International Journal of Pharmaceutics, 81 (1992) 89–99

IJP 02693

Physical model approach to understanding finite dose transport and uptake of hydrocortisone in hairless guinea-pig skin

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(Received 22 March 1991)
(Modified version received 1 November 1991)
(Accepted 4 November 1991)

Key words: Physical model; Finite dose; Hairless guinea-pig skin; Hydrocortisone uptake; Hydrocortisone transport

Summary

For the purpose of obtaining a mechanistic understanding of drug transport through skin in the finite dose case, the permeation of radiolabeled hydrocortisone (^{3}H-HC) through hairless guinea-pig skin and layerwise retention in the skin have been studied using Franz diffusion cells. Steady-state permeation and retention were investigated for the infinite dose case with full-thickness and stripped skin to obtain basic parameter values for establishing a model. A 3 μl volume of the solution in Miglyol 840 as a vehicle was applied per cm^2 of diffusion area in the finite dose case. ^{3}H-HC retention as a function of time in the epidermal membrane and dermis was determined by separating full-thickness skin into the two layers using a microwave irradiation technique; then each layer was analyzed after digestion with sodium hydroxide. A physical model approach was developed to predict the drug retention profiles for the finite dose case using the parameters (diffusion coefficient, partition coefficient, and thickness of the membranes) obtained from independent experiments (infinite dose experiments). Satisfactory agreement was found between the prediction of the model for the finite dose case and the experimental results (drug concentrations in the epidermal membrane and dermis and the flux into the receiver chamber).

Introduction

There have been a number of publications recently (Franz, 1975, 1978; Nakagawa et al., 1976; Fox et al., 1979; Hadgraft, 1980; Okamoto et al., 1989; Addicks et al., 1989, 1990; Kubota and Yamada, 1990) addressing the 'finite dose' situation in topical drug therapy. The finite dose situation differs from the infinite dose problem (e.g., transdermal drug delivery) in that transient effects (non-stationary state effects) may be much more important and may, in fact, dominate the bioavailability issues. The understanding of the problem of drug permeation, uptake and target-site delivery in the finite dose situation is therefore much more complicated and new strategies need to be considered. The objective of the present research was to explore a quantitative ap-

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proach for modelling drug permeation and retention in the skin for the finite dose case. The physical model considers the skin to be composed of three layers with different physiological and physicochemical properties.

In the present study, a model case has been examined with the goal of attempting to explain and/or to describe drug (hydrocortisone) uptake and transport from a finite dose applied to the skin surface (hairless guinea-pig skin) in in vitro experiments. The principal strategy involves (a) experimentally determining the drug uptake vs time in the epidermal membrane and in the dermis, (b) determining the drug appearance in the receiver chamber with time, and (c) attempting to describe this behavior via a physical diffusion model. The input parameters in this model were the diffusion coefficients and the partition coefficients (for the stratum corneum, epidermis, and dermis) and thickness of the stratum corneum, the viable epidermis, and the dermis. The diffusion coefficient of each membrane component was calculated from the partition coefficient and the permeability coefficient; the former was determined directly with the individual membrane component and the latter was evaluated via steady-state permeation experiments with full-thickness skin, stripped skin, and dermis. The thicknesses of the stratum corneum and the viable epidermis were estimated by weighing the epidermal membrane before and after digestion with trypsin. Drug solubility experiments were conducted to assess relative thermodynamic activities.

Materials and Methods

Animal

A female hairless guinea pig (4–8 weeks old, Charles River, Wilmington, MA) was anesthetized by sodium pentobarbital injection and killed by a saturated potassium chloride injection. Six pieces of skin were obtained from the abdominal region and freed from fat and other debris.

Drug

[^{3}H]Hydrocortisone (40–60 Ci/mmol) was obtained in ethanol solution from New England Nuclear Products (Boston, MA) with a radiochemical purity of 99%. The ethanol was evaporated with the aid of a nitrogen stream and then the radiolabeled drug (^{3}H-HC) was reconstituted into the vehicle employed in the experiment.

Permeability experiments

Permeability experiments were carried out using the Franz diffusion cell with an effective diffusional area of approx. 1.0 cm^2. The skin membrane was mounted on the diffusion cell with the dermis side facing the receptor chamber. ^{3}H-HC in Miglyol 840 (Dynamit Nobel of America, Kay-Fries, Inc., Stony Point, NY) was added into the donor chamber using 1.0 ml (for the infinite dose case) or 3.0 μl (for the finite dose case) and spread evenly onto the skin with the help of a preweighed glass-rod. Saline containing 0.01% gentamicin sulfate was the vehicle for the receiver chamber (5–6 ml) maintained at 37 °C and magnetically stirred at 300 rpm. At predetermined time intervals, 1 ml aliquots were taken from the receiver chamber and mixed with 10 ml of scintillation fluid (Opti-Fluor, Packard Instrument Company, Inc., Meriden, CT) and analyzed for total radioactivity using a liquid scintillation counter (Model LS 7500, Beckman Instruments, Inc., Redmond, WA). The same volume of saline solution was added back to the receiver chamber to keep a constant volume. The initial donor concentration was determined from the ^{3}H-HC concentration in the stock solution by assaying 3 μl aliquots. Permeability experiments were conducted with both full-thickness skin and stripped skin (tape stripping was repeated 25–30 times with 3M Scotch tape on the abdominal skin).

