In Vitro Permeation Test (IVPT) for Pharmacokinetic Assessment of Topical Dermatological Formulations

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In vitro assessment of topical (dermal) pharmacokinetics is a critical aspect of the drug development process for semi-solid products (e.g., solutions, foams, sprays, creams, gels, lotions, ointments), allowing for informed selection of new chemical entities, optimization of prototype formulations during the non-clinical stage, and determination of bioequivalence of generics. It can also serve as a tool to further understand the impact of different excipients on drug delivery, product quality, and formulation microstructure when used in parallel with other techniques, such as analyses of rheology, viscosity, microscopic characteristics, release rate, particle size, and oil droplet size distribution. The in vitro permeation test (IVPT), also known as in vitro skin penetration/permeation test, typically uses ex vivo human skin in conjunction with diffusion cells, such as Franz (or vertical) or Bronaugh (or flow-through) diffusion cells, and is the technique of choice for dermal pharmacokinetics assessment. Successful execution of the IVPT also involves the development and use of fit-for-purpose bioanalytical methods and procedures. The protocols described herein provide detailed steps for execution of the IVPT utilizing flow-through diffusion cells and for key aspects of the development of a liquid chromatography–tandem mass spectrometry method intended for analysis of the generated samples (epidermis, dermis, and receptor solution). © 2020 Wiley Periodicals LLC.

Basic Protocol 1: In vitro permeation test
Support Protocol: Dermatoming of ex vivo human skin
Basic Protocol 2: Bioanalytical methodology in the context of the in vitro permeation test

Keywords: dermal drug delivery • in vitro permeation test (IVPT) • pharmacokinetics • skin permeability • topical formulation

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INTRODUCTION

The skin is the largest organ of the body and has the primary functions of protecting against xenobiotics, microorganisms, radiation, and mechanical injury and regulating

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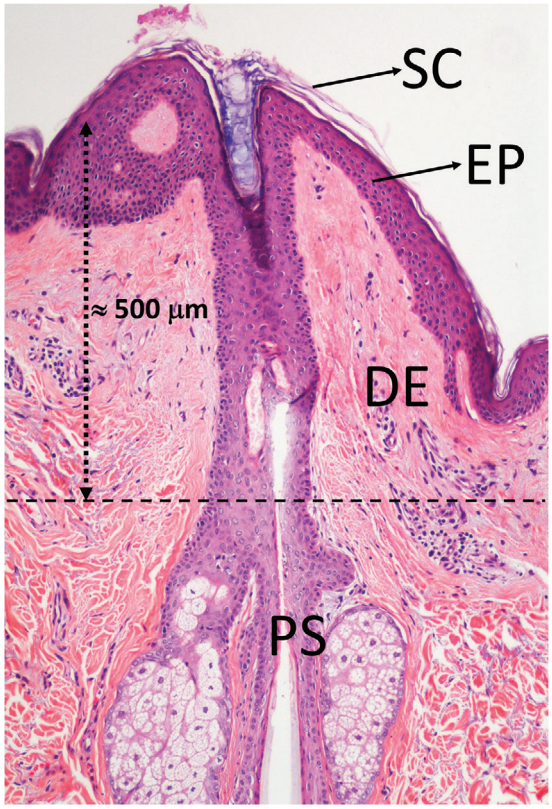

Figure 1 Histology of human skin, highlighting stratum corneum (SC), viable epidermis (EP), dermis (DE), and a pilosebaceous unit (PS). For scale, 500-μm thickness is shown; note that the skin section can fold during tissue preparation, resulting in an apparent variability in thickness.

the loss of nutrients and water. It is complex in nature, with four main layers: stratum corneum, viable epidermis, dermis, and hypodermis (Fig. 1). In addition to presenting a number of appendages and structures, such as sweat glands, sebaceous glands, nerve endings, and hair, the skin also contains a variety of immune cells (Biederman, Rocken, & Carballido, 2004). The skin is an attractive organ for drug delivery, either for treatment of localized diseases (e.g., acne, plaque psoriasis, atopic dermatitis), permitting low systemic exposure (thus decreasing the likelihood of adverse events), or for treatment of systemic diseases, avoiding first-pass metabolism. The intrinsic challenge of dermal delivery of a compound is penetration through the stratum corneum, the outermost skin layer, comprising proteins and lipids and the primary barrier to pharmacologically active agents. There are various ways in which the stratum corneum can be disrupted to allow drug penetration, either by active procedures, such as mechanical disruption via microneedles or iontophoresis, or by passive mechanisms due to chemical interaction with certain excipients present in topical formulations.

A critical aspect of dermal drug development involves the selection of a molecule with physicochemical properties amenable to skin permeation, good solubility and stability in the semi-solid formulation of interest (e.g., solutions, foams, sprays, creams, gels, lotions, ointments), and the ability to reach the biological target site at concentrations sufficient to elicit a therapeutic response (Mitra et al., 2015; Smith et al., 2016; Zane et al., 2016). Additionally, the topical formulation should demonstrate good physical stability and adequate aesthetics (e.g., an absence of color and odor, easy to apply on skin), leading to improved patient compliance during treatment (Hernandez, Jain, Sharma, Lam, & Sonti, 2020).

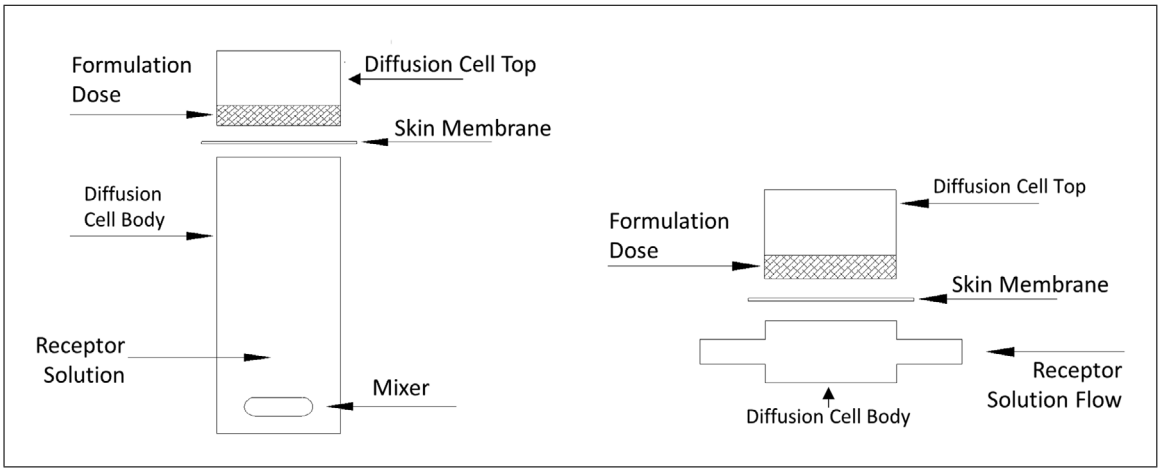

Figure 2 Schematics of diffusion cells, with the Franz type (or vertical diffusion cell) on the left and the Bronaugh type (or flow-through diffusion cell) on the right.

Selection of a new chemical entity (NCE) and subsequent assessment of prototype formulations require a variety of assays across several discovery and development functions, including chemistry, pharmacology, formulation development, toxicology, and pharmacokinetics. One critical aspect of the pharmacokinetic (PK) evaluation is drug delivery to the target site, and the most commonly used technique for its assessment is the in vitro permeation test (IVPT) (Barker et al., 2018; Flaten et al., 2015; Mitra et al., 2015).

The IVPT involves dosing of the semi-solid product under evaluation on a biological membrane placed within a diffusion cell containing receptor solution (also known as receptor/receiving fluid). The purpose of the test is to evaluate the drug permeation through the stratum corneum and subsequent skin layers (skin distribution), as well as the amount of compound collected in the receptor solution (skin penetration), allowing calculation of the compound's skin flux. The IVPT is suitable for the relative ranking of different semi-solid formulations and is not intended to provide an absolute prediction of in vivo/clinical dermal levels or systemic exposure for the compound under consideration.

Basic Protocol 1 describes the setup and execution of an IVPT using customized flow-through diffusion cells; the underlying principles are not specific and can be applied to studies employing vertical diffusion cells (Franz cells) or conventional flow-through diffusion cells (Bronaugh cells) (Fig. 2). The Support Protocol describes ex vivo human skin dermatoming and further information on skin sourcing, as the quality of the biological membrane used in the IVPT can significantly impact the experimental outcomes. Basic Protocol 2 outlines procedures that require specific assessment when developing a bioanalytical method for analysis of IVPT samples.

IN VITRO PERMEATION TEST

BASIC PROTOCOL 1

The IVPT is conducted by placing sections of the biological membrane of choice (see Support Protocol), generally dermatomed ex vivo human abdominal skin (with the stratum corneum side up), across flow-through diffusion cells and dosing the formulation(s) of interest on top (Fig. 3). Receptor solution will flow underneath the skin section and is collected into a 96-well plate for analysis of unbound compound penetrating through the skin section (Fig. 4). Further, at the end of the experiment, the skin sections can be harvested (Fig. 5) for analysis of total compound amounts in the epidermis and dermis (Fig. 6). Sample analysis is performed via a fit-for-purpose bioanalytical method (typically LC-MS/MS) to determine compound levels in the epidermis, dermis, and receptor solution and to subsequently calculate parameters such as skin flux, lag phase time, and

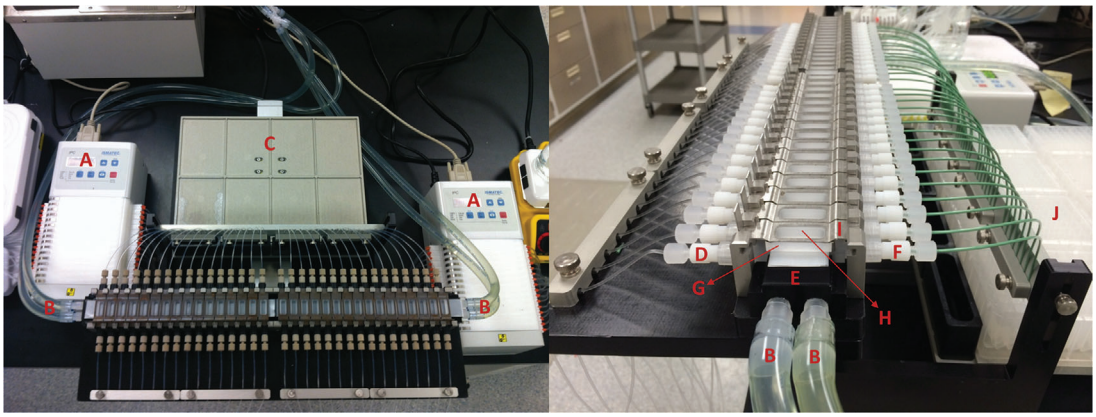

Figure 3 Photographs of the top view (left) and the side view (right) of the system with 32 customized flow-through diffusion cells, denoting a few components: two 16-channel peristaltic pumps (**A**), circulating water bath lines (**B**), the 96-well plate sample collection holder with capacity for eight plates (**C**), diffusion cell inlet tubing (connected to the peristaltic pumps) and luer lock (**D**), the cell-warming block (**E**), the diffusion cell outlet with luer lock and PEEK tubing (**F**), the donor compartment block/diffusion cell top (**G**), the donor chamber (where the skin membrane is located and the formulation is dosed) (**H**), the stainless-steel spring (**I**), and 96-well plates for sample collection (**J**).

