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Development and Validation of a Franz Diffusion Cell *In Vitro* Release Test Method for a Glycosaminoglycan-Derived API Gel Formulation

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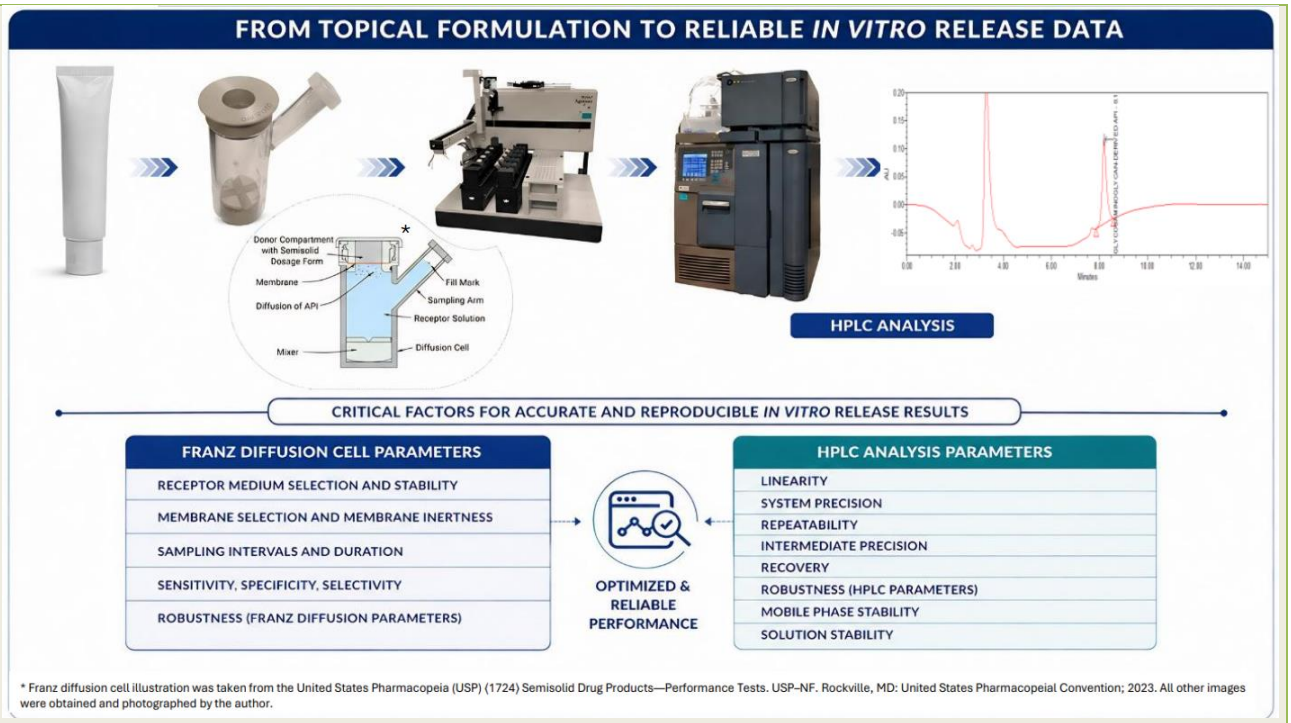
Abstract | **Original Research Article**



Graphical Abstract

This study aimed to develop and validate an in vitro release test (IVRT) method for a gel formulation containing a glycosaminoglycan-derived active pharmaceutical ingredient (API) using Franz diffusion cells, in accordance with ICH Q2(R2), the FDA guidance "In Vitro Release Test Studies for Topical Drug Products Submitted in ANDAs" and USP <1724>. Two receptor media, pH 7.4 phosphate buffer and pH 7.4 phosphate buffer containing 20% ethanol, and two membrane types, 0.45 µm cellulose acetate (CA) and a 300 kDa dialysis membrane, were comparatively evaluated for their impact on API release. Quantitative analysis of the released API was performed by HPLC using a USP L81 column and an ionic mobile phase, with UV/PDA detection. Optimal IVRT conditions for both test and reference products were established at 400 rpm using the 300 kDa dialysis membrane in pH 7.4 phosphate buffer, with sampling at 0.5, 1, 2, 3, 4 and 5 hours. The cumulative release profiles were statistically compared using the Mann-Whitney U test, demonstrating no significant difference between the test and reference products. In conclusion, the validated IVRT procedure provides a robust, discriminatory and regulatory-compliant analytical tool for the quality control and in vitro sameness (in vitro equivalence) evaluation of gel formulations containing a glycosaminoglycan-derived API.

Keywords: Glycosaminoglycan (GAG), In Vitro Release Test (IVRT), Franz Diffusion Cell, Gel Formulation, Method Validation, HPLC Method Development, IVRT Method Development.

1. INTRODUCTION

In vitro release testing (IVRT) is a fundamental analytical method that evaluates the release kinetics of active ingredients from semi-solid formulations under controlled conditions. The rate of drug release from a dosage form is a critical parameter for understanding dermal absorption and bioavailability (Tiffner *et al.,* 2021). IVRT methods began to be systematically standardized in the 1970s, based on Franz-diffusion cell systems developed to assess the performance of topical formulations and comply with regulatory requirements (Franz, 1975). This methodology gained significant scientific attention when Dr. Richard Stoughton published a study in 1987 questioning the equivalence of generic topical glucocorticoids with the original product (Stoughton, 1987). The significant role played by dissolution tests in determining bioavailability for solid oral formulations suggested that a similar approach could be applied to topical drugs (Guy *et al.,* 1986). In the early stages, the limited ability of the method to simulate clinical conditions, combined with the variability of the skin as a biological barrier, created significant challenges (Caron *et al.,* 1990). In this regard, the Franz diffusion cell system was modified with synthetic membranes offering more reproducible properties instead of cadaver skin, which enabled the development of the IVRT method for topical semi-solid formulations (Shah *et al.,* 1989). Officially defined in the 1997 FDA SUPAC-SS guidance, "Nonsterile Semisolid Dosage Forms; Scale-Up and Post-Approval Changes: Chemistry, Manufacturing and Controls; In Vitro Release Testing and In Vivo Bioequivalence Documentation," for the purpose of evaluating product equivalence and contributing to the growing acceptance of IVRT in the field, this method was initially regarded with doubt by both academia and industry (FDA, 1997). However, IVRT has demonstrated the ability to distinguish variations in composition and performance between different formulations, and studies conducted with corticosteroid creams have provided results consistent with clinical outcomes (Shah *et al.,* 1991, 1992).

Although IVRT cannot fully reflect the complexity of the clinical context, it plays a critical role in the drug development process as a sensitive and reproducible comparative test for evaluating the quality, efficacy, and bioequivalence of topical products (Flynn *et al.,* 1999). IVRT is recognized by the FDA as a recommended method for evaluating pharmaceutical equivalence and sameness in pre- and post-approval product changes (FDA, 2022)Similarly, current guidelines issued by the European Medicines Agency (EMA) emphasize the use of IVRT in the approval processes of generic products (EMA, 2024). Regulatory expectations for quality and equivalence assessment of topical semi-solid products have been significantly updated in recent years. As of 2023, the United States Pharmacopeia (USP) has revised standards in Chapter <1724>, covering performance tests such as IVRT and in vitro permeation test (IVPT) (USP <1724>, 2023)Furthermore, in October 2022, the FDA published the guidance document "In Vitro Release Test Studies for Topical Drug Products Submitted in ANDAs: Guidance for Industry," which provides detailed instructions on the application of IVRT in the evaluation of generic topical products (USP <1724>, 2023). In parallel, the EMA finalized and published its guidance on "Quality and Equivalence of Topical Products" in 2024. The guidance is scheduled to come into effect in 2025 and defines in vitro tests such as IVRT and IVPT as fundamental tools for demonstrating equivalence using a structured and stepwise approach (EMA, 2024).

