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Acyclovir permeation through rat skin: mathematical modelling and in vitro experiments

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Abstract

The aim of this work is to characterise the skin permeation properties of a male rat by means of a purely diffusive mathematical model based on Fick's second law. Additionally, in the attempt of proposing a reliable tool allowing the skin permeability (or resistance) determination on the basis of experimental data, the model automatically accounts also for two typical experimental conditions. In particular, drug dissolution in the donor environment and receiver sampling technique (part of the receiver volume is withdrawn and immediately replaced by fresh solvent) are considered. The results of this characterisation are then compared with those coming from a common simplified approach.

Acyclovir is chosen as model drug and a thermostatic (37 °C) Franz cell apparatus is used to perform permeation experiments.

This study suggests that Acyclovir permeation through the rat skin can be well described by the proposed model and that some differences arise in the evaluation of the full-skin resistance performed by means of our model or the usual simpler approach.

Keywords: Mathematical modelling; Acyclovir; Skin permeation

1. Introduction

Topical application of drugs for treatment of skin diseases or pathology has for a long time studied (Zanowiak and Jacobs, 1982) as the skin is one of the most readily accessible organs of the body. Indeed, it separates the vital organs from the outside environment and serves as a protective barrier against physical, chemical and microbial attacks. Though these good protective properties, often topical applications represent a successful drug delivery strategy and that is why mechanisms governing drug delivery from the topical or transdermal formulation have to be understood.

Skin may be considered as a multilayered membrane composed by the stratum corneum (the outermost layer), the epidermis and the dermis, each characterised by physiologically and physiochemically different properties (Ritschel and Hussain, 1988; Cevc, 1997). Stratum corneum consists of many layers of compacted hydrated and keratinized cells and it is known as the most impermeable layer to the majority of the drugs, while viable epidermis and dermis exhibit their barrier function only for lipophilic penetrants (Ritschel and Hussain, 1988).

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The aim of this work is to verify whether a purely diffusive mechanism is suitable for the description of drug transport through rat skin and to characterise both the full-thickness skin (stratum corneum, epidermis and dermis) and the one-layer skin (epidermis and dermis) by means of proper diffusion coefficients. Accordingly, a mathematical model based on Fick's second law will be built on. Moreover, in the attempt of getting a calculation tool as reliable as possible, many aspects of the entire permeation phenomenon are carefully considered so that the model contains many physical parameters that must be determined in advance by independent experiments. In particular, a critical re-examination of the permeation apparatus (Franz cells) reveals that, usually, the donor compartment contains an amount of not dissolved drug aimed to maintain the drug concentration at a constant level (saturation) during the whole permeation experiment. This assumption being true, the mathematical treatment of the experimental data is considerable simplified but, at our knowledge, the validity of this hypothesis is rarely verified. Moreover, regardless the hydrodynamic conditions imposed in the receiver compartment, the presence of a stagnant layer faced to the membrane is unavoidable. Also in this case, the negligibility of this layer is usually assumed, but seldom verified. That is why in this paper we measure the drug dissolution constant (dissolution from a powder) in the donor compartment fluid jointly with the thickness of the stagnant layer and the drug diffusion coefficient in it (this, obviously, coincides with the drug diffusion coefficient in the receiver fluid). Indeed, only if the solid dissolution rate (represented by the dissolution constant) is greater than the leaving drug flux (depending upon membrane permeability), drug concentration in the donor compartment can be assumed constant. Accordingly, another aim of this work is to establish a threshold value for the permeability/dissolution constant ratio above which the dissolution flux can not balance the permeation flux.

Moreover, the measurement of the stagnant layer thickness and the drug diffusion coefficient in it allows the estimation of the effect of this further diffusive resistance on the membrane permeability determination. Finally, the determination of the drug solubility and the drug partition coefficient in the full skin and in the dermis–epidermis layer completes the number of physical parameters required by the model. The only fitting parameters are then drug diffusion coefficient in the dermis–epidermis layer and in the stratum corneum. Nevertheless, it is worth mentioning that we firstly determine the drug diffusion coefficient in the dermis–epidermis layer resorting to proper experiments and then, on the basis of this result, the drug diffusion coefficient in the stratum corneum is calculated by model fitting on drug permeation data referred to full skin. In this manner, we always perform a one parameter fitting, being all other parameters known from independent experiments. Obviously, this strategy represents a severe test for the model and for all the hypotheses on which it has been built on. The results of our analysis are then compared with those coming from a common, simplified, approach (Chien, 1987a,b) based on the pseudo steady state approximation.

Acyclovir (9-hydroxyethoxymethyl, guanine selective antiviral agent) is employed as model drug for its large employ in this field (Dorsky and Crumpacker, 1987; Gonsho et al., 1990; Okamoto et al., 1990), while male hairless rat (5–7 weeks old) skin is considered as permeating membrane. The receiver compartment of the Franz cells apparatus is filled by phosphate buffered saline (PBS solution pH 7.4, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride), while the donor one contains an Acyclovir saturated PBS solution in presence of a not dissolved Acyclovir amount.

2. Modelling

Due to the complexity of drug permeation through skin, many attempts were performed in order to mathematically model this phenomenon (Flynn et al., 1974; Michaels et al., 1975; Chien, 1987b; Parry et al., 1990, 1992; Yamashita et al., 1990, 1993; Lieckfeldt et al., 1993; Aguilella et al., 1994; Williams and Riviere, 1995; Lee et al., 1997; Sugibayashi et al., 1999). Indeed, drug flux through the skin can, in principle, take place according to several pathways (Cevc, 1997) as, for instance, stratum corneum can be thought as an heterogeneous membrane made up by flattened, interdigitated, keratinized epidermal cells separated by a lipid phase (Michaels et al., 1975). Moreover, the presence of follicles makes the situation more complex as they can represent a preferential way for

