Unusual Reduction of the *In Vitro* Skin Permeation of [^{3}H]dexetimide by Atropine

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INTRODUCTION

Transdermal drug delivery of anticholinergics may be useful in the treatment of obstructive airways diseases because sustained, constant and controlled levels of the drug in the blood may result in a prolonged duration of action (1). It will also lead to better patient compliance by eliminating frequent dosing. Therefore, we performed *in vitro* experiments with anticholinergics to determine their permeation characteristics through the skin (2). Seven anticholinergics were selected because of their high affinity towards the muscarinic receptor. Because large variations in permeability between skin samples are often observed, a standardization procedure was developed to correct for the large variations in permeability between skin samples. 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, thus showing 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, the influence of atropine on the permeation of [^{3}H]dexetimide was studied in *more* detail. 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 (3–7).

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MATERIALS AND METHODS

Materials

[^{3}H]Dexetimide hydrochloride ([^{3}H]dex, 15 Ci/mmol) was obtained from Janssen Pharmaceutica N.V. (Beerse, Belgium). [*N-methyl*-^{3}H]Scopolamine methyl chloride ([^{3}H]NMS, 81.5 Ci/mmol) was obtained from Du Pont NEN (Du Pont, Wilmington, DE, USA). Atropine base was obtained from Merck (Darmstadt, Germany). Dexetimide hydrochloride was of pharmaceutical quality and obtained from a local wholesaler. Sigmacoat® was obtained from Sigma (St. Louis, Missouri, USA). 1-Dodecylazacycloheptan-2-one (Azone®) was kindly supplied by Nelson Research (Irvine, California, 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). Polyethylene tubes (12 ml) were obtained from Greiner (Alphen a/d Rijn, The Netherlands). The GF/B glassfibre filters were from Whatman (Maidstone, UK). Rialuma was used as scintillation liquid, obtained from Lumac (Olen, Belgium), in combination with mini-scintillation counting vials from Packard (Groningen, The Netherlands).

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_2PO_4 and 1.44 g $Na_2HPO_4.2H_2O$ in 1 l distilled water. PBS-buffer was used as the receptor solution.

The 50 mM sodium phosphate buffer pH 7.4 (assay buffer) was prepared by dissolving 1.38 g $NaH_2PO_4.H_2O$ and 7.12 g $Na_2HPO_4.2H_2O$ in 1 l distilled water.

Stock solutions (1×10^{-3}M) of the anticholinergic were prepared in ethanol and stored at −20°C. Ethanol / propylene glycol / PBS-buffer / Azone® 60:20:15:5 (v/v) was used as donor solution (vehicle) (8).

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 [^{3}H]dex (3 MBq/ml).

The tissue suspension was prepared by dissolving 5 mg of lyophilized receptors (9) in 1 ml assay buffer.

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 (3,5). The

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Table I. Permeation of [^{3}H]dexetimide Through Fresh Pig Epidermal Membranes in the Absence and Presence of Atropine: Means and Standard Errors of the Means

Sampling time (hours)	Without atropine[a]	With atropine[b]			
	Permeation [^{3}H]dex (%)	Permeation [^{3}H]dex (%)	Permeation atropine (%)	Ratio atropine/ [^{3}H]dex	Ratio [^{3}H]dex$_{without}$/ [^{3}H]dex$_{with}$
1	0.37 (0.36)	0.01 (0.00)	0.01	1.14	26.4
3	0.75 (0.70)	0.04 (0.01)	0.03	0.77	18.7
5	1.04 (0.95)	0.08 (0.02)	0.10	1.24	12.7
15	3.77 (2.76)	0.97 (0.34)	3.24	2.87	3.89
17	4.81 (3.22)	1.41 (0.42)	5.77	3.60	3.41
19	6.35 (3.71)	1.99 (0.50)	11.89	5.40	3.19
21	7.78 (4.03)	2.58 (0.56)	18.11	6.48	3.01
23	9.39 (4.37)	3.24 (0.59)	28.65	8.53	2.90
25	14.68 (7.91)	3.96 (0.64)	40.08	9.80	3.70

[a] Permeation experiments in the absence of atropine but in the presence of dexetimide: n = 3.
[b] Permeation experiments in the presence of atropine: n = 4.

frozen whole membranes were thawed before epidermal membranes were prepared.

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 (7).

Permeation Experiments

Permeation experiments were performed using Franz diffusion cells (6,10). These cells were made of glass with a contact area of 1.35 cm^2 (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

Table II. Permeation of [^{3}H]dexetimide Through Frozen Pig Epidermal Membranes in the Absence and Presence of Atropine: Means and Standard Errors of the Means

Sampling time (hours)	Without atropine[a]	With atropine[b]			
	Permeation [^{3}H]dex (%)	Permeation [^{3}H]dex (%)	Permeation atropine (%)	Ratio atropine/ [^{3}H]dex	Ratio [^{3}H]dex$_{without}$/ [^{3}H]dex$_{with}$
1	0.65 (0.16)	0.92 (0.81)	1.98	2.48	0.71
3	13.12 (10.90)	3.51 (2.22)	10.88	3.13	3.74
5	20.40 (14.80)	5.66 (2.65)	19.68	4.21	3.60
7	24.76 (15.25)	7.96 (3.31)	33.03	5.02	3.11
17	34.00[c] (10.39)	10.64 (3.59)	48.24	6.12	3.20
19	37.06[c] (10.15)	11.80 (3.78)	55.95	6.61	3.14
21	39.67[c] (10.58)	12.83 (4.07)	58.41	6.15	3.09
23	42.56[c] (9.93)	13.92 (4.10)	63.60	5.91	3.06
25	45.25[c] (10.26)	15.30 (4.20)	68.88	5.62	2.96

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

until analysis. The cells were refilled with receptor solution to keep the volume constant during the experiment. The experiments were run for 25 hours.

