RESEARCH PAPER

Development and Validation of a High-Performance Liquid Chromatographic (HPLC) Method for the Simultaneous Estimation of Five Related Pyridine Compounds Using a Mixed-Mode Primesep 100 Column.

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Received: 30th June, Revised: 6th July, Accepted: 11th July, Available online: 13th July

ABSTRACT

A robust, precise, and highly specific High-Performance Liquid Chromatographic (HPLC) method has been developed and validated for the simultaneous determination of five structurally related pyridine compounds: Pyridinedicarboxylic acid (PDCA), Nicotinic Acid, Ethyl Nicotinate, Pyridine, and 2-Methyl-5-ethylpyridine (MEP). Chromatographic separation was achieved on a mixed-mode Primesep 100 column at a controlled temperature of 30°C. This specific mixed-mode column eliminated the traditional requirement for tedious ion-pairing reagents by combining reversed-phase and cation-exchange mechanisms within a single stationary phase. The mobile phase components consisted of Mobile Phase A (400 mL Acetonitrile + 599.5 mL Water + 0.5 mL H2SO4 and Mobile Phase B (900 mL Acetonitrile + 99.5 mL Water + 0.5 mL H2SO4, executed via an optimized linear gradient program at a flow rate of 1.0 mL/min. Ultraviolet detection was performed at 250 nm with an injection volume of 5 µL. The method was validated according to International Council for Harmonisation (ICH) Q2(R1) guidelines. Specificity was demonstrated with resolved peaks across a tight 7-minute analytical runtime: PDCA (1.98 min), Nicotinic Acid (3.54 min), Ethyl Nicotinate (4.84 min), Pyridine (5.53 min), and MEP (6.60 min). Linearity was confirmed across a concentration range of 50 to 150 ppm for all compounds with correlation coefficients (R2 0.997). Method precision was verified via multi-day analysis, consistently exhibiting relative standard deviations (%RSD) well below 2.0%. The limits of detection (LOD) and quantitation (LOQ) were established at 1 ppm and 10 ppm, respectively. Accuracy evaluations yielded high recovery values ranging from 95.82% to 100.81%. Robustness testing involving deliberate flow perturbations (±0.1 mL/min) confirmed system suitability compliance, demonstrating that this mixed-mode strategy is highly reliable for routine quality control and regulatory analysis of nitrogenous heterocycles.

Keywords: HPLC Method Validation; Primesep 100 Column; Mixed-Mode Chromatography; Pyridine Derivatives; Nicotinic Acid; ICH Guidelines.

How to cite this article: Pardeshi AA, Philip BO, Ghalsasi Y, Mulik N, Dhakane V. Development and Validation of a High-Performance Liquid Chromatographic (HPLC) Method for the Simultaneous Estimation of Five Related Pyridine Compounds Using a Mixed-Mode Primesep 100 Column. Int J Drug Deliv Technol. 2026;16(69s):98-108. DOI: 10.25258/ijddt.16.69s.11

1. INTRODUCTION

Pyridine rings and their various alkylated, oxidized, or esterified derivatives represent foundational chemical architecture across agrochemical synthesis, fine chemical processing, and pharmaceutical manufacturing(1). Compounds such as Pyridinedicarboxylic acid (PDCA), Nicotinic Acid (Vitamin B3), Ethyl Nicotinate, Pyridine, and 2-Methyl-5-ethylpyridine (MEP) routinely co-exist as synthetic precursors, structural isomers, side-

reaction impurities, or stability degradants within active pharmaceutical ingredients (APIs) (2, 3). Quantifying and resolving these components simultaneously presents an extreme analytical challenge because of their high polarity, overlapping ultraviolet (UV) profiles, and disparate acid-base characteristics across wide pKa boundaries (4).

