QUALIMETRIX
Analytical Laboratories - Quality Control

In-vitro permeation studies

by QUALIMETRIX

Contents

QMx stepwise approach

Qualimetrix is a customer-driven CRO that employs the Six Sigma philosophy in order to design and implement optimized processes with the aim of transforming customer inputs and requirements into “customer value”. As such, the first and probably the most critical factor for a successful project is its proper definition in terms of both customer and technical requirements. To this end, a comprehensive study request form is provided to the customer with the following objectives:

- The definition of the type and scope of the study
- The provision of critical product information
- The determination of the most suitable, expedient and cost-effective approach

Locally applied products – Advantages and Therapeutic Equivalence establishment

Topical products are exemplified by medicines for cutaneous use; but in broadest scope, they are locally applied, locally acting products. They can be applied to any of the diverse external surfaces of the body that may present a physiological barrier to drug absorption e.g. skin, eye, ear.

Apart from topical products, transdermal patches, containing one or more active substances, intended for systemic absorption, are designed to provide a controlled delivery of the active substance(s) through the skin, principally by diffusion, resulting in a defined rate and extent of systemic delivery of active substance. Transdermal and topical drug delivery have the following advantages:

- Administration avoids gastrointestinal drug absorption difficulties caused by gastrointestinal pH; enzymatic activity; and drug interactions with food, drink and other orally administered drugs.
- Delivery provides a substitute for oral administration of medication when that route is unsuitable, as with vomiting and diarrhea.
- Administration avoids the “first-pass effect”, that is, the initial pass of a drug substance through the systemic and portal circulation following gastrointestinal absorption, possibly avoiding the deactivation by digestive and liver enzymes.
- Delivery is non-invasive, avoiding the inconvenience of parenteral therapy.
- Extended therapy is provided with a single application, improving compliance over other dosage forms requiring more frequent dose administration.
- Activity of drugs having a short half-life is extended through the reservoir of drug in the therapeutic delivery system and its controlled release.

- Drug therapy may be terminated rapidly by removal of the application from the surface of the skin.
- Locally applied products achieve the delivery onto the target organ at an optimal concentration with a rapid onset of action and the minimization of systemic effects

However:

The bioavailability of the active substance at the site of action from topical products is known to be affected mainly by:

- The active substance's physicochemical properties
- The topical formulation design
- The manufacturing process

To this end, small changes in formulation, dosage form, administration or manufacturing process may significantly influence the efficacy and/or safety and this presents challenges to the prediction of therapeutic equivalence.

In assessing generic formulations, regulatory agencies require the demonstration of bioequivalence (BE) to a reference drug product (RP / RLD). The US Food and Drug Administration (FDA) guidelines note that, taken together with the confirmation of pharmaceutical equivalence, establishing BE allows for a regulatory conclusion of therapeutic equivalence.

For the majority of topical drug products, comparative clinical endpoint studies are used to demonstrate BE to the RLD. The use of clinical endpoints to determine BE of topical products, although providing a direct assessment in patients that is reassuring to clinicians, is associated with a number of challenges such as the ones presented in Figure 1 below:

Figure 1: Challenges of Clinical endpoint studies

It is evident from the above that there is a clear need for BE studies using alternate approaches which are faster, less expensive, more reproducible and sensitive to differences in locally applied products. This need for suitable surrogates seems to be "embraced", despite the skepticism, by the regulatory authorities (i.e. FDA, EMA) as reflected by recent guidance documents. However, these efforts are far from flawless considering the quite restrictive acceptance criteria discussed in the following paragraphs.

Regulatory Framework for Topical Semisolid Pharmaceutical Products

Since 2012, U.S. Food and Drug Administration (FDA) has continuously published non-binding, product-specific guidelines for generic product development, to identify the appropriate methodology for developing drugs and generating evidence needed to support abbreviated new drug application (ANDA) approval. Over the past five years, a number of relevant guidelines were made public, including an in vitro option to establish bioequivalence of topical semisolid drug products. On the other hand, in October 2018, the European Medicines Agency (EMA) published for public consultation a universal guideline for topical generic product submission entitled "Draft Guideline on Quality and Equivalence of Topical Products". Due to the high diversity of topical products, the complex range of skin conditions that should be treated and the variety of patient needs, this guideline does not provide a single procedure, but states that general recommendations should be adopted on a case-by-case basis. Despite the clear differences on the guidelines' applicability, generally to grant a waiver of clinical endpoint studies, a modular framework for BE documentation is often accepted.

First, the qualitative composition (Q1) of the Test Product (TP) should be equivalent to the Reference Product (RP). This is to be followed by the quantitative equivalence (Q2) sameness. To achieve this, reverse engineering procedures are usually 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 IVRT methods, should likewise be evidenced. Finally, studies on local availability of the product should also be submitted. According to EMA, these can be further divided into two categories: permeation kinetic studies and pharmacodynamic studies. The first category includes:

i. dermatopharmacokinetic studies for drugs that present limited diffusion and predominantly target the skin surface

ii. IVPT studies for drugs that present a quantifiable permeation profile; and finally

iii. pharmacokinetic studies for drugs that are systemically bioavailable.

In this context, the selection of permeation kinetic studies to be used depends mainly on the "site" where the drug can be quantified. The second category refers to pharmacodynamic methods. The most common methodology regards the vasoconstriction assay, which is solely applied to corticosteroids because of the respective skin bleaching properties.

According to EMA, regarding simple formulations (e.g. cutaneous solutions, single phase gels and ointments), product equivalence can be based on Q1 – Q4 similarity while for more complex dosage forms the applicant should additionally provide evidence on product safety equivalence (Fig.1)

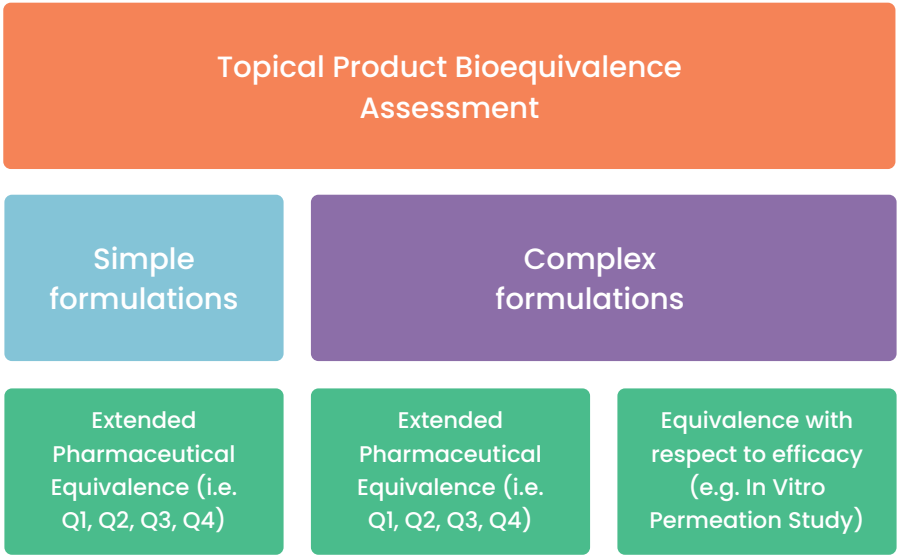


Figure 2: EMA's modular framework for Equivalence Assessment

Establishing topical bioequivalence is undoubtedly an extremely complex process which is dependent on the formulation's technological features and the significant intrinsic variability associated with this class of products. Despite the fact that both FDA's product-specific guidance documents and EMA's draft guideline constitute a regulatory opening of paramount importance, the establishment of realistic acceptance criteria which are, at