The rate of in vitro release is shaped by a combination of formulation-related and physicochemical parameters. Factors such as the solubility of the active substance, its particle size, and the rheological (Raghavan *et al.,* 2019)behavior of the dosage form play significant roles. Additionally, specific characteristics of the formulation, including droplet or particle size, viscosity, microstructure, and overall rheological profile, further contribute to how the drug is released(Shah *et al.,* 2022)IVRT, performed using the Franz diffusion cell, is a controlled in vitro test method that measures the time-dependent transfer of the active compound from a semi-solid formulation applied to the donor phase through a membrane into the receptor phase, enabling quantitative determination of release kinetics (Szoleczky *et al.,* 2024). Kinetic data are obtained by monitoring the time-dependent diffusion of the drug through the membrane located between the donor and receptor compartments (Li *et al.,* 2023). Due to its ease of application and high reproducibility, the Franz cell has become the standard method for the characterization of semi-solid dosage forms(Salamanca *et al.,* 2018a)According to EMA, FDA, and USP guidelines, this method is considered a reliable tool both for confirming formulation performance during development as well as for evaluating equivalence (sameness) in post-approval changes (EMA, 2024; FDA, 2022; USP <1724>, 2023). This method becomes particularly important in semi-solid formulations containing complex polyanionic molecules, as it allows accurate measurement of release kinetics and precise determination of formulation differences (Ciftci *et al.,* 2024). Compounds with high molecular weight and polyanionic structure, such as glycosaminoglycans, present specific challenges in pharmaceutical formulations with respect to dermal absorption and release profiles**.** Literature reports indicate that high molecular weight can slow membrane permeation and cause irregularities in diffusion profiles (Kızılçay *et al.,* 2021). The polyanionic nature of these compounds (Zhang *et al.,* 2022). increases polymer–membrane and polymer–drug interactions, thereby complicating reproducibility in in vitro release studies (Neupane *et al.,* 2020). Consequently, IVRT studies using the Franz cell for topical gels containing glycosaminoglycan-derived APIs must be carefully optimized. Additionally, high molecular weight and

strongly anionic structures, require method optimization for chromatographic measurements; in particular, column selection, the ionic strength of the mobile phase, and the type of detector play critical roles (Kızılçay *et al.,* 2021; Ng, Rouse, Sanderson, & Eccleston, 2010). The combination of a weak UV chromophore with the high molecular weight and polyanionic nature of glycosaminoglycans, may result in peak broadening, reduced detection sensitivity, and other analytical challenges during chromatographic analysis (Kızılçay *et al.,* 2021; Gómez-Lázaro *et al.,* 2024a). These considerations require careful optimization of both Franz cell-based IVRT parameters such as membrane selection, diffusion conditions, and sampling strategies and the chromatographic methodologies (e.g., HPLC) employed for quantitative analysis of release profiles, ensuring accuracy, selectivity, and recovery Kızılçay *et al.,* 2021; Shivatare *et al.,* 2024).

This study aims to evaluate the in vitro release profile and validate the method for a gel containing a glycosaminoglycan-derived API using a Franz diffusion cell, in accordance with the parameters specified in the FDA guidance *In Vitro Release Test Studies for Topical Drug Products Submitted in ANDAs: Guidance for Industry*, USP <1724>, and the ICH Q2(R2) validation guideline. In this context, the HPLC method reported by Ozgen et al. was adapted and applied within a Franz diffusion cell-based IVRT system. This approach ensures the accuracy and reproducibility of the method, thereby enabling reliable assessment of the pharmaceutical performance of the formulation (ICH), 2023; USP <1724>, 2023).

2. MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

Disodium Hydrogen Phosphate Dihydrate, Orthophosphoric Acid (85%), Sodium Chloride, Potassium Dihydrogen Phosphate, Potassium Chloride, and 0.45 µm CA membranes were obtained from Merck (Schuchardt OHG, Darmstadt, Germany). Sodium Dihydrogen Phosphate Anhydrous was purchased from Thermo; Sodium Perchlorate Monohydrate from Supelco; and Sodium Hydroxide from Isoloab. The study API standard was supplied by the API provider. High-purity water was generated using a Millipore Milli-Q Plus system. The 0.45 µm RC, PTFE, and Nylon filters were obtained from Millex Millipore and Sartorius, and the 300 kDa cellulose ester dialysis membrane was obtained from Repligen.

2.1.2 DEVICES

Two high-performance liquid chromatography (HPLC) systems with different detectors were used during the analytical studies: a Waters HPLC (e2695) equipped with a UV and PDA detector. The in vitro release experiments were conducted using a Phoenix RDS Automated Diffusion System.

2.2 METHODS

2.2.1 CHROMATOGRAPHIC CONDITIONS

Chromatographic analyses were performed using a Waters HPLC system equipped with both UV and PDA detectors. USP L81 column were employed. The mobile phase flow rate was set at 0.22 mL/min, injection volume at 10 µL, and column temperature maintained at 40 °C. Sample temperature was kept at 25 °C, and the total run time was 15 minutes.

Two aqueous mobile phases with different ionic strengths were used to ensure adequate selectivity and analyte retention. Both Mobile Phase A and B were adjusted to pH 3.0 ± 0.05, with A formulated at a low ionic strength (~3.6×10^{-3} mol·L^{-1}) and B at a high ionic strength (~1.0 mol·L^{-1}) to enable effective gradient modulation. Both mobile phases were filtered through 0.45 µm membrane filters and degassed prior to use(Ozgen *et al.,* 2024).

2.2.2 CALCULATION OF CUMULATIVE RELEASE

$$A_R = \left(\frac{A_{Un}}{A_S}\right) x\ C_S\, x\ 1000\ x\ \left(\frac{V_c}{A_0}\right) + \left[\left(\frac{V_S}{V_C}\right) x \sum_{i=1}^{n-1} \frac{A_{U\,(n-1)}}{A_S}\ x\ C_S\, x\ 1000\ x \left(\frac{V_c}{A_0}\right)\right]$$

A_R = Amount of drug released (µg/cm^2)
A_U = Peak area of Glycosaminoglycan-derivated ingredient obtained from Sample Solution.
A_S = Peak area of Glycosaminoglycan-derivated ingredient obtained from Standard Solution.
C_S = Concentration of the Standard solution (mg/ml).
V_C = Volume of the diffusion cell (22 ml).
A_0 = Area of the orifice (1.77 cm^2).
V_S = Volume of sample transferred into the cell (1 ml).

2.2.3 PREPARATION OF RECEPTOR MEDIUM

For the preparation of pH:7.4 phosphate buffer; 8.1 g sodium chloride (NaCl), 0.2 g potassium chloride (KCl), 0.191 g potassium dihydrogen phosphate (KH_2PO_4), and 1.53 g disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) were dissolved in 1000 mL of purified water. The pH was adjusted to 7.4 ± 0.05 using phosphoric acid. The pH7.4 phosphate buffer containing 20% ethanol (v/v) was prepared by mixing ethanol and pH 7.4 phosphate buffer in a 20:80 (v/v) ratio.