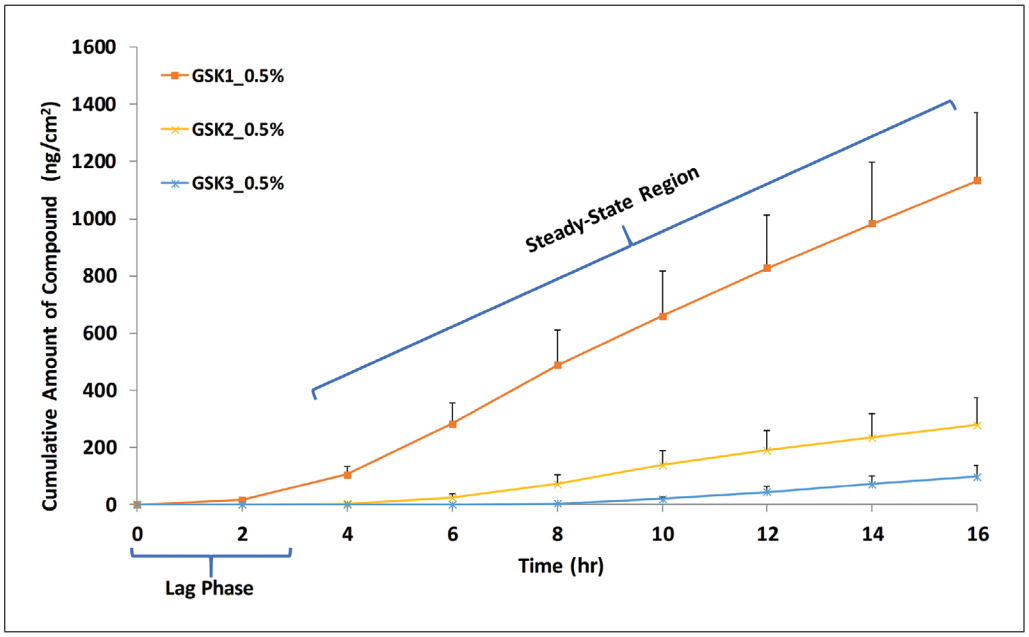

Figure 4 Example IVPT data showing the cumulative amount of compound (over dosed area, ng/cm^2) in the receptor solution over 16 hr post-dosing. Lines represent the cumulative mean amount from 11 to 13 replicates from two donors plus the standard error of the mean (SEM). Annotations for the steady-state region and the lag phase of the curve for GSK1 are shown. The dose consisted of 10 µl of a solution at 0.5% (w/v) of the test article over a 1-cm^2 dosing area.

total cumulative amount. Further details on ex vivo human skin sourcing and processing are outlined in the Support Protocol.

The results of the IVPT can have a high degree of variability, mainly attributed to the assay complexity and dependency on non-standardized skin samples and the assessment of prototypes/formulations that may not yet be available commercially (thus not yet fully characterized). It is suggested that each laboratory have at their disposal easily available and fully characterized (e.g., confirmed physical and chemical stability) prototype or commercial formulations (latter preferred) that can be used to confirm the

Figure 5 Photograph of the skin laying on aluminum foil (left) prior to incubation for epidermis/dermis splitting and photograph of dermis homogenate (right).

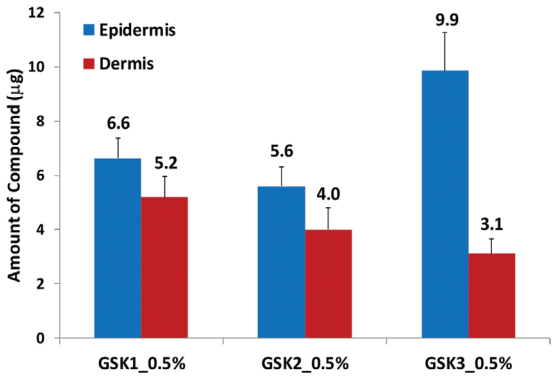

Figure 6 Example IVPT data showing the amount of compound (µg) delivered into the epidermis (blue) and dermis (red) 16 hr post-dosing. Bars represent the mean amount of each compound from 11 to 13 replicates from two donors plus the standard error of the mean (SEM). The dose consisted of 10 µl of a solution at 0.5% (w/v) of the test article over a 1-cm^2 dosing area.

consistency of the IVPT workflows over time. Some examples of commercial formulations are Hydrocortisone Cream USP 1%, Clobetasol Propionate Cream USP 0.05%, Desoximetasone Gel USP 0.05%, Fluocinonide Gel USP 0.05%, Acyclovir Ointment USP 5%, Lidocaine Ointment USP 5%, Minoxidil Topical Solution USP 5%, and Fluocinolone Acetonide Topical Solution USP 0.01%, among others. It is highly recommended that for each project (e.g., NCEs for the same biological target or lead molecule being evaluated in several prototype formulations), a "bridge" formulation (also with characterized physical/chemical stability) be used across IVPT experiments where a direct data comparison is desired. A reliable, fit-for-purpose (or validated) bioanalytical method can help decrease variability and minimize the frequency of outlier results (see Basic Protocol 2).

NOTE: The human biological samples used in the examples here were sourced ethically, and their research use was in accord with the terms of informed consent under an IRB/EC-approved protocol. Collection and use of ex vivo human skin must adhere to all institutional and governmental guidelines and regulations and be reviewed and approved by the relevant human research ethics committee (HREC). Appropriate occupational health and safety procedures (e.g., for disposal of biological waste) and guidelines for handling of

biological specimens (e.g., use of protective clothing, gloves) should be followed when handling human tissues. Further details on ex vivo human skin sourcing and processing are outlined in the Support Protocol.

Materials

10× phosphate-buffered saline (PBS), pH 7.4 (MilliporeSigma™, 65061L)
Purified and deionized water (e.g., Milli-Q® water)
Dermatomed (split-thickness) ex vivo human abdominal skin sheets (e.g., BioIVT, or see Support Protocol; store at −80°C)
Formulation prototypes to be tested [compounds dissolved in proprietary solution at 0.5% (w/v)]
LC-MS-grade organic solvent [acetonitrile (LC-MS grade; e.g., Honeywell Chromasolv™, 394676) or methanol (LC-MS grade; e.g., Honeywell Chromasolv™, 394666); for compound extraction and sample dilution]
9:1 acetonitrile/hexane mixture (HPLC grade or higher)
Homogenization solution (see step 27)
70:30 water/organic solvent (typically acetonitrile or methanol; see above)

2-L glass bottles (dedicated for IVPT use to avoid cross-contamination)
Ultrasonic bath/degasser (e.g., Branson Ultrasonics™, CPX952339R)
37°C water or bead/dry bath (e.g., Lab Armor™, 74309714)
Circulating water bath (Fisher Scientific, 13-874-173)
Diffusion cell system, including flow-through diffusion cells, cell-warming blocks, donor compartment blocks, stainless streel springs, dosing chambers, and associated PEEK/plastic tubing (see Fig. 3, which depicts customized diffusion cells that we used; alternate suppliers are PermeGear and Logan Instruments)
Digital micrometer (Mitutoyo, 547-500S)
Razor blades (Fisher Scientific, 12-640)
Laboratory cutting board (e.g., high-density polyethylene, Fisher Scientific, 09-002-24B)
Forceps (e.g., Fisher Scientific, 12-000-132)
Peristaltic pumps (two pumps; 16 channels each; Ismatec/Cole-Parmer, IPC 16 ISM 933),
1.5-ml Eppendorf® tubes (Eppendorf™, 022379224)
Positive-displacement pipet (e.g., Gilson™ MICROMAN™ E) and pipet tips/capillary pistons (e.g., Gilson capillary pistons CP100, F148314)
Receptor-collection 96-well plates (see Basic Protocol 2)
LC-MS/MS-compatible 96-well plates (Nunc® 96 DeepWell™ plate, non-treated)
Cotton swabs (e.g., Puritan™, 25806 10WC)
Adhesive tape (e.g., 3M™ Scotch® Transparent Film Tape 600, S-6748)
Aluminum foil (Fisher Scientific, 14-648-236)
Dry-heat incubator (e.g., Fisherbrand™ Isotemp™ Microbiological Incubator, 75 L, Stainless Steel, Fisher Scientific, 15-103-0513), 60°C
Reinforced homogenization tubes containing stainless steel beads (e.g., Omni International, Hard Tissue Grinding Mix, 2-ml Reinforced Tubes, SKU 19-620)
Homogenizer (e.g., Omni International Bead Ruptor Elite, SKU 19-040E)
Repeater pipet (Eppendorf™, 4897000398) and pipet tips (25- or 50-ml; Eppendor™, 0030089472)
Refrigerated centrifuge with temperature control (Thermo Scientific™ Sorvall™ Legend™ X1R), 5°C
LC-MS/MS system (Waters® Xevo® TQ-XS with Waters® Acquity UPLC™ system or similar)
Vendor-specific software (e.g., MassLynx™ for Waters® systems)

Prepare receptor solution

1. Prepare 2 L receptor solution (receiving/receptor fluid) by mixing 200 ml of 10× PBS with 1800 ml of purified and deionized water.

   Volumes can be scaled proportionally based on the number of diffusion cells used in the experiment, the receptor solution flow rate, and the length of the experiment. This protocol involves 32 diffusion cells and an experiment length of 16 hr (flow rate of 10 μl/min).

   Receptor solution composition will vary depending on the solubility and chemical stability of the analyte(s) under consideration (see Basic Protocol 2).

   IMPORTANT NOTE: *It is critical to ensure that all glassware used to prepare the receptor solution is clean and free of detergents; it is generally advised to keep such glassware allocated solely for the receptor solution preparation.*

2. Degas 2-L glass bottles containing the receptor solution for 60 min using an ultrasonic bath/degasser.

   Residual air bubbles present in the receptor solution may impact the continuous flow or result in air bubbles accumulating underneath the skin throughout the experiment, resulting in higher experimental variability.

3. Move receptor solution bottles from the ultrasonic bath/degasser to a 37°C water or bead/dry bath adjacent to the diffusion cell system and allow to warm to 37°C.

   Note that the water or bead/dry bath mentioned here is used to keep the receptor solution temperature at 37°C. The combination of the circulating water bath (mentioned in step 4) and the water or bead/dry bath temperatures will affect the skin surface temperature, which should be at 32 ± 2°C for the duration of the experiment.

Prepare diffusion cells

4. Place flow-through diffusion cells within cell-warming blocks and set circulating water bath to 38°C.

   Depending on the laboratory temperature and air flow, among other factors that may impact heat transfer, the circulating water bath temperature may need to be adjusted accordingly to ensure that the skin surface temperature will be kept at 32 ± 2°C (via a digital thermometer with a probe; e.g., Fisherbrand™ Traceable™ Digital Thermometer, Fisher Scientific, 15-077-8) for the duration of the experiment. Allow the blocks to equilibrate for ≥30 min before placing the skin sections to ensure that all blocks are at a consistent temperature.

5. Connect outlet of the diffusion cell system to the associated PEEK/plastic tubing using the attached luer lock fittings.

   Ensure that the PEEK tubes are properly secured and aligned with the appropriate locations in the guide to allow proper sample collection and prevent cross-contamination. This step is not applicable for other types of diffusion cell systems, which may use automated sample collection in scintillation vials or HPLC vials or manual collection.

Prepare ex vivo human skin sections

6. Remove dermatomed ex vivo human abdominal skin sheets from the −80°C freezer and let them thaw at room temperature.

   For more details on the dermatomed skin, see the Support Protocol.

   It is recommended that at least two but preferably three or more different skin donors are evaluated, with a minimum of four replicates for each formulation per skin donor. The skin-thawing process should not be accelerated by using external heating sources. It is advised to not freeze/thaw the same skin sheet more than once; therefore, estimate the amount of skin to be used in the study beforehand and remove only the necessary number of skin sheets.

7. Once the skin sheets are thawed and equilibrated to room temperature, measure skin thickness using a digital micrometer.

