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Research paper

Drilling down the bioequivalence assessment of topical antifungal products: Microstructure and release

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ABSTRACT

In recent years, the regulatory mechanisms for topical generic product bioequivalence (BE) assessment have been subjected to noteworthy changes, with the FDA issuing product specific guidances, and the EMA adopting a more universal approach with the quality and equivalence of topical products draft guideline. The agencies advise on a modular strategy for BE documentation. Nevertheless, their scope, data analysis and criteria are rather distinct.

This study aims to tackle bioequivalence assessment issues of complex topical formulations starting by statistical implications of the EMA/FDA approaches concerning the documentation of qualitative (Q1), quantitative (Q2), microstructure (Q3) and performance requirements (Q4). As a model drug product, a bifonazole 10 mg/g cream formulation was selected. For this specific formulation, the commercially available Reference Product (RP) was compared with two comparator products, also commercially available, referred to as comparator product A (CPA) and comparator product B (CPB). The former displays Q1 sameness and Q2 differences, whilst CPB is categorically considered as Q1/Q2 different. Furthermore, intending to establish a regulatory rationale for the submission of a generic product according to the updated regulatory requirements, the RP was likewise compared with a Test Product (TP). This formulation was designed to display equal Q1/Q2 profile to that of the RP. Validated rheology and *in vitro* release test (IVRT) methods were used to infer on Q3/Q4 characteristics.

During rheology studies, statistically significant RP batch to batch differences were observed. Therefore, in an attempt to surpass this heterogeneity, the initial pool of RP batches was expanded to include RP product batches at different lifecycle stages. Despite this effort, it was not possible to classify the RP/TP, RP/CPA or RP/CPB as rheologically equivalent products. Nevertheless, product performance results, retrieved from IVRT, were able to sustain equivalence between the RP and the formulations exhibiting Q1 sameness (TP and CPA). It should however be mentioned, that for some RP batch combinations, the IVRT results failed to demonstrate equivalence according to the EMA requirements. Enlarging the RP batch pool was then a critical step in further understanding an optimum statistical approach for establishing equivalence in product performance.

This study highlights the need to that a 'one-fits-all approach' may not be an optimum path way for establishing the regulatory strategy and requirements to support generic product bioequivalence.

Abbreviations: API, Active Pharmaceutical ingredient; BE, Bioequivalence; BFZ, Bifonazole 10 mg/g cream; C, Compliant; CI, Confidence interval; CQA, Critical Quality Attribute; CPA, Comparator product A; CPB, Comparator product B; EMA, European Medicine Agency; FDA, US-Food and Drug Administration; Ġ, Storage modulus; Ġ, Loss modulus; HPLC, High Performance Liquid Chromatography; IVRR, *In Vitro* Release Rate; IVRT, *In Vitro* Release Testing; LVR plateau, Linear Viscoelastic Region plateau; η_0, Zero-shear viscosity; η_∞, Infinite shear viscosity; NC, Non-compliant; Osc. Tests, Oscillatory tests; Q1, Quantitative sameness; Q2, Qualitative sameness; Q3, Microstructure sameness; Q4, Performance sameness; Qf, Cumulative amount of drug released in the end of the IVRT study; Rot. Tests, Rotational tests; RP, Reference Product; RSD%, Relative Standard Deviation; Sensi., Sensitivity; S_R, Relative thixotropic Area; SUPAC-SS, Scale-Up and Postapproval Changes: Chemistry, Manufacturing, and Controls, *In Vitro* Release Testing and In Vivo Bioequivalence Documentation. FDA guidance for Industry; TGP, Topical Generic Product; TP, Test Product; $\tau_{0.OSC}$, Yield point obtained through oscillatory methods; $\tau_{0.ROT}$, Yield point obtained through rotational methods; τ_f, Flow point.

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1. Introduction

Superficial fungal infections are becoming more common, affecting 20–25% of people worldwide at any given time. This may be ascribed to changes in socioeconomic conditions, lifestyle, migration, but also to medical practices. Within the available pharmacotherapeutic classes, topical azoles, such as bifonazole, are particularly used, due to their proven efficacy, affordability, and over-the-counter (OTC) availability [1]. The growing popularity of OTCs, aligned with an increase in onychomycosis cases is also expected to increase the market growth of topical antifungals by 3.5% (2022–2027) [2]. Nowadays, however, the readiness, and consequently the affordability of such products is linked to the scientific and regulatory mechanisms involved in the development and approval of topical generic products (TGP) [3]. Over the past decade, topical dermatological products have experienced extraordinary price increases. According to a report from the FDA's Government Accountability Office, this trend is directly related to the shortage of multisource generic drugs [4,5]. A major reason for this shortage is the difficulty in documenting topical bioequivalence (BE). Two main factors contribute to this difficulty: (i) As the skin is the target site of most topical semisolid formulations, undetectable or extremely low drug levels can be measured systemically. In this context, the gold standard method for establishing BE of TGP significantly relied on comparative clinical efficacy studies. However, such studies are often complex, time consuming and prohibitively expensive when compared to the market size and likely project sales [4,6-9]. Traditionally, the only other established BE approach has been the vasoconstrictor assay, which is exclusively applicable to topical steroids [10]; (ii) Multiphasic systems, such as creams, can be considered as highly complex dosage forms because their quality attributes exhibit a myriad of interdependencies. These are influenced by the physicochemical properties of both the active pharmaceutical ingredient (API) and excipients, by the formulation characteristics, and also by the manufacturing process itself.

In the past, it was not uncommon for TGPs, often submitted as bibliographic applications, to have a different Q1 qualitative profile compared to the reference product (RP). Consequently, these "older generation" products often presented different organoleptic characteristics [4]. Nowadays, both American as well as European agencies, are increasingly reluctant in approving such applications. For this reason, and to encourage market competition, the FDA is dynamically sponsoring several scientific and regulatory initiatives with the sole objective of developing surrogate approaches for demonstrating topical products BE. These have resulted in the FDA non-binding product-specific guidances for the development of TGPs, which recommend specific approaches depending on product features. In Europe, the EMA draft guideline on quality and equivalence of topical products, through a different perspective, also laid the groundwork to provide alternative *in vitro* methods to clinical endpoint studies to demonstrate BE. Both agencies intend to pave the way for a new generation of TGP that are not only therapeutically equivalent to their reference products, but also comparable in appearance, feel, smell, and application/drying behaviour [4]. To achieve such similarity, the applicant is encouraged, by both FDA and EMA, to pursue a modular framework to establish BE. The first steps, Q1 and Q2 sameness, relate to the qualitative and quantitative profile of the TP, respectively. These should be consistent with those of the RP. The next steps comprise Q3 and Q4 sameness documentation, which regard the microstructure and the performance of the TP. These, likewise, should be equivalent to the RP. Altogether and according to EMA, the demonstration of Q1 to Q3 sameness can be defined as the documentation of the *(extended)* pharmaceutical equivalence. The final steps of the flowchart for BE establishment *in lieu* of clinical endpoint studies, is generally solely applicable to multiphasic formulations, and it focuses on the documentation of the local drug skin availability, herein termed as TP efficacy profile. This can be determined through *in vitro* permeation tests (IVPT), dermatopharmacokinetic methods or vasoconstrictor studies, the latter solely applicable to corticosteroid products (European Medicines [11]. Efficacy studies represent the cornerstone of BE documentation for more complex formulations, as they are designed to directly relate to the clinical characteristics of the TP. Nevertheless, it should be pointed out that establishing the extended pharmaceutical equivalence through validated methodologies as prescribed in the guidance can also be a long and laborious task.

Q3 equivalence should be demonstrated by submitting data on pH, droplet/particle size, product metamorphosis, rheological behaviour analysis, among other parameters. However, since rheological parameters are particularly representative of the microstructure of topical products, the EMA draft guideline specifies that numerical data should be produced to describe and compare the rheological profiles of the formulations (European Medicines [11,12]. Then, Q4 sameness, supported by IVRT methods, should likewise be evidenced.

Based on previous research conducted by our group, a bifonazole 10 mg/g cream RP was found to be a complex formulation with pronounced variability [13]. Based on these experimental observations, an expanded pool of batches for this specific RP was considered in this study. All RP batches were analysed for their rheology and Q4 profile. Two comparator products, referred to as comparator product A (CPA) and comparator product B (CPB) were also included. The former displayed Q1 sameness and Q2 differences, whilst the latter is categorically considered as both Q1 and Q2 different. Both comparator formulations are commercially available. Furthermore, in order to replicate the development and submission of a truly generic formulation, a test product (TP) with Q1/Q2 sameness to the RP was manufactured and compared with the RP.