Drug uptake and retention experiments

These were started as in the permeability experiments (above), but at predetermined times the experiment was terminated and the uptake and retention of ^{3}H-HC in the epidermal membrane and dermis measured in the following way. For the finite dose experiments, the donor side surface of the skin membrane was then immedi-

ately wiped several times with pieces of soft tissue paper which were then set aside for later analysis of total radioactivity. As quickly as possible, the diffusional area of the skin membrane was cut out with a pair of scissors, the skin sample was then exposed to microwave energy for 6–10 s (Microwave Oven, Sharp Carousel II, Model R-1M50), and the epidermal membrane was peeled off the dermis layer with forceps (Kumar et al., 1989). Both layers were weighed and assayed for ^{3}H-HC after digestion with 0.3 N sodium hydroxide at 60 °C for 10–12 h.

For the infinite dose experiments, the donor phase was pipetted out of the donor chamber and the fluid adhering to the skin membrane surface was wiped from the surface with soft tissue paper several times. The remainder of the procedure was the same as that for the finite dose experiment.

Partition coefficient determinations

A portion of skin of known weight was equilibrated with ^{3}H-HC solution in Miglyol (1 ml) for 10 h at 37 °C and blotted on tissue paper to remove the adhering liquid. The epidermal membrane was separated from the dermis by microwave radiation; then both the epidermal membrane and the dermis were weighed and analyzed for ^{3}H-HC after treatment with sodium hydroxide, and the partition coefficients for the two membranes calculated using Eqn 1.

$$K_{app} = [(\text{radioactivity in skin membrane}) / (\text{weight of skin membrane})] / [(\text{radioactivity in vehicle}) / (\text{volume of vehicle})] \quad (1)$$

The partition coefficient for the stratum corneum was similarly determined after removal of the viable epidermis by digestion of the epidermal membrane in the following manner. Full-thickness skin was first treated with microwave irradiation and the epidermal membrane was removed as described above, then treated with a trypsin solution by placing the epidermis side down on a filter paper saturated with 0.1% trypsin solution in phosphate buffer at pH 8.0 for an overnight period.

TABLE 1

Effect of epidermal membrane separation technique and the possibility of hydrocortisone migration on the skin / saline partition coefficients

Technique	Partition coefficient			
	Epidermal membrane		Dermis	
Separation by microwave irradiation				
After Equilibration	3.4	3.0	2.1	1.8
Before Equilibration	3.0	3.1	1.8	1.4
Separation by hydration	3.0	–	2.1	–

Evaluation of the microwave technique

To rule out the possibility of significant HC migration during microwave treatment, the following test was conducted. The epidermal membrane and dermis were first separated by microwave radiation, then each layer was weighed, equilibrated with HC solution, analyzed for HC content, and K_{app} calculated as described above. These results were found to be similar to those obtained when the order of equilibration with the HC solution and microwave irradiation was reversed as shown in Table 1. We were also able to separate the epidermal membrane from the dermis without microwave application after 24 h soaking in saline (hydration). The K_{app} values obtained with these membranes were found to be comparable to those determined after microwave separation. It is evident from Table 1 that the microwave technique is suitable for determining drug distribution during evaluation of topical/transdermal drug delivery.

Theoretical

The rate of change in concentration with time may be treated using Fick's second law

$$\frac{\partial C}{\partial t} = D\frac{\partial^2 C}{\partial x^2} \quad (2)$$

where C is the drug concentration, D denotes the diffusion coefficient, x is position, and t represents time. The complexities involved in finding an exact solution to Eqn 2 were overcome in our calculations by taking a numerical approach. The skin is considered to be a three-layer membrane (see Fig. 1) consisting of stratum corneum, viable epidermis and dermis, and each layer is divided into N hypothetical thin elements.