   Thickness measurement of at least six different areas of the skin sheet is recommended. Areas that deviate from the expected thickness (500 ± 100 μm) should be discarded to minimize experimental variability. The average (± standard deviation) skin thickness of each skin donor should be reported in the final study report.

8. Cut skin sheets into approximately 2.5 × 1.0–cm sections (n = 32) with a razor blade on a laboratory cutting board and use forceps to spread each section on a diffusion cell, making sure to cover entire recessed area.

   The required amount of skin should always account for an area ≥30% in excess of the theoretical sum of the dosing areas of the diffusion cells. Each diffusion cell will require excess skin to allow the donor compartment block to effectively seal the receptor solution compartment, preventing leaks.

Finalize diffusion cell setup and start system equilibration

9. As soon as each skin section is spread, place donor compartment block on the skin section and secure it with stainless steel spring, leaving the dosing area (1 cm^2) unoccluded.

   IMPORTANT NOTE: *Skin sections dry over time, causing shrinking; therefore, if the skin is not quickly and properly secured, it may result in receptor solution leakages coming from the receptor compartment.*

10. Connect each tubing connection to a peristaltic pump and place each peristaltic pump inlet end into receptor solution bottles.

11. Prime each peristaltic pump to ensure that all tubing connections have a steady flow of receptor solution.

12. Connect luer lock on each tubing connection to the inlet of the diffusion cells and set each peristaltic pump to 30 μl/min for a ≥5 min to allow diffusion cells to equilibrate with receptor solution.

    This step can be performed by using the "prime" setting of the peristaltic pump (for the peristaltic pumps used, the "prime" flow rate is 44 μl/min); however, beware that extended exposure (typically >10 min) to the fast-flowing receptor solution may cause damage to the skin section.

13. Once all diffusion cells have receptor solution dripping from the PEEK tubing outlet, set each peristaltic pump to the experimental flow rate (10 μl/min) and allow to equilibrate for ≥30 min before dosing the formulation prototypes to be tested (see steps 14 and 15).

    In Figure 3, photographs of the top and side views of the customized flow-through diffusion cells are shown.

Dose test formulation(s) and start the study run

14. Transfer an aliquot of test formulation(s) from the original container into appropriately labeled 1.5-ml Eppendorf® tube(s).

    Some semi-solid formulations such as creams, suspensions, and high-concentration or saturated solutions may separate (or crystallize) over time. It is important to ensure that test formulation(s) are homogeneous prior to transferring to the Eppendorf® tube(s) and performing dosing on the skin membrane. Transfer to Eppendorf® tubes prior to dosing is intended to minimize the potential to cross-contaminate the original formulation container.

15. Using a positive-displacement pipet and respective pipet tip/capillary piston, 10 μl of each test formulation on dosing area (1 cm^2) of the skin membrane,

ensuring the formulation is as evenly spread as possible. Leave dosing chamber unoccluded.

The test formulations should be randomized across the 32 diffusion cells to minimize bias. Note that occlusion of the dosing chamber (with Parafilm®, for instance) can be done in instances where its effect on increasing compound penetration is of relevance; however, this is an exception and therefore not described herein.

16. Once all formulations are dosed, begin run with a 5-min diversion of the receptor solution flow to waste to allow the diffusion cells to re-equilibrate, followed by automated receptor solution collection into receptor-collection 96-well plates at the desired intervals for 16 hr.

    A study length of 16 hr is adequate (start the run in the late afternoon, with study completion in the early morning of the next day), with receptor solution samples collected every hour (if sample capacity is an issue, collection every 2 hr is acceptable). For the purposes of screening, a 16- to 24-hr experiment should allow the majority of compounds/formulations to display a linear/steady-state phase (constant skin flux) after a lag time period (the time required to achieve steady state); Figure 4 displays an example of the steady-state phase and lag time period for a compound. Longer experiments (sometimes up to 4 days) may be necessary depending on the goal of the study; however, the receptor solution should contain 1% (w/v) antibiotic-antimycotic solution (see recipe in Reagents and Solution section) to prevent microorganism growth and deterioration of the skin section.

Receptor solution sample collection and processing

17. At the end of the 16-hr experiment, transfer receptor solution samples to LC-MS/MS-compatible 96-well plates and dilute with LC-MS-grade organic solvent.

    The receptor solution compound levels are generally in the pg/ml to low ng/ml range, and adding organic solvent (typically acetonitrile or methanol) will help prevent precipitation and nonspecific binding during storage. A general dilution scheme uses 175 µl receptor solution and 75 µl organic solvent, resulting in diluted samples at a ratio 70:30 aqueous/organic solvent mixture; this is done to minimize the matrix effect during LC-MS/MS analysis.

    Supporting Information Video 1 demonstrates use of an automated liquid handler to accomplish this step.

18. Store diluted samples at 4°C until analysis.

    A laboratory refrigerator unit (2° to 8°C; e.g., Fisherbrand™ Isotemp™ General Purpose Laboratory Refrigerators, Sliding Glass Door, Fisher Scientific) may be used for storage.

    The length of time for which receptor solution samples can be stored is dependent upon the analyte stability in the aqueous/organic solvent mixture. Analyte stability should be assessed during bioanalytical method development (see Basic Protocol 2).

Epidermis and dermis sample collection and processing

19. Remove excess test formulation from surface of the skin using a dry cotton swab, being careful not to disrupt the integrity of the stratum corneum.

20. Remove spring and donor compartment block from the diffusion cells.

21. Clean remaining test formulation from the surface of the skin by using three consecutive cotton swabs:

    a. First, use a dry cotton swab to ensure that bulk of the remaining formulation is removed.
    b. Second, use a cotton swab dipped in 9:1 acetonitrile/hexane mixture to dissolve and remove additional residual formulation.

c. Finally, use another dry cotton swab to remove any excess solvent from skin surface.

22. Remove skin sections from the diffusion cells and transfer to a clean laboratory cutting board, with dermis side down, maintaining ≥1 in. of space between samples to prevent cross-contamination.

23. Use three consecutive adhesive tape strips to remove superficial layers of the stratum corneum.

    Compound in this layer is considered to have not penetrated into the skin barrier and will not be analyzed.

24. Transfer skin samples to a sheet of aluminum foil and place them in a dry-heat incubator (equilibrated at 60°C) for 2.5 min to help break down epidermal-dermal junction (Fig. 5).

25. Use clean forceps to separate (via scraping) epidermis from the dermis, being careful to prevent cross-contamination of the samples during skin splitting.

    IMPORTANT NOTE: *Epidermis typically has significantly higher compound levels than the dermis; therefore, skin sample handling should be done with that aspect in mind. Cross-contamination can happen when the epidermis is not well separated from the dermis or when the forceps cause substantial compound smearing from the epidermis to the dermis. Skin section-to-section cross-contamination is also possible during the splitting process, so it is recommended to clean the forceps with alcohol swabs (e.g., Medique, 22133) after splitting each section.*

26. Place each sample into a separate reinforced homogenization tube containing stainless steel beads.

27. Add 500 µl homogenization solution to each tube.

    The homogenization solution is typically a mixture of 1:1 aqueous/organic solvent, often acidified to increase extraction efficiency [e.g., 1:1 water/acetonitrile, with 0.1% (v/v) formic acid (LC-MS grade, Fisher Scientific, A1171-AMP)]. Depending on the analyte of interest, the homogenization solution can be modified to increase extraction efficiency, which is an important parameter to be evaluated during method development (see Basic Protocol 2).

28. Prepare samples using a homogenizer following the cycles below for each skin layer. After homogenization is complete, wait ∼3 min before opening tubes.

    a. Epidermis: 5.8 M/s for 15 s, 1 cycle.
    b. Dermis: 7.1 M/s for 30 s, one round of 2 cycles, 90-s dwell time in between cycles.

    In some cases, the dermis samples may not be fully homogenized, so step 28b should be repeated after the samples have cooled for ≥1 min. The homogenization procedure increases the internal sample temperature; therefore, it is necessary to wait ≥3 min before opening the tubes.

    Photographs of the skin prior to incubation at 60°C and of dermis homogenate are shown in Figure 5.

29. Using a repeater pipet and pipet tips, add 1000 µl organic solvent (acetonitrile or methanol, depending on the solvent of choice during method development; see Basic Protocol 2) to each tube of homogenized epidermis or dermis. Close tubes after the solvent is added.

30. Briefly mix samples using the homogenizer (5.8 m/s for 15 s, 1 cycle) to ensure interaction of the organic solvent with the epidermis/dermis homogenates and efficient protein precipitation.

31. Centrifuge tubes for 5 min at 10,000 × *g*, 5°C.

32. Transfer 10 µl of each sample into 490 µl of 70:30 water/organic solvent (typically acetonitrile or methanol, depending on the choice during method development; see Basic Protocol 2).

    In some instances, the epidermis or dermis samples may need to be further diluted for the compound concentrations to be within the analytical range of the bioanalytical method previously developed (see Basic Protocol 2).

    Supporting Information Video 2 demonstrates use of an automated liquid handler to accomplish this step.

33. Store these samples at 4°C until analysis.

    The length of time for which skin homogenate samples can be stored is dependent upon the analyte stability in the aqueous/organic solvent mixture. Analyte stability should be assessed during bioanalytical method development (see Basic Protocol 2).

Analyze receptor solution, epidermis, and dermis samples

34. Analyze samples using a validated or fit-for-purpose LC-MS/MS analytical method with an LC-MS/MS system.

    A freshly prepared calibration curve should always be used, and it is good practice to include analytical quality controls (with low, middle, and high concentrations within the calibration curve range) throughout the run to ensure good LC-MS/MS performance.

35. Use vendor-specific software for mass spectrometric data analysis and data extraction.

    Most mass spectrometer vendors provide quantitation software (e.g., MassLynx™ for Waters® systems) for data analysis and calculation of analyte concentration. The results can then be extracted into a Microsoft® Excel® file for further analysis of the IVPT-specific data.

    Further data analysis is described in the Understanding Results section of the Commentary.

DERMATOMING OF EX VIVO HUMAN SKIN

SUPPORT PROTOCOL

Dermatomed (split-thickness, typically 500-µm) human skin, sourced from a cadaver or elective surgery (typically abdominoplasties), is the most appropriate membrane of choice for Basic Protocol 1 due to its intrinsic relevance to the clinical setting and ease of handling during experimental procedures (see Fig. 7). Dermatomed porcine skin has a relatively similar morphology to human skin and can be used as a secondary option. Use of rodent skin is discouraged due to significant differences from human skin in terms of morphology and stratum corneum thickness. Use of human or porcine full-thickness (non-dermatomed) skin can pose experimental challenges and is generally not recommended. Epidermal sheets and isolated stratum corneum are described in the literature but do not appear to offer advantages over split-thickness skin.