2. Materials and methods

2.1. Materials

Bifonazole cream products were acquired from the European Market. All the products displayed the same active pharmaceutical ingredient, same dosage form and strength. Four different products were included in this study (Tables 1 and 2):

(i) The RP (Bayer). Five distinct batches of the RP were sourced in different markets: The Portuguese (Canesten Unidia®, which were then defined to as RP1 and RP3), the Spanish (CanesMycospor®, RP2), and the German market (Canesten Extra®, RP4 and RP5). The different commercial names are due to the market source; please note that all RP batches were collectively considered as the RP.
(ii) A qualitative and quantitative formulation similar to the RP was manufactured. This formulation was defined to as the TP. Three batches were considered (TP1-TP3);
(iii) A commercially available Q1 formulation to the RP was also studied – Comparator product A (CPA) – Amycor® 1% (Merck Serono);
(iv) A commercially available Q1/Q2 RP different formulation – Comparator product B (CPB) – Levelina® crema (Ern Laboratories).

Due to market availability, only one batch was considered for CPA and CPB formulations.

The qualitative composition of all products is shown in Table 2.

Table 1
Study design.

Reference formulations	Test products		
RP	TP – TP1, TP2, TP3	CPA	CPB
RP1, RP2, RP3, RP4, RP5	Q1, Q2 equivalent	Q1 equivalent	Q1, Q2 non-equivalent

Phosphate buffered saline (PBS) was purchased from Sigma. Water was purified using a Millipore MILLI-Q reagent water system and filtered through a 0.22 μm nylon filter before use. All other chemicals were of analytical grade or equivalent.

2.2. Methods

2.2.1. Formulation production

To evaluate the discriminatory ability of the methods used to assess microstructure and performance, different bifonazole cream formulations were manufactured and included a 5 mg/g, a 20 mg/g, and a placebo cream formulation. Furthermore, based on formulation development studies, it was determined that a critical excipient responsible for the viscosity profile was cetostearyl alcohol, therefore its content was reduced by half to obtain a formulation with different rheology/microstructure. For the TP, the Q1/Q2 composition of the RP (Table 2, note that for confidentiality reasons, the quantitative composition cannot be disclosed) was replicated.

In the manufacturing process, both the aqueous and lipophilic phases were prepared separately and heated to 68 ± 2 °C. Afterward, bifonazole was added to the dispersed phase. Both phases were then combined and cooled to 20–25 °C. All formulations were prepared conventionally resorting to an Ultra-Turrax X 10/25 (Ystral GmbH, Dottingen, Germany) as blending equipment. The optimal experimental settings in terms of speed, duration and temperature of the manufacturing processes were carefully optimized during the formulation studies (data not shown).

2.2.2. Microstructure evaluation

Comparative microstructure studies constitute a key element for establishing Q3 equivalence [14,15] and there are a plethora of parameters/methods that should be evaluated together to draw conclusions about semisolid microstructure. Among these, rheology methods are particularly important due to their regulatory status, *as per* EMA draft guideline requirements [14,15].

Previous studies by our group using bifonazole 10 mg/g cream RP have shown that the differences observed in pH and in globule size were not as meaningful as those registered in rheological endpoints. For this reason, microstructure evaluation was directed towards a detailed characterization of the formulations rheological behaviour. Rheology studies were performed according to [16]. Briefly, rotational tests were performed with a C35/2°/Ti cone geometry. For the flow curve [$\eta = f(\tau)$], a linear CS flow ramp ranging from 0.01 to 100 Pa was measured for 300 s. On the other hand, to assess the thixotropic behaviour (Pa/s), a shear rate from 0.01 to 300 s^{-1} and again down to 0.01 s^{-1}, during 180 s was used. In all tests, approximately 0.3 g of each formulation were used. Regarding oscillatory measurements, a plate geometry (P35/Ti) was used and approximately 1 g of the formulations were placed in the *peltier* plate. An amplitude sweep ranging from 0.01 and 500 Pa at 1 Hz was firstly conducted to estimate the linear viscoelastic region (LVR), as well as the flow point (τ_f). Afterward, a frequency sweep analysis was performed within the LVR range to determine the storage modulus (G) and loss modulus ($\acute{G}$) from 100 to 0.1 Hz. Results are presented for 1 Hz. All measurements were performed in triplicate at 32 °C, the physiological skin temperature. A sample hood was used to minimize sample volatilization and a positive displacement syringe was used to place the formulations in a lower TMP35 plate. A preset gap of 0.1 mm was considered for all samples. A HAAKE MARS 606,000 (Thermo Scientific, Karlsruhe, Germany) equipped with a MARS controlled temperature module was employed. Data analysis was performed with the Haake RheoWin Data Manager software (Thermo Scientific, Karlsruhe, Germany).

2.2.2.1. Rheology method validation. The suitability and discriminatory capacity of rheological methods should be adequately established. To document so, a formulation with different rheology profile was manufactured. As previously detailed, one of the excipients that plays a central role on the formulation viscosity profile is cetostearyl alcohol. Therefore, the concentration of this excipient was reduced by half to obtain a formulation with significantly different viscosity. By tracing the rheological profile of the altered formulations, the sensitivity and selectivity of the proposed methods can be substantiated, as the differences in microstructure are highly dependent on excipient concentration [14,17–21]. The methods were considered sensitive if the rheological endpoints obtained with the RP were different when compared to altered formulations. To assess the selectivity of the method, i.e. the ability of the method to show that the rheology profile of the nominal (reference) and the varied (negative control) formulations are inequivalent, the 75–133% confidence interval (CI) were determined for each rheological endpoint. The method was considered to show selectivity if the attained CI was outside the 75–133% range.

Table 2
General information and qualitative composition of the products used in the present study. Batch age is given in months (M). The RP has an expiry date of 5 years, CPA of 3 years and CPB of 4 years.

Studied products		RP	TP	CPA	CPB
	Used batches	RP1: 32 M RP2: 32 M RP3: 26 M RP4: 14 M RP5: 12 M	TP1: 12 M TP2: 17 M TP3: 17 M	CPA: 11 M	CPB: 11 M
Excipient	Function				
Benzyl alcohol	Preservative	X	X	X	
Cetostearyl alcohol	Emulsifier	X	X	X	X
Cetyl palmitate	Thickener	X	X	X	
Disodium EDTA	Chelating agent				X
Methyl parahydroxybenzoate	Preservative				X
Mineral oil	Emollient / emulsifier				X
Octyldodecanol	Emulsifier	X	X	X	
Polyoxyethylene stearate 40	Emulsifier				X
Polysorbate 60	Emulsifier	X	X	X	
Propyl parahydroxybenzoate	Preservative				X
Polyethylene glycol 400	Co-solvent				X
Purified water	Solvent	X	X	X	X
Sodium Hydroxide	Buffering agent				X
Sorbitan monostearate	Emulsifier	X	X	X	
Vaseline	Thickener				X

Key: RP – Reference Product; TP – Test Product; CPA – Comparator Product A, and CPB – Comparator Product B.

2.2.3. Product performance evaluation – IVRT studies

IVRT studies were performed in a static vertical Franz diffusion cell equipment, with a diffusion area of 0.636 cm^2 and a receptor volume of 5 mL (PermeGear, Inc., PA, USA). Diffusional cell system and laboratory qualification studies were carried out as described in [18]. Table 3 summarizes the specific IVRT conditions used for the bifonazole 10 mg/g cream formulations.

The cumulative amount of drug released as a function of time was calculated in relation to the amount of formulation placed in the donor compartment using the following equation

$$Qn = (Cn \times Vo + \sum_{i=1}^{n=1} Ci \times Vi)/A \quad (1)$$

where C_n corresponds to the drug concentration of the receptor medium at each sampling time, C_i to the drug concentration of the i^{th} sample, A to the effective diffusion area, and V_0 and V_i to the volumes of the receptor compartment and the collected sample, respectively. The release rates were calculated from the Higuchi's equation, according to SUPAC guidance, and the EMA draft guideline. Higuchi first described the drug release from topical ointments, as a pseudo-steady state, in which the diffusion coefficient remains constant when the drug content in the matrix exceeds solubility [14,22–24]. The percentage of bifonazole released was also calculated by the average cumulative amount of drug released at the last sampling point (Qf), divided by the actual amount of active substance placed in the diffusion cell, and multiplied by 100. According to the European regulatory requirements an n = 12 was in all cases considered.

The concentrations of bifonazole in IVRT samples were determined with a qualified HPLC method. The instrumentation consisted of a Shimadzu LC-2010HT apparatus equipped with a quaternary pump (LC-20AD), an autosampler unit (SIL-20AHT), an oven (CTO-10AS), and a detector (SPD-M2OA). Chromatographic separation was performed using a XBridge™ C18 column (2.1 × 150 mm, 5 μm particles) held at 30 °C with a mobile phase composed of a buffer solution (2 mL of phosphoric acid with 980 mL of ultra-purified water, adjusted to pH 3.2 ± 0.05 with trimethylamine) and acetonitrile (68:32, v/v), at a flow rate of 0.5 mL/min. The total run time was 5.5 min and the detection wavelength was set at 210 nm. The quantitation range was 0.1 – 150 μg/mL. Analytical method validation results are carefully detailed in the supplementary material (Tables S1-S5 and Figure S1) [25].

