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Original scientific paper

Development and validation of an *in* vitro release test and use of Strat-M synthetic membrane as a predictor for the *in vitro* permeation test of topical gel formulations

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Abstract

The purpose of this research was to use the vertical diffusion cell method to produce an appropriate *in vitro* release test for topical gel formulations. In order to occur a topical drug product's pharmacological activity, the active pharmaceutical ingredient (API) must first be released from the vehicle and be available to penetrate the skin layers and reach the site of action. Formulation properties (characteristics / factors) have the greatest influence on drug release. Concepts presented in the EMA's draft guideline on topical product quality and equivalence were utilized to analyze critical qualification factors and validate *in vitro* release test (IVRT) methods. Factors that may influence the test, such as the receptor medium and membrane qualification, were appropriately evaluated, followed by an evaluation of method accuracy and method robustness. With 50% and 400% (200% for 2.32% gel) API formulations, the linearity of the IVRT technique as a function of drug concentration in the formulation was studied. Furthermore, the method's discriminatory power was proven using a formulation with variable viscosity as a significant quality parameter.

A permeation study of API at 24 h and drug release rate was performed based on Strat-M membrane permeability parameters in order to predict the outcome of the *in vitro* permeation testing (IVPT). API release profile comparison fulfilled the applicable acceptance criteria for the 90% confidence interval (CI) for the ratio of means of the pairwise comparisons.

Key words: IVRT, IVPT, STRAT-M membrane, topical formulations, method validation.

Introduction

Used API is BCS class II, proven and commonly prescribed nonsteroidal anti-inflammatory drug (NSAID) that has analgesic, anti-inflammatory, and antipyretic properties, and has been shown to be effective in treating a variety of acute and chronic pain and inflammatory conditions. API is considered as a non-selective NSAID, which is associated with serious adverse effects. Their anti-inflammatory effects occur through the inhibition of the cyclooxygenase (COX) enzymes (Ulubay et al., 2018).

The high demand for this medicine has generated a lot of interest from generic medicine manufacturing companies. Nowadays, importance of drug administration via the skin for quick therapeutic effects has increased. In

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contrast to the traditional oral route, the use of transdermal drug delivery bypasses first-pass metabolism of the liver, the acidic environment of the gastrointestinal tract and the problems of absorption in the stomach (Upadhyay et al., 2020).

For a topical drug product after dosage form application, the first step is the drug release from the dosage form followed by drug partition or penetration to or thru *stratum corneum* (Guy et al, 1986). Depending on the physicochemical properties of the active pharmaceutical ingredient, the formulations factors and the intended site of action, the specific pharmacodynamic action may be expressed at a specific, outermost or deeper layer of the skin (Shah et al., 2022). The permeation through the skin can be measured by *in vitro* techniques.

Pharmaceutical equivalence and bioequivalence (BE) of the test product towards the reference product should be adequately documented to ensure therapeutic equivalence and interchangeability (Lukic et al., 2020). Comparative pharmacokinetic studies represent the gold standard for BE assessment. Nevertheless, for the vast majority of topically applied and locally acting drugs, alternative approaches must be equated due to the absence of systemic drug absorption (Miranda et al., 2022).

The qualitative composition of the test product should be equivalent to the reference product (EMA`s Draft guideline on quality and equivalence of topical products, 2018). Although this may seem unproblematic, difficulties may arise in selecting the excipient grade, as this information is not publicly available. This is to be followed by the quantitative equivalence (Q2) sameness. To achieve this, reverse engineering procedures may be required. Microstructure equivalence (Q3) should also be documented. Within this analysis, data on pH, droplet/particle size, product metamorphosis, rheological behavior analysis, stability profile, among other parameters, should be provided. Product performance equivalence (Q4), mainly supported by *in vitro* release test (IVRT) methods, should likewise be evidenced (Miranda et al., 2022). The *in vitro* release profile was performed for generic and originator products and its acquisition was done by means of a static vertical Franz diffusion cell equipment.

In vitro drug release test has become one of the most important tools for drug development and approval process of semisolid dosage forms. IVRT has the ability to reflect the combined effects of several physicochemical characteristics, particle or droplet size, viscosity, microstructure arrangement of the matter and state of aggregation of dosage form (Shah et al., 2022).

Historically, IVRT has been frequently employed to ensure product sameness. Consequently, the test may be used to determine how well a topical treatment performs *in vivo* (Manian et al., 2022).

Therefore, the purpose of this study was to develop and validate an IVRT method that was sensitive, specific and selective for evaluation of topical gel formulations. For IVPT prediction, the use of synthetic Strat-M membrane proved quite beneficial. The results of this investigation showed that there was a good correlation coefficient between the artificial membrane and the human skin for the penetration of API.

Material and methods

Chemical and standards

Working standard was used, supplied by Alkaloid AD, North Macedonia. The mobile phase for HPLC analysis was prepared using sodium dihydrogen phosphate dihydrate (Merck, Germany) and acetonitrile isocratic grade from Supelco, Germany. The column for HPLC analysis was supplied by Bischoff, Germany. Phosphate Buffered Saline (PBS) pH 7.4 supplied by VWR Chemicals, USA and methanol acquired from Supelco, Germany, were used to prepare the receptor medium solution for the IVRT test. Regenerated Cellulose filter membranes with pore sizes of 0.2 µm and diameters of 25 mm supplied by Sartoirius, Germany and Strat-M membranes with diameters of 25 mm and thickness of 300 µm, supplied by Merck Millipore Ltd., Germany were the membranes used during the development and validation of the IVRT study.

Drug formulations

1.16% and 2.32% topical gel formulations containing 11.6 mg and 23.2 mg API utilized in the method development and evaluation were obtained from Alkaloid AD, North Macedonia. Samples from originator were procured from GSK, Germany.

Vertical Diffusion Cell (VDC) system

In vitro release method for topical dosage forms is based on an open chamber diffusion cell system such as a Franz cell system, fitted usually with a synthetic membrane. The test product is placed on the upper side of the membrane in the open donor chamber of the diffusion cell and a sampling fluid is placed on the other side of the membrane in a receptor cell. Diffusion of drug from the topical product to and across the membrane is monitored by assay of sequentially collected samples of the receptor fluid (US Food and Drug Administration, 1997).

An automated Hanson Vision Microette Vertical Diffusion Cell Apparatus with 6 cells was used as the VDC system in the study. The Type A diffusion cells used in the VDC system are specifically discussed in USP chapter 1724. 9 mL cells were utilized, with the exact volume noted on each cell. The metallic helix and magnetic stirrer at the bottom of the cells are used to continuously agitate the receptor media inside the cells. A circulating water bath is supplied with the apparatus to keep the receptor medium at a consistent temperature. Dosage wafers with a dose of roughly 600 mg were employed for the dosage delivery. The process for applying the gel was validated in order to verify that a proper amount of drug - a dose of roughly 600 mg - was applied with each trial, in each cell.