For the general situation, the drug concentration change in an element n (sandwiched between elements $n-1$ and $n+1$) in one of the skin layers (i.e., the stratum corneum, the viable epidermis, or the dermis) is given by Eqn 3:

$$\frac{(V_n \cdot \Delta C_n)}{t} = \frac{\Delta A_n}{t} = J_{n-1,n} - J_{n,n+1} \tag{3}$$

where V_n is the volume of element n, C_n represents the concentration in element n, t is time, A_n corresponds to the amount of drug in element n, and $J_{n-1,n}$ and $J_{n,n+1}$ are the fluxes between elements $n-1$ and n and between n and $n+1$, respectively. If we are within one skin layer, then the properties of all the elements within that layer are assumed to be the same and $D_{n-1} = D_n = D_{n+1}$; $K_{n-1} = K_n = K_{n+1}$; $h_{n-1} = h_n = h_{n+1}$. For this situation we have:

$$J_{n-1,n} = \frac{D}{h}(C_{n-1} - C_n) \tag{4}$$

where D is the diffusion coefficient, K represents the (donor solution-to-skin) partition coefficient, and h is the element thickness for that layer. When we are at the interface of two skin layers (e.g., between the stratum corneum and the viable epidermis or between the viable epidermis and dermis) the interfacial flux between these two layers (say, layers A and B) is given by $J_{A,B}$

$$J_{A,B} = \frac{2(C_{A,N} - C_{B,1}/K_r)}{\frac{h_A}{D_A} + \frac{h_B}{(D_B \cdot K_r)}} \tag{5}$$

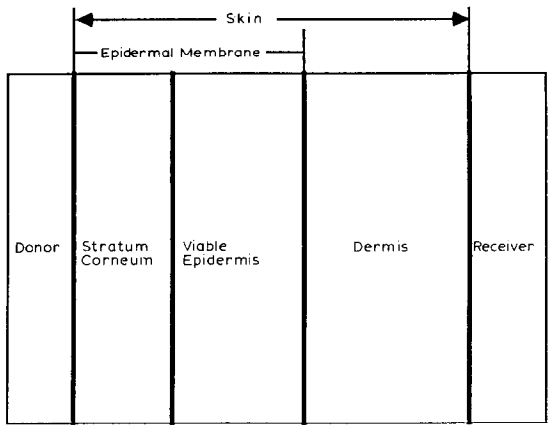

Fig. 1. Basic construction of skin layers used in the model calculations. Each component of the skin is divided into N hypothetical thin elements.

where $C_{A,N}$ is the drug concentration in the last element ($n = N$) of layer A, $C_{B,1}$ denotes the drug concentration in the first element of layer B, and $K_r = K_B/K_A$ where K_A and K_B are the partition coefficients for the elements in layer A and layer B.

For a typical calculation, at zero time, the drug concentrations are assumed to be zero in all skin layers and in the receiver solution, and an appropriate concentration is assigned to the donor phase. The concentration changes for each element in all three layers and the donor and receiver phases may then be calculated iteratively from time 0 to 12 h by choosing sufficiently small Δt values. The donor phase and the receiver phase are assumed to be well-mixed compartments. Calculations were carried out employing different values for N (the total number of elements in a layer); for values greater than $N = 4$, the outcomes were essentially the same.

Results and Discussion

Input data required for the physical model analysis

All the input parameters required for the physical model analysis were determined experimentally and are summarized in Table 2. The permeability coefficients for full-thickness skin and stripped skin were determined at steady-state for

the infinite dose situation using 1 ml of ^{3}H-HC in Miglyol as the donor solution. The permeability coefficient of the stratum corneum (P_{sc}) was calculated from the P values for full-thickness skin (P_T) and stripped skin (P_{ss}) using Eqn 6:

$$1/P_T = 1/P_{sc} + 1/P_{ss} \quad (6)$$

The permeability coefficient of the viable epidermis (P_{ve}) was calculated using the equation:

$$P_{ve} = \frac{K_{ve} \cdot D_{dermis}}{h_{ve}} \quad (7)$$

assuming that the diffusion coefficient in the viable epidermis (D_{ve}) is the same as that for the dermis ($D_{dermis} = D_{ve}$). The permeability coefficient of the dermis (P_{dermis}) was assumed to be essentially the same as the stripped skin value (P_{ss}) but corrected for the thickness difference as follows

$$P_{dermis} = P_{ss}(h_{SS}/h_{dermis}) \quad (8)$$

where h_{ss} is the thickness of stripped skin and h_{dermis} is the thickness of dermis.

The partition coefficients of the stratum corneum (K_{sc}), epidermal membrane (K_{ep}), der-

TABLE 2

Input data used for the physical model analysis

Hairless guinea-pig skin	Permeability coefficient, P (cm/s)($\times 10^7$)	Partition coefficient, K	Thickness, h (μm)	Diffusion coefficient, D[j] (cm^2/s)
Full-thickness	3.0 ± 2.0 (n = 12) [a]	–	720.9 ± 120 [g] (n = 12)	–
Stratum corneum	3.1 ± 2.1 (n = 12) [b]	3.15 ± 0.51 [e] (n = 10)	13.4 ± 5.9 [h] (n = 12)	8.38×10^{-11}
Viable epidermis	1010 [c]	2.7 [f]	73.0 ± 18.6 [i] (n = 12)	2.85×10^{-7}
Epidermal membrane	–	2.8 ± 0.34 [e] (n = 12)	85.7 ± 27.5 [h] (n = 12)	–
Dermis	82.5 [d]	1.8 ± 0.16 [e] (n = 12)	635.2 ± 113 [h] (n = 12)	2.85×10^{-7}
Stripped skin	73.5 ± 14.9 (n = 6) [a]	1.6 ± 0.2 [e] (n = 6)	–	–

[a] Directly determined experimentally from infinite dose experiment (1 ml Miglyol in donor chamber; 5.5 ml saline in receiver chamber).

