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In vivo percutaneous absorption: a key role for stratum corneum/vehicle partitioning

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Summary. Percutaneous absorption of five compounds was studied in the hairless rat in vivo: benzoic acid, caffeine, hydrocortisone, inulin and thiourea. The results clearly demonstrate that, as with in vitro experiments, a steady-state flux can be achieved in vivo. This steady-state flux is strongly molecule dependent. Thus, the values for inulin and benzoic acid differ by a factor of about 40. In contrast, although the physicochemical properties of the studied compounds vary widely, their lag times were not significantly different. The mean lag time was 11 ± 2 min. Different compounds could be considered to have approximately the same apparent diffusion coefficient with regard to their percutaneous absorption in vivo. Thus, for a given thickness of stratum corneum and a given anatomical site, the penetration flux value of a substance depends only on its stratum corneum/vehicle partition coefficient. Using a classical model, we have demonstrated that the amount of substance present in the stratum corneum (Q_{sc}) at equilibrium (30 min) is related to this partition coefficient. There is also a linear relationship between steady-state flux and Q_{sc}. In practice, the in vivo steady-state flux of penetration of a compound can be predicted from the simple measurement of the amount present in the stratum corneum after a contact time of 30 min.

Key words: Percutaneous absorption – Steady-state flux – Stratum corneum – Vehicle – Partition coefficient

Over the past two decades considerable attention has been paid to understanding the mechanisms and routes by which chemical compounds penetrate the skin. Irrespective of the different theories on mechanisms relating to percutaneous absorption, it is well established that the stratum corneum (SC) constitutes the main barrier [14, 16, 29, 32]. Thus, it is to be expected that the overall kinetic process will depend mainly on the pharmacokinetic parameters governing the penetration of compounds through this membrane.

The interaction between the drug, the vehicle and the SC as a consequence of their physicochemical properties is likely to be an important pharmacokinetic parameter in an early step of the absorption process. In studies in rats [20] and humans [9], we have demonstrated a correlation between the amount of the test substance found in the SC at the end of a 30-min application and the total amount absorbed over 4 days. The predictive aspect of the so-called 'stripping method' was found to take into account the main factors influencing percutaneous absorption [10, 21, 22, 23].

It has been suggested that the amount of chemical absorbed within the SC after 30 min of application could reflect its SC vehicle partitioning, and also its rate of entry into the skin [10]. Previous studies in hairless rats [21] showed clearly that the amount of various compounds which penetrated in vivo was strictly proportional to the time of application, thus providing indirect evidence that a constant flux of penetration really does exist in vivo.

In the light of these results, the present study was carried out to determine whether the stripping method could also be used to predict the in vivo steady state flux of a test compound.

Materials and methods

Five radiolabelled compounds (The Radiochemical Centre, Amersham, UK) with very different physicochemical properties and belonging to different chemical classes were compared: benzoic acid, caffeine, thiourea, hydrocortisone and inulin (Table 1).

A group of six, 12-week-old hairless Sprague Dawley female rats (IFFA-CREDO, Lyon, France) weighing 200 ± 20 g was used for each compound and each application time.

Percutaneous absorption measurements

Each compound (1000 nmol) was applied to a 1 cm^2 area of the back of anaesthetized animals (IP injection of gammabutyrolactone, 0.5 ml/kg) in 20 µl ethylene glycol/Triton X100 mixtures (Table 1).

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Table 1. Application conditions

Compound	Specific activity	Purity (%)	Molecular weight (Da)	Vehicle
Benzoic acid 7 – ^{14}C	21.3 mCi/mmol	> 98	122	Ethylene glycol/TritonX100 (90/10)
Caffeine 8 – ^{3}H	24 Ci/mmol	> 98	194	Ethylene glycol/TritonX100/water (45/5/50)
Thiourea ^{14}C	58 mCi/mmol	> 98	76	Ethylene glycol/TritonX100 (90/10)
Hydrocortisone (1,2,6,7) ^{3}H	98.5 Ci/mmol	> 99	362	Ethylene glycol/TritonX100/isopropanol (72/8/20)
Inulin ^{3}H	3.2 Ci/mmol	> 98	5200	Ethylene glycol/TritonX100 (90/10)

Dose applied for all compounds: 1000 nmol/cm^2 in 20 µl vehicle

The treated area was delimited by an open circular cell attached with silicone glue in order to prevent spreading. The application times were 1, 2, 3, 4 and 5 h.

At the end of the application time, the treated area was washed twice (2 × 300 µl) with ethanol/water (95/5), rinsed twice with water and dried with cotton wool in order to remove excess product. We considered that the compounds had effectively penetrated when they had crossed the SC and reached the viable epidermis. The SC was then stripped ten times (3M adhesive tape 810, USA) in order to exclude any compound which had not penetrated during the time of application (previous histological studies in hairless rat have shown that this procedure almost totally removes the SC). The remaining skin (epidermis plus dermis) was sampled and counted by liquid scintillation (Packard 360, Packard Instruments, Downers Grove, Ill., USA) after digestion in Soluene 350 (United Technology, Packard, USA).

The carcasses were then lyophilized, homogenized, and samples counted by liquid scintillation after combustion (Oxidizer 306, Packard Instruments, USA). In the case of tritiated molecules (caffeine, hydrocortisone, inulin), the water resulting from the lyophilization of the carcasses was sampled and assayed for radioactivity. The radioactivity found was added to that detected in the carcasses, thus obtaining the overall percutaneous absorption values. The urine excreted during the time of application was collected to take into account any product contained therein in the overall absorption. The total amount of each compound penetrating at each application time was determined by summation of the amount found in the epidermis, dermis and the carcass.

Stratum corneum/vehicle partitioning measurements

Six strippings (3M adhesive tape 810, USA) were performed on the treated area of each group of six animals for each compound after a fixed application time of 30 min (the reasons for this choice of time is discussed below). The amount of product within the SC was assessed, after complete digestion of the keratin material in Soluene 350, by liquid scintillation counting.

Measurement of the thickness of the stratum corneum

The thickness of the SC was measured in biopsies from the backs of six rats according to the technique described by McKenzie [17]. Each biopsy was placed on a strip of acetyl cellulose coated with Tekt issue (Miles Scientific, Naperville, Ill., USA), then frozen in dry ice. Transverse sections (8 µm) were then cut with the aid of a cryomicrotome (HRLM, Slee, London, UK). The sections obtained were fixed for 10 min in a 70% alcohol bath, then stained for 30 s with a 0.5% aqueous solution of methylene blue. After rinsing with distilled water the sections were mounted between a slide and a coverslip with the aid of aquamount (hydrophilic mounting medium). The thickness of the horny layer was measured at 20 different points on each section by means of a semi-automatic image analyser (Digipet, Reichert, Wien, Austria) connected to a microscope (Polyvar, Reichert, Austria), and hence a mean thickness could be calculated.

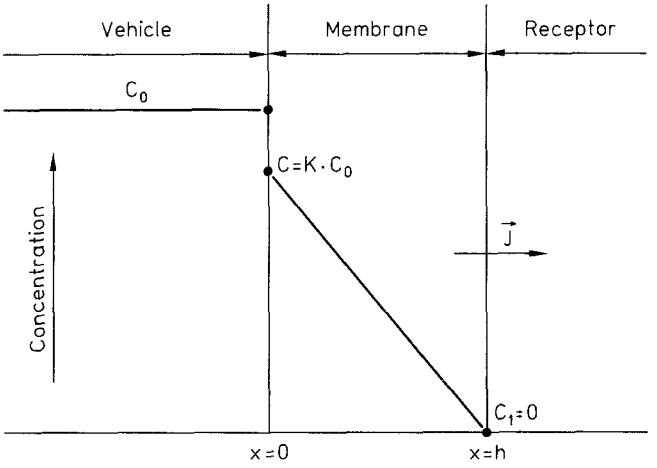

Fig. 1. Concentration profile across homogeneous membrane at steady-state (zero order flux case)

Effect of vehicles used on the stratum corneum integrity

After anaesthesia, 20 µl of each of the vehicles used was applied to a 1 cm^2 area on the backs of groups of five rats. The area was delimited by an open circular cell as described previously. After 5 h of contact, the treated area was washed twice (2 × 300 µl) with water and dried with cotton wool in order to remove excess vehicle. One hour after completion of the vehicle treatment, transepidermal water loss (TEWL) was measured with an Evaporimeter EP1 (ServoMed, Stockholm, Sweden). The hand-held probe was fitted with a 1 cm tail chimney, to reduce air turbulence around the hydrosensors, and a metallic shield (supplied by ServoMed), to minimize the possibility of sensor contamination. Measurements (g/m^2 per h) stabilized within 1 min. The effects of total destruction of the SC have also been studied by measuring TEWL from the backs of a group of five animals, 5 h after a series of ten strippings (3M adhesive tape 810, USA).

Theory and data treatment

The mass transfer of compounds from the surface of the skin to the interior of the body through the SC is generally considered to be due to passive diffusion. A classical but oversimplified description of the transport process is represented in Fig. 1. The SC is assumed

Table 2. Amount of chemical penetrating through the stratum corneum (nmol/cm^2) measured at the end of the application time, and their steady-state parameters

Compound	Application time (h)					Steady-state flux (J) (nmol/cm^2/h)	Lag time (h)
	1	2	3	4	5		
Benzoic acid	12.5 (SE 3)	30 (SE 2)	39 (SE 10)	64 (SE 6)	78 (SE 7)	16.4 (SE 0.8)	0.25
Caffeine	7.4 (SE 0.8)	18.6 (SE 2.9)	29.5 (SE 2)	45 (SE 5)	52 (SE 8.5)	10.2 (SE 0.6)	0.17
Thiourea	3 (SE 0.7)	6 (SE 2)	10.7 (SE 2)	13.4 (SE 1.8)	17 (SE 2.2)	3.5 (SE 0.2)	0.17
Hydrocortisone	0.49 (SE 0.05)	0.9 (SE 0.1)	1.5 (SE 0.1)	1.8 (SE 0.3)	2.6 (SE 0.4)	0.5 (SE 0.04)	0.16
Inulin	0.32 (SE 0.04)	0.63 (SE 0.04)	1.1 (SE 0.2)	1.5 (SE 0.2)	1.8 (SE 0.3)	0.38 (SE 0.02)	0.20
						Mean	0.19

to be a homogeneous membrane (thickness h). D is the diffusion coefficient of the solute through the membrane. The concentration of solute (C) within the outermost layer of the membrane ($x = 0$) depends on the concentration within the vehicle (C_0) and the partition coefficient (K) between the membrane and the vehicle:

$$C = KC_0 \quad (1)$$

For all values of time (t) the concentration of solute within the innermost layer of the membrane ($x = h$) is assumed to be negligible (sink condition). The validity of such an assumption will be discussed in the results section. The change in the cumulative amount of solute (Q) which passes through the membrane per unit area as a function of time is represented in Fig. 2. When a steady state is reached, the curve $Q(t)$ is linear and can be described by the equation:

$$Q = J_s(t - L) \quad (2)$$

where J_s corresponds to the steady-state flux (the slope of the straight line):

$$J_s = KC_0 D/