Application of Dermal Microdialysis for the Determination of Bioavailability of Clobetasol Propionate Applied to the Skin of Human Subjects

W.L. Au, M.F. Skinner, E. Benfeldt, R.K. Verbeeck, I. Kanfer

Introduction

The determination of bioavailability of topical drug products has been quite challenging for a number of reasons such as poor aqueous solubility of many topical drugs, difficulty in monitoring the penetration through the stratum corneum and limited access to biological samples following application to the skin, amongst others. Dermal microdialysis (MD) has been developed as one of several promising in vivo methods which may be used to assess cutaneous drug penetration since it facilitates continuous sampling of unbound drug fraction in extracellular fluid in the skin and other tissues after either topical or systemic drug delivery [1]. This technique first emerged from the neuroscience field [2] where it was developed for neurotransmitter sampling in the rat brain. It has been successfully used to investigate endogenous substances in extracellular fluid in the skin [3, 4] and for studies of the absorption of exogenous substances as well [5–7]. The MD technique can be used to assess the permeation of topically applied drugs across both healthy and diseased or perturbed skin [8–10]. More recently, it has been shown to be a useful technique to assess bioequivalence of some topical formulations [11–14]. For a
review of MD in topical drug studies, see Holmgaard et al. [15] and Kanfer [16].

The application of dermal MD to study drug penetration through the skin is based on the use of a hollow probe structure consisting of a thin semi-permeable membrane which can be introduced into the skin and which will imitate the function of a capillary blood vessel [17]. The dialysis membrane is continuously perfused with a suitable physiological solution (perfusate) throughout the experiment. A concentration gradient is created between the extracellular fluid in the skin and the perfusate which allows for passive diffusion of endogenous and exogenous substances across the membrane and into the perfusion medium (dialysate) and vice versa [18, 19]. Samples of the dialysate can then be collected at specific time intervals and analyzed for the solutes of interest.

Since many topical drugs are lipophilic, their poor aqueous solubility coupled with binding/adherence of these drugs to the membrane and other components of the MD system [20–23] necessitates the development of a MD system which can overcome these limitations. Accordingly, Intralipid® (IL) 20% was investigated as an alternative perfusate for the usual aqueous physiological fluids such as isotonic saline and buffered electrolyte solutions and was successfully utilized to recover concentrations of the lipophilic topical corticosteroid, clobetasol propionate (CP), in vivo MD.

The use of this relatively novel MD approach was evaluated as a possible tool to assess the in vivo bioavailability of a topical corticosteroid, CP, following application of a solution of CP on the volar aspect of the forearms in healthy human volunteers.

Subjects and Methods

Chemicals

CP and desoxycorticosterone acetate were purchased from Symbiotec Pharmalab P.V.T. Ltd. (Pigdamber, Maharashtra, India). HPLC-grade water was purified by reverse osmosis followed by filtration utilizing a Milli-Q® system (Millipore Co., Bedford, Mass., USA). Saline solution was prepared by dissolving 9 g of NaCl (Rochelle Chemicals, Johannesburg, South Africa) in one litre of HPLC-grade water. The NaCl used to prepare the saline solution was of analytical reagent grade. HPLC-grade acetonitrile (UV cutoff 200 nm) was purchased from Romil Ltd. (Waterbeach, Cambridge, UK). IL, a sterile lipid emulsion consisting of purifying soybean oil (200 g), purified egg phospholipids (12 g), glycerol anhydrous (22 g), sodium hydroxide (used to adjust the pH of the emulsion to 8) and water for injection were purchased from Fresenius Kabi (Midrand, South Africa). MD probes were sterilized by immersion in ethanol (70% v/v) for 20 min, while all other MD equipment was left in ethanol (70% v/v) over night.

CP Solution

CP (Symbiotec Pharmalab P.V.T. Ltd., Pigdamber, Maharashtra, India) was dissolved in ethanol to achieve a 4% m/v ethanolic solution.

Subjects

Ten healthy human subjects (4 males and 6 females, aged 18–26) who met the necessary inclusion/exclusion criteria with skin phototype I–III [24] were enrolled to participate in the in vivo MD study. Certain exclusion criteria such as shaving of the arms and the use of moisturizers (48 h prior to the start of the study) were deemed necessary for such a study since these could affect the penetration of the drug through the skin surface. Written informed consent was obtained from each volunteer and the research with human subjects followed the recommended guidelines as set out in the Declaration of Helsinki (1964) and its amendments. The study protocol was approved by the Rhodes University Ethical Standards Committee (Grahamstown, South Africa).