2.2.4 IVRT METHOD DEVELOPMENT BY FRANZ DIFFUSION

2.2.4.1 RECEPTOR MEDIUM SELECTION

To evaluate the effect of different receptor media on the in vitro release of the active pharmaceutical ingredient, two receptor media were prepared: pH:7.4 phosphate buffer and pH:7.4 phosphate buffer containing 20% ethanol (v/v). Release experiments were performed using both 0.45 µm CA membranes and 300 kDa dialysis membranes. The studies were conducted at 32 °C with a stirring rate of 300 rpm over a period of 24 hours.

For the medium showing an appropriate release profile, sink conditions and pH stability were further evaluated. For pH stability, the prepared receptor media were placed in Franz diffusion cells in triplicate and incubated under the same conditions as the release study. The pH of the receptor media was measured and monitored at predetermined sampling intervals throughout the experiment. Sink condition studies were carried out to determine the solubility of API.(Ng, Rouse, Sanderson, Meidan, *et al.,* 2010). For this purpose, 178 mg of the raw material was added to 100 mL of preheated (32 ± 1 °C) receptor media, pH 7.4 phosphate buffer and pH 7.4 phosphate buffer containing 20% ethanol and the mixtures were kept in a shaking water bath. All experiments were performed in triplicate.

2.2.4.2 MEMBRAN SELECTION AND MEMBRAN INERTNESS STUDY

To evaluate membrane inertness, a stock solution was prepared by dissolving 40.40 mg of API in 200 mL of in vitro release medium, from which test solutions corresponding to 25%, 20%, and 15% concentration levels were prepared and tested in triplicate. Each solution was placed into Franz diffusion cells configured as follows: three cells without membrane, three with a 300 kDa dialysis membrane, and three with a 0.45 µm CA membrane. All cells were incubated for 6 hours, after which samples were collected before and after incubation, filtered, and analyzed by HPLC. The same procedure was applied for each concentration level (Sarıbey *et al.,* 2024). For membrane selection, in vitro release studies were conducted using 0.45 µm CA and 300 kDa dialysis membranes with two receptor media (pH 7.4 phosphate buffer and pH 7.4 phosphate buffer containing 20% ethanol) at 32 °C and 300 rpm.

2.2.4.3 ‘’SAMENESS’’ ASSESSMENT

The equivalence between the reference product and the test gel formulation was evaluated in accordance with FDA guidelines (FDA, 1997, 2022) and the USP ⟨1724⟩ “Semisolid Drug Products-Performance Tests” monograph. The results were statistically analyzed using the Mann-Whitney U test. According to this approach, the T/R slope ratios obtained from the Mann-Whitney U test were ranked from the lowest to the highest, and if the 8th and 29th data points fall within the 75%–133.33% range, the two products are considered to demonstrate equivalence (“sameness”).(USP <1724>, 2023)

2.2.4.4 SAMPLING INTERVALS AND DURATION

In order to determine the appropriate sampling intervals and incubation duration, in vitro release studies were conducted at different stirring speeds (300, 400, and 600 rpm). Samples were collected at 0.5, 1, 2, 3, 4, 5, 6, 7, 12, 18, and 24 hours under each condition and analyzed accordingly. The obtained release profiles were subjected to linear regression analysis, and the corresponding coefficient of determination (R^2) were calculated. Based on the R^2 values, the sampling points most representative of the method’s kinetic profile were identified, and the incubation time used in the release studies was optimized. The selected sampling points and durations were chosen in accordance with FDA and USP guidelines to adequately cover the initial, linear, and plateau phases of the release profile (FDA, 2022; USP <1724>, 2023)

2.2.4.5 SENSITIVITY, SPECIFICITY, SELECTIVITY

The sensitivity, specificity, and selectivity parameters of the IVRT method were evaluated by comparing the release profiles of gel formulations containing the API at 50%, 100%, and 150% of the nominal concentration. For each formulation, six parallel Franz diffusion cells were used to perform the in vitro release studies (Wellington *et al.,* 2024a). For sensitivity assessment, the variation in cumulative release rates in response to changes in API concentration was examined. (Klein *et al.,* 2010)Specificity was evaluated by testing the linear relationship between cumulative release rate and API concentration using regression analysis, and the coefficient of determination (R^2) was calculated. In the selectivity assessment, the release rates of the low (50%) and high (150%) concentration formulations were statistically compared to that of the reference formulation (100%) to confirm the method’s ability to distinguish between different API levels(Tiffner *et al.,* 2018)

2.2.5 METHOD VALIDATION FOR DETERMINATION OF RELEASE

2.2.5.1 SELECTIVITY

For the selectivity assessment, mobile phase A, mobile phase B, the in-vitro release medium, placebo, standard solution, and the sample solution collected at the final time point (5th hour) of the release study were injected into the HPLC system, and their corresponding chromatographic spectra were recorded(Bhujbal *et al.,* 2024)

2.2.5.2 LINEARITY

To evaluate the linearity of the method, a 2.02 mg/mL stock standard solution was prepared using the in vitro release medium. To verify the linear response relationship, this stock solution was diluted to obtain concentrations corresponding to 1%, 5%, 10%, 20%,

40%, 80%, 100%, 120%, and 150% of the working level. A calibration curve was constructed using the resulting solutions.

2.2.5.3 PRECISION

For system precision, six consecutive injections (n=6) of the standard solution at the 100% working concentration were performed, and the relative standard deviation (%RSD) of the peak areas was calculated. To assess repeatability, in vitro release analyses were conducted using six gel samples (n=6) at a stirring speed of 400 rpm. For the intermediate precision study, another set of six gel samples (n=6) was analyzed under the same conditions on a different day, by a different analyst, using a different HPLC system and a column from a different lot.

2.2.5.4 RECOVERY

To evaluate the accuracy of the method, recovery samples were prepared by spiking 1 g of gel placebo with the API at 1%, 100%, and 150% levels. For this purpose, a 4.444 mg/mL stock API solution was prepared using the in vitro release medium. Aliquots of 0.01 mL, 1.0 mL, and 1.5 mL of this stock solution were added to Franz diffusion cells containing 1 g of placebo gel to obtain the respective 1%, 100%, and 150% levels, and the total volume was adjusted to 22 mL with the in vitro release medium. The cells were incubated at 32 °C and 400 rpm for 5 hours, after which the solutions were filtered through 0.45 µm RC filters and analyzed.

2.2.5.5 ROBUSTNESS

To evaluate the robustness of the method, small deliberate variations were introduced in critical analytical parameters on both the HPLC system and the Franz diffusion cell. For HPLC analysis, flow rates of 0.20, 0.22, and 0.24 mL/min were applied. Standard and sample solutions were analyzed at column temperatures of 38 °C, 40 °C, and 42 °C and at wavelengths of 200, 202, and 204 nm. The percentage difference (%Difference) was calculated. For the Franz cell experiments, release studies were conducted using gel samples at stirring rates of 380, 400, and 420 rpm, and at temperature conditions of 30.0 °C ± 0.5, 32.0 °C ± 0.5, and 34.0 °C ± 0.5. The release data were statistically evaluated using the Mann–Whitney U test.

2.2.5.6 SOLUTION STABILITY AND FILTER COMPATIBILITY

To evaluate the stability of the solution, a standard solution was prepared at a concentration of 0.202 mg/mL in pH 7.4 phosphate buffer. The gel sample solution was prepared using the sample obtained at the 5th hour of the release study. Both the prepared standard and sample solutions were analyzed at predetermined time intervals to evaluate their stability. For the filter compatibility studies, 0.45 µm PTFE, 0.45 µm RC, and 0.45 µm nylon filters were employed. Filtrates of 3, 5, and 7 mL were collected for each filter, respectively, and analyzed. The obtained results were compared, and the most suitable filter for subsequent studies was selected.

