Revolutionizing Topical Drug Evaluation: A Discriminative and Validated In-Vitro Release Testing Paradigm for Mupirocin Cream 2%

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Abstract:
The development of a robust and discriminative in-vitro release testing (IVRT) method for Mupirocin Cream 2% w/w was undertaken to support regulatory expectations for Q1/Q2 sameness and Q3 similarity in topical semisolid formulations. The method was designed using a vertical Franz diffusion cell system with a synthetic nylon membrane and optimized for sensitivity, specificity, and reproducibility. A comprehensive screening of receptor media identified USP buffer (pH 6.8): ethanol (70:30 v/v) as the most suitable medium, offering high solubility (675.88 µg/mL) and stability for Mupirocin. Membrane binding studies confirmed minimal drug retention, and pretreatment was deemed unnecessary. The method was validated using HPLC-UV detection at 222 nm, with a linearity range of 2.70–205.24 µg/mL ($R^2 \geq 0.999$). Accuracy ranged from 95.56% to 101.48%, and precision (%RSD) was consistently below 3%. The method demonstrated excellent specificity, with no interference from placebo or blank matrices. Discriminatory power was established by evaluating release profiles of 50%, 100%, and 150% strength formulations. Confidence interval analysis confirmed equivalence for Test-02 and Test-03 with the reference product, while Test-01 was non-equivalent. Mass balance studies showed recovery between 91.1% and 99.4%, with drug depletion ranging from 11.5% to 13.3%. This validated IVRT method is suitable for routine quality control, formulation development, and regulatory submissions. It provides a sensitive and reliable tool for assessing the release characteristics of Mupirocin from semisolid matrices and supports the demonstration of bioequivalence in generic product development.

Keywords: Mupirocin; In-vitro release testing; Franz diffusion cell; HPLC; Topical semisolid