drug permeation even if their contribution is hard to be quantified (in human skin their contribute is negligible) as follicles number per unit area is not equal for the different body parts both in humans and animals (Michaels et al., 1975; Cevc, 1997). Whether, in principle, all these aspects could be accounted for, the resulting mathematical model (the concept of parallel pathways should be recalled (Flynn et al., 1974), for instance) would be characterised by many parameters whose determination would be very difficult (if not impossible) and the result would be at least questionable. Accordingly, in order to overcome these problems, we believe that a completely diffusive mechanism can be assumed for the description of drug permeation through the skin (Michaels et al., 1975; Anissimov and Roberts, 1999), provided that the skin is thought as the sum of two homogeneous membranes (the stratum corneum and the dermis–epidermis layer (Okamoto et al., 1989; Young et al., 1994)) each one characterised by a proper effective diffusion coefficient (Peppas, 1984; Bird et al., 1960) accounting for every membrane irregularities. In other words, the effect of drug transport through follicles (and through other possible preferential channels) is embedded in the diffusion coefficient value that, for this reason, is called "effective". Furthermore, the presence of a stagnant layer faced on the dermis–epidermis layer (receiver compartment side) is considered in order to account for a possible lack of mixing (Kokubo et al., 1992; Grassi et al., 1995, 1996; Grassi and Colombo, 1999). As we also consider the presence of an amount of not dissolved drug in donor solution (directly in contact with the membrane), the existence of a possible concentration gradient on the donor side (that would be the effect of the stagnant layer on the donor side) is not considered (see Fig. 1).

The model requires the solution, in one dimension, of Fick's second law inside the trilaminate made up by stratum corneum, dermis–epidermis and stagnant layer, provided that the pertinent initial and boundary conditions are considered. Fick's second law for stratum corneum, dermis–epidermis and stagnant layer reads, respectively:

$$\frac{\partial C_{sc}}{\partial t} = \frac{\partial}{\partial X}\left(D_{sc}\frac{\partial C_{sc}}{\partial X}\right) \quad (1)$$

$$\frac{\partial C_{de}}{\partial t} = \frac{\partial}{\partial X}\left(D_{de}\frac{\partial C_{de}}{\partial X}\right) \quad (2)$$

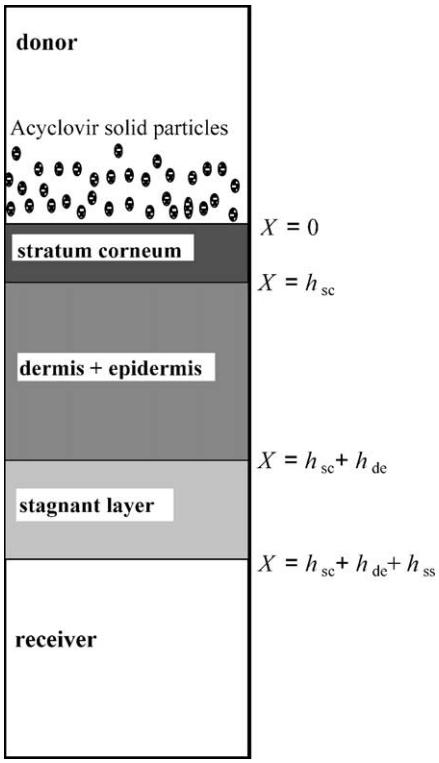

Fig. 1. Schematic representation of the skin sandwiched between the donor and receiver compartment.

$$\frac{\partial C_{ss}}{\partial t} = \frac{\partial}{\partial X}\left(D_{ss}\frac{\partial C_{ss}}{\partial X}\right) \quad (3)$$

where t is time, X is abscissa, D_{sc}, D_{de}, D_{ss}, C_{sc}, C_{de} and C_{ss} are, respectively, the drug effective diffusion coefficient (Bird et al., 1960; Peppas, 1984) and concentration in the stratum corneum, in the dermis–epidermis and in the stagnant layer. The above equations must be solved with the following boundary conditions:

$$V_d\frac{dC_d}{dt} = V_d K_t(C_s - C_d) + D_{sc}S\left.\frac{\partial C_{sc}}{\partial X}\right|_{X=0} \quad (4)$$

$$D_{sc}\left.\frac{\partial C_{sc}}{\partial X}\right|_{X=h_{sc}} = D_{de}\left.\frac{\partial C_{de}}{\partial X}\right|_{X=h_{sc}} \quad (5)$$

$$D_{de}\left.\frac{\partial C_{de}}{\partial X}\right|_{X=h_{sc}+h_{de}} = D_{ss}\left.\frac{\partial C_{ss}}{\partial X}\right|_{X=h_{sc}+h_{de}} \quad (6)$$

$$V_r \frac{dC_r}{dt} = - D_{ss} S \frac{\partial C_{ss}}{\partial X}\bigg|_{X=h_{sc}+h_{de}+h_{ss}} \quad (7)$$

$$K_{p1} = \frac{C_{sc}}{C_d}, \quad K_{p2} = \frac{C_{de}}{C_{sc}},$$

$$K_{p3} = \frac{C_{de}}{C_{ss}}, \quad K_{p4} = \frac{C_{ss}}{C_r} = 1 \quad (8)$$

and the following initial conditions:

$$C_d = C_{d0}, \quad C_r = C_{sc} = C_{de} = C_{ss} = 0,$$
$$M = M_0 \quad (9)$$

where V_d and V_r are the volume of the donor and receiver environment, respectively, K_t is the dissolution rate constant, C_d and C_r are the drug concentration in the donor and receiver compartment, respectively, C_{d0} and M_0 are the starting drug concentration in the donor compartment and the starting amount of not dissolved drug in the donor compartment, C_s is drug solubility in the fluid filling the donor compartment, S is the permeation area and K_{p1}, K_{p2}, K_{p3} and K_{p4} are the partition coefficients.

Eq. (4) represents the drug mass balance made up on the donor compartment: the first right hand side term takes into account powder dissolution, while the second represents the matter flux leaving the donor trough the stratum corneum.

Eq. (5) imposes that the matter flux leaving the stratum corneum is equal to that entering the dermis–epidermis ($X = h_{sc}$), while Eq. (6) equals the matter flux leaving the dermis–epidermis layer and that entering the stagnant layer ($X = h_{sc} + h_{de}$). Eq. (7) represents the drug mass balance made up on the receiver compartment where the right hand side term is the entering drug flux coming from the stagnant layer. While Eq. (8) expresses partitioning conditions, Eq. (9) states that, at the beginning, trilaminate and receiver are drug free, while donor compartment contains a solution characterised by a C_{d0} drug concentration and an amount M_0 of not dissolved drug.