3. RESULTS AND DISCUSSIONS

3.1 IVRT METHOD DEVELOPMENT BY FRANZ DIFFUSION

3.1.1 RECEPTOR MEDIUM SELECTION

According to the FDA's *In Vitro Release Test Studies for Topical Drug Products* guidance, the receptor medium should ensure the solubility of the studied molecule at levels sufficient to maintain sink conditions, remain stable in terms of pH and composition throughout the test, and be compatible with the analytical method (FDA, 2022) Accordingly, the pH 7.4 phosphate buffer provides a stable buffering capacity at physiological pH and, due to its isotonic nature, allows the diffusion kinetics to be reliably monitored without causing ionic or osmotic stress on the membrane system. These properties enable accurate measurement of water-soluble compounds, such as glycosaminoglycan-derived API, in Franz-cell IVRT release studies without restriction by the membrane (Gómez-Lázaro *et al.,* 2024b).

Table 1: Sink Conditions and Solubilities Results in Different Reseptor Mediums for API.

Sample Concentration	Sink Condition (3× Sample Concentration)	Sink Condition (10× Sample Concentration)	Receptor Medium	Solubility	Results
0.202 mg/ml	0.606 mg/ml	2.02 mg/ml	pH:7.4 Phosphate buffer	39.6 mg/ml	39.6 mg/ml > 0.606 mg/ml and 39.6 mg/ml > 2.02 mg/ml Sink conditions are achieved.
			pH:7.4 Phosphate buffer: %20 Ethanol	29.7 mg/ml	29.7 mg/ml > 0.606 mg/ml and 29.7 mg/ml > 2.02 mg/ml Sink conditions are achieved.

As shown in Table 1, both receptor media met sink conditions, indicating that solubility was not a limiting factor and that either medium could in principle be used for IVRT. Under IVRT conditions, pH 7.4

phosphate buffer and the same buffer containing 20% ethanol remained stable over 24 hours with pH variations within ±0.5, consistent with literature reports that pH 7.4 phosphate buffer is thermally robust (Shah *et al.,* 1999)This stability minimizes pH related variability and supports the suitability of these media for release studies.

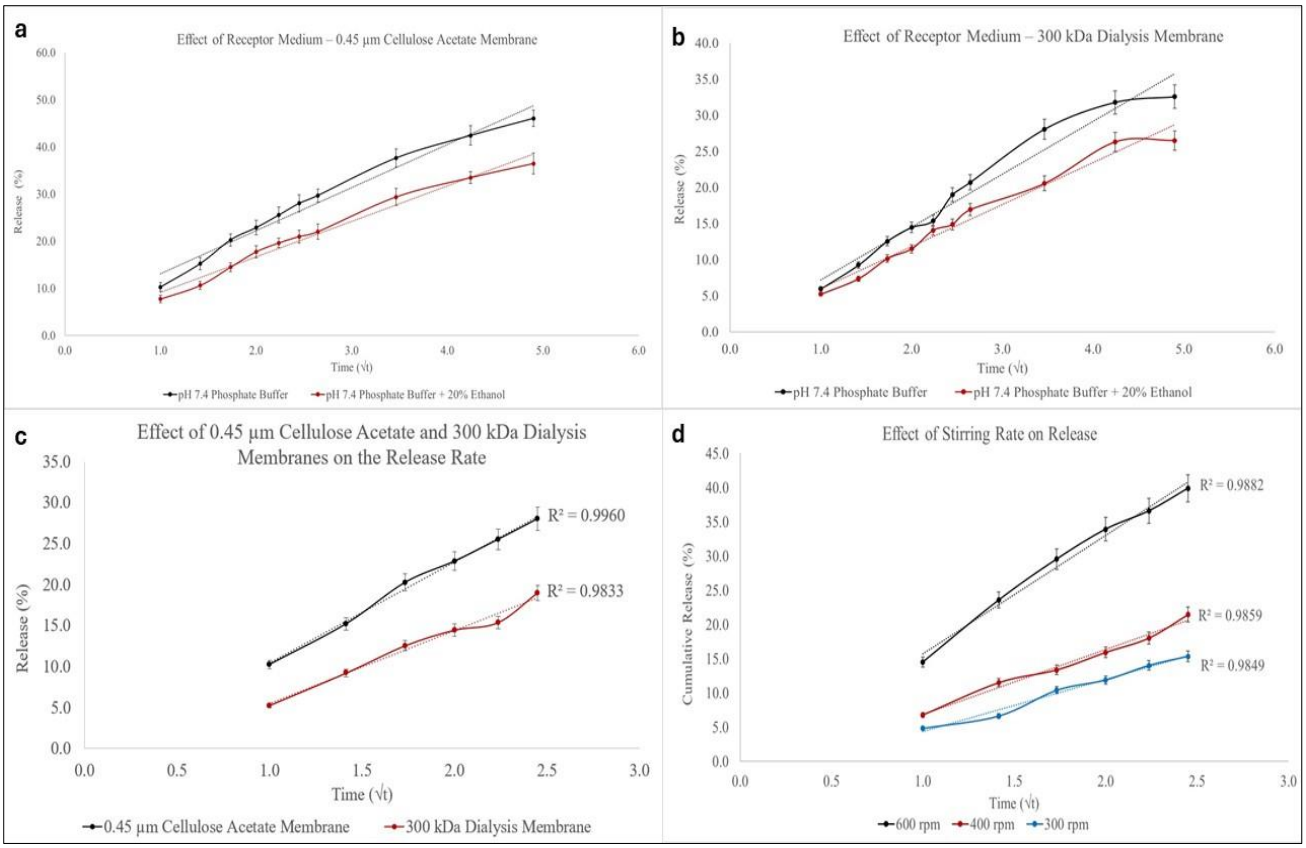


Figure 1: 1a: Release Study Profile- 0.45 µm Cellulose Acetate Membrane at 300 rpm and 32 °C, 1b: Release Study Profile- 300 kDa Dialysis Membrane at 300 rpm and 32 °C, 1c: In vitro release profiles at pH 7.4 in phosphate buffer using 0.45 µm cellulose acetate and 300 kDa dialysis membranes at 300 rpm, 1d: Cumulative release profiles obtained at different stirring rates

To assess the impact of the receptor media on API release, IVRT experiments were performed for 24 hours in pH 7.4 phosphate buffer and in pH 7.4 phosphate buffer containing 20% ethanol using 0.45 µm CA and 300 kDa dialysis membranes at 300 rpm. As shown in Figures 1a and 1b, in phosphate buffer the release with the CA membrane reached approximately 30 percent within 6 hours, whereas in the ethanol containing buffer it remained around 20 percent. With the 300 kDa membrane, the corresponding values were about 20 percent and 15 percent, respectively.

These results indicate that ethanol in the receptor medium reduces diffusion and lowers the extent of release, in line with data showing that organic solvents can decrease membrane permeability and slow the diffusion of hydrophilic macromolecules (Shah *et al.,* 1999). To obtain more biopharmaceutically relevant profiles, pH 7.4 phosphate buffer was therefore selected as the receptor medium. It provides adequate solubility and sink conditions and maintains pH and ionic composition throughout the experiment, and is well supported in the literature as a physiologically relevant receptor phase (Salamanca *et al.,* 2018b)

3.2 MEMBRAN SELECTION AND MEMBRAN INERTNESS STUDY

The validity of the method is directly determined by the membrane's non-interaction with the active substance, the absence of excessive interaction with the formulation or receptor medium, and its maintenance of structural stability throughout the test to provide an inert environment (Mekjaruskul *et al.,* 2021). For this purpose, membrane inertness studies were conducted using 0.45 µm CA and 300 kDa dialysis membranes for in vitro release experiments.

