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Original Article

DEVELOPMENT AND VALIDATION OF ANALYTICAL METHOD BY RP-HPLC FOR SIMULTANEOUSLY ESTIMATION OF ANTIBIOTICS IN A HOSPITAL ETP SLUDGE AND SOIL AS PER ICH GUIDELINE

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ABSTRACT

Objective: The study aimed to develop and validate a simple, rapid, precise, and eco-friendly reversed-phase high-performance liquid chromatography (RP-HPLC) method for the simultaneous estimation of metronidazole (MTZ), mupirocin (MPC), and chloramphenicol (CPL) in hospital effluent treatment plant (ETP) sludge and soil samples, with emphasis on compliance with green analytical chemistry (GAC) principles using the blue applicability grade index (BAGI) tool.

Methods: Chromatographic separation was achieved using a C18 column with a mobile phase comprising phosphate buffer and acetonitrile (72:28 v/v) at a flow rate of 1.5 ml/min and detection at 220 nm. A detection wavelength of 220 nm was selected after UV spectral evaluation and used consistently throughout method validation. The method was validated as per international-council-for-harmonisation (ICH Q2(R1) guidelines for parameters including system suitability, precision, accuracy, linearity, robustness, ruggedness, limit-of-quantitation (LOQ), limit-of-detection (LOD), solution stability, and forced degradation. Green assessment was conducted using the BAGI tool to evaluate solvent use, energy efficiency, waste generation, and sustainability.

Results: The method showed excellent linearity for all three antibiotics (R^2>0.999). The %RSD for precision and intermediate precision was below 2%, indicating high reproducibility. LOD and LOQ values confirmed the method's sensitivity. Forced degradation studies revealed significant degradation under acidic and oxidative conditions, confirming its stability-indicating nature. BAGI assessment demonstrated complete compliance with green chemistry principles, indicating minimal solvent and energy consumption, reduced waste generation, and environmental safety.

Conclusion: The developed RP-HPLC method is accurate, precise, and environmentally sustainable. It can be effectively applied for routine analysis and monitoring of antibiotic contaminants in hospital ETP sludge and soil, supporting green analytical practices.

Keywords: Method development, Validation, Reverse-phase-high-performance-liquid-chromatography, Metronidazole, Mupirocin, Chloramphenicol, ICH-guidelines, Blue applicability grade index, Hospitals effluent treatment plant sludge and soil

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INTRODUCTION

Antibiotics are essential therapeutic agents that have revolutionized modern healthcare by effectively preventing and treating bacterial infections through mechanisms such as inhibition of cell wall synthesis, protein synthesis, or nucleic acid replication [1-5]. Despite their undeniable medical importance, the extensive and often unregulated use of antibiotics in hospitals, community settings, and agriculture has resulted in their continuous release into the environment. A significant portion of administered antibiotics is excreted as unchanged or as active metabolites, entering wastewater systems through hospital discharges and sewage. The mismanagement of unused pharmaceuticals makes this problem even more serious, as it leads to the fact that antibiotic residues remain in different environmental matrices, among which are water, sludge, and soil [6-8]. Pharmaceutical contaminants have been identified to be concentrated sources of effluents released by hospitals and the capacity to treat these sources remains limited in most hospitals. As a result, antibiotics are deposited in effluent treatment plant sludges and the adjacent soils posing serious ecological and personal health threats. The presence of the residues not only disturbs the microbial communities but also contributes to the emergence and spread of antimicrobial resistance (AMR) in the environmental ecosystems, which highlights the importance of attentive monitoring and control of antibiotic contamination of hospital-generated waste streams [9-12].

The continued presence of antibiotic residues in the environment like water, sludge and soil has been a major issue of concern around the world because of their longevity and dissimilarity to natural decomposition processes. When emitted into the environment by hospital effluents, wastewater, or the application of sludge, these compounds may be long lasting, and hence they will continuously selectively stress out populations of microbes [13-15]. This chronic exposure can facilitate the emergence and spread of AMR and allow the growth of resistant bacteria and resistance genes in aquatic and terrestrial environments. In addition to AMR, antibiotic residues may cause ecological toxicity by altering the balance of the microbial community, preventing the cycling of nutrients, and altering the growth and reproduction of aquatic life. In addition, they add to the level of bioaccumulation and possible entry into the food chain when accumulated in sediments and soils thus presenting an indirect risk to human health [16-19]. The acknowledgement of these menaces has made international organisations like the World Health Organization (WHO) and the United Nations Environment Programme (UNEP) to identify antibiotic pollution by the hospital effluents as a serious environmental and human health concern. They have emphasized the great need of constant surveillance, effective wastewater management, and the tightening of the belt on the regulatory measures to prevent further spread of antibiotics into the environment and to curb the increasing problem of antimicrobial resistance [20-23].

The antibiotics that are commonly used in the hospital context are metronidazole, mupirocin, and chloramphenicol, and they belong to different classes of chemicals. Metronidazole is a broad-spectrum antibacterial and antiprotozoal prescribed to treat anaerobic infections; mupirocin is a topical

antibiotic with low biodegradability and chloramphenicol is a long-duration antibiotic that is environmentally stable. Their common presence at hospitals and environmental stability underpin the concomitant determination of the compounds in an effluent, sludge, and soil sample [24-27].

It is also necessary to monitor such antibiotic residues to determine the quality of hospital effluent treatment and environmental safety. There are many benefits of simultaneous estimation of multiple antibiotics, among them is the application of reduced solvents, decreased time on analysis and decreasing operational costs and a detailed insight into the extent of contamination and treatment efficiency in complex environmental matrices [28-31].

A variety of analytical methods have been reported in analysis of antibiotics such as spectrophotometry, microbiological assays, high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-M S). Nevertheless, several of the current methods are single-analyte-oriented, demanding costly equipment, or have not been shown to work in complex matrices (sludge and soil). Reverse-phase high-performance liquid chromatography (RP-HPLC) is an effective, yet cost-effective alternative since it has high sensitivity, selectivity, reproducibility as well as being ubiquitous in the analytical laboratories [32-40]. This is crucial to ensure the reliability, accuracy and reproducibility of analytical results especially when the results are to be used in environmental monitoring application [41-44]. Although the problem of environmental antibiotic contamination is gaining momentum, few studies have documented any validated RP-HPLC procedures of the concomitant determination of metronidazole, mupirocin, and chloramphenicol in hospital effluent treatment plant sludge and soil matrices [45-52].

Thus, the objective of the current research is to design and to confirm an easy, correct, precise, and powerful RP-HPLC technique to determine simultaneously metronidazole, mupirocin, and chloramphenicol in hospital effluent treatment plant sludge and soil samples as per the ICH directions. It is expected that the suggested approach will help to conduct regular environmental monitoring, as well as to aid the evaluation of treatment effectiveness and contribute towards methods of antibiotic pollution control and reduction of antimicrobial resistance transmission.