the same time, feasible for generic manufacturers and suitable for ensuring the efficacy and safety of topical products, is still a "work in progress".

The following sections aim to briefly present the shortcomings of the regulatory landscape with respect to the studies required to establish equivalence, the differences between FDA and EMA and how Qualimetrix can be a reliable partner in formulating a "totality of evidence" strategy to generic topical product approval.

Reverse Engineering (aka Deformulation) Studies

One of the most critical aspects regarding the therapeutic efficacy of a topical product is the formulation composition. To this end, both European and American regulatory authorities require the demonstration of acceptable **Q1** and **Q2** sameness (i.e., to document that the test product contains the same excipients in the same quantitative composition as the comparator medicinal product (differences not greater than ±5% are acceptable). According to EMA draft guideline, only excipients whose function is not related to product performance and administration (i.e., antioxidants, preservatives, coloring agents) could be qualitatively and quantitatively different (not more than ±10% is acceptable).

Considering that the excipients in the reference product are available in the public domain (i.e. listed in the patient information leaflet), establishing the Q1 sameness seems to be rather straightforward. On the other hand, the establishment of Q2 equivalence involves an initial stage of **Reverse Engineering** studies in order to reveal the quantitative composition of the comparator. Concentration ranges can be established by suitable analytical methods that span a wide range of analytical techniques. Excipients used in topical products often show batch and source variation e.g. homologue composition of hydrocarbon chains, the degree of unsaturation, molecular weight, polymorphism. This in turn may lead to unforeseen variability in the product's rheological properties, microstructure/physical properties, crystallisation of the active substance or other ingredient, stability, or bioavailability. To this end, special attention needs to be directed to also defining the grade of excipient. The latter is certainly one of the most

challenging tasks and obstacles that both generic product manufacturers and analytical labs undertaking such studies need to tackle with and overcome in order to set a solid basis for successful formulation development.

The following table summarizes the issues pertaining to Q1, Q2 sameness associated with the complexity of the task itself that is aggravated by strict guideline requirements and how Qualimetrix can engage in overcoming these hurdles with its state-of-the-art instrumentation and its proven analytical expertise and experience.

Table 1: Problems and Solutions regarding Q1/Q2 similarity

Regulatory expectations		Practical issues / limitations	QMx
FDA	EMA		
Strict acceptance criterion (i.e. ± 5%)		Physicochemical diversity of compounds included in the formulation	• Wide range of analytical techniques and database of available methodologies for each type of excipient
		Uncertainty of quantitative determination	• Analytical expertise and experience that ensure the development of a suitable methodology • Verification of suitability by analyzing lab-scale samples of known composition
N/A	**Excipient grade and source determination**	Challenging due to patent protection issues and need to isolate the excipient and proceed with extensive characterization	• Database of excipient grades employed in similar cases (if available) • Expertise and experience with isolation and characterization of formulation constituents

Microstructure Evaluation Studies

Comparative microstructure studies are of paramount importance for demonstrating Q3 equivalence. It should be noted that although the criteria for Q1/Q2 sameness may be met, the generic formulation may exhibit significant differences in the arrangement of matter compared to the reference product. This is mainly attributed to the complexity of formulation composition and manufacturing process parameters. The importance of formulation microstructure is highlighted in EMA's draft guideline which states that:

> *"Evidence should be provided to characterize the microstructure/physical properties in terms of bulk physical CQAs that influence bioavailability, usability or indicate variability in the manufacturing process and product instability".*

There are numerous tests that should be performed within this scope when addressing semisolid dosage forms, such as visual and microscopy appearance, particle/globule size, API polymorphic form, vehicle metamorphosis, pH, API distribution, among others. Similar requirements are set out in the FDA product-specific guidance documents regarding the in vitro option of bioequivalence assessment. However, EMA stresses the particular importance of rheological properties by defining specific rheological parameters that should be documented when characterizing the rheological profile of a given semi-solid formulation. More specifically:

> *"Non-Newtonian rheological behaviour should be characterized using an appropriate absolute rheometer and include:*
>
> - *A complete flow curve of shear stress (or viscosity) versus shear rate, comprising multiple data points across the range of increasing and decreasing shear rates…*
> - *Yield stress and creep testing*
> - *The linear viscoelastic response (storage and loss modulus vs. frequency)*

Rheograms should be provided and the product's behaviour classified according to shear and time effects e.g. pseudoplastic, dilatant, thixotropic, and characterized using appropriate metrics. For example: viscosities at specified shear rates across the rheograms (e.g. η100); plastic flow yield stress values; thixotropic relative area (SR); viscoelastic storage and loss moduli (G' and G"), apparent viscosity, loss tangent (tan δ)"

In order to demonstrate microstructure equivalence, the 90% confidence interval (CI) for the difference of means of the test and reference products should be included within the acceptance limits of ±10% of the reference product mean, assuming normal distribution of data.

This requirement has been extensively discussed in the literature and criticized as overly restrictive, because it does not consider the intrinsic variability of topical semisolids. There are numerous publications comparing rheological data of Q1/Q2 equivalent test and reference products with the results obtained from in vivo pharmacokinetic studies. The outcome of the latter (i.e. demonstration of bioequivalence) does not concord with the statistically significant difference with respect to the rheological parameters which may suggest that differences greater than 10% do not necessarily translate into clinically significant differences. What is even more interesting and indicative of the rather unrealistic criterion set by EMA, is the fact that marked differences have been reported even within reference products.

"Statistical analysis demonstrated that if EMA criteria are applied, none of the same product batches can be considered as equivalent"

Variability is caused by various contributing factors associated to the 6 M's: **M**achine, **M**anpower, **M**aterials, **M**easurements, **M**anufacturing methods, and **M**other nature. In drug product manufacturing, it relates to processing equipment, personnel, raw materials, analytical method, manufacturing process, and facility/environmental controls as depicted in the following figure.