In accordance with the FDA guidance 'In Vitro Release Test Studies for Topical Drug Products', membrane inertness requires that the tested active substance can pass into the receptor phase without adsorption or binding to the membrane material, and that the recovery values are within the range of 100% ± 5% (FDA, 2022). The membrane inertness study was conducted using membrane-free vessels, 0.45 µm CA membranes, and 300 kDa dialysis membranes, with three parallel measurements at three different release levels (15%, 20%, and 25%). As shown in Table 2, based on the membrane-free vessel results, the active substance

did not experience any loss in the Franz diffusion cell environment and was not affected by external factors, and no adverse effect on recovery was observed during the incubation period in the membrane-free systems. Recovery values in the membrane-containing cells were determined as shown in Table 2. These findings are in compliance with the membrane inertness criteria outlined in the FDA guidance 'In Vitro Release Test Studies for Topical Drug Products'. All measurements in our study were within these limits, indicating that the membranes do not significantly interact with the active substance and can therefore be reliably used in in vitro release experiments.

Table 2: Membrane Inertness Results with 300 kDa Dialysis and 0.45 µm Cellulose Acetate Membrane

	Membrane-Free Vessel: Active Substance Recovery (%)	**300 kDa Membrane-Containing Vessel: Active Substance Recovery (%)**	**0.45 µm Cellulose Acetate Membrane-Containing Vessel: Active Substance Recovery (%)**
15% Release Level	100.8	99.1	99.2
20% Release Level	100.4	98.8	99.5
25% Release Level	100.4	100.3	99.2
Mean	100.5	99.4	99.3
SD	0.2	0.8	0.2
RSD %	0.2	0.8	0.2
Confidence Interval	100.5 ± 0.3	99.4 ± 0.9	99.3 ± 0.2

To evaluate the effect of membranes on the release profile, in vitro release studies were conducted in pH 7.4 phosphate buffer at 300 rpm using 0.45 µm CA and 300 kDa dialysis membranes. As shown in Figure 1c, a 6-hour in vitro release analysis was performed, and the results were assessed as a function of the square root of time. In the study using the CA filter, the release reached approximately 30% at the end of 6 hours, with a coefficient of determination (R^2) of 0.996. In the release study using the 300 kDa dialysis membrane, the release reached approximately 20%, with an r^2 value of 0.983. Data obtained with both membranes indicate that the release was linear. To evaluate the suitability of membrane selection, a 'sameness' study was conducted using both the CA filter and the 300 kDa dialysis membrane (Figure 2a and 2b). In vitro release studies were performed at 300 rpm with the 0.45 µm CA membrane for both the test and reference products, and at 400 rpm with the 300 kDa dialysis membrane for both products. The results were analyzed using the Mann-Whitney U test.

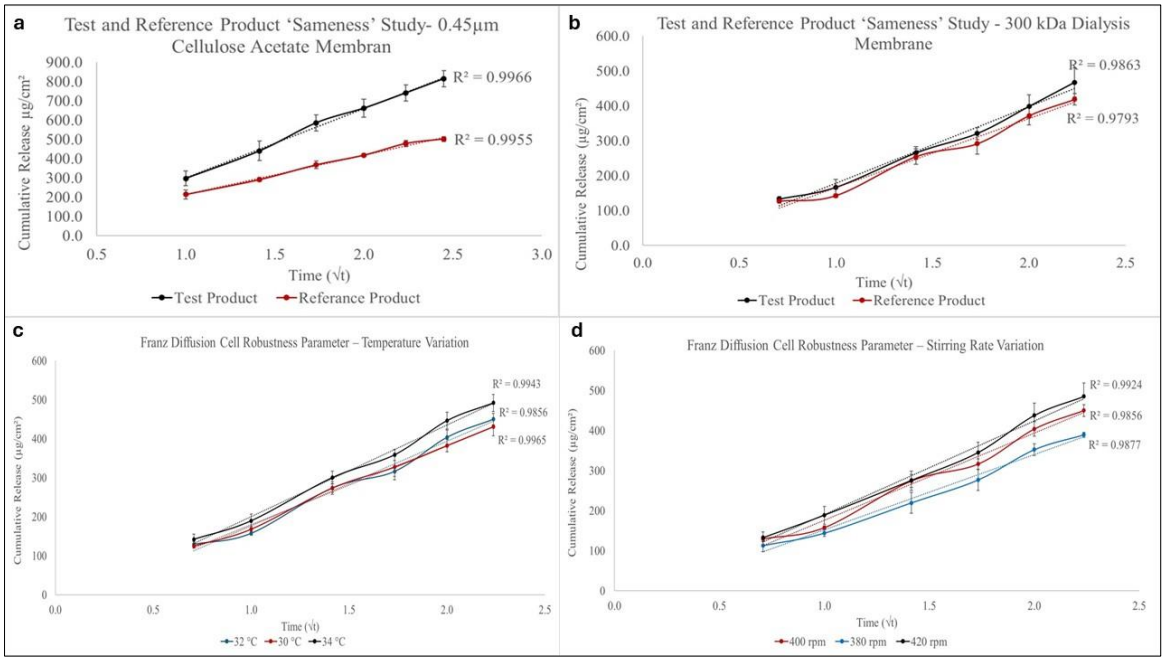


Figure 2: 2a: ''Sameness'' Study Profiles of Test and Reference Product Using 0.45 µm Cellulose Acetate Filter, 2b: 'Sameness' Study Profiles of Test and Reference Products Using 300 kDa Dialysis Membrane Filter, 2c: In-vitro release profiles from the robustness study in the Franz diffusion cell at 30 °C, 32 °C, and 34 °C, 2d: In-vitro release profiles from the robustness study in the Franz diffusion cell at 380 rpm, 400 rpm, and 420 rpm

The 'sameness' assessment of the gel formulations of the test and reference products was conducted in accordance with USP <1724> Semisolid Drug Products-Performance Tests. For the 'sameness'

studies, a 0.45 µm CA membrane was initially used (Table 3). The results showed that the T/R ratios between the 8th and 29th values were not within the 75%–133.33% range, and therefore, the similarity criteria between the two products were not met (Table 3, Figure 2a). In particular, comparison of the release profiles between the reference product and the test sample indicated that the test product exhibited approximately 1.5-fold higher release rate. This observation demonstrates that when using the CA membrane, the release kinetics of the two products were not similar.

Table 3: 'Sameness' Study Results of the Test Product and Reference Product

	0.45 µm Cellulose Acetate Filter, 300 rpm	**300 kDa Dialysis Membrane Filter, 400 rpm**
8th Value	157.0%	99.8%
29th Value	146.9%	82.5%
Values 8th and 29th Limit at 90% Confidence Interval: 75%–133.33%		

In our study, the IVRT/sameness assessment performed with the 0.45 µm CA membrane revealed significant differences between the release profiles of the reference and test products. The main mechanistic explanations for the failure of the CA membrane in the sameness study are as follows: membrane-drug adsorption, where the CA surface can adsorb certain ionic or macromolecular components, thereby reducing the total amount transferred from the donor to the receptor phase; pore/porosity mismatch, which may limit the true diffusion path for high molecular weight polymeric APIs such as glycosaminoglycans; and surface fouling/boundary layer formation, where accumulation of gel matrix components on the membrane surface reduces the effective permeable area, artificially decreasing the measured release. These mechanisms have been reported both in experimental studies and in the review literature (Abu-Zurayk *et al.,* 2023; Jayadev & Ismail, 2023). Literature data indicate that the type of membrane used can significantly affect in vitro release profiles, with these differences depending on membrane pore structure, hydrophobic/hydrophilic character, and interaction capacity with the active substance (Torlak *et al.,* 2025).

