Effects of holes and sample removal on diffusion through excised *stratum corneum* membranes

A. Göpferich and G. Lee
Institute for Pharmaceutical Technology and Biopharmaceutics, Heidelberg University

The effects of two sources of experimental error on measurements of drug permeation through excised human stratum corneum *have been considered, namely the presence of holes in the membrane and the removal of samples from the system for analysis. The influence of holes introduced in skin membranes during their preparation was simulated using a numerical solution to Fick's Second Law. This showed quantitatively the errors in flux and lag time which can occur. Experimental measurements of the* in vitro *diffusion of the drug clenbuterol through perforated* stratum corneum *membranes corroborated these theoretical findings. Fluorescence-marked dextran of molecular weight 60,000 could be used to identify the presence of holes during permeation experiments. These macromolecular substances are water-soluble and do not penetrate through intact* stratum corneum. *The error introduced by the removal of samples from the system was also simulated. Although small for diffusion through* stratum corneum *by virtue of the low diffusivities typically found for this membrane, the error could be substantial for synthetic membranes where diffusivities are greater.*

Keywords : Stratum corneum - *Holes - Permeation - Dextrans.*

Les effets de deux sources d'erreurs expérimentales sur la mesure de l'absorption des principes actifs à travers du stratum corneum *humain excisé sont étudiés : il s'agit de la présence de perforations dans la membrane et du prélèvement d'échantillons pour analyse à partir du système. L'influence des perforations faites au cours de la préparation des membranes de peau a été simulée par l'emploi d'une résolution numérique de la seconde loi de Fick. Ceci met en évidence les erreurs de flux et de temps de latence qui peuvent survenir. Les déterminations expérimentales de diffusion* in vitro *de clenbutérol à travers des membranes de* stratum corneum *perforées corroborent ces résultats théoriques. La fluorescence de dextran de poids moléculaire 60.000 peut être utilisée pour mettre en évidence la présence de perforations au cours d'expériences de perméation. Ces substances macro-moléculaires sont solubles dans l'eau et ne pénètrent pas au travers d'un* stratum corneum *intact. Les erreurs induites par le prélèvement d'échantillons à partir du système ont aussi été simulées. Bien que faibles pour la diffusion à travers le* stratum corneum *du fait de la faible diffusivité constatée pour ces membranes, elles peuvent être appréciables pour des membranes synthétiques dont la diffusivité est beaucoup plus grande.*

Mots clefs : Stratum corneum - *Perforations - Perméation - Dextrans.*

The percutaneous route of drug delivery can be investigated quantitatively using *in vitro* techniques with excised skin samples taken from man or animals. The excised skin is attached within some sort of diffusion cell (of which numerous designs are available [1]) such that it contacts the drug preparation on its outer side and an acceptor medium on its inner side. The permeation of the drug through the skin is then characterized directly from the rate of appearance of the drug in the acceptor medium. The relevance of such *in vitro* data is assured provided the permeation of the drug through the outermost layer of the skin, the *stratum corneum*, is the rate-limiting step for the *in vivo* process of percutaneous absorption [2]. Care must, however, be taken with the preparation and use of the excised skin if the *in vitro* studies are to yield reliable results. Starting from the original excised skin tissue, one proceeds with the removal of the subcutaneous fat to leave so-called « whole skin », comprising the *stratum corneum*, the viable epidermis, and a part of the dermis. The two underlying layers

of this preparation can be partially removed by heat treatment at 60°C to yield a membrane *ca.* 100 µm thick composed of the *stratum corneum* plus attached epidermis (SCE) [3]. This can then be treated with trypsin, which digests the viable epidermis leaving a membrane of isolated *stratum corneum* (SC) [4]. Studies have shown that both SCE and SC so prepared act as discriminating barriers to drug permeation [5]. Of critical importance, however, is the integrity of the membrane, since holes can easily be introduced unnoticed during the delicate preparation process. From our experience, holes larger than *ca.* 0.2 mm in diameter can certainly be detected with the naked eye. Smaller holes can be identified microscopically, but this is time-consuming and moreover not an absolute guarantee of an intact membrane. The magnitude of the errors arising in measured flux and lag time due to the presence of small holes is, however, unclear.