HPLC method for evaluation of analytical results

Across Barriers (Germany) developed and thoroughly validated the HPLC method for evaluation of the analytical data, which was then successfully transferred to Alkaloid AD, North Macedonia. A complete validation of the transferred method was as well performed in Alkaloid AD, North Macedonia.

RP-HPLC method was performed using ProntoSIL120-3-C18 H (75mm x 4.6mm, 3µm) column and pre-column SecurityGuard Cartridge C18 (4mm x 2mm) with 10 mM dihydrogen phosphate dehydrate buffer pH 3.3 in combination with acetonitrile 60 : 40 (% *v/v*) as a mobile phase at a flow rate of 1.0 mL/min. The injection volume is 10 µL and detection wavelength of 277 nm. Column temperature was set on 40 ºC with the retention time of API around 2.7 minutes. Thermo Ultimate HPLC model was used.

IVRT Parameters

A set of parameters related to the IVRT apparatus were used for the evaluation of 1.16% and 2.32% gel formulations. Table 1.

The set of parametars were chosen based on general recommendations for performing IVRT test (EMA`s Draft guideline on quality and equivalence of topical products, 2018). 10 % PBS pH 7.4 : methanol 80 : 20 (% v/v) is used as receptor medium with sirring speed of 400 rpm and temperature of 32 °C. Regenerate celulose 0.2µm is used as membrane. The test was scheduled to last 3 hours. To cover the complete release profile of the API from the drug product, the sampling schedule was set to gather samples every 30 minutes. Twelve samples of each formulation were evaluated from two separate experiments of six samples applied.

Table 1. IVRT Tested Parametars

Release Medium	10% PBS pH 7.4 : MeOH = 80:20 (%, v/v)
Franz Cells	9 mL volume (ca.1.8 cm² diffusion area)
Membrane	Regenerated Cellulose, 0.2 µm
Stirring speed	400 rpm
Temperature	32.0 °C ± 1 °C
Duration	3 hours
Time points	0.5h; 1h; 1.5h; 2h; 2.5h; 3h

Results and discussion

In general, semisolid dosage forms are complex formulations having complex structural elements.

The EMA and FDA have emphasized the importance of completing IVRT as part of the development and evaluation of semi-solid formulations. An *in vitro* release rate can reflect the combined effect of several physical and chemical parameters, including solubility and particle size of the API and rheological properties of the dosage form. In most cases, *in vitro* release rate is a useful test to assess product sameness. The guidelines provide basic approaches that would lead to the appropriate development of an IVRT method that is both, reliable and effective (EMA`s Draft guideline on quality and equivalence of topical products, 2018). This study for development and validation of the IVRT method for topical gel formulation includes appropriate evaluation of the receptor medium based on sink conditions criteria, membrane qualification, evaluation of method precision, linearity, discriminatory power, and method robustness. The method was developed and validated using 1.16% and 2.32% topical gel formulations. Later, the IVRT method was used to compare the similarity of 1.16% and 2.32% gel formulations *vs* originator's product.

Receptor medium selection

The most important factor for the selection of receptor medium is the solubility of the API in the medium. The receptor medium should provide a "diffusional sink" for the API released from the semisolid formulation. The relationship of cumulative amount released versus square root of time is derived from the Higuchi model with the assumption that there is a reservoir of the drug always available to diffuse thru. There should be no more than 30% of the total amount of the dose applied released into the medium at the end of the experiment (Higuchi, 1960).

One practical consideration is to choose a receptor medium that allows sufficient amounts of API released within a reasonable time period to ensure accurate analysis of the release rate samples.

Since one purpose of IVRT is to, if possible, serve as a surrogate for *in vivo* testing, the medium most resembling the relevant physiological fluid is preferred, which is why PBS is often a first choice. However, for APl's with low aqueous solubility, a modification of PBS

is necessary to increase the solubility sufficiently to approach sink conditions. Alcohol and/or a surfactant may be incorporated into the receptor medium if the API shows solubility issues (Kanfer et al., 2017).

The solubility of API in methanol-buffered media is sufficient to meet all of the requirements discussed above. After a few preliminary experiments, a 10% PBS with pH 7.4 mixed with 20% methanol (MeOH) was selected as the receptor medium. PBS with 20% methanol allowed sufficient levels of API to diffuse through the membrane from formulations for accurate analysis.

To determine the solubility of API, two receptor medias were analyzed. Receptor medium 1 which was a mixture of 10% PBS/MeOH in ratio 60:40 *v/v%* and receptor medium 2 which was a mixture of 10% PBS/MeOH in ratio 80:20 *v/v%*. To analyze the API solubility in receptor medium two weightings of standard in both preselected receptor media were prepared in a transparent glass vial and stored for 4 hours at 32 °C on a stirrer. Three samples were taken at time 0 h, centrifuged, diluted to linear range of the calibration curve with either receptor medium 1 or 2 and measured. For both receptor medias, after 4 hours storage at 32 °C, the weighed amount was completely dissolved. No remarkable differences in the amount of dissolved API between the two evaluated receptor media were observed. Results from solubility are presented in Table 2.

Sink conditions are ensured if the maximum concentration of the API in the receptor medium, achieved during the experiment, does not exceed 30% of its maximum solubility in the receptor medium. Sink conditions normally occur in a volume of medium that is at least 3-10 times of the saturation volume. The maximum release (concentration in the Franz diffusion cell) from the 4.64% formulation during method validation was 304 μg/mL. This is 1.37% of the solubility determined during IVRT method development. Theoretically, if 600 mg of the formulation with the highest API content (4.64%) is applied using a 12 mL Franz cell, and 100% of the applied API is released, the measured concentration would be 2320 μg/mL. Sink conditions are ensured if 30% of the maximum solubility is not exceeded. 2320 μg/mL is 10% of the maximum solubility (approx. 21000 to 22500 μg/mL) of API in both pre-selected medias. Therefore, with both media sink conditions are ensured. The analysis in Alkaloid AD is carried out using 9 mL Franz cells. Sink conditions are also maintained in 9 mL cells because if 600 mg of the formulation with the highest drug content (4.64%) is released 100%, the measured concentration will be 15% of the maximum API solubility.

Since the release rate with receptor medium 2 (10% PBS/MeOH 80:20 (*v/v%*)) was lower than receptor medium 1 (10% PBS/MeOH 60:40 (v/v%)), this medium seems to be better suited for the IVRT experiments. A slower release rate can lead to a higher discriminative power. Furthermore, receptor medium 2 showed a better linear trend for all different membranes.