Different anatomical sites are likely to demonstrate differences in compounds' skin flux, considering that the biophysical properties (e.g., hydration; transepidermal water loss, pH) of the skin can vary across the body (Kleesz, Darlenski, & Fluhr, 2012). Ex vivo abdominal skin offers two main advantages: it is more easily sourced (due to the frequency of elective surgeries intended to remove excess skin, although cadavers can also be a source) than skin from other anatomical sites, and it provides larger areas, suitable for use in experiments that necessitate several replicates/diffusion cells for evaluation of different formulations. Another factor that may impact compounds' skin flux is the skin thickness, which can vary within the same skin sheet; therefore, the thickness of

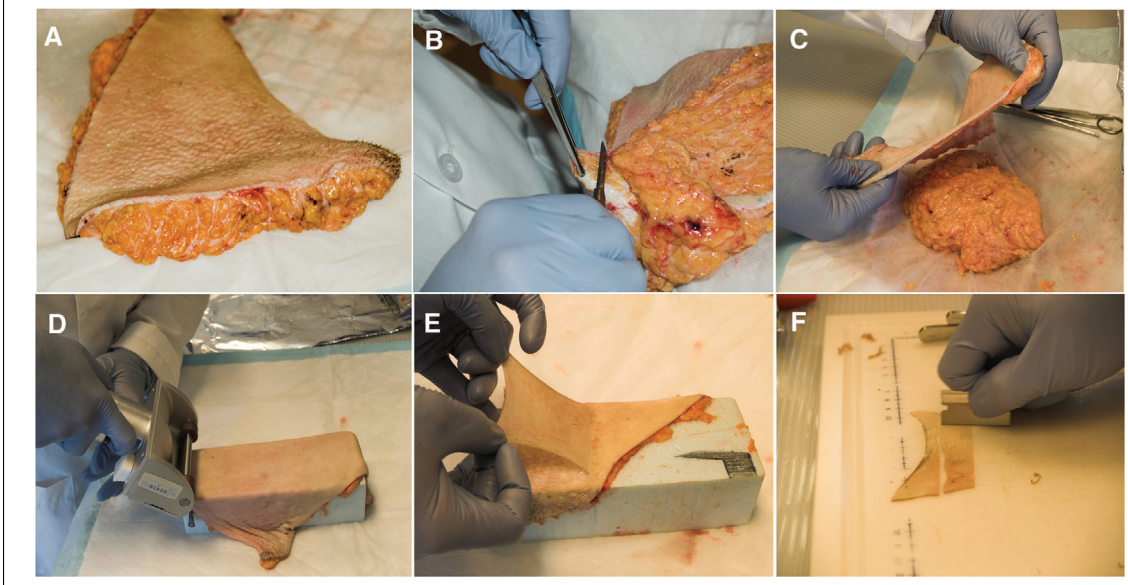

Figure 7 Photographs of ex vivo human abdominal skin (**A**); the defatting procedure (**B**); removal of the full-thickness layer (**C**); dermatoming using an electrical dermatome (**D**); removal of split-thickness, 500-μm skin (**E**); and cutting of skin sections prior to placement in the diffusion cells (**F**).

dermatomed skin should be confirmed prior to assembling the skin membrane in the diffusion cell, and it is recommended that the variability does not exceed 20% of the targeted value (e.g., 500 ± 100 μm) to minimize experimental outliers (Wilkinson et al., 2006).

It is important to note that the gender, age, race, and health state of the human skin donor can affect the permeability of the biological specimen (Darlenski & Fluhr, 2012). Although local regulations may limit the amount of donor information provided for each skin specimen, the collection of biometric data is recommended if allowed. Because skin sourcing can be costly and sometimes supply may not be ample, investigators should not be restrictive regarding skin donor age, gender, or race. Instead, use of a centralized database that enables the allocation of unique skin donor numbers and storage of relevant donor information can be a useful tool to ensure quality, keep track of potential data trends, and provide accurate data for biological sample management audits. Other useful information to store includes average skin thickness, commercial supplier name, and inventory data (e.g., sample location inside a −80°C freezer). It has been observed that commercially sourced cadaver skin samples generally have less donor information than skin sourced from elective surgery and that the quality of cadaver samples is not always comparable. If possible and not cost prohibitive for the IVPT laboratory, skin from elective surgeries (typically abdominoplasties) should be chosen over cadaver skin.

Investigators with limited experience in skin processing may encounter difficulties in dermatoming ex vivo abdominal skin (full thickness), which is the process presently described. An alternative is the sourcing of already dermatomed (split-thickness, generally 500-μm) skin from local vendors, such as BioIVT. Full-thickness skin can be obtained from a variety of vendors: BioIVT, ZenBio, and Tissue Solutions, among others.

CAUTION: The dermatoming process should be performed with a high degree of caution because of the handling of blades and the electrical dermatome; therefore, wearing personal protective equipment, including use of Kevlar® gloves under conventional nitrile gloves, is highly recommended.

NOTE: Collection and use of ex vivo human skin must adhere to all institutional and governmental guidelines and regulations and be reviewed and approved by the relevant HREC. Appropriate occupational health and safety procedures (e.g., for disposal of biological waste) and guidelines for handling of biological specimens (e.g., use of protective clothing, gloves) should be followed when handling human tissues.

Materials

Full-thickness ex vivo human abdominal skin (e.g., BioIVT)
1% (w/v) antibiotic/antimycotic solution (see recipe)

Disposable blue underpads (e.g., Medline™ Protection Plus Disposable Underpads, Fisher Scientific, 23-666-062)
Scalpel (e.g., Integra™ Miltex™ Sterile Safety Scalpels, 12-460-459)
Forceps (e.g., Fisher Scientific, 12-000-132)
High-density foam blocks (approximate dimensions of 2 × 4 × 7 in.)
Electrical dermatome (e.g., Integra® Padgett® Dermatome Model B)
Dermatome blades (e.g., Robbins Instruments, 718776)
Aluminum foil (Fisher Scientific, 14-648-236)
Water-impermeable plastic bags (e.g., Ampac™ Flexibles SealPAK Heavy-Duty Pouches, VWR, 01-812-76)
Heat sealer (e.g., Heat Sealer Ampac™, VWR, 11214-107)
Roller (optional)

Obtain, defat, and rinse ex vivo human skin specimen

1. Obtain full-thickness ex vivo human abdominal skin.

   Immediately after collection at the surgery center/hospital, skin is transferred to a plastic container with a small volume (just enough to keep the skin moist) of 1× PBS, pH 7.4 (from 10×; MilliporeSigma™, 65061L), and kept at 4°C using gel packs (e.g., Sonoco™ ThermoSafe U-tek™, 03-528-1) during storage and shipment (generally same-day or overnight delivery).

   It is recommended to work directly with commercial suppliers of biological specimens, which generally have agreements in place with several hospitals and surgery clinics and can deliver human skin (full thickness or dermatomed) to the IVPT laboratory to meet experimental demand. Full-thickness skin should never be frozen during shipment, as this may affect the tissue quality upon thawing. Note that the full-thickness skin should be processed as soon as it is received by the IVPT laboratory.

2. Place full-thickness skin on a secured bench covered with disposable blue underpads and remove subcutaneous fat from the skin samples using a scalpel and forceps, working from a corner edge.

   CAUTION: *For additional protection against accidental cuts, steps 2 to 4 should be done while wearing Kevlar® gloves (e.g., SHOWA™ Atlas™ Grip Aramid Fiber KV300 Gloves, 19-270-234) under disposable nitrile gloves (e.g., Kimberly-Clark Professional™, 55082).*

3. Briefly rinse skin with 1% antibiotic/antimycotic solution.

Dermatome and store defatted skin

4. Place full-thickness skin on a high-density foam block and dermatome it to the specified thickness (i.e., 500 ± 100 μm) using an electrical dermatome and dermatome blades.

   It is recommended to confirm the thickness of the dermatomed skin by using a digital micrometer (e.g., Mitutoyo, 547-500S). Skin thickness should be assessed across different areas of the skin section to confirm consistency.

5. If necessary, store split-thickness skin for later use by spreading the tissue out on aluminum foil and placing it in a water-impermeable plastic bag.

6. Remove air from the water-impermeable plastic bag using a roller or gloved hands and seal bag using a heat sealer. Store skin sheets at −80°C until the time of the experiment (see Basic Protocol 1).

   A –80°C freezer (e.g., PHCbi TwinGuard® Series –86°C ULT Freezer, MDF-DU302VX-PA) may be used for dermatomed skin storage.

   Previous experiments have shown that skin samples can be stored this way for up to 4 years without damaging the stratum corneum, i.e., the physical barrier that is the rate-limiting step for skin penetration.

BASIC PROTOCOL 2

BIOANALYTICAL METHODOLOGY IN THE CONTEXT OF THE IN VITRO PERMEATION TEST

Although the IVPT experiments described in Basic Protocol 1 are generally applied in the drug discovery setting and thus are not subject to the FDA Bioanalytical Method Validation Guidance for Industry (see Internet Resources), some pre-experiment method development should take place to maximize data quality. Sufficient selectivity, precision, and accuracy of the LC-MS/MS method should be established prior to sample analysis; sample-to-sample carryover and sample stability should also be assessed. Nonspecific binding of all analytes to the containers (labware) used and skin (epidermis and dermis) extraction efficiency for analytes should be evaluated. Each of these assessments provides increased confidence in the reported analytical concentrations, thus de-risking the selection of NCEs and/or formulations for development into later stages.

NOTE: The methods described herein do not make use of an internal standard, which is commonly included in validated bioanalytical methods. The main reason for not describing internal standardization in this protocol is that an isotope-labeled material is generally not available in earlier stages of nonclinical development, when the majority of the IVPT studies will be performed. Because of the high dilution factors typically used in analysis of samples from an IVPT experiment, matrix effects are generally limited, especially if adequate LC-MS/MS conditions are used. Therefore, if an appropriate internal standard is unavailable, IVPT studies can be completed using peak area count rather than the more traditional peak area ratio for quantitation.

Additional Materials *(also see Basic Protocol 1)*

1× PBS, pH 7.4 (from 10×; MilliporeSigma™, 65061L)
Acidified LC-MS-grade water (Honeywell Chromasolv™, 392534), pH 4.0
Acetonitrile (LC-MS grade; e.g., Honeywell Chromasolv™, 394676)
50 µg/ml compound stock solution
Homogenization solution: 1:1 acetonitrile/water (LC-MS grade; Honeywell Chromasolv™, 392534) + 0.1% (v/v) formic acid (LC-MS grade; Fisher Scientific, A1171-AMP) or 1:1 methanol/water (LC-MS grade; Honeywell Chromasolv™, 392534) + 0.1% (v/v) formic acid (LC-MS grade; Fisher Scientific, A1171-AMP)
Methanol (LC-MS grade; e.g., Honeywell Chromasolv™, 394666)

1-L glass flasks (dedicated for LC-MS/MS analysis)
LC-MS-compatible 96-well plates (Nunc® 96 DeepWell™ plate, non-treated)
Dry-heat incubator (e.g., Fisherbrand™ Isotemp™ Microbiological Incubator, 75 L, Stainless Steel, Fisher Scientific, 15-103-0513), 37°C
20-ml glass vials (e.g., DWK Life Sciences, 986562)

Plastic square-well receptor-collection 96-well plates (Nunc™ 96-Well Polypropylene DeepWell™ Storage Plates)

Silanized square-well receptor-collection 96-well plates (Thermo Scientific™, 60180-P308)

Silanized LC-MS/MS-compatible HPLC vials (DWK Life Sciences, 110000300S)

Prepare calibration curves and quality controls in three different aqueous matrices (potential receptor solutions)

1. In three clean 1-L glass flasks, allocate 350 ml of (i) 1× PBS, (ii) purified and deionized water, and (iii) acidified LC-MS-grade water (pH 4.0).

   IMPORTANT NOTE: *Due to the sensitivity of LC-MS/MS instruments, there are multiple recommendations to be followed to minimize cross-contamination. It is suggested that disposable nitrile gloves (e.g., Kimberly-Clark Professional™, 55082) or equivalent be worn during all the work described in Basic Protocol 2 to avoid contamination of the samples by the investigator. Additionally, worn gloves should be frequently discarded and exchanged for clean pairs to avoid sample cross-contamination. Dedicated glassware should also be utilized for LC-MS/MS analyses to minimize the risk of cross-contamination from inadequately cleaned glassware.*

2. Separately dilute each of the solutions above with 150 ml LC-MS-grade organic solvent (acetonitrile or methanol) to generate a mixture of 70:30 receptor solution/organic solvent in an LC-MS-compatible 96-well plate.

   The organic solvent of choice will be based on the analyte solubility and stability. Acetonitrile works in most cases, but sometimes methanol is preferred due to its relatively lower cost or improved analyte extraction from skin samples.