1. Introduction:

Skin and soft-tissue infections (SSTIs) are among the most prevalent infectious illnesses globally. They make life hard for people, businesses, and society as a whole [1–3]. When dangerous bacteria or viruses enter into the skin and create inflammation in one location or all over the body, this is called an infection. Impetigo, cellulitis, folliculitis, dermatophytosis, candidiasis, herpes simplex, and varicella-zoster are some of the most common diseases [4–7]. SSTIs can be caused by bacteria, fungi, or viruses. Global epidemiological research indicate that this burden is substantial. The Global Burden of Disease (GBD) Study [8,9] found that skin and subcutaneous disorders were among the most common non-fatal illnesses in the globe in 2019, with more than 4.8 billion new cases reported. In low- and middle-income countries, fungal and bacterial infections were the primary causes of these cases [10]. If SSTIs aren't treated or managed properly, they can cause serious complications like cellulitis, necrotizing fasciitis, bacteremia, and sepsis. This is especially true for older adults and people with weak immune systems [11–13]. Recurrent and chronic dermatological disorders are strongly linked to social stigma, a lower quality of life, and psychological anguish. This makes them even more harmful to public health [14–16]. Topical antimicrobial therapy remains the most effective treatment for mild to severe localized bacterial skin infections, as it minimizes systemic exposure while providing high concentrations of drug directly to the infection site [17–19]. Mupirocin is still the best topical antibiotic for treating superficial bacterial infections like impetigo and secondary infected traumatic wounds because it works so well against Staphylococcus aureus and Streptococcus pyogenes [20–22]. When infections are really serious or hard to treat, systemic medications such β-lactams, cephalosporins, and macrolides are usually the last option [23,24]. Mupirocin is an antibiotic that works differently than other antibiotics. It stops bacteria from producing proteins by blocking their isoleucyl-tRNA synthetase enzyme. This does not confer resistance to other critical antibiotics in clinical settings [25–27]. Most of the time, it is used on the skin as a 2% w/w cream or ointment two or three times a day for up to 10 days [28]. Mupirocin's chemical formula ($C_{26}H_{44}O_9$; molecular weight = 500.63 g/mol) has a lot of ester and hydroxyl groups. These groups alter how well it dissolves, how stable it is, and how it comes out of semisolid matrices [29,30]. Mupirocin treatment is beneficial in clinical settings; however it possesses certain limitations. Long-term treatment may make patients more likely to experience fungal overgrowth again [31–33]. There have also been reports of localized side effects as burning, irritation, and itching. The increasing prevalence of both low- and high-level mupirocin resistance, particularly in methicillin-resistant Staphylococcus aureus (MRSA) strains, complicates therapy and management further [34–37]. To stop these difficulties and make sure that products work the same way every time, you need good formulation strategies and strong analytical procedures that can determine the difference. It is still hard to prove from a regulatory point of view that generic topical semisolid pharmaceuticals are the same as other medicines. Standard physicochemical characterization does not consistently demonstrate Q1 (qualitative), Q2 (quantitative), and Q3 (microstructural and performance) similarities, all of which are mandated by regulatory authorities [38–41]. Clinical endpoint bioequivalence studies are the best way to do it, but they can be very costly, time-consuming, and energy-draining, and they can also pose huge ethical questions and not be sensitive enough to identify changes in formulations [42–44]. The biorelevance of in vitro permeation testing (IVPT) using excised skin is enhanced; nevertheless, this approach is limited by tissue scarcity, operational complexity, and considerable variability [45–47]. Regulators support In-Vitro Release Testing (IVRT) as a good way to find out how well drugs come out of topical semisolid formulations [48–50]. IVRT is particularly sensitive to changes in the way things are made and the way they are put together. It allows you to measure how quickly drugs are released in a controlled and repeatable method, especially when you use Franz diffusion cell devices [51–53]. Quality control, making new drugs, making changes after approval, and submitting abbreviated new drug applications (ANDAs) all officially recognize IVRT under USP <1724>, FDA, and EMA guidelines [54–58].In order to create a reliable IVRT method, the choice of membranes, the makeup of receptor media, the properties of diffusion cells, the sampling strategy, and the analytical quantification must all be tuned in a systematic way [59–62]. Method validation prove invalidated method should be more specific, linear, accurate, precise, sensitive, robust, and able to identify the difference between different things, in conformity with current regulatory standards which lead to patient safety and USP <1724>, ICH Q2(R2), and USP <1724> [63-67]. In this study, we use Franz diffusion cells and HPLC-UV analysis to create and test an IVRT method that can distinguish the difference between Mupirocin Cream 2% w/w. The approach was made to make a good sink environment, stop membrane binding without pretreatment, and always distinct formulations with strengths of 50%, 100%, and 150%. The validation tests suggest that the method works well for monitoring the quality of medicines on a regular basis, enhancing formulations, and making sure that topical semisolid medicines are the same as those that are already on the market [68–75].

2. Materials and Methods:

2.1 Chemicals and Reagents

All reagents used were of analytical grade. Mupirocin working standard was obtained from Raptim Research Ltd. Solvents and chemicals included acetonitrile, ethanol, sodium acetate, acetic acid, sodium hydroxide, dipotassium phosphate, and potassium dihydrogen phosphate. Nylon membrane filters (0.45 µm, 25 mm) were used as the diffusion barrier. The receptor medium was prepared using USP buffer (pH 6.8) and ethanol in a 70:30 v/v ratio.

2.2 Instrumentation

Chromatographic analysis was performed using an Agilent HPLC system equipped with a UV detector. The Franz diffusion cell system (Logan FDC manual) was used for IVRT, with a cell volume of 12 mL and an orifice area of 1.77 cm^2. Additional equipment included an autosampler, vortex mixer, sonicator, and analytical balance.

2.3 Chromatographic Conditions

The quantification of Mupirocin in IVRT samples was performed using a validated high-performance liquid chromatography (HPLC) method with UV detection. The method was optimized to ensure high sensitivity, specificity, and reproducibility, in line with ICH Q2(R1) guidelines and USP <621>.

2.5 Column Selection: A Phenomenex Gemini C18 column (150 × 4.6 mm, 5 µm) was selected for its excellent retention and peak shape for moderately polar compounds like Mupirocin. The C18 stationary phase provides adequate hydrophobic interaction, ensuring good resolution and reproducibility.

