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Standardization procedure for the in vitro skin permeation of anticholinergics

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Abstract

The permeation of seven anticholinergics was studied in vitro on pig epidermal membranes, using static Franz diffusion cells. The donor solution consisted of isotonic phosphate-buffered saline, pH 7.4 with ethanol, propylene glycol and Azone®. Tritium-labelled dexetimide was added as an internal standard. Ratios were calculated by dividing the percentage of permeated anticholinergic by the percentage of permeated [^{3}H]dexetimide. For all anticholinergics, the use of ratios decreased the variations which shows the usefulness of [^{3}H]dexetimide as an internal standard to correct for variations in the skin. For all anticholinergics, the lag times were comparable; however, the fluxes differed by about a factor of 6 between the highest and lowest values. These differences in permeation data were found not to correlate with the molecular weight and octanol/water partition coefficient or octanol/buffer partition coefficient. The differences in permeation between atropine base and atropine sulphate might be explained by differences in solubility and pH of the donor solution. The use of pig skin which had been frozen and stored for 2 months at − 80°C, resulted in a higher permeability without any lag time. Therefore only fresh skin should be used.

Keywords: Anticholinergics; [^{3}H]Dexetimide; In vitro permeation; Transdermal delivery; Internal standard

1. 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 (Guy and Hadgraft, 1986). It will also lead to better patient

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compliance by eliminating frequent dosing. Therefore, we performed in vitro experiments with anticholinergics to determine their permeation characteristics through the skin. Seven anticholinergics were selected for these experiments because of their high affinity towards the muscarinic receptor (Bosman et al., 1998).

The permeation of chemicals through the skin can be studied using in vitro techniques. It is widely believed that in vitro permeation is a good representation of the in vivo situation (Behl et al., 1990; Bronaugh and Collier, 1990; Friend, 1992). However, in vitro experiments with human skin are difficult to conduct due to the scarcity of this material and the fact that gender, race, site, age and skin condition of the donor cannot be controlled satisfactorily (Behl et al., 1990). Therefore, various animal skin alternatives have been used to predict the percutaneous absorption through human skin.

We selected pig skin for the in vitro experiments, because previous reports indicate that it has histological properties comparable to human skin with similarities in epidermal thickness and composition, pelage density, dermal structure, lipid content, and general morphology (Montagna and Yun, 1964; Marcarian and Calhoun, 1966; Nicolaides et al., 1968; Gray and Yardley, 1975; Meyer et al., 1978; Monteiro-Riviere, 1986). Furthermore, in a number of permeation studies, pig skin showed to be a good model for human skin permeability (Bartek et al., 1972; Bronaugh et al., 1982; Hawkins and Reifenrath, 1984, 1986; Chang and Riviere, 1991; Riviere and Monteiro-Riviere, 1991; Dick and Scott, 1992; Woolfson et al., 1992). Because the dermis in full-thickness skin may act as an additional barrier to lipophilic drugs, we prepared epidermal membranes from the pig ear (Bhatti et al., 1988; Dick and Scott, 1992) and used these in the in vitro experiments.

The present study describes the in vitro permeation of seven anticholinergics through pig epidermal membranes using static Franz diffusion cells. The donor solution consisted of isotonic phosphate-buffered saline, pH 7.4, with ethanol, propylene glycol and Azone® as additional solvent components and as penetration enhancers. Because large variations in permeability between skin samples are often observed, we decided to use an internal standard (tritium-labelled dexetimide, which is also an anticholinergic drug) to correct for variations in the skin. This standardization is discussed and the permeation data are examined for their correlation with a number of physicochemical parameters (Bosman et al., 1998). The suitability of pig skin after frozen storage is also studied and compared with fresh skin.

2. Materials and methods

2.1. 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). The anticholinergics atropine, atropine sulphate monohydrate, benztropine mesylate, dexetimide hydrochloride, oxyphencyclimine hydrochloride, scopolamine hydrobromide trihydrate, and tropicamide were all of pharmaceutical quality and obtained from local wholesalers. Sigmacoat® was obtained from Sigma (St. Louis, MO). 1-Dodecylazacycloheptan-2-one (Azone®) was kindly supplied by Nelson Research (Irvine, CA). 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).