3. Use dilution scheme below, in duplicate, to generate two calibration curves for each of the 70:30 receptor solution/organic solvent mixtures to be evaluated:

   a. Spike 50 µl of 50 µg/ml compound stock solution into 4950 µl acetonitrile, generating a 500 ng/ml stock ("Intermediate stock solution," or "Intermediate").
   b. Spike 25 µl of "Intermediate" into 600 µl diluent to make (1) (20.0 ng/ml).
   c. Spike 450 µl of (1) into 150 µl diluent to make (2) (15.0 ng/ml).
   d. Spike 200 µl of (2) into 300 µl diluent to make (3) (6.00 ng/ml).
   e. Spike 200 µl of (3) into 300 µl diluent to make (4) (2.40 ng/ml).
   f. Spike 200 µl of (4) into 300 µl diluent to make (5) (0.960 ng/ml).
   g. Spike 200 µl of (5) into 300 µl diluent to make (6) (0.384 ng/ml).
   h. Spike 200 µl of (6) into 300 µl diluent to make (7) (0.154 ng/ml).
   i. Spike 100 µl of (7) into 200 µl diluent to make (8) (0.102 ng/ml).

   Ensure that thorough mixing (vortexing) is complete prior to each subsequent dilution.

   The dilution scheme represented here is an example for guidance, with typical calibration curves for IVPT experiments ranging from 100 pg/ml to 20 ng/ml. The suggested dynamic range described here is only 200-fold, as opposed to in most of the bioanalytical methods intended for analysis of compound levels in plasma, where a calibration curve spanning at least three orders of magnitude is generally used. The narrower range for the IVPT is to ensure minimal to no carryover (especially for more lipophilic or charged molecules), which can substantially increase the analytical variability. Carryover is a source of analytical error and is caused when analyte from a previously analyzed sample are still present in the analytical system and co-elute with a subsequent sample.

4. Use dilution scheme below, in triplicate, to generate quality-control samples in each of the 70:30 receptor solution/organic solvent mixtures to be evaluated:

   a. Spike 20 µl of "Intermediate" (see step 3) into 580 µl diluent and mix thoroughly (QC1; 16.7 ng/ml).

b. Spike 150 µl of (2) (see step 3) into 500 µl diluent and mix thoroughly (QC2; 3.85 ng/ml).
c. Spike 40 µl of (3) (see step 3) into 800 µl diluent and mix thoroughly (QC3; 0.183 ng/ml).

A separately weighed quantity of the test article is generally used for preparing solutions as quality controls; because this is a fit-for-purpose method, used in the context of discovery, the solutions are prepared using the same compound stock solution.

Evaluate baseline, carryover, precision, and accuracy

5. Inject a blank 70:30 receptor solution/organic solvent sample from the first potential receptor solution (see step 2) into the LC-MS/MS system a sufficient number of times to establish background signal (typically 3 to 5 injections).

   This is particularly important after equilibrating the LC-MS/MS system after startup due to the potential accumulation of contaminants (which may cause analytical background noise) on the LC column under starting conditions.

6. For that same 70:30 receptor solution/organic solvent mixture, starting at the lowest concentration calibrator and working to higher concentrations (see step 3), inject each analyte-containing well five successive times.

   Injecting from low to high concentration minimizes the potential impact of sample-to-sample carryover during this initial evaluation, when carryover is yet to be determined.

7. Inject blank sample (see step 2) five successive times to establish carryover.

   If the carryover signal from the highest calibration curve point is >20% of the signal from the lowest calibrator, consider modifying the wash solutions and procedures or the calibration curve linearity range. Carryover that is >20% of the signal from the lowest calibrator can significantly impact data quality, especially for the receptor solution samples, where concentrations are often in the pg/ml to low ng/ml range.

   Ensure that the signal from the blank samples has been reduced to <20% of the lowest calibrator prior to moving on to step 8.

8. Repeat precision and accuracy analysis (see steps 5 to 7) with each remaining potential receptor solution.

   Accuracy is represented as a percentage of the nominal concentration, wherein a calculated concentration below the nominal concentration will be <100% (e.g., 95%) and a calculated concentration above the nominal concentration will be >100% (e.g., 105%). It is recommended that the average of the five replicates at each concentration level be within 15% of the nominal concentration (i.e., between 85% and 115%), except at the lower limit of quantification, where an average accuracy within 20% (i.e., between 80% and 120%) is acceptable.

   Precision is represented by the relative standard deviation (RSD) at each calibration point [(average concentration/standard deviation of concentration) × 100] and is expressed as a percentage. %RSD should be <15% at all concentration levels, except at the lower limit of quantification, where %RSD around 20% is acceptable.

   An example of a dataset for precision and accuracy analysis of a compound in a generic mixture of 70:30 receptor solution/organic solvent is shown in Table 1.

Evaluate analyte stability in receptor solution

9. Use dilution scheme from step 3 to generate two calibration curves for each of the 70:30 receptor solution/organic solvent mixtures to be evaluated.

   Receptor solution/organic solvent mixtures used in stability evaluation should have demonstrated sufficient accuracy/precision during steps 5 to 8.

Table 1 Precision and Accuracy Dataset for a Compound in a Generic 70:30 Receptor Solution/Organic Mixture

Nominal (ng/ml)	Experimental (ng/ml)	Precision (%)	Accuracy (%)
0.0826	0.073 ± 0.01	8.55	88.0
0.124	0.124 ± 0.01	11.5	99.8
0.310	0.312 ± 0.03	10.8	101
0.774	0.808 ± 0.03	3.72	104
1.94	2.05 ± 0.1	5.12	106
4.84	5.00 ± 0.2	3.56	103
12.1	12.2 ± 0.5	3.96	101
16.1	15.7 ± 0.6	3.61	97.3

10. Use dilution scheme from step 4 to generate quality-control samples, in triplicate, in each of the 70:30 receptor solution/organic solvent mixtures to be evaluated.
11. Prepare solvent blanks (i.e., no compound) in each of the 70:30 receptor solution/organic solvent mixtures to be evaluated.
12. On Day 0, inject solvent blank sample of the first potential receptor solution (see step 11) a sufficient number of times to establish background signal (typically 3 to 5 injections).
13. For that same potential receptor solution, inject each calibrator well (see step 9) once and each quality-control well (see step 10) in triplicate, starting at the lowest concentration calibrator and working to higher concentrations.
14. Repeat analysis (see steps 12 and 13), including the solvent blanks, with each remaining potential receptor solution.
15. On each subsequent day, perform the following steps:
    a. Inject solvent blank sample of the first potential receptor solution (see step 11) a sufficient number of times to establish background signal.
    b. Prepare (as per step 3) and inject two calibration curves (one injection per well).
    c. Inject each quality-control well from Day 0 (see step 10; kept inside refrigerated UPLC sample manager, generally between 5° and 10°C, until use) in triplicate.
    d. Repeat for each potential 70:30 receptor solution/organic solvent mixture.

    As the numbers of analytes, formulations, and replicates increase, the analytical run time for an IVPT study can extend over multiple days. Therefore, it is recommended that stability is evaluated for ≥48 hr (72 hr is preferred).

    It is recommended to inject from low to high concentrations (intermixing calibrators and quality controls as needed) to minimize the potential impact of sample-to-sample carryover.
16. Analyze intra-day and inter-day precision and accuracy of the quality-control samples.

    Intra-day precision and accuracy are evaluated as described above in step 8. This value is meant to represent the precision and accuracy during any one analytical run, and values from multiple days or runs should not be combined.

    Inter-day precision and accuracy can be determined by extending the calculations from step 8 across all analytical runs during the stability experiment. These are meant to use each individual calculated concentration across the stability study, and not the averages of the intra-day values. The inter-day evaluation is meant to account for day-to-day ana-

Table 2 Dataset for the 48-hr Stability of a Compound in a Generic 70:30 Receptor Solution/Organic Mixture

	Day 0		Day 1		Day 2		Inter-day (Days 0-2)	
70:30 PBS/ acetonitrile	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Low (134 pg/ml)	7.70	113	8.45	103	5.81	118	9.21	111
Mid (6.59 ng/ml)	3.60	102	4.62	99.9	6.12	105	5.18	102
High (23.1 ng/ml)	1.19	85.3	2.36	81.3	1.66	87.3	3.43	84.6

lytical variability, and samples that pass the precision and accuracy acceptance criteria are defined as stable.

An example of the 48-hr stability results for a compound in a generic 70:30 receptor solution/organic solvent mixture is shown in Table 2.

Evaluate extraction of analyte from skin samples

17. Label 66 reinforced homogenization tubes for skin extraction samples:
    a. Three tubes for each combination of acetonitrile/methanol, dermis/epidermis/control, and low/middle/high concentration (54 tubes).
    b. Three tubes for each combination of acetonitrile/methanol and dermis/epidermis (blanks; 12 tubes).

    When multiple analytes are being evaluated, a pilot extraction procedure can be utilized to save time during method development and prior to selection of a lead molecule. Following the same general protocol, only the "mid" concentration samples are prepared and analyzed. Once a lead molecule is selected, it is suggested to confirm the extraction efficiency by running the assessment outlined in steps 17 to 37.

    Generally, acetonitrile provides sufficient extraction efficiency (generally considered as >90%) and has been viewed as the preferred solvent because it facilitates matching the final sample composition to that needed for most commonly used LC-MS/MS methods. However, methanol provides alternative chemical properties (e.g., polarity, lipophilicity) that may improve extraction efficiency for some analytes and should be also evaluated.

18. Make two diluted solutions from compound stock solution:
    a. Spike 300 µl of 50 µg/ml compound stock solution into 600 µl acetonitrile, generating a "mid" concentration solution.
    b. Spike 100 µl of 50 µg/ml compound stock solution into 900 µl acetonitrile, generating a "low" concentration solution.

    Stock solution (50 µg/ml) will be used as the "high" concentration solution.

19. Remove dermatomed ex vivo human abdominal skin sheets from −80°C freezer and let thaw at room temperature.
20. Use a razor blade to cut skin into 2.5 × 1.0–cm sections.
21. Use forceps to lay skin pieces flat, dermis down, onto a clean aluminum foil sheet.
22. Incubate skin on aluminum foil in a dry-heat incubator (equilibrated at 60°C) for 2.5 min to help break down the epidermal-dermal junction.
23. Remove aluminum foil from the incubator.
24. Use forceps to separate (via scraping) epidermis and dermis skin sections and place sections in the appropriately labeled homogenization tubes from step 17.

25. Spike 10 µl "high" solution (50 µg/ml compound stock solution), "mid" solution (step 18a), or "low" solution (step 18b) directly onto each corresponding dermis or epidermis sample.

    Take care to spike the solution directly onto the skin to allow for maximum absorption of the compound into the skin section and avoid nonspecific binding to the homogenization tube.

    Dermis and epidermis blank-control skin sections are not spiked with the compound of interest but are incubated.

26. Incubate spiked skin samples and controls for 4 hr in a dry-heat incubator (set at 37°C).

27. At the 3.5-hour mark generate spiked solvent-control solutions in triplicate:

    a. Use a repeater pipet to add 500 µl homogenization solution to corresponding control samples.
    b. Spike 10 µl "high" solution (50 µg/ml compound stock solution), "mid" solution (step 18a), or "low" solution (step 18b) directly into each corresponding solvent-control sample.

28. Remove skin samples from the incubator.

29. Use a repeater pipet to add 500 µl homogenization solution to corresponding skin samples.

30. Homogenize samples, controls, and blanks at the following settings using a homogenizer:

    a. Epidermis: 5.8 M/s for 15 s, 1 cycle.
    b. Dermis: 7.1 M/s for 30 s, one round of 2 cycles and 90-s dwell time in between cycles.