Conventional reversed-phase liquid chromatography (RP-HPLC) utilizing standard octadecyl-silica columns (C18) struggles significantly when dealing with polar, basic nitrogenous compounds (5). On a traditional C18 matrix, these basic chemical entities exhibit poor, erratic retention, leading to severe peak tailing caused by secondary silanol interactions (6). To mitigate these issues, chromatographers have historically resorted to adding mobile-phase ion-pairing agents such as heptanesulfonic acid or trifluoroacetic acid (7). However, ion-pairing techniques introduce major disadvantages, including prolonged column equilibration requirements, poor baseline reproducibility, irreversible stationary-phase alteration, and absolute incompatibility with downstream liquid chromatography-mass spectrometry (LC-MS) instrumentation (8). Hydrophilic interaction liquid chromatography (HILIC) offers an alternative for highly polar matrices but suffers from matrix sensitivity and slow equilibration periods (9).

To bypass these systemic limitations, mixed-mode liquid chromatography has emerged as a high-efficiency alternative for pharmaceutical method development (10). The Primesep 100 column incorporates a highly advanced stationary phase configuration that provides a dual mechanism of interaction: it combines a highly hydrophobic alkyl chain backbone with an embedded, hydrophilic acidic ion-pairing functional group covalently non-toxic acidic modifiers (14).

While independent methodologies exist for isolated selections of these chemicals, single-run method capable of simultaneously separating and quantifying this complex five-component mixture under strict regulatory validation standards. This research presents the development and full validation of a high-throughput gradient HPLC-UV method utilizing a Primesep 100 column. Validation runs were executed under strict adherence to the International Council for Harmonisation (ICH) Q2(R1) protocol to ensure compliance regarding specificity, linearity, precision, sensitivity, accuracy, and operational robustness for academic or industrial deployment (15, 16).

2. MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

Certified reference standards for Pyridinedicarboxylic acid (PDCA), Nicotinic Acid, Ethyl Nicotinate, Pyridine, and 2-Methyl-5-ethylpyridine (MEP) were acquired at high purity (>99.5%). HPLC-grade Acetonitrile (ACN) and ultra-pure deionized water were utilized throughout the experimental work. Concentrated Sulfuric Acid (H_2SO_4 Analytical Reagent Grade) served as the acidic modifier to modulate stationary-phase ionization and mobile phase ionic strength.

2.2 CHROMATOGRAPHIC SYSTEM AND INSTRUMENTAL CONDITIONS

Separations were executed using an automated Agilent 1260 Infinity II HPLC system equipped with a quaternary gradient pump, a high-precision autosampler, a column thermostatic chamber, and an ultraviolet (VWD) detector.

bonded to a high-purity silica matrix (11, 12). By functioning as both a reversed-phase media and a strong cation-exchanger, the Primesep 100 column allows analytical methods to capitalize on multiple retention mechanisms simultaneously (13). Hydrophobic neutral components are retained via standard partition pathways, while polar basic nitrogen heterocycles are securely retained and resolved via electrostatic ionic interactions with the embedded functional groups, driven by simple,

- **Column:** Primesep 100 Column (150mmx4.6mmx5um)
- **Column Temperature:** 30°C
- **Detection Wavelength:** UV 250 nm
- **Injection Volume:** 5 µL
- **Column Flow Rate:** 1.0 mL/min
- **Diluent Matrix:** Acetonitrile : Water (50:50 v/v)
- **Total Gradient Runtime:** 32 minutes

Table 1. Optimized Gradient Elution Time-Profile

Time (Minutes)	Mobile Phase A (%)	Mobile Phase B (%)
0.0	100	0
10.0	0	100
17.0	0	100
19.0	100	0
24.0	100	0
32.0	100	0

The mobile phases were prepared as follows:

- **Mobile Phase A:** 400 mL Acetonitrile + 599.5 mL Purified Water + 0.5 mL H_2SO_4
- **Mobile Phase B:** 900 mL Acetonitrile + 99.5 mL Purified Water + 0.5 mL H_2SO_4

2.3 PREPARATION OF STANDARDS AND VALIDATION PROTOCOL

Stock standards were accurately weighed and dissolved in the diluent matrix to prepare individual standard components. Standard working blends (100 ppm target mixture) were evaluated across successive days by two independent analytical operators to assess system suitability and intermediate precision. Linearity evaluations spanned five precisely mapped levels: 50, 80, 100, 120, and 150 ppm. Analytical accuracy recovery experiments were established at low (80%), medium (100%), and high (120%) targeted theoretical response regions.