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_2PO_4 and 1.44 g

$Na_2HPO_4 \cdot 2H_2O$ 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 $NaH_2PO_4 \cdot H_2O$ and 7.12 g $Na_2HPO_4 \cdot 2H_2O$ 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) (Swart et al., 1992).

The drug solution was prepared by mixing 500 μl of a solution of the anticholinergic (15 mg/ml in 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 (Ensing et al., 1994) in 1 ml assay buffer.

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). Permeation experiments were performed with epidermal membranes which were prepared by soaking the whole skin membranes in water for 120 s at 60°C, followed by blunt dissection (Bhatti et al., 1988; Dick and Scott, 1992). The frozen whole membranes were thawed before epidermal membranes were prepared.

2.4. Permeation experiments

Permeation experiments were performed using Franz diffusion cells (Barry, 1983; Tojo, 1987; Frantz, 1990). These cells were made of glass with a contact area of 1.35 cm^2 (University Centre for Pharmacy, Groningen, The Netherlands) and pre-treated with a silanizing agent (Sigmacoat®). The Franz diffusion cell consists of a donor compartment and a receptor compartment. Epidermal 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 $t = 1$, 3, 5, 7 or 15, 17, 19, 21, 23 and 25 h, and stored at −20°C until analysis. The cells were refilled with receptor solution to keep the volume constant during the experiment.

2.5. 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 40000 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 (Ensing et al., 1994). From the anticholinergic stock solutions, appropriate dilutions were made in assay buffer, concentrations ranging from 1×10^{-9} to 1×10^{-5} M (calibration curve). The calibration samples, together with the receptor solution samples, were analysed using RRA with pre-incubation at 0°C for tropicamide and benztropine mesylate and using RRA under equilibrium con-

ditions for the other anticholinergics (Bosman et al., 1998).

Calibration curves were fitted with the ligand curve-fitting program (Munson and Rodbard, 1980). The obtained binding values (Bq) of the receptor solution samples were introduced in the calibration curves and the unknown concentrations of the anticholinergics were calculated. When the final concentration of the receptor solution samples exceeded the upper limit of quantitation of the calibration curves, the receptor solution samples were diluted (10–10000-fold) and reanalysed.

2.6. Data analysis

The amount of drug permeated through the pig epidermis at a certain time interval was calculated based on the measured concentrations in the receptor compartment, which were corrected for the sampling dilution, and volume. The results of the permeation experiments were plotted in graphs showing the percentage of permeated anticholinergic versus time or the percentage of permeated [^{3}H]dexetimide versus time.

Flux values were calculated by linear regression from the steady-state portion of the permeation curve and expressed in nmol cm^{-2} h^{-1}. The lag time was determined by extrapolation of the steady-state portion of the curve to the intercept of the time axis. Permeability coefficients of the anticholinergics were calculated as the flux divided by the applied concentration in the donor compartment (Williams and Barry, 1992).

3. Results and discussion

3.1. Permeation experiments: use of an internal standard

The mean cumulative permeation percentages of the anticholinergics and of radiolabelled [^{3}H]dexetimide are plotted in Fig. 1. Table 1 shows the corresponding standard errors of the mean (S.E.M.) at $t = 25$ h. The percentages of permeation show considerable variations at all time intervals for all anticholinergics; however,