    After homogenization is complete, wait ∼3 min before opening the tubes to allow the samples to cool. In some cases, the dermis samples may not be fully homogenized, and step 30b should be repeated after the sample has cooled for ≥1 min.

31. Using a repeater pipet, add 1000 µl acetonitrile or methanol (organic solvent matching homogenization solution) as a protein precipitation solvent to all samples.

    All homogenization tubes will now have 1500 µl of total volume, utilizing either acetonitrile or methanol as the organic component.

32. Briefly mix all samples and controls using homogenizer (5.8 M/s for 15 s, 1 cycle).

33. Centrifuge all samples and controls for 5 min at 10,000 × *g*, 5°C.

34. Transfer 10 µl supernatant from each sample and control into 490 µl of the appropriate 70:30 receptor solution/organic solvent mixture.

35. Use dilution scheme from step 3 to generate calibration curves, in duplicate, in the appropriate 70:30 receptor solution/organic solvent mixture.

36. Use dilution scheme from step 4 to generate quality-control samples, in triplicate, in the appropriate 70:30 receptor solution/organic solvent mixture.

37. Quantitatively analyze samples via LC-MS/MS using calibration curves and quality controls and determine extraction efficiency compared to the control samples, which are considered to have a recovery of 100%.

    An example epidermis and dermis extraction dataset is shown in Table 3. In this case, acetonitrile and methanol gave similar extraction efficiencies. Acetonitrile was selected

Table 3 Epidermis and Dermis Dataset Comparing the Extraction Efficiency of Acetonitrile/Water and Methanol/Water Mixtures

	(1:1 acetonitrile/water) + 0.1% formic acid mixture			(1:1 methanol/water) + 0.1% formic acid mixture		
		LOW			LOW	
	Mean ± SD (ng)	Precision (%)	Recovery (%)	Mean ± SD (ng)	Precision (%)	Recovery (%)
Control	37.7 ± 2	4.71	100	37.2 ± 23	7.51	100
Dermis	29.7 ± 2	5.36	78.9	30.0 ± 1	4.59	80.6
Epidermis	30.6 ± 1	3.85	81.3	30.1 ± 2	7.28	80.8
		MID			MID	
	Mean ± SD (ng)	Precision (%)	Recovery (%)	Mean ± SD (ng)	Precision (%)	Recovery (%)
Control	133.3 ± 7.57	5.68	100.0	124.8 ± 4.66	3.74	100.0
Dermis	108.3 ± 4.43	4.09	81.2	101.5 ± 5.38	5.30	81.4
Epidermis	105.7 ± 6.12	5.79	79.2	100.5 ± 3.74	3.72	80.6
		HIGH			HIGH	
	Mean ± SD (ng)	Precision (%)	Recovery (%)	Mean ± SD (ng)	Precision (%)	Recovery (%)
Control	387.5 ± 16.58	4.28	100.0	375.9 ± 7.19	1.91	100.0
Dermis	335.4 ± 8.58	2.56	86.6	304.9 ± 19.88	6.52	81.1
Epidermis	323 ± 4	1.34	83.4	311.6 ± 7.34	2.36	82.9
	(1:1 acetonitrile/water) + 0.1% formic acid Average recovery (%)			(1:1 methanol/water) + 0.1% formic acid Average recovery (%)		
Control	100			100		
Dermis	82.2			81.0		
Epidermis	81.3			81.4		

as it tends to produce a more compact "pellet" of precipitated homogenized epidermis or dermis after centrifugation, facilitating automated liquid handling (with a lower probability of clogging pipet tips during volume transfer).

Evaluate nonspecific binding of analyte to assay components

38. Generate necessary solutions in 20-ml glass vials for binding assessment:

    a. Spike 50 µl of 50 µg/ml compound stock solution into 4950 µl acetonitrile, generating a 500 ng/ml "Intermediate" stock solution.
    b. Spike 500 µl of "Intermediate" into 9500 µl of the selected receptor solution, generating a 25 ng/ml "high" standard solution.
    c. Spike 300 µl of "Intermediate" stock into 9700 µl of the selected receptor solution, generating a 15 ng/ml "mid" standard solution.
    d. Spike 60 µl of "Intermediate" stock into 9940 µl of the selected receptor solution, generating a 3 ng/ml "low" standard solution.

39. Pipet 500 µl of each of the standard solutions, in triplicate, into each of the following containers:

    a. Plastic LC-MS/MS-compatible 96-well plate to be used for LC-MS/MS analysis.
    b. Plastic square-well receptor-collection 96-well plate used for IVPT receptor solution collection.
    c. Silanized square-well receptor-collection 96-well plate used for IVPT receptor solution collection.

    Nonspecific binding is a concentration-dependent process and can have a prominent effect on compounds or formulations that demonstrate a relatively low skin flux (e.g., receptor solution concentrations in the pg/ml to low ng/ml range).

    Additional labware that can be analyzed for nonspecific binding includes glass-coated LC-MS/MS 96-well plates (following same protocol as above) and diffusion cells (via submersion of a cell in each of the stock solutions after performing step 40).

40. Pipet 1000 µl of each of the standard solutions from step 38, in triplicate, into silanized LC-MS/MS-compatible HPLC vials.

41. Incubate samples for 30 min at room temperature.

42. Add 214 µl organic solvent to each 96-well plate incubation well.

    For consistency across the different bioanalytical steps, it is recommended that the organic solvent of choice is the same as that selected for the skin extraction procedure (see steps 29 and 31).

43. Add 428 µl organic solvent to each silanized HPLC vial.

44. Use dilution scheme from step 3 to generate calibration curves, in duplicate, in the appropriate 70:30 receptor solution/organic solvent mixture.

45. Use dilution scheme from step 4 to generate quality-control samples, in triplicate, in the appropriate 70:30 receptor solution/organic solvent mixture.

46. Quantitatively analyze samples via LC-MS/MS using calibration curves and quality controls and determine nonspecific binding compared to that in the silanized HPLC vials, which are considered controls with 100% recovery.

    Alternatively, samples can be analyzed without calibration curves and quality controls, comparing the peak area of the different incubation mixtures to that of the contents of the silanized glass HPLC vials, which are considered controls with a 100% recovery.

    An example of a nonspecific binding dataset is shown in Table 4. In this case, no significant nonspecific binding was observed, and typical labware was utilized for the

Table 4 Example of a Nonspecific Binding Dataset across Different Types of Labware

	LOW			MID			HIGH			
Container	Mean ± SD (ng/ml)	Precision (%)	Recovery (%)	Mean ± SD (ng/ml)	Precision (%)	Recovery (%)	Mean ± SD (ng/ml)	Precision (%)	Recovery (%)	Average recovery (%)
Silanized HPLC vial	1.30 ± 0.117	9.00	100	6.50 ± 0.343	5.27	100	10.0 ± 0.261	2.61	100	100
Glass IVPT 96-well plate	1.17 ± 0.0876	7.51	89.5	5.50 ± 0.228	4.13	84.7	8.47 ± 0.190	2.25	84.4	86.2
Plastic IVPT 96-well plate	1.13 ± 0.0634	5.60	86.9	6.16 ± 0.279	4.53	94.8	9.51 ± 0.270	2.84	94.7	92.1
Plastic LC-MS 96-well plate	1.17 ± 0.0789	6.69	90.5	6.49 ± 0.255	3.93	99.9	9.23 ± 0.990	10.7	92.0	94.1

IVPT experiment. Some molecules may display substantial binding to plastic, especially at low ng/ml concentrations. The nonspecific binding evaluation in the earlier stages of bioanalytical method development can help in the selection of proper labware.

REAGENTS AND SOLUTIONS

Antibiotic/antimycotic solution, 1%

500 ml 1× PBS, pH 7.4 (from 10×; MilliporeSigma™, 65061L)
2.5 ml Fungizone (Invitrogen®, 15290018; store at −20°C)
2.5 ml penicillin-streptomycin-glutamine (PSG; Fisher®, BW17718R; store at −20°C)
0.5 ml gentamicin (Invitrogen®, 15750060; store at room temperature)
Store ≤3 months at −5°C

COMMENTARY

Background Information

Initial assessments of the IVPT using specific apparatuses started with the development of the Franz cells, or VDCs (Franz, 1975), followed by the Bronaugh cells, or flow-through diffusion cells (Bronaugh, Stewart, & Congdon, 1982). The main differences between the two types are related to their configuration, vertical versus horizontal, and the flow of receptor solution, which is "static" or continuous (see Fig. 2). These differences have an impact on certain aspects of the experimental procedure, such as receptor solution composition (to ensure sink conditions), the frequency of time-point collection (flow-through cells provide an easier sampling mechanism), the need for more sensitive bioanalytical methods (VDCs yield comparatively higher compound concentrations in the receptor solution due to their static nature, thus requiring less sensitive methods than flow-through cells), and the ability to automate the experimental procedure (flow-through cells are more amenable to sample collection in 96-well plates).

The IVPT can be used for selection of NCEs, optimization of prototype formulations during the nonclinical stage, and determination of bioequivalence of generics. Although in silico models have been proposed for prediction of skin flux of molecules dissolved in water (Brown et al., 2012; Mitragotri et al., 2011), there are limitations when evaluating complex semi-solid formulations, where the dermal pharmacokinetics profile will be a result of the interplay between several excipients and their interactions with the compound(s) of interest and the skin barrier. For these reasons, use of synthetic membranes or skin surrogates (Flaten et al., 2015) is discouraged and is likely to result in data that are not representative of the in vivo/clinical behavior of the semi-solid product under development. Additionally, IVPT results have been shown to correlate with clinical data, especially when comparing formulations (Lehman, Raney, & Franz, 2011), thus demonstrating the value of in vitro data using ex vivo human skin as the biological membrane of choice.

Some of the limitations of the IVPT are the intrinsic biological variability of ex vivo human skin (Akomeah, Martin, & Brown, 2007; Williams, Cornwell, & Barry, 1992), its relatively lower throughput compared to other screening techniques, and the relatively high level of assay complexity (Akomeah, Nazir, Martin, & Brown, 2004; Henning, Schaefer, & Neumann, 2009; Selzer, Abdel-Mottaleb, Hanh, Schaefer, & Neumann, 2013). Furthermore, it is a purely PK assay and therefore does not provide insight into pharmacodynamic (PD) aspects or the differences between healthy and diseased skin, although some assays attempting to make such assessments have been reported (Fang et al., 2016; Smith et al., 2016). The IVPT should not be used to evaluate skin metabolism, which requires a proper experimental setup for its assessment (Manevski et al., 2015); it is relevant to note that the relative level of metabolizing enzymes in the skin is substantially lower than in the liver (Eijl et al., 2012; Hewitt et al., 2013). The IVPT should be used in the context of other complementary in vitro and in vivo techniques, with the goal of de-risking topical product development and ensure the selection of the best formulation for first-time-in-human (FTIH) studies.

Assessment of IVPT samples with matrix-assisted laser desorption ionization (MALDI) imaging mass spectrometry is useful for providing spatial resolution and a better

understanding of the drug concentration gradients across skin layers (Bonnel et al., 2018). MALDI can also be employed in nonclinical in vivo studies and clinical studies (Hochart, Bonnel, Stauber, & Stamatas, 2019) and for concomitant analysis of drug(s) and biomarkers (Schulz, Becker, Groseclose, Schadt, & Hopf, 2019).