As part of a study of the permeation of the drug clenbuterol across excised human *stratum corneum*, we examined the error caused by the presence of holes in the membrane. A theoretical model was first constructed for non-steady state diffusion through a perforated membrane, and the effect of hole size on permeation simulated. The permeation of clenbuterol through both intact and artificially-perforated *stratum corneum* membranes was then measured experimentally, and the results obtained compared with the simulated data. We also examined the suitability of macromolecular dextrans as marker substances to test the integrity of a *stratum corneum* membrane being used for a routine *in vitro* permeation experiment.

As a further aspect of this study, we also considered the problems associated with the removal of samples from the diffusion cell. These are taken for analysis from the acceptor solution, whose volume is then maintained by adding an equivalent amount of blank buffer. The process dilutes the acceptor solution to an extent depending on the volume of the sample removed. Some published sampling procedures require the removal of large volumes, which may lead to a substantial error in the measured permeation rate. We were able to examine the magnitude of this source of error using the theoretical model developed for the first part of this study.

I. MATERIALS AND METHODS

1. Simulation of *in vitro* skin permeation

Figure 1 illustrates the theoretical model used to simulate non-steady state diffusion of a drug through a perforated membrane ; the applicable initial and boundary conditions are also given. With this model it was possible to simulate the effects of the presence of holes of any size and the removal of samples of any volume on the permeation rate. We assumed a linear diffusion with constant diffusivity, D, through a finite, isotropic membrane :

$$c(x,t)_t - D\ c(x,t)_{xx} = 0 \qquad \textbf{Eq. 1}$$

where c(x,t) is the concentration at position x after time t, and

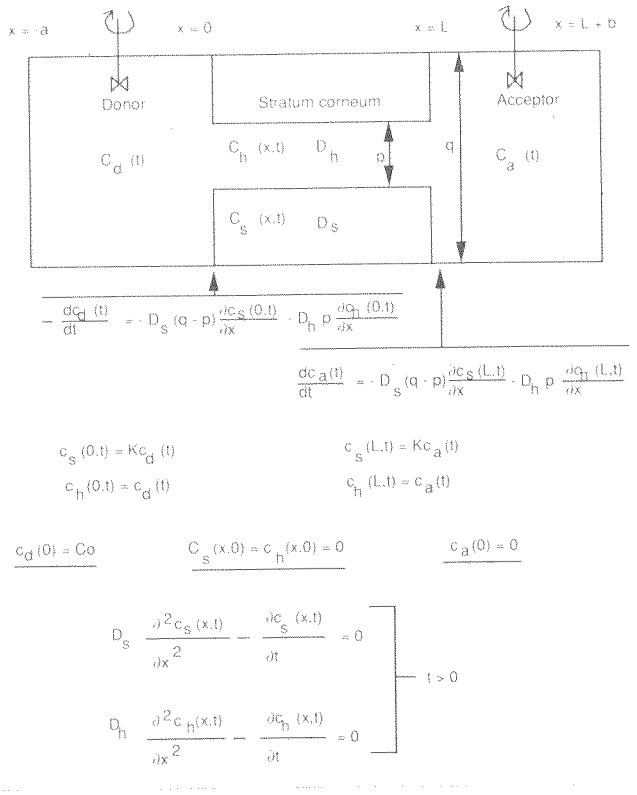
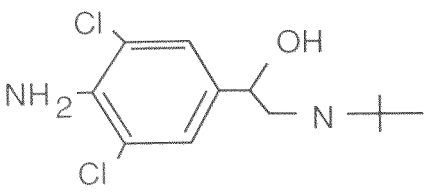


Figure 1 - Model used to simulate diffusion through a perforated *stratum corneum* membrane.

the subscripts denote partial derivatives. *Equation 1* was solved numerically using CRANK and NICOLSON's finite difference method [6]. To this end a matrix comprising *equation 1* and the initial and boundary conditions in finite difference form was solved on an Epson PC AX3 personal computer using Pascal. This yielded the theoretical mass of drug in the acceptor solution, $m_a(t)_{th}$, as a function of time for any values of diffusivity within *stratum corneum* and hole, D_s and D_h, respectively, membrane thickness, L, partition coefficient, K, and percentage hole size to membrane size, α. A smooth curve was drawn through the coordinates of $m_a(t)_{th}$ using third-degree polynomial splines.