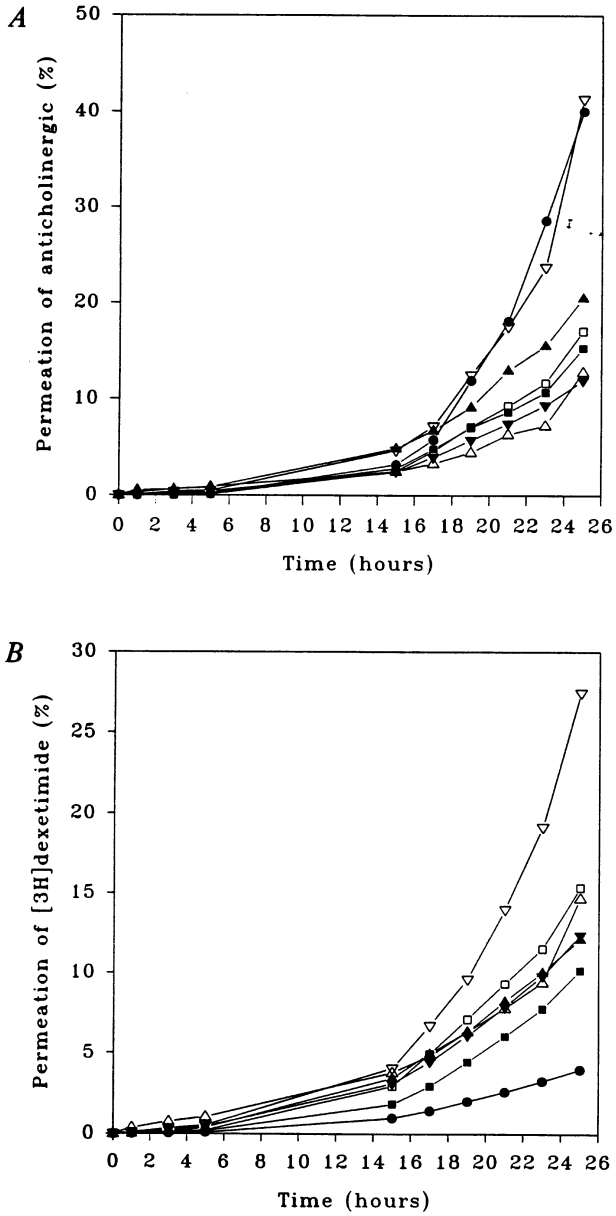

Fig. 1. Permeation of the anticholinergics in the presence of [^{3}H]dexetimide (A) and permeation of the internal standard [^{3}H]dexetimide in the presence of the anticholinergics (B) through fresh pig epidermal membranes. (●) Atropine; (□) atropine sulphate monohydrate; (▽) benztropine mesylate; (△) dexetimide hydrochloride; (▼) oxyphencyclimine hydrochloride; (■) scopolamine hydrobromide trihydrate; (▲) tropicamide.

Table 1
Percentage permeation of the anticholinergics and internal standard [^{3}H]dexetimide, and anticholinergic/[^{3}H]dexetimide ratios at $t = 25$ h, using fresh pig epidermal membranes

Anticholinergic	Permeation anticholinergic (%)	Permeation [^{3}H]dexetimide (%)	Ratio[a]	CV_A^2/CV_R^2 [b]	n
Atropine	40.1 (9.8)	4.0 (0.6)	9.8 (1.4)	2.9	4
Atropine sulphate·H_2O	17.1 (3.5)	15.4 (2.3)	1.1 (0.1)	3.3	4
Benztropine mesylate	41.4 (10.3)	27.5 (3.2)	1.5 (0.2)	3.4	2
Dexetimide HCl	12.8 (8.0)	14.7 (7.9)	0.8 (0.1)	30.2*	3
Oxyphencyclimine HCl	11.9 (5.8)	12.4 (4.7)	0.8 (0.2)	6.2**	4
Scopolamine HBr·$3H_2O$	15.3 (7.9)	10.1 (5.1)	1.5 (0.2)	10.4**	3
Tropicamide	20.5 (8.1)	12.1 (2.7)	1.6 (0.3)	4.3	2

Data represent mean values ± standard error of the mean.
[a] Ratio = permeation of anticholinergic (%) divided by permeation of [^{3}H]dexetimide (%).
[b] CV_A^2 = variance in permeation of anticholinergic corrected for the mean; CV_R^2 = variance in ratio corrected for the mean.
*F-test, $p < 0.05$; **F-test, $p < 0.10$.

this is not exceptional for biological membranes (Southwell et al., 1984; Smith and Haigh, 1992). Fig. 2 gives an example of the variations in permeation (S.E.M.), at all time intervals, of the unlabelled drug dexetimide as well as of the radiolabelled drug.