From results it can be concluded that satisfactory solubility is obtained in PBS/MeOH 80:20 (*v/v*) so this medium was chosen as the most suitable receptor medium for the IVRT evaluation, as it complies with the sink conditions acceptance criteria.

Membrane selection

The membrane selected should provide an inert holding surface for the formulation, but not a barrier. The membrane of choice should allow the API to readily diffuse into the receptor medium as it is "released" from the dosage form. It is important to confirm that there is no physical or chemical interaction between the membrane and the formulation (Thakker et al. 2003,).

Appropriate inert and commercially available synthetic membranes with a diameter of ca. 25 mm were used. The selection criteria for membrane were:

1) A permeability rate to achieve a comparative differentiation of the formulations which will be tested;

2) The membrane should be compatible with the drug product formulation and not bind to the API;

3) The membrane should ensure that the product and the receptor medium remain separate to ensure the tested formulation remains unchanged throughout the testing period;

4) The membrane should not be rate-limiting to API release.

Since the major constituent of many semisolid products is water, hydrophilic/hydrophilized synthetic membranes are typically used. During the IVRT development, three different membranes in two receptor media were tested:

Table 2. Solubility of API obtained in receptor medium 1 and receptor medium 2

Storage time (h)	Theoretical Conc.	0	1	4
Storage temperature		Room Temp.	32 °C	32 °C
Receptor 1: 10% PBS/MeOH 60:40 (*v/v*)	23009 $\mu g \cdot mL^{-1}$	21179 $\mu g \cdot mL^{-1}$	22306 $\mu g \cdot mL^{-1}$	21797 $\mu g \cdot mL^{-1}$
Receptor 2: 10% PBS/MeOH 80:20 (*v/v*)	23076 $\mu g \cdot mL^{-1}$	22128 $\mu g \cdot mL^{-1}$	22204 $\mu g \cdot mL^{-1}$	22505 $\mu g \cdot mL^{-1}$
Appearance of solution		Small particles	Clear, colorless	Clear, colorless

Table 3. Recoveries from membrane absorption with API solution in receptor medium 1 and 2

	Recovery (%)			
Solution	Receptor medium 1: 60:40 10%PBS:MeOH_0h	Receptor medium 1: 60:40 10%PBS:MeOH_4h	Receptor medium 2: 80:20 10%PBS:MeOH_0h	Receptor medium 2: 80:20 10%PBS:MeOH_4h
Durapore PVDF 0.45µm	99.95	99.65	99.88	100.05
Nylon GNWP 0.20µm	99.19	97.14	97.75	92.17
Regenerated cellulose 0.2µm	100.67	100.15	99.50	100.05

1) Durapore PVDF – hydrophilic 0.45 µm pore size
2) Nylon GNWP - hydrophilic 0.20 µm pore size
3) Sartorius regenerate cellulose – hydrophilic 0.2 µm pore size.

Membranes ability to bind the API was evaluated. The membranes of interest were incubated with API solutions in the desired receptor media for 4 hours at 32 °C ± 1 °C. As control, the API solutions without membrane were incubated and evaluated. Samples were withdrawn before and after incubation to assess any decrease of API in solution. Additionally, the membranes were incubated in the same manner with the pure receptor medium (without API) to exclude any analytical interferences. The results of the recoveries are presented in Table 3.

The only membrane that showed a slight decreased recovery after four hours of incubation was the Millipore GNWP membrane (recovery of 92.17%) in receptor medium 2. However, this is still within the set specifications of ± 10%. For the other investigated membranes and media, a physical or chemical interaction of the API with the membrane was not observed (recovery ranges between 97 and 100%).

For further membrane selection the different membranes were used to evaluate the release kinetics. During this step the duration of the experiment, the sampling points, the discriminative ability of the membrane and the linearity of the Higuchi plot were evaluated. Gels with different API content (1.16%, 4.64% and 1.16% with low or high viscosity) were used. For each membrane the Higuchi plot was created. The release profile of API from receptor medium PBS/MeOH 60:40 (v/v%) and receptor medium PBS/MeOH 80:20 (v/v%) are presented in Table 4 and Table 5, respectively.

Table 4. Release of API from originator 1.16% gel formulation, 4.64% generic gel formulation and 1.16% low viscosity generic gel formulation with different membranes in acceptor medium 10% PBS/MeOH 60:40 (*v/v%)*

Formulation	Membrane	Cumulative amount of API after 4 hours ($\mu g^{*}cm^{-2}$)	Drug release rate, Slope ($\mu g/cm^2/h^{-0.5}$)	RSD of Cumulative transport of API	RSD of slopes	r^2
Originator 1.16% gel	Durapore PVDF 0.45µm	918.69	493.37	7.96	6.28	0.994
	Nylon GNWP 0.20µm	987.26	580.71	17.39	15.92	0.998
	Regenerated cellulose 0.2µm	1244.21	739.92	5.99	7.25	0.999
4.64% generic gel formulation	Durapore PVDF 0.45µm	3463.24	1931.83	10.76	9.05	1.000
	Nylon GNWP 0.20µm	3478.20	2012.52	6.40	7.33	0.997
	Regenerated cellulose 0.2µm	3517.56	2068.90	10.75	10.63	0.999
1.16% low viscosity generic gel formulation	Durapore PVDF 0.45µm	1404.37	749.29	10.19	9.78	0.974
	Nylon GNWP 0.20µm	1501.41	892.10	/	/	1.000
	Regenerated cellulose 0.2µm	1162.88	703.32	14.17	11.17	0.996

Table 5. Release of API from originator 1.16% gel formulation, 4.64% generic gel formulation and 1.16% low viscosity generic gel formulation with different membranes in acceptor medium 10% PBS/MeOH 80:20 (*v/v%*)

Formulation	Membrane	Cumulative amount of API after 4 hours ($\mu g^{*}cm^{-2}$)	Drug release rate, Slope ($\mu g/cm^2/h^{-0.5}$)	RSD of Cumulative amount of API	RSD of slopes	r^2
Originator 1.16% gel	Durapore PVDF 0.45µm	794.93	466.57	14.15	18.41	0.994
	Nylon GNWP 0.20µm	677.29	416.20	8.34	9.07	0.997
	Regenerated cellulose 0.2µm	705.98	431.65	0.69	1.32	0.997
4.64% generic gel formulation	Durapore PVDF 0.45µm	2773.24	1510.79	2.34	4.11	0.994
	Nylon GNWP 0.20µm	2478.59	1401.34	2.21	4.32	0.993
	Regenerated cellulose 0.2µm	2066.84	1167.63	8.05	12.98	0.990
1.16% low viscosity generic gel formulation	Durapore PVDF 0.45µm	1184.25	652.68	8.89	6.88	0.996
	Nylon GNWP 0.20µm	984.98	574.25	5.19	0.97	0.999
	Regenerated cellulose 0.2µm	1066.74	645.74	0.49	0.97	0.998

When comparing the different membranes from the experiment with receptor medium 2, the membranes show no difference in the linear trend. However, the Millipore Durapore HVLP membrane has a high RSD of the slope (18.41%). Therefore, this membrane was excluded. The other two membranes do not show significant differences. However, as shown in the membrane inertness experiment the Millipore GNWP membrane has a lower recovery than the Sartorius regenerated cellulose membrane. Therefore, the Sartorius regenerated cellulose membrane was selected for further development.