2. Measurement of *in vitro* skin permeation

The water-soluble, basic drug clenbuterol (M.Wt. = 277 ; pKa = 9.5) was used for these studies :



Stratum corneum membranes (SC) were prepared from whole human skin excised from the inner thighs of cadavers within 48 h *post mortem* by sequential heat and trypsin treat-

ments [3, 4]. After drying to 25% RH, some of the SC samples were then artificially perforated in their geometric centres using a fine, hot needle. *Figure 2* shows a typical preparation viewed under the light microscope ; the diameter (60 µm for the preparation shown in *figure 2*) could be readily measured. The permeation of clenbuterol through both the intact and perforated SC membranes was then measured at 35 ± 0.2°C using a glass diffusion cell of standard design [7]. A pH 8 phosphate buffer solution was used in both donor and acceptor compartments, from the latter of which samples were removed at regular intervals and assayed by reverse-phase HPLC with UV detection (error ≤ 2%). The results were expressed as coordinates of the mass of drug in the acceptor solution, $m_a(t)_{exp}$, versus time, t.

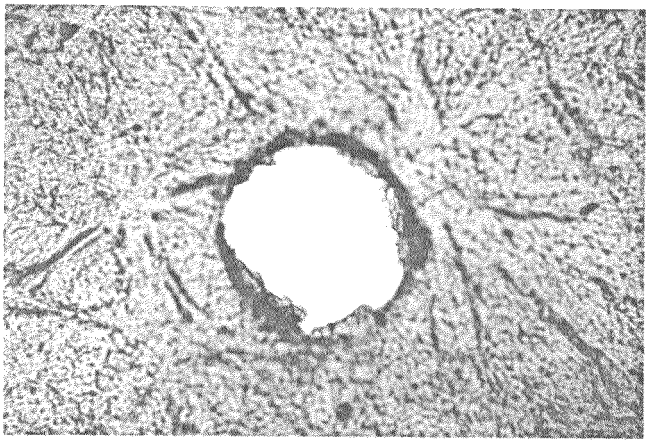


Figure 2 - Perforated *stratum corneum* preparation viewed from its outer side.

The integrity of the SC membranes was examined by using a fluorescence-marked, macromolecular dextran of molecular weight 60,000 (Sigma Chemicals, München, FRG) as marker substance. This was dissolved together with the drug in the donor solution used for the permeation experiment. The samples removed from the acceptor solution were then analyzed for drug by HPLC as before and for dextran by fluorescence spectroscopy (Kontron Analytical SFM 25 Fluorimeter). For each individual piece of perforated *stratum corneum*, a plot of $m_a(t)_{exp}$ versus t was constructed.

II. RESULTS AND DISCUSSION

For simulations of the effects of hole size on permeation rate we used the classic [8] dimensionless plots of $m_a(t)_{th}/m_\infty$ versus $D_s t/L^2$, where m_∞ is the total mass of drug in the system, D_s is the diffusivity within the *stratum corneum*, t is the time, and L the thickness of the *stratum corneum*. *Figure 3* shows such data for the simulation of diffusion through perforated SC. Each of the simulated curves shows the lag phase typical for membrane diffusion, followed by a pseudo-steady state phase which is sigmoid in shape owing to the non-sink boundary conditions existing at both sides of the membrane. The effect of the holes on the shape of these curves is quite marked for α (percentage hole size relative to membrane size) greater than 0.001%. For this simulation we assumed D_h to be 10^6 greater than D_s, as