MATERIALS AND METHODS

Materials

Chemical and reagents

Acetonitrile of HPLC grade (Advent Chembio Private Limited, 99% purity), Potassium dihydrogen phosphate (Merck Life Science Private Limited, 99% purity), Sodium hydroxide Pellets (Avantor Performance Materials India Private Limited, 99% purity) procured were of analytical grade and used without further purification. All active pharmaceutical ingredients (API) like Metronidazole (99.15% of potency), Mupirocin (99.68% of potency) and Chloramphenicol (99.35% of potency) were provided by ADPL, Uttarakhand as gift samples. All other required chemicals and solvents are also gifted by ADPL.

Software and instrumentation

We used an ARC-2998 UV PDA HPLC system from Waters, which has a quaternary pump, UV-PDA detector and Empower software version 03. Ultrasonic cleaner (PCI), Analytical balance (Radwag, AS 120. X2 PLUS), pH Meter (Spectra lab, Accu pH-3) were also used during this research work.

Methods

Method development with chromatographic conditions

Chromatographic separation was achieved using an Accellis C18 column (250 × 4.6 mm, 5 µm) under isocratic conditions (table 1). Sample pre-treatment involved filtration and Solid-Phase Extraction (SPE) using C18 cartridges to concentrate of all analytes and remove matrix impurities.

Table 1: Chromatographic conditions

Column and packing details	Accellis C18 column (250 × 4.6 mm, 5 µm of particle size, Part no. ACL18-25046-5)
Preparation of aqueous solution for the mobile phase	0.1 M buffer solution (Buffer solution was prepared by dissolving 13.68 gm Potassium Dihydrogen phosphate in 1000 ml in Millipore water).
Mobile phase used	72:28 (Phosphate buffer, pH 6.3: Acetonitrile)
Detector set at	220 nm
Flow-rate set at	1.5 ml per min.
Volume of injection	20 µl of injection
Column-oven temperature set	25 °C of Temperature
Auto sampler-temperature set	25 °C of Temperature
Run time set at	12 min
Diluent used	Millipore water

Method validation

The developed RP-HPLC method was validated according to ICH Q2(R1) and ICH Q1A(R2) guidelines, confirming specificity, linearity, accuracy, precision, sensitivity, robustness, and system suitability [53, 54].

Solubility study

Based on the solubility study, Metronidazole, Mupirocin, and Chloramphenicol were found to be highly soluble in water but showed limited solubility in methanol and acetonitrile. Therefore, water was chosen as the most suitable component of the mobile phase as well as the diluent for preparing both reference and test solutions.

Sample preparation and matrix-effect study

Sludge samples were collected from the outlet of the hospital effluent treatment plant at a depth of 1–2 cm, while soil samples were obtained within 5–10 m of the discharge zone at a depth of 0–15 cm, using sterile polypropylene containers and stored at 4 °C until analysis. Sludge was dried at 40 °C, and soil was air-dried, after which both matrices were homogenized and sieved through a 2 mm mesh. For extraction, 2.0 g of each sample was mixed with 10 ml of methanol, vortexed for 2 min, ultrasonicated for 20 min, shaken for 15 min, and centrifuged at 5000 rpm for 10 min; the residue was re-extracted with an additional 5 ml of solvent, and the supernatants were pooled. The extract was purified using C18 SPE cartridges (500 mg/6

ml) that were conditioned with 5 ml methanol and 5 ml water, after which 5 ml of sample extract was loaded, washed with 2 ml water, and eluted with 5 ml acetonitrile. The eluate was evaporated at 40 °C under nitrogen, reconstituted in 1 ml Millipore water, filtered (0.22 µm), and injected into the RP-HPLC system. To assess matrix effects, blank sludge and soil extracts prepared by the same procedure were post-spiked with known quantities of metronidazole, mupirocin, and chloramphenicol, and their responses were compared with pure solvent standards at identical concentrations; the matrix factor (MF = A/B) and % matrix effect (%ME = (MF − 1) × 100) were calculated.

Wavelength selection process

All three antibiotics Metronidazole, Mupirocin, and Chloramphenicol were individually scanned using a Ultraviolet (UV) spectrophotometer over the wavelength range of 200 to 400 nm. The obtained spectra exhibited a common absorption maximum (λmax) near 220 nm, where all analytes showed strong absorbance with minimal interference. Consequently, a detection wavelength of 220 nm was selected for the development and validation of the RP-HPLC method to enable accurate and simultaneous quantification of all three antibiotics, as summarized in table 1.

System suitability evaluation

A sequence of injections was performed, consisting of a single blank injection (20 µl**), six replicate injections of the standard solution, one injection of the test solution, and one repeat injection of the standard solution as a bracketing standard. The system suitability parameters were evaluated and maintained in accordance with ICH guidelines, as presented in Tables 2 and 3.

Table 2: System-suitability parameters limits

Evaluated-parameters	Limit maintained
RSD for all six replicate Std. solution of the estimated antibiotic	NMT 2.0 %
USP-Tailing Value for every estimated antibiotic	NMT 2.0
USP-Resolution set between every estimated antibiotic	NLT 3.0
Theoretical plate maintains for each estimated antibiotic	NLT 2000

RSD – Relative standard deviation, USP – United States pharmacopeia, NMT – Not more than and NLT – Not less than.

Table 3: Retention time obtained

Different antibiotic components	Retention time (min)
Metronidazole	About 2.7
Mupirocin	About 5.0
Chloramphenicol	About 8.4

Specificity

For the evaluation of specificity, a sequence of injections (20 µl** each) was performed as follows: a single blank, six replicate injections of the reference mixed API solution, individual injections of MTZ, MPC and CPL, one injection of the test solution, and finally a single bracketing injection of the reference solution.

Peak-purity analysis

Peak purity was assessed using Empower-03 liquid Chromatography software equipped with a PDA detector, and the purity of the main peaks in both the sample and reference preparations was evaluated and compared.

Linearity

The linearity of the developed RP-HPLC method was assessed to verify the direct proportionality between the concentration of each antibiotic and its detector response. Standard solutions of metronidazole, mupirocin, and chloramphenicol were prepared at five to seven concentration levels, ranging from the LOQ to 150% of the target concentration, and each level was injected in triplicate. The mean peak areas were recorded and plotted against concentration to construct calibration curves. Regression analysis yielded correlation coefficients (R^2) ≥ 0.99 for all analytes, confirming excellent linearity. The slope and intercept values were consistent across the concentration range, indicating uniform detector sensitivity. Residual plots showed random distribution around zero, further supporting the accuracy and linearity of the relationship. These findings demonstrate that the developed method provides precise and reliable quantitative estimation of the selected antibiotics in hospital ETP sludge and soil samples.

Precision

The precision of the proposed analytical method was evaluated by assessing how closely the data values agreed across multiple determinations. This was examined through system precision, method precision, and intermediate precision studies using different analytical conditions.

System-precision

For the evaluation of system precision, 20 µl** injections were performed in the following sequence: a single blank injection followed by six replicate injections of the reference solution using the proposed method. All results were recorded. The acceptance criteria required the relative standard deviation (RSD) of all components across six replicate injections to be not more than (NMT) 2%, tailing factor NMT 2, theoretical plates not less than (NLT) 2000, and resolution between all peaks maintained at NLT 1.5.