Since the variations observed may be due to differences in skin preparations, we hypothesized that the use of an internal standard may eliminate these differences. Yet, this concept would be valid only if the anticholinergic and internal standard have similar permeation characteristics, i.e. if they follow similar transdermal routes. This can be tested by plotting ratios over time, as shown in Fig. 3. The ratios were calculated by dividing the

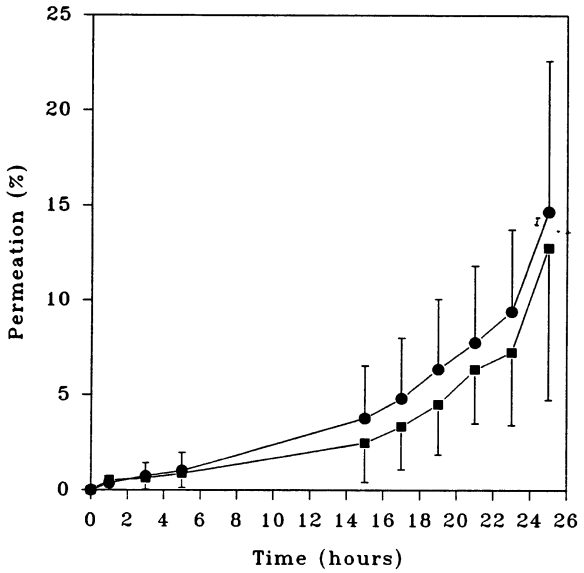

Fig. 2. Permeation of dexetimide (■) and [^{3}H]dexetimide (●) through fresh pig epidermal membranes; each point represents mean and standard error of the mean ($n = 3$).

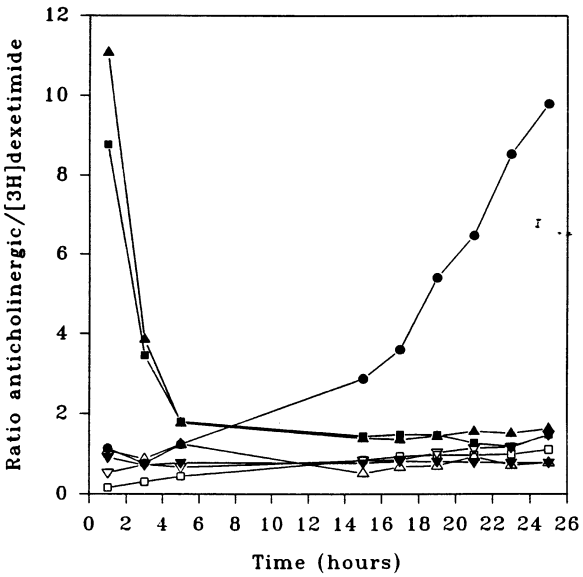

Fig. 3. Ratios of anticholinergic and [^{3}H]dexetimide versus time. (●) atropine; (□) atropine sulphate monohydrate; (▽) benztropine mesylate; (△) dexetimide hydrochloride; (▼) oxyphencyclimine hydrochloride; (■) scopolamine hydrobromide trihydrate; (▲) tropicamide.

Table 2
Permeation data of the anticholinergics using fresh pig epidermal membranes

Anticholinergic	Lag time (h)	Flux (nmol cm^{-2} h^{-1})	Permeability coefficient ($\times 10^6$ cm s^{-1})	n
Atropine	16.1 (0.9)	321 (82)	1.76 (0.45)	4
Atropine sulphate·H_2O	14.1 (0.4)	46 (11)	0.61 (0.14)	4
Benztropine mesylate	16.1 (1.9)	215 (24)	1.64 (0.18)	2
Dexetimide HCl	13.7 (1.7)	59 (35)	0.44 (0.26)	3
Oxyphencyclimine HCl	14.1 (0.7)	55 (31)	0.40 (0.22)	4
Scopolamine HBr·$3H_2O$	13.2 (0.5)	61 (34)	0.51 (0.28)	3
Tropicamide	10.1 (5.1)	130 (84)	0.70 (0.45)	2

Data represent mean values ± standard error of the mean.

percentage of permeation of unlabelled anticholinergic by the percentage of permeation of the radiolabelled drug.

