Transdermal delivery of anticholinergic bronchodilators
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Chapter 4

Unusual reduction of the in vitro permeation of \([^3\text{H}]\text{dexetimide}\) by atropine

4.0. Summary

The influence of atropine on the permeation of \([^3\text{H}]\text{dexetimide}\) is studied using fresh and frozen pig epidermal membranes and artificial membranes (Silastic®). In previous experiments with fresh pig epidermal membranes, \([^3\text{H}]\text{dexetimide}\) was used as an internal standard to correct for variations in the skin. However, we noticed that in the presence of atropine the permeation of \([^3\text{H}]\text{dexetimide}\) decreased whereas no influence was seen using other anticholinergics, including atropine sulphate. We also noticed that the ratios became constant for all anticholinergics, except for atropine. To investigate whether this effect is caused by atropine or by the type of membrane, experiments with frozen epidermal membranes and Silastic® membranes were performed.

The experiments show that atropine reduces the permeation of \([^3\text{H}]\text{dexetimide}\) through fresh as well as frozen pig epidermal membranes. This decrease in permeation also occurs with Silastic® membranes, however to a lesser extent. This seemed to suggest that the transepidermal routes for atropine and \([^3\text{H}]\text{dexetimide}\) are different and that some interaction between the two drugs may occur at the surface of the membrane. However, it should be noted that atropine sulphate did not affect the permeation of \([^3\text{H}]\text{dexetimide}\). Because this contradicts the above hypotheses, further studies are necessary. These results show that one should remain aware of unexpected drug-drug interactions and/or drug-vehicle interactions in transdermal permeation experiments.
4.1. Introduction

To measure the in vitro permeation of seven anticholinergics, we developed a standardization procedure to correct for the large variations in permeability between skin samples (Chapter 3). Radiolabelled $[^3]$H]dexetimide was added to the donor solution as an internal standard and ratios were calculated by dividing the percentage of permeated anticholinergic by the percentage of permeated $[^3]$H]dexetimide. For all anticholinergics studied, the use of ratios decreased the variations which showed the usefulness of an internal standard to correct for variations in the skin. However, we noticed that in the presence of atropine the permeation of $[^3]$H]dexetimide decreased substantially compared to the $[^3]$H]dexetimide permeation in the presence of the other anticholinergics, including atropine sulphate. Therefore, we studied the influence of atropine on the permeation of $[^3]$H]dexetimide in more detail in this chapter. Experiments were performed using a donor solution of $[^3]$H]dexetimide with or without atropine, and using three types of membranes. The tested membranes were fresh and frozen pig epidermal membranes, and artificial membranes (Silastic®). Pig skin was chosen because it has histological properties and permeabilities comparable to human skin, and Silastic® was used as a synthetic alternative to skin [1-18].

4.2. Materials and Methods

4.2.1. Materials

$[^3]$H]Dexetimide hydrochloride ($[^3]$H]dex, 15 Ci/mmol) was obtained from Janssen Pharmaceutica N.V.(Beerse, Belgium). Atropine base was obtained from Merck (Darmstadt, Germany). Dexetimide hydrochloride was of pharmaceutical quality and obtained from a local wholesaler. 1-Dodecylazacycloheptan-2-one (Azone®) was kindly supplied by Nelson Research (Irvine, CA, USA). Sigmacoat® was obtained from Sigma (St.Louis, MO, USA). Propylene glycol was purchased from Brocacef (Maarssen, The Netherlands). All other chemicals and solvents were of analytical grade and obtained from Merck (Darmstadt, Germany).

4.2.2. Preparation of solutions

Isotonic phosphate buffered saline pH 7.4 (PBS-buffer) was prepared by dissolving 8.00 g NaCl, 0.20 g KCl, 0.20 g KH$_2$PO$_4$ and 1.44 g Na$_2$HPO$_4$.2H$_2$O in 1 l distilled water. PBS-buffer was used as the receptor solution. Ethanol / propylene glycol / PBS-buffer / Azone® 60:20:15:5 (v/v) was used as donor solution (vehicle) [19]. Drug solutions with atropine or unlabelled dexetimide were prepared by mixing 500 µl of a solution of atropine or unlabelled dexetimide (15 mg/ml in vehicle) with 10 µl of an ethanolic stock solution of $[^3]$H]dex (3 MBq/ml).
The drug solution without atropine or unlabelled dexetimide was prepared by mixing 500 µl vehicle with 10 µl of an ethanolic stock solution of [3H]dex (3 MBq/ml).