2.2.3.1. IVRT method validation. According to the regulatory requirements, membrane inertness, linearity, precision, and robustness studies should be carried out to validate the IVRT method. Moreover, the IVRT discriminatory power should also be documented [14,26,27]. To evaluate whether there are interactions between tuffryn membranes and bifonazole, membrane inertness studies were performed by incubating the membrane in a 35 μg/mL bifonazole solution, under the same experimental conditions as IVRT studies and then measuring the drug recovery. A negative control (solution without membrane) was in all cases prepared. The membrane was considered to be inert, if at least, a 95% drug recovery was achieved [14,18,27,28].

To test linearity, the amount of drug released per unit area should be linear to the square root of time. In this context, a coefficient of determination (R^2) in excess of 0.9 was considered acceptable. Cells that did not exhibit the adequate linearity were not considered.

For method precision and reproducibility studies, three IVRT runs were conducted, on three different days, by two different operators, each with a set of 12 vertical diffusion cells. For this analysis, the same TP batch was used (TP1). Intra- and inter-run variability were estimated for IVRR and Qf. Because these studies were performed by two operators, this assessment also enabled the determination of operator variability. Even though a RSD of less than 10% is required by EMA, in this work a RSD of less than 15% was considered acceptable based on the intrinsic variability linked to IVRT and also since this threshold is considered suitable by the FDA [27–29].

To assess whether the IVRT method had an adequate discriminatory power, sensitivity, specificity and selectivity were evaluated. For this, the IVRT endpoints – IVRR and Qf were determined using formulations with different strengths and rheology profiles. The IVRT method was considered sensitive when the IVRR/Qf of the lower strength formulation were significantly lower than the nominal formulation and the IVRR/Qf of the higher strength formulation were higher than the nominal formulation. On the other hand, the method was considered specific when a linear relationship (at least R^2 greater than 0.9) was achieved between formulations with different drug concentration levels [6,14,18,30].

The supplemental selectivity of the IVRT method was also investigated by tracing the release profile of a formulation with half of the cetostearyl alcohol concentration, when compared to the RP/TP. As noted in the rheology method validation studies, changing the concentration of thickening agents is expected to lead to formulations with a distinctive rheology profile and, consequently, with a different release behaviour. In this context, the formulations prepared with a distinct rheological profile were also examined during the IVRT studies to further document this relationship and also to assess whether the purposed IVRT methodology was selective. Considering this information, the selectivity of the method could be stated if the confidence interval (CI) determined with the endpoints retrieved from all altered formulations (strength and rheology profile) fall outside the range of 90–111%. For IVRT discriminatory studies, 12 replicates of each formulation were also performed.

Finally, to investigate the robustness of the IVRT method, two IVRT runs of 12 replicates each were performed at slight temperature differences, +2°C and −2°C, from the nominal temperature of 37 °C specified by the IVRT. The method was considered robust, if the IVRR and Qf did not deviate more than 15% from the mean release rate at nominal method parameter settings [14,27]

Table 3
Receptor solution, sampling times, and donor drug loading used for IVRT studies.

Receptor phase	PBS-Ethanol (50:50, v/v) pH = 7.4
Donor drug loading technique	Positive displacement syringe
Applied formulation	150 mg, evenly placed over the membrane. Efforts were made to ensure a reproducible and consistent formulation application procedure (±5%)
Sampling times (h)	0.25, 0.5, 1, 2, 3, 4, 6, 8, 10 and 24 h
Temperature	37 °C, to assure 32 °C at the membrane surface
Membrane	SUPOR 450 pore size 0.45 μm, Pall Corporation, USA The membrane was previously soaked in purified water for 30 min
Agitation	600 rpm
Equilibration period	30 min
Sampling and replacement volume	300 μL
Occlusion	Performed with Parafilm® in the donor compartment, as well as in the sampling arm

2.2.4. Data analysis and statistics

To perform the equivalence test, as well as the sensitivity analysis of quantitative physicochemical parameters, the 90–111% or the 75–133% confidence intervals (CI), both calculated at a 90% confidence level, were determined.

For rheology and IVRT data, the CI was obtained by calculating the ratio of means between the test/reference formulations, following the EMA draft guideline approach. The data was natural log transformed. and then the means and the standard deviations were calculated. This was followed by obtaining the ratio of the two back-transformed averages

Equations (2) and (3) were then used for CI calculations.

$$\frac{\overline{X_1}}{\overline{X_2}} \pm t_{1-\alpha/2,n_1+n_2-2,} S_p \sqrt{\frac{1}{n_1}+\frac{1}{n_2}} \quad (2)$$

$$S_p = \sqrt{\frac{(n_1-1)\times s_1^2 + (n_2-1)\times s_2^2}{n_1+n_2-2}} \quad (3)$$

where $\overline{X}$ is the mean value to evaluate the test ($\overline{X_1}$) or reference product ($\overline{X_2}$), $t_{1}.\alpha_{/2}$ is the student's t value for $\alpha = 0.90$, s is the standard deviation, and n the number of observations.

Additionally, solely for IVRT data, the 75–133% CI calculated at a 90% confidence level was determined following the Wilcoxon Rank Sum/Mann-Whitney rank test recommended by the SUPAC.

Accordingly, the first step involved in the computation of this CI was to form a 144-element matrix, which corresponded to the 12 × 12 individual TP/RP ratios. Then, these individual ratios were ranked from lowest to highest. The 43rd and the 121st ordered individual ratios regarded the lower and upper limits, respectively, of the CI.

Data analysis was conducted using Microsoft Office Excel®.

3. Results and discussion

3.1. Rheology and IVRT method validation

According to the EMA guideline, evidence on the discriminatory power of the product characterization methods should be properly justified. To this end, both rheology and IVRT methods validation studies were carried out. Even though US and European directives are clear on the level of validation required for IVRT studies, the same still does not occur for rheology method validation. Concerning rheology data, from a regulatory point of view, and following the recent guidance issued by FDA on the physicochemical and structural (Q3) characterization of topical drug products submitted in ANDAs, the following information must be submitted: (i) complete flow curves, (ii) yield stress values whenever applicable, and finally (iii) the respective linear viscoelastic response. These evaluations should ideally be performing considering a minimum of three batches of the test topical product and three batches (whenever available) of the reference standard [31]. Note, however, that no information on the number of repetitions is provided, therefore only a minimum number of three was considered in this work to infer on the statistical meaning. In an attempt to present a validation protocol directed towards this technique, the strategy proposed by Miranda et al. was herein adapted [16]. Rheology method validation was performed in terms of precision, selectivity and sensitivity. All RP formulations were considered in this analysis, as well as the negative control formulation.

With regards to the rheological total variability (rheological method precision, i.e., the closeness of the repeated individual measures between all RP batches), it should be noted that extremely high RSD were registered in the RP (11–512%) [32]. However, if the intra-batch RSD values are considered instead, method precision can overall be supported, see Table 4.

To document the sensitivity of the method, the rheological endpoints obtained with the negative control formulation should be lower when compared to the RP. For 7 out of 9 endpoints this condition was registered, nevertheless, a lack of compliance was observed for the relative thixotropic area (S_R) and for the yield point estimated through oscillatory endpoints (τ_{OSC}).

The relative thixotropic area (S_R) derived from the thixotropic rheograms provides information on the formulation breakdown and recovery and after the shearing process, respectively. The S_R, also referred to as the hysteresis loop area, is generally regarded as the measure of the thixotropy in the formulation [17,19].

As shown in the Fig. 1, all RP batches revealed a full thixotropic recovery, which is regarded as a good stability indicator, since it reflects the capacity of the cream microstructure to fully recover after the shear termination. The high variability herein denoted may be associated with batch age, since the batches with more prolonged shelf life (RP1, RP2 and RP3) displayed an inferior S_R (4492, 6290, 3269P/s, respectively) when compared with the batches at an early life cycle stage (RP4 + RP5 = 12750, 15,563P/s, respectively). The thixotropic properties appear to reflect a time dependent structure degradation, as batch age increased, the S_R decreased, which is indicative of a weaker internal structure and a lower tolerance to stress when compared to the "younger" batches [17,19].

The yield point estimated through oscillatory measurements regards the minimum shear stress that must be applied to induce material flow. In this work, this parameter was retrieved from amplitude sweep measurements and corresponded to shear stress value in which the LVR *plateau* ceased. No obvious correlation between batch age and yield

Table 4
Rheology method validation results. Rheological endpoint results pertaining to the RP regard an n = 15. For altered rheology formulations, an n = 3 was considered. To assess method selectivity, the 75–133% confidence interval (CI) calculated at a 90% confidence level of the ratio (altered rheology/RP) is presented. If the CI of the considered rheological endpoints was not within the 75–133% range, selectivity could be inferred.