[b] Calculated from P values for full-thickness skin and stripped skin.

[c] Calculated value assuming the diffusion coefficient in the viable epidermis is the same as that for dermis.

[d] Assumed to be essentially the same as stripped skin value but corrected for thickness difference.

[e] Directly determined experimental partition coefficient (membrane-to-Miglyol).

[f] Calculated from partition coefficient values of stratum corneum and epidermal membrane using the following equation

$$K_{ve} = (K_{ep} - r_{sc} \cdot K_{sc})/(1 - r_{sc})$$

where

K_{ep} = partition coefficient of epidermal membrane, K_{ve} = partition coefficient of viable epidermis, K_{sc} = partition coefficient of stratum corneum and r_{sc} = volume ratio of stratum corneum to epidermal membrane.

[g] Directly measured by micrometer.

[h] Thickness was estimated by weighing (assuming specific gravity = 1 for the stratum corneum, epidermal membrane and dermis).

[i] Obtained from the difference in thicknesses of epidermal membrane and stratum corneum.

[j] Diffusion coefficient values were calculated using $D_0 = P \cdot h/K_0$ (h values used in the calculation depend on the thickness ratio of stratum corneum-to-viable epidermis under consideration).

mis (K_{dermis}) and stripped skin (K_{ss}) were determined experimentally. The partition coefficient of the viable epidermis (K_{ve}) was calculated from the partition coefficient values of the stratum corneum and the epidermal membrane using the following equation

$$K_{ve} = (K_{ep} - r_{sc} \cdot K_{sc})/(1 - r_{sc}) \quad (9)$$

where r_{sc} is the stratum corneum-to-epidermal membrane volume ratio.

The thickness (h_T) of full-thickness skin was directly measured by a micrometer. The thicknesses of the stratum corneum (h_{sc}), epidermal membrane (h_{ep}) and dermis (h_{dermis}) were estimated by weighing a known area of the respective membranes and assuming their specific gravity equals unity. The thickness of the viable epidermis (h_{ve}) was obtained from the difference in the thickness of the epidermal membrane and the stratum corneum.

The parameters described in the preceding section were then used to calculate the diffusion coefficients for the three layers (stratum corneum, viable epidermis and dermis) using the relationship

$$D_i = (P_i \cdot h_i)/K_i \quad (10)$$

where i represents the layer (stratum corneum; viable epidermis; dermis) under consideration.

Analysis of the infinite dose case

The experimental results for the infinite dose studies are presented in Fig. 2 and compared with a 'best-fit' theoretical calculation based on parameter values given in Table 2. We consider the fit of the experimental results to the theoreti-