Particular care has been devoted to the dissolution process estimation as it can heavily affect drug concentration C_d in the donor compartment (Byun et al., 1990; Kurnik and Potts, 1997; Grassi and Colombo, 1999). Whether this can be, in principle, a complex mechanism affected by several factors such as solid particles size distribution, shape and aggregation, we believe that the key point is represented by solid particles radius reduction upon dissolution. Indeed, dissolution is ruled by parameter K_t that, sometimes, may be considered time independent but, generally, it is function of time. Indeed, remembering K_t definition (Nogami et al., 1969):

$$K_t = D \frac{S_p}{h_b V_d}, \quad K_d = \frac{D}{h_b} \quad (10)$$

where D is the drug diffusion coefficient in the donor compartment fluid, h_b is the thickness of the boundary layer arising between the dissolving solid surface and the dissolution medium (h_b strongly depends on the stirring conditions as stated by Levich (1962)) and S_p is the area of the solid/liquid interface, it is evident the K_t dependency on S_p and, consequently, on time, as soon as the dissolution surface is not constant. If we suppose that the solid drug in the donor compartment can be considered as a powder made by N_p all equal spherical particles, K_t expression becomes:

$$K_t = 4\pi R^2 \frac{K_d}{V_d} \quad (11)$$

where R is the particle radius. Of course, as dissolution develops, R reduction causes a K_t decrease. In order to evaluate R reduction and, thus, K_t decrease, we resort to a drug mass balance made up on the trilaminate, the donor and receiver compartments (Grassi and Colombo, 1999):

$$M = M_0 + V_d(C_{d0} - C_d) - V_r C_r - \int_0^{h_{sc}} C_{cs} S \, dX - \int_{h_{sc}}^{h_{sc}+h_{de}} C_{de} S \, dX - \int_{h_{sc}+h_{de}}^{h_{sc}+h_{de}+h_{ss}} C_{ss} S \, dX \quad (12)$$

where M is the drug amount not yet dissolved at time t. Bearing in mind that:

$$M = N_p M_p = N_p \left(\frac{4}{3}\right) \pi \rho R^3 \quad (13)$$

we have, in accordance with the Hixon–Crowell law (Martin et al., 1983):

$$R = \sqrt[3]{\frac{3M}{N_p 4\pi\rho}} = R_0 \sqrt[3]{\frac{M}{M_0}} \quad (14)$$

where ρ is the drug density and M_0 is the M starting value. By means of Eqs. (12) and (14), it is possible to calculate K_t reduction. The effects of K_t decrease

are more evident when the dissolution phenomenon implies a considerable variation of the particle radius. Indeed, in this case, the solid surface will be strongly reduced and, as a consequence, the drug flux feeding the donor compartment will be decreased.

In order to make the model more adherent to experimental conditions, we also consider the receiver compartment sampling technique. Indeed, it requires to withdraw, at fixed times, part of the receiver volume (ΔV_r) and immediately replacing it with an equal amount of fresh solvent. This determines a sharp variation (reduction) of the drug receiver concentration (we suppose that the receiver volume, stagnant layer apart, is well stirred) that can be accounted for by the following equation:

$$C_r^a = \frac{C_r^b(V_r - \Delta V_r)}{V_r} \quad (15)$$

where C_r^b and C_r^a represent, respectively, the receiver drug concentration just before and just after the sampling. The numerical solution of the model makes easy the incorporation of Eq. (15). Indeed, the three layers composing the trilaminate can be subdivided, respectively, in N_1, N_2 and N_3 elementary grid volumes on which Fick's second law is integrated in the space and over the time, in order to get the drug concentration in each grid volume. We chose, as solving technique, the control volume approach (Patankar, 1980) that is an implicit finite differences method suitable to solve such kind of problems (Grassi et al., 1999).

Of course, the one-layer skin case (dermis–epidermis) is treated in an analogous manner, with the only difference of considering a two layers membrane (dermis–epidermis plus the stagnant layer).

Due to the numerical nature of the solution, the model is not very user friendly and that is why a Microsoft Excel file enabling the use of our model is available.[1]

3. Experimental

With the aim of severely test the model, we decide to minimize its degrees of freedom reducing the fitting parameters to D_{de} in the one-layer skin case, and to D_{sc} for the full-skin case. Accordingly, Acyclovir powder surface and mean radius, its solubility, its diffusion coefficient and its dissolution constant have been measured by means of independent experiments. Moreover, the stagnant layer thickness and the Acyclovir partition coefficient in the full skin and in the one-layer skin have been determined in advance.

3.1. Permeation studies

Permeation studies are performed using a Franz cells thermostatic bank (eight cells) at 37 °C, equipped with two compartment cells (type FDC 400, nominal diameter 21 mm; Crown Glass Co., Sommerville, USA). The donor compartment, characterised by a 3 cm^3 volume V_d and a ≈3.5-cm^2 permeation area S, is filled by an Acyclovir saturated PBS solution (phosphate buffered saline solution pH 7.4, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride (BDH-Poole, UK)) obtained by stirring buffer PBS pH 7.4 at 37 °C for 24 h in presence of an Acyclovir excess amount. Receiver compartment is filled by $V_r = 14.5\,cm^3$ of pure PBS solution mixed by means of a magnetic stirrer at 500 rpm. Full and one-layer skin pieces are mounted on Franz cells with the dermis–epidermis on the lower side and are equilibrated before starting the permeation experiments.

Receiver fluid sampling ($\Delta V_r = 1.5\,cm^3$), performed throughout the whole experiment (9 h), is filtered with 0.45 μm filter (type Millex, HA, Millipore, France) and analysed by High Performance Liquid Chromatography (HPLC). Sampled volumes are immediately replaced with an equal amount of fresh buffer.

3.2. High Performance Liquid Chromatography (HPLC)

A Model Varian 9010 liquid chromatograph (Varian, Walnut Crick, CA) equipped with injection valve fitted with a 20 μl loop and a model 9050 UV-Vis detector (Varian) set at 254 nm is used to measure the receiver Acyclovir concentration. A stainless steel reversed phase column is used (C18 3.9 mm × 300 mm Waters , Milford, MA). The chromatograms are registered by a model 4290 (Varian) recorder. Elution is done at room temperature by means of a mobile phase

[1] Upon request to Mario Grassi (mariog@dicamp.univ.trieste.it), a Microsoft Excel file for permeation data analysis is available free of charge, 2002.

consisting of a sodium hydroxide solution 0.01N, and the flux rate was 2 ml/min.