	Reference Product							Negative control formulation		
Rheological endpoints	**Mean**	**RSD% overall**	**RSD% RP1**	**RSD% RP2**	**RSD% RP3**	**RSD% RP4**	**RSD% RP5**	**Mean**	**RSD%**	**90% CI**
η_0 (Pa.s)	18,905	13.0	13.0	3.19	0.93	0.65	7.24	9779	1.0	45.12 – 60.35
τ_{ROT} (Pa)	43.3	26.40	2.44	4.15	1.02	6.61	4.47	24.2	2.7	42.46 – 79.85
η_∞ (Pa.s)	16.2	55.14	45.3	11.6	14.8	17.6	4.23	0.041	2.868	0.13 – 0.89
S_R (Pa/s)	8473	60.52	35.3	18.0	6.68	16.7	16.7	8556	19	61.24 – 240.64
LVR (Pa)	1037	32.38	6.88	4.04	8.14	4.47	13.2	420	3.0	31.47 – 56.46
τ_f (Pa)	329	48.04	11.2	0.00	13.2	4.77	4.66	103	32	21.97 – 59.07
τ_{OSC} (Pa)	25.6	23.93	10.0	7.87	23.62	10.7	5.06	27.7	19.8	81.72 – 147.87
G – **1 Hz (Pa)**	828	11.0	4.00	1.00	1.00	1.00	0.13	449	2.0	48.71 – 60.84
G – **1 Hz (Pa)**	233	22	10.0	10.0	2.00	9.0	9.0	115	9.0	40.17 – 63.17
Validation parameter	Acceptance criteria									
Sensitivity	Acceptance criteria: RP > Altered Rheology formulation Rotational endpoints: Compliant except for S_R Oscillatory endpoints: Compliant except for τ_{OSC}									
Selectivity	RP ≠ Altered Rheology formulation Rotational endpoints: Compliant for all endpoints Oscillatory endpoints: Compliant for all endpoints									

Key: η_0 (Pa.s) – Zero-shear viscosity; η_∞ (Pa.s) – Infinite-shear viscosity; $\tau_{0.ROT}$ (Pa) – Yield point obtained through rotational methods; S_R (Pa/s – Relative thixotropic Area; $\tau_{0.OSC}$ (Pa) – Yield point obtained through oscillatory methods; LVR plateau (Pa) – Linear Viscoelastic Region plateau; τ_f (Pa) – Flow point; G – Storage modulus; Ġ – Loss modulus; Sensi – Sensitivity; Green label – Compliant results; Red label – Non-compliant results; CI – Confidence interval. To interpret the color references in the table caption, the reader is referred to the web version of this article.

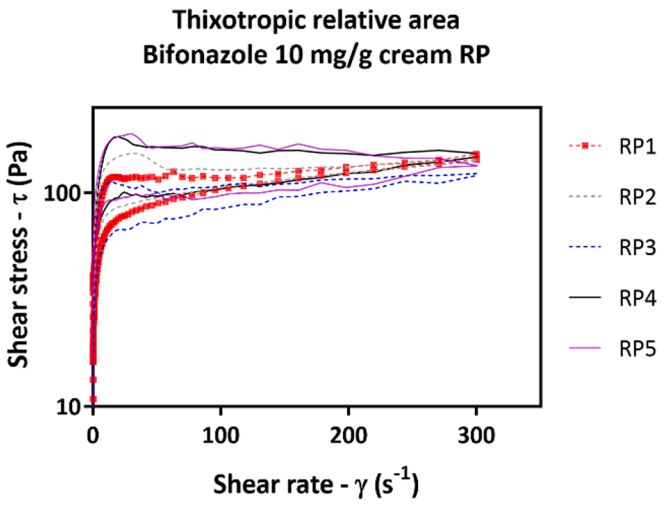

Fig. 1. Thixotropy profile of the reference products. The results report to mean values. Three replicates were used per batch formulation.

point was observed. Despite the lack of sensitivity reporting to the τ_{OSC}, the remaining amplitude sweep indicators (τ_F and LVR plateau) adequately documented compliance with the established criteria.

For method selectivity, it should be noted that a direct application of EMA criteria is not possible, since (i) several rheological parameters did not follow a normal distribution and (ii) a higher than 10% RSD is registered between the different RP batches. In this context, and similarly to the published work by Maria Pleguezuelos-Villa, a larger criterion [75–133%] was selected for selectivity studies, as well as for equivalence studies [14,20]. To support method selectivity, statistical inequivalence between the RP and the negative control formulation should be observed. Even though the calculated CI were outside the 75–133% criteria (Table 4), the CI of the τ_{OSC} endpoint was partially inside this range. This occurrence is in line with the previously discussed sensitivity results.

Taking all the information into account, the rheological methods were considered fit for the purpose of this study since an adequate precision and discriminatory power were overall demonstrated.

IVRT method validation studies were likewise performed according to US and European guidelines and the results are summarized in Table 5 [14,27].

The release medium revealed to be suitable according to regulatory requirements, as sink conditions were registered [13]. Historically, ethanol based solutions are commonly employed as a release medium in IVRT experiments due to its miscibility profile with aqueous solutions, and overall suitability for analytical processing [33]. The selection of a diffusion membrane is also a key parameter of the IVRT method. A suitable IVRT membrane should provide an inert holding surface, but not constitute a barrier for drug release. The membrane must display chemical compatibility with both formulations, as well as release medium, and should not contain any leachables. Furthermore, a reduced back diffusion should be observed, in order to avoid product transformation. Membrane inertness studies revealed that the selected SUPOR membrane fulfils these requirements, as a bifonazole recovery of 99.63% was attained (Table 5). The selection of the release medium, membrane and overall experimental setup (sampling points, speed, temperature, amount of formulation applied) should enable the acquisition of a linear release profile [23]. As linearity was observed in every diffusion cell, it was demonstrated that steady-state kinetics conditions were achieved with these experimental conditions (Table 5). Even though the guidelines recommend $R^2 \geq 0.90$ over the entire IVRT time range, the correlation coefficient is not a very discriminatory parameter, therefore a higher R^2 ($R^2 > 0.97$) should be registered to demonstrate adequate linearity of release [14,27,33]. This refers to the Higuchi

Table 5
Acceptance criteria for bifonazole 10 mg/g cream IVRT method validation studies based on regulatory requirements [14,27]. The same TP batch was used during IVRT validation studies.

Parameter	Results	Acceptance criteria	Status
Membrane inertness	Bifonazole recovery: 99.63%	Recovery ≥ 95%	Compliant
Linearity	R^2 = 0.979 ± 0.017 (n = 36)	R^2 greater than 0.90	Compliant
Precision and reproducibility, including operator variability (IVRR data reports to µg/cm²/√h and Qf to µg/cm²)	Run 1 (operator A): IVRR = 310 (6%) | Qf = 1258 (8%) Run 2 (operator B): IVRR = 243 (8%) | Qf = 967 (7%) Run 3 (operator B): IVRR = 326 (6%) | Qf = 1277 (6%) **Intra-run variability** (n = 36) RSD IVRR = 6.75% |RSD Qf = 7.07% **Inter-run variability** (n = 36) RSD IVRR = 14.04% | RSD Qf = 14.10%	**Intra-run and inter-run variability:** RSD Qf ≤ 15% RSD IVRR ≤ 15%	Compliant
Selectivity (Data reports to IVRR, µg/cm²/√h)	n = 12 0.5% vs. 1% → CI = [14.05 – 17.11%] 1% vs. 2% → CI = [273.29 – 329.53%] 1% vs. ≠ CQA → CI = [39.60 – 49.97%]	CI between different strength products falls outside the limits [90–111] %	Compliant
Robustness (Data reports to IVRR, µg/cm²/√h)	n = 12 Mean IVRR 37 °C = 310 (6%) Mean IVRR 35 °C = 305 (8%) Mean IVRR 39 °C = 317 (6%)	Mean IVRR of runs with minor temperature fluctuations should not deviate more than 15% from the IVRR of the nominal method parameter settings	Compliant

equation which is based on Fick's first law of diffusion. Under this paradigm, it is assumed that the percentage of drug released from the applied dose is lower than 30%. As such, although the current EMA guidance states the requirement for 70% release, this is practically impractical and thus the maintenance of sink conditions should be followed [7,14,34]).

Precision, reproducibility and operator variability studies results met the established acceptance criteria. The maximum RSD was attained when estimating the inter-run variability (14.10%). Although this value is still compliant with the 15% RSD acceptance threshold defined by the FDA, it should be noted that EMA criterion is far stricter only allowing a maximum of 10% deviation [14,27]. Variability in IVRT results may be related to air entrapment, inability to uniformly spread the formulation upon the membrane and difficulty in reproducing the exact amount of formulation loaded in the system [29]. Nevertheless, several papers report similar RSD results in IVRT precision studies (less than15%) [30].