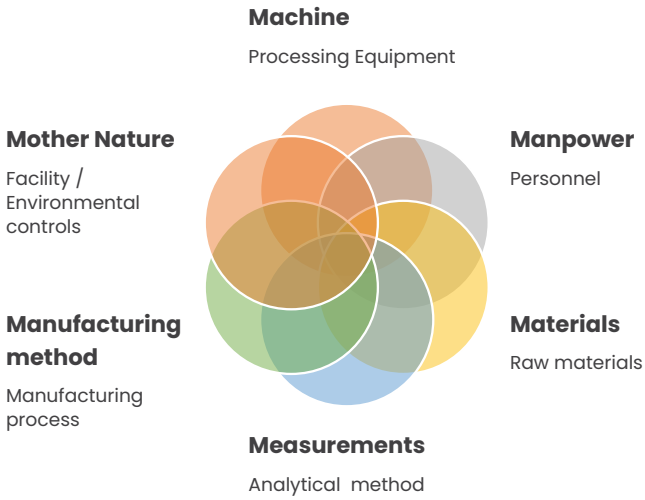


Figure 3: Sources of product variability

The microstructure of semisolid products is highly sensitive to these variability sources especially to interchanges between suppliers and manufacturing processes. Regarding the former, there are several cases on excipient intra-supplier variability that eventually led to differences in the final product. Another significant factor that in many cases seems to affect the rheology of the formulation, is "batch age" and it should therefore be taken into consideration in the process of selection prior to proceeding with comparative studies.

The following table summarizes the issues regarding Q3 sameness associated with the probable root causes outlined above and the tight guideline requirements and how Qualimetrix can engage in overcoming these hurdles.

Table 2: Problems and Solutions regarding Q3 similarity

Regulatory expectations		Practical issues / limitations	QMx
FDA	EMA		
N/A	**Restrictive acceptance criterion** (i.e. the 90% confidence interval 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)	• Intrinsic wide variability of topical semi-solid formulations (i.e. batch-to-batch variation of reference product) • Non-normal distribution for the majority of rheological parameter data	• Well-founded study design to minimize sources of variation — preliminary screening of batches to select those that will minimize the probability of failure — sample size (i.e. number of batches) calculation to achieve the desired statistical power based on batch-to-batch variability — Strict control of experimental parameters to minimize analytical measurement variability • Participation in scientific advice meetings with authorities to support the study design and criteria • Justification of wider acceptance criteria for reference products exhibiting high variability (e.g. 75 – 133%) or scaled according to within-reference product variability
Extensive physicochemical characterization (e.g. particle / droplet size distribution, API polymorphism, rheological parameters)		Diversity of analytical techniques that normally requires the collaboration with several analytical laboratories with different areas of expertise and a high level of knowledge and understanding of regulatory expectations	• Integrated services covering the total of the studies required • Experienced personnel with in-depth knowledge and understanding of regulatory expectations in terms of study design / execution and statistical processing

In Vitro Release (IVR) Studies

According to the FDA's SUPAC-SS guidance, an in-vitro release rate can reflect the combined effect of several physical and chemical parameters, including solubility and particle size of the active ingredient and rheological properties of the dosage form. To this end, IVR testing is a useful test to assess product "sameness" under certain scale-up and post approval changes for semisolid products. Following this rationale EMA's draft guideline defines the release rate as a Critical Quality Attribute (CQA) to be specified in the final product release and shelf-life specification. Moreover, a validated IVR test, as a method for product performance characterization, is required to support extended pharmaceutical equivalence.

Qualimetrix can provide IVR testing for semisolid preparations (i.e. creams, ointments, lotions and gels) by considering the requirements of the relevant EMA's draft guideline and FDA's product-specific guidance documents and by following the general principles of USP General Chapter <1724> Semisolid Drug Products – Performance Tests. The Diffusion cell (Fig.2) is a reliable and reproducible means of measuring drug release from semisolid dosage forms.

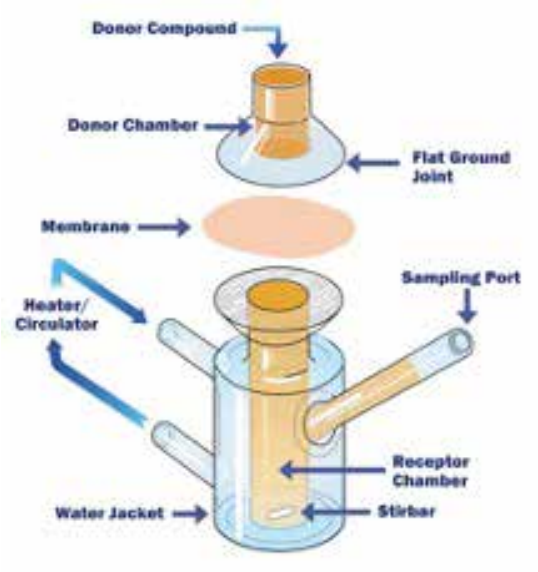


Figure 9: Sources of nitrosamines

The process involves the application of the semisolid product in the donor chamber which is placed in contact with a medium in a reservoir (i.e. receptor chamber). The latter acts as a receptor when the drug substance diffuses through the formulation, across an inert, highly permeable support membrane, and into the reservoir. Samples are then withdrawn from the receptor chamber at predefined time intervals. For each cell, the amount of drug released (μg/cm2) at each sampling time is determined and the cumulative amount released plotted versus √t. The slope of the resulting line is a measure of the rate of drug release.

According to the recent regulatory requirements, during the marketing authorization procedure, adequate evidence should be provided to demonstrate that IVRT method is properly validated. The requirements of European and American regulatory authorities are similar, but significantly more details regarding procedure validation can be found in the FDA product-specific guideline on Acyclovir. Prior to that stage, all method variables should be optimized in the frame of a method development activity that will ensure the successful outcome of method validation. Both FDA and EMA require the submission of method development and validation data and the omission of such reports is often among the main deficiencies compromising the approval of generic semisolid products.

IVR Method Development and Validation

The first and probably the most critical step for setting up a suitable IVR test is method development. Both guidelines highlight the importance of several features that need to be carefully studied and optimized in order to ensure that the applied methodology is fit for its intended purpose.

Analytical lifecycle management (ALM) is a novel approach which derives its basic principle from the combination of **ICH** guidelines **Q8**, **Q9** and **Q10**. It has several benefits over the traditional approach as it integrates validation, transfer and verification of procedure. This approach is divided into three stages starting with:

1. Procedure design, which includes defining the **analytical target profile (ATP) and critical analytical attributes (CAAs)**. Once the ATP and CAAs are defined, the quality risk management (QRM) tools like fish-bone diagram, control-noise- experimental (C-N-X) approaches are best utilized to identify the **critical method variables (CMVs)** demanding further studies. The identified variables are investigated using the design of experiments (DoE) to minimize the risks and optimize the experimental conditions.