For all anticholinergics the use of ratios decreased the variations in comparison with the variations in percentage permeation of the anticholinergic alone (Table 1, Figs. 2 and 3). However, in the beginning of the curves ($t = 1–5$ h), this decrease in variation was negligible. After 15 h, the ratios became constant which implies that the permeation routes of unlabelled and labelled drug through the skin were comparable. However, atropine base was an exception in that the ratio still increased after 15 h, which may be explained by differences in transdermal routes of atropine and [^{3}H]dexetimide. Another explanation may be a competition of the two drugs for a certain permeation route because in the presence of atropine the permeation of [^{3}H]dexetimide decreased compared to the [^{3}H]dexetimide permeation in the presence of other anticholinergics (Bosman et al., 1998b).

3.2. Permeation experiments: correlation with physicochemical parameters

The permeation data of the anticholinergics are shown in Fig. 1 and Table 2. The lag times of the curves were comparable for all anticholinergics, which means that the diffusivities would be more or less the same. However, the fluxes differed by about a factor of 6 between the highest and lowest values, which may be explained by differences in the physicochemical characteristics of the anticholinergics (Bosman et al., 1998).

Various models, based on Fick's first law of diffusion, have been described to predict the relationship between percutaneous absorption and physicochemical properties of drugs (Tayar et al., 1991; Guy and Potts, 1992; Potts and Guy, 1992; Ridout et al., 1992). Potts and Guy (1992) analysed permeability data using a model which depends only upon the size of the drug and its octanol/water partition coefficient. The following equation was used to predict skin permeability:

$$\log K_p = \log \frac{D^0}{d} + f \cdot \log K_{o/w} - \beta'' \cdot MW$$

where K_p = the permeability coefficient; D^0 = the diffusivity of a hypothetical molecule having zero molecular volume; d = the diffusion path length; f = constant which accounts for the difference between the partitioning domain presented by octanol and that presented by the stratum corneum lipids; $K_{o/w}$ = the octanol/water partition coefficient; β'' = a constant which includes a conversion factor for the substitution of molecular weight for molecular volume; MW = the molecular weight.

We used this model to examine the permeability data of the anticholinergics, although the model was initially used to describe skin permeabilities of non-ionic drugs dissolved in water. Multiple regression analysis of $\log K_p$ upon $\log K_{o/w}$ and MW was required to provide values for f, β'' and $\log(D^0/d)$. Octanol/water partition coefficients ($\log K_{o/w}$) and molecular weights of the bases (MW) were found not to correlate with permeability coefficients ($\log K_p$). Using the octanol/buffer partition coefficients ($\log K_{o/b}$), instead of $\log K_{o/w}$, we still did not find any correlation.

This lack of correlation may be due to the penetration enhancers added to the donor solution. The actions of these enhancers may explain why the model does not fit our data, providing values for f, β'' and D^0/d which have no physicochemical significance. Ethanol has been proposed to increase skin permeability of polar solutes, propylene glycol probably solvates the intracellular proteins and occupies hydrogen bonding sites, whereas Azone® reduces the order of the intercellular lipids (Wotton et al., 1985; Barry, 1987; Guy and Hadgraft, 1987; Williams and Barry, 1992). Also, it is known that propylene glycol promotes Azone® penetration and vice versa (Williams and Barry, 1992). Apparently, the combination of these enhancers resulted in a high permeation for all anticholinergics because the passage of both hydrophilic and lipophilic drugs can be enhanced using this combination. However, for any drug/enhancer/vehicle combination, it will be difficult to predict which mechanism will predominate, and how this will interfere with the model. Another reason for the lack of correlation may be the ionic character of the anticholinergics, because only non-ionic drugs were predicted with this model.