In the second phase of the study, the same 'sameness' assessment was conducted using the 300 kDa dialysis membrane. Under these conditions, the T/R ratios obtained were within the 75%–133.33% range, and no statistically significant differences were observed between the two products (Table 3, Figure 2b).

These results indicate that the 300 kDa dialysis membrane provides a suitable in vitro model for the 'sameness' assessment of the test product. The suitability of the Franz cell diffusion IVRT method for assessing formulation sameness has also been well established in the literature. (Wellington *et al.,* 2024a)This finding demonstrates that the membrane material and its physicochemical properties play a direct and critical role in IVRT outcomes. Literature reports clearly show that different membrane materials (e.g., cellulose acetate, regenerated cellulose, PES, nylon) can impose varying diffusion resistances depending on pore size, surface hydrophilicity/hydrophobicity, and adsorption tendencies, resulting in different flux and kinetic profiles for the same formulation across different membranes (T. Higuchi, 1961; Klein *et al.,* 2018; Sarıbey *et al.,* 2024)

3.3 SAMPLING INTERVALS AND DURATION

According to the FDA guideline "In Vitro Release Test Studies for Topical Drug Products", the sampling schedule and total study duration should be set to accurately and reproducibly describe the in vitro release kinetics of the drug. The release profile should remain linear and the total duration generally should not exceed 6 hours, with sampling points selected to cover the initial, linear, and plateau phases while taking into account dosage form properties, API solubility, and analytical sensitivity (FDA, 2022)

In IVRT, a cumulative release of about 30% has been recommended to maintain linear kinetics and ensure accurate and reproducible measurements. This limit preserves infinite dose conditions in the donor compartment so that the release rate is not restricted by drug depletion and remains compatible with the assumptions of the Higuchi model, preventing solubility or membrane related constraints from biasing the results (T. Higuchi, 1961, 1963; W. I. Higuchi, 1962; Klein *et al.,* 2018; Siepmann & Peppas, 2011)

To define appropriate experimental conditions, the effect of stirring speed (300, 400, and 600 rpm) on drug release was investigated (Figure 1d). In all cases, the cumulative amount released increased linearly with the square root of time ($\sqrt{t}$), with coefficients of determination above 0.98, confirming a diffusion controlled mechanism. Higher stirring speeds increased the amount of drug released. At 300 rpm, the release reached only about 20% at 6 hours, which is at the lower limit of the target 20–30% range and indicates limited mass transfer. At 600 rpm, the release exceeded 30% within 6 hours, violating Higuchi model assumptions and potentially reducing the physiological relevance of the test because of excessive hydrodynamic agitation.

Therefore, 400 rpm was selected as a suitable compromise, providing 20–30% release within the first 6 hours and maintaining high linearity (R^2>0.98) in accordance with FDA recommendations. Validation

studies were conducted at 400 rpm using sampling times of 0.5, 1, 2, 3, 4, and 5 hours. This scheme adequately characterizes the initial and linear phases of the release profile while keeping total release below 30%, and was considered appropriate for IVRT characterization of the glycosaminoglycan-derived API containing gel formulation.

3.4 METHOD VALIDATION

3.4.1 SELECTIVITY

No peaks originating from mobile phase A, mobile phase B, the in-vitro release medium, or the placebo were observed at the API retention time in the chromatograms of the standard and sample solutions. The spectral evaluation confirmed that the API peak in both the standard and sample chromatograms exhibited no interference from other components. Furthermore, in both chromatograms, the purity angle was found to be lower than the corresponding purity threshold (purity angle<purity threshold*)*, demonstrating adequate peak purity for the API. The selectivity of the analytical method was successfully demonstrated.

3.4.2 LINEARITY – SYSTEM PRECISION – REPEATABILITY – INTERMEDIATE PRECISION

The calibration curve obtained in the study, covering the concentration range of 0.00202–0.30345 mg/mL (Table 4), exhibited strong linearity, with a coefficient of determination (R^2). The high coefficient of determination, together with the consistent slope and the low intercept value, further confirms the sensitivity and appropriateness of the analytical method for quantification.

Table 4: Linearity Table

Linearity Level (%)	Concentration (mg/ml)	Mean Area
1	0.0020	19357
5	0.0101	82925
10	0.0202	182836
20	0.0404	365289
40	0.0808	750878
80	0.1616	1591968
100	0.2020	1879183
120	0.2424	2242955
150	0.3030	2781889
Slope		9269349.1266
y-intercept		3753.7926
Coefficient of Determination (R^2)		0.999449626
y-intercept / 100% level area × 100		0.2
Sum Of Squared Residuals		9562158375.10636

These results demonstrate that the method performs reliably across the measurement range and exhibits a linear response. The obtained data indicate that the HPLC method is suitable for accuracy, reproducibility, and quantitative analyses.

Table 5: System Precision Results Table

Injection No.	Symmetry Factor	Plate Count	Retention Time (min)	Area
1	0.98	6238	8.683	1695324
2	1.00	6186	8.650	1747400
3	1.02	6437	8.656	1766713
4	1.00	6711	8.661	1757861
5	1.00	6621	8.645	1787990
6	0.99	6634	8.646	1780859
Mean	**1.00**	**6471**	**8.657**	**1756024**
Standard Deviation			**0.01**	**33211.78**
% RSD			**0.16**	**1.89**
Confidence İnterval (CI) (% 95)			**8.657 ± 0.011**	**1756025 ± 26574**

The chromatographic data obtained from six replicate injections confirm the system precision of the method, as presented in Table 5. As shown in the Table 5, the results indicate adequate separation efficiency, consistent peak symmetry, and high reproducibility. According to the ICH Q2(R2) guideline, the system precision criteria are satisfied, demonstrating the reliability of the method for quantitative analyses.(International Council for Harmonisation (ICH), 2023)

Table 6: Repeatability and Intermediate Precision Results

	Repeatability			**Intermediate Precision**		
Sample No	**Cumulative Release (µg/cm²)**	**Slope**	**R^2**	**Cumulative Release (µg/cm²)**	**Slope**	**R^2**
1	472.2	229.2	0.9769	435.9	218.0	0.9721
2	453.3	220.7	0.9931	434.0	211.1	0.9865
3	440.3	211.6	0.9801	454.6	207.2	0.9842
4	449.0	220.1	0.9755	445.3	230.8	0.9779
5	457.7	220.9	0.9886	437.3	204.7	0.9803
6	429.2	202.6	0.9851	498.4	231.7	0.9818
Mean	**450.3**	**217.5**	**0.9832**	**450.9**	**217.3**	**0.9805**
Standard Deviation	**14.8**	**9.2**	**0.01**	**24.5**	**11.7**	**0.01**
RSD%	**% 3.3**	**4.2**	**0.70**	**5.4**	**5.4**	**0.52**
Confidence Interval (CI) (95%)	**450.3 ± 11.8**	**217.5 ±7.3**	**0.9832 ± 0.01**	**450.9 ± 19.6**	**217.3 ± 9.4**	**0.9805 ± 0.004**
The 12-point data for repeatability and intermediate precision of cumulative release results						
Mean						450.6 µg/cm²
Standard Deviation						19.30
RSD%						4.28
Confidence İnterval (CI) (%95)						450.6 ±10.9

The cumulative release data obtained from six samples in the IVRT study demonstrate that the developed method exhibits high repeatability. The mean release value among the samples was 450.3 µg/cm² as shown in Table 6. These results are in compliance with the acceptance criteria outlined in the FDA's "In Vitro Release Test Studies for Topical Drug Products" guidance, which specifies that the %RSD among samples should be below 15% (FDA, 2022). The observed %RSD of 3.3% is well below this limit, indicating that the method operates with high accuracy and consistency.

