Chapter 10
Application of Microdialysis in Assessing Cutaneous Bioavailability

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10.1   Introduction

In this chapter the focus is on microdialysis (MD) methodology with emphasis on cutaneous, i.e., dermal microdialysis (DMD) sampling. MD is an *in vivo* technique used for sampling endogenous and exogenous substances in the extracellular space, routinely used in the clinic as well as in research settings [13].

MD sampling methodology in the skin and subcutaneous tissue introduces a unique opportunity for *in vivo, ex vivo,* and *in vitro* studies of topical drug penetration, and the DMD method has gained ground in the last decade (for an in-depth review, see [20].

MD as a technique provides *in vivo* chronological, real-time information about the pharmacokinetics of drugs, obtained from the extracellular fluid phase at the site of action, i.e., the target tissue.

Today, there are over 14,000 publications describing the applications of MD in numerous tissues and therapeutic areas and there are over 600 publications on the applications of MD in the skin.

This chapter aims to provide a background for understanding the current position of DMD as one of several eligible methodologies for evaluation of bioequivalence of topical products, proposed by regulatory experts [29, 32].

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10.2   The Methodology (Brief Description)

MD is a sampling technique that involves insertion of a MD probe in the tissue of interest with the aim of sampling and subsequently measuring local concentrations of the analyte in the sample.

A probe occurs in various sizes and shapes and consists of a cylindrical semi-permeable membrane. The membrane is affixed on to nonpermeable tubings. The semipermeable membrane offers an exchange of solutes through the pores of the membrane. The probe is perfused with a physiological solution (called "perfusate"), which is pumped through the probe at a very slow flow-rate—typically between 1 and 5 μL/min. The slow flow rate is selected to prevent excessive periprobe drainage and/or loss of perfusate volume as well as drainage of molecules into or out of the tissue, thus minimizing any disturbance in the tissue microenvironment. The recovery (by dialysis) of a given drug or substance is independent of the concentration of the drug or substance in the tissue. The resulting solution that exits the probe is called "dialysate" and can be directly analyzed using a well validated bioanalytical method. For most probe types this dialysate is free of cells, larger molecules such as enzymes, and protein.

For a detailed review of the methodology, the probe types available, the calibration of probe efficacy, recovery evaluation, and analytical considerations, please consult Holmgaard et al. [20] for a comprehensive technical review.

10.3   Planning an *In Vivo* Bioequivalence Study

There are many choices while preparing for *in vivo* experimentation. The most important one is probably the study design itself, as the between-subjects variability will, for some topical applications, be much larger than the inter-subject variability. Whenever possible, a design using the opportunity for several test and reference areas in the same person should be chosen. Furthermore, the opportunity of pooling samples in collecting vials for larger sample volume as well as optimizing probe length/membrane area should be considered for low-recovery drugs.

10.3.1   Choice of Probe Type and Perfusate

Different types of probes for DMD are available [20] and the majority of these probes are inserted in the skin through a small guide cannula as the probes are made of fragile material. The linear probe has a unidirectional flow and compared to the other probe types has a very small diameter—down to approximately 200 μm for some structures. Linear probes will, however, need to penetrate the skin twice when inserted, since they have both an inlet and an outlet. The typical probe used in clinical studies relating to systemic drugs is the concentric probe which, like the

side-by-side and loop design probes, has an inlet and outlet placed in parallel with each other. The latter probe types require guide cannulas of larger dimensions (e.g., 500 μm) than those necessary for insertion of the linear probe, which leads to a larger insertion trauma (see later)—and this is a drawback.

In cutaneous MD, the perfusate is most often an isotonic saline solution or a Ringer solution. Depending on the lipophilicity of the drug, the perfusate medium may have to be modified to allow more lipophilic substances to enter the probe. Substances such as albumin, Intralipid®, and Encapsin® have been used [22, 46]. The enhancing effect of adding binding agents such as α, β, γ-cyclodextrins to the perfusate has been evaluated by studying the recovery of several eicosanoids *in vitro*. Similarly, the effect of adding small organic molecules such as ethanol, propylene glycol, and dimethylsulfoxide has been studied, and the inclusion of arachidonic acid in the perfusate has been shown to increase *in vitro* relative recovery for hydrophilic analytes [42].