Method-precision

In the assessment of method precision, 20 uL injections were performed in the following order: a single blank injection, six replica injections of the reference solution, single injections of test samples obtained by six different hospital sources and a final bracketing reference injection. All results were recorded. The acceptance criteria were that the relative standard deviation (RSD) of all 6 replicate injections of the main active pharmaceutical ingredients should not exceed 2%, the tailing factor should not exceed 2, the theoretical plates should not fall below 2000, and all the peaks should have a resolution of not less than 1.5.

Intermediate precision

The intermediate precision was tested to determine the reproducibility of the analytical method in different conditions within the laboratory. To ensure consistency in the results, the changes included in the study were the change in analysts, the change in instruments, the change in days, and the change in columns. The results indicated the strength of the created approach that meant that any slight changes in operation did not affect accuracy. Intermediate precision was reported as the percentage RSD of replicate analysis, all of which were within acceptable limits, which proved the reliability of the method. The acceptance criteria were that the % RSD of all antibiotics in six replicate injections must not exceed 2%, the tailing factor must not exceed 2, the theoretical number of plates must not be less than 2000 and the resolution between all peaks should not be less than 1.5.

LOQ and LOD

Limit of detection (LOD) and limit of quantification (LOQ) were calculated to find out the lowest concentrations of antibiotics that can be reliably detected and measured through the developed RP-HPLC method. A sample of low-concentration standard solutions of MTZ, MPC and CPL were made beneath the following envisaged detection limit and were subjected to triplicate analysis. The LOD definitions were based on signal-to-noise ratio (S/N) at which the signal to noise ratio of 3:1 was considered detectable presence, and 10:1 was the limit of quantification (LOQ). Chromatograms had good separation between baseline noise and the peptide and the percentage of relative standard deviation in the acceptable limit of less than or equal to 2%. The sensitivity, precision and reliability of the developed method were confirmed because all the analyses were done under the same chromatographic conditions, which proved that the method could detect trace quantities of antibiotics in the sludge and soil samples of hospital effluent treatment plants.

Robustness

The stability of the developed RP-HPLC procedure was tested to ensure its stability under small intentional changes in the conditions of analysis. Systematic alterations were performed on variables such as mobile phase composition, flow rate (±0.1 ml/min), column temperature (±5 C), mobile phase pH (±0.2 units) and detection wavelength (±2 nm). The metronidazole (MTZ), mupirocin (MPC) and chloramphenicol (CPL) standard solutions were analyzed under each modified condition, and the chromatographic performance measures, such as retention time, peak resolution, and symmetry of the chromatographic performance, were compared to those of the optimized approach. These findings showed that there were consistent peak areas, retention times of less than 2%, and tailing factors ≤ 2 in all the changes, which showed that the small changes in the operation did not make any major alterations to the separation and quantification. The findings validate the strength, durability, and applicability of the method to the normal environmental assessment of antibiotics in sludge and soil samples of the hospital effluent treatment plant.

Ruggedness

The ruggedness of the developed RP-HPLC method was determined to ensure consistency and reproducibility in a variety of analytical conditions, such as diverse analysts, instruments, and chromatographic columns. Independent preparations of standard solutions of MTZ, MPC and CPL were analyzed by the various technicians in separate chromatographic systems with the same operating procedures. Chromatographic parameters crucial to include retention time, peak area, resolution and tailing factor were compared in all conditions. The difference in retention times was not too big, and the difference in the retention time was within the range of ±2 per cent alongside values of the per cent relative standard deviation within the acceptable range, thus, indicating high reproducibility. The findings of these studies support the fact that the technique provides reliable and uniform data regardless of the variability of the analyst or instrument, which makes it rugged, reliable and applicable in routine environmental monitoring of antibiotics in hospital wastewater samples under different laboratory conditions.

Spiking of APIs into actual soil and sludge samples

To verify the applicability of the method and assess the recovery, soil and sludge samples on the hospital effluent treatment plant site were fortified with known amounts of MTZ, MPC and CPL. Each matrix (2.0g) was spiked with a mixed standard solution to attain a final concentration of 10ugg-1 of each antibiotic, and equilibrated after 30 min to ensure that the contents were homogenously distributed. The fortified matrices were then treated to undergo the same extraction process and solid-phase extraction cleanup and RP-HPLC analysis as those of unspiked samples. The values of recoveries were determined by contrasting the peak spiked samples with solvent standards so that the method performance in real environmental matrices could be assessed.

Solution-stability study

The stability of the solution was also tested to make sure that the standard and sample solutions of MTZ, MPC and CPL were stable during the analytical period. Optimized aqueous diluent was used to prepare the standard solutions and kept at ambient temperature and refrigeration conditions (2-8 C). The analyses were conducted at 0, 24, 48 and 72h to identify any alteration in the concentration, retention time or peak area. The comparison of chromatograms to fresh standards was done to reveal any degradation, precipitation or loss of the analyte response. There were no significant differences (<5 percent) in the analyte concentrations, impurity profiles or chromatographic parameters: the resolution and the peak symmetry. These data prove that the solutions can be considered to be steady at least in 72 h under the conditions of the tests, showing the importance and applicability of the invented RP-HPLC technique to perform routine analyses of the soil samples and sludge of the hospital effluent treatment plants.

Forced-degradation studies

Experiments on forced degradation were conducted to determine the stability of MTZ, MPC and CPL and to determine the potential presence of degradation products under various stress factors. All the three antibiotics were prepared in standard forms in optimized diluent (buffer and acetonitrile, 72:28) and subjected to acid hydrolysis (1 N HCl), base hydrolysis (1 N NaOH), oxidative degradation (30 per cent H_2O_2), thermal degradation (60 C in 24 h), and photolytic degradation (exposure to light for 48 h). After every stress treatment, neutralisation (acid/base) of the samples was carried out, then the samples were filtered and evaluated through the developed RP-HPLC technique. The interpretation of chromatograms involved the modification of peak area, retention time and the existence of other peaks that were degradation products. The extent of degradation was great in acidic and oxidative conditions, but thermal and photolytic stresses led to moderate changes. The strategy was able to overcome all degradation products of the parent compounds as a result of which its specificity, stability-indicating and environmental monitoring capacity of the pharmacokinetic plant of hospital effluents were confirmed.

Blue-applicability-grade index (BAGI)

The BAGI tool was used to implement the concept of green chemistry to optimise chromatography in the analysis of MTZ, MPC and CPL in sludge and soil in hospital effluent treatment plants. The BAGI test focused on the environmental benignity of solvents, reduction of chemical hazards, energy usage reduction and waste. In this regard, solvent choice and chromatographic conditions were optimised to maximise the sustainability of the method

without affecting accuracy, precision or sensitivity. The resulting technique follows the principles of Green Analytical Chemistry in the use of a water-acetonitrile mobile phase and avoids the use of toxic reagents. This environmentally conscious practice not only reduces the environmental impact of the analytical process but also delivers strength, dependability, and regulatory effectiveness, thereby creating sustainable analytical practices in both the pharmaceutical and environmental settings.