3.3. Skin samples preparation

Male hairless rats (Rnu eutimic, Charles River, MI, Italy) of 5–7 weeks old are used in permeability experiments. The rats were sacrificed by cervical dislocation and full-thickness skin removed from abdomen, by incision of the outermost layer with a surgical bisturi. Stratum corneum was separated from the dermis–epidermis by placing the full-thickness skin (dermis-side down) on a filter paper saturated with a 1% trypsin (Sigma Chemical, purified bovine pancreas, type III) solution at the temperature of 37 °C for 4 h (Knutson et al., 1985). Trypsin solution was prepared with PBS at pH 8.0 (potassium dihydrogen phosphate and sodium hydroxide, BDH-Poole, UK). Stratum corneum is separated from dermis–epidermis via slight vortexing in de-ionised water. This manipulation determines dermis–epidermis part swelling and that is why it results ticker than the full skin (see Table 1) (conversely, no appreciable swelling of both full and one-layer skin was observed during permeation tests as we used isotonic solution in the receiver compartment). To match the same conditions for drug permeation, full skin should have been treated in the same manner as the one-layer skin (but without trypsin), but we believe that this operation cannot substantially modify the structure of stratum corneum, the most important barrier to diffusion among the skin layers. The thickness of full and one-layer skin (only dermis–epidermis) is measured with an electronic calibre (Mitutojo, type IDC 112MCB, Japan).

3.4. Acyclovir

Acyclovir (Recordati, MI, Italy) powder surface area A_S (=3370 cm^2/g) and mean drug particles radius R_0 (=5.7 μm) are determined by mercury porosimetry (Mod. 2000 Carlo Erba Strumentazione, Milano, Italy) and applying the method of Mayer and Stone (Carli and Motta, 1984).

Acyclovir PBS solubility C_s (2.62 mg/cm^3) (T = 37 °C) is determined by measuring the concentration of a saturated PBS solution in presence of an Acyclovir amount excess. After 24 h, part of the stirred solution is analysed by an UV detector (λ = 251 nm; UV-Vis Spectrophotometer, Perkin-Elmer) to get C_s.

Acyclovir diffusion coefficient D at 37 °C in PBS pH 7.4 is determined resorting to Acyclovir intrinsic dissolution rate (IDR) (Martin et al., 1983). A 1-cm diameter cylindrical Acyclovir disk (300 mg, compressed at 5 tonnes) is attached, by means of liquefied paraffin, to a rotating stainless steel disk of the same diameter. As soon as the whole apparatus is immersed in a 100 cm^3 thermostatic PBS solution (37 °C), the disk rotation is immediately started and the Acyclovir concentration is collected by means of an UV detector (λ = 251 nm, UV-Vis Spectrophotometer, Perkin-Elmer). Dissolutions are performed at four different rotating speeds ω and for each test the dissolution constant K_{dd} (defined according to Eq. (10)) is determined by fitting the experimental data according to the usual dissolution equation (Banakar, 1992):

$$C = C_s\left(1 - e^{(-K_{dd}S_c/V_{rd}t)}\right) \quad (16)$$

where C is Acyclovir concentration, t is time, S_c is the surface of the cylindrical disk basis and V_{rd} is

Table 1
Permeation characteristics of the nine performed tests

Experiment number identifier (N)	Animal age (weeks)	Thickness (cm)	Permeation area (cm^2)
Dermis–epidermis			
1	5	0.110	3.80
2	5	0.093	3.80
3	5	0.119	3.46
4	7	0.130	3.97
5	7	0.140	3.62
Full skin			
6	5	0.070	3.80
7	5	0.077	3.46
8	5	0.080	3.46
9	7	0.048	3.62

the dissolution environment volume. Accordingly, it is possible to determine the experimental dependence of K_{dd} on $\omega^{1/2}$ and, consequently, it is possible to get the D value resorting to the Levich (1962) equation fitting on the experimental data:

$$K_{dd} = 0.6203\frac{D^{2/3}}{\nu^{1/6}}\omega^{1/2} + A_0 \quad (17)$$

where ν is the distilled water kinematic viscosity, D is the fitting parameter and A_0 is an adjustable parameter accounting for the experimental evidence that also for $\omega = 0$ an even though small dissolution takes place. This fitting yields $D = 7.81 \times 10^{-6}\,cm^2/s$.

The powder dissolution constant ($K_d = 5.01 \times 10^{-5}$ cm/s) is determined by fitting the Hixon–Crowell (Martin et al., 1983) equation on the experimental Acyclovir concentration data obtained by a powder dissolution test performed in a not stirred 38 cm^3 PBS pH 7.4 volume at 37 °C. Concentration measurement is achieved by means of an UV detector (UV-Vis Spectrophotometer, Perkin-Elmer) set at 251 nm for 5 min.

3.5. Stagnant layer thickness

In order to estimate the thickness of the stagnant layer faced on the dermis–epidermis layer (receiver side) in the desired hydrodynamic conditions, we resort to the dimensionless relation between the Sherwood, Schimdt and Reynolds numbers *Sh*, *Re* and *Sc* (Kydonieus, 1992):

$$Sh = f\,Re^m\,Sc^n, \quad Sh = \frac{K_d d}{D} = \frac{d}{h_{ss}},$$
$$Sc = \frac{\eta}{\rho D}, \quad Re = \frac{\omega d^2 \rho}{\eta} \quad (18)$$

where η and ρ are the PBS pH 7.4 viscosity and density, respectively; ω and d are the rotating speed and the diameter of the magnetic stirrer, respectively; f, m and n are three fitting parameters characterising the Franz cell and h_{ss} is the stagnant layer thickness. Due to the mathematical nature of Eq. (18), the fitting parameters calculation requires to experimentally determine *Sh*, *Sc* and *Re* at different temperatures and rotating speeds. Then, a non linear regression of Eq. (18) on these data yields the fitting parameters values. Accordingly we get:

$$h_{ss} = \frac{d\,(=1\,cm)}{3.24 \times 10^{-6}\,Re^{1.2}\,Sc^{0.953}} = 0.011\,cm,$$
$$Sc = 1147, \quad Re = 5737 \quad (19)$$

3.6. Partition coefficient

The full skin/Acyclovir solution and the one-layer skin/Acyclovir solution partition coefficients K_{skin} and K_{de} are determined by immersion of both fragments in the Acyclovir solution (PBS pH 7.4) for 4 h at 37 °C (longer equilibrium times would have provoked the skin cells swelling and disruption). Acyclovir residue is calculated on samples referring to the initial concentration ($t = 0$). Measurements, performed in triplicate, give $K_{skin} = 0.547$ and $K_{de} = 0.9523$.