10.3.2 Application Site

In cutaneous MD, the most frequently used area of application is the dermis of the volar forearm [3, 4, 6–8, 16–18, 35, 45].

The reason for this is twofold—this is the "standard area" for investigations into noninvasive measurements of skin blood flow and barrier perturbation. Secondly, the area is easily accessible, usually not hairy and not very convex. If there is a need for shaving, this should be done preferably 3 days before the experiment to prevent an artifact by skin barrier perturbation.

Only one arm is used for cutaneous MD at a time, since that arm must be at rest and tied up during the experiment. It is uncomfortable to have both arms tied up. Blood drawn from the other arm can provide measurements of plasma drug concentrations and thus the systemic drug delivery, if relevant.

10.3.3 Insertion Procedure, Trauma, and Exclusion Criteria

The probes are inserted under clean or sterile procedures after gentle wash of the skin area. To minimize the discomfort during insertion, local anesthesia [18] and application of ice packs onto the skin can be used [1, 24, 45]. The probes are inserted using a 19–23 G cannula as a guide, which is inserted horizontally in the dermis or subcutis. The probe is subsequently inserted in the opposite direction through the open tip of the hollow cannula and tested to secure the functionality. Subsequently, the guide cannula is withdrawn with the probe still in place. The accuracy of placing the probe at the intended depth in the skin depends on the training and experience of the laboratory personnel, and the depth should be measured routinely by ultrasound scanning as part of the experimental protocol, see Sect. 10.6.3.

MD is a minimally invasive technique, but the skin trauma and histamine release evoked by the insertion will cause a reversible increase in the local blood flow, increased skin thickness, and hyperemia in both animals and humans. The tissue trauma needs to subside before sampling can begin and an appropriate equilibration period of minimum 60–90 min in human skin is advisable. The hyperemia reaction lasts between 90 and 135 min, but complete normalization of the skin perfusion may not occur [28]. The use of local anesthesia has been found to reduce the trauma reaction in man [18].

The presence of a MD probe will elicit an inflammatory response after approximately 12 h; infiltration of lymphocytes will begin and after 32 h scar tissue may appear [2]. Following insertion of concentric probes more extensive tissue disruption compared to a linear probe has been reported. The larger reaction could be caused by the larger diameter of the guide cannula used for implantation of concentric probes (e.g., ~ 500 μm).

The tissue reactions are generally reversible and lasting tissue damage is negligible. However, all the cytokines and cells associated with, e.g., inflammatory disease are involved in the trauma reaction [41]. In my experience the development of skin changes, visible at clinical examination after, e.g., 3 months, is very rare. One exception is if the volunteer or patient is prone to keloid formation, and for this reason we recommend screening for this as an exclusion criterion of the study protocol, thus avoiding DMD in keloid-prone individuals.

10.4   Bioavailability

The bioavailability of a topically applied drug is defined as "the rate and extent to which the active ingredient or active moiety is absorbed and becomes available at the target site. For drugs that are not intended to be absorbed into the bloodstream, bioavailability reflects the rate and extent to which the active ingredient becomes available at the relevant site within the skin" as per FDA "Guidance for Industry" 2002. A recent study demonstrates the use of MD to determine the bioavailability in a study comparing orally administered diclofenac with topically administered diclofenac. Thus, the bioavailability at the target site was measured by placing the MD probe in the subcutaneous tissue as well as the muscular tissue. The relative bioavailability in both muscular and subcutaneous tissue after topical application was significantly higher than after oral administration and the measured plasma levels were much lower when the drug was topically administered compared to oral administration [3, 43, 44]. Similarly, the diffusion of ketoprofen from transdermal patch application to the knee joint fluid has been demonstrated in rats and pigs [38]. A recent study explores, by MD sampling in subcutaneous tissues and skeletal muscle, the effect of alterations in the galenic composition of four novel topical diclofenac formulations under development. An improved drug delivery to the tissue (2.7-fold improved) over the reference product, a commercially available diclofenac gel, was demonstrated by Brunner [11].