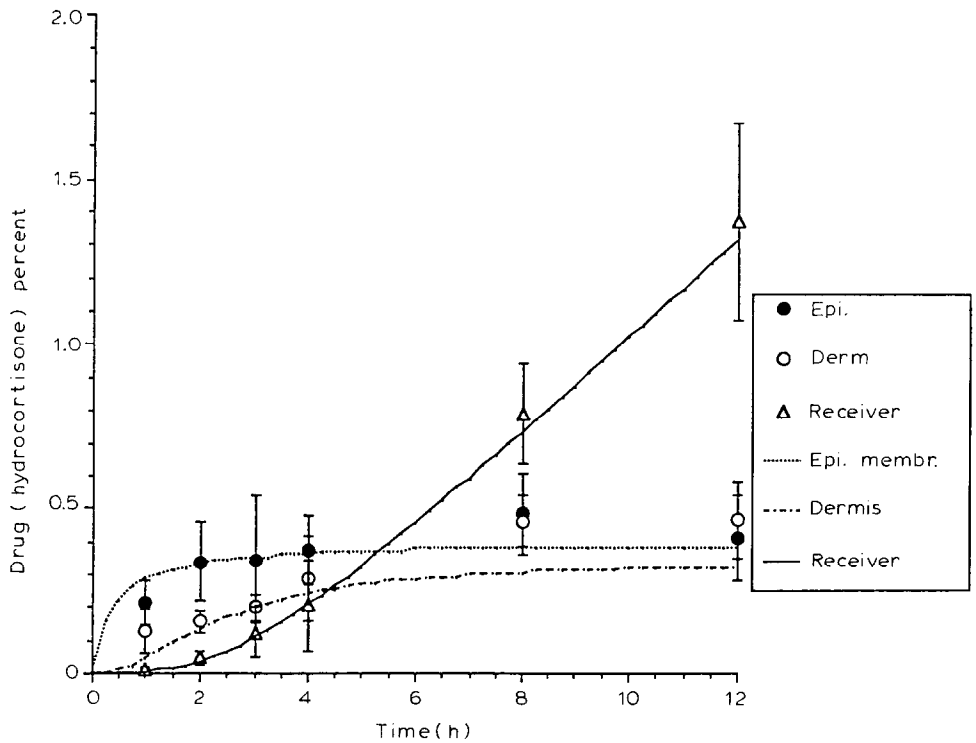

Fig. 2. Infinite dose case. Comparison of experimental results (symbols; $n = 4$; 1.0 ml hydrocortisone solution in Miglyol is the donor phase; 5.5 ml saline is the receiver phase) with the theoretically calculated values (curves). Parameter values used in the theoretical calculations: P value for the stratum corneum = 4.5×10^{-7} cm/s; thickness ratio (stratum corneum-to-viable epidermis) = 1 : 4; all other values taken from Table 2.

cal calculations to be good considering that, except for the full-thickness skin P value, all of the input parameters in Table 2 were obtained independently of the infinite dose experiments with full-thickness skin. Although the parameter values in Table 2 all exhibited relatively large variations, the P value for full-thickness skin (and therefore the P value for the stratum corneum) and the thickness (h_{sc}) for the stratum corneum showed the largest percent variations. A P value for the stratum corneum of 4.5×10^{-7} cm/s and a thickness ratio of 1:4 (stratum corneum-to-viable epidermis) were used in the calculations presented in Fig. 2. As can be seen from Table 2, these two values are within the range found from independent experiments. Other calculations (not reported here) show that P values for the stratum corneum from around 3×10^{-7} to 6×10^{-7} cm/s would give reasonably satisfactory fits of the theory to experimental results when the other parameter values are kept the same as those used in Fig. 2.

Fig. 3 shows the effect of changing the thickness ratio from 1:4 to 1:9, keeping the other parameter values the same as in Fig. 2. It is seen that the fit between the experimental results and the theoretical calculations is still rather good; however, the deviations of the experiment from theory for the epidermal membrane are somewhat greater here than for the case of the 1:4 thickness ratio (Fig. 2). Note that, from Table 2, both the 1:4 and 1:9 thickness ratios would be in the range of the experimentally estimated thickness ratios.

Analysis of the finite dose case

A comparison of the experimental results for the finite dose experiments with a best-fit theoretical calculation is presented in Fig. 4. A P value of 1×10^{-7} cm/s for the stratum corneum