2.6 Mobile Phase Composition: The mobile phase consisted of 25 mM potassium dihydrogen phosphate buffer (pH 6.8) and acetonitrile in a 75:25 v/v ratio. This composition was optimized to balance retention time (~8.0 minutes), peak symmetry, and baseline stability. The pH was chosen to maintain Mupirocin in its unionized form, enhancing chromatographic performance.

2.7 Detection Wavelength: UV detection at 222 nm was selected based on the maximum absorbance (λmax) of Mupirocin, ensuring optimal sensitivity and minimal interference from excipients or receptor media components.

2.8 System Parameters:

Flow rate: 1.0 mL/min, Injection volume: 10 µL, Column oven temperature: 30°C, Autosampler temperature: 10°C, Run time: 12 minutes. These conditions provided a robust and reproducible method with a linear response over the range of 2.70–205.24 µg/mL, suitable for quantifying Mupirocin in IVRT samples across all time points.

2.8.1 IVRT Method Development

The IVRT method was developed using a vertical Franz diffusion cell system, following the principles outlined in USP <1724> and FDA product-specific guidance for semisolid topical dosage forms. The goal was to establish a method that is not only reproducible but also capable of discriminating between formulations with different release characteristics.

2.8.2 Receptor Medium Selection: Several receptor media were screened for solubility and stability of Mupirocin. The final selection—USP buffer (pH 6.8): ethanol (70:30 v/v)—was based on High solubility (675.88 µg/mL), Chemical stability over 8 hours, Compatibility with membrane and analytical method, Minimal foaming or precipitation

2.8.3 Membrane Selection and Binding Studies:

Nylon membranes (0.45 µm, 25 mm) were chosen for their low binding affinity and high permeability. Binding studies at LLOQ and ULOQ levels showed >99% recovery, confirming negligible drug retention. Pretreatment was found unnecessary, simplifying the procedure and reducing variability.

2.8.4 Dose Application and Sampling:

A dose of ~300 mg was applied to each cell, ensuring uniform coverage of the membrane surface (1.77 cm^2). Sampling was performed at 0.5, 1, 2, 3, 4, 6, and 8 hours, capturing both the initial burst and steady-state release phases. Receptor fluid was partially replaced after each sampling to maintain sink conditions.

2.8.5 Temperature and Agitation:

The receptor compartment was maintained at 32 ± 1°C to simulate skin surface temperature.

Stirring at 500 rpm ensured uniform mixing and minimized boundary layer effects.

2.8.6 Discriminatory Power

The method was evaluated using 50%, 100%, and 150% strength formulations. Regression analysis and confidence interval comparisons confirmed the method's ability to detect formulation differences, fulfilling the criteria for sensitivity and selectivity.

2.8.7 Sample Preparation

Sample preparation is a critical step in the in-vitro release testing (IVRT) workflow, as it directly influences the accuracy, precision, and reproducibility of the analytical results. In this study, a standardized and validated procedure was established to ensure consistent handling of samples collected from the Franz diffusion cell system. At each predefined time point (0.5, 1, 2, 3, 4, 6, and 8 hours), a 100 µL aliquot of the receptor medium was withdrawn from each diffusion cell. This volume was selected to maintain sink conditions while ensuring sufficient analyte concentration for quantification. Each sample was transferred into a pre-labeled polypropylene (PP) tube. To ensure matrix compatibility and maintain the integrity of the analyte, 400 µL of the mobile phase (25 mM potassium dihydrogen phosphate buffer, pH 6.8: acetonitrile, 75:25 v/v) was added to the sample. This 1:5 dilution ratio was optimized to bring the sample concentration within the validated linear range of the HPLC method. The mixture was vortexed for approximately 30 seconds to ensure complete homogenization and dissolution of the analyte. The prepared samples were then transferred to autosampler vials and stored at 10°C in the autosampler tray until analysis. Calibration standards and quality control (QC) samples were prepared using Mupirocin stock solutions diluted in the same receptor medium used during IVRT. This matrix-matching approach minimizes potential matrix effects and ensures analytical consistency. Calibration Standards: A series of eight non-zero standards were prepared to span the full analytical range. These were used to construct the calibration curve for quantification. Quality Control Samples: Four QC levels were prepared—LLOQ, LQC, MQC, and HQC—to assess method performance across the concentration range. All standards and