Therefore, setting a broader acceptance criterion is warranted from both a scientific and experimental perspective.

From a regulatory point of view, it should be noted that there are slight differences in what concerns method precision documentation, according to EMA draft guideline, ICH guidelines and the FDA acyclovir guidance [14,27,35]. By the EMA guideline, IVRT methods should present an adequate intermediate precision. For this, studies should be conducted with the same batch product, by different operators, on different days. In this work, these procedures were considered for validation purposes. Conversely, according to ICH guidelines, the method intermediate precision is a part of the precision assessment, which also includes the documentation of the repeatability and reproducibility [35]. According to this guideline, intermediate precision documentation can be sustained by submitting the method to specific variations that might occur during routine analysis, such as different days, analysts (similarly to what was previously exposed for ICH requirements), but also including equipment variations. This rationale is also supported by the acyclovir FDA guidance [27].

The IVRT method discriminatory capacity was successfully documented, (Fig. 2 and Table 5). The IVRT method sensitivity was established since the following occurrence was registered: IVRR/Qf 0.5% bifonazole cream < IVRR/Qf 1% bifonazole cream < IVRR/Qf 2% bifonazole cream. Furthermore, both IVRT endpoints retrieved from the different strength formulations, presented a linear relationship ($R^2 \geq 0.9$) (Table 5). In what concerns method selectivity, the CI reporting to the IVRR of the lower vs. nominal strength and higher vs. nominal strength formulations were outside the range 90–111%; hence, the method was considered selective to establish differences in release rates. Moreover, the supplemental selectivity was also adequately demonstrated, as statistical inequivalence between the TP and the altered rheology formulation was registered. These assumptions would also be maintained if the FDA criteria was used instead (75–133%) [27].

The ability of the method to be unaffected by minor variations in the experimental conditions was also supported, as the mean IVRR did not deviate more than 15% from the IVRR of the nominal method parameter settings (Table 5). Therefore, the method robustness was documented.

3.2. Microstructure and product performance equivalence demonstration

After establishing suitable methodologies for both rheology/IVRT experiments, comparative studies between the different bifonazole cream formulations were carried out. A previous work by our group with this specific RP (batches from RP1 to RP3) highlighted the statistically significant rheological variability between batches [34]. Reasons attributed to this variability were related to the observed differences in the formulations pH, as well as globule size. Other aspects, such as batch age was also thought to be a possible explanation although in this specific case, batch age was similar.

As such, in an attempt to optimise RP characterization, a larger pool of batches was considered, with two extra batches being added to the analysis (Fig. 3). An effort was made to select batches at an early life cycle stage, when compared to the previous ones, please see Table 2.

The added batches (RP4 and RP5) proved to have an intermediate viscosity profile when compared to the initial pool of batches. Moreover, both formulations presented closer viscosity behaviour, when compared to the initial products.

As previously described in the materials section, 5 RP batches were compared with the three formulations: (i) Comparator product A (CPA); (ii) Comparator product B (CPB) and iii) Test product (TP). The first two formulations are commercially available, however, even though they share the same API, strength and dosage form of the RP, there are some differences in the Q1/Q2 attributes. CPA displays the same qualitative composition of the RP, whilst CPB presents several distinct excipients when compared to the reference formulation (mineral oil, polyoxyethylene stearate 40, polyethylene glycol 400, vaseline, sodium hydroxide, disodium EDTA, methyl parahydroxybenzoate and propyl parahydroxybenzoate).

A single batch of each comparator product was studied. These products were selected due to their market availability. CPA has been available in France since 1984, and CPB have been available in Spain since 1990. These aspects further reinforces their safety, as well as therapeutic efficacy [36].

In addition, the RP was compared with a third, not commercially available, formulation (TP). This specific formulation was designed to match the Q1/Q2 profile of the RP. Three independent batches were manufactured.

The results for comparative rheological analysis, are summarized in Fig. 4, and Table 6.

As previously observed during rheology validation studies, the viscosity curves of all formulations displayed a zero-shear *plateau* followed by a shear-thinning region and an infinite shear *plateau* (Fig. 4A). A variable shear stress application has been observed when dispensing doses from a container and applying them into the skin. It should be denoted that the container itself may cause variable shear stress on the formulation, as well as the patient may induce a wide range of stress upon product application. In this context, the zero-shear viscosity is related to the ease of formulation dispensing from the container. On the other hand, the infinite-shear viscosity pertains to the spreadability of the product to the application site.

The overall RSD attained with the RP were higher when compared to the remaining formulations. This was expected due to the pronounced RP inter-batch variability. The rotational endpoints retrieved from the

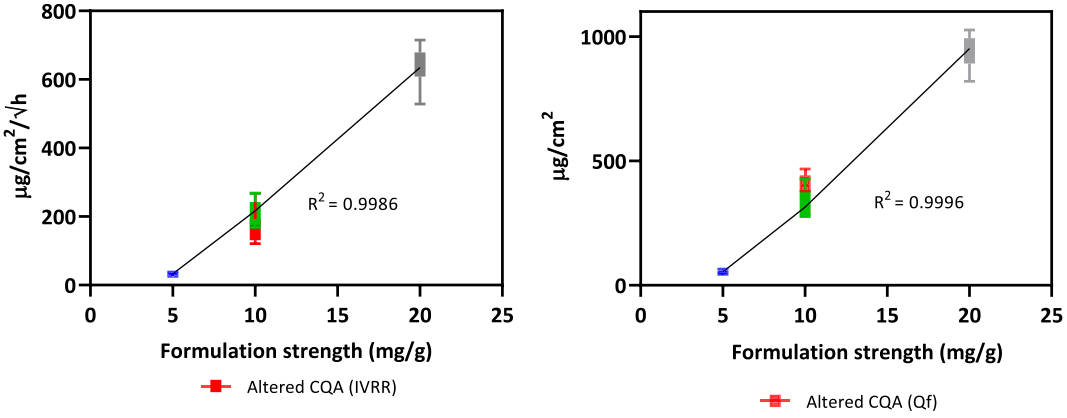

Fig. 2. IVRT sensitivity and specificity studies. Box and Whiskers plots of the measured release rates/cumulative amount released for the different strength and altered rheology formulations. The altered rheology formulation is signalled in red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

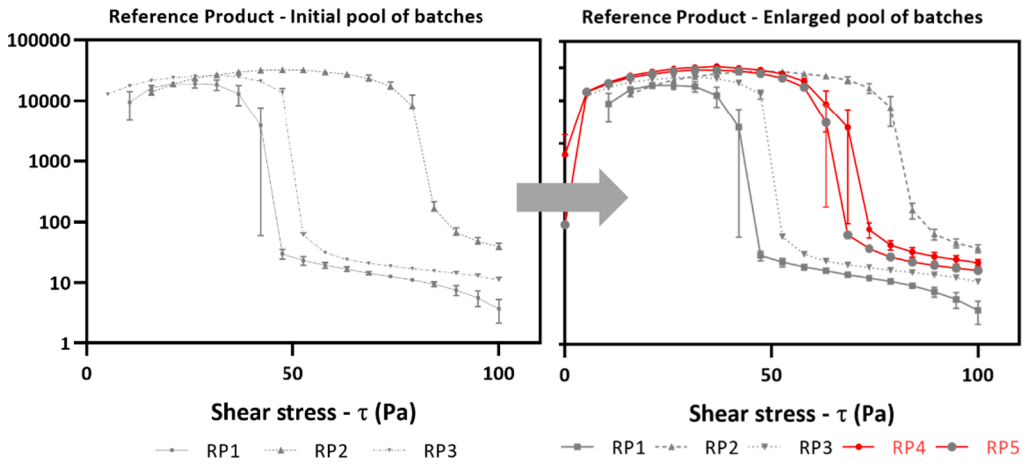

Fig. 3. Viscosity curves of bifonazole 10 mg/g cream reference products. All results report to mean ± SEM. Three replicates were used per batch formulation.