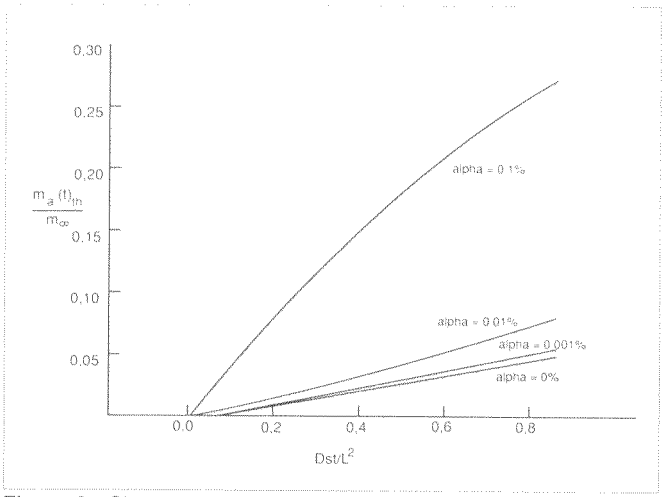


Figure 3 - Simulated curves of $m_a(t)_{th}/m_\infty$ versus $D_s t/L^2$ for increasing percentage relative hole size (α).

would be typical for drug diffusion through *stratum corneum* where diffusivities of *ca.* 10^{-11} to 10^{-12} cm² s⁻¹ are found. *Table I* shows for each of the four simulated curves the values for flux through the membrane (J, µg/cm² per h) as measured at the point of inflexion and lag time (τ, hours) determined by extrapolating back from this point to be time axis. For these calculations, we used a typical SC data of $D_s = 5 \times 10^{-12}$ cm² s⁻¹ and L = 10 µm. The increase in the flux observed is clearly proportional to the hole size. Although even the presence of a small hole (α = 0.001% is equivalent to a hole diameter of 35 µm in a 1 cm² membrane) increases the flux, its existence would probably not be obvious when examining experimental data as the associated error in the curve (*figure 3*) is quite small. The lag time is also influenced by the presence of the holes, being reduced even for the smallest value of α simulated here. Indeed, for α = 0.1%, the lag time completely vanishes. Major errors would, therefore, certainly occur were diffusivity calculated from the lag time of such data, since the presence of even a small hole substantially alters the extrapolated lag time, but may not be obvious from the shape of the curve. The wisdom of using a marker substance to check the integrity of *stratum corneum* membranes during routine permeation experiments is thus evident.

Table I - Flux (J) and extrapolated lag time (τ) for simulated diffusion of a drug through perforated *stratum corneum*.

Relative hole size α (%)	Flux J (µg cm⁻² h⁻¹)	Lag time τ (h)
0	4.80	8.16
0.001	5.36	7.66
0.01	7.14	4.70
0.1	26.5	0

The influence of hole size on the permeation of clenbuterol through the artificially-perforated SC membranes could be determined experimentally, and is shown in *figure 4* as plots of $m_a(t)_{exp}$ versus t. To maintain clarity, the curve for the intact SC membrane is not shown ; it is marginally less steep than that for the smallest hole size. These results compare well with the

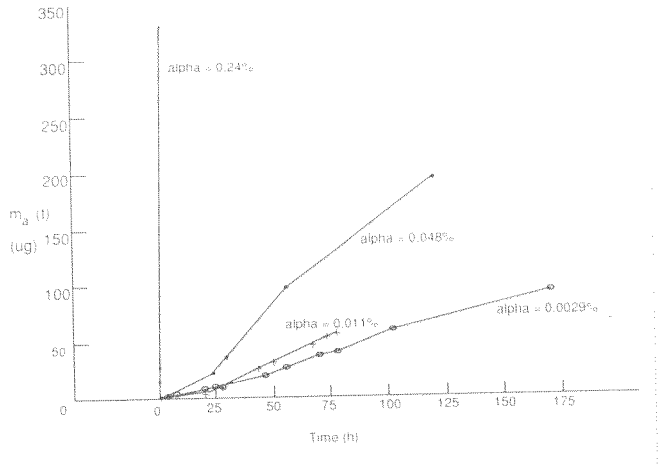


Figure 4 - Experimentally-determined influence of percentage relative hole size (α) on the diffusion of clenbuterol through perforated *stratum corneum*.