The high-resolution separation was governed by the optimized multi-step linear gradient profile outlined in Table 1.

3. RESULTS

3.1 SPECIFICITY AND CHROMATOGRAPHIC RESOLUTION

Method specificity was evaluated by evaluating individual component injections against blanks to guarantee zero co-elution, matrix interference, or baseline artifact anomalies. The unique dual-action mechanism of the Primesep 100 column provided an exceptionally well-resolved profile, yielding high peak symmetry and definitive resolution margins between all five components within a tight 7-minute separation window (Table 2).

Table 2. Component Specificity and Nominal Chromatographic Baseline Configurations

Peak ID	Target Analytes	Retention Time (min)	Peak Area
1	Pyridinedicarboxylic acid (PDCA)	1.980	522.8
2	Nicotinic Acid	3.542	854.1
3	Ethyl Nicotinate	4.838	694.8
4	Pyridine	5.529	1517.5
5	2-Methyl-5-ethylpyridine (MEP)	6.603	417.0

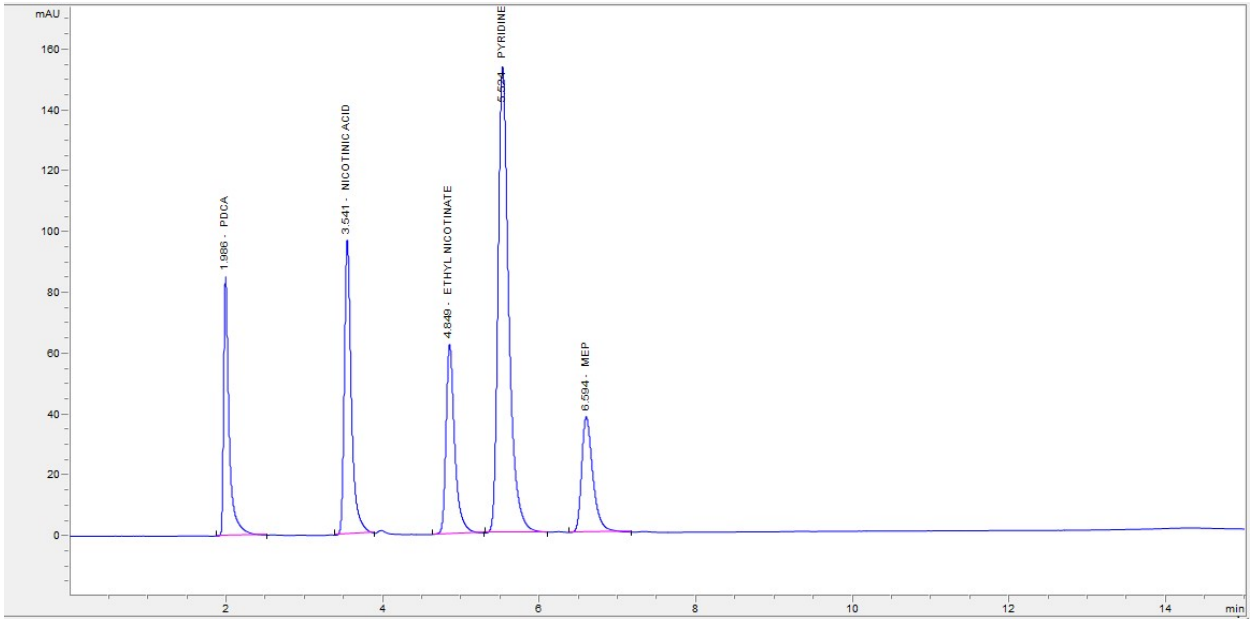


Figure 1. Reference high-performance liquid chromatogram layout detailing high-resolution baseline separation for the five related pyridine derivatives on the mixed-mode column.