MD System

Linear MD probes (fig. 1) were fabricated in-house no more than 24 h prior to the study. The MD probes consisted of a dialysis membrane fibre (Haemophan fibre dialysis cartridges, Alwall GPS plus 12, Gambio, Leuven, Belgium) into which was inserted a stainless steel guidewire (inner diameter 0.5 mm and outer diameter 0.63 mm; Scientific Laboratory Suppliers Ltd., Nottingham, UK) to glass microsyringes (Exmire microsyringes, Aurora Borealis Control BV, Schoonebeek, The Netherlands) with blue tubing adapters (CMA MD AB, Stockholm, Sweden), and attached to a MD pump (CMA 400, Chromatography Sciences Company, Quebec, Canada). The membrane’s external diameter was 210 μm with a molecular weight cut-off value of 2 kDa. The components of the MD probes were assembled with the use of cyanoacrylate glue (Bostik®, Ltd., Swindon, England). The outlet end of each probe was placed into a pre-weighed 1.5-ml polypropylene centrifuge tube to collect the dialysate samples.

In vitro Recovery

Lipophilic drugs tend to adhere to various components of MD systems [21–23], hence a retrodialysis experiment was undertaken to determine possible CP interactions with components. Standard CP solutions of 1 μg/ml in IL and 3 μg/ml in saline were studied by perfusing the respective solutions through the MD system with the probes suspended in air as the surrounding medium (n = 4). Subsequently, an in vitro MD study was conducted to assess the relative recovery (RR) from standard CP solutions of 0.5, 0.75, 1.5 and 3 μg/ml in saline as the surrounding medium and IL was used as the perfusate (n = 4). The perfusate (IL) was pumped at a flow rate of 1.0 μl/min and the system allowed to equilibrate for 30 min prior to sample collection. Dialysate samples were collected hourly over a period of 5 h.

The RR of CP was calculated according to the following relationship [13]:

\[
\text{Relative recovery (RR)} = \frac{C_{\text{dialysate}}}{C_{\text{medium/tissue}}}
\]

where \(C_{\text{dialysate}}\) and \(C_{\text{medium/tissue}}\) are the concentrations of CP in the dialysate and in the medium/tissue surrounding the MD probe, respectively.
In vivo MD Study Design

A total of four sites were used per subject on one arm and one linear MD probe was inserted into each site under the skin using a 23-gauge cannula (Crown®, Pretoria, South Africa) as a guide to facilitate the insertion of the probes. Entry and exit points were marked ensuring that a length of 30 mm of the membrane of the MD probe was placed intradermally. An ice pack (Medac (Pty) Ltd., Cape Town, South Africa) was placed directly on each insertion site on the skin for a few minutes to induce a local anaesthetic effect [25] and subsequently removed prior to inserting the needle guide. The probes which were previously sterilized in ethanol (70% v/v) were inserted through the cannula and the latter was removed once the MD probe was in place. The IL, used as the perfusate, was perfused at 1.0 ml/min for approximately 1 h to allow for equilibration and for the insertion trauma to subside. During the equilibration period, the probes were secured in place with an epoxy-based glue gel (Super Glue, Genkem®, Johannesburg, South Africa) and custom-made PVC reservoirs (3.5 x 2.3 x 2.5 cm) which were used to act as containers for the CP solution were glued to the application sites on the skin using clear adhesive glue (Bostik, Cape Town, South Africa) as shown in figure 2. Immediately following the equilibration period, the study was initiated by filling the PVC chambers with ~4 ml of 4% m/v CP ethanolic solution and perfusion with IL was initiated at a rate of 1.0 µl/min.

Dialysate samples were collected every 30 min for 4 h and samples were immediately extracted and analysed using a validated HPLC method.

At the end of the sampling period, the probes were detached from the pump (but not removed from the skin) and the remaining CP ethanolic solutions along with the PVC reservoirs were carefully removed. The skin sites were then gently wiped with alcohol swabs and prior to the removal of the MD probes from the skin, the depths of the probes were measured three times (at the middle and close to the entrance and exit of the inserted probe) for each probe by ultrasound scanning at 20 MHz using the A-mode of a Dermascan C® ultrasound scanner (Cortex Technologies, Hadsund, Denmark). The probes were then carefully removed from the skin and the sites were gently wiped with alcohol swabs and cotton pads to remove any remaining residue. Biocort® cream (1% m/m hydrocortisone acetate; Adcock Ingram Ltd, Bryanston, South Africa) was provided to each subject to treat the application sites on the arm as a prophylactic measure against any possible post-traumatic skin inflammation. A post-study medical follow-up examination of the sites of each subject was performed twice weekly for a month.