10.5   Bioequivalence

FDA describes bioequivalence as a comparative test between two products using specified criteria. Bioequivalence is defined as "the absence of a significant difference in the rate and extent to which the active ingredient or active moiety in pharmaceutical equivalents or pharmaceutical alternatives becomes available at the site of drug action when administered at the same molar dose under similar conditions in an appropriately designed study." However, the assessment of bioequivalence for locally acting and targeted delivery drugs has presented great challenges to science in the approval of generic drugs.

Kreilgaard et al. published the first human study demonstrating the potential of cutaneous MD for bioequivalence studies of topically applied drugs in 2001 [27]. The aim of that study was to evaluate the cutaneous bioequivalence of a lipophilic drug (lidocaine) applied in a novel topical microemulsion vehicle, compared to a conventional oil-in-water emulsion. Earlier, Kreilgaard had published an experimental study in rats documenting that dermal drug delivery of hydrophilic and lipophilic drugs was improved by microemulsion vehicles [26]. Subsequently, Kreilgaard proved that microemulsion vehicles can increase dermal drug delivery of lipophilic drugs in humans, and that the MD technique combined with an appropriate pharmacokinetic model provides high sensitivity in this bioequivalence study of a topically applied formulation.

When addressing the issue of DMD variability the source of variability in these studies can be extracted from data obtained in hairless rats (which are genetically inbred and of the same sex and age and thus have minimum variability). The increased variability observed in dermal sampling of the topically applied drugs can be seen to arise from interindividual variability in skin penetration kinetics/barrier function and or microcirculation, unrelated to the MD sampling methodology, which is followed by an internal calibrator simultaneously with the ongoing penetration process [40].

The comparability of dermato-pharmacokinetics, so-called tape-stripping methodology, and DMD was demonstrated in a recent study evaluating bioequivalence of lidocaine in ointment and cream [8]. The study showed agreement between the two methods, finding a 3–5 fold higher lidocaine absorption from the cream formulation over the ointment formulation. Statistical calculations from this study indicated that bioequivalence evaluation of topical formulations based on DMD sampling may be conducted using 27 subjects and applying two probes in each application site or 18 subjects using three probes in each application site with 90% confidence interval and 80–125% bioequivalence limits, which are the limits applicable to topical bioequivalence evaluations as per FDA protocols [38].

A theoretical-statistical MD paper found, based on data in the literature, that an evaluation of topical bioequivalence, conducted by duplicate sampling of both formulations in the same human volunteer, could be expected to be conclusive within 80–125% confidence limits when a population of approximately 20 subjects was to be used [30]. This number of participants was similar to what was calculated

in the study on topical lidocaine formulations [8]. In the latter study, 61 % of the experimental variability could be ascribed to inter-subject variability—an important finding for planning of future studies. This result was corroborated by Tettey-Amlalo et al. who demonstrated a very low variability using the exact same probe structure for sampling drug penetration from a topical ketoprofen gel formulation [45]. However, different formulations may demonstrate different variabilities when the cutaneous penetration is studied by DMD; in a study of topical metronidazole creams the variability was higher and the number of participants for a conclusive bioequivalence evaluation would have been 34 [17]. Nevertheless, in comparison with the 6–700 subjects needed for a clinical comparative study, these numbers are small [29].

Another recent study has compared topical penetration of two commercially available tetracycline formulations and also found high variability [23], whereas a study of an aggressive ethanol solution of clobetasol propionate—a drug which it has previously not been feasible to sample by MD in the dermis—showed that using Intralipid ® as the perfusate the drug could now be reproducibly sampled [1].

Dermal MD sampling may also enable bioequivalence studies in diseased skin [6, 16, 36], which can be argued to be closer to the clinical situation than bioequivalence evaluations conducted in healthy subjects. However, variability is likely to be increased and possibly too problematic, see Sect. 10.6.1 below.