3.2 SYSTEM SUITABILITY AND MULTI-DAY PRECISION PROFILE- System precision parameters were evaluated by executing six replicate injections of the 100 ppm blended standard sample across distinct analysis dates.

The intermediate precision matrix compiled by distinct laboratory analysts verified excellent reproducibility (Table 3).

Table 3. Precision Profile Evaluated via Analytical Run Sequences (100 ppm Concentration)

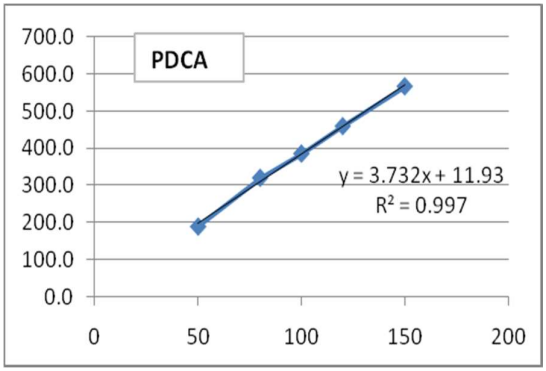
System Parameters / Run ID	PDCA	Nicotinic Acid	Ethyl Nicotinate	Pyridine	MEP
Day 1: Mean Retention Time (min)	2.004	3.547	4.867	5.549	6.631
RT Standard Deviation (%RSD)	0.55%	0.26%	0.26%	0.28%	0.28%
Day 1: Mean Peak Area	432.18	610.63	483.83	1461.98	372.07
Area Standard Deviation (%RSD)	1.65%	0.32%	0.28%	0.36%	1.51%
Day 1: Mean Area Percent (%)	12.86	18.17	14.40	43.50	11.07
Area% Standard Deviation (%RSD)	1.60%	0.38%	0.20%	0.21%	1.52%
Day 2: Mean Retention Time (min)	2.098	3.589	4.924	5.618	6.698
RT Standard Deviation (%RSD)	0.20%	0.11%	0.10%	0.14%	0.15%

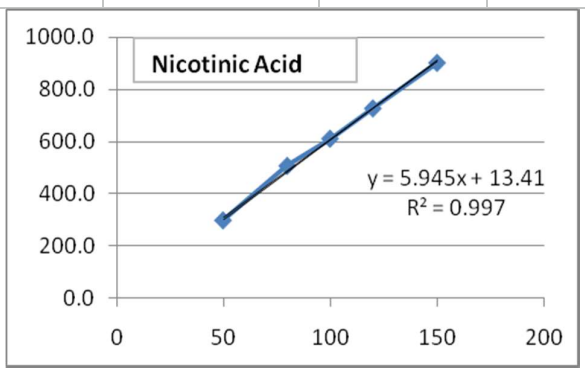
Day 2: Mean Peak Area	390.47	618.05	496.13	1471.78	424.50
Area Standard Deviation (%RSD)	0.59%	0.19%	0.38%	0.46%	1.82%
Day 2: Mean Area Percent (%)	11.48	18.17	14.59	43.28	12.48
Area% Standard Deviation (%RSD)	0.28%	0.41%	0.25%	0.43%	1.54%
System Suitability Status	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**

3.3 LINEARITY AND SENSITIVITY THRESHOLD LIMITS (LOD / LOQ) -The method's capability to deliver proportional peak area responses directly matching sample mass concentrations was verified across an extensive standard concentration window from 50 ppm to 150 ppm (Table 4).