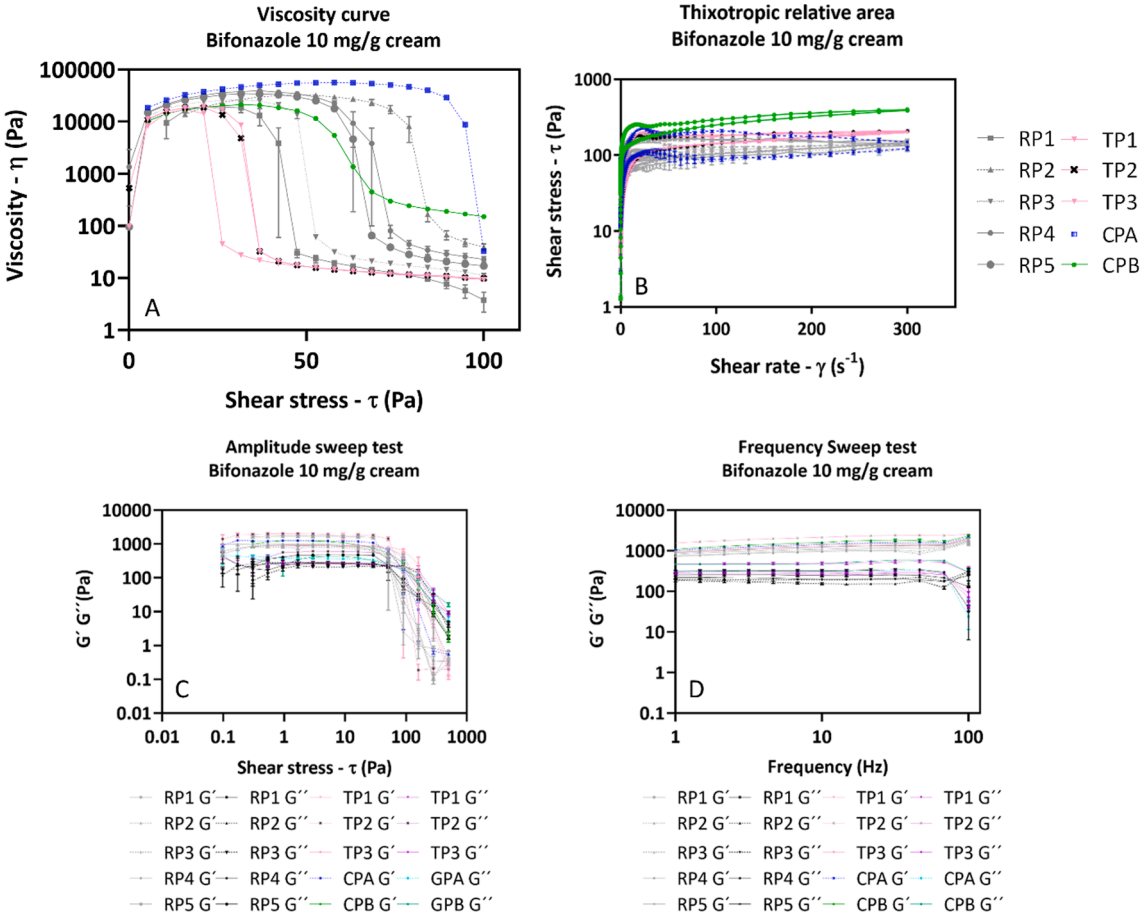

Fig. 4. Rheology profile of the bifonazole 10 mg/g cream products. All results report to mean ± SEM. Three replicates were used per batch formulation. Key: A – Viscosity curve; B – Thixotropic behaviour; C – Amplitude sweep test; D – Frequency sweep test.

CPA, the qualitative equivalent formulation, were the highest, suggesting a firmer consistency. The opposite scenario was overall observed with the TP, which presented the lowest rotational endpoints, thus highlighting a more fluid consistency (Fig. 4A). This, however, was not registered when addressing the TP oscillatory profile, where higher values were denoted when comparing to the remaining formulations, please see Table 6. The RP, on the other hand, presented intermediate rheological endpoint values. To compare the formulations, three statistical scenarios were drawn: (i) comparison of the RP itself, considering the two batches with more different rheology profile; (ii) comparison of RP batches with closer batch age of TP; (iii) comparison of CPA *versus* RP4/RP5 and CPB *versus* RP4/RP5 formulations, since they were tested at a similar lifecycle point of the TP. The rationale for these analyses is explained below.

In light of the RP variability, first, a statistical comparison between the batches with rather opposite rheology behaviour (RP1 vs. RP2) was carried out, see Table 6. The main purpose of this analysis was to investigate whether the rheological equivalence between the RP batches

Table 6
Statistical analysis of the rheological endpoints retrieved from the bifonazole 10 mg/g cream formulations. The 75–133% confidence intervals were calculated for the following ratios (i) TP/RP; (ii) CPA/RP and; (iii) CPB/RP. For the RP, 15 replicates were considered, for the TP, 9 replicates were considered instead. Finally, for the comparator products (CPA and CPB) 3 replicates were regarded.

Rheological endpoints	η_0 (Pa.s)	τ_{ROT} (Pa)	η_∞ (Pa.s)	S_R (Pa/s)	LVR (Pa)	τ_f (Pa)	τ_{OSC} (Pa)	G – 1 Hz (Pa)	G – 1 Hz (Pa)
RP1 Mean (RSD %)	15,573 (13.0%)	25.1 (2.44%)	2.83 (45.3%)	4492 (35%)	870 (6.88%)	233 (12%)	19.0 (10.05%)	955 (4%)	312 (10.0%)
RP2 Mean (RSD %)	18,933 (3.19%)	56.1 (4.15%)	27.2 (11.6%)	6290 (18%)	878 (4.04%)	169 (0%)	31.7 (7.87%)	729 (1%)	184 (10.0%)
RP3 Mean (RSD %)	17,730 (0.93%)	35.89 (1.02%)	11.2 (14.8%)	3269 (7%)	800 (8%)	220 (13%)	20 (23.62%)	747 (1%)	193 (2%)
RP4 Mean (RSD %)	21,606 (0.65%)	50.41 (6.61%)	22.80 (17.6%)	2134 (17%)	975 (4%)	310 (5%)	27 (10.75%)	80 (1%)	255 (9%)
RP5 Mean (RSD %)	20,683 (7.24%)	48.81 (4.47%)	17.03 (4.2%)	2607 (17%)	1663 (13%)	604 (5%)	31 (5.06%)	821 (0.1%)	220 (9%)
RP (overall) Mean (RSD %)	18,905 (13.0%)	43.3 (27.3%)	16.2 (59.1%)	8473 (63.0%)	1037 (512%)	307 (53.0%)	25.6 (23.93%)	828 (11.0%)	233 (22%)
TP1 Mean (RSD %)	14,596 (19%)	17.57 (3.09%)	9.62 (1.99%)	11,200 (8.35%)	910 (7%)	239 (1%)	35 (6.65%)	909 (1%)	282 (9%)
TP2 Mean (RSD %)	15,798 (5%)	19.19 (1.92%)	9.81 (6.76%)	10,268 (10%)	1892 (14%)	628 (14%)	38 (13.6%)	1554 (0%)	469 (7%)
TP3 Mean (RSD %)	16,016 (8%)	19.56 (2.02%)	9.26 (1.53%)	10,250 (2%)	910 (4%)	246 (5%)	53 (19.10%)	900 (0%)	253 (8%)
TP Mean (RSD %)	15,470 (13.0%)	18.8 (5.5%)	9.6 (5.2%)	10,573 (32.6%)	1238 (42.0%)	371 (54.0%)	41.7 (26.5%)	1121 (29.0%)	335 (31.0%)
CPA Mean (RSD %)	25,779 (2.00%)	78.3 (0.60%)	32.6 (65.8%)	24,877 (8.00%)	1228 (4.00%)	307 (26.0%)	25.4 (6.20%)	1034 (1.00%)	301 (12.0%)
CPB Mean (RSD %)	14,187 (14.0%)	44.7 (0.10%)	150 (12.0%)	12,433 (17.0%)	1189 (2.00%)	160 (10.0%)	13.1 (23.5%)	1081 (2.00%)	466 (2.00%)
90% CI									
RP1 vs. RP2	100.6–149.2	207.5 – 240.3	436 – 2846.7	80.50 – 265.2	89.32 – 114.1	61.23 – 87.01	138.2 – 202.7	71.89 – 81.34	48.02 – 72.73
RP 4/5 vs. TP	65.32–80.87	35.83–39.99	43.20–55.27	66.02–86.94	65.70–126.71	50.97–116.0	117.00–170.59	103.81–154.78	108.08–171.06
RP vs. CPA	113.8–131.0	147.16–169.8	89.1–239.0	140.3–227.2	68.2–137.6	44.0–109.6	76.5–101.6	114.9–127.3	107.5–149.4
RP vs. CPB	73.95–76.40	103.9–111.4	1124.72–1312.20	163.5–190.7	115.50–123.4	54.8–61.0	49.8–53.8	129.6–132.9	199.5–209.6

Key: CI – Confidence Interval; η_0 – Zero-shear viscosity; τ_{ROT} – Rotational yield point; η_∞ - Infinite-shear viscosity; S_R – Relative thixotropic area; LVR – Linear Viscoelastic Region; τ_f – flow point; τ_{OSC} – Oscillatory Yield point; $\dot{G}$- Storage modulus; $\acute{G}$ – Loss modulus; Green label – Compliant results; Red label – non-compliant results. To interpret the color references in the figure caption, the reader is referred to the web version of this article.

can be supported even when a more permissive acceptance criterion is considered.