simulated profiles shown in *figure 3*, with increasing hole size leading to increase flux and reduced lag time. These effects are, however, greater in magnitude than those obtained from the simulation for comparable α values. For example, there is already no discernible lag time for $\alpha = 0.01\%$, although the equivalent curve in *figure 3* still shows an evident lag phase. In an effort to resolve this anomaly, we attempted to fit the experimental $m_a(t)_{exp}$ values to the corresponding, theoretical $m_a(t)_{th}$ values obtained from the numerical simulation using an improved simplex method, as discussed fully elsewhere [7]. This was, however, only successful for the data obtained from the intact SC membrane ($\alpha = 0$), which yielded for D_s a value of $3.39 \times 10^{-12} \pm 1.76 \times 10^{-12}$ cm^2 s^{-1} and for K a value of 205 ± 90 (n = 5) : these compare well with literature values [7]. The experimental data obtained for the perforated SC membranes could not be fitted adequately to the simulated data. The simplex residual sum of squares did not converge satisfactorily, indicating that the model does not describe adequately the process of diffusion through a perforated membrane. This is most likely due to the existence of forced convectional transport of the drug through the hole, as caused by the strong convection currents existing within the stirred acceptor fluid. This effect would then account for the greater diffusion rates and smaller lag times obtained experimentally as compared with the simulated data of corresponding α . Although this finding limits the use of the model for the evaluation of data, the model provides a useful, quantitative description of the magnitude of the errors to be expected due to the presence of holes.

The fluorescence-marked dextran proved itself to be a very effective indicator of SC membrane integrity. It has the advantage of being macromolecular, yet also water-soluble, and cannot, therefore, diffuse through intact SC membranes. Indeed, its presence could not be detected in the acceptor solution 7 days after starting a diffusion experiment. Some of the dextran molecules from the donor solution were clearly adsorbed on to the outer surface of the SC, as can be seen from the photograph in *figure 5*. They appear to aggregate preferably in the regions of the hair follicles. Despite this phenomenon, the

Figure 5 - Photograph of the external side of *stratum corneum* used for a permeation experiment showing adsorbed fluorescing dextran molecules.

diffusion of clenbuterol was not quantitatively influenced by the dextran, for which the above-mentioned fitting procedure yielded a D_s of 2.64×10^{-12} cm^2 s^{-1} and a K of 251. The dextran molecules were able to pass through perforated SC membranes, as illustrated in *figure 6* for the smallest hole size examined (α = 0.0029%), thereby clearly identifying the presence of a hole. The observed pseudo-steady state diffusion rate for the clenbuterol (0.64 µg/cm^2 per h) is marginally higher than that seen with intact SC (0.60 µg/cm^2 per h), the hole being so small in this case. The measured lag time is shorter. As before, however, it was not possible to fit these results to the equivalent simulated data, since diffusion through the hole is influenced by the convection currents existing within the acceptor solution.

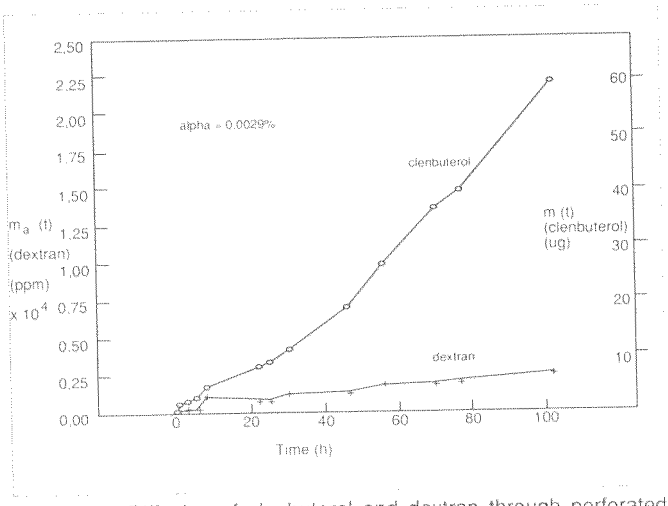


Figure 6 - Diffusion of clenbuterol and dextran through perforated *stratum corneum*.