2. The second stage is known as procedure performance qualification which includes experimentation based on optimized conditions and suitable analytical control strategies are derived.

3. In the conclusive stage i.e. procedure verification, the compliance with analytical control strategy is monitored continuously to improve the method performance.

The general framework outlined above has been adopted by QMx as a useful and cost-effective tool for the development and validation of IVRT methods. One of the most significant benefits of this approach is the establishment of the **design space** of the method, also known as the **Method Operable Design Region (MODR)**. The latter defines the acceptable ranges of the method's parameters and thus ensures the robustness of the method and the quality of the results.

To facilitate risk identification, the analytical procedure can be described using a process flow, map, or summary, and each part of the procedure can be broken down into detailed sub-steps. It is important to consider all steps, from sample and standard preparation to analyte testing to quantitation. The procedure process map can then be used to identify variables associated with the analytical procedure. Tools such as Ishikawa diagrams can be used in conjunction with the process maps to identify potential variables associated with each step in the analytical procedure.

The following figure depicts such a diagram with an indicative list of variables that could have an impact on the outcome of IVRT. The majority of these variables correspond to the experimental conditions mentioned in FDA's Draft Guidance on Acyclovir and EMA's draft guideline.

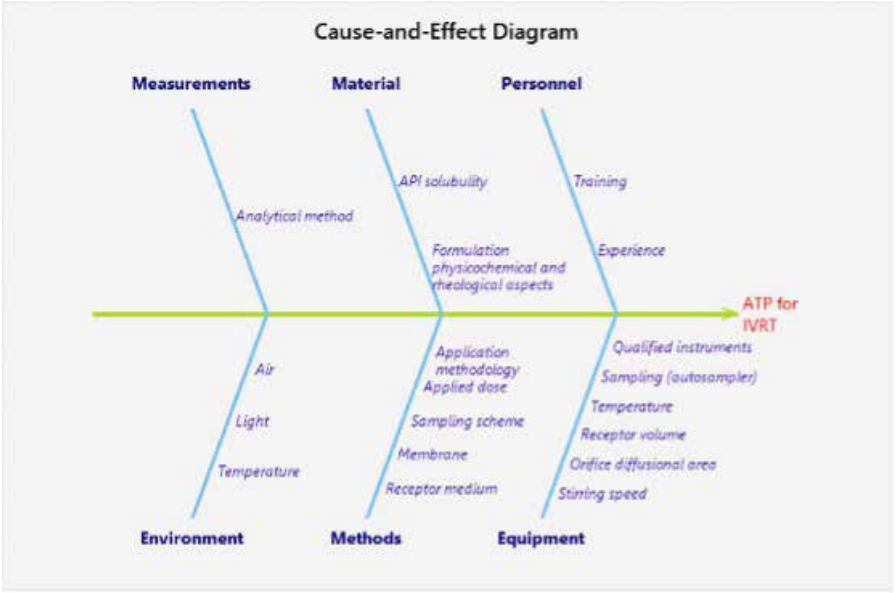


Figure 5: IVRT method variables

The next stage following the identification of potential CMVs is to assess their actual impact on the **CAAs** (i.e. In Vitro Release Rate, Cumulative amount released) as well as possible interactions. This is achieved by means of DoE studies whose aim is to establish mechanistic models (i.e. suitable mathematical models) and calculate coefficient values that are indicative of the magnitude of their influence on method performance.

Based on the above, it is evident that the application of the principles of ALM can greatly reduce experimental work and cost and provide a deep understanding of the main risks intrinsic to the method. The significance of method variables on IVRT analytical endpoints, along with possible interactions, can be explained through mathematical equations that define the region within which method performance will remain “acceptable”. The true value of this approach is that the selection of the “optimal settings” is achieved and justified by means of a scientifically-sound and regulatory-oriented process.

According to the scientific literature, it is of paramount importance that the apparatus, methodology and study conditions utilized in the IVR study are appropriately validated and qualified for their intended purpose. Detailed protocols and well-controlled study procedures should be developed for each project to ensure the precise control of dosing, sampling, and other IVRT study variables or potential sources of experimental bias.

The following table summarizes the regulatory requirements with respect to the validation scheme that should be followed for demonstrating the suitability of an IVRT method.

Table 3: Acceptance criteria for IVRT method validation studies (EMA vs FDA)

Parameter	Description	EMA acceptance criteria	FDA acceptance criteria
Membrane inertness	Evaluation of drug binding to membrane should be performed by immersing membrane in solution of drug at concentration relevant to average drug concentration in the receptor solution at the end of the test.	Not defined	The recovery of drug in solution should be within the range **100%±5%** at the end of the test duration
Drug solubility in the receptor medium	Evaluation of drug solubility in the receptor mediums should be performed to confirm its suitability to maintain sink conditions during the study.	Drug concentration in the receptor medium should not exceed **30%** of its maximum solubility in the receptor medium	Drug concentration in the receptor medium should ideally not exceed **10%** of its maximum solubility in the receptor medium
Linearity	The R2 value of the in vitro release rate (IVRR) (slope) should be calculated across the sampling times throughout the IVRT study duration.	Not explicitly defined but implied from the requirement that *"For extended pharmaceutical equivalence testing: The cumulative amount of active substance released versus the square root of time should be linear"*	Linearity: R2 ≥ **0.90** across the study duration

Parameter	Description	EMA acceptance criteria	FDA acceptance criteria
Precision and /intermediate precision (reproducibility)	Precision and reproducibility should be assessed from intra-/inter-run data analysis.	Intermediate precision should be < **10%**	CV for the intra- and inter-run variability should be ≤ **15%**
Discriminative power	The IVRT method should be able to discriminate drug substance release rates from similar formulations	Sensitivity: Not defined Specificity: minimum R^2 value > **0.90** for the correlation of formulation concentration to the IVR rate Selectivity: It is implied that the CI between altered product formulations should fall outside the limits **90–111%**	Sensitivity: mean IVRR (low drug concentration) < mean IVRR (nominal drug concentration) < mean IVRR (high drug concentration) Specificity: R^2 value ≥ **0.90** for the correlation of formulation concentration to average IVRR Selectivity: CI between altered product formulations should fall outside the limits **75.00–133.33%**
Robustness	Robustness testing should include minor variations in the method parameters (mixing rate, temperature, amount of formulation applied and receptor medium composition)	Not defined	The IVRT method may be considered robust if the average slope of the IVRT run (under altered conditions) is within ± **15%** of the average slope of the Precision & Reproducibility IVRT runs
Application	The amount and method of formulation application should be shown to be consistent and validated to ensure homogeneous spreading of the formulation over the membrane	**±5 %** between samples	Not defined

In accordance with the issues highlighted above, regarding the strict regulatory criteria for demonstrating equivalence, the same observations and conclusions are valid for IVRT, considering that there are numerous studies showing that the equivalence criteria cannot be consistently met even for different batches of the reference product. This holds especially true for the criteria set by EMA as they do not account for semisolid dosage form and IVRT method intrinsic variability and therefore they do not provide a viable alternative to clinical endpoint studies.