Dialysate Preparation and Analysis

Sixty microlitres of acetonitrile (containing 2 µg/ml of internal standard, desoxycorticosterone acetate) was added to the 30 µl IL dialysate sample. The sample was vortexed and centrifuged for 20 min at 12,000 rpm on an Eppendorf centrifuge. The supernatant was removed using a 100-µl pipette (Pipetman, Gilson Inc., Middleton, Wisc., USA) and placed into a 300-µl glass micro-insert seated in an amber HPLC vial.

CP concentrations were quantitatively determined using an HPLC-UV system comprising of a Waters Alliance® system (Waters Separation module model 2690), a PDA detector (Waters model 2996), and an autosampler (Waters® Co., Milford, Mass., USA). Separation was achieved using a Luna® C8 5 µm 150 x 2.0 mm reversed phase column (Phenomenex, Torrance, Calif., USA) at a column temperature of 22 ± 0.5°C. A mobile phase of aceto-
nitrile and water (46:54% v/v) was pumped through the system at 0.5 ml/min. Twenty-microlitre sample extracts were injected onto the column and CP was monitored by UV detection at a wavelength of 238 nm.

The calibration curve was linear over the range of 0.5–100 μg/ml using spiked, extracted IL containing CP. The extraction recovery of CP from IL was found to be 79.08 ± 1.43%. This was determined by comparing the concentration of an acetonitrile standard of known CP concentration to an extracted spiked IL sample of the same CP concentration. The accuracy of the extracted IL samples was between 89.64 and 101.75% and precision <5.5% RSD. Accuracy and precision were determined using blank IL samples spiked with CP as quality control samples according to FDA guidelines [26] and the LOD and LOQ for CP in IL perfusate was 0.5 and 0.25 μg/ml.

**Statistical Analysis**

The mean, standard deviation and intra- and inter-individual variability following the in vivo MD study using 4% m/v ethanolic solution of CP in 10 subjects were determined. The AUCs from 0 to 4 h from plots of CP concentrations versus time were determined and differences in penetration of CP between subjects, gender and application sites on the forearms were assessed using one-way ANOVA and the two-tailed t test (95% confidence interval).

**Results**

**In vitro Recovery Study**

The retrodialysis experiment where a saline solution containing CP (3 μg/ml) was perfused through the MD probe with air as the surrounding medium resulted in no drug being found in the dialysate over a period of 5 h (n = 4). This was most probably due to adherence to the membrane and other components of the MD probe. When IL containing CP (1 μg/ml) was used to perfuse through the MD probe with air as the surrounding medium, 102.6% of the drug was recovered in the dialysates (%RSD = 2.9; n = 4) which indicated the absence of binding.

Using a MD system similar to Kurosaki et al. [27] and Carneheim and Ståhle [28] and IL as the perfusate, the dialysis probe was inserted into a surrounding medium consisting of a solution of CP in saline, and the resulting RR was found to be in the range of 211–215% (table 1).

**In vivo MD Study**

Following application of a 4% m/v CP ethanolic solution to the skin of 10 volunteers, concentrations of CP were detected in the MD dialysate samples at various times. Figure 3 depicts the penetration of CP following topical application of an ethanolic solution of CP on the forearms of human subjects. Concentrations of CP were readily detected in the initial dialysate samples.

**Table 1.** RR of CP using in vitro MD (perfusate: IL; surrounding medium: saline solution of CP)

<table>
<thead>
<tr>
<th>Concentrations, μg/ml</th>
<th>RR, %</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>209.23</td>
<td>5.96</td>
</tr>
<tr>
<td>0.75</td>
<td>215.06</td>
<td>1.38</td>
</tr>
<tr>
<td>1.5</td>
<td>214.42</td>
<td>3.66</td>
</tr>
<tr>
<td>3.0</td>
<td>210.66</td>
<td>6.73</td>
</tr>
</tbody>
</table>
As shown in Table 2, the intra-individual variation (CV%) ranged between 10.17–121.65% and the inter-individual variation was found to be 50.56%. The inter-individual AUCs from 0–4 ranged from 0.74 to 3.30 μg/ml·h. No significant differences were found when comparing the penetration profiles of CP between subjects (p value > 0.05).

Figure 4 illustrates the penetration of CP into the skin when comparing male and female subjects. No differences were observed in the dermal penetration of CP between genders (p value = 0.1058).

The depth of each MD membrane was measured using a Dermascan® ultrasound probe which showed that the MD probes were inserted at a mean depth of 0.692 ± 0.128 mm from the surface of the skin as depicted in Table 3. All probes were confined to the dermis.