It is important to note that a direct application of EMA criteria – "the 90–111% confidence interval calculated at a 90% confidence level for the difference of means of the test and comparator products should be contained within the acceptance criteria of +/-10% of the comparator product mean, assuming a normal distribution of data [14]" – does not apply to any of the considered endpoints. There was a difference of more than 10% between the rheological endpoints attained with the RP batches, and there was a lack of compliance with the 90–111% confidence interval. Based on these results, and similarly to the published work by Maria Pleguezuelos-Villa *et al*, a larger criterion [75–133%] was selected to assess rheological equivalence. Nevertheless, even when considering this broader criterion, solely equivalence pertaining to the LVR was observed (Table 6). Although borderline results were obtained for the η_0, τ_F and G endpoints, the statistical analysis is consistent with the obtained rheograms. Taking this information into account, equivalence pertaining to the rheology profile between the RP itself cannot be inferred.

As detailed in Fig. 1, bifonazole RP cream formulations appear to exhibit a time-dependent behaviour for some rheological endpoints. Taking into account the pronounced RP batch-to-batch variability, the calculation of the RP/TP as well as RP/comparator products equivalence confidence intervals was performed considering only the products with the closer RP batch age. As previously displayed in Table 1, these correspond to RP4 and RP5.

When comparing RP4 and RP5 vs. the TP, equivalence was not registered for any of the endpoints.

This scenario slightly differed when addressing the comparator formulations. For the CPA formulation (which displayed the same qualitative composition of the RP), equivalence was successfully established in terms of the zero-shear viscosity and oscillatory yield point. Furthermore, borderline values were registered for the LVR plateau. In what concerns the CPB formulation, 3 endpoints proved to be equivalent towards the RP - τ_{ROT}, LVR and G. This behaviour was not unexpected, given that rheology profiles depicted in Fig. 4.

Taking all the results into account, the comprehensive rheological analysis herein described suggests that rheologically, none of the formulations can be categorically considered as equivalent. Inter-batch variability in terms of rheological parameters can be ascribed to multiple factors, such as excipient or manufacturing process, storage conditions and aging of the formulation [37]. A recent study by Mangas-

Sanjuán *et al* compared the rheology profile of different batches of a calcipotriol and betamethasone reference ointment, in an attempt to define a fit for purpose acceptance range that would enable to establish equivalence between RP batches. The authors concluded that equivalent microstructure between batches cannot be demonstrated without an enlarged sample size due to batch-to-batch differences, when following EMA requirements. According to the authors, rheological studies should be performed to compare RP and TP formulations. However, to fulfil EMA requirements and in order to surpass the variability observed in complex semisolid systems, a larger number of batches and replicates need to be considered. Such an increase would materially increase the complexity of these studies, and poses as a significant constraint to topical product generic development [37]. Facing the rheological variability described herein, product performance (IVRT) was evaluated for all products, see Fig. 5.

A careful analysis of the release profiles suggests that the impact of these rheological differences is not perceptible, except when addressing the CPB formulation, which corresponds to the bifonazole product with a distinctively different (Q1) qualitative composition. However, this formulation in rheological studies revealed an intermediate viscosity profile of those presented by the RP, (Fig. 4) which suggests that rheological differences for the bifonazole cream 10 mg/g are not indicative of significant differences in product performance. On the other hand, differences in the qualitative composition, especially when contemplating thickening agents, highly influenced the release profile, thus proving the discriminatory capacity the IVRT method.

Correlation between qualitative composition and IVRT outputs was already described by Goebel and collaborators, who investigated diclofenac diethylamine *in vitro* release from gel formulations [38]. In their research, four approved generic products, with Q1/Q2 differences and probably different manufacturing processes, were compared towards the RP. Their results demonstrated that solely one generic product, with closer Q1/Q2 profile to the RP, revealed a similar release profile. The formulation with an additional co-emulsifier, cetostearyl alcohol, resulted in lower drug release compared to the reference formulation [17,38].

In the present study, all bifonazole products have cetostearyl alcohol as an emulsifying agent, nevertheless, CPB presented several excipients with an emollient function, namely white vaseline and mineral oil. Moreover, this product also presented an additional emulsifier agent – polyoxyethylene stearate 40. This excipient by contributing to an increase of the formulation viscosity may lead to a reduced release of the API and consequently to a lower IVRR [17,39].

Despite these observations, compliance with the 90–111% requirement is the key outcome as *per* EMA draft guideline criteria, and even though the release profiles of the RP are similar, there are still some batch-to-batch differences that should be addressed in more detail.

Tables 7 and 8 present the limits of the 90% confidence intervals calculated for the IVRR. Table 7 shows if the EMA criteria of 90–111%, mentioned in the draft guideline of quality and equivalence of topical products is respected, whilst Table 8 is focused on FDA criteria instead (75–133%). The results clearly show that if EMA criteria to IVRT is applied, several RP batch to-batch pairwise comparisons would fail to meet the requirements, as the 90% CI are several times not within the 90–111% limits.

Table 7
Statistical analysis of the IVRR of each RP batch, according to EMA criteria. The 90% CI attained for the mean ratio between all RP batches pairwise comparisons, was compared with the acceptance interval of 90 – 111%. An n = 12 was considered per batch.

	RP1	RP2	RP3	RP4	RP5
RP1		97.9–112.1	93.2–112.3	97.0–113.1	102.0–117.2
RP2	89.2–102.2		90.4–105.5	94.5–105.8	99.6–109.2
RP3	89.0–107.3	94.7–110.6		93.9–111.5	98.6–115.6
RP4	88.4–103.1	94.6–105.8	89.7–106.4		98.35–110.6
RP5	85.4–98.2	91.5–100.4	86.5–101.4	90.4–101.7	

Key: Green label – Compliant results; Red label – non-compliant results. To interpret the color references in the table caption the reader is referred to the web version of this article.

Table 8
Statistical analysis of the IVRR of each RP batch, according to FDA criteria. The 90% CI attained for the mean ratio between all RP batches pairwise comparisons, was compared with the acceptance interval of 75 – 133%. An n = 12 was considered per batch.

	RP1	RP2	RP3	RP4	RP5
RP1		96.1–112.5	94.1 – 117.4	93.8–114.0	91.0–108.9
RP2	92.9–113.2		93.9–116.3	93.8–114.0	91.7–105.4
RP3	89.9–116.0	92.6–111.8		89.7–114.4	89.3–107.3
RP4	91.1–115.4	93.4–111.5	94.1–118.4		90.4–108.1
RP5	95.1–119.4	97.7–114.1	98.1–121.5	98.4–116.6	

Key: Green label – Compliant results; Red label – non-compliant results. To interpret the color references in the table, caption the reader is referred to the web version of this article.

On the other hand, if the FDA criteria is selected instead (acceptance interval of 75–133%), all RP batches would be considered to have an equivalent performance (see Table 8).

Despite these batch-to-batch differences, the main aim of this study was to infer on the performance equivalence between the RP/CPA, RP/CPB and RP/TP. To achieve so, the 5 RP batches were collectively considered and statistically compared with the above-mentioned formulations. The results are displayed in Table 9.

When pooling all RP batches and comparing them with the CPA and TP formulations, product performance equivalence is registered, as *per* EMA requirements. This, however, was not the case for CPB.

When considering the EMA approach to IVRT data, increasing the number of RP batches was a successful strategy to overcome the RP batch-to-batch variability. The 90% confidence interval, generally yielded uncompliant results when pooling RP1, as these surpassed the 90–111% limits. However, by considering a broader RP panel, the variability of the RP was more taken into account. According to these results, the selection of RP batches is extremely important, especially whenever involved in a topical generic product R&D program aiming at

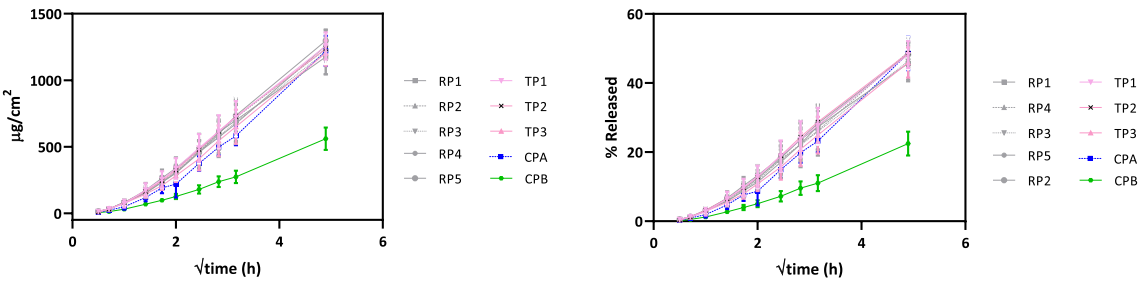

Fig. 5. IVRT profiles of all bifonazole products. Results report to n = 12 mean ± SD. For the RP, 5 batches were considered, for TP, 3 batches were selected, and for the comparator formulations (CPA and CPB) solely one batch was considered *per* product.