Assuming the partition coefficient of the stratum corneum $K_{p1} = 0.5$ (Parry et al., 1992), remembering that the following relations hold:

$$K_{p2} = \frac{K_{skin}(1+G) - K_{p1}}{GK_{p1}}, \quad K_{p3} = K_{p2}K_{p1} \quad (20)$$

where G is the ratio of the dermis–epidermis thickness/stratum corneum thickness ($G = 11.7$), we get $K_{p2} = 1.1$ and $K_{p3} = 0.55$.

4. Results and discussion

Of the nine performed experiments (see Table 1), the first five deal with permeation through the dermis–epidermis membrane, while the last four are about the full-skin permeation. Moreover, tests 1, 2, 3, 6, 7 and 8 refer to 5 weeks old animals, while tests 4, 5 and 9 refer to 7 weeks old animals. The determination of the Acyclovir diffusion coefficient in the dermis–epidermis layer (D_{de}) can be achieved by model fitting (Eqs. (1)–(15)) on tests 1, 2, 3, for the younger animal and on tests 4, 5 for the older animal, provided that the correct model parameters are considered ($D_{ss} = D = 7.81 \times 10^{-6}\,cm^2/s$; $h_{ss} = 0.011$ cm, $K_{de} = 0.9523$; $K_d = 5.01 \times 10^{-5}$ cm/s, $R_0 = 5.7\,\mu m$; $C_{d0} = C_s = 2.62\,mg/cm^3$).

Due to the biological nature of the examined membranes, it is practically impossible to perform different tests in the same conditions as, for both the full skin and the one-layer skin, each membrane is

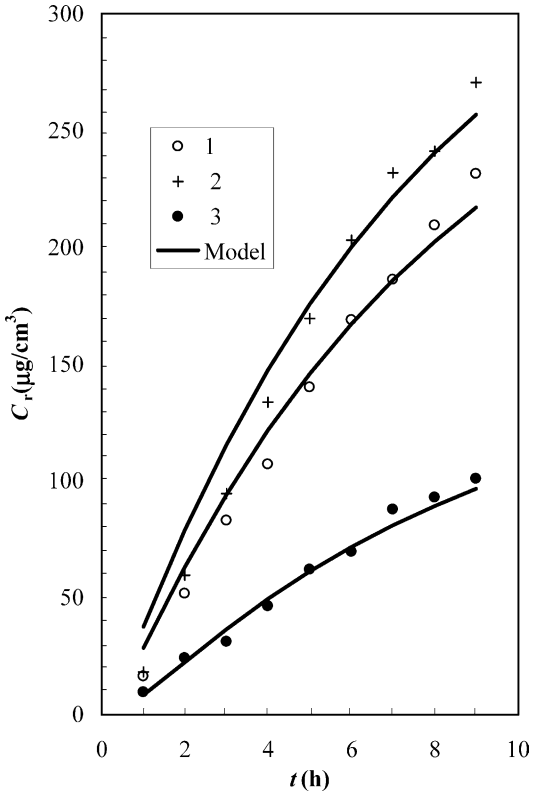

Fig. 2. Comparison between the permeation data (crosses, open and filled circles) and the model fitting (solid line) referring to tests 1, 2, 3 (5 weeks old animal, one-layer skin).

characterised by different thickness and permeation surface (see Table 1). Accordingly, the evaluation of data standard errors is not possible and data variability can be seen only through the standard deviation of the fitting parameters.

An inspection of Fig. 2, showing the trend of the Acyclovir receiver concentration C_r versus time t, reveals that the agreement between the model best fitting (solid lines) and the experimental data (open circles, crosses and filled circles) is reasonably good. This impression is also substantiated by the F-test (Draper and Smith, 1966) results shown in Table 2 [$F_{\text{calculated}}(8, 8, 0.99) = 6.03$]. Consequently, we can affirm that our model is able to properly take into account the main physical phenomena ruling Acyclovir permeation through the dermis–epidermis membrane. Table 2 shows the calculated values of D_{de} (±S.D. estimated according to Press et al. (1992)) corresponding to tests 1, 2 and 3, jointly with the mean value $\bar{D}_{de}$ (±S.D.). While very little D_{de} standard deviations prove the statistically validity of the fitting procedure performed, the greater $\bar{D}_{de}$ standard deviation derives from skin intrinsic variability and represents an estimation of experimental data variability.

Fig. 3 reports the comparison between the model best fitting (solid curves) and the experimental data referred to tests 4 and 5 (filled and open circles, respectively). Also in this case, the model is able to well describe the experimental data (see F-test, Draper and Smith, 1966) values in Table 2; [$F_{\text{calculated}}(8, 8, 0.99) = 6.03$]) even if the permeated amount is lower with respect to the data of Fig. 2. Table 2, showing the D_{de} calculated values referred to tests 4 and 5 jointly with the mean value (±S.D. estimated according to Press et al. (1992)), seems to indicate that the dermis–epidermis membrane of the older animal is less permeable to Acyclovir than that of younger animals this being probably due to ageing. Although this difference is statistically significant only for a relatively low probability value P ($= 1 - \alpha = 0.85$) ($t = 1.9$; d.f. $= 3$) and could be simply ascribed to natural membrane variability, it is in line with what Dick (Dick and Scott, 1992) observed studying water and mannitol permeation through Alderly Park rats epidermal membranes. Again, D_{de} standard deviations are small, while the greater $\bar{D}_{de}$ standard deviation represents the intrinsic biological variability of the dermis + epidermis layer.