RESULTS AND DISCUSSION

The developed RP-HPLC method for the simultaneous estimation of metronidazole, mupirocin, and chloramphenicol showed satisfactory chromatographic performance with well-resolved, sharp, and symmetrical peaks under optimized conditions. In accordance with ICH guidelines, the method was successfully developed and validated to ensure reliability and regulatory compliance [53, 54]. Consistent retention times, low %RSD values, acceptable tailing factors, and high theoretical plate counts confirmed good system suitability, precision, and stability. Adequate resolution without matrix interference demonstrated effective separation in sludge and soil samples. Furthermore, purity angle values lower than purity thresholds confirmed peak homogeneity and absence of co-eluting impurities, establishing the selectivity and stability-indicating capability of the developed method.

System suitability evaluation

The retention times with trailing factors, plate count values, system appropriateness injecting standard solution and USP (United States Pharmacopoeia) values were reported in table 4. Replicate injections of standard solutions showed consistent retention times and peak areas with low %RSD values, indicating excellent repeatability. Theoretical plate counts were within acceptable limits, confirming good column efficiency, while tailing factors were below the recommended threshold, indicating peak symmetry [55].

Table 4: System suitability evaluation

S. No.	Metronidazole (MTZ)					Mupirocin (MPC)					Chloramphenicol (CPL)				
	Area observed (mAU)	Retention time (min)	Tailing factor	USP plate count	USP resolution	Area observed	Retention time (min)	Tailing factor	USP plate count	USP resolution	Area observed	Retention time (min)	Tailing Factor	USP plate count	USP resolution
1	28676	2.723	1.25	23455	-	36958	5.048	1.32	4257	11.36	33550	8.458	1.42	6052	5.93
2	28747	2.732	1.22	23547	-	37124	4.988	1.39	4589	11.42	34572	8.485	1.38	6078	5.96
3	28647	2.739	1.19	23457	-	36958	5.024	1.25	4358	11.28	33457	8.512	1.34	6056	5.85
4	28597	2.713	1.18	23547	-	37124	4.990	1.28	4458	11.65	34254	8.507	1.35	6125	5.75
5	28647	2.723	1.21	23517	-	37187	5.011	1.29	4512	11.25	35248	8.498	1.36	6098	5.81
6	29124	2.729	1.25	23478	-	37087	5.009	1.27	4589	11.36	36257	8.495	1.42	6457	5.83
Mean	28739.7	2.727	-	-	-	37073	5.012	-	-	-	6 78340	8.493	-	-	-
Stdev.	194.6	0.0089	-	-	-	95	0.0224	-	-	-	3059.35	0.0194	-	-	-
%RSD	0.01	0.0033	-	-	-	0.00	0.0045	-	-	-	0.45	0.0023	-	-	-

MTZ – Metronidazole, MPC – Mupirocin, CPL – Chloramphenicol, S. no. – Serial Number, mAU – milli-Absorbance Units, min – Minute, USP – United States Pharmacopeia, and RSD – Relative Standard Deviation. The results were reported as mean±SD (n =6)

Specificity

The interference was examined by separately analyzing the placebo, standard, and sample solutions in this test procedure. All the results were summarised in table 5 and represented in different chromatograms (fig. 1-fig. 6). Resolution between adjacent peaks was found to be adequate, confirming effective separation without interference from excipients, degradation products, or matrix components present in sludge and soil extracts [56].

Table 5: Specificity of different components

Components	Observation
Blank injection	No interference was detected.
Metronidazole API* solution	No interference was detected.
Mupirocin API solution	No interference was detected.
Chloramphenicol API solution	No interference was detected.
Mixed reference-Solution	No interference was detected.
Test Solution	No interference was detected.

*Active Pharmaceutical Ingredient.