Table 9
IVRR and Qf 90% confidence intervals (CI) for the ratio between TP/RP, CPA/RP and CPB/RP. An n = 60 was considered for the RP, n = 36 for the TP, n = 12 for both CPA and CPB (EMA approach).

	IVRR T/R (μg/cm^2/√t) 90% CI	Total cumulative amount T/R (μg/cm^2) 90% CI	Acceptance criteria	Status
RP vs. TP	96.3 – 103.2	96.6 – 103.4	Within 90–111%	C
RP vs. CPA	90.7 – 100.3	93.7 – 104.2		C
RP vs. CPB	41.0 – 45.8	42.5 – 47.8		NC

Key: Green label (C) – Compliant results; Red label (NC) – Non-compliant results. To interpret the color references in the figure caption the reader is referred to the web version of this article.

an abridged bioequivalence demonstration. Furthermore, the statistical approach used is also critical.

Documenting the extended pharmaceutical equivalence of TGP is a cornerstone in EMA draft guideline. If equivalence fails to register, RP variability should be evaluated since semisolid dosage forms, especially those that comprise of multiphasic systems, exhibit intrinsic variability. To better characterize the RP, enlarging the pool of batches can be a reliable strategy. However, in our opinion, to be able to consider a biowaiver from clinical endpoint studies for topical generic products, further work and discussion with EMA are required. The document in its current form does not take into account the intrinsic variability of these products, since the statistical criteria regarding microstructure and performance fail to register even when only addressing the RP.

Despite the attained experimental data, the general conclusions on RP variability and the strictness of the regulatory criteria imposed, it is essential to establish a workflow that seeks to surpass these constraints.

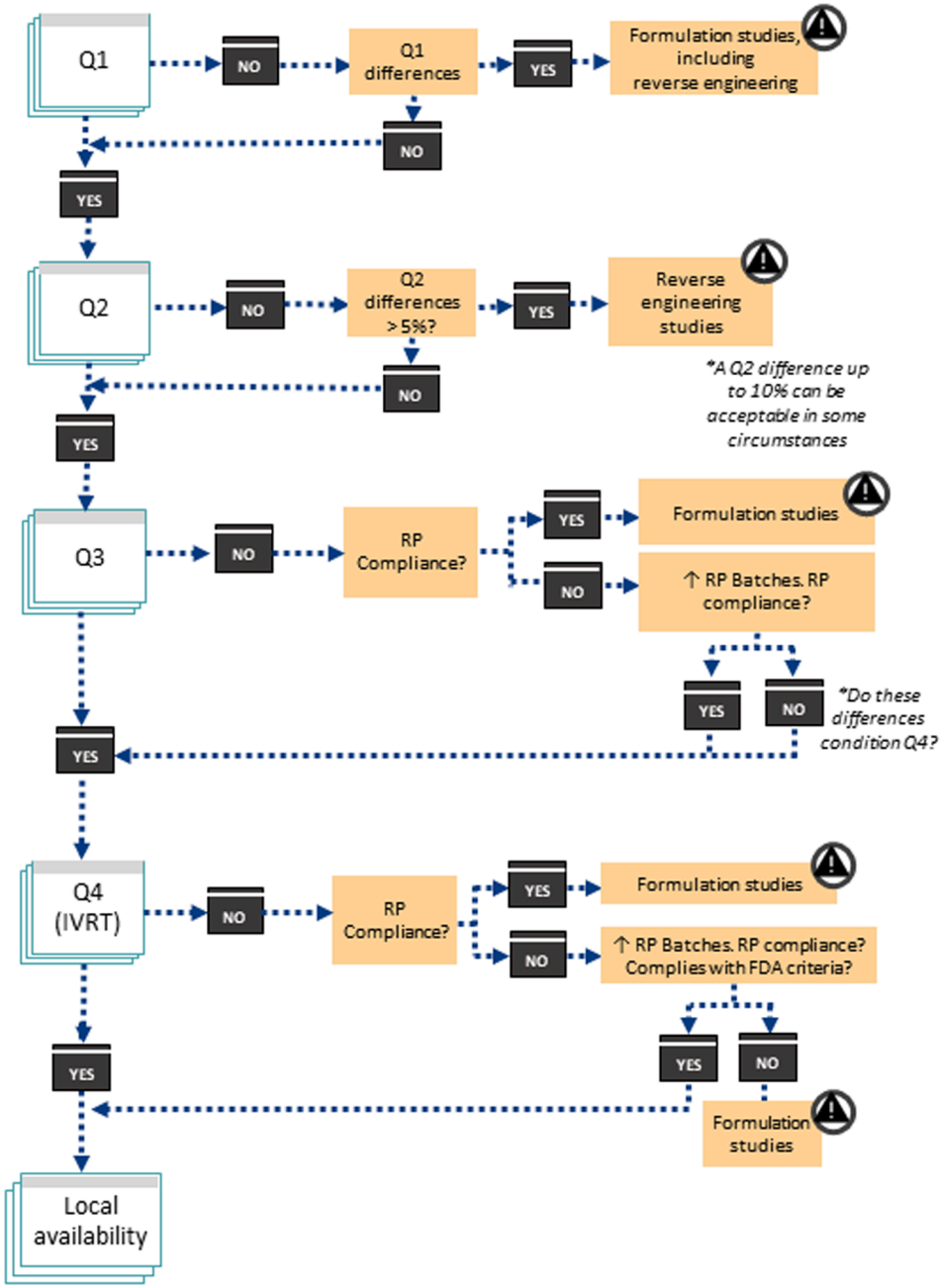

Fig. 6. Proposed bioequivalence assessment flowchart.

In an attempt to do so, the following flow chart is proposed (see Fig. 6).

Since creams are complex dosage forms, local availability studies should be performed to provide proof of equivalence in terms of the product efficacy. Such studies constitute the last step within the modular framework for BE of topical multiphasic formulations. Due to the complexity of these local availability studies, a strategy involving product efficacy equivalence may be required. *In vitro/ex vivo* disease models may be useful tools to address the mechanisms of action involved in the therapeutic responses.

As antifungals are expected to display limited diffusion characteristics, the use of surrogate pharmacodynamic tests capable of characterizing the efficacy profile of products could also be of value. These would provide an alternative, complementary and product-specific approach to assessing therapeutic efficacy. Several interesting examples, specially tailored to topical antifungals, such as the TurChub, ChubTur, TurSh and RoMar *in vitro* models, have proven useful for this application. [40]

4. Concluding remarks

In this work, we have investigated both the experimental procedures and regulatory mechanisms underlying the bioequivalence assessment of bifonazole 10 mg/g cream formulations.

According to European and American regulatory agencies, semisolid dosage forms that exhibit a complex microstructure, such as creams, should present Q1-Q4 equivalence, in addition to local availability assessment. In order to comprehensively address several scenarios that may occur in daily practice, an initial sample of RP batches was considered, together with a Q1/Q2 formulation (TP), a Q1 formulation (CPA), and finally, a bifonazole cream formulation with Q1/Q2 differences (CPB).

The product microstructure was evaluated in rheological studies. As high rheological variability was observed in the RP, the initial pool of RP batches was strategically enlarged to enable a more detailed characterization of the product at different lifecycle stages. In this analysis, the rheological equivalence reporting to the RP itself failed to be supported. Not surprisingly, the rheology profiles between the RP and CPA/CPB/TP were also not equivalent.

The high variability registered in the rheological studies motivated the determination of the release profile for all formulations. Interestingly, these results were not consistent with data from rheology, as product performance showed equivalent results between the RP and the formulations with the same qualitative composition (TP and CPA). It should be pointed out that equivalence was not generally supported when comparing RP batches amongst themselves, if EMA requirements were considered. However, the scenario totally differed when FDA criteria were applied. By applying the Mann-Whitney test, as well as a more permissive confidence interval, to IVRT results, equivalence could be inferred for all RP batch-to-batch combinations. Compliant results, following EMA approach were solely attained when pooling all RP batches together. Enlarging the RP batch pool was a critical step in establishing equivalence of product performance according to the EMA requirements. Furthermore, the data herein presented highlighted the importance of the statistical approach to follow.

Taking these results into account, a reflection on the real importance of documenting statistical equivalence of rheology outputs between test and reference formulations can also be made. The release profile of the formulations being compared, attained through a validated method, revealed equivalent results except for the product that displayed a different qualitative profile. As such, from an efficacy and safety perspective to the patient, are there really any risks in documenting the formulations as extended pharmaceutical equivalent, even if rheological sameness in not a guarantee?

Facing these challenges, product specific guidelines may provide a better solution to tackle product age and variability and the respective equivalence criteria, ultimately encouraging topical generics entry into the market.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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