Method Precision

Method precision was studied with different operators on different days. The intra-run and inter-run precision and reproducibility were compared for the release rate (slopes) and cumulative amount at the end of the IVRT experiments calculated for each diffusion cell. The mean and relative standard deviation among slopes and cumulative amount at the end of the IVRT experiments were calculated within and across all runs. Inter/Intraday variability and inter/intra operator variability were also demonstrated. The results of the cumulative amount of API released per unit area at the end of the test and the slope representing the release rate of the API are given in Table 6. The acceptance criteria for a suitable reproducibility of the method were RSD < 10% for both, the cumulative amount and the slope of the release curve.

The IVRT method is precise because intra-runs and inter-run precision was ≤ 10% which is acceptable according to EMA, Draft guideline on quality and equivalence of topical products, 2018.

Discrimination Sensitivity, Specificity and Selectivity

The IVRT method has to be able to discriminate API release rates from non-similar formulations. For evaluation of these parameters, modulation in the formulation regarding the dose amount (one formulation with a higher strength, 4.64% and one with a lower strength 0.58%) were made. Release rate as a function of the drug concentration was observed for these formulations. The discrimination ability of the IVRT method was described using three concepts: sensitivity, specificity and selectivity.

IVRT sensitivity is the ability of the IVRT method to detect changes in the release rate as a function of API concentration in the formulation.

Table 6. IVRT of 1.16% and 2.32% topical gel formulations on Regenerated Cellulose 0.2 µm – Method Precision

Formulation	Precision	Cumulative amount of API after 3 hours ($\mu g/cm^2$) (Average of 12 samples tested)	Drug release rate, Slope ($\mu g/cm^2/h^{-0.5}$) (Average of 12 samples tested)	RSD of Cumulative amount of API	RSD of slopes
1.16% topical gel formulation	Analyst I	865.62	688.53	8.54	8.85
	Analyst II	835.85	694.10	2.28	2.86
2.32% topical gel formulation	Analyst I	1743.50	1371.82	3.1	3.9
	Analyst II	1691.65	1339.75	5.6	6.1

Table 7. IVRT of 50%, 100% and 400% API Formulation of 1.16% gel formulation

	Cumulative Amount of API after 3 hours (µg/cm²) (Average of 12 samples tested)	Drug release rate, Slope (µg/cm²/h$^{-0.5}$) (Average of 12 samples tested)	RSD of Cumulative amount of API	RSD of slopes
50% API Formulation	386.70	316.92	2.29	3.75
100% API Formulation	865.62	688.54	8.06	7.62
400% API Formulation	2835.65	2348.03	2.46	2.18

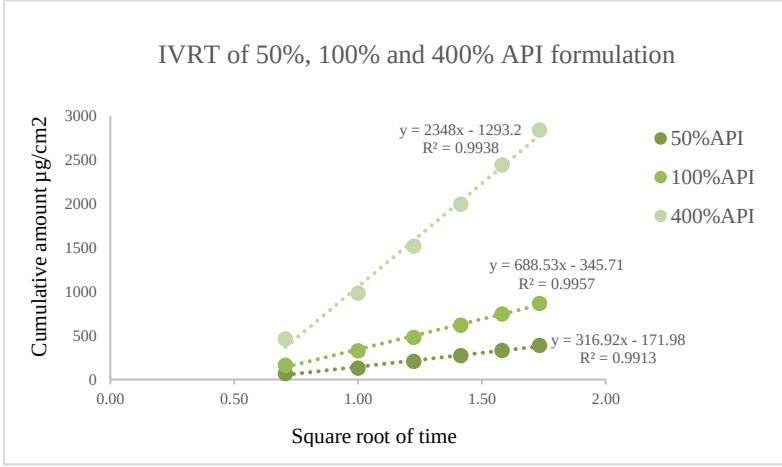

Fig. 1. Cumulative amount of topical gel released from 50%, 100% and 400% API Formulation of 1.16% gel formulation.

The summarized results from 12 samples are given in Table 7, and the Highuchi plot for each formulation is presented in Figure 1.

The results have showed that the IVRT method consistently identifies higher or lower rates of release for test formulations with increased or decreased API concentrations, respectively, relative to the reference formulation which confirms that the method has suitable sensitivity.

IVRT specificity is the ability to accurately monitor the proportionality of changes in the release rate as a function of drug concentration in the formulation. The mean release rate (slope of curve) was plotted against the formulation concentration. According EMA draft guideline on quality and equivalence of topical products, the release rate as a function of drug concentration (at least three strengths) in the formulation should be investigated. The linearity (r2>0.90) of the correlation of formulation concentration to rate of drug release (R) should be confirmed when the drug is fully dissolved.

The linearity of the curve was evaluated: r² should be ≥ 0.90 for the correlation of formulation concentration and average IVRT release slope. Highuchi plot for each formulation is presented in Fig. 2.

The method was found to be specific and linear, as the amount of API released was proportional to the concentration of API in the formulation.

IVRT selectivity is the ability of the IVRT method to be sufficiently selective to identify that the different API release rates from the altered concentration test formulations are in-equivalent with respect the API release rate from the reference product. In order to evaluate the method selectivity, a comparison was made between the three formulations in terms of the cumulative amount and the slope of the release curve of API, representing the release rate. The results of the 90% CI are presented in a Table 8.

The 90% CI falls out of the specified limits 90 - 111%, showing that there is a significant difference between the formulations, which confirmed that the IVRT method is selective.

Discrimination: Formulation changes

As amended in EMA's draft guideline on quality and equivalence of topical products, altered product formulations with changes in critical quality attributes should be analyzed in order to prove discriminative power of the proposed method. Formulation changes in the

amount of excipient composition were made, that would result in changes of the critical quality attributes of the formulation such as the rheological properties. Formulations with higher and lower viscosity than that of the proposed were made. The release profile of API form discriminatory formulations was compared to 1.16% and 2.32% topical gel formulations with evaluation of the 90% CI (Table 9). The Highuchi plots for the discriminatory formulations vs. 1.16% and 2.32% gel formulations are presented in Fig. 3 and 4.