Analytical Procedure

To determine the amount of [^{3}H]dexetimide present in the receptor solution, 1 ml of the receptor solution sample was added to mini-scintillation vials and mixed with 3.5 ml Rialuma. The vials were counted for 40,000 counts or 5 min in a liquid scintillation counter (Minaxi, Packard, Groningen, The Netherlands), whatever came first.

The concentrations of the unlabelled anticholinergics in the receptor solution samples were determined by means of a radioreceptor assay (9). From the anticholinergic stock solutions, appropriate dilutions were made in assay buffer, concentrations ranging from 1×10^{-9}M to 1×10^{-5}M (calibration curve). The calibration samples, together with the receptor solution samples, were analysed using radioreceptor assays under equilibrium conditions (2).

RESULTS AND DISCUSSION

Table I shows the mean cumulative percentage permeation of radiolabelled [^{3}H]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}H]dexetimide in the presence of atropine is about a factor 3 lower in comparison with the [^{3}H]dexetimide permeation in the presence of dexetimide (Table I).

This decrease was only observed with atropine and not with other tested anticholinergics (atropine sulphate monohydrate, benztropine mesylate, dexetimide hydrochloride, oxyphencyclimine 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}H]dexetimide, became constant for all anticholinergics after 15 hours, except for atropine (2). This seems to indicate that the permeation routes of atropine and [^{3}H]dexetimide are different. If so, [^{3}H]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}H]dexetimide through frozen pig epidermal membranes in the absence and presence of atropine is presented in Table II. The results show that 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 (12–13). Again, the permeation of [^{3}H]dexetimide decreased about a factor 3 in the presence of atropine, however the ratios became relatively constant. No influence was seen using other anticholinergics. These results indicate that although the properties of the skin may be altered due to freezing and thawing, the unusual behaviour of [^{3}H]dexetimide in the presence of atropine still exists.

Table III shows the influence of atropine on the permeation of [^{3}H]dexetimide using Silastic® membranes. In the presence of atropine the permeation of [^{3}H]dexetimide is decreased, however the influence of atropine is smaller compared to the results with pig epidermal membranes: After 17 hours the decrease of [^{3}H]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

Table III. Permeation of [^{3}H]dexetimide Through Silastic® Membranes in the Absence and Presence of Atropine: Means and Standard Errors of the Means

Sampling time (hours)	Without atropine[a]	With atropine[b]			
	Permeation [^{3}H]dex (%)	Permeation [^{3}H]dex (%)	Permeation atropine (%)	Ratio atropine/ [^{3}H]dex	Ratio [^{3}H]dex$_{without}$/ [^{3}H]dex$_{with}$
1	0.11 (0.02)	0.11 (0.01)	0.00	0.01	1.00
2	0.29 (0.03)	0.31 (0.01)	0.06	0.19	0.93
3	0.58 (0.03)	0.55 (0.02)	0.14	0.25	1.05
4	0.98 (0.05)	0.84 (0.04)	0.25	0.29	1.17
5	1.42[c] (0.05)	1.16 (0.05)	0.37	0.32	1.22
6	1.91[c] (0.08)	1.53 (0.06)	0.55	0.36	1.25
7	2.38[c] (0.09)	1.89 (0.07)	0.72	0.38	1.26
17	5.87[d] (0.48)	4.18 (0.16)	3.41	0.81	1.40
19	7.05[d] (0.55)	5.05 (0.19)	4.41	0.87	1.40
21	8.18[c] (0.68)	5.74 (0.19)	5.85	1.01	1.43
23	9.33[c] (0.81)	6.30 (0.23)	6.67	1.05	1.48
25	10.75[c] (0.88)	7.29 (0.26)	8.29	1.13	1.47

[a] Permeation experiments in the absence of atropine: n = 6.

[b] Permeation experiments in the presence of atropine: n = 6.

[c] Significantly higher than [^{3}H]dex permeation in the presence of atropine: $p < 0.01$ (Student t-test).

[d] Significantly higher than [^{3}H]dex permeation in the presence of atropine: $p < 0.01$ (Mann-Whitney rank sum test).

through the intracellular spaces, or the intercellular spaces, or through both (14). In addition, there is the transappendageal route, but this route is considered of minor importance and only relevant for very hydrophilic drugs (14,15). With Silastic® membranes which are considered to be more hydrophobic than skin, only the more lipophilic transepidermal pathway is mimicked (6,15). It can be noted from Table III that the permeation of [^{3}H]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 intercellular route whereas atropine permeates via the intracellular route. This hypothesis seems to be corroborated by the finding that the ratio of atropine/[^{3}H]dexetimide does not become constant for fresh pig epidermal membranes (Table I). However, using frozen skin and Silastic® membranes, the ratio of atropine/[^{3}H]dexetimide became relatively constant.

Besides, the assumption of different pathways for atropine and [^{3}H]dexetimide does not explain the observation that atropine reduced the permeation of [^{3}H]dexetimide through epidermal membranes by a factor 3 and that the other anticholinergics do not affect [^{3}H]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.

Although the mechanism for the reduced permeation of [^{3}H]dexetimide in the presence of atropine cannot be explained properly at this moment, the results indicate that one should remain aware of unexpected drug-drug interactions and/or drug-vehicle inetractions in transdermal permeation experiments.

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