Table 4. Target Analyte Calibration Responses Across Concentrations

Target Calibration Point	Conc. (ppm)	PDCA Area	Nicotinic Acid Area	Ethyl Nicotinate Area	Pyridine Area	MEP Area
Linearity Level 1	50	190.15	298.10	237.55	704.45	234.15
Linearity Level 2	80	321.20	506.15	405.60	1195.95	364.35
Linearity Level 3	100	386.55	609.85	491.20	1459.55	429.55
Linearity Level 4	120	460.35	726.10	584.15	1741.40	496.65
Linearity Level 5	150	567.40	899.85	728.45	2163.20	599.40
Regression	**(R^2)**	**0.997**	**0.997**	**0.997**	**0.998**	**0.996**





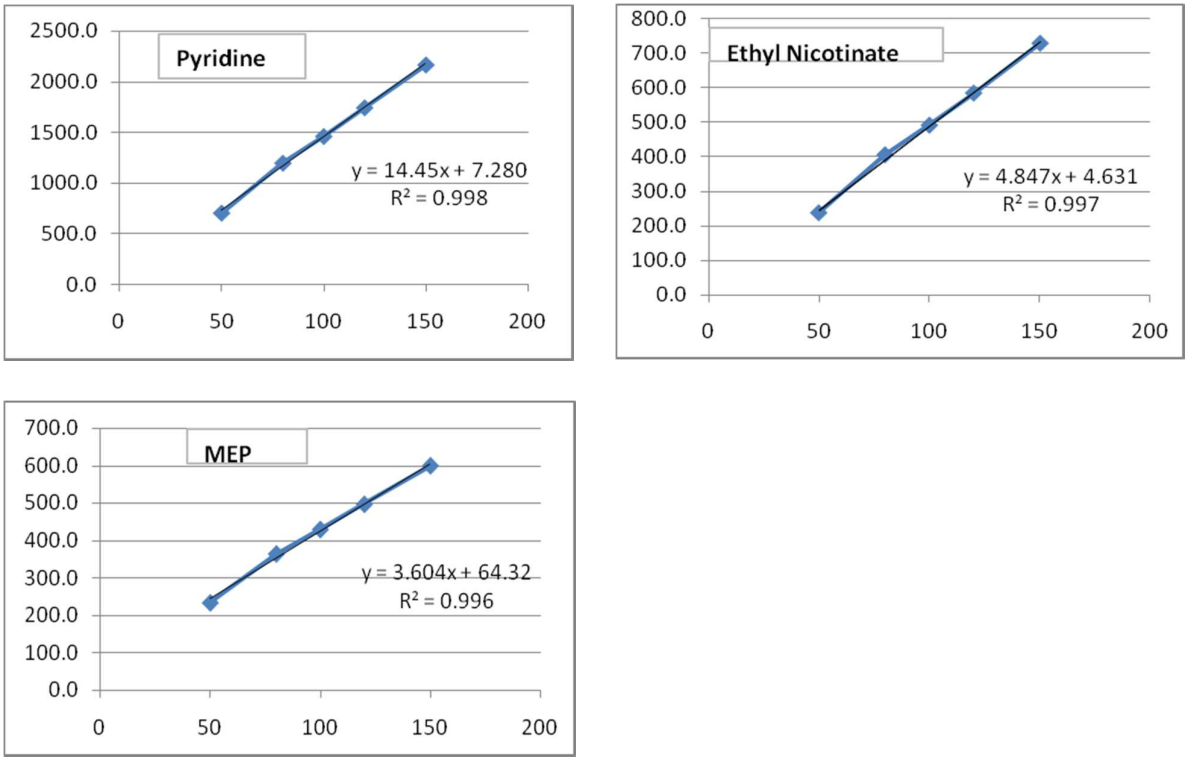


Figure 2. Calibration curve trends demonstrating strict detector linearity across the 50 to 150 ppm working concentration range.