QC samples were prepared fresh or stored under refrigerated conditions (2–8°C) and protected from light to prevent degradation. Matrix Compatibility: Using the receptor medium for dilution ensures that the sample matrix closely resembles the calibration matrix, reducing variability and enhancing method robustness. Light Sensitivity: Mupirocin is known to be light-sensitive; therefore, all sample handling was performed under sodium vapor lamps to minimize photodegradation. Temperature Control: Autosampler storage at 10°C helps maintain sample stability during the analytical run, especially for extended sequences. This sample preparation protocol was designed to align with regulatory expectations and best practices for IVRT studies, ensuring that the analytical results accurately reflect the release characteristics of the topical formulation.

3. Results and Discussions:

3.1 Formulation Screening

Three test formulations (Test-01, Test-02, and Test-03) were evaluated against a reference formulation using Franz diffusion cells. The cumulative drug release profiles were analyzed, and slopes were calculated for each cell. Test-01 showed a mean slope of 78.98 µg/cm²/hr^0.5 with a %RSD of 7.63%, and a 90% confidence interval (CI) of 63.16–77.55%, indicating non-equivalence. In contrast, Test-02 and Test-03 had mean slopes of 115.48 and 129.39 µg/cm²/hr^0.5, respectively, with %RSD values of 5.09% and 5.71%. Their 90% CIs (104.47–120.22% for Test-02 and 116.34–131.31% for Test-03) confirmed equivalence with the reference. Based on these results, Test-02 was selected for further method development due to its optimal performance and tighter confidence interval.

3.2 Analytical Method Development

3.2.1 Specificity

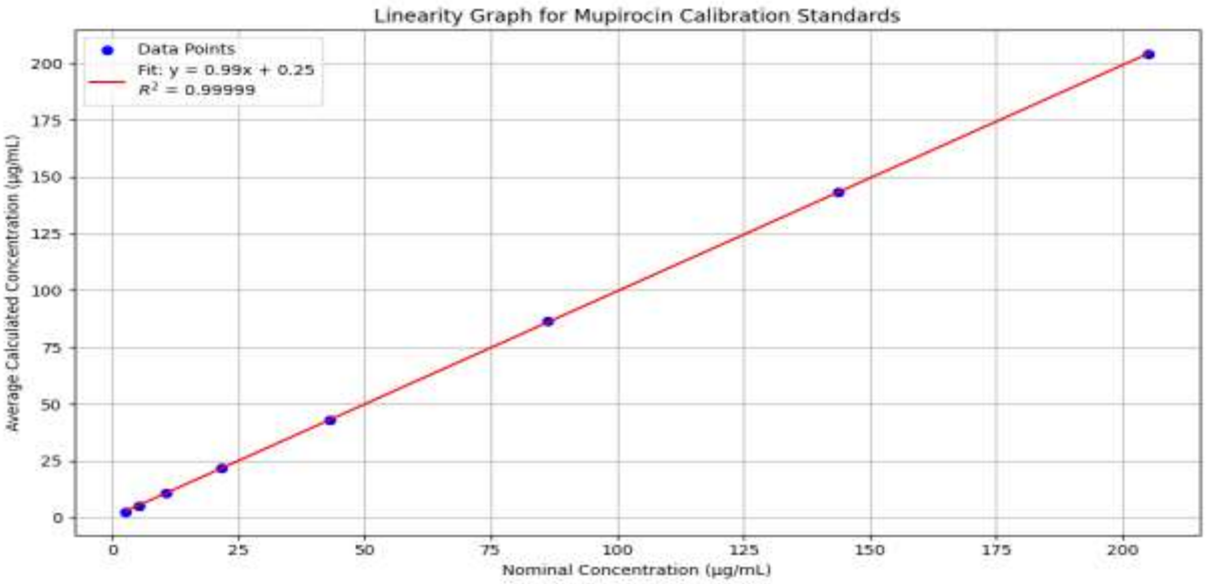
No interference was observed at the retention time of Mupirocin in placebo or blank samples. The % interference was 0.00%, confirming the method's specificity.