Evaluation of the 90% CI (Table 10) shows that there is a significant difference between the two formulations and the IVRT method is efficient in discriminating between formulations with changed critical quality attributes.

Method Robustness

Variations in mixing rate, amount of formulation applied, receptor mediums and temperature should be studied for method robustness according EMA's draft guideline on quality and equivalence of topical products. In order to assess method robustness, small deliberate changes in the method were made. The parameter IVRT robustness is performed by making changes of the bath temperature (± 0.5 °C), rpm (± 40 rpm) and in the receptor medium composition (75 – 90% MeOH) in comparison with the original method.

The IVRT method is considered robust to some variations in the test method if the average slope of that IVRT run (under altered conditions) is within ± 10% of the average slope of the precision and reproducibility IVRT runs. The results are presented in Table 11. The 90% CI were evaluated as well and are presented in Table 12.

The method was found to be robust and small changes in the parameters did not affect the method performance.

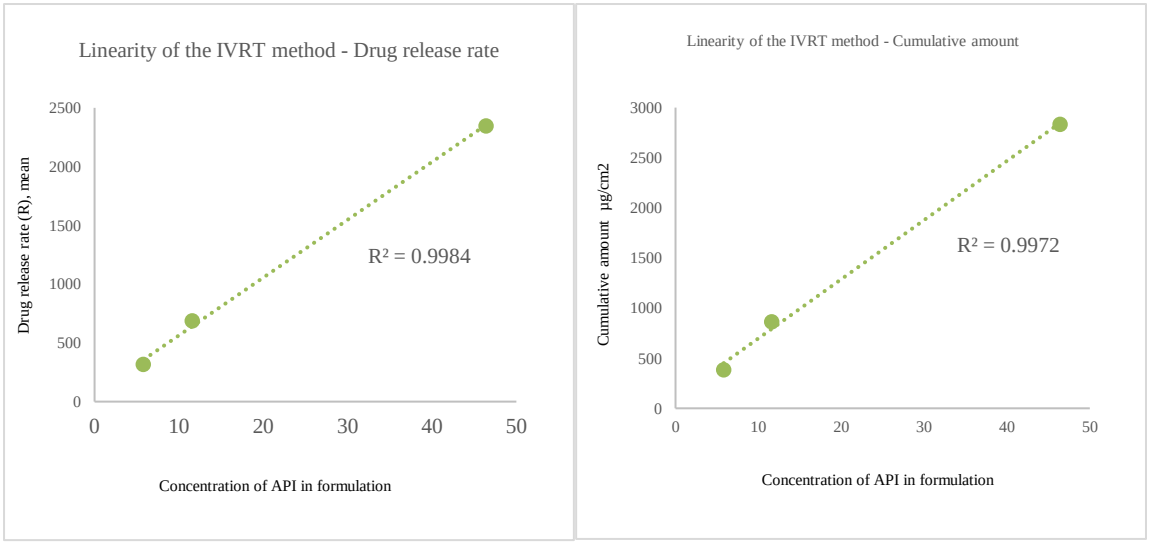

Fig. 2. Linearity of the IVRT method evaluated trough the cumulative amount and the slope of the release curve.

Table 8. Evaluation of IVRT Method Selectivity

Formulation	90% Confidence Interval for cumulative amount		90% Confidence Interval for slopes		Significant difference
	Lower limit	Upper limit	Lower limit	Upper limit	
5.8% gel formulation vs 1.16% gel formulation	44.40	45.63	45.73	47.09	YES
4.64% gel formulation vs 1.16% gel formulation	325.50	334.70	338.84	348.88	YES
1.16% gel formulation vs 2.32% gel formulation	47.07	47.83	47.17	48.01	YES
2.32% gel formulation vs 4.64% gel formulation	197.36	199.62	195.41	197.87	YES

Table 9. IVRT of Discriminatory formulations vs. 1.16% and 2.32% gel formulation

Formulation	Cumulative amount of API after 3 hours (µg/cm²) (Average of 12 samples tested)	Drug release rate, Slope (µg/cm²/h$^{-0.5}$) (Average of 12 samples tested)	RSD of Cumulative transport of API	RSD of slopes
Low viscosity 1.16% gel	1138.28	889.21	1.83	2.34
High viscosity 1.16% gel	690.79	579.94	1.85	1.56
1.16% gel formulation	865.62	688.54	8.06	7.62
Low viscosity 2.32% gel	2094.07	1625.94	5.15	4.24
High viscosity 2.32% gel	1492.05	1227.07	4.83	4.75
2.32% gel formulation	1743.50	1371.82	1.88	3.86

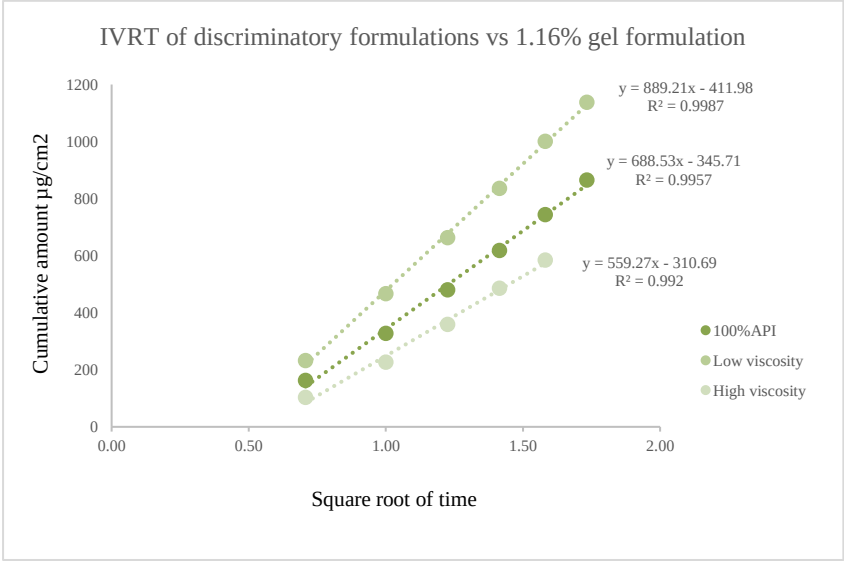

Fig. 3. ***Cumulative*** amount of API released from discriminatory Formulations vs. 1.16% topical gel formulation.