Sensitivity parameters were formally mapped using signal-to-noise evaluations at trace calibration points:

- **Limit of Detection (LOD) at 1 ppm:** Distinct peak recognition was confirmed with low variance. The average peak area responses observed at 1 ppm were: PDCA (4.23), Nicotinic Acid (7.00), Ethyl Nicotinate (4.37), Pyridine (13.43), and MEP (73.87). Replicate injection area attributes achieved high stability (%RSD < 5.0%).
- **Limit of Quantitation (LOQ) at 10 ppm:** Definitive quantitative precision was verified at the 10 ppm marker. The mean peak area footprints captured were: PDCA (38.75), Nicotinic Acid (61.95), Ethyl Nicotinate (48.43), Pyridine (143.18), and MEP (39.18). Replicate injection parameters tracked entirely within acceptable regulatory thresholds (%RSD < 2.0%).

3.4 ACCURACY RECOVERY EVALUATIONS

Method analytical recovery parameters were evaluated by verifying observed experimental concentrations against theoretical target points across low (80%), medium (100%), and high (120%) levels (Table 5).

Table 5. Percent Recovery Determinations for Target Analytes

Compound Name	Conc. Level (%)	Theoretical Area	Observed Area	Recovery (%)
PDCA	80% Level	365.04	365.75	100.19%
	100% Level	456.3	456.3	100.00%
	120% Level	547.56	524.65	95.82%
Nicotinic Acid	80% Level	482.96	483.8	100.17%

	100% Level	603.7	603.7	100.00%
	120% Level	724.44	729.7	100.73%
Ethyl Nicotinate	80% Level	384.8	384.7	99.97%
	100% Level	481	481	100.00%
	120% Level	577.2	579.45	100.39%
Pyridine	80% Level	1150.84	1149.95	99.92%
	100% Level	1438.55	1438.55	100.00%
	120% Level	1726.26	1740.3	100.81%
MEP	80% Level	347.6	338.75	97.45%
	100% Level	434.5	434.5	100.00%
	120% Level	521.4	508.05	97.44%

3.5 METHOD ROBUSTNESS UNDER FLOW RATE VARIATIONS

Method robustness was evaluated by introducing deliberate perturbations of ±0.1 mL/min around the nominal 1.0 mL/min flow rate. The system maintained system suitability guidelines under all conditions, with critical peak area precision parameters remaining well below the 2.0% RSD margin (Table 6).

Table 6. Robustness Performance Trends During Flow Rate Modifications (100 ppm Standard)

Robustness Parameters	PDCA	Nicotinic Acid	Ethyl Nicotinate	Pyridine	MEP
0.9 mL/min Flow Condition					
Mean Retention Time (min)	2.258	3.972	5.440	6.231	7.391
Mean Peak Area	492.15	685.43	535.95	1634.67	404.98
Peak Area Variance (%RSD)	0.38%	0.38%	0.39%	0.39%	1.46%
1.1 mL/min Flow Condition					
Mean Retention Time (min)	1.837	3.243	4.462	5.066	6.079
Mean Peak Area	400.23	560.88	444.07	1327.93	342.38

Peak Area Variance (%RSD)	0.27%	0.29%	0.14%	0.38%	0.63%
Acceptance Evaluation	**PASS**	**PASS**	**PASS**	**PASS**	**PASS**

4. DISCUSSION

4.1 ROLE OF THE PRIMESEP 100 COLUMN IN METHOD DEVELOPMENT

The primary catalyst for achieving full baseline resolution of these five related substances without high-pH environments or complex ion-pairing chemicals was the dual-action architecture of the mixed-mode Primesep 100 column. In traditional reversed-phase settings, highly polar carboxylic and basic entities such as Pyridinedicarboxylic acid and Nicotinic Acid elute nearly immediately within the void volume, because water-rich mobile phases fail to partition them onto standard C18 chains (5, 6). Conversely, strongly basic molecules like 2-Methyl-5-ethylpyridine (MEP) can experience severe peak tailing due to uncontrollable silanol interactions with the unreacted silica underbed (10, 14).