3.2.2 Linearity

Linearity was established over a range of 2.70–205.24 µg/mL. The correlation coefficient (R^2) was ≥ 0.9999 across all calibration batches. %RSD values for all calibration standards were below 1%, with the lowest at 0.21% and the highest at 1.00%.

3.2.3 Linearity results:

Calibration Standard ID	Nominal Concentration (µg/mL)	Average Calculated Concentration (µg/mL)
CS 1	2.70	2.65
CS 2	5.39	5.36
CS 3	10.78	11.02
CS 4	21.55	21.82
CS 5	43.10	43.18
CS 6	86.20	86.43
CS 7	143.67	143.18
CS 8	205.24	204.11



3.2.4 Calibration and QC Samples

All calibration standards and QC samples met acceptance criteria. For example, CS-1 (2.70 µg/mL) had an average measured concentration of 2.62 µg/mL with a %RSD of 2.89%. HQC (153.93 µg/mL) had an average of 151.87 µg/mL with a %RSD of 0.48%. These results confirm the method's accuracy and precision.

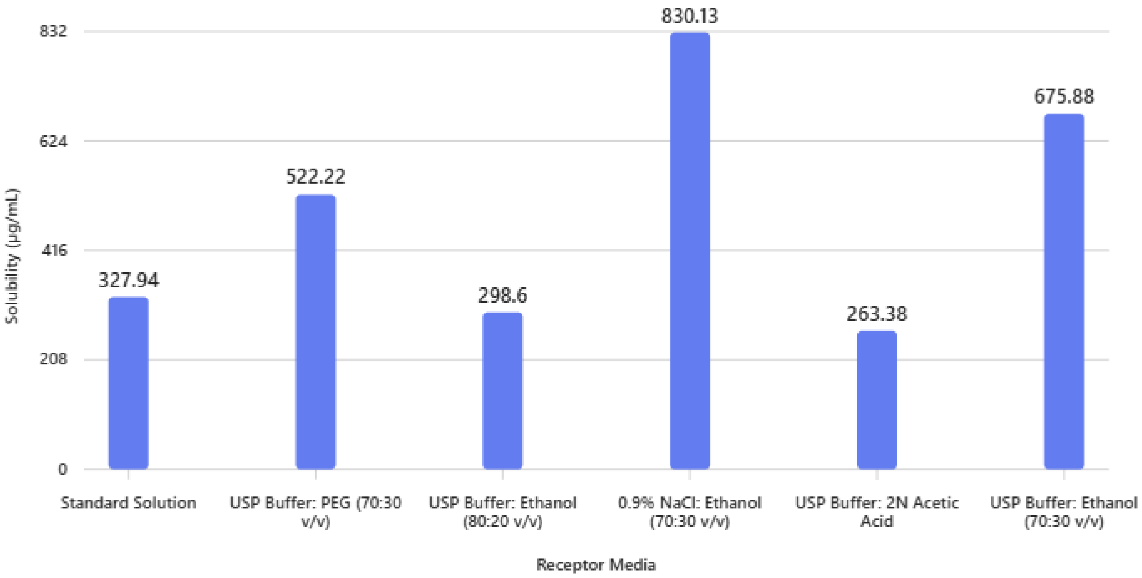
3.3 IVRT Method Development

3.3.1 Solubility Studies

During IVRT method development, solubility studies play a critical role in selecting an appropriate receptor medium that ensures sink conditions and drug stability throughout the test. Among various media evaluated, the highest solubility of mupirocin was observed in 0.9% NaCl: Ethanol (70:30 v/v) at approximately 830.13 µg/mL, followed by USP Buffer: Ethanol (70:30 v/v) at 675.88 µg/mL. The latter was chosen for further testing due to its superior performance in screening experiments and better compatibility with analytical procedures. Selection of receptor medium must consider drug solubility, chemical stability, and absence of precipitation, as recommended by USP <1724> and FDA guidance documents (Miranda et al., 2020; Szoleczky et al., 2024; Grozdanoska et al., 2023; Klein et al., 2018; Frontage Labs, 2020) [33–37].