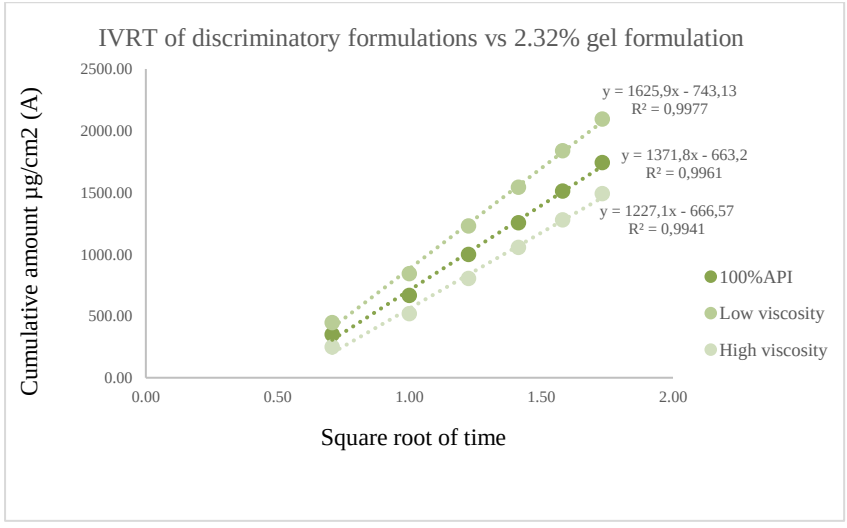

Fig. 4. Cumulative amount of API released from discriminatory Formulations vs. 2.32% topical gel formulation.

Table 10. 90% Evaluation of IVRT Method-Discriminative power

Formulation	90% Confidence Interval for cumulative amount		90% Confidence Interval for slopes		Significant difference
	Lower limit	Upper limit	Lower limit	Upper limit	
1.16% low viscosity gel formulation vs. 1.16% gel formulation	79.31	81.52	83.69	86.17	YES
1.16% high viscosity gel formulation vs. 1.16% gel formulation	130.76	134.26	128.36	132.09	YES
2.32% low viscosity gel formulation vs. 2.32% gel formulation	119.20	121.22	117.79	119.59	YES
2.32% high viscosity gel formulation vs. 2.32% gel formulation	84.99	86.31	88.85	90.30	YES

Table 11. IVRT – Method Robustnes

	Cumulative amount (µg/cm²)	Slopes (µg/cm²/h$^{-0.5}$)
Nominal method	865.62	688.53
Altered temperature (31.5 °C)	835.57	684.69
Altered temperature (32.5 °C)	872.35	713.88
Altered rpm (360 rpm)	851.99	699.05
Altered rpm (440 rpm)	873.36	717.47
Altered RM composition 75:25	913.95	739.78
Altered RM composition 90:10	821.49	683.44

Table 12. 90% Evaluation of IVRT Method – Robustness

Formulation	90% Confidence Interval for cumulative amount		90% Confidence Interval for slopes		Significant difference
	Lower limit	Upper limit	Lower limit	Upper limit	
31.5 °C vs. 32 °C	95.98	98.56	98.89	101.65	NO
32.5 °C vs. 32 °C	100.18	102.93	103.07	106.02	NO
340 rpm vs. 400 rpm	97.87	100.49	100.92	103.83	NO
440 rpm vs. 400 rpm	100.27	103.07	103.60	106.55	NO
75:25 vs. 80:20	104.78	108.00	106.76	109.92	NO
90:10 vs. 80:20	94.38	96.88	98.70	101.48	NO

Comparison of the drug product formulations

In summary, the developed IVRT method illustrated the additional important and critical properties of being able to accurately discriminate between *in vitro* release rates, which could reflect the similarity and/or differences in product performance. Furthermore, the results indicate that the developed IVRT method has the valuable ability to also detect changes in a formulation, which may be due to Q1/Q2/Q3 properties. The validated IVRT system provides evidence that the experimental design, equipment, and

methodology has the necessary requirements to accurately evaluate the release of API from topical gel products for both innovator and originator formulations.

Three different batches of 1.16 and 2.32% topical gel formulations of generic product were compared with three different batches of 11.6 mg/g and 23.2 mg/g originator gel formulations.

1.16% gel formulations showed a cumulative amount released of 907.93 μg/cm^2, 905.13 μg/cm^2, 837.75 μg/cm^2 of API after 3 hours respectively, with slopes of the release curve 723.72 μg/cm^2/h, 729.95 μg/cm^2/h and 697.26 μg/cm^2/h. The 11.6 mg/g originator gel formulations showed a cumulative amount released of 833.19 μg/cm^2, 850.59 μg/cm^2, 879.86 μg/cm^2 of API after 3 hours and slope of the release curve 679.98 μg/cm^2/h, 692.14 μg/cm^2/h and 718.37 μg/cm^2/h. The 90% CI was calculated for these formulations and evaluation of the results was performed (Table 13).

2.32% gel formulations showed a cumulative amount released of 1587.92 μg/cm^2, 1597.53 μg/cm^2, 1550.02 μg/cm^2 of API after 3 hours respectively, with slopes of the release curve 1298.66 μg/cm^2/h, 1319.48 μg/cm^2/h and 1270.42 μg/cm^2/h. The 23.2 mg/g originator gel formulations showed a cumulative amount released of 1488.48 μg/cm^2, 1471.45 μg/cm^2, 1567.33 μg/cm^2 of API after 3 hours and slope of the release curve 1229.35 μg/cm^2/h, 1243.43 μg/cm^2/h and 1299.59 μg/cm^2/h. The 90% CI was calculated for these formulations and evaluation of the results was performed (Table 14).

Results confirm the suitability of the IVRT method to measure the release rate of API from gel formulations in a reliable and reproducible manner. The calculated upper and lower limits for the pairwise comparisons were inside the CI limits of 90 - 111%. All three batches of the 1.16% gel formulation were found to be similar with 11.6 mg/g originator gel formulation. All three batches of the 2.32% gel formulation were found to be similar with 23.2 mg/g originator gel formulation. As both formulations have same qualitative and quantitative excipients and penetration of API trough skin is not relevant to efficacy, the *in vitro* release test can be used as a nonclinical approach of demonstration of similarity of a generic drug product with a reference drug product, based on the concept of extended pharmaceutical equivalence for topical products (EMA`s Draft guideline on quality and equivalence of topical products, 2018).