Dermal bioavailability cannot be reliably estimated from IVPT samples, as factors such as drug clearance from tissue into systemic circulation (Ibrahim, Nitsche, & Kasting, 2012; Kapoor, Milewski, Mitra, & Kasting, 2016) and skin metabolism (Manevski et al., 2015; Oesch, Fabian, Oesch-Bartlomowicz, Werner, & Landsiedel, 2007; Svensson, 2009) may not be easily determined or mathematically modeled. Use of dermal open-flow microperfusion (dOFM), with the measurement of drug levels in the dermal interstitial fluid, has been reported as a suitable option for assessment of dermal bioavailability (Bodenlenz, Dragatin, et al., 2016; Bodenlenz, Tiffner, et al., 2017); dermal microdialysis has also been used for such a purpose (Erdo, Hashimoto, Karvaly, Nakamichi, & Kato, 2016; Holmgaard et al., 2012; see Current Protocols article; Voelkner, Voelkner, & Derendorf, 2019).

Use of the IVPT for selection of a "lead" formulation is generally followed by nonclinical safety, toxicology, and pharmacokinetics studies, which use the minipig as the model of choice (Mitra et al., 2015; Stricker-Krongrad, Shoemake, Liu, Brocksmith, & Bouchard, 2016; Thombre et al., 2020) due to similarities in skin morphology with humans (Barbero & Frasch, 2009; Qvist, Hoeck, Kreilgaard, Madsen, & Frokjaer, 2000). Rodent models should *not* be used to understand dermal penetration and distribution, as most of the compound will be available systemically given that rodent skin is much more permeable than human or minipig skin (Schmook, Meingassner, & Billich, 2001).

The IVPT protocol described here (Basic Protocol 1), in conjunction with the key bioanalytical parameters related to sample analysis (Basic Protocol 2), provides guidance on the successful execution of this assay and should aid in the process of selection of NCEs and semi-solid formulations for further development.

Critical Parameters

There are key aspects of the IVPT methodology that can deviate from what has been described herein; although effort has been made to highlight important points in each step, different laboratories may not have direct access to some of the equipment mentioned in the protocols.

A key parameter in the IVPT methodology is the selection of the diffusion cell type. There are two key types of diffusion cells used in IVPTs: (i) the Franz cell (also known as static or vertical) and (ii) the Bronaugh cell (also known as flow-through). The choice of either will be dependent on the required experimental throughput (including the ability to automate sample collection), the bioanalytical methodology of choice, reproducibility, and cost. Franz cells have been the choice of regulatory agencies for bioequivalence assessments, whereas Bronaugh cells have been a more adequate choice to increase throughput and automation, aspects typically required during topical formulation development and optimization.

Additionally, the compound concentration in the formulation can result in broad differences between two seemingly comparable formulations of the same active ingredient. The thermodynamic activity of the compound(s) present in the topical formulation will affect the rate and extent of skin permeation/penetration, independently of the nominal compound concentration in the formulation (Moser, Kriwet, Kalia, & Guy, 2001). It is advised to have an understanding of the saturation solubility of the compound(s) in the solvents/excipients of interest and in the formulation prototypes under evaluation.

Dose may also alter the kinetics of compound penetration through the stratum corneum. The typical finite dose ranges from 5 to 15 mg of formulation per cm^2, with 10 mg/cm^2 being the most common (Selzer et al., 2013). Ensuring accurate dispensation of the formulation and uniform coverage of the skin section placed in the diffusion cell will minimize experimental variability. Use of positive-displacement pipets is recommended, especially for more fluidic solutions, gels, and creams. Formulations that are viscous (e.g., ointment, viscous gels/creams) may require different dosing techniques, such as using a glass rod or the bottom of a glass HPLC vial to apply the formulation, as follows: (i) apply the formulation on a clean glass rod/glass HPLC vial and weigh it; (ii) spread the formulation on top of a skin section; and (iii) weigh the glass rod/glass HPLC vial again and record the actual formulation dose dispensed on the skin section.

The receptor solution composition, although expected to include PBS in most cases, may need to be modified to ensure successful study execution. The receptor solution should ensure sink conditions throughout the experiment. Sink conditions are determined based on the solubility of the analyte(s) of interest in the receptor solution and are achieved when the highest concentration of the permeant(s) does not exceed 10% of its saturation solubility. Organic solvents, such as ethanol, should be avoided due to their potential to disrupt the skin barrier or affect the donor concentration (by back diffusion from the receptor compartment). Lipophilic molecules may require the addition of a small amount (typically <0.1%) of surfactant (e.g., polysorbate 80, polyethylene glycol, Volpo™ N20) to the receptor solution to ensure sink conditions. Addition of bovine serum albumin (BSA) to the receptor solution, though described in the literature, may lead to analytical challenges. Moreover, labile molecules may require modifications in the receptor solution, including the addition of antioxidants and/or chelating agents (e.g., EDTA) or the use of acidified water (e.g., pH 4.0) instead of PBS. Any receptor solution solubility/stability modifier under consideration should be evaluated for its potential to cause analytical issues or to disrupt the skin barrier.

Biological membrane integrity assessment was not discussed in the protocols; however, it is commonly described as part of IVPT workflows. Based on the authors' experience, assessing membrane integrity for every skin section has proven to be extremely time consuming in the context of the high-throughput laboratory setting where the experiments are conducted (with availability of 128 diffusion cells, three mass spectrometers, and two liquid handlers, with an average of 200,000 samples analyzed per year). It was observed that the combination of rigorous quality control of the sourced ex vivo human skin, in addition to consistent dermatoming procedures, resulted in a low number of problematic skin sections. Additionally, assessment of data outliers based on an internally developed workflow resulted in efficient detection of problematic skin sections. Although each IVPT laboratory should choose the most adequate workflows to suit their demand, it is generally recommended that a membrane integrity assessment be performed with techniques such as transepidermal water loss, electrical impedance/conductance, or tritiated water permeation and sections replaced as required (Guth, Schafer-Korting, Fabian, Landsiedel, & Van Ravenzwaay, 2015).

Full mass balance was not described herein as for the purposes of formulation or NCE screening, it does not appear to add significant value. Full mass balance assessment requires more intensive bioanalytical method development to ensure that the compound(s) of interest are efficiently extracted from each sample matrix type, such as the cotton swabs used to remove the excess formulation present on the skin surface at the end of the experiment, the tape strips used to remove the stratum corneum, and homogenized epidermis and dermis. Alternatively, full mass balance can be accomplished using radiolabeled compounds, which minimizes the need for developing different extraction procedures for each sample matrix type.

As dilutions for the large number of samples generated in these studies (for example, a 24-hr study with time points collected every hour can generate up to 26 samples per replicate, including receptor solution time points and epidermis/dermis homogenates) can be laborious and error prone when performed by hand, it is generally advisable to use an automated liquid handler to perform these steps. This allows for greater reproducibility across the experiment compared to a user performing these dilutions manually. Based on the labware used (a mix of homogenization tubes in a 24-well-format holder and 96-well plates for receptor solution capture), a combination Span-8 and 96-head would be ideal, with the Span-8 performing dilutions from tubes and the 96-head performing whole-plate operations. Examples of systems available with this configuration are the Tecan® EVO® or Fluent™ series, the Hamilton® STAR™ or VANTAGE™ systems, and many others available from various manufacturers. On top of these base systems, other integrations can assist with increasing walk-away time, such as a decapper for the homogenization tubes, hotels for storing samples not actively being processed, and a centrifuge to allow for automated extraction of homogenized samples. Time-lapse videos of automated liquid handling processes in action can be seen in Supporting Information Videos 1 and 2.

Troubleshooting

Described in Table 5 and 6 is some troubleshooting guidance for problems that may be encountered during the respective execution of the IVPT and bioanalytical method development protocols (Basic Protocols 1 and 2).

Table 5 Troubleshooting Guide for IVPT

Problem	Possible cause	Possible solution
Receptor solution leaking into donor compartment	Perforation in skin section	Exclude or replace replicate and ensure membrane integrity prior to next experiment
	Skin improperly spread	Exclude or replace replicate and ensure skin is spread to completely cover donor block area
No receptor solution in collection 96-well plate	Pump not started	Re-run experiment and ensure pumps properly start at beginning of next experiment
	Clog in lines	Exclude replicate and ensure lines are cleared prior to next experiment
Bubble present under skin in diffusion cell	Receptor solution not sufficiently primed	Ensure receptor solution is primed until steady flow of liquid is seen dripping from PEEK tubing
	Receptor solution not degassed	Ensure receptor solution is properly and fully degassed prior to experiment
Epidermis and dermis will not separate after incubation at 60°C	Insufficient incubation time	Incubate for an additional minute
	Skin drying out or degrading due to length of experiment	Re-run for shorter time if skin amounts are important
Extremely high amounts of compound in receptor solution at early time points	Perforation in skin section	Exclude replicate and ensure membrane integrity prior to next experiment
	Skin improperly spread	Exclude replicate and ensure skin is spread to completely cover donor block
	Cross-contamination of glassware used to prepare receptor solution	Ensure glassware is thoroughly cleaned and free of surfactants and ensure glassware intended for receptor solution preparation is kept separate and used only for its intended purpose
	Analytical carryover	Ensure bioanalytical method is not causing carryover by injecting solvent samples several times and assessing analytical response
Higher amount of compound in dermis than epidermis	Incomplete splitting of skin samples	Ensure complete removal of epidermis from dermis sample
	Cross-contamination during skin splitting	Ensure more careful separation of epidermis from dermis and ensure forceps are cleaned as needed using alcohol swabs

Understanding Results

The key IVPT results that should be used for comparison of NCEs or prototype formulations are compound amounts in the epidermis and dermis (generally at the μg level), cumulative amount in the receptor solution (generally expressed as ng/cm^2), steady-state skin flux (ng/cm^2·hr), and respective lag time (hr). The compound amounts in the epidermis and dermis should be calculated accounting for the dilution steps performed prior to LC-MS/MS analysis. Due to the intrinsic challenges of weighing low amounts of epidermis and dermis for a large number of samples (while also avoiding cross-contamination), it is assumed that the tissue weight across samples is fairly constant, and sample mass need not be obtained prior to homogenization. The IVPT results for a selection of proprietary molecules (generically named GSK1, GSK2, and GSK3) dissolved in the same prototype solution are shown in Figures 4 and 6. It is relevant to note that most (≥90%) of the applied compound will remain on the skin surface (i.e., compound not penetrating the stratum corneum), with 5% to 10% distributed between epidermis and dermis and typically <1% present in the receptor solution; these values are approximate and depend on the compound/formulation under consideration, but results on the same

Table 6 Troubleshooting Guide for Bioanalytical Method Development

Problem	Possible cause	Possible solution
High background signal at analyte retention time	Contaminated solvents (with analyte or otherwise) or matrix interferents	Utilize fresh, high-purity solvents, alter chromatography in attempt to separate analyte and matrix interferents, and/or utilize alternative MS/MS transitions
High analytical carryover	Insufficient LC syringe wash volumes	Optimize LC syringe wash volumes (vendor specific)
	Inappropriate LC syringe washes	Optimize wash solvents based on analyte lipophilicity/pKa
Nonlinear calibration curve	Ionization or detector saturation	Shift calibration curve to lower concentrations
	Drug precipitation	Alter receptor solution/organic composition (different or higher organic)
	Nonlinear matrix effects	Optimize MS source conditions and/or assess matrix effects and optimize chromatography to minimize
Low extraction efficiency	High drug-skin protein binding or drug precipitation	Remove acidic modifier from extraction solution and/or add basic modifier such as ammonium hydroxide (pH 9-10) in extraction solution, which may improve extraction efficiency in rare cases
High nonspecific binding	Use of low-grade plastics with high number of binding sites	Change labware supplier ((e.g., for LC-MS/MS-compatible 96-well plates)) and/or use glass-coated options
Low LC-MS/MS precision	Insufficient chromatographic retention	Use longer gradient hold at beginning of LC run
	Overly steep LC gradient	Use more gradual gradient
	Inconsistent ionization	Optimize MS source conditions

order of magnitude have been previously reported (Gschwind, Waldmeier, Zollinger, Schweitzer, & Garssberger, 2008).