The Primesep 100 column addresses this by utilizing embedded acidic ion-pairing/cation-exchange functional groups integrated directly into its long hydrophobic alkyl chains (11). When basic target analytes enter the column under acidic conditions (mediated by the H_2SO_4 modifier), the nitrogen atoms of the pyridine rings become positively protonated. These protonated heterocycles then interact via strong electrostatic attraction with the negatively charged embedded stationary phase groups (12, 13). This ionic interaction provides a secondary retention mechanism independent of hydrophobic partition. This dual retention allows polar molecules like PDCA and Nicotinic Acid to be retained effectively beyond the column void volume.

Simultaneously, adjusting the organic modifier concentration (Acetonitrile) across the linear gradient controls the elution of the less polar compounds (Ethyl Nicotinate, Pyridine, and MEP) through traditional reversed-phase mechanisms (14). Consequently, the Primesep 100 column simplifies mobile phase preparation, ensures high batch-to-batch reproducibility, and preserves long-term instrumentation durability—achieving excellent performance without the limitations of traditional ion-pairing techniques.

4.2 ANALYTICAL PERFORMANCE EVALUATION

The experimental data demonstrates that the developed gradient method fulfills all predefined ICH Q2(R1) regulatory validation thresholds (15, 16). The method exhibited high specificity, with zero baseline noise from the blank diluent matrix interfering with the target analytes. System suitability testing yielded low peak area variations across multiple analysis dates and operating technicians, with all intermediate precision %RSD margins falling well below the 2.0% ceiling mandated for pharmaceutical assays (17).

Detector linearity was confirmed over a working concentration range of 50 to 150 ppm, yielding correlation coefficients (R^2) exceeding 0.997 for all five compounds. Method sensitivity evaluations demonstrated low detection limits, establishing an LOD of 1 ppm and an LOQ of 10 ppm. These sensitivity thresholds confirm that the method is suitable for tracking low-level process impurities or decomposition products in active substances.

The analytical accuracy recovery metrics remained tightly clustered around the nominal theoretical points, yielding recovery values between 95.82% and 100.81%. Deliberate flow rate adjustments (±0.1 mL/min) during robustness testing led to predictable shifts in absolute retention times; however, system resolution factors and overall area reproducibility remained unaffected. This demonstrates that minor physical fluctuations during routine operation do not compromise the quantitative integrity of the method, confirming its suitability for routine quality control and industrial applications (18).

5. CONCLUSION

A reliable, high-resolution gradient HPLC-UV method has been developed and validated for the simultaneous determination of

Pyridinedicarboxylic acid, Nicotinic Acid, Ethyl Nicotinate, Pyridine, and 2-Methyl-5-ethylpyridine using a mixed-mode Primesep 100 column. By leveraging the simultaneous reversed-phase and cation-exchange mechanisms of the stationary phase, the method successfully resolves highly polar and basic pyridine derivatives without the need for traditional, complex ion-pairing additives. The method was validated in compliance with ICH Q2(R1) guidelines, showing excellent specificity, linearity, precision, sensitivity, accuracy, and operational robustness. This streamlined approach provides an efficient tool for routine quality control, stability monitoring, and impurity profiling of pyridine-based compounds in both pharmaceutical research and manufacturing environments.

ACKNOWLEDGEMENTS

The authors express their sincere gratitude to Godavari Biorefineries Limited and K. J. Somaiya College of Science and Commerce, Mumbai, for providing the research laboratory infrastructure, instrumentation, and analytical support. The constant encouragement and excellent facilities provided by both institutions played a vital role in the successful development and verification of this analytical method.

We also gratefully acknowledge the valuable contributions of the research and development (R&D) team and analytical technicians who assisted with method optimization, sample preparation, and data acquisition. Their expertise and technical assistance were essential to ensuring the accuracy and reliability of the experimental results presented in this manuscript.

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