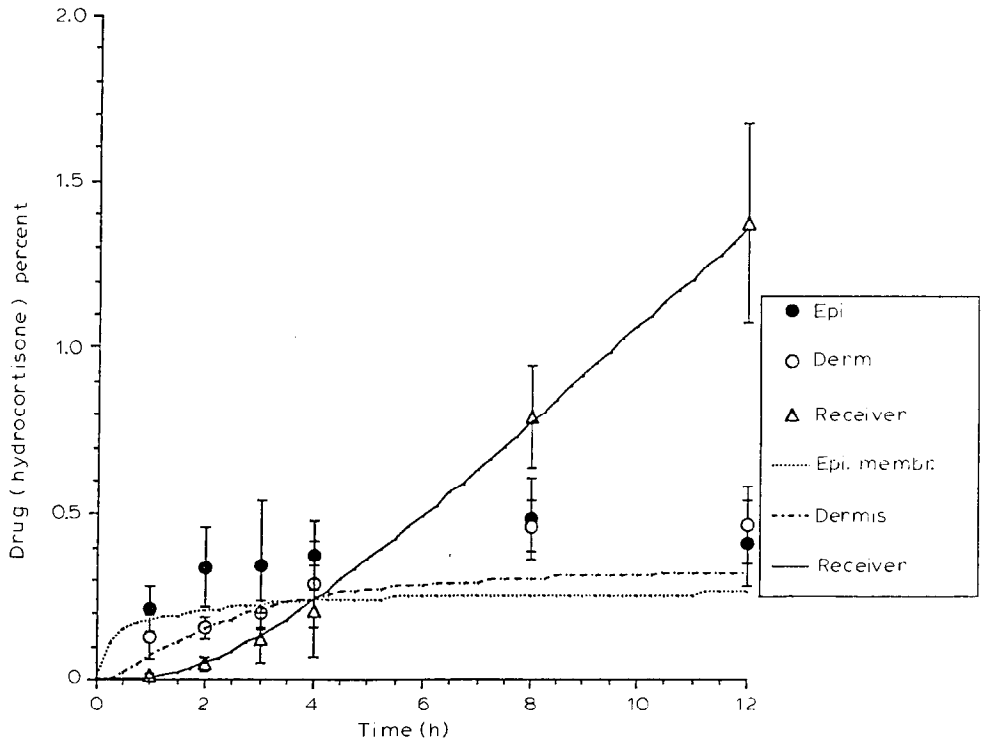

Fig. 3. Infinite dose case. Comparison of experimental results (symbols; $n = 4$; 1.0 ml hydrocortisone solution in Miglyol is the donor phase; 5.5 ml saline is the receiver phase) with the theoretically calculated values (curves). Parameter values used in the theoretical calculations: P value for the stratum corneum = 4.5×10^{-7} cm/s; thickness ratio (stratum corneum-to-viable epidermis) = 1:9; all other values taken from Table 2.

and a thickness ratio of 1:9 (stratum corneum-to-viable epidermis) were used in these theoretical calculations. All of the other parameters were the same as those given in Table 2 and which were used in the infinite dose calculations (Figs 2 and 3). Both the P value (1×10^{-7} cm/s) and the thickness ratio (1:9) lie within the range of values given in Table 2, and, therefore, good agreement between experiment and theory (Fig. 4) may be acknowledged. It does appear, however, that the 'best-fit' stratum corneum P value of around 1×10^{-7} cm/s for the finite dose case (Fig. 4) is 2–3 times smaller than the best-fit stratum corneum P value for the infinite dose case. It also appears that the thickness ratio of 1:4 gives a somewhat poorer fit between theory and experiment for the finite dose case (see Fig. 5) while the fit was quite good for the infinite dose case using this value for the thickness ratio; it was found that a thickness ratio of around 1:12 (stratum corneum-to-viable epidermis) gives an even better fit between experiment and theory for the finite dose case (as shown in Fig. 6).

An assessment of the present approach

The above analysis has demonstrated that the drug transport and uptake in the finite dose case for hydrocortisone in Miglyol (as the vehicle) may be roughly predicted by the same parameter values as obtained for the infinite dose case. It was found, however, that a lower P value for the stratum corneum of 1×10^{-7} cm/s (vs 4.5×10^{-7} cm/s for the infinite dose case) and a smaller thickness ratio of 1:12 (vs 1:4 for the infinite dose case) were needed along with the other parameter values (Table 2) for obtaining close

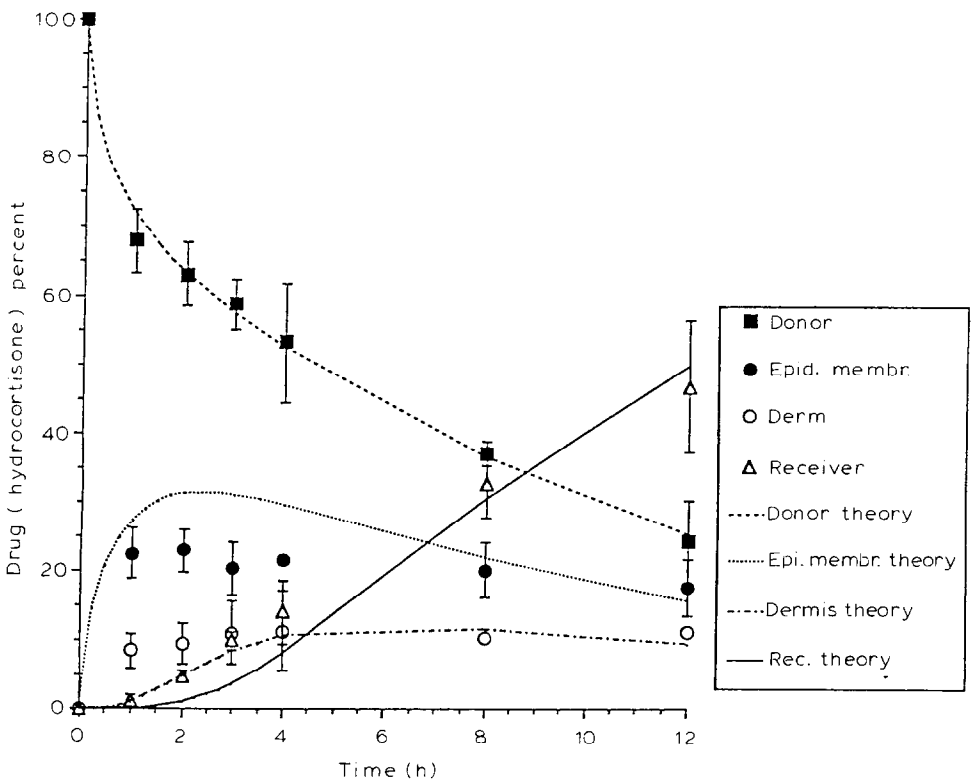

Fig. 4. Finite dose case. Comparison of experimental results (symbols; $n = 4$; 3.0 μl hydrocortisone solution in Miglyol is the donor phase; 5.5 ml saline is the receiver phase) with the theoretically calculated values (curves). Parameter values used in the theoretical calculations: P value for the stratum corneum = 1.0×10^{-7} cm/s; thickness ratio (stratum corneum-to-viable epidermis) = 1:9; all other values taken from Table 2.

agreement between experiment and theory. Using a P value of 4.5×10^{-7} cm/s for the stratum corneum generally gave somewhat poorer fits between theoretical calculations and the finite dose experiments.