10.6   Sources of Variability

10.6.1   Skin Barrier Function

Damage of the skin barrier implies an increase in trans-epidermal water loss (TEWL) which can be quantified by measurements above the skin surface. In a number of *in vivo* DMD studies the impact of experimentally induced skin barrier perturbation on the cutaneous penetration of different substances has been demonstrated [8, 25, 31, 36]. Benfeldt et al. have studied the effect of different barrier disruption methods on cutaneous penetration in hairless rats as well as humans [5, 7]. The studies demonstrated highly increased drug penetration in tape stripped skin (157- and 170-fold respectively, in comparison to the penetration in unmodified skin) and in skin with irritant dermatitis (46- and 80-fold increased penetration). Since the same probe type, perfusate, flow rate, and topical drug solution were used in these studies, a direct comparison between drug penetration in hairless rats and human volunteers could be made. This showed a 46-fold increase in penetration across rat skin when compared with human skin, while increases in penetration, induced by barrier perturbation were of the same order of magnitude [3].

Other more recent studies have compared, e.g., the penetration of acyclovir and salicylic acid on disrupted skin barriers using MD in the dermis and tape stripping [25], and the penetration of a metronidazole cream formulation (1 %) applied to the

forearm skin in areas of both irritant dermatitis and normal skin [36]. Furthermore, DMD sampling showed a significant threefold increased penetration of topically applied metronidazole in skin with atopic dermatitis compared with unaffected skin [16].

10.6.2   Microcirculation

The bioavailability of topically applied products in skin and underlying tissues is not only dependent on the integrity of the skin barrier, but also on the local blood flow. Vasodilatation as well as vasoconstriction can be physiologically or pharmacologically induced and will have a large influence on the local blood flow. The skin concentration of a topically applied drug will increase if the blood flow is diminished, whereas an increased blood flow enhances the uptake and subsequent systemic distribution and elimination of the drug from the skin [2]. Experimental studies which induce vasoconstriction or vasodilatation have demonstrated that the bioavailability of topically as well as systemically applied test substances is highly influenced by changes in the microcirculation of the skin [9, 12, 37]—an influence much stronger than the influence of variations in probe depth.

Topically administered substances have been studied, with added noradrenaline for vasoconstriction and the nitric oxide donor glyceryltrinitrate for vasodilatation, delivered by the MD probe. The changes seen in the dialysate concentration reflected the changes in the microcirculation [10, 14].

10.6.3   Probe Depth in the Skin

An influence of probe depth on the drug levels sampled following topical drug application is likely from a theoretical point of view. The recommendation is to measure (normally by 20 MHz ultrasound scanning) the probe depth in three separate scans along the length of the probe *in situ.* With experience, probes can be inserted with great accuracy and low variability [8, 45], e.g., 0.7 ± 0.15 mm mean $\pm$ SD [36]. The preferable insertion depth is 0.6–1.0 mm in the dermis.

The original study regarding transdermal delivery of nicotine showed a correlation between depth and drug concentration, but only when the analysis included different skin layers (both dermal and subcutaneous probe placement) [33]. Following this, several studies have been unable to show a correlation between the depth of the probe and the drug concentration [8, 17, 19, 35, 36]. However, in a very recent MD study, conducted in *ex vivo* human donor skin, an inverse relationship between the depth of the probe in the dermis and the amount of drug sampled following topical penetration is demonstrated [21]. The result is of relevance to the *in vivo* situation, and it can be predicted that the differences in sampling at different probe depths will have a more significant impact in the beginning of a study or in studies of short duration.

Based on this study it can be recommended that studies of topical drug penetration using DMD sampling should include measurements of probe depth and that efforts should be made to minimize probe depth variability (e.g., to have few and similarly trained persons undertaking insertion as well as ultrasound scanning or other imaging technique for feedback of probe depth achieved) [21].

10.7 Advantages and Limitations

The methodological challenges that may influence *in vivo* experiments may typically be identified through well-planned *in vitro* experiments as a part of the planning phase. Founded on pre-experimental troubleshooting, conducted previous to *in vivo* experimentation, reproducible results with acceptable variability and validated analysis can be achieved for most drugs.