Considering that the EMA guideline is not yet official and the assessment approach followed by the authorities is not harmonized, the scientific advice option, offered by many authorities is a useful tool in order to ensure that any reasonable (i.e. justified based on a scientifically sound rationale) deviations from the strict acceptance criteria will not trigger major objections. The following table summarizes the issues pertaining to the demonstration of IVR sameness by meeting the tight regulatory requirements and possible ways out.

Table 4: Experimental scheme for procedure qualification

Regulatory expectations		Practical issues / limitations	QMx
FDA	EMA		
Wider criteria and approach of USP <1724> (i.e. 90% confidence interval for the ratio of test to reference release rates must be within the range of 75%–133.33%.	**Restrictive Confidence Interval** (i.e. The 90% confidence interval for the ratio of means of the test and comparator products for the parameters (R), (A) should be contained within the acceptance interval of 90 – 111%.)	• Intrinsic wide variability of topical semi-solid formulations (i.e. batch-to-batch variation of reference product) • Despite the fact that the FDA proposes broader criteria, attaining these may also prove to be challenging when dealing with complex formulations	• Well-founded study design to minimize sources of variation — preliminary screening of batches to select those that will minimize the probability of failure — sample size (i.e. number of batches) calculation to achieve the desired statistical power based on batch-to-batch variability — Strict control of experimental parameters to minimize analytical measurement variability • Participation in scientific advice meetings with authorities to support the study design and criteria • Justification of wider acceptance criteria for reference products exhibiting high variability or scaled according to within-reference product variability

Regulatory expectations		Practical issues / limitations	QMx
FDA	EMA		
N/A	**Dose depletion of at least 70%**	• The majority of topical products do not attain a 70% release of drug substance throughout the duration of an IVRT experiment. Prolonged testing durations would be required that are not representative of in vivo conditions • Literature data indicate that deviations from linearity are usually observed when more than approximately 35-45% of the API in the dosage form is released from the semisolid sample	• The exact phrasing of the guideline is that "The duration of IVRT should be sufficient to characterize the release profile, **ideally at least 70%** of the active substance applied is released. It is therefore not very prescriptive and therefore the submission of a proper rationale, supported by data demonstrating that the release profile has been adequately captured (i.e. including at least 6 time points in the linear portion including the first sample immediately after drug diffusion has reached a steady state) is adequate to justify such a deviation.
Acceptance criterion for Intermediate Precision (i.e. %CV ≤ 15%)	**Acceptance criterion for Intermediate Precision** (i.e. CV<10%)	• The acceptance criteria, especially those of EMA, do not account for IVRT method intrinsic variability	• Analytical method development and validation focusing on the identification and control of Critical Method Variables and the establishment of Method Operable Design Region.

IVR test Applications

Assessment of product "sameness" under certain scale-up and post-approval changes

Optimization of product performance (i.e. release profile) during formulation development

Assessment of product stability / Batch-to-batch uniformity QC test

Initial screening of the in-vivo performance of lead candidates prior to proceeding with clinical end-point / in-vitro permeation studies

Figure 6: IVR Test Applications

In Vitro Permeation (IVP) Studies

Clinical end point studies for the assessment of “bioequivalence” of locally applied products are often characterized by high variability and low sensitivity that make such studies less reliable and less efficient. Furthermore, they are also cumbersome, invasive, time-consuming and expensive. To this end, in vitro drug absorption into and across excised human skin mounted on diffusion cells can serve as a powerful and sensitive tool.

IVP Test principle

The test formulation is applied to the surface of a tissue (e.g. skin, cornea) sample separating the two chambers of a diffusion cell. The formulation remains on the tissue for a specified time under specified conditions. The receptor fluid is sampled at time points throughout the experiment and analysed for the test chemical and/or metabolites.

Using appropriate conditions, which are described in the study protocol, the absorption of a test substance during a given time period is measured by analysis of the receptor fluid and the treated tissue. Analysis of the other components (material remaining in the donor chamber, applicator, and tissue layers) allows for further data evaluation, including total test substance disposition and percentage recovery.

IVP Study project management process

In vitro permeation studies are carefully designed according to the client’s requirements, the purpose of the study and the provisions of the relevant guidelines.
The studies that are intended for submission to the authorities are performed under GLP environment according to an approved by the client written protocol that clearly indicates the objectives and the methods to be employed. The general step-wise approach followed for each IVP study is schematically presented in Figure 8 along with a brief description of each stage

Review of request

Following the submission of the sponsor's request for conducting an in-vitro permeation study, a preliminary project assessment is performed by the lab. Based on the technical aspects / method complexity and the resources required for the study, a quotation is issued and sent to the client.

Bioanalytical and IVP Method Development

The first and most critical step is the development of methods that will be suitable for their intended purpose. During this stage all critical method variables are optimized (e.g. dosing amount and application, IVP study duration and sampling schedule, receptor medium, bioanalytical method parameters)

Bioanalytical Method Validation Study

The in-vitro permeation method should be suitably discriminating (demonstrated during both the pilot and pivotal study) while the analytical methods for determining the content of the test substance in the receptor fluid should be validated according to ICH guideline M10 on bioanalytical method validation.

In-vitro permeation pilot study

In cases where the purpose of the in-vitro permeation study is the pharmacokinetic comparison (i.e. comparison of the rate and extent of in vitro permeation) between a test and a reference product, a pilot study should preferably be performed in order to validate the IVP methodology and estimate the number of donors required for the pivotal study and optimize the sampling scheme

In-vitro permeation pivotal study

The purpose of a pivotal study is to compare the rate and extent of in vitro permeation between a reference and a test formulation in order to support submissions claiming equivalence. Its design (sampling times, number of time points, number of donors / Lots, etc.) highly depends on the outcome of the pilot study.

Figure 7: IVP study stages

IVP Method Development, Validation and Implementation

The utility of the in vitro permeation test (IVPT) methodology for the documentation of bioequivalence has been supported by a substantial body of evidence showing that in vitro results correlate well with and are predictive of human in vivo bioavailability data. However, due to high variability of human skin (related to gender, race, age and anatomical site), the method standardization and verification of reproducibility is a quite challenging task. To this end, EMA provides certain, generalized recommendations for a number of variables that could significantly influence the performance of the applied methodology.