Figure 5 depicts the comparison of the AUC from 0–4 values between the four sites, A to D, with the sites arranged from the elbow to the wrist. No differences were observed between the sites (p value = 0.8159). Figure 6 depicts the flux of CP from the extracellular fluid of the skin through the membrane of the MD probe into the perfusate. To determine the flux of CP from the surrounding medium into the perfusate, the slope of the plot (fig. 6) was determined and the flux of CP was found to be 0.15 μg/cm²·h. The permeation coefficient was calculated from the equation P = J/C⁰ and found to be 3.68 × 10⁻⁶ cm/h where P is the permeation coefficient, J is the flux and C⁰ is the

**Table 2.** Intra- and inter-individual variability in MD pharmacokinetics: AUC values from the concentration-time profiles

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>AUC ± SD, μg/ml-h</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.13 ± 1.52</td>
<td>71.34</td>
</tr>
<tr>
<td>2</td>
<td>1.41 ± 0.14</td>
<td>10.17</td>
</tr>
<tr>
<td>3</td>
<td>2.96 ± 1.64</td>
<td>55.43</td>
</tr>
<tr>
<td>4</td>
<td>3.30 ± 1.24</td>
<td>37.60</td>
</tr>
<tr>
<td>5</td>
<td>2.78 ± 0.37</td>
<td>13.20</td>
</tr>
<tr>
<td>6</td>
<td>1.23 ± 0.29</td>
<td>23.67</td>
</tr>
<tr>
<td>7</td>
<td>1.58 ± 1.62</td>
<td>102.90</td>
</tr>
<tr>
<td>8</td>
<td>0.74 ± 0.90</td>
<td>121.65</td>
</tr>
<tr>
<td>9</td>
<td>1.04 ± 0.94</td>
<td>90.39</td>
</tr>
<tr>
<td>10</td>
<td>0.97 ± 1.07</td>
<td>109.46</td>
</tr>
<tr>
<td>Mean data of 10 subjects</td>
<td>1.81 ± 0.92</td>
<td>50.56</td>
</tr>
</tbody>
</table>

**Table 3.** Probe depth measurement by ultrasound scanning (n = 4 for each subject)

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Mean depth, mm</th>
<th>SD</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.81</td>
<td>0.051</td>
<td>6.27</td>
</tr>
<tr>
<td>2</td>
<td>0.67</td>
<td>0.034</td>
<td>4.96</td>
</tr>
<tr>
<td>3</td>
<td>0.95</td>
<td>0.17</td>
<td>18.01</td>
</tr>
<tr>
<td>4</td>
<td>0.66</td>
<td>0.12</td>
<td>18.76</td>
</tr>
<tr>
<td>5</td>
<td>0.79</td>
<td>0.040</td>
<td>4.99</td>
</tr>
<tr>
<td>6</td>
<td>0.63</td>
<td>0.046</td>
<td>7.23</td>
</tr>
<tr>
<td>7</td>
<td>0.63</td>
<td>0.066</td>
<td>10.34</td>
</tr>
<tr>
<td>8</td>
<td>0.59</td>
<td>0.098</td>
<td>16.63</td>
</tr>
<tr>
<td>9</td>
<td>0.64</td>
<td>0.038</td>
<td>5.94</td>
</tr>
<tr>
<td>10</td>
<td>0.518</td>
<td>0.024</td>
<td>4.609</td>
</tr>
<tr>
<td>All subjects</td>
<td>0.692</td>
<td>0.128</td>
<td>18.42</td>
</tr>
</tbody>
</table>

**Fig. 5.** Comparison of AUC from 0–4 between the four sites.

**Fig. 6.** Flux of CP through the membrane of the MD probe in vivo.
starting concentration which was based on the concentration of CP applied at the site.

The post-study medical follow-up examination of the sites of each subject showed a rapid decrease in erythema and bruising produced from the introduction of the cannula into the skin. The final follow-up examination of the subjects’ forearm showed complete recovery from the erythema and bruising, and slight hyperpigmentation along the needle insertion site. No adverse reactions were observed during and after the study.

Discussion

In vitro Recovery Study

Since no CP was found in the retrodialysis experiment where a saline solution containing CP was perfused through the MD probe with air as the surrounding medium, it is assumed that CP, an extremely lipophilic drug with a log P of 3.5 [29], adhered aggressively to the MD probe and/or components of the MD system as previously shown by others [21–23]. To overcome this adsorption/interaction by CP, IL containing CP was investigated as an alternative medium and perfused through the MD probe with air as the surrounding medium. The 102.6% recovery of CP clearly indicated the preference of CP for IL compared to its affinity for either the membrane or other system components such as the tubing, etc.