It is believed that an important difference between the infinite dose and finite dose experiments reported here may be the small volume (3 μl) of the vehicle (Miglyol) used in the finite dose experiments (vs 1.0 ml used in the infinite dose experiments). Other experiments (not reported here) show that, under infinite dose conditions, Miglyol may act as a modest enhancer (4–8-fold) for hydrocortisone transport across guinea-pig skin stratum corneum compared to saline. It is possible that, in the finite dose situation, because the volume of Miglyol is very small, the effective stratum corneum diffusion coefficient may be smaller than for the infinite dose case, this situation arising as the result of either insufficient Miglyol, to begin with, interacting with the stratum corneum or the limited amount of Miglyol diffusing out of the stratum corneum and into the viable epidermis and dermis during experimental times.

There is an aspect of the theoretical calculation that calls for some discussion. Both Eqns 7 and 8 imply that the properties of the viable epidermis may be approximated by those for the dermis. In the present study, because independent data for the dermis were not obtainable, the dermis was approximated by the data for stripped skin, corrected for thickness (Eqn 8), and the viable epidermis was assumed to be an extension of the dermis (Eqn 7). It is suggested that, for the present situation, this approximation is reasonable: within experimental variability in hairless mouse skin studies (Inamori et al., unpublished data), the P values of many permeants are comparable for the dermis and for stripped skin and

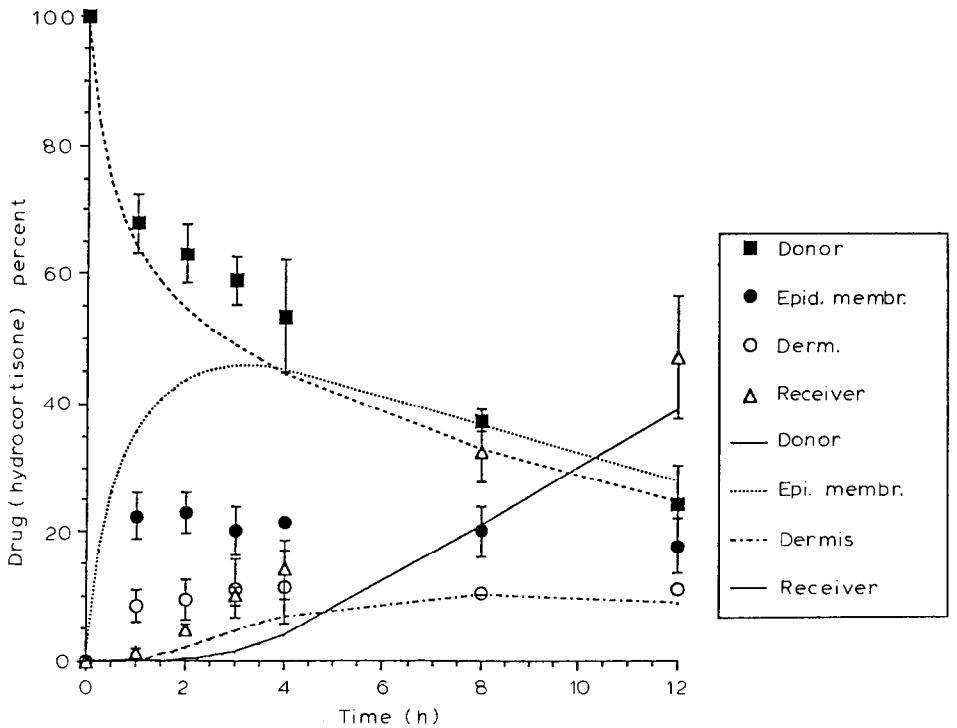

Fig. 6. Finite dose case. Comparison of experimental results (symbols; $n = 4$; 3.0 μl hydrocortisone solution in Miglyol is the donor phase; 5.5 ml saline is the receiver phase) with the theoretically calculated values (curves). Parameter values used in the theoretical calculations: P value for the stratum corneum = 1.0×10^{-7} cm/s; thickness ratio (stratum corneum-to-viable epidermis) = 1 : 12; all other values taken from Table 2.