Table 13. Evaluation of similarity between 1.16% generic gel formulation vs. originator 11.6 mg/g gel formulation

Formulation	90% Confidence Interval for cumulative amount		90% Confidence Interval for slopes		Significant difference
	Lower limit	Upper limit	Lower limit	Upper limit	
1.16% generic gel formulation, B.No: 1 vs. Originator 11.6 mg/g gel, B.No: 1	108.34%	109.77%	105.80%	107.24%	NO
1.16% generic gel formulation, B.No: 1 vs. Originator 11.6 mg/g gel, B.No: 2	106.14%	107.45%	103.97%	105.25%	NO
1.16% generic gel formulation, B.No: 1 vs. Originator 11.6 mg/g gel, B.No: 3	102.55%	104.54%	100.11%	101.99%	NO
1.16% generic gel formulation, B.No: 2 vs. Originator 11.6 mg/g gel, B.No: 1	108.19%	109.25%	106.86%	108.02%	NO
1.16% generic gel formulation, B.No: 2 vs. Originator 11.6 mg/g gel, B.No: 2	106.01%	106.93%	105.03%	105.99%	NO
1.16% generic gel formulation, B.No: 2 vs. Originator 11.6 mg/g gel, B.No: 3	102.34%	104.11%	101.07%	102.77%	NO
1.16% generic gel formulation, B.No: 3 vs. Originator 11.6 mg/g gel, B.No: 1	99.82%	101.44%	101.80%	103.45%	NO
1.16% generic gel formulation, B.No: 3 vs. Originator 11.6 mg/g gel, B.No: 2	97.78%	99.31%	100.03%	101.54%	NO
1.16% generic gel formulation, B.No: 3 vs. Originator 11.6 mg/g gel, B.No: 3	94.52%	96.57%	96.36%	98.35%	NO

Table 14. Evaluation of similarity between 2.32% generic gel formulation vs. originator 23.2 mg/g gel formulation

Formulation	90% Confidence Interval for cumulative amount		90% Confidence Interval for slopes		Significant difference
	Lower limit	Upper limit	Lower limit	Upper limit	
2.32% generic gel formulation B.No: 1 vs. Originator 23.2 mg/g gel B.No: 1	106.00%	107.55%	105.05%	106.36%	NO
2.32% generic gel formulation B.No: 1 vs. Originator 23.2 mg/g gel B.No: 2	106.72%	108.12%	106.78%	108.02%	NO
2.32% generic gel formulation B.No: 1 vs. Originator 23.2 mg/g gel B.No: 3	103.67%	104.78%	102.91%	103.90%	NO
2.32% generic gel formulation B.No: 2 vs. Originator 23.2 mg/g gel B.No: 1	107.22%	108.82%	103.85%	105.24%	NO
2.32% generic gel formulation B.No: 2 vs. Originator 23.2 mg/g gel B.No: 2	107.95%	109.40%	105.55%	106.89%	NO
2.32% generic gel formulation B.No: 2 vs. Originator 23.2 mg/g gel B.No: 3	104.86%	106.03%	101.72%	102.82%	NO
2.32% generic gel formulation B.No: 3 vs. Originator 23.2 mg/g gel B.No: 1	100.64%	102.37%	99.33%	100.90%	NO
2.32% generic gel formulation B.No: 3 vs. Originator 23.2 mg/g gel B.No: 2	101.32%	102.92%	100.95%	102.48%	NO
2.32% generic gel formulation B.No: 3 vs. Originator 23.2 mg/g gel B.No: 3	98.40%	99.77%	97.28%	98.60%	NO

Using artificial membrane Strat-M as IVPT prediction

In the direction of accurate dermal permeation, a synthetic membrane with structural and chemical characteristics similar to those of human skin was selected for performing an IVRT testing. Strat-M membranes were designed with the intent to share similar structural and chemical characteristics found in the human skin. Additionally, the Strat-M membrane contains a combination of lipids in a specific ratio similar to what is found in the human *stratum corneum*. The human *stratum corneum* is commonly the rate limiting step for successful API delivery (Haq et al.,2018).

Understanding the physiochemical relationship of API/vehicle interactions through a membrane barrier is critical for selection of optimal formulation penetration enhancement efficacy (Flaten et al., 2015). In this regard, during development evaluation of the of Strat-M synthetic membrane permeability and its correlation with human cadaver skin was performed. Synthetic membranes for in vitro permeation studies were originally developed to be used as an alternative to using a human skin models (SUPAC, 1997). Determination of drug permeation of formulations using ex vivo human skin methods possesses several drawbacks which hinder reproducibility data of drug candidate screening including: variations of skin thickness from skin donors, diseased skin states, skin storage conditions, membrane preparation complexity, density of hair follicles, age of donor, and high laboratory costs (Mathes et al., 2014; Semlin et al., 2011). Some advantages of using a synthetic membrane are: controlled membrane thickness, faster membrane preparation time, low storage space, and relatively low cost.

Strat-M is an artificial membrane envisioned as an alternative to animal skin. This multi-layer artificial membrane possesses a tight top layer coated with a lipid blend resembling the lipid chemistry of the human SC and a porous lower layer resembling the epidermis and dermis layers. Other advantages associated with the Strat-M membrane are its simplicity of handling, low-cost, and low variability of lot to lot quality as opposed to animal-based models. The use of Strat-M as an alternative membrane for development, product optimization, regulatory compliance, and safety assessments has been encouraged by several reports (Arce et al., 2020).

Table 15. Evaluation of similarity between 1.16% generic gel formulation vs. originator 11.6 mg/g gel formulation and 2.32% generic gel formulation vs. originator 23.2 mg/g gel formulation using Strat-M artificial membrane

Formulation	90% Confidence Interval for cumulative transport		90% Confidence Interval for slopes		Significant difference
	Lower limit	Upper limit	Lower limit	Upper limit	
1.16% generic gel formulation vs. Originator 11.6 mg/g gel	102.44	111.32	102.47	112.78	NO
2.32% generic gel formulation vs. Originator 23.2 mg/g gel	97.11	102.58	97.51	103.27	NO

IVRT experiment was performed on both, innovator and generic formulations, using artificial Strat-M membrane with Phosphate Buffer Saline (PBS) pH 7.4, for 24 hours, stirring rate of 400 rpm and temperature of 32 °C. Obtained results for CI are presented in Table 15.

The Strat-M membrane similarity to skin features was taken into consideration while evaluating the results. Because the CI for the IVPT study is substantially wider than the CI for IVRT study, obtained results close to the upper limit for 1.16% gel formulation are unconcerned. The variability in the results obtained from the Strat-M membrane approximates the variability of the skin results during the IVPT study. The results support the IVRT method's applicability for measuring the release rate of API from gel formulations in a reliable and reproducible way. A good correlation coefficient for the permeation of API was found between the artificial membrane and the skin.