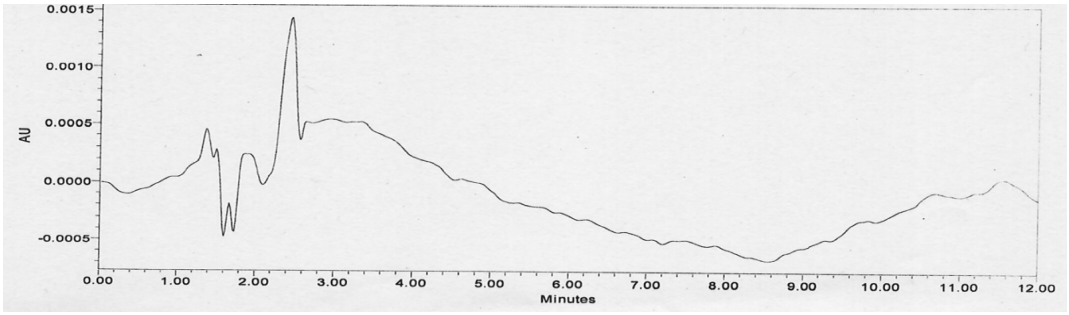

Fig. 1: Blank chromatogram

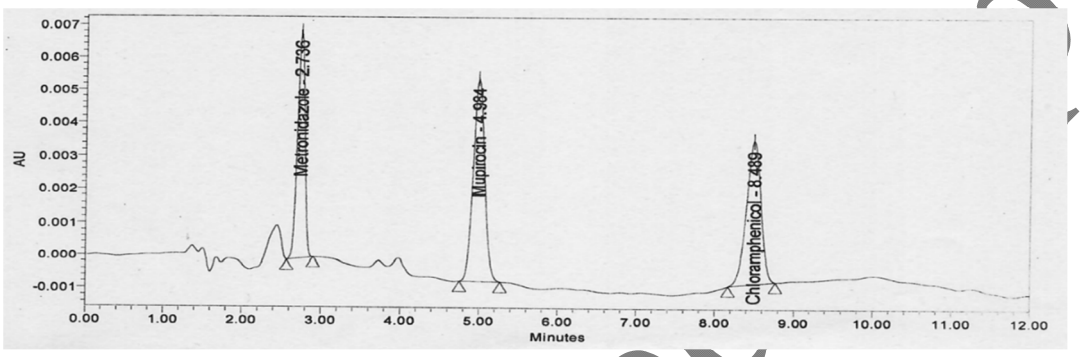

Fig. 2: Reference (mixed) chromatogram

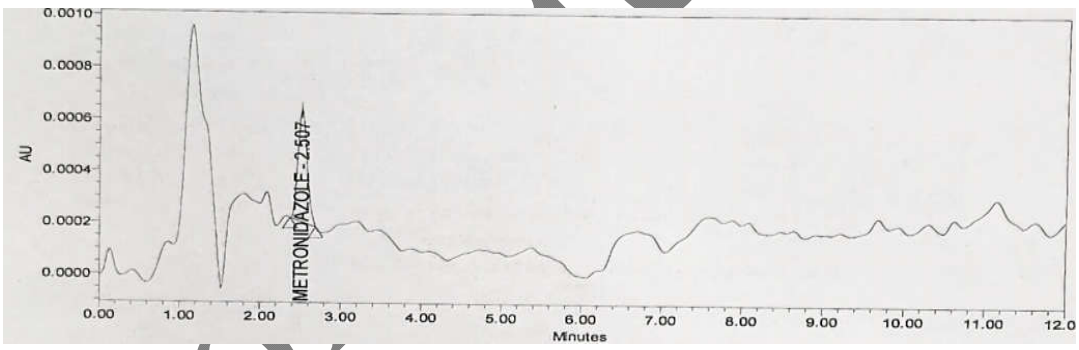

Fig. 3: Test chromatogram

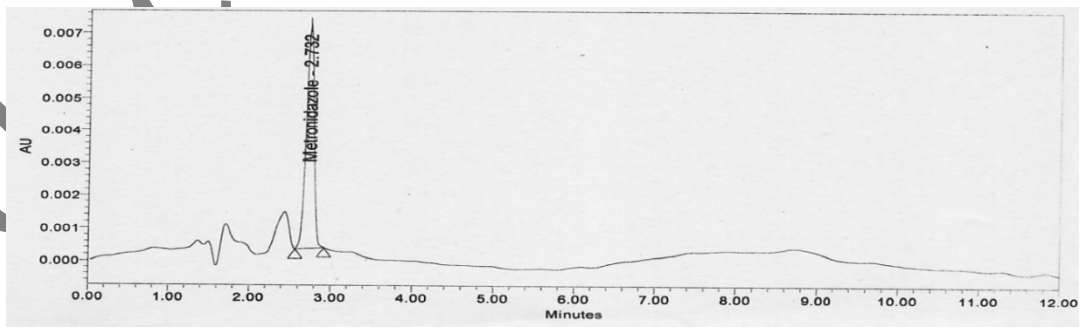

Fig. 4: Chromatogram of metronidazole solution

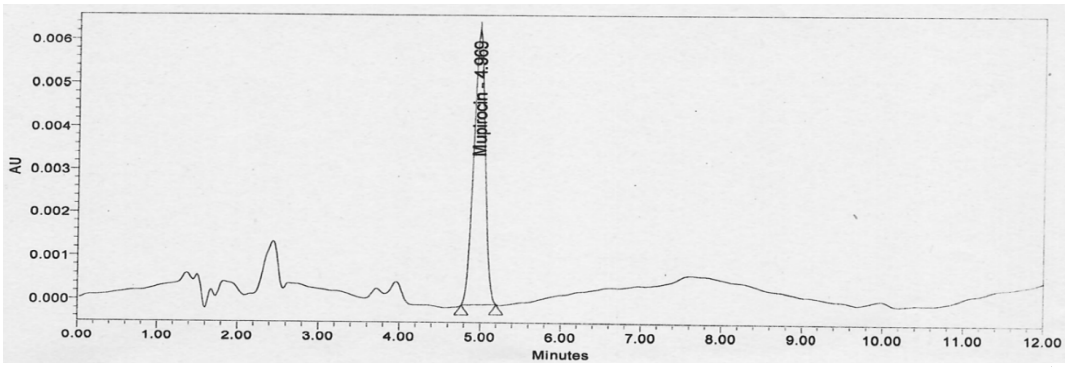

Fig. 5: Chromatogram of mupirocin solution

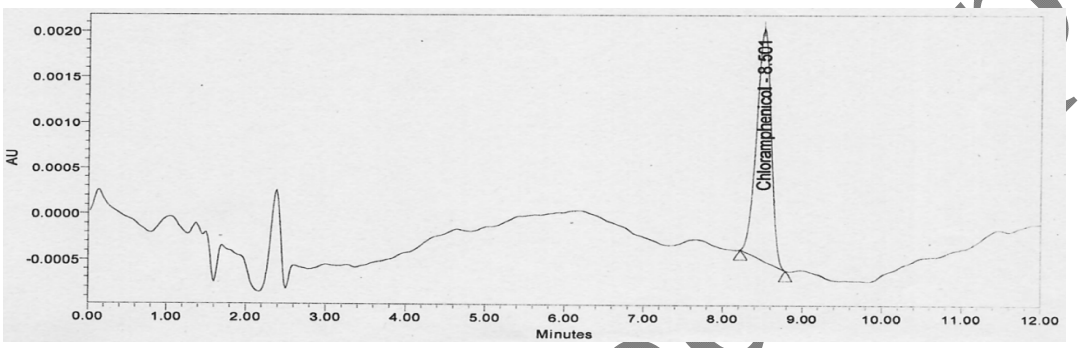

Fig. 6: Chromatogram of chloramphenicol solution

Peak-purity analysis

The results were assessed using photodiode array (PDA) detection by comparing the purity angle with the corresponding purity threshold values for each analyte peak (table 6 and fig. 7). For all three antibiotics, the purity angle was found to be lower than the respective purity threshold, confirming peak homogeneity and the absence of co-eluting impurities or matrix interferences [57].

Table 6: Peak-purity results observed for different antibiotics

Peak purity	MTZ[a]	MPC[b]	CPL[c]
Purity angle	0.877	1.018	0.743
Purity Threshold	0.911	7.023	4.196

[a]Metronidazole, [b]Mupirocin, [c]Chloramphenicol.

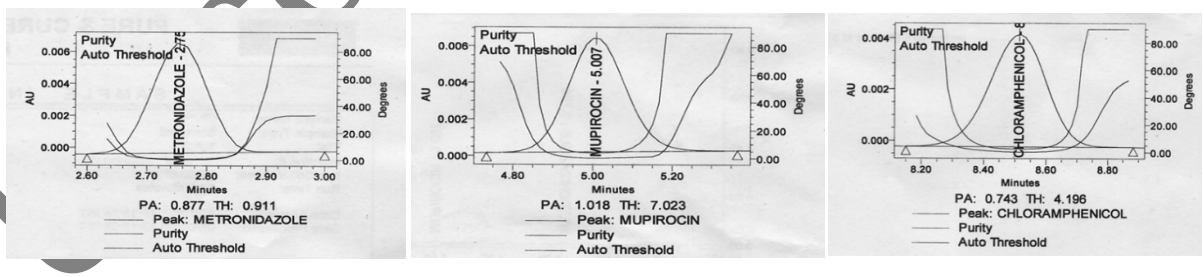

Fig. 7: Peak-purity chromatograms of different antibiotics

Linearity

Excellent linearity was demonstrated across the studied concentration range, with high regression coefficients (R^2>0.999) and minimal % bias at all levels (table 7-8 and fig. 8). These results confirm a strong proportional relationship between concentration and response, supporting accurate and reliable quantification of all analytes [55].