10.7.1 Advantages

- MD captures the pharmacological events where they take place in the tissues, providing high-resolution real-time details.
- There is no fluid extraction from the tissue.
- DMD sampling allows testing of both test and reference product at the same time in the same individuals.
- Both the drug of interest and the metabolites.
- The method can provide protein-free samples, which is often an analytical advantage.
- Cessation of enzyme degradation in the samples.
- DMD is a relatively inexpensive method to use once the MD pumps have been acquired.
- The probes allow sampling as well as delivery of substances.
- Multiple application sites.
- Good reproducibility.
- DMD sampling of topical drug formulation in the bioavailability/bioequivalence setting does not depend on drug concentrations in the formulations being the same.
- DMD sampling can be used in the presence of barrier perturbation or skin disease (unlike other methods for skin penetration assessment).

10.7.2 Limitations

- Drugs with a very high lipophilicity are excellent for topical application but less favorable for sampling by MD (the tape-stripping method will often be more suitable).

- An *in vitro* relative recovery of less than 4% will most often characterize a compound as unsuitable for MD studies due to an expected even lower *in vivo* recovery.
- Some topical drug formulations are of very low drug concentrations—the analysis of the dialysate will unavoidably be very challenging.
- For protein-bound substances it is often necessary to add a protein to the perfusate to increase recovery, which will often result in more complex analytical procedures.
- Low variability in probe insertion and probe manufacturing depends on experienced personnel.
- The dialysate concentration will decrease with increasing flow rate and vice versa since the relative recovery of substances is flow-dependent. However, if very low flow rates are used,the time resolution can be compromised.
- Analytical procedures may require extensive modifications prior to *in vivo* experiments.
- Both drug concentrations in the tissue as well as recovery by MD sampling are known to be influenced by blood flow and this must be considered in the planning stage of an experiment.
- The duration of a DMD experiment is in many instances limited for practical reasons, and this creates a limitation for slowly penetrating substances (this can be overcome by the use of portable pumps, which in turn typically have relatively few syringe spaces).
- As a consequence of the above-mentioned relationships between topical dose, dermal concentration, and the ensuing concentration in the microdialysates, MD sampling in the skin will hardly ever be the right method for toxicological studies of low dose/real life skin exposures—other methods will be more relevant.

10.8   Future Research

A key issue in the development of a standardized protocol is the reproducible insertion of the probe at a consistent depth within the skin. Reproducible probe insertion is a skill which is only improved with practice. It is evident that implantation in either the superficial dermis or the subcutaneous tissue will affect the data collected. A thorough evaluation of this methodological issue, preferably studied under human *in vivo* conditions, using drugs of varying MW and lipophilicity, is needed.

Most MD studies are of limited time duration, typically less than 8 h, and histological studies of the skin response to probe implantation have not showed signs of tissue inflammation [36]. Studies with more extended observation and sampling periods have, however, demonstrated that infiltration of lymphocytes and even development of scar tissue may be observed over [36, 41].

Another concern, particularly relevant to DMD, is the use of MD in studies of inflammatory and immune-mediated diseases. Here the molecules of interest will very often be the same as those generated during the insertion trauma and

subsequent wound repair. Care should be taken to incorporate the proper controls in order to confirm the correct relation between molecule, implantation trauma, and disease process [41] Studies of the exact relation between probe implantation (depth, diameter, time since implantation) and the ensuing tissue damage will be important in the further development of the MD methodology, also from an ethical point of view.

In vitro drug penetration studies using static or flow-through diffusion cells have been extensively used in the past, and there is a pertinent need to establish a correlation between results from *in vitro* penetration studies of topical drugs and *in vivo* data obtained by MD methodology.

Only few MD studies in diseased skin have been performed. An improved knowledge of the impact of the structural changes in diseased or otherwise impaired skin on topical drug penetration *in vivo* will improve the development of topical therapies. Furthermore, the relevance is supported by the fact that a substantial fraction of the general population suffer from different skin conditions that make the skin barrier potentially more permeable—an aspect relevant also to the occupational setting [15].

MD methodology has a place among other methodologies employed in drug research, preclinical test phases, and clinical studies as well as for use in industry for regulatory approval purposes. The potential for studies of bioequivalence of topical formulations and in evaluation of line extensions of topical is huge.

Whether the method is employed for sampling of pharmacokinetic or pharmacodynamic information, MD in the skin as a technique offers a unique opportunity for real-time chronological sampling in the target tissue.

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