During the MD study where IL was used as the perfusate and the probe immersed into a surrounding medium consisting of a solution of CP in saline, the resulting RR was found to be very high (210.66–215.05%). This is again probably due to the higher affinity of CP for the IL emulsion (perfusate) than for an aqueous saline solution. Hence this finding corroborates the suitability of IL for use as perfusate for in vivo MD studies.

In vivo MD Study

The CP concentration versus time profile (fig. 3) illustrates that CP penetrates the skin from ethanol at a fairly constant rate.

It was interesting to note that soon after (~15 min) applying the 4% m/v ethanolic solution onto the skin sites, the inflammatory response caused by the needle insertion trauma faded and the skin began to whiten indicating that skin blanching was occurring. This is consistent with the skin blanching (pallor) response observed following application of topical corticosteroids [30]. It can be assumed that this blanching will have a diminishing effect on the wash-out of CP by the dermal capillaries, since vasoconstriction induced by adrenaline has been shown to diminish dermal drug wash-out by several authors [9, 31, 32].

Although a relatively high concentration of CP was applied to the skin sites, the concentration of CP recovered from the skin was relatively low. The reasons for this low recovery could be due to a number of factors such as protein binding [33], very slow diffusion of the bulk amount entering the stratum corneum and the exertion of a ‘barrier’ effect within the stratum corneum. From figure 3, it is seen that the average concentration of CP appears to be quite steady up to 4 h. It was interesting to note that CP was recovered in the dialysate at the first sampling time (0.5 h) indicating the relatively rapid penetration of CP from the ethanolic solution into the skin.

The intra-individual variation was found to be highly variable with the coefficient of variation ranging from 10 to 121% (table 2). Even so, it is comparable to that seen by García Ortiz et al. [14]. It is thus apparent that even within the same subject and using a confined area of the body (volar aspect of the forearm) the penetration of CP is quite variable. In spite of this, when the data were statistically analyzed according to the one-way ANOVA, no significant differences were found when comparing the penetration profiles of CP between subjects. Also, no significant differences were observed in the dermal penetration of CP between genders.

CP recovered from the extracellular medium in the skin utilizing MD indicated that considerably less than the total amount applied had been collected up to the end of the MD sampling time of 4 h, implying that not all the CP from the ethanolic solution had penetrated through the skin in that time. In this regard, it has been acknowledged that MD is not a technique where mass balance can be obtained [34]. Although the in vitro recovery of CP by IL as a perfusate was exceptionally high, the same recovery is not obtained in vivo – in which case a ‘wash-out’ phenomenon would have had to be expected. The marked difference between in vivo versus in vitro recovery of lipophilic drugs has been shown [20] for e.g. betamethasone 17-valerate. Finally, it was seen that the application sites and depth of each MD membrane do not seem to affect the amount of CP recovered.

Conclusions

The in vitro retrodialysis experiments indicated that there was significant and complete adsorption of CP to the MD probe system when saline was used as the perfus-
ate, but when IL replaced saline as the perfusate, CP was completely recovered in the dialysate. Since lipophilic substances are known to have high affinity to bind to membranes and other components, the use of IL therefore has the additional advantage of overcoming those limitations. Further in vitro experiments to investigate CP recovery confirmed that IL was an acceptable perfusate for use in MD studies on CP.

In vivo MD was used to assess the bioavailability of CP penetration into the skin following the topical application of an ethanolic solution of CP. Whilst previous reports have described the use of in vivo MD to assess bioequivalence of topical dosage forms [11, 12], one of the most important challenges to assess bioequivalence using in vivo MD is the necessity to have a sufficiently sensitive analytical method to quantitatively measure extremely low concentrations following application of topical drug products. In the case of CP products, for example, the CP concentration in the commercial topical CP products is extremely low (0.05%). Hence, an extemporaneously prepared solution of CP containing a relatively high CP concentration of 4% was used in order to measure CP concentrations in dialysate samples. The findings in this study have clearly indicated that bioavailability/bioequivalence assessment of CP formulations, whilst feasible, will be highly dependent on the availability of a sufficiently sensitive analytical method to detect and measure the extremely low concentrations of CP which penetrate the skin from commercial CP topical formulations. Furthermore, the usual perfusates which consist of saline solutions or similar physiologically acceptable electrolyte/buffer solutions, etc. could not be used due to the insolubility of CP in such media. Hence, we have demonstrated that IL, a lipid emulsion which is used in total parenteral nutrition and is a sterile preparation which is physiologically acceptable, can be used as a suitable alternative perfusate and in which the solubility of CP was found to be in the order required.

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