Table 7: Linearity data for metronidazole, mupirocin and chloramphenicol

Concentration % against STD	MTZ Area observed (mAU)	MTZ % bias	MPC area observed (mAU)	MPC % bias	CPL area observed (mAU)	CPL % bias
50	14 627	−0.05	18,623	0.07	16 577	−0.04
80	23 403	0.84	29,796	−0.01	26 523	0.06
100	29 254	−0.20	37,245	−0.03	33 154	−0.02
120	35 105	−0.88	44,694	−0.05	39 785	0.04
150	44 251	−0.09	55,925	0.04	49 087	−0.06

STD – Standard, MTZ – Metronidazole, MPC – Mupirocin, CPL – Chloramphenicol, mAU – milli-Absorbance Units, % – Percentage, and Bias (%)-[(Measured concentration – Nominal concentration)/Nominal concentration] ×100

Table 8: Regression statistics of metronidazole, mupirocin and chloramphenicol

Parameter	MTZ[a]	MPC[b]	CPL[c]
Slope	295.73	372.94	325.99
Intercept	−244.97	−37.54	426.23
Regression Coefficient (R^2)	0.99995	0.99996	0.99986

[a]Metronidazole, [b]Mupirocin, [c]Chloramphenicol

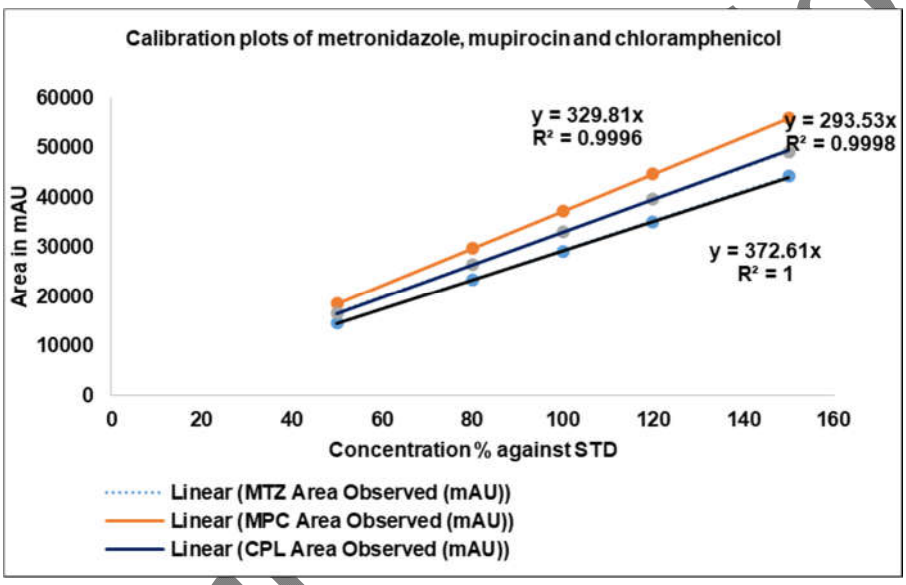

Fig. 8: Calibration plots of metronidazole, mupirocin and chloramphenicol

Method and system precision

Recoveries ranging from 98.7–101.2% with %RSD values below 2% demonstrate accurate quantification and good precision in complex soil and sludge matrices (table 9-11). These results indicate minimal matrix interference and confirm the applicability of the method for reliable analysis of antibiotics in environmental samples [58].

Table 9: Method precision (repeatability) in spiked sludge and soil samples

Matrix	API	Spiked level (µg/g, dry wt.)	Mean measured conc. (µg/g)	Recovery (%)	%RSD (n = 6)	Sample type
Soil	MTZ	10	9.87	98.7	1.25	Spiked
Soil	MPC	10	9.92	99.2	1.18	Spiked
Soil	CPL	10	10.05	100.5	1.32	Spiked
Sludge	MTZ	10	9.81	98.1	1.27	Spiked
Sludge	MPC	10	9.89	98.9	1.21	Spiked
Sludge	CPL	10	10.12	101.2	1.34	Spiked

API – Active Pharmaceutical Ingredient, µg/g – Microgram per g, RSD – Relative Standard Deviation. The results were reported as mean±SD (n =6).

Table 10: System-suitability obtained during method precision

RSD% of six replicate injections for the Mixed standard solution	MTZ[a]	MPC[b]	CPL[c]
	0.265	0.007	0.005
Tailing Factor observed	1.242	1.35	1.452

Theoretical Plates count	23457	4457	6247
Resolution obtained	-	11.45	5.94

[a]Metronidazole, [b]Mupirocin, [c]Chloramphenicol

Table 11: Peak purity results of different antibodies in the method precision

Peak purity	MTZ[a]	MPC[b]	CPL[c]
Purity angle	0.267	3.427	0.652
Purity threshold	0.857	4.062	2.624

[a]Metronidazole, [b]Mupirocin, [c]Chloramphenicol

Intermediate precision

Inter-day and analyst-to-analyst precision showed recoveries near 100% with %RSD values below 2%, confirming good method reproducibility (table 12-13). Comparable system suitability parameters during intermediate precision demonstrate instrument stability over time and between analysts, supporting the robustness and reliability of the developed RP-HPLC method [57].

Table 12: Intermediate precision (inter-day/analyst-to-analyst precision)

Matrix	API	Spiked level (µg/g, dry wt.)	Mean recovery (%)	%RSD (n = 12)	Day/Analyst variation	Sample type
Soil	MTZ	10	98.9	1.45	Day 1 vs Day 2	Spiked
Soil	MPC	10	99.1	1.39	Analyst A vs B	Spiked
Soil	CPL	10	100.3	1.52	Day 1 vs Day 2	Spiked
Sludge	MTZ	10	98.5	1.48	Analyst A vs B	Spiked
Sludge	MPC	10	99.0	1.41	Day 1 vs Day 2	Spiked
Sludge	CPL	10	101.0	1.56	Analyst A vs B	Spiked

API – Active Pharmaceutical Ingredient, µg/g – Microgram per g, RSD – Relative Standard Deviation, MTZ – Metronidazole, MPC – Mupirocin, and CPL – Chloramphenicol. The results were reported as mean±SD (n =6).

Table 13: System suitability found during the intermediate of the method precision

RSD% of six replicate injections for the mixed standard solution	MTZ[a]	MPC[b]	CPL[c]
	0.275	0.006	0.010
Tailing Factor observed	1.248	1.325	1.448
Theoretical Plates count	23478	4325	6357
Resolution obtained	-	11.35	5.457

[a]Metronidazole, [b]Mupirocin, [c]Chloramphenicol

LOQ and LOD

The low LOD and LOQ values obtained for all antibiotics indicate high method sensitivity, enabling reliable detection and quantification at trace levels (table 14). Signal-to-noise–based estimation confirms the suitability of the method for monitoring low-level residues in environmental matrices [59].

Table 14: LOD and LOQ results of different antibiotics

Different components	Concentration level (% of target concentration) S/N value	LOQ conc. in µg/ml	LOD level S/N value	LOD conc. in µg/ml
MTZ	10.14	3.73	3.32	1.24
MPC	9.89	4.98	3.69	1.67
CPL	11.02	13.25	4.12	4.53

MTZ – Metronidazole, MPC – Mupirocin, CPL – Chloramphenicol, S/N – Signal-to-Noise Ratio, LOQ – Limit of Quantitation, LOD – Limit of Detection, and µg/ml – Microgram per millilitre. LOD and LOQ were calculated using the signal-to-noise approach, where LOD corresponds to S/N ≥ 3:1 and LOQ corresponds to S/N ≥ 10:1.

Robustness

Minor deliberate variations in flow rate, column temperature, wavelength, mobile phase composition, and buffer pH produced %RSD values within acceptable limits (<2%) (table 15). This indicates that the chromatographic performance and quantitative response are not significantly affected by small method variations, confirming good method robustness [60].