Furthermore, it is required to demonstrate the appropriate discriminatory power of IVPT using the batches with significant alterations compared to the finished product (e.g., by changing the product strength, quantitative composition, CQA and process parameters). A similar procedure for IVPT is also described in the FDA product-specific guideline (Draft Guidance on Acyclovir), but with substantially more attention to detail regarding the method development, validation, and statistical data analysis. The following table summarizes the requirements of both guidance documents with respect to the "elements" of study design and execution, method development and validation.

Table 5: EMA vs FDA IVPT study "elements"

Parameter	EMA	FDA
Study Design	• ISingle dose, cross-over study design • Test, comparator and negative control formulations should be tested using the same donor skin, ideally from adjacent sites, per replicate. • To minimize risk of bias, the study protocol should specify methods of blinding and randomization in line with ICH E8	• Parallel, single-dose, multiple replicate per treatment group. Balanced design • A detailed description of the blinding procedure is to be provided in the study protocol and final report. The method of randomization should be described in the protocol and the randomization schedule provided
Number of Donors	The number of skin donors should not be less than 12, with at least 2 replicates per donor.	It is the responsibility of the applicant to determine the number of donors to adequately power the IVPT pivotal study, however, a minimum of 4 dosed replicates per donor per treatment group (RLD or test) is recommended
Apparatus	The apparatus should ensure consistent temperature control throughout the duration of the experiment. The skin surface temperature should be stable at 32±1ºC	The laboratory qualification of each diffusion cell should, at minimum, qualify the diffusional area of the orifice, the volume of the receptor solution compartment in each diffusion cell, the control of a 32ºC ± 1ºC temperature (at the skin surface), and the control of the rate of stirring or flow rate, as applicable.
Skin	• Excised human skin • Skin barrier integrity tests (e.g. TEWL, electrical impedance/conductance) to ensure validity • Skin thickness measured and reported	• Ex vivo adult human skin • The skin integrity should be checked prior to and after each experiment • The skin thickness and separation technique should be described
Dosing	• Range of 2 – 15 mg/cm2 • Dose application should be validated to ensure reproducibility (±5 %) and homogeneous spreading of the formulation over the skin membrane.	• Range of 5 – 15 mg/cm2 • Control of procedures related to the dose include the control of the area of dose application, the dose amount, the dosing technique, the dose duration, and the blinding and randomization procedures for dosing

Parameter	EMA	FDA
Receptor medium	• Compatibility with skin • Minimum solubility exceeding highest study concentration ideally by an order of magnitude • Inclusion of anti-microbial agent recommended • Stability of analyte should be validated	• Receptor medium does not compromise skin barrier integrity • Sink conditions • Inclusion of anti-microbial agent is conditionally acceptable • The stability of the active substance in the receptor solution over the duration of IVPT study, and sample storage prior to analysis, should be confirmed.
Receptor Solution Sampling	N/A	The accuracy and precision of receptor solution sample collection at each time point should be appropriately qualified
Study Duration	The number of sampling time points should be sufficient to obtain meaningful profiles, i.e. capturing the maximal rate of absorption and a decline in the rate of absorption thereafter, with more frequent sampling during the period of greatest change. The duration for testing should be 24 hours. If the study duration is longer than 24 hours, it should be shown that skin barrier function and integrity is adequately maintained	The study duration should be sufficient to characterize the cutaneous pharmacokinetics of the drug substance, including a sufficiently complete flux profile to identify the maximum (peak) flux and a decline in the flux thereafter across multiple subsequent time points. The sampling frequency should be selected to provide suitable resolution for the flux profile, and a minimum of 8 non-zero sampling time points is recommended across the study duration (e.g. 48 hours).
Contamination / Interference	To identify potential contamination and/or interferences, pre-dose samples collected from each diffusion cell and a parallel non-dosed blank control skin experiment are recommended.	Control of procedures related to study should include a non-dosed control skin section from each skin donor to ensure that drug substance concentrations in the receptor solution are associated with the dose applied and not drug substance contamination in the skin from that donor. A pre-dose “zero” sample collected from each diffusion cell is also recommended, which may identify potential contamination associated with each skin section and/or each diffusion cell.
Analytical Methods	The analytical methods should comply with the Guideline on bioanalytical method validation (EMEA/CHMP/EWP/192217/2009 Rev. 1 Corr. 2)	The receptor sample analysis procedures should be validated in a manner compatible with the current FDA Guidance for Industry on Bioanalytical Method Validation.

Parameter	EMA	FDA
IVPT Precision and Reproducibility	Not specifically mentioned but suggested from the following: IVPT data should be provided in tabular and graphical formats. All individual data and parameters should be listed by formulation together with summary statistics	The pilot study flux and cumulative permeation results should be tabulated for each diffusion cell and time point, with summary statistics to describe the intra-donor average, standard deviation, and %CV among replicates as well as the inter-donor average, standard error, and %CV.
IVPT Robustness	N/A	The variability inherent in the permeability of human skin, whether in vitro or in vivo, may not be compatible with the primary assumption related to the consistency of the test system. Therefore, it may be challenging to qualify broad operational ranges, and study procedures should be controlled as precisely as possible. Relevant results from studies during IVPT method development that appear to support the robustness of the IVPT system may be reported and discussed.
Discrimination ability	The suitability of the test conditions should be demonstrated using batches with different quality attributes (a negative control), such as a drug formulation with 50% of the proposed product strength, that is shown to be statistically different and non-equivalent to the comparator product. The 90% confidence interval for the ratio of means of the comparator and negative control products should be entirely outside the interval of 80.00-125.00%	IVPT Sensitivity is the ability of the IVPT method to detect changes in the cutaneous pharmacokinetics of the drug substance as a function of differences in delivery. IVPT Selectivity is the ability of the IVPT method to discriminate that the cutaneous pharmacokinetics of the drug substance from a product or formulation that exhibits differences in delivery is not equivalent to the cutaneous pharmacokinetics of the drug substance from the RLD product.
Mass balance and dose depletion	The mass balance should be determined. The cumulative amount of the active substance permeated into the receptor medium (A_{total}), the total amount of active substance retained (S_{total}) in the skin samples and amount of active substance retained on the cleaning or experimental equipment (R_{total}) should be presented. The overall recovery of the active substance of 90-110% would be acceptable without justification, larger variation should be fully justified and explained.	The recovery of permeated drug in the receptor solution may be characterized in each diffusion cell as the cumulative total permeation of drug substance in the receptor solution over the IVPT duration. This may be expressed as a percentage of the amount of drug substance in the applied dose. The minimum amount of dose depletion (not accounting for skin content) may thereby be estimated and should be reported.