4.2.3. Preparation of pig skin

Pigs (CDL, Groningen, The Netherlands) weighing 20 kg, 8 weeks old, previously used for experimental surgery and sacrificed by termination of the resuscitation, were used. Pig ears were obtained within 30 min after termination of the resuscitation and cleaned under cold running water before whole skin membranes were removed from the underlying cartilage. Hairs were cut and the whole membranes were used immediately (fresh skin) or frozen in liquid nitrogen and stored at -80°C until further use (frozen skin). Epidermal membranes were prepared by soaking the whole skin membranes in water for 120 sec at 60°C, followed by blunt dissection [7, 15]. The frozen whole membranes were thawed before epidermal membranes were prepared.

4.2.4. Preparation of Silastic® membranes

Non-reinforced silicone membrane (Silastic®, polydimethyl siloxane, type 500-1, Laboratoire Perouse Implant, Bornel, France) of 0.125 mm thickness was extensively rinsed in hot distilled water (60°C) until all sodium bicarbonate (present on the surface to facilitate handling) was removed. This was followed by a thorough rinse in distilled water (20°C) for one hour [17].

4.2.5. Permeation experiments

Permeation experiments were performed using Franz diffusion cells [16, 20, 21]. These cells were made of glass with a contact area of 1.35 cm² (University Centre for Pharmacy, Groningen, The Netherlands) and pretreated with a silanizing agent (Sigmacoat®). The Franz diffusion cell consists of a donor compartment and a receptor compartment. The membranes were mounted between the cell compartments with the stratum corneum towards the donor compartment and an O-ring was used to position the membrane. The two cell compartments were held together with a clamp. The receptor compartment has a volume of 4.3 ml and was filled with PBS-buffer. It was kept at 37°C by circulating water through an external water jacket. After 30 min of equilibration of the membrane with the receptor solution, 200 µl of the drug solution was applied in the donor compartment by means of a pipet. The donor compartment was covered with parafilm to prevent evaporation of the solvent. The receptor fluid was continuously stirred by means of a spinning bar magnet, at 400 rpm (Multipoint HP 15, Variomag, München, Germany). Receptor solution samples, 2.0 ml aliquots, were withdrawn through the sampling port of the receptor compartment at various time intervals and stored at -20°C until analysis. The cells were refilled with receptor
Table 4.1. Permeation of [\(^3\text{H}\)]dexetimide through fresh pig epidermal membranes in the absence and presence of atropine.

<table>
<thead>
<tr>
<th>Sampling time (hours)</th>
<th>Without atropine(^1)</th>
<th>With atropine(^2)</th>
<th>Ratio atropine / [(^3\text{H})]dex</th>
<th>Ratio [(^3\text{H})]dex(<em>{\text{without}}) / [(^3\text{H})]dex(</em>{\text{with}})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permeation [(^3\text{H})]dex (%)</td>
<td>Permeation [(^3\text{H})]dex (%)</td>
<td>Permeation atropine (%)</td>
<td>[(^3\text{H})]dex</td>
</tr>
<tr>
<td>1</td>
<td>0.37</td>
<td>0.01</td>
<td>0.01</td>
<td>1.14</td>
</tr>
<tr>
<td>3</td>
<td>0.75</td>
<td>0.04</td>
<td>0.03</td>
<td>0.77</td>
</tr>
<tr>
<td>5</td>
<td>1.04</td>
<td>0.08</td>
<td>0.10</td>
<td>1.24</td>
</tr>
<tr>
<td>15</td>
<td>3.77</td>
<td>0.97</td>
<td>3.24</td>
<td>2.87</td>
</tr>
<tr>
<td>17</td>
<td>4.81</td>
<td>1.41</td>
<td>5.77</td>
<td>3.60</td>
</tr>
<tr>
<td>19</td>
<td>6.35</td>
<td>1.99</td>
<td>11.89</td>
<td>5.40</td>
</tr>
<tr>
<td>21</td>
<td>7.78</td>
<td>2.58</td>
<td>18.11</td>
<td>6.48</td>
</tr>
<tr>
<td>23</td>
<td>9.39</td>
<td>3.24</td>
<td>28.65</td>
<td>8.53</td>
</tr>
<tr>
<td>25</td>
<td>14.68</td>
<td>3.96</td>
<td>40.08</td>
<td>9.80</td>
</tr>
</tbody>
</table>

*1 Permeation experiments in the absence of atropine but in the presence of dexetimide: n=3.
*2 Permeation experiments in the presence of atropine: n=4.

Figure 4.1. Permeation of [\(^3\text{H}\)]dexetimide through fresh pig epidermal membranes in the presence of atropine or dexetimide; each point represents the mean and standard error of the mean.

- ■ = [\(^3\text{H}\)]dex in the presence of dexetimide (n=3);
- ● = [\(^3\text{H}\)]dex in the presence of atropine (n=4).
solution to keep the volume constant during the experiment. The experiments were run for 25 hours. The amounts of \[^{3}\text{H}]\text{dexetimide}\) and atropine or dexetimide present in the receptor solution, were measured by liquid scintillation counting and radioreceptor assay under equilibrium conditions, respectively, according to previously described procedures (Chapter 3).