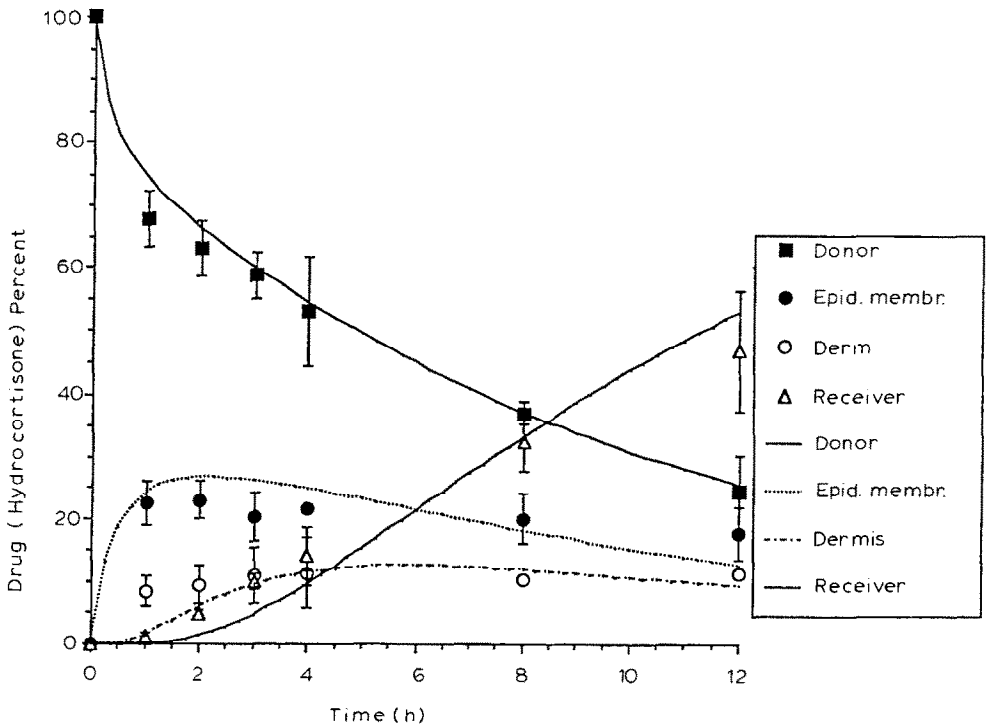

Fig. 6. Finite dose case. Comparison of experimental results (symbols; $n = 4$; 3.0 μl hydrocortisone solution in Miglyol is the donor phase; 5.5 ml saline is the receiver phase) with the theoretically calculated values (curves). Parameter values used in the theoretical calculations: P value for the stratum corneum = 1.0×10^{-7} cm/s; thickness ratio (stratum corneum-to-viable epidermis) = 1 : 12; all other values taken from Table 2.

the P value of 8.3×10^{-6} cm/s for hairless guinea-pig dermis (Table 2) is in the range for hairless mouse dermis P values when corrected for thickness. Also, the theoretical drug percent in the dermis is very sensitive to the P value used in the calculations; the good agreement between experiment (dermis) and theory (dermis) in Figs 4–6 thus supports the choice of $P = 8.3 \times 10^{-6}$ cm/s.

A final matter of concern in applying the physical model approach for the experimental data presented here for both the infinite dose and the finite dose cases is that the donor phase and the receiver phase are not the same. A rigorous theo-

TABLE 3

Activity coefficient ratio (R) for hydrocortisone in Miglyol and in saline

	Miglyol	Saline	R
Partition coefficient data [a]			
Epidermal membrane	2.8 $\pm$ 0.34 ($n = 12$)	2.7 $\pm$ 0.29 ($n = 12$)	1.04
Dermis	1.8 $\pm$ 0.16 ($n = 12$)	2.1 $\pm$ 0.07 ($n = 12$)	0.86
Stripped skin	1.6 $\pm$ 0.2 ($n = 6$)	1.8 $\pm$ 0.30 ($n = 6$)	0.89
Solubility data [b] (mg/ml)	0.460 $\pm$ 0.008 ($n = 6$)	0.369 $\pm$ 0.003 ($n = 6$)	1.25

[a] Membrane-to-Miglyol (or saline) partition coefficient determined by shaking for 10 h.

[b] Obtained by shaking excess hydrocortisone for 72 h, centrifugation and HPLC assay.

retical treatment of the problem in which there is a gradient of the solvent across the stratum corneum is extremely complicated (Liu, 1989). For the theoretical calculations presented here, all the partition coefficients (Table 2) employed in the calculations are based upon Miglyol as the solvent, even though it is likely, if not certain, that the solvent phase interacting with the dermis (in the present experiments) is saline. Fortunately, the activity coefficient for hydrocortisone in Miglyol and that for hydrocortisone in saline are nearly the same (see Table 3). Therefore, it is believed that the calculations carried out using the Table 2 parameter values should apply to the present case.

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