Table 2
Fitting results referred to the dermis + epidermis permeation

Experiment number identifier (N)	Animal age (weeks)	F-value	D_{de} (cm^2/s)	$\bar{D}_{de}$ (cm^2/s)
1	5	374.3	$(2.00 \pm 0.005) \times 10^{-6}$	$(1.68 \pm 0.6) \times 10^{-6}$
2	5	275.3	$(2.05 \pm 0.004) \times 10^{-6}$	
3	5	527.3	$(1.00 \pm 0.005) \times 10^{-6}$	
4	7	464.3	$(6.50 \pm 0.5) \times 10^{-7}$	$(8.00 \pm 2.1) \times 10^{-7}$
5	7	436.7	$(9.50 \pm 0.5) \times 10^{-7}$	

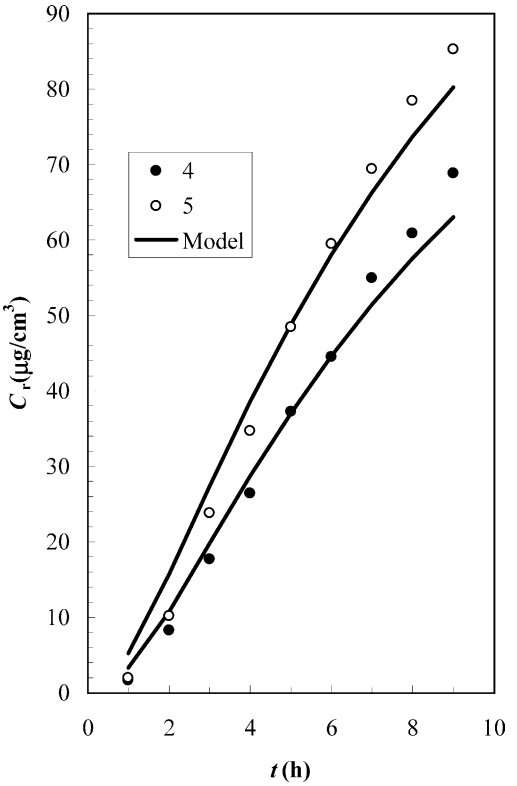

Fig. 3. Comparison between the permeation data (open and filled circles) and the model fitting (solid line) referring to tests 4 and 5 (7 weeks old animal, one-layer skin).

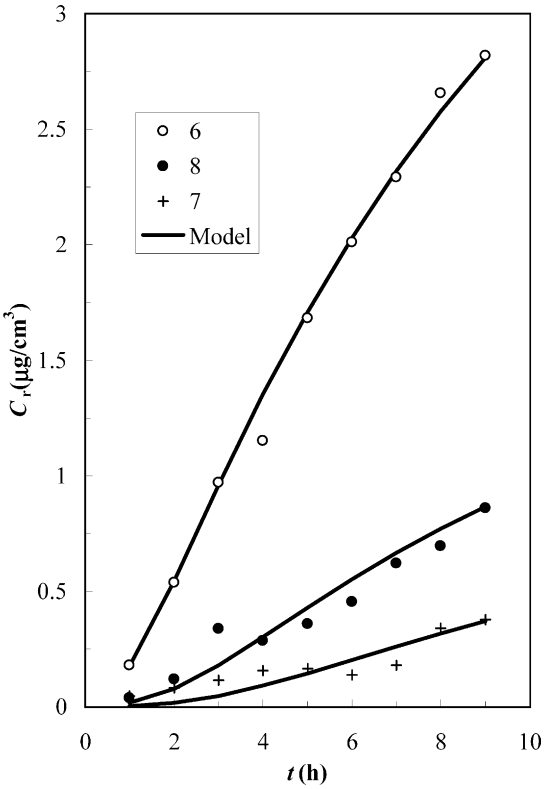

Fig. 4. Comparison between the permeation data (crosses, open and filled circles) and the model fitting (solid line) referring to tests 6, 7, 8 (5 weeks old animal, full skin).

On the basis of the calculated $\bar{D}_{de}$ referred to the dermis–epidermis layer, it is possible to determine the Acyclovir diffusion coefficient in the stratum corneum D_{sc}. Consequently, D_{sc} is now the only unknown model parameter and it can be determined by means of a model fitting on the full-skin permeation data. Fig. 4 shows the good agreement (see also F-test values in Table 3) between the model best fitting (solid lines) and the experimental data (filled and open circles, crosses) coming from the full-skin permeation of the younger animal. At the same time, Fig. 5 reports the comparison between the model fitting and the permeation data referred to the older animal. It can be seen that the agreement, at least statistically, is satisfactory even if the first two points are missed on fitting (see also F-test values in Table 3; $[F_{calculated}(8, 8, 0.99) = 6.03]$). Except for test 7 (relatively high standard deviation), standard deviations testify about the statistical validity of the fitting parameter D_{sc}, while the higher $\bar{D}_{de}$ standard deviation

Table 3
Fitting results referred to the full-skin permeation

Experiment number identifier (N)	Animal age (weeks)	F-value	D_{sc} (cm^2/s)	$\bar{D}_{de}$ (cm^2/s)
6	5	1152.2	$(2.50 \pm 0.39) \times 10^{-9}$	$(1.33 \pm 1.0) \times 10^{-9}$
7	5	21.7	$(5.00 \pm 4.7) \times 10^{-10}$	
8	5	84.1	$(1.00 \pm 0.49) \times 10^{-9}$	
9	7	236.2	$(6.50 \pm 2.66) \times 10^{-10}$	$(6.50 \pm 2.66) \times 10^{-10}$

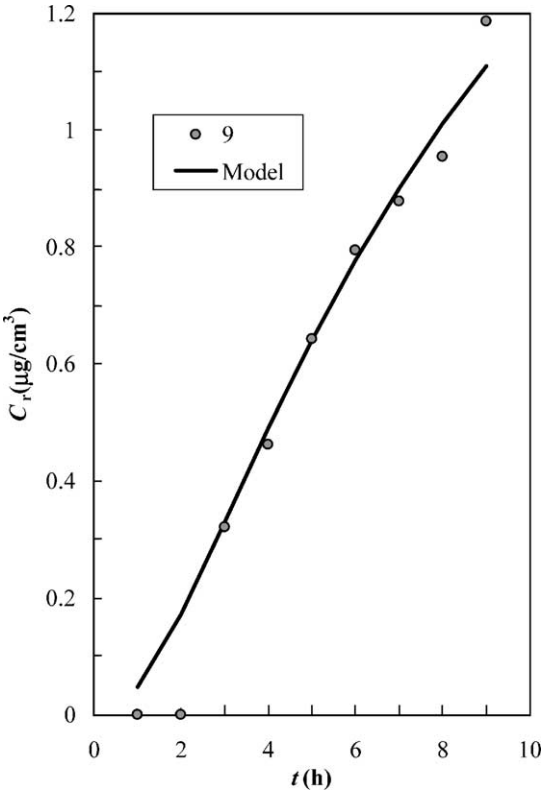

Fig. 5. Comparison between the permeation data (filled circles) and the model fitting (solid line) referring to test 9 (7 weeks old animal, full skin).

is attributable to skin variability and it represents an estimation of experimental data variability.