The cumulative release of the test sample was evaluated in terms of repeatability and intermediate precision using a 12-point dataset as presented Table 6. The mean cumulative release was found to be 450.6 µg/cm², corresponding to a %RSD of 4.28%. The 95% confidence interval was calculated as 450.6 ± 10.9 µg/cm², indicating the precision and reliability of validation.

These findings confirm that the developed IVRT method can be reliably used for in-vitro release studies. Consequently, the method can be considered analytically reliable, repeatable, and a suitable tool for the evaluation of preclinical release profiles.

3.4.3 SENSITIVITY, SPECIFICITY AND SELECTIVITY OF RELEASE

To evaluate the sensitivity, specificity, and selectivity parameters of the IVRT method, gel test formulations containing the glycosaminoglycan-derivative API were prepared at 50%, 100%, and 150% of the nominal API concentration. For each formulation, in-vitro release studies were performed using six parallel Franz diffusion cells (n=6) under the validated experimental conditions. The cumulative release rates were calculated for each concentration level and compared to assess the method's capability to discriminate between formulation strengths. The results demonstrated that the developed IVRT method exhibited adequate sensitivity to detect variations in API concentration, as well as specificity and selectivity towards the active substance without interference from excipients, as shown in Figure 3.

Sensitivity demonstrates whether the method's response is affected by changes in the API concentration. As shown in Figure 3, the release rates increased with increasing concentration (50% < 100% < 150%). This trend confirms that the method's signal responds appropriately and predictably to changes in formulation strength. Therefore, the IVRT method meets the sensitivity criterion.

In the specificity study, the method was evaluated based on the demonstration that the release rate responds proportionally to the API concentration (linear relationship). As shown in Figure 3, linear regression analysis between the concentration levels (50%, 100%, 150%) and the release rates yielded an $R^2 = 0.9925$. This value is well above the threshold of $R^2 > 0.90$, which is commonly used by the FDA to assess linearity. Accordingly, the method exhibits a specific and linear response.

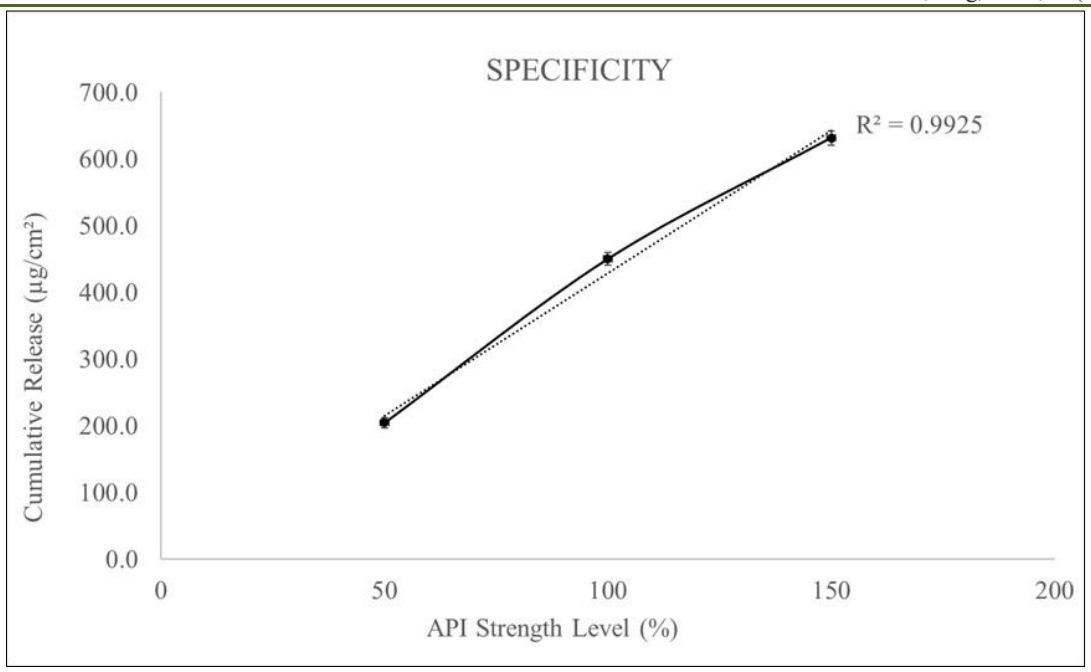


Figure 3: Linearity of Specificity

To evaluate the selectivity parameter, the method's ability to statistically differentiate between formulations of varying strengths was assessed. The 50% and 150% strength gels were compared to the 100% reference using the Mann–Whitney U test to determine whether they met the "sameness criterion." According to USP <1724> and FDA guidance for sameness evaluation, the acceptance window is defined as 75–133.33%. Both of the calculated confidence intervals exceeded this range, indicating that the method can clearly distinguish between both the low-strength (50%) and high-strength (150%) formulations from the 100% reference as presented Table 7. These findings, consistent with the literature, demonstrate that the IVRT method robustly meets the selectivity/discriminatory requirement (Jayadev *et al.,* 2025) Consistent with the literature, IVRT has been reported to provide discriminatory power for formulations of different strengths or dosage forms, reliably detecting formulation changes (Purazi *et al.,* 2020; Wellington *et al.,* 2024b).

Table 7: "Sameness" Evaluation of 50% and 150% Strength Formulations Compared to the 100% Gel formulation

	50% Gel Formulation	**150% Gel Formulation**
8th Value	173.3	61.9
29th Value	197.2	67.3
Values 8th and 29th Limit at 90% Confidence Interval: 75%–133.33%		

3.4.3 RECOVERY

The recovery studies presented in Table 8 were conducted at three different concentration levels (1%, 100%, and 150%) using three parallel samples for each level. The mean recoveries were found to be 100.9%, 101.8%, and 101.8%, respectively. All levels fall within the 100 ± 2% range, demonstrating that the analytical method meets international accuracy criteria (98–102%). %RSD values ranged from 0.3% to 1.4%, supporting high repeatability and consistency.

Table 8: Recovery Results Using pH 7.4 Phosphate Buffer

Recovery Level (%)	**Mean Recovery (%)**	**Standard Deviation (SD)**	**% RSD**	**Confidence Interval (CI) (95%)**	**% Bias**
1	100.9	0.3	0.3	100.9 ± 0.3	0.9
100	101.8	0.6	0.6	101.8 ± 0.7	1.8
150	101.8	1.4	1.4	101.8 ± 1.6	1.8

These results indicate that the method provides reliable recovery across different concentration levels without systematic bias, fulfilling the validation criteria and confirming the method's reliability for analytical measurements.

3.4.4. ROBUSTNESS

To evaluate the robustness of the HPLC method with respect to critical chromatographic parameters, 5-hour release studies were conducted under variations in wavelength, column temperature, and flow rate, compared to the validation condition. The results, as presented in Table 9, indicate that variations in wavelength had minimal impact on the release values, while changes in column temperature and flow rate affected the results more noticeably. Overall, the method was found to be generally reliable and reproducible; however, strict control of column temperature and flow rate is recommended to ensure consistent performance.