Table 15: Robustness results found

Table showing robustness results of reference solution				
Parameters	**Variation**	**MTZ[a]**	**MPC[b]**	**CPL[c]**
Retention Time RSD (limit NMT 2%)				
Flow Rate	Standard Flow Rate (1.50 ml/min)	1.25	1.13	1.20
	less by 10% Flow Rate (1.20 ml/min)	1.33	1.25	0.98
	More by 10% Flow Rate (1.32 ml/min)	1.30	1.24	1.33
Retention Time RSD (limit NMT 2%)				
Column Temperature	Normal Column Temp. (25 C)	1.25	1.18	1.26
	Lower Column Temp. (20 C)	1.22	1.13	1.23
	Higher Column Temp. (30 C)	1.23	1.21	1.22
Peak area, %RSD (limit NMT 2%)				
Wavelength	Normal Wavelength (220 nm)	1.23	1.19	1.23
	less by 2 nm (218 nm)	1.23	1.15	1.19
	More by 2 nm (222 nm)	1.27	1.14	1.21
Peak area, %RSD (limit NMT 2%)				
Mobile Phase Ratio	more by 2%	1.15	1.19	1.24
(±2% Acetonitrile)	less by 2%	1.19	1.22	1.27
Peak area, %RSD (limit NMT 2%)				
pH of Buffer	6.1	1.25	1.29	1.26
(pH within ±0.2 units)	6.5	1.32	1.31	1.27

MTZ – Metronidazole, MPC – Mupirocin, CPL – Chloramphenicol, RSD – Relative Standard Deviation, NMT – Not More Than, ml/min – Millilitre per minute, °C – Degree Celsius, and nm – Nanometre.

Ruggedness

Low %RSD values (<1.5%) observed across analyst, instrument, and day-to-day variations demonstrate that the method performance remains consistent under normal laboratory changes (table 16). These findings confirm the ruggedness of the developed RP-HPLC method for routine analysis [60].

Table 16: Ruggedness results found.

Different types of comparison	Variation	MTZ[a]	MPC[b]	CPL[c]
Among Analyst difference	%RSD values	1.29	1.24	1.31
Within Instrument difference	%RSD values	1.19	1.21	1.33
On different days	%RSD values	1.22	1.27	1.25

[a]Metronidazole, [b]Mupirocin, [c]Chloramphenicol

Spiking of APIs into actual soil and sludge samples

Recovery and precision studies for soil and sludge samples spiked with MTZ, MPC, and CPL (10 µg/g) showed satisfactory percentage recoveries within acceptable limits, indicating good extraction efficiency and minimal matrix interference. The %RSD values were low for both matrices, demonstrating excellent repeatability and intermediate precision (table 17). These results confirm the accuracy, reliability, and suitability of the method for environmental analysis [61].

Table 17: Recovery and precision results for soil and sludge samples spiked with MTZ, MPC and CPL (10 µg/g)

Matrix	API	Spiked Level (µg/g)	Observed concentration (µg/g)	Recovery (%)	%RSD (n = 6)
Soil	Metronidazole (MTZ)	10	9.68±0.21	96.8	1.92
	Mupirocin (MPC)	10	9.82±0.17	98.2	1.73
	Chloramphenicol (CPL)	10	10.12±0.15	101.2	1.48
Sludge	Metronidazole (MTZ)	10	9.75±0.19	97.5	1.85
	Mupirocin (MPC)	10	9.88±0.14	98.8	1.42
	Chloramphenicol (CPL)	10	10.05±0.18	100.5	1.79

Note: API – Active Pharmaceutical Ingredient, MTZ – Metronidazole, MPC – Mupirocin, CPL – Chloramphenicol, µg/g – Microgram per g, and RSD – Relative Standard Deviation. The results were reported as mean±SD (n =6)

Solution-stability study

Assay values for all analytes (table 18) remained within acceptance limits (95–105%) under room temperature, refrigerated, and elevated temperature conditions for up to 72 h. These findings confirm adequate short-term solution stability, ensuring reliability of analytical results during routine sample preparation and analysis [62].

Table 18: All used solution stability study results

Different condition	Testing time Interval	Assay Limits	MTZ[a]	MPC[b]	CPL[c]
	Initial	95 to 105%	99.26	99.25	99.68

In room temperature	After 24h		98.69	99.32	100.33
	After 48h		99.23	97.98	99.67
	After 72h		98.67	99.68	100.25
In refrigerated (2–8 °C)	Initial	95 to 105%	99.67	100.47	99.67
	After 24h		99.38	100.19	100.14
	After 48h		99.62	99.67	100.29
	After 72h		99.67	100.67	100.36
In stress 40 °C	Initial	95 to 105%	99.48	99.14	100.36
	After 24h		100.67	100.27	100.23
	After 48h		99.87	100.62	100.37
	After 72h		100.95	100.38	100.67

[a]Metronidazole, [b]Mupirocin, [c]Chloramphenicol.

Forced-degradation studies

The analytes exhibited controlled degradation under acid, base, oxidative, thermal, and photolytic stress, while maintaining clear peak resolution and acceptable system suitability (table 19). These results confirm that the method effectively separates degradation products from the main peaks, demonstrating its stability-indicating capability [63].

Table 19: Forced degradation results of different solutions

Different stress condition evaluated	Different antibiotics	% Degradation observed	Peak resolution	System suitability observed (Tailing factor, theoretical plates etc.)	Remarks
Acid Hydrolysis degradation	MTZ	9.23	Observed with high clarity	System suitability acceptable	Medium degradation
	MPC	8.67	Observed with high clarity	System suitability acceptable	
	CPL	12.97	Observed with high clarity	System suitability acceptable	
Base Hydrolysis degradation	MTZ	14.58	Observed with high clarity	System suitability acceptable	High degradation observed
	MPC	19.68	Observed with high clarity	System suitability acceptable	
	CPL	20.65	Observed with high clarity	System suitability acceptable	
Oxidative Degradation	MTZ	14.67	Observed with high clarity	System suitability acceptable	High degradation observed
	MPC	15.37	Observed with high clarity	System suitability acceptable	
	CPL	16.37	Observed with high clarity	System suitability acceptable	
Thermal Stress degradation (60 °C)	MTZ	5.67	Observed with high clarity	System suitability acceptable	Noticeable degradation observed
	MPC	6.57	Observed with high clarity	System suitability acceptable	
	CPL	5.92	Observed with high clarity	System suitability acceptable	
Photolytic Degradation	MTZ	0.98	Observed with high clarity	System suitability acceptable	Less degradation observed
	MPC	1.02	Observed with high clarity	System suitability acceptable	
	CPL	1.05	Observed with high clarity	System suitability acceptable	

MTZ – Metronidazole, MPC – Mupirocin, CPL – Chloramphenicol, °C – Degree Celsius, and % – Percentage. "System suitability acceptable" indicates that tailing factor, theoretical plates, and resolution met predefined acceptance criteria despite degradation, consistent with stability-indicating method requirements

Blue-applicability-grade index (BAGI)

The favourable BAGI outcomes indicate efficient solvent usage, minimal waste generation, low energy consumption, and high analytical throughput, demonstrating that the developed RP-HPLC method effectively balances analytical performance with environmental responsibility and is suitable for sustainable routine analysis (table 20). Although AGREE and GAPI tools were not formally applied, the BAGI assessment sufficiently captured solvent toxicity, waste volume, energy demand, and analytical efficiency, making it appropriate for this study [64].