Parameter	EMA	FDA
Equivalence parameters / Cutaneous pharmacokinetic endpoints and acceptance criteria **Quality Management System / Accreditation**	• Relevant permeation parameters, e.g., the maximal rate of absorption (Jmax) and total amount permeated at the end of experiment (Atotal) should be determined and compared. Additional permeation parameters, such as the time of maximal rate of absorption (tmax) and lag-times, should also be reported. • The 90% confidence interval for the ratio of means of the test and comparator products should be contained within the acceptance interval of 80.00- 125.00%, unless justified. Wider confidence interval limits may be accepted in the case of high variability (detailed discussion below) • The lag-times between the test and comparator products should be the same (i.e. within ± 10%) if present.	• The rate of drug permeation is characterized by the flux (J) and the extent of permeation is characterized by the total cumulative amount of the drug substance permeated into the receptor solution across the study duration. • The 90% confidence interval for the ratio of means of the test and comparator products should be contained within the limits of 80.00-125.00%. Scaled criterion in case of high within-reference variability (detailed discussion below)
	It should be ensured that the performing laboratory is qualified to undertake the studies and that an effective quality system is in place.	Compatible with applicable principles of GLP described in 21 CFR 58

IVP Statistical Considerations

A paired comparison is recommended by both the EMA and FDA. It is important to understand the assumptions in which these statistical methods are based.

In order to perform the paired comparison, the difference between the Test Product (TP) and the Reference Product (RP) is calculated considering each individual donor. Considering that IVPT data do not follow a normal distribution, they should be natural log-transformed prior to any calculation. In the EMA approach, the arithmetic mean of all individual TP-RP differences is calculated. On the other hand, in the FDA approach, a similar rationale to that presented in the EMA guideline on the investigation of equivalence for highly variable drugs is used. In other words, there is an attempt to standardize the difference due to the observed variability in the reference product. Under this paradigm, the within-subject standard deviation (S_{WR}) is evaluated for each IVPT endpoint attained with the RP formulation. If SWR > 0.294, the product is considered highly variable, and the scaled average bioequivalence (SABE) methodology can be used.

In the SABE approach, bioequivalence can be inferred if the geometric mean ratio (GMR) falls within the range [0.8, 1.25] for the selected bioequivalence margin and if the upper bound of the 90% confidence interval for the quantity, $(\mu_T - \mu_R)^2 - \sigma_{WR}^2$, is less than or equal to zero, where θ is equal to $\frac{(\ln(1.25))^2}{(0.25)^2}$. The implementation of SABE analysis enables the capitalization upon the ability of IVPT methodology to discriminate differences in drug permeation through the skin from any single individual, while compensating for the variability from one individual compared to another. According to recent literature this statistical approach has been shown to improve the power of comparative IVPT studies, thus reducing the number of skin donors (i.e. 16 donors with four replicates per donor per treatment group) compared to traditional average bioequivalence analysis requiring almost 40 donors. More specifically, the authors determined the number of donors that would adequately power an IVPT BE study, by conducting power simulations for both PK parameters (Jmax and AUC) using an ABE analysis as well as a SABE

analysis, and using the BE limits of 0.8–1.25 as well as 0.75–1.33 (as recommended by the EMA). The more permissive BE limits of 0.75–1.33 were also considered in the power simulations comparing ABE and SABE so as to illustrate that the power (and efficiency) of an IVPT study is increased to a greater magnitude by a SABE statistical analysis of the results than by widening the BE limits for an ABE analysis. Instead, using a SABE analysis when the SWR is >0.294, while maintaining the traditional BE limits of 0.8–1.25, increases the power of the study to an even greater degree than by widening the BE limits to 0.75–1.33 for an ABE analysis.

Considering that the EMA guideline is not yet official and the assessment approach followed by the authorities is not harmonized, the scientific advice option offered by many authorities is a useful tool in order to ensure that any reasonable (i.e. justified based on a scientifically sound rationale) deviations from the strict acceptance criteria will not trigger major objections. The following table summarizes the issues pertaining to the demonstration of IVP sameness by meeting the tight regulatory requirements and possible ways out.

Table 6: Problems and Solutions regarding IVPT

Regulatory expectations		Practical issues / limitations	QMx
FDA	EMA		
Restrictive Confidence Interval (presented in Table 5 and discussed in "IVP Statistical considerations")		Intrinsic wide variability of topical semi-solid formulations (i.e. batch-to-batch variation of reference product), IVPT methodology and inter-, intra-individual variability of human skin.	• Well-founded study design to minimize sources of variation — preliminary screening of batches to select those that will minimize the probability of failure (based on the data obtained with IVR testing) — Balancing of the distribution of skin thicknesses and barrier function values in each treatment group (test or reference) by a procedure specified in the study protocol — Strict control of experimental parameters to minimize analytical measurement variability • Participation in scientific advice meetings with authorities to support the study design and criteria
Sufficient number of donors to adequately power the study (i.e. a minimum of 4 dosed replicates per donor per treatment group)	**High number of skin donors** (i.e. at least 12, at least 2 replicates per donor)	Extremely difficult to procure a sufficient amount of ex vivo sections	• Pilot study to determine the number of donors required to adequately power the pivotal study based on the estimated ratio of endpoint means and 'within-reference' variability. • Collaboration with several skin tissue banks that meet specific quality and ethical standards to ensure both tissue quality and availability at the time of the pivotal study. • Use of skin surrogates, either artificially cultured human skin models or animal skin models (e.g. porcine skin) provided that they have previously been considered as acceptable by the authorities in the frame of a scientific advice meeting
Dosing amount (clinically relevant)		The therapeutic dose for most topical products corresponds to a very small amount that is difficult to apply in a manner that will allow the complete and consistent coverage of the entire permeation area without introducing high variability	• Well-established (i.e. validated methodology) for dose application • Scientifically sound justification, supported by relevant data, in cases where the application of a dose specified in the SmPC is not feasible
Equivalence parameters / endpoints		There are cases where a Jmax is not observed or cannot be unequivocally determined	• Use of steady-state flux (i.e. release rate)

Tissues / Models

Tissues from human or animal sources can be used. Either epidermal membranes (enzymically, heat or chemically separated) or split-thickness skin (typically 200-400 µm thick) prepared with a dermatome, are acceptable.

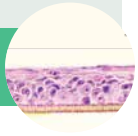
Other tissue models, based in artificial membranes, provided by MatTek Corporation, can also be employed. These models include but are not limited to the following examples:

Also known generically as a Reconstructed Human Epidermis (RHE), EpiDerm is a ready-to-use, highly differentiated 3D tissue model consisting of normal, human-derived epidermal keratinocytes (NHEK) cultured on specially prepared tissue culture inserts.