Table 9: HPLC Parameters Robustness Results

	Validation Condition (202 nm, 40°C, 0.22 ml/min)	**Different Wavelength**		**Different Column Temperature**		**Different Flow Rate**	
		200 nm	**204 nm**	**38 °C**	**42 °C**	**0.20 ml/min**	**0.24 ml/min**
Mean Cumulative Release (µg/cm²)	395.7	453.5	431.8	400.5	477.7	484.1	457.0
Standard Deviation	13.10	23.47	16.56	34.91	40.36	17.43	29.99
% RSD	3.3	5.2	3.8	8.7	8.4	3.6	6.6
Confidence Interval (CI) (%95%)	395.7 ±10.5	453.5 ±18.8	431.8 ±13.3	400.5 ±27.9	477.7 ±32.3	484.1 ±14.0	457.0 ±24.0
% Differences	-	%14.6	%9.1	%1.2	%20.7	%22.3	%15.5

To evaluate the robustness of the method with respect to Franz diffusion cell parameters, in-vitro release studies were conducted under four different conditions; 400 rpm at 30 °C, 400 rpm at 34 °C, 380 rpm at 32 °C, and 420 rpm at 32 °C, against the validation condition of 400 rpm and 32 °C. The profiles shown in Figures 2c and 2d, along with the cumulative release data presented in Table 9, indicate that the results at 30 °C and 34 °C did not differ significantly from the reference values obtained at 32 °C, and this was confirmed by the Mann-Whitney U test. Similarly, the release measurements at 380 rpm and 420 rpm were consistent with the reference values at 400 rpm.

Table 10: Statistical evaluation of robustness parameters in the Franz diffusion cell

	300 kDa Dialysis membrane filter 400 rpm, 30°C	**300 kDa Dialysis membrane filter 400 rpm, 34°C**	**300 kDa Dialysis membrane filter 32°C, 380 rpm**	**300 kDa Dialysis membrane filter 32°C, 420 rpm**
Mean Cumulative Release Results (µg/cm²)	430.7	491.8	389.5	485.1
8th Value	100.5	88.1	102.7	87.6
29th Value	115.3	97.5	120.4	101.2
Values 8th and 29th Limit at 90% Confidence Interval: 75%–133.33%				

A Mann–Whitney U test was applied to evaluate whether variations in robustness conditions resulted in differences in release performance, as shown in Table 10. The statistical assessment was based on the 8th and 29th values, all of which remained within the predefined acceptance limits (75%–133.33%). These results indicate that the tested changes in temperature and stirring rate did not lead to statistically meaningful differences, confirming the robustness of the method within the studied condition

The findings, as demonstrated by the robustness parameters evaluated in the Franz diffusion cell, indicate that ±2 °C temperature and ±20 rpm stirring speed variations do not have a significant effect on the in-vitro release profile, confirming that the developed IVRT method is a robust and reliable analytical approach against minor variations.

3.4.5 MOBILE PHASE STABILITY, SOLUTION STABILITY AND FILTER COMPATIBILITY

In Table 11, the results of standard and sample analyses, along with the mobile phase stability, solution stability, and filter compatibility studies, are presented. In the mobile phase stability study, indicating that the mobile phase remained stable for 8 days. For solution stability, results ranging from 99.3–101.5% were obtained at all sampling points over 48 hours, demonstrating that both the standard and sample solutions remained stable at room temperature throughout this period. These results indicate that the analytical method can be safely used for sample analysis during routine daily work and, in particular, that it maintains stability without degradation during 24-hour in-vitro release studies in Franz diffusion experiments.

Table 11: Results of mobile phase stability, solution stability, and filter compatibility studies

Mobile Phase Stability Results **Acceptance Criteria: 95.0–105.0%**		
Day	Standard Solution Results (%)	Sample Solution Results (%)
3th day	103.2	103.7
8th day	104.5	98.7
Solution Stability Results **Acceptance Criteria: 98.0–102.0%**		
Time (hour)	Standard Solution Results (%)	Sample Solution Results (%)
4th hour	100.9	100.4
8th hour	99.3	100.5
12th hour	101.5	100.3
16th hour	99.3	99.5
24th hour	99.3	100.6
36th hour	100.4	100.2
48th hour	100.4	100.3
Filter Compatibility Study Results **Acceptance Criteria: 98.0–102.0%**		
Filter/Filtrate	Standard Solution Results (%)	Sample Solution Results (%)
0.45 µm PTFE-3 ml	100.3	100.6
0.45 µm PTFE-7 ml	101.3	100.7
0.45 µm RC- 3 ml	99.8	101.7
0.45 µm RC- 7 ml	101.7	101.4
0.45 µm Naylon-3 ml	100.9	101.0
0.45 µm Naylon-7 ml	101.5	100.2

The filter compatibility study was conducted using PTFE, RC, and Nylon membranes. Both 3 mL and 7 mL filtrates were analyzed for standard and sample solutions. The results which presented at Table VII, demonstrated that all obtained values remained within the acceptance range of 98.0–102.0%, independent of the filtrate volume. These findings indicate that the tested filters do not retain the active pharmaceutical ingredient and can be safely employed during the validation studies, regardless of the filtrate volume. Therefore, the study confirms that PTFE, RC, and Nylon filters are suitable for use within the scope of the developed analytical method.

4. CONCLUSION

In this study, a comprehensive in-vitro release test (IVRT) method was developed for a glycosaminoglycan-derived gel formulation using Franz diffusion cells, and validated in accordance with ICH Q2(R2), FDA "In Vitro Release Test Studies for Topical Drug Products Submitted in ANDAs: Guidance for Industry", and USP <1724> guidelines (FDA, 2022; International Council for Harmonisation (ICH), 2023; USP <1724>, 2023)

Among the evaluated receptor media, phosphate buffer (pH 7.4) was identified as the most suitable system, providing optimal sink conditions and release performance for both test and reference products. Regarding membrane selection, the 300 kDa dialysis membrane demonstrated a more appropriate release profile for the glycosaminoglycan-based API, effectively confirming the similarity between the test and reference formulations through statistical evaluation.

The optimized IVRT conditions were established at a stirring rate of 400 rpm with sampling at 0.5, 1, 2, 3, 4, and 5 hours, ensuring linearity of release without deviation from the Higuchi kinetic model. The study clearly showed that membrane selection directly affects the accuracy of IVRT outcomes and sameness assessments; while the 0.45 µm cellulose acetate (CA) membrane led to detectable differences between the formulations, the 300 kDa dialysis membrane minimized these discrepancies.

Therefore, for high-molecular-weight and polyanionic active pharmaceutical ingredient, careful evaluation of membrane porosity and material characteristics is crucial, and a properly validated membrane selection must be ensured.(Żyłła *et al.,* 2022)

The method validation comprehensively fulfilled the criteria for linearity, system precision, repeatability, recovery, and discriminatory power. The results confirm that IVRT is not only a valuable tool for formulation optimization but also a reliable method for generic equivalence (sameness) assessment. Although IVRT alone is not sufficient to establish clinical translatability (in vivo–in vitro correlation), when properly validated with an appropriate membrane and receptor medium, it serves as a robust preclinical indicator of product performance and equivalence.(Dhudashia & Patel, 2024)

In conclusion, the validated IVRT method is regulatory-compliant, reproducible, discriminatory, and reliable, offering a practical analytical approach for the quality control and sameness evaluation of glycosaminoglycan-based gel formulations.

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DECLARATIONS

Competing interests: The authors are employees of Abdi İbrahim Pharmaceuticals. The company had no role in the study design, data analysis, or interpretation of the results.

Availability of data and materials: The data used and evaluated during the present study can be made available from the corresponding author, Didem AKTAS, upon reasonable request.

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