Finally, the theoretical influence of the removal of samples from the acceptor solution on the diffusion rate is illustrated in *figure 7*. The most extreme case of the complete exchange of the acceptor solution with blank buffer every four hours is shown. The observed increases in diffusion rate occur as a result of the sudden increases in the concentration gradient across the membrane associated with sampling. *Figure 7* shows that this

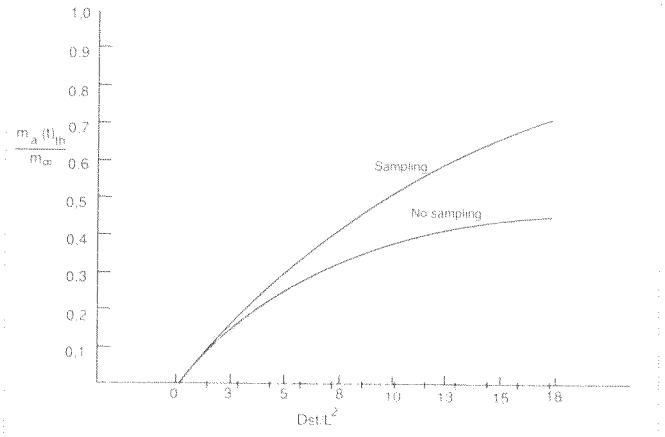


Figure 7 - Simulated curves of $m_a(t)_{th}/m_\infty$ versus $D_s t/L^2$ for the complete exchange of the acceptor phase every 4 h.

error is, however, only substantial when diffusivities are > 10^{-10} $cm^2 s^{-1}$. Although not of great magnitude for experiments with stratum corneum (where diffusivities are typically an order of magnitude smaller), care should be taken when measuring diffusion through artificial membranes, where lower diffusivities exist and the error associated with sampling can be substantial.

*

* *

In conclusion, we have shown quantitatively that the presence of holes within prepared SC membranes can lead to substantial error in measured flux and lag time. This error can go unnoticed when evaluating diffusional data and, particularly, can lead to incorrect calculations of the diffusivity within the SC. Fluorescence-marked dextrans can, however, be very effectively used to check the integrity of the SC membranes. The removal of samples from the system does not cause substantial error for diffusion through an SC membrane. ◆

REFERENCES

1. TOJO K. - Design and calibration of *in vitro* permeation apparatus. - In : Transdermal controlled systemic medications, Y. Chien, Marcel Dekker Inc., New York, 1987, pp. 127-158.
2. TOJO K. - Concentration profile in plasma after transdermal drug delivery. - Int. J. Pharm., **43**, 201-205, 1988.
3. LEE G. and PALICHARLA P. - An examination of excised skin tissues used for *in vitro* membrane permeation studies. - Pharm. Research, **3**, 356-359, 1986.
4. KLIGMAN A. and CHRISTOPHERS E. Preparation of isolated sheets of human *stratum corneum*. - Arch. Dermatol., **88**, 702-710, 1963.
5. SWARBRICK J., LEE G., BROM J. and GENSMANTEL N. - Drug permeation through human skin. II. Permeability of ionizable compounds. - J. Pharm. Sci., **73**, 1352-1355, 1984.
6. CRANK J. and NICOLSON P. - A practical method for the numerical evaluation of solutions of partial differential equations of the heat conduction type. - Proc. Camb. Phil. Soc., **43**, 50-67, 1947.
7. GÖPFERICH A. and LEE G. - Measurement of drug diffusivity in stratum corneum membranes and a polyacrylate matrix. - Int. J. Pharm., in press.
8. CRANK J. - The mathematics of diffusion. - 2nd ed., Oxford University Press, London, 1976.

ACKNOWLEDGEMENTS

We gratefully acknowledge the generous financial support of Boehringer Ingelheim KG.

AUTHORS' ADDRESS

Achim Göpferich and Geoffrey Lee, Institut fuer Pharmazeutische Technologie und Biopharmazie, INF 366, 6900 Heidelberg, Germany.
Correspondence : Prof. Geoffrey Lee.

MANUSCRIPT

Received june 26, 1990, accepted for publication october 24, 1990.