3.3.2 Solubility study results

Receptor Media	Solubility (µg/mL)
Standard Solution	327.94
USP Buffer: PEG (70:30 v/v)	522.22
USP Buffer: Ethanol (80:20 v/v)	298.60
0.9% NaCl: Ethanol (70:30 v/v)	830.13
USP Buffer: 2N Acetic Acid	263.38
USP Buffer: Ethanol (70:30 v/v)	675.88



3.3.3 Membrane Binding and Pretreatment

Membrane binding studies showed recoveries above 90% at both LLOQ and ULOQ levels, with or without pretreatment. For example, at ULOQ, recovery was 93.95% with pretreatment and 105.14% without. Hence, untreated Nylon 0.45 µm membranes were used.

3.3.4 Selectivity, Specificity & Sensitivity

Slopes for 50%, 100%, and 150% test concentrations were 89.14, 150.13, and 230.43 µg/cm²/hr^0.5, respectively. The method demonstrated clear discrimination between concentrations. Regression values were ≥ 0.997, and %RSD values were within 15%, confirming the method's robustness.

3.3.5 Time Point Selection and Mass Balance

Drug release was linear up to 8 hours, with steady-state observed after 10 hours. Sampling time points were finalized at 0.5, 1, 2, 3, 4, 6, and 8 hours. Drug release ranged from 11.48% to 13.33%, and retained drug ranged from 79.40% to 86.59%. Mass balance was within 91.09% to 99.35%, meeting the acceptance range of 70–130%.

3.3.6 Final Optimized Parameters

The final chromatographic conditions included a Phenomenex Gemini C18 column, mobile phase of 25 mM KH_2PO_4 (pH 6.8): Acetonitrile (75:25 v/v), flow rate of 1.0 mL/min, and UV detection at 222 nm. The retention time was ~8.00 minutes. Diffusion parameters included a receptor medium of USP Buffer: Ethanol (70:30 v/v), temperature of 32 ± 1°C, dose of ~300 mg, and sampling up to 8 hours.

4. Conclusion:

The development of an in vitro release testing (IVRT) method for Mupirocin Cream 2% w/w was successfully accomplished through a systematic evaluation of formulation performance, analytical robustness, and diffusion characteristics. The screening phase demonstrated that among the three test formulations, Test-02 and Test-03 were statistically equivalent to the reference formulation, with 90% confidence intervals falling within the acceptable bioequivalence range of 75%–133.33%. Test-02 was selected for further method development due to its tighter confidence interval and consistent release profile. The analytical method was rigorously validated for specificity, linearity, accuracy, and precision. Specificity studies confirmed no interference at the retention time of Mupirocin (8.68 minutes), while linearity was established across a wide concentration range (2.70–205.24 µg/mL) with correlation coefficients consistently ≥ 0.999. Quality control samples at LLOQ, LQC, MQC, and HQC levels showed %RSD values well below 2%, and mean recoveries within 95–101%, confirming the method's reliability. Solubility studies identified USP Buffer: Ethanol (70:30 v/v) as the optimal receptor medium, providing high solubility and consistent release kinetics. Membrane binding experiments demonstrated that Nylon 0.45 µm membranes did not significantly retain Mupirocin, and pretreatment was deemed unnecessary. Selectivity and sensitivity assessments showed that the method could clearly discriminate between 50%, 100%, and 150% concentrations, with regression values ≥ 0.99 and %RSD < 15%, validating the method's robustness and discriminatory power. Time point selection experiments revealed linear drug release up to 8 hours, with a plateau observed beyond 10 hours. Sampling intervals were optimized accordingly. Mass balance studies confirmed that drug recovery ranged from 91.09% to 99.35%, and drug release ranged from 11.48% to 13.33%, with retained drug levels between 79.40% and 86.59%, all within acceptable limits. The final optimized method employed a Phenomenex Gemini C18 column with an isocratic mobile phase of 25 mM KH_2PO_4 (pH 6.8): Acetonitrile (75:25 v/v), a flow rate of 1.0 mL/min, and UV detection at 222 nm. The method was precise, reproducible, and capable of detecting formulation differences, making it suitable for routine quality control, formulation development, and regulatory bioequivalence assessments. In summary, the IVRT method developed for Mupirocin Cream 2% w/w is scientifically sound, regulatory-compliant, and well-suited for its intended applications.

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