3.3. Comparison of atropine and atropine sulphate

Fig. 1 and Table 2 present the permeation characteristics of atropine and atropine sulphate. The lag times of the two curves, 16 h for atropine and 14 h for atropine sulphate, indicate that the diffusion coefficients of the two drugs are comparable. However, the flux of atropine is approximately six times higher compared to the flux of atropine sulphate, and the permeability coefficient of the former is approximately three times higher.

These differences in permeation may be explained by differences in solubility and pH of the donor solution. The apparent pH of atropine base in the donor solution was 9.8 compared to 6.5 for atropine sulphate (Bosman et al., 1998) which means that the concentration of non-ionized species in the donor solution of atropine base will be increased compared to atropine sulphate ($pK_a = 9.9$). This may explain the higher flux and permeability of atropine base, because in general the flux of the non-ionic species will be greater. However, it should be realized that the measured flux is the flux of both ionic and non-ionic species and therefore the differences between atropine base and salt can be smaller than expected on the basis of pH (Wiechers, 1989).

3.4. Effects of freezing on permeation

Where some authors suggest that freezing affects skin permeation, other authors show no effect of freezing on permeability data (Harrison et al., 1984; Rosenquist et al., 1988; Kasting and Bowman, 1990; Hadzija et al., 1992; Rhoads et al., 1993; Weber, 1993). To examine the effects of frozen storage, the experiments with anticholinergics and internal standard were also performed using pig skin which had been frozen and thawed. An example is given in Fig. 4, which shows the permeation of dexetimide and [^{3}H]dexetimide using pig skin which had been stored for 2 months at -80°C. When we compare these profiles with the results on fresh skin as depicted in Fig. 2, the storage of pig skin resulted in a higher permeability without any lag time. This indicates a loss of viability of the skin and changes in physical and

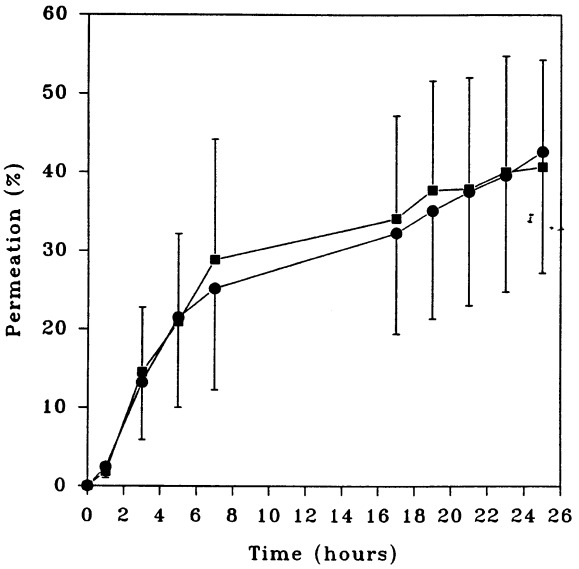

Fig. 4. Permeation of dexetimide (■) and [^{3}H]dexetimide (●) through frozen pig epidermal membranes; each point represents the mean and standard error of the mean ($n = 4$).

chemical properties (Kasting and Bowman, 1990; Hadzija et al., 1992; Rhoads et al., 1993; Weber, 1993). It has been suggested that the greater the storage times, the greater the permeation is (Hawkins and Reifenrath, 1984). These results show that, at least for anticholinergics, only fresh pig skin should be used, because frozen storage clearly affects the permeation.

4. Conclusions

The use of an internal standard and expressing the permeation as the ratio of drug permeated over internal standard permeated decreased the variations in comparison with the variations in percentage of permeated anticholinergic alone. This shows the usefulness of [^{3}H]dexetimide as an internal standard for anticholinergics to correct for variations in the skin. The permeability coefficients of the anticholinergics were found not to correlate with the molecular weight, and the octanol/water partition coefficient or the octanol/buffer partition coefficient. The differences between the permeation of atropine base and atropine sulphate might be explained by differences in solubility and pH of the donor solution. The use of pig skin which had been frozen and stored for 2 months at -80°C, resulted in a higher permeability without any lag time indicating a loss of skin viability. Therefore only fresh skin should be used.

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