It is evident how the stratum corneum is the effective limiting step of the whole permeation process, as the Acyclovir diffusion coefficient in it is approximately 10^3 times lower than that in the dermis–epidermis membrane ($t_{young} = 4.8$; $t_{old} = 5.1$). It is interesting to notice again that the only fitting parameter of the new model is D_{de} (for the one-layer skin data fitting) and D_{sc} (for the full-skin data fitting), all other parameters being determined by independent experiments. Although this considerably reduces the model "degrees of freedom" in terms of data fitting capability, the satisfactory data description and the reasonable D_{de} and D_{sc} values obtained, guarantee about the reliability of both the experimental and theoretical approaches developed.

Interestingly, a rapid inspection of our data (see Figs. 2–5) could erroneously lead to the conclusion that a fickian diffusion process does not take place inside our membrane as data do not collocate on a straight line and the pseudo steady state seems to be never attained. The explanation of this apparent paradox lies on the fact that, in all figures, we report data not corrected for receiver dilution (receiver dilution is due to the sampling technique adopted) as, our model, for its nature, does not require to correct data for dilution, this improving data fitting reliability. It is easy to verify that this correction would confer the typical fickian aspect to our data.

In this light and on the basis of the above performed diffusive skin characterization (both full and one-layer skin cases), it is possible to verify by means of our model that pseudo-steady conditions are attained in our experiments from 6 h on. Indeed, subdividing the experimental tests in four groups (group 1: tests 1, 2, 3; group 2: tests 4, 5; group 3: tests 6, 7, 8; group 4: test 9), assuming the mean values of the diffusion coefficient (see Tables 2 and 3), membrane thickness and permeation area (see Table 1) relatively to each group, the model can predict the temporary trend of Acyclovir flux (automatically accounting also for the data dilution correction). Accordingly we have that for group 1 (tests 1, 2, 3) Acyclovir flux can be approximately retained constant in the time range 2–9 h (flux variation is lower than 9%), while for all other groups Acyclovir flux is absolutely constant in the time range 6–9 h. Moreover, it is also easy to determine the lag time t_L simply fitting by a straight line the temporary trend of the Acyclovir concentration C_r, calculated according to our model. Accordingly, we have: group 1 $t_L = 0.31$ h; group 2 $t_L = 0.77$ h; group 3 $t_L = 1.5$ h; group 4 $t_L = 1.25$ h.

In order to better understand model features, Fig. 6 shows the temporary trend of the Acyclovir donor and receiver concentration C_d and C_r in the full skin (stratum corneum, dermis, epidermis; crosses) and in the dermis + epidermis (solid line) cases. This simulation is performed assuming $D_{de} = 8.0 \times 10^{-7}$ cm^2/s, $D_{sc} = 6.5 \times 10^{-10}$ cm^2/s, all other parameters being those referring to tests 9 and 5. Interestingly, while in the full-skin case C_d is practically constant and C_r is slightly increasing with time, in the dermis+epidermis case, C_d and C_r have a different behaviour. Indeed, wherever C_d shows an initial decrease followed by

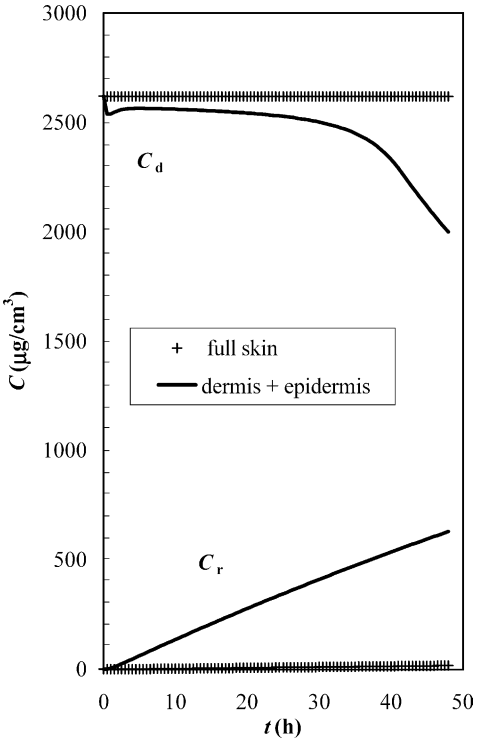

Fig. 6. Prediction of the temporary trend of the Acyclovir donor (C_d) and receiver (C_r) concentration in the case of the full skin (crosses) and dermis + epidermis (solid line) permeation. The parameters adopted refer to tests 5 and 9.

a relative constant region and a final considerable decrease, C_r monotonically increases. This behaviour underlines that, in the dermis + epidermis case, the strategy of keeping C_d constant and close to C_s by the addition of solid drug fails. Obviously, this is connected to the relative speed of solid dissolution (K_d) and membrane permeability: when the permeability/K_d ratio is not close to zero (in the dermis + epidermis case this ratio is around 0.14) such behaviour should appear. Simulations performed in the Fig. 6 conditions indicate that more pronounced initial C_d decreases take place when smaller and smaller donor volumes are considered.

It is now interesting to compare the results above obtained with those coming from a common method (Chien, 1987a) employed to estimate drug permeation through membranes (skin, in our case) when each concentration measurement needs to withdraw part of the receiver volume that is immediately replaced with an equal volume of fresh solvent (PBS in our case). Briefly, this technique needs for a permeation data correction according to the following equation:

$$C_{rc}^{n} = C_{r}^{n} + \frac{\Delta V_r}{V_r}\sum_{i=0}^{n-1} C_{r}^{i} \tag{21}$$

where n indicates the nth concentration measurement, V_r is the receiver volume, ΔV_r is the withdrawn volume at each measurement, C_r^n is the receiver drug concentration at the nth measurement, C_{rc}^n is the corrected drug concentration in the receiver due to the introduction of a volume ΔV_r of fresh solvent, and $C_r^{i=0} = 0$.