The receptor solution data are displayed by plotting time (hours) on the "x" axis and the cumulative amount (over area) on the "y" axis. The cumulative amount is calculated by converting each time-point concentration (e.g., ng/ml) into mass (e.g., ng) by considering the volume collected for that specific time point (e.g., flow rate versus time-point length; in this case, 10 μl/min $\times$ 60 min = 600 μl). Any dilution factor should also be considered. The calculated analyte mass at each time point is divided by the dosing area (e.g., 1 cm^2) and summed up sequentially. The flux can be calculated at each time point by dividing the compound amount/area by the specific time. Additionally, there is a region of the curve where flux will remain relatively constant; this is the steady-state skin flux value and is a key parameter to compare analytes and/or formulations. The steady-state flux can also be calculated by identifying a somewhat linear region in the cumulative-amount curve and calculating its slope ($\Delta y/\Delta x$). By extrapolating this linear region to the "x" axis, the lag time can be determined, which is the time necessary for the formulation to achieve the steady-state flux; the lag time is another important parameter used to characterize and compare analytes and/or formulations and may provide a relative comparison of the time necessary for the onset of pharmacological action.

A typical project in nonclinical development may include upward of five NCEs prior to selection of a lead candidate and over 30 formulation prototypes (containing the selected compound) prior to identification of the lead formulation for FTIH studies. The IVPT is a critical assay throughout those steps, and the laboratory should design experiments that allow retrospective data comparison. There are various ways of comparing the data (including statistical analyses comparing pairs of formulations, which are not presented here),

and some guidance is available in Table 7. It is generally helpful to choose a prototype as the reference formulation (for ease, it can be the "bridge" formulation to be tested in each different IVPT experiment to allow a direct comparison); in our case, GSK1 0.5% was selected. Based on the data, GSK1 achieves steady state faster when compared to the other NCEs (2.6 hr versus 5.0 and 8.0 hr for GSK2 0.5% and GSK3 0.5%, respectively) and yields a skin flux 3.3- and 7.1-fold higher than GSK2 and GSK3, respectively, with cumulative amounts following a similar trend. It is relevant that GSK3 showed epidermal levels 1.5-fold higher than GSK1 but that dermal levels were 1.7-fold lower. Such behavior is not unusual and can be attributed to various reasons, such as cross-contamination, physicochemical properties that allow better penetration through the lipophilic layer of the stratum corneum but not necessarily through the more hydrophilic dermal layer, or higher nonspecific binding to the stratum corneum and/or epidermis components. The rate and extent of skin permeation/penetration will be a result of the physicochemical properties of the molecule, such as molecular weight, log P, hydrogen bonding (Sun et al., 2011), and the thermodynamic activity of the molecule in the formulation being evaluated (Moser et al., 2001).

It is also relevant to consider the biological target site of the disease under evaluation. For example, in acne, the target site for inflammatory pathways is the sebaceous glands, typically localized at a skin depth of around 800 to 1200 μm. It would be expected that a molecule with high skin flux and dermal levels would be more optimal (although more likely to result in higher systemic exposure). In contrast, diseases such as vitiligo or atopic dermatitis are generally associated with immunological imbalance at a more superficial depth of the skin (i.e., epidermis and/or upper dermis) and thus do not necessarily require a compound with very high skin flux. MALDI imaging mass spectrometry, as discussed earlier, can be a useful technique to better understand drug distribution across skin layers if a sufficient level of spatial resolution (typically 20 to 50 μm) is utilized.

Data extrapolation using epidermal and dermal amounts for comparison with pharmacological potency can be done, but several caveats must be factored in. By considering that in a split-thickness skin section (thickness ~500 μm), ~150 μm comprises the stratum corneum/viable epidermis, with the remainder

Table 7 IVPT Data Compilation for GSK1, GSK2, and GSK3 and Relative Comparison

	Receptor solution data				Skin levels		Comparison relative to GSK1 (fold)			
Formulation	Flux ($ng/cm^2 \cdot hr$)	Lag (hr)	R^2	Cumulative amount ± SEM (ng/cm^2)	Epidermis ± SEM (μg)	Dermis ± SEM (μg)	Flux	Cumulative amount	Epidermis	Dermis
GSK1 0.5%	86.2	2.6	0.998	1134 ± 238.2	6.6 ± 0.74	5.2 ± 0.78	1.0	1.0	1.0	1.0
GSK2 0.5%	12.9	5.0	0.994	278.1 ± 97.05	5.6 ± 0.70	4.0 ± 0.81	0.30	0.25	0.85	0.77
GSK3 0.5%	12.1	8.0	0.992	98.69 ± 38.83	9.9 ± 1.4	3.1 ± 0.54	0.14	0.087	1.5	0.60

(~350 μm) being dermis, with a dosing area of 1.0 cm^2, the theoretical volume of these two compartments will be ~0.015 cm^3 (or ml) and 0.035 cm^3 (or ml), respectively. Using the epidermis and dermis levels for GSK1 cited above (6.63 and 5.19 μg, respectively), the respective compound concentrations will be 442 and 148 μg/ml. Thus, with an average (and hypothetical) molecular weight of 400 Da, the epidermal and dermal concentrations will be respectively 1105 and 370 μM. These numbers assume homogeneous distribution of the compound within the two compartments, that the compound will behave in skin in a similar way as if it were in blood, and that dermal bioavailability and systemic bioavailability are similar. Even assuming average protein binding (either skin or keratin binding) of 98%, the unbound fraction in epidermis and dermis will be approximately 22 and 7 μM, respectively. These numbers are still at least 1 to 3 orders of magnitude above the average potency (e.g., IC_{50} or IC_{90} value) of most NCEs (depending on if enzymatic or cellular potency values are used as a reference). Such calculations should be done only in the context of a PD assay or if using actual dermal bioavailability (via dOFM) data from in vivo studies.

Finally, a critical aspect is the correlation between PK levels (and the dermal bioavailability) and the PD response, even if assessed via in vitro models using human skin. The combination of PK and PD data is crucial in the selection of the optimal NCE and prototype formulation; PK or PD data should never be considered separately during dermal drug development. In summary, IVPT data alone should not be used to decide if sufficient dermal delivery (to elicit a clinical pharmacological response) has been achieved for a new formulation or NCE. This is especially critical if no comparison with another semi-solid formulation with the same molecule (or a molecule with the same mechanism of action), previously shown to be efficacious in a statistically powered clinical study, can be made. It is relevant to highlight that the IVPT cannot provide a direct correlation with dermal bioavailability (unbound drug levels in the dermis), typically considered as unbound drug levels in the dermal interstitial fluid (which should be evaluated by dOFM). Additionally, compound levels in the receptor solution should not be considered as a measure of systemic exposure, given that the IVPT uses split-thickness ex vivo skin and cannot account for dermal drug clearance (or, in some rare cases, significant dermal drug metabolism). Proper assessment of systemic and dermal exposure upon administration of semi-solid formulations should be done via in vivo minipig/pig repeat-dose PK studies.

Time Considerations

Setup of IVPT experiment

Experimental setup for an IVPT will vary depending on the total number of samples but typically takes a team of two scientists ~2 hr to complete. This covers steps 1 to 16 of Basic Protocol 1.

Sample preparation for LC-MS/MS analysis

Sample preparation for LC-MS/MS analysis will vary based on the total number of samples and the use of automated liquid handlers but typically takes a team of two scientists ~2 hr to complete with the assistance of automated liquid handling. This covers steps 17 to 33 of Basic Protocol 1. Without automation, sample preparation time will vary significantly based on the number of samples but could take up to 4 hr using multichannel pipets for a typical experiment with 32 diffusion cells.

LC-MS/MS sample analysis and data workup

Setup of the LC-MS/MS system (including cleaning the MS source and system equilibration) will take 1 to 3 hr, depending on the instrument (Basic Protocol 1, step 34). Sample analysis times will vary based on the total number of samples as well as the LC-MS/MS method run time. A rough estimate for 10 samples each for 32 diffusion cells using a 3-min run time would be ~24 hr, including running calibration-curve and quality-control samples. The data workup (Basic Protocol 1, step 35) would take one scientist ~2 hr, including calculation of cumulative amounts in the receptor solution and formatting the appropriate figures.

Skin dermatoming

Upon receipt of the skin specimen as described in step 1 of the Support Protocol, it should take one scientist ~2 hr to dermatome the skin sheets (Support Protocol, steps 2 to 6) necessary to supply an experiment requiring 32 diffusion cells.

LC-MS/MS method assessment

All time considerations for LC-MS/MS method assessment are based on scientists' hands-on experimental activities and do not

consider the analytical run time, which will largely depend on the method being utilized.

Preparation of calibration curves and quality controls in each potential receptor solution and submission of the accuracy/precision run typically take one scientist ∼2 hr to complete (steps 1 to 8 of Basic Protocol 2). Data analysis should take one scientist 1 to 2 hr.

Evaluating receptor solution stability is expected to take one scientist ∼8 hr across 4 days (steps 9 to 16 of Basic Protocol 2). Data analysis should take one scientist 2 to 4 hr.

The extraction efficiency evaluation (steps 17 to 37 of Basic Protocol 2) is expected to take one scientist ∼5 hr (plus the 4-hr incubation in the middle of the experiment), depending on the use of automated liquid handlers, but this time can be reduced through the support of an additional scientist at key bottlenecks, such as the skin-splitting step (step 24 of Basic Protocol 2). Data analysis should take one scientist 1 to 2 hr.

The evaluation of nonspecific binding generally takes one scientist ∼1 hr to complete (steps 38 to 46 of Basic Protocol 2). Data analysis should take one scientist <1 hr.

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Author Contributions

Leandro L. Santos: Conceptualization; methodology; project administration; supervision; writing-original draft; writing-review & editing. **Nathaniel J. Swofford:** Data curation; investigation; methodology; writing-original draft; writing-review & editing. **Brandon G. Santiago:** Data curation; investigation; methodology; writing-original draft; writing-review & editing.

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Roberts, M. S. (Ed.) (2013). Skin delivery – to scheuplein and beyond. *Skin Pharmacology and Physiology*, *26*(4–6), 179–372. doi: 10.1159/000351922.

Compilation providing a historical view of skin penetration and the contributions of key opinion leaders over the years.

Internet Resources

https://www.fda.gov/regulatory-information/search-fda-guidance-documents/bioanalytical-method-validation-guidance-industry

FDA guidance on bioanalytical method development providing further scientific background.

https://www.fda.gov/drugs/news-events-human-drugs/topical-dermatological-generic-drug-products-overcoming-barriers-development-and-improving-patient

Several presentations (video and PDF files) on the IVPT technique, with a focus on the development of generics.

https://www.accessdata.fda.gov/scripts/cder/psg/index.cfm

https://www.accessdata.fda.gov/drugsatfda_docs/psg/Acyclovir_topical%20cream_RLD%2021478_RV12-16.pdf

Repository of guidance for generic development (first link) and an Acyclovir cream 5%–specific document (second link) containing the FDA's recommendations regarding IVPT method validation and execution to demonstrate in vitro bioequivalence.

https://www.oecd.org/env/ehs/testing/48532204.pdf

https://www.oecd.org/chemicalsafety/testing/Guidance%20Notes%20Dermal%20Absorption%20156_Oct2019_clean.pdf

Document on practical aspects of dermal absorption. At the time of the present publication, this guidance is under (second link) review, aiming to update the first version originally released in 2011 (first link).

https://online.uspnf.com/uspnf/document/1_GUID-94DCFABD-D687-4F3A-A43B-09CB5D5B0B5E_1_en-US?source=TOC

Compendial chapter providing general information on performance testing of semi-solid drug products (subscription is required).