4.3. Results and Discussion

Figure 4.1 shows the mean cumulative percentage permeation of radiolabelled \[^{3}\text{H}]\text{dexetimide}\) through fresh pig epidermal membranes in the presence of atropine or dexetimide, respectively. During steady state which is reached after approximately 17 hours, the permeation of \[^{3}\text{H}]\text{dexetimide}\) in the presence of atropine is about a factor 3 lower in comparison with the \[^{3}\text{H}]\text{dexetimide}\) permeation in the presence of dexetimide (Table 4.1). This decrease was only observed with atropine and not with the other tested anticholinergics (atropine sulphate monohydrate, benztropine mesylate, dexetimide hydrochloride, oxyphencyclidine hydrochloride, scopolamine hydrobromide trihydrate, tropicamide). We also noticed that the ratios, calculated by dividing the percentage of permeation of anticholinergic by the permeation of the internal standard \[^{3}\text{H}]\text{dexetimide}\), became constant for all anticholinergics after 15 hours, except for atropine (Chapter 3) which seems to indicate that the permeation routes of atropine and \[^{3}\text{H}]\text{dexetimide}\) are different (Table 4.1). If so, \[^{3}\text{H}]\text{dexetimide}\) would not be appropriate to serve as an internal standard for atropine permeation studies. To investigate these phenomena further, we performed in vitro experiments with frozen pig epidermal membranes and Silastic® membranes as well.

The permeation of \[^{3}\text{H}]\text{dexetimide}\) through frozen pig epidermal membranes in the absence and presence of atropine is presented in Figure 4.2 and Table 4.2. Again, the permeation of \[^{3}\text{H}]\text{dexetimide}\) decreased about a factor 3 in the presence of atropine whereas no influence was seen using other anticholinergics, as shown in Figure 4.3 for dexetimide. Apparently, storage of pig skin for 2 months at -80°C resulted in a higher permeability compared to fresh epidermal membranes, which is probably caused by a loss of barrier function of the skin and changes in physical and chemical properties [22-25]. These results indicate that although the properties of the skin may be altered due to freezing and thawing, the unusual behaviour of \[^{3}\text{H}]\text{dexetimide}\) in the presence of atropine still exists.

Figure 4.4 shows the influence of atropine on the permeation of \[^{3}\text{H}]\text{dexetimide}\) using Silastic® membranes. In the presence of atropine the permeation of \[^{3}\text{H}]\text{dexetimide}\) is decreased, however the influence of atropine is small compared to the results with pig epidermal membranes: After 17 hours the decrease of \[^{3}\text{H}]\text{dexetimide}\) permeation in the presence of atropine is only about a factor 1.4.

It is generally assumed that most drugs permeate through biological membranes via the transepidermal route, either through the intracellular spaces, or the intercellular spaces, or through both (Chapter 1). In addition, there is the transappendageal
Table 4.2.  Permeation of [$^3$H]dextimide through frozen pig epidermal membranes in the absence and presence of atropine.

<table>
<thead>
<tr>
<th>Sampling time (hours)</th>
<th>Without atropine $^1$</th>
<th>With atropine $^2$</th>
<th>Ratio [3H]dex / [3H]dex without atropine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Permeation [3H]dex (%)</td>
<td>Permeation [3H]dex (%)</td>
<td>Permeation atropine / [3H]dex</td>
</tr>
<tr>
<td>1</td>
<td>0.65</td>
<td>0.92</td>
<td>1.98</td>
</tr>
<tr>
<td>3</td>
<td>13.12</td>
<td>3.51</td>
<td>10.88</td>
</tr>
<tr>
<td>5</td>
<td>20.40</td>
<td>5.66</td>
<td>19.68</td>
</tr>
<tr>
<td>7</td>
<td>24.76</td>
<td>7.96</td>
<td>33.03</td>
</tr>
<tr>
<td>17</td>
<td>34.00$^3$</td>
<td>10.64</td>
<td>48.24</td>
</tr>
<tr>
<td>19</td>
<td>37.06$^3$</td>
<td>11.80</td>
<td>55.95</td>
</tr>
<tr>
<td>21</td>
<td>39.67$^3$</td>
<td>12.83</td>
<td>58.41</td>
</tr>
<tr>
<td>23</td>
<td>42.56$^3$</td>
<td>13.92</td>
<td>63.60</td>
</tr>
<tr>
<td>25</td>
<td>45.25$^3$</td>
<td>15.30</td>
<td>68.88</td>
</tr>
</tbody>
</table>

$^1$ Permeation experiments in the absence of atropine: n=2.
$^2$ Permeation experiments in the presence of atropine: n=4.
$^3$ Significantly higher than \[3H\]dex permeation in the presence of atropine: p < 0.05 (Student t-test).