These new permeation data are then fitted by means of the following equation:

$$C_r = mt + q, \quad m = \frac{SK_pC_0D_m}{V_rh}, \quad q = \frac{SK_pC_0h}{6V_r} \tag{22}$$

where S is the permeation surface, K_p is the partition coefficient, V_r is the receiver volume, D_m is the drug diffusion coefficient in the membrane. This equation represents the asymptotic solution of Fick's second law in a membrane of thickness h, sandwiched between a donor compartment, characterised by a constant drug concentration C_0, and a receiver compartment in which the sink conditions are attained (Flynn et al., 1974). Although Eq. (22) should be one parameter model (D_m, or m), usually (Chien, 1987a) it is employed as a two parameters model (m, q) by means of which D_m can be determined according to:

$$D_m = \frac{mV_rh}{SK_pC_0} \tag{23}$$

Due to the asymptotic nature of Eq. (22), its regression is performed on experimental data collected from the sixth hour on, as we previously demonstrated that pseudo-steady state is, in our experimental tests, attained after 6 h, this reflecting in a linear trend of the permeation curve (Chien, 1987a; Grassi and Colombo, 1999; Grassi et al., 1999; Crank, 1975).

The main problem arising in the comparison of our model and Eq. (22) consists in the impossibility of directly compare the values of the calculated drug diffusion coefficient for the existence of a multilayered condition for both the full and the one-layer skin situation. Accordingly, membrane resistance

Table 4
Comparison between of our model and Eq. (22) on the basis of membrane resistance to diffusion. (R_0: our model resistance; R_m: Eq. (22) resistance)

Experiment number identifier (N)	Animal age (weeks)	F-value (Eq. (22))	R_0 (s/cm)	R_m (s/cm)
Dermis–epidermis (one-layer skin)				
1	5	732	$(5.9 \pm 0.014) \times 10^4$	$(6.1 \pm 0.016) \times 10^4$
2	5	261	$(4.9 \pm 0.001) \times 10^4$	$(5.5 \pm 0.012) \times 10^4$
3	5	136	$(12.6 \pm 0.06) \times 10^4$	$(12.1 \pm 0.059) \times 10^4$
4	7	864	$(21.1 \pm 1.62) \times 10^4$	$(19.2 \pm 1.35) \times 10^4$
5	7	5906	$(15.6 \pm 0.814) \times 10^4$	$(15.0 \pm 0.765) \times 10^4$
Full skin				
6	5	595	$(4.83 \pm 0.75) \times 10^6$	$(2.49 \pm 0.175) \times 10^6$
7	5	45	$(26.4 \pm 24.7) \times 10^6$	$(10.7 \pm 2.96) \times 10^6$
8	5	99965	$(13.7 \pm 6.7) \times 10^6$	$(7.68 \pm 0.014) \times 10^6$
9	7	57	$(12.7 \pm 5.2) \times 10^6$	$(5.76 \pm 0.13) \times 10^6$

to diffusion seems to be the proper discriminating parameter:

$$R_0 = \frac{h_{sc}}{D_{sc}K_{p1}} + \frac{h_{de}}{D_{de}K_{de}} + \frac{h_{ss}}{D_{ss} \times 1},$$

$$\sigma_{R_0} = \sqrt{\left(\frac{\partial R_0}{\partial D_{sc}}\right)^2 \sigma_{D_{sc}}^2} = \left|\frac{\partial R_0}{\partial D_{sc}}\right| \sigma_{D_{sc}} = \frac{h_{sc}\sigma_{D_{sc}}}{D_{sc}^2 K_{p1}} \quad (24)$$

$$R_m = \frac{h}{D_m K_p},$$

$$\sigma_{R_m} = \sqrt{\left(\frac{\partial R_m}{\partial D_m}\right)^2 \sigma_{D_m}^2} = \left|\frac{\partial R_m}{\partial D_m}\right| \sigma D_m = \frac{h\sigma_{D_m}}{D_m^2 K_p} \quad (25)$$

where R_0 and R_m represent membrane resistance expression according to our model and Eq. (22), respectively, σ_{R_0} and σ_{R_m} are the estimated standard deviations according to the error propagation law (Press et al., 1992) and K_p indicates both K_{de} or K_{skin}. It is obvious the Eq. (24) modification for the one-layer skin case. Table 4 showing R_0 and R_m values (jointly with standard deviations) for all the experimental tests performed, evidences how small differences arise between the two models for what concerns the one-layer skin case. On the contrary, for the full-skin case, Eq. (22) predicts a membrane resistance that is approximately one half of that predicted by our model. This qualitative observation is substantiated by the use of the Pearson correlation coefficient r_{xy} (=0.968) revealing the existence of a positive linear relation between R_0 and R_m characterised by a slope $b = 2.5 \pm 0.45$. If b had been statistically equal to 1, we should have concluded that R_0 and R_m are equal. A robust fitting analysis (Press et al., 1992) further confirms the calculated b value.

Finally, it can be noticed that the simpler method (Eq. (22)) requires individuating the linear part of the experimental curve on which the linear regression has to be performed. Undoubtedly, this decision may be a little bit subjective and it obliges to not consider all the information coming from the beginning permeation data.

5. Conclusions

The main conclusion of this work regards the fact that Acyclovir permeation through rat skin can be well described by the purely diffusive model employed, provided that all the model parameters (partition coefficient, drug diffusion coefficient in the release environment, thickness of the stagnant layers facing on the skin, Acyclovir dissolution constant and Acyclovir solubility in PBS) except for the drug diffusion coefficient in the stratum corneum and in the dermis–epidermis layer, are measured in advance by means of independent experiments. Additionally, the model automatically accounts for the receiver volume sampling technique consisting in the withdrawal of part of receiver volume, immediately replaced by

fresh drug free solvent. As a consequence, regardless membrane nature, our model is suitable for the description of drug permeation provided that the main transport mechanism is represented by diffusion. Interestingly, this should be the case of permeation through human skin as, in this case, drug transportation through follicles is negligible as stated by Cevc and Michaels (Michaels et al., 1975; Cevc, 1997) as the surface covered by follicles is less than 0.5% of the total skin surface.

Particular care has been devoted to the dissolution properties of the dissolving Acyclovir powder present in the donor compartment since the beginning of the permeation experiment. These characteristics, in conjunction with membrane permeability, play an important role in determining the donor drug concentration variation during the whole permeation experiment. We found that in the full-skin case the drug dissolution flux is higher than the flux due to drug permeation so that drug concentration in the donor compartment remains constant during the experiment. On the contrary, in the one-layer case the opposite situation takes place. Moreover, we demonstrated that a simpler common method based on the pseudo steady state approach yields to incorrect results in the estimation of membrane resistance for what concerns the full-skin situation. All findings are supported by statistical analysis.

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