EpiDerm™

The EpiOcular tissue construct is a nonkeratinized epithelium prepared from normal human keratinocytes (MatTek). It models the cornea epithelium with progressively stratified, but not cornified cells

EpiOcular™



EpiIntestinal is a highly differentiated 3D tissue model that closely recapitulates the physiology, tissue structure, and function of the epithelium of the small intestine.

EpiIntestinal™

MatTek's EpiOral tissues consist of normal, human-derived epithelial cells. The tissues, which are cultured on specially prepared cell culture inserts using serum free medium, attain levels of differentiation on the cutting edge of in vitro cell culture technology. Morphologically, the tissue models closely parallel native buccal human tissues

EpiOral™

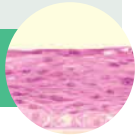


Figure 8: Artificial membranes

Although these artificial skin surrogates offer numerous advantages (e.g., defined thickness, composition, ease in handling and storage, and reproducibility in the permeation data), the correlation with the human data is often poor, due to the inability to completely recreate the heterogeneous nature of the skin, including cell metabolism and skin appendages. Consequently, skin surrogates are currently recommended for the early screening of different formulations, while human skin should be used for the in vitro permeation testing of finished drug products.

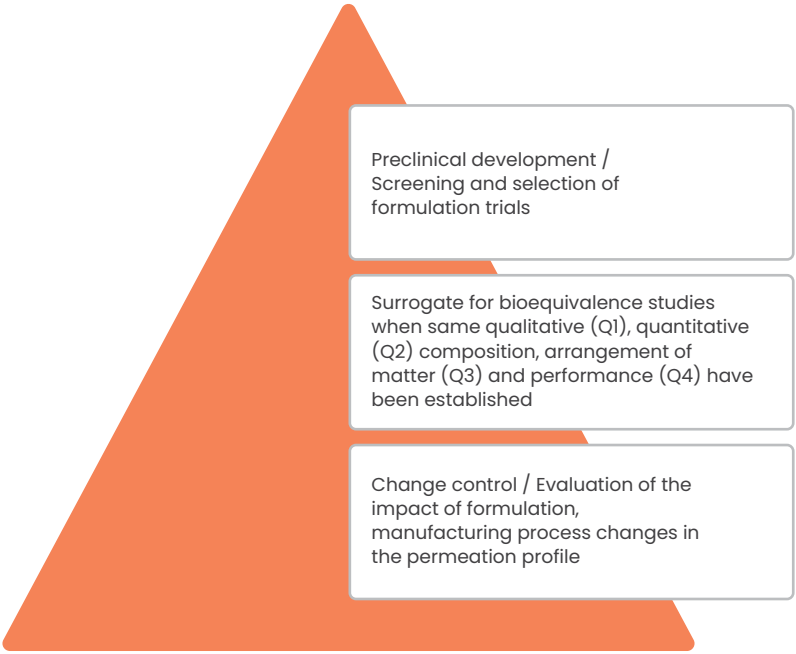
Tissue Integrity Tests

Any physical deterioration in the tissue preparations (e.g. due to time at ambient temperature or hydration, pretreatment) may result in an overestimate of permeability. Pre-study evaluation is always performed in order to ensure that damaged tissue will be eliminated before performing the test. The methods employed are the following:

- checking that trans-epidermal water loss (TEWL) from the stratum corneum is in the normal range for the skin type,
- measurement of transepithelial electrical resistance (TEER) which provides a convenient indicator of tight junction development and barrier function
- measuring the penetration characteristics of a reference material

IVP test Applications

The major advantage of in vitro studies is the possibility for controlling the conditions of the experiment and therefore changes in permeation should only arise from changes in the formulation and / or the tissue employed. The IVP test is a valuable tool for demonstrating equivalence with respect to efficacy. To this end, it can serve as a valuable tool for the following applications:



QUALIMETRIX
Analytical Laboratories - Quality Control

Equipment

Qualimetrix is equipped with state-of-the-art instrumentation spanning a wide range of analytical techniques combined with analytical expertise and experience.

UPLC-MS/MS (triple quadrupole, QqQ)

Several triple quadrupole mass spectrometers hyphenated with UPLC chromatographic systems are employed within the CRO. The latter is the technique of choice for the reliable identification and quantitation of known analytes that are contained within a complex matrix, such as the IVPT analytes and excipients that permeate through human skin and end up in the receptor medium. Through the Multiple Reaction Monitoring (MRM) mode, it provides higher Signal-to-Noise, allowing thus selective and sensitive identification and quantitation, as well as wide linear range. Analyte determination in the frame of IVRT testing is a significantly less challenging task in terms of sensitivity and selectivity and it can therefore be performed by means of the less sophisticated PDA detector.

UPLC-MS/MS (triple quadrupole, QqQ)

The Phoenix RDS Robotic Diffusion Station available at QMx provides the capability for state-of-the-art diffusion testing. Th system features Teledyne Hanson's breakthroughs in four areas:

a. diffusion cell design

b. heating and stirring;

c. automated sampling and collection and

d. computerized control

The dry heat diffusion cell delivers significantly improved test results as compared to traditional water jacketed, displacement sampling systems. The precision heating and stirring components built into each of the six cell blocks provide outstanding control of temperature and speed. Automatic sampling and collection are accomplished through a syringe driven probe on an XYZ platform controlled by Teledyne Hanson's sophisticated Diffusion Master software. The automated system mimics the way sampling, collection, and media replace are performed by laboratory analysts when working manually, while simultaneously reducing the potential for variances due to procedural inconsistencies.

Other Instruments

The following is an indicative list of other instruments that are employed for establishing "extended pharmaceutical equivalence" between a reference and a generic topical product

- HPLC/UPLC – UV/Vis/PDA/FLD/ELSD/RID/CAD
- Ion Chromatography Sys. – Conductivity/PAD
- GC – MS, GC – MS/MS
- GC – FID/ECD (split-splitless and head space)
- ICP – MS
- Conductivity meter
- Karl Fischer titrator
- UV – Vis spectrophotometer
- ATR– Fourier Transform Infrared Spectroscopy System (ATR-FTIR)
- PSD Analyser Malvern Mastersizer 3000
- XRD
- Microplate reader
- Viscometer
- Cryoscopic Osmometer
- Optical Microscope
- Cytation 5 multimode reader

QMx Authorizations and Certifications

Qualimetrix S.A. is a service laboratory authorized by the Greek Health Authorities (National Organization for Medicines, EOF) for batch certification and quality control testing activities of human medicinal products - sterile and non-sterile - and human or animal extracted biological medicinal products. All In Vitro Release tests in the frame of post-approval quality control and product changes are performed under **GMP**. Furthermore, Qualimetrix is regularly inspected by the General Chemical State Laboratory of Greece (official member of the OECD) and holds a **GLP** Certification for conducting In Vitro Permeation studies.

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