Evaluation of results

One of the most important and challenging areas in the drug delivery field is to predict the release of the API as a function of time using both simple and sophisticated mathematical models (Gurny et al., 1982). The importance of such models lies in their utility during both the design stage of a pharmaceutical formulation and the experimental verification of a release mechanism (Siepmann et al., 2011).

The API release from matrix systems such as gels, is trough diffusion mechanism. The best way to explain the diffusion mechanism is using the Higuchi model. The Higuchi model for the rate of drug release from matrix devices has been an invaluable tool in developing the modern controlled drug delivery industry (Paul, 2011). Based on a pseudo-steady-state approach, a direct proportionality between the cumulative amount of drug released and the square root of time can be demonstrated. In contrast to various other "square root of time" release kinetics, the constant of proportionality in the classical Higuchi equation has a specific, physically realistic meaning (Siepmann, 2011).

According this mathematical model, drug transport through the gel base is rate limiting, whereas drug transport within the skin is rapid. The skin acts like a "perfect sink". The drug concentration in this compartment can be considered to be negligible.

According to Higuchi's assumptions, less than 30% of the total amount of API applied should be released from the gel base in order to apply this linear model. Higuchi mathematical model was used for calculation of the analytical results. The individual amount of API released per unit area is plotted against the square root of time, which results in a straight line. The Higuchi plot is generated for each cell. The slope of the release curve of API obtained is the rate of release. The drug release rate of API from the dosage form is represented by the average of 12 slopes for the tested formulation. The slope of the release curve reflecting the API release rate and the average cumulative amount of API released per unit area (μg/cm²) after 3 hours from 12 samples were used to evaluate the results. The statistical method used to compare the obtained results for these two parameters was the determination of the 90% CI for the ratio of means of the pairwise comparisons. The obtained upper and lower limits of the interval should fall between the 90-111% ranges specified by the EMA's draft guidance on topical product quality and equivalence.

In vitro permeation study (IVPT) results

In accordance to the EMA draft guideline on quality and equivalence of topical products (2018) the excised human skin from surgery, inserted into a Franz diffusion cell is an appropriate *in vitro* test system to simulate the permeation and penetration processes of an API into and across human skin under *in vivo* conditions. For the permeation study the human skin of twelve donors which was dermatonized to a mean thickness of 500 ± 100 μm was used.

The amount of API which was absorbed into human organism was determined using human excised skin as an *in vitro* model for dermal absorption. The permeated amount of API was quantified over a time period of 48 hours (3, 6, 9, 24, 27, 30, 33 and 48 h). After the permeation

experiment the amount of API taken up into the skin was determined. For this penetration study the SC was stripped by the so called "tape stripping technology".

Both test and reference products exhibit the same permeation characteristics through human skin. In accordance to the EMA draft guideline the originator and test product exhibit no significant difference for cumulative transport and J_{max} (limits of 90% CI lay between 80 and 125%).

Conclusion

To meet the requirements for evaluating the release profile of the active pharmaceutical ingredient in concentrations of 1.16% and 2.32% in topical gel formulations a suitable IVRT method was developed and validated. The procedure was carried out in accordance with the requirements of the EMA's draft guideline on quality and equivalence of topical products. The method employed provides an effective and simple evaluation of the important validation parameters of an IVRT method. Following satisfactory validation, the IVRT approach was used to determine the similarity of the formulations. Both, 1.16% and 2.32% topical gel formulations were found to be similar with its originators. The use of the Strat-M membrane as a predictor of IVPT study was demonstrated to be a good strategy due to its similarity and variability to human skin. Positive outcome of IVPT study approved the Strat-M prediction as well as similarity of both gel formulations with its originators.

The developed and validated IVRT method has the potential to be used as a useful tool for evaluating different topical gel formulations as well as verifying the quality of ongoing manufacturing process and batch to batch performance. IVRT method can be used as a cross effective tool for evaluation of the quality of topical gel formulations utilized in place of a BE study.

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Table 16. IVPT results for 90% Confidence Interval for evaluation of similarity between 1.16% generic gel formulation vs. originator 11.6 mg/g gel formulation and 2.32% generic gel formulation vs. originator 23.2 mg/g gel formulation

Formulation	Cumulative transport after 48 h (μg/cm^{-2})		J_{max} values (μg/cm^{-2}/h^{-1})		Significant difference
	Lower limit	Upper limit	Lower limit	Upper limit	
1.16% generic gel formulation vs. Originator 11.6 mg/g gel formulation	99.95	113.76	93.87	108.75	NO
2.32% generic gel formulation vs. Originator 23.2 mg/g gel formulation	97.31	112.23	98.69	114.69	NO

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Резиме

Развој и валидација на тест за *in vitro* евалуација на профилот на ослободена активна супстанција и употреба на синтетска мембрана *Strat-M* како предикција за *in vitro* тестот за пропусливост на гел за надворешна употреба

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Клучни зборови: IVRT, IVPT, алтернативна *Strat-M* мембрана, гел за надворешна употреба, валидација на метод

Целта на ова истражување беше развој на *in vitro* метод што би бил соодветен за евалуација на профилот на ослободена активна супстанција (IVRT) од гел за надворешна употреба. За да може да се манифестира фармаколошката активност на производ за надворешна употреба, активната супстанција мора прво да се ослободи од матриксот, да навлезе во слоевите на кожата и да стигне до местото на делување. Физичко-хемиските карактеристики на формулацијата имаат најголемо влијание врз ослободувањето на активната супстанција.

Насоките дадени во водичот на Европската агенција за лекови (EMA) за евалуација на производи за надворешна употреба беа следени за евалуација на критичните карактеристики што влијаат врз перформансот на полуцврстите дозирани форми како и при процесот на валидација на IVRT методот. Во тек на развојот факторите што можат да влијаат на тестот како што се изборот на рецепторен медиум и квалификација на мембрана беа соодветно анализирани, проследени со евалуација на точност и робустност на методот. Преку анализирање на гел формулации кои содржат 50% и 400% (односно 200% за 2,32% гел) активна супстанција беше потврдена линеарноста на методот како функција на концентрацијата на активната супстанција во формулацијата. Дискриминаторната моќ на методот беше потврдена преку анализирање на формулации со променета вискозност (помала/поголема вискозност) како критичен атрибут за квалитет.

Студија за пропустливост на активната супстанција во тек на 24 часа беше изведена со користење на синтетска *Strat-M* мембрана земајќи ги во предвид нејзините параметри за пропустливост со цел да се предвиди исходот од студијата за *in vitro* пропустливост (IVPT). За евалуација на резултатите од профилот на ослободена активна супстанција беше користен статистички пристап преку пресметка на 90% интервал на доверба. По развојот и валидацијата на IVRT методот, двата производи на диклофенак гел беа споредени при што беше потврдена нивната сличност.