Table 20: Greenness assessment of the developed RP-HPLC method using the blue applicability grade index

BAGI criterion	Quantitative parameter considered	Measured/estimated value	Justification for the developed method	Assessment outcome
Solvent Selection	Type and volume of organic solvent per run	~3.4 ml acetonitrile per analysis (12 min run, 1.5 ml/min, 28% ACN)	Acetonitrile–water system avoids chlorinated solvents; organic solvent usage is significantly lower than conventional gradient HPLC methods	Favourable
Sample Preparation	Number of steps and reagent hazard	Methanol (15 ml)+SPE cleanup; no derivatization	Simple extraction and SPE cleanup without toxic reagents or chemical modification	Favourable
Energy Consumption	Instrument power × runtime	~0.05–0.08 kWh per run	Short isocratic runtime (~12 min) with UV detection minimizes electricity demand	Favourable
Waste Generation	Total liquid waste per analysis	<12 ml aqueous–organic waste	Waste volume is low and composed of biodegradable aqueous–organic mixtures	Favourable
Chemical Hazard	Toxicity class of reagents	Low–moderate (ACN, phosphate buffer)	No carcinogenic, mutagenic, or highly toxic reagents used	Favourable
Analytical Efficiency	Number of analytes per run	3 antibiotics simultaneously	Single-run multi-analyte determination reduces solvent, energy, and analysis time	Highly favourable

Occupational Safety	Analyst exposure risk	Minimal (routine solvents, closed system)	Limited solvent handling and standard laboratory safety controls	Favourable
Method Sustainability (Overall)	Combined greenness score (qualitative BAGI scale)	High compliance	Balanced solvent usage, low energy demand, minimal waste, and high throughput meet BAGI sustainability expectations	High compliance

ACN – Acetonitrile, kWh – Kilowatt-hour, UV – Ultraviolet, SPE – Solid Phase Extraction, and BAGI – Analytical Greenness (Green Analytical Procedure Index) Criterion.

Analysis of unspiked hospital ETP sludge and adjacent soil samples confirmed the applicability of the developed RP-HPLC method to real environmental matrices. Chromatographic peaks corresponding to metronidazole, mupirocin, and chloramphenicol were observed at their respective retention times, as illustrated by the representative chromatogram in fig. 3. However, the observed peak responses were below the validated LOQ and were therefore reported as detected (<LOQ) in table 21 in accordance with ICH Q2 (R1) guidelines. The presence of detectable but non-quantifiable residues is consistent with extensive dilution, degradation, and adsorption processes occurring during wastewater treatment and environmental transport. Importantly, successful recovery at low spiking levels demonstrates that the method possesses adequate sensitivity and selectivity for environmental screening. Thus, the combined qualitative detection in real samples and quantitative validation through recovery studies confirms the suitability of the developed method for routine monitoring of trace-level antibiotic residues in environmental samples.

Table 21: Detection status of antibiotics in unspiked hospital ETP sludge and soil samples

Sampling site	Sample matrix	Metronidazole (MTZ)	Mupirocin (MPC)	Chloramphenicol (CPL)
Hospital A	ETP Sludge	Detected (<LOQ)	ND	Detected (<LOQ)
Hospital B	ETP Sludge	Detected (<LOQ)	Detected (<LOQ)	Detected (<LOQ)
Hospital C	ETP Sludge	Detected (<LOQ)	Detected (<LOQ)	Detected (<LOQ)
Hospital A	Adjacent Soil	Detected (<LOQ)	ND	Detected (<LOQ)
Hospital B	Adjacent Soil	Detected (<LOQ)	Detected (<LOQ)	Detected (<LOQ)
Hospital C	Adjacent Soil	Detected (<LOQ)	Detected (<LOQ)	Detected (<LOQ)

MTZ – Metronidazole, MPC – Mupirocin, CPL – Chloramphenicol, ETP – Effluent Treatment Plant, LOQ – Limit of Quantitation, and ND – Not Detected.<LOQ-less than limit of qualification; ND: less than limit of detection; Hospital A, B and C are three different hospitals of Dehradun district of Uttarakhand

CONCLUSION

The developed RP-HPLC method for the simultaneous determination of metronidazole, mupirocin, and chloramphenicol in hospital ETP sludge and soil proved to be accurate, precise, robust, and environmentally sustainable. The application of the BAGI ensured adherence to green analytical chemistry principles by minimizing solvent and energy use, reducing waste generation, and avoiding hazardous reagents. Validation parameters, including system suitability, linearity, precision, robustness, ruggedness, and stability, confirmed the method's reliability and reproducibility. Forced degradation studies demonstrated its stability-indicating nature, while the use of eco-friendly solvents contributed to a lower environmental footprint. Overall, the method provides a cost-effective, efficient, and sustainable analytical approach for monitoring pharmaceutical residues in environmental samples. It supports regulatory compliance and promotes green laboratory practices, making it an ideal tool for sustainable pharmaceutical quality control and environmental impact assessment.

LIST OF ABBREVIATIONS

API: Active-Pharmaceutical-Ingredient, HPLC: High-Performance-Liquid-Chromatography, ICH: International-Council-For-Harmonisation, BAGI: Blue Applicability Gade Index, GAC: Green Analytical Chemistry, SPE: Solid-Phase Extraction, MTZ: Metronidazole, MPC: Mupirocin, CPL: Chloramphenicol, LC: Liquid-Chromatography, RSD: Relative-Standard-Deviation, AMR: Antimicrobial Resistance, WHO: World Health Organization, LOQ: Limit-Of-Quantification, LOD: Limit-Of-Detection, UNEP: United National Environmental Programme, UNDP: United National Development Programme, NMT: Not More Than, NLT: Not Less Than, S/N: Signal To Noise Ratio, ETP's: Effluent Treatment Plants, UV: Ultra Violet, RP-HPLC: Reversed-phase High-performance Liquid Chromatography, u/l: Microgram per litre, n/l: Nanogram per litre, HETPs: Hospital Effluent Treatment Plants, LLE: Liquid-Liquid Extraction, LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry, UV/PDA: Ultraviolet/Photodiode Array, nm: Nanometre, PDA: Photodiode Array, 1N HCL: One Normality of Hydrochloric acid, ^{1}N NAOH: One Normality of Sodium Hydroxide, R^2: Correlation coefficients, AGREE: Analytical Greenness, GAPI: Green Analytical Procedure Index

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AUTHORS CONTRIBUTIONS

Omprakash Uniyal conducted the research, performed the experiments, and analysed the data. Sanjeev Kumar Sahu and Manoj Kumar supervised the study, provided guidance in experimental design, and contributed to the critical review and interpretation of results. All authors read, reviewed, and approved the final version of the manuscript.

CONFLICT OF INTERESTS

There is no conflict of interest to be declared

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