Figure 4.2.  Permeation of [$^3$H]dextimide through frozen pig epidermal membranes in the absence and presence of atropine; each point represents the mean and standard error of the mean.

- ■ = [$^3$H]dex (n=2); ● = [$^3$H]dex in the presence of atropine (n=4).
Figure 4.3. Permeation of $[^3]$H]dexetimide through frozen pig epidermal membranes in the absence and presence of dexetimide; each point represents the mean and standard error of the mean.


Figure 4.4. Permeation of $[^3]$H]dexetimide through Silastic® membranes in the absence and presence of atropine; each point represents the mean and standard error of the mean.

Table 4.3. Permeation of [3H]dexetimide through Silastic® membranes in the absence and presence of atropine.

<table>
<thead>
<tr>
<th>Sampling time (hours)</th>
<th>Without atropine*1</th>
<th>With atropine*2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.11</td>
<td>0.11</td>
</tr>
<tr>
<td>2</td>
<td>0.29</td>
<td>0.31</td>
</tr>
<tr>
<td>3</td>
<td>0.58</td>
<td>0.55</td>
</tr>
<tr>
<td>4</td>
<td>0.98</td>
<td>0.84</td>
</tr>
<tr>
<td>5</td>
<td>1.42*3</td>
<td>1.16</td>
</tr>
<tr>
<td>6</td>
<td>1.91*3</td>
<td>1.53</td>
</tr>
<tr>
<td>7</td>
<td>2.38*3</td>
<td>1.89</td>
</tr>
<tr>
<td>17</td>
<td>5.87*4</td>
<td>4.18</td>
</tr>
<tr>
<td>19</td>
<td>7.05*4</td>
<td>5.05</td>
</tr>
<tr>
<td>21</td>
<td>8.18*3</td>
<td>5.74</td>
</tr>
<tr>
<td>23</td>
<td>9.33*3</td>
<td>6.30</td>
</tr>
<tr>
<td>25</td>
<td>10.75*3</td>
<td>7.29</td>
</tr>
</tbody>
</table>

*1 Permeation experiments in the absence of atropine: n=6.
*2 Permeation experiments in the presence of atropine: n=6.
*3 Significantly higher than [3H]dex permeation in the presence of atropine:
  p < 0.01 (Student t-test).
*4 Significantly higher than [3H]dex permeation in the presence of atropine:
  p < 0.01 (Mann-Whitney rank sum test).

route, but this route is considered of minor importance and only relevant for very hydrophilic drugs [26, 27]. With Silastic® membranes which are considered to be more hydrophobic than skin, only the more lipophilic transepidermal pathway is mimicked [16, 26]. It can be noted from Table 4.3 that the permeation of [3H]dexetimide alone through Silastic® membranes is comparable with the permeation through fresh pig epidermal membranes. Yet, for atropine, permeation through Silastic® membranes decreased by a factor 5 compared to the epidermal membranes. This is in line with the more lipophilic character of dexetimide, and may indicate that dexetimide preferably permeates via the more lipophilic intercellular route whereas atropine permeates via the intracellular route. This hypothesis seems to be corroborated by the finding that the ratio of atropine / [3H]dexetimide does not become constant for fresh pig epidermal membranes (Table 4.1). However, the assumption of different pathways for atropine and [3H]dexetimide does not explain the observation that atropine reduced the permeation of [3H]dexetimide through epidermal membranes by a factor 3 and that the other anticholinergics do not affect [3H]dexetimide permeation. This would suggest some
sort of interaction between atropine and $[^3]$H$dexetimide at the membrane surface. Competition for a common transport carrier may also be possible, but this would imply common permeation routes, rather than different routes. On the other hand, it should be noted that the experiments with atropine sulphate in Chapter 3 showed that the permeation of $[^3]$H$dexetimide in the presence of atropine sulphate did not reduce and that the ratio atropine sulphate / $[^3]$H$dexetimide was constant at steady state. These results do not indicate an interaction or competition, nor do they point to different permeation routes.

4.4. Conclusions

The experiments show that atropine reduces the permeation of $[^3]$H$dexetimide through fresh and frozen pig epidermal membranes. This decrease in permeation also occurs with Silastic® membranes, but to a lesser extent. The mechanism for this phenomenon cannot be explained properly at this moment. The results of the present studies show that care should be taken when dealing with more than one drug in transdermal permeation studies. Also, it cannot be excluded that vehicle components may interfere with the permeation of the active ingredient(s).

4.5. References

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