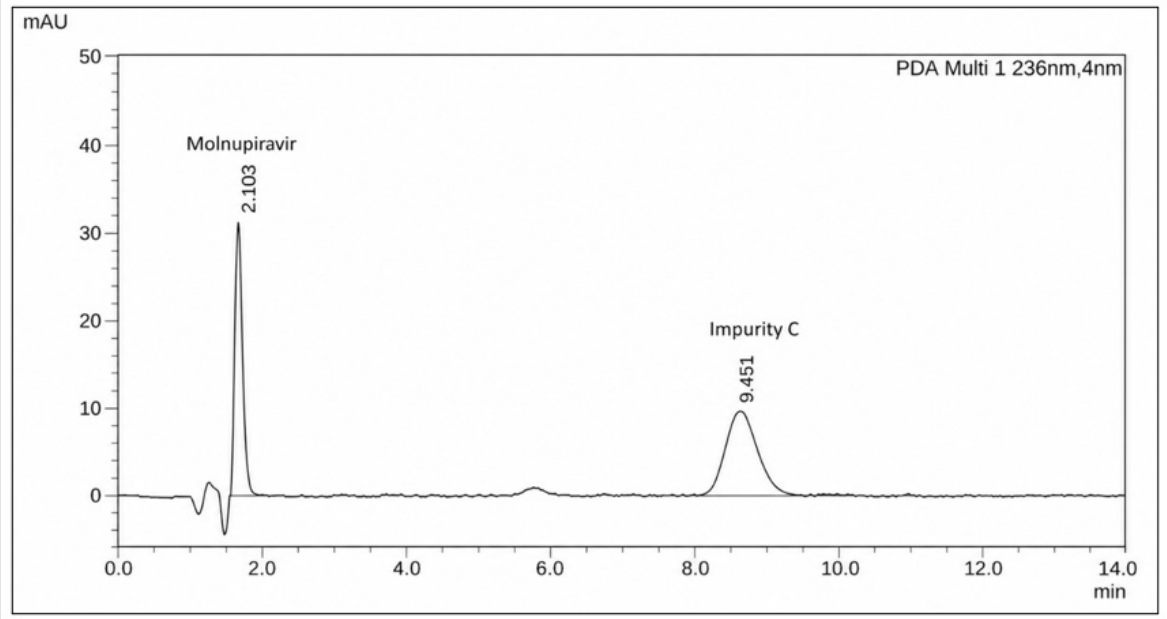

Figure 2: Optimized RP-HPLC chromatogram showing separation of Molnupiravir (RT ≈ 2.10 min) and Impurity C (RT ≈ 9.45 min) under ethanol-based mobile phase conditions (0.1% formic acid:ethanol, 48:52 v/v).

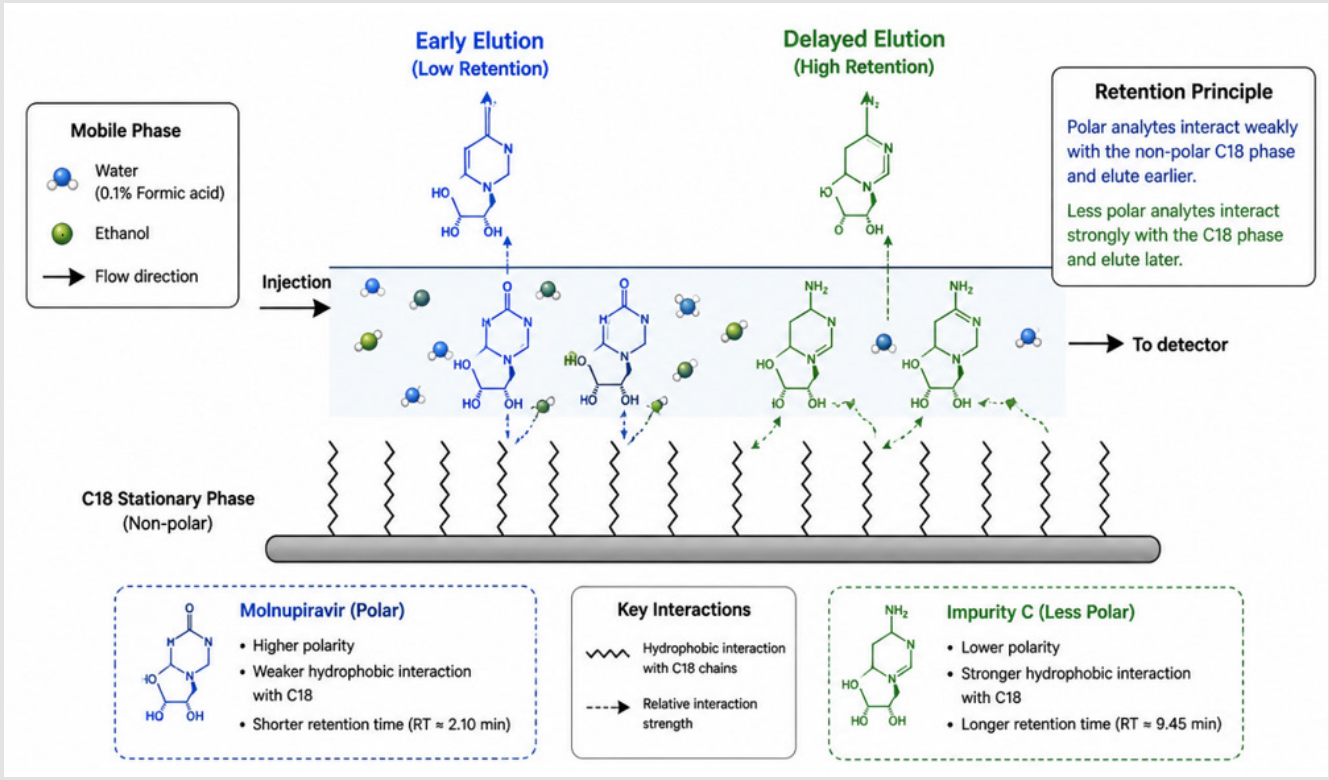

Figure 3: Schematic representation of chromatographic retention behavior in reversed-phase HPLC, illustrating early elution of polar Molnupiravir and delayed elution of less polar Impurity C due to stronger interaction with the C18 stationary phase.

Results and Discussion

Method Development and Optimization

Optimization studies were focused on obtaining efficient chromatographic separation of Molnupiravir and Impurity C together with reduction of solvent-associated environmental burden. Preliminary experiments performed with commonly used organic modifiers generated longer retention behavior and comparatively higher solvent hazards. Introduction of ethanol as the principal organic component provided improved sustainability while maintaining acceptable chromatographic performance. Variation in ethanol proportion significantly affected analyte migration behavior during chromatographic separation. Increasing ethanol proportion enhanced mobile phase elution strength, thereby reducing retention of Impurity C. However, excessive organic composition adversely affected chromatographic resolution. A mobile phase containing 0.1% formic acid and ethanol (48:52, v/v) provided the most suitable compromise between runtime, selectivity, and peak symmetry. Thermal optimization demonstrated that operation at 30 °C improved analyte mass transfer and minimized peak broadening effects. Likewise, a flow rate of 0.8 mL/min produced satisfactory separation efficiency without compromising chromatographic resolution. Under optimized conditions, Molnupiravir exhibited earlier elution because of its comparatively higher polarity, whereas Impurity C demonstrated prolonged retention resulting from stronger hydrophobic interaction with the C18 stationary phase.

System Suitability

Chromatographic performance characteristics were examined to verify system adequacy prior to validation studies under optimized conditions. The results demonstrated that the system met all predefined acceptance criteria (Table 1). The resolution between Molnupiravir and Impurity C exceeded the minimum requirement, confirming adequate separation. Peak symmetry and plate count further indicated efficient column performance and method suitability. The retention behavior is schematically represented in (Figure 4).

Table 1: System Suitability Parameters.

Parameter	Molnupiravir	Impurity C	Acceptance Criteria
Retention time (min)	2.10	9.45	–
Tailing factor	1.18	1.22	≤ 2.0
Theoretical plates	4200	6800	≥ 2000
Resolution	–	3.12	≥ 2.0

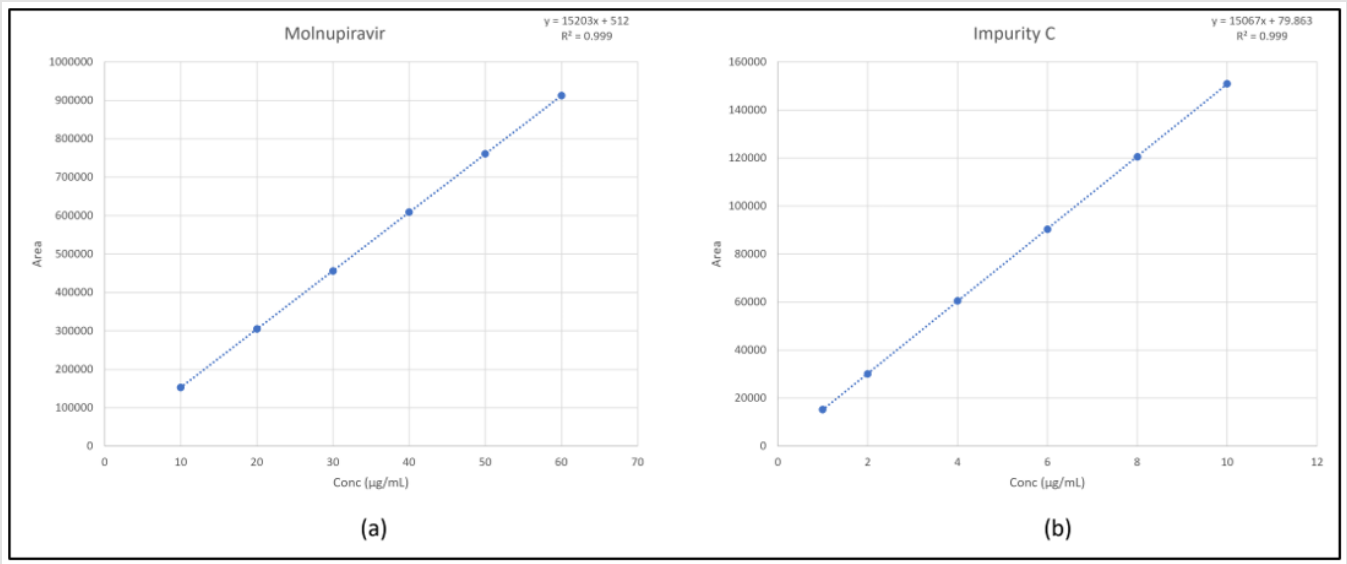

Figure 4: Calibration curves for (a) Molnupiravir (10–60 µg/mL) and (b) Impurity C (1–10 µg/mL), showing linear relationship between concentration and peak area with correlation coefficients exceeding 0.999.

Method Validation

Method validation studies were conducted following ICH Q2(R2) recommendations using the datasets embedded in the accompanying workbook [17]. The method demonstrated acceptable performance across all evaluated parameters for both Molnupiravir and Impurity C.

Linearity: Linearity studies were carried out by analyzing a series of standard solutions covering working concentration intervals of 10–60 µg/mL for Molnupiravir and 1–10 µg/mL for Impurity C. Calibration plots were generated by correlating analyte concentration with corresponding chromatographic peak responses. Regression analysis demonstrated excellent proportionality between con-

centration and detector response throughout the investigated ranges. Correlation coefficient values exceeding 0.999 confirmed the suitability of the developed procedure for quantitative estimation of both analytes (Tables 2 & 3). Evaluation of calibration data demonstrated consistent proportionality between analyte concentration and detector response throughout the investigated ranges. Regression analysis showed minimal deviation from linearity for both compounds. The calculated regression equations and corresponding correlation coefficients confirmed reliable quantitative performance of the developed analytical procedure across the selected concentration intervals.

Table 2: Linearity Data (Molnupiravir).

Concentration (µg/mL)	Peak Area
10	152340
20	304820
30	456210
40	609100
50	760950
60	912300

Note: Regression equation: y = 15203x + 512
Correlation coefficient (r^2): 0.9993.

Table 3: Linearity Data (Impurity C).

Concentration (µg/mL)	Peak Area
1	15230
2	30120
4	60510
6	90300
8	120500
10	150900

Note: Regression equation: y = 15067x + 79.863
Correlation coefficient (r^2): 0.9991.

Accuracy: Method accuracy was investigated through recovery experiments performed at 80%, 100%, and 120% concentration levels. The obtained recovery values for both analytes remained within pharmaceutically acceptable limits, demonstrating the reliability of the proposed analytical procedure for quantitative determination without interference from formulation components or analytical conditions (Table 4).

Table 4: Accuracy (% Recovery).

Level (%)	Molnupiravir (% Recovery)	Impurity C (% Recovery)
80%	99.2	99.5
100%	100.3	100.1
120%	100.6	99.8

Precision: Precision studies were performed by evaluating repeatability and intermediate precision under identical analytical conditions. The percentage relative standard deviation values obtained for Molnupiravir and Impurity C remained below 2%, indicating excellent reproducibility of chromatographic response. Minimal variability among replicate measurements confirmed the consistency and reliability of the analytical system during routine operation (Table 5).

Table 5: Precision (%RSD).

Parameter	Molnupiravir	Impurity C
Repeatability (%RSD)	0.82	0.95
Intermediate precision (%RSD)	1.14	1.2

Sensitivity (LOD and LOQ): Sensitivity of the analytical procedure was estimated through determination of detection and quantification limits using calibration slope and response variability data. The comparatively low LOD and LOQ values obtained for both analytes demonstrated the capability of the developed method to detect and quantify trace impurity levels with adequate sensitivity (Table 6).

Table 6: LOD and LOQ.

Analyte	LOD (µg/mL)	LOQ (µg/mL)
Molnupiravir	0.25	0.75
Impurity C	0.08	0.24

Robustness: Robustness evaluation was performed by introducing controlled variations in chromatographic variables, including minor changes in flow rate and column temperature. These deliberate modifications produced no meaningful deterioration in retention characteristics, chromatographic resolution, or peak symmetry. The observations confirmed that the developed RP-HPLC method remains reliable under small experimental variations likely to occur during routine laboratory application (Table 7).

Table 7: Robustness Study.

Condition	RT (Molnupiravir)	RT (Impurity C)	Resolution
Normal	2.10	9.45	3.12
Flow +0.1 mL/min	2.05	9.10	3.05
Flow −0.1 mL/min	2.20	9.80	3.18
Temp +2°C	2.08	9.20	3.10
Temp −2°C	2.15	9.70	3.16

Green Analytical Assessment

Environmental sustainability of the proposed analytical procedure was investigated using multiple greenness assessment metrics, including Analytical Eco-scale, AGREE, and MoGAPI approaches [18,19]. Incorporation of ethanol as the principal organic modifier

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substantially decreased solvent-associated hazards and reduced the environmental burden commonly associated with conventional RP-HPLC methodologies. The comparative environmental impact is illustrated in (Figure 5), while the overall green analytical assessment is presented in (Figure 6).

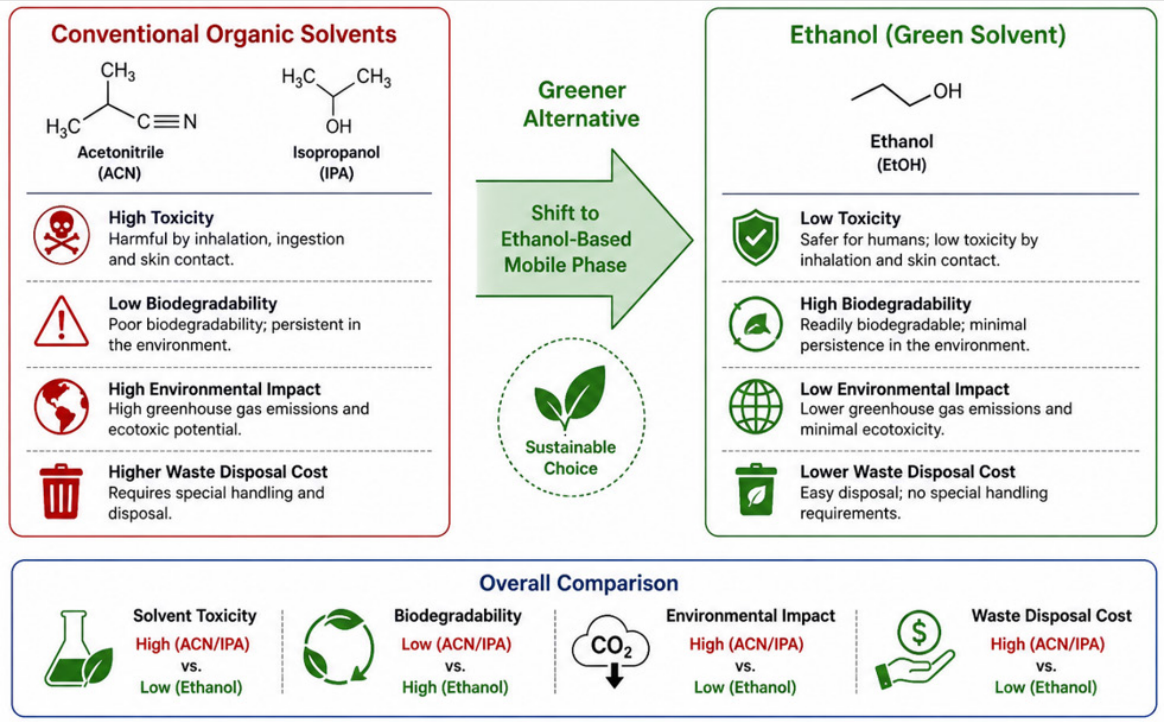

Figure 5: Comparative representation of ethanol and conventional organic solvents used in RP-HPLC, highlighting reduced toxicity, improved biodegradability, and lower environmental impact associated with ethanol-based mobile phases.

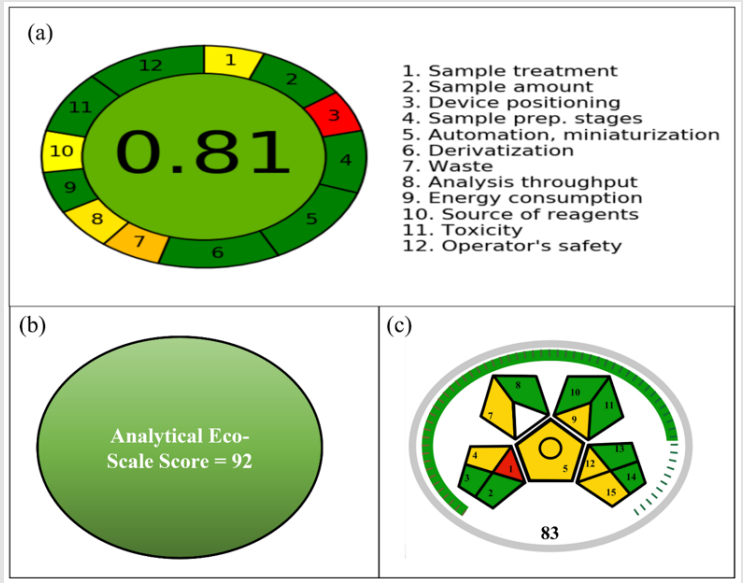

Figure 6: Graphical representation of green analytical assessment using

a. AGREE principles,
b. Eco-scale, and
c. MoGAPI, highlighting the reduced environmental impact of the ethanol-based RP-HPLC method.

Analytical Eco-Scale Evaluation: Eco-scale evaluation was performed through assignment of penalty points for factors such as solvent type, reagent hazards, energy consumption, and waste generation [20]. The detailed Eco-scale evaluation parameters and corresponding penalty points are presented in Table 8. The high Eco-scale score reflects the minimal environmental burden associated with the method, primarily due to reduced solvent toxicity and lower solvent consumption per analysis.

Table 8: Analytical Eco-Scale Assessment.

Parameter	Penalty Points
Ethanol (green solvent)	2
Formic acid (low hazard)	2
Energy consumption	1
Waste generation	2
Occupational hazard	1
Total penalty points	8

Note: Eco-scale score = 100 − 8 = 92

Interpretation: A score >75 indicates an excellent green analytical method.

AGREE Assessment: The method was further evaluated based on AGREE principles, which consider the 12 principles of environmentally sustainable analytical practice [21]. The ethanol-based mobile phase, reduced runtime, and minimal hazardous waste contributed to a high overall greenness profile. The estimated AGREE score 0.81 indicates strong compliance with environmentally sustainable analytical concepts, further supporting the environmental suitability of the developed method.

MoGAPI Assessment: Additional environmental compatibility assessment of the developed RP-HPLC procedure was performed using the MoGAPI approach [22]. The method achieved an overall score of 83, reflecting a high level of environmental compatibility. The assessment highlights favorable attributes such as minimal sample preparation, use of low-toxicity solvents, and controlled waste generation, while moderate contributions from solvent consumption and instrumental energy requirements were observed. Overall, the method demonstrates good compliance with green analytical principles. The low cumulative penalty points primarily arise from the use of ethanol as a low-toxicity organic modifier and reduced analysis time, both of which contribute significantly to lowering the overall environmental burden.

Conclusion

A sustainable RP-HPLC analytical procedure was successfully established for selective determination of Molnupiravir together with its related Impurity C. Adoption of an ethanol-containing mobile phase enabled effective chromatographic separation while decreasing dependence on environmentally hazardous organic solvents commonly employed in reversed-phase liquid chromatography. The developed procedure demonstrated satisfactory validation characteristics including acceptable linearity, precision, accuracy, robustness, and sensitivity according to ICH recommendations. Retention behavior observed during chromatographic separation corresponded with differences in analyte polarity and interaction with the stationary phase. Greenness investigations using Eco-scale, AGREE, and MoGAPI assessment tools confirmed the environmentally favorable nature of the analytical procedure. The proposed method can therefore serve as an effective alternative for routine pharmaceutical quality control and impurity profiling applications.

Conflict of Interest

The authors declare no conflict of interest.

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Ethical Approval

This study did not involve human participants or experimental animals.

Author Contributions

Rajat R. Durbule: Experimental work, data analysis, manuscript preparation, and editing.

Dr. Pallavi M. Patil: Conceptualization, supervision, and manuscript review.

Prathmesh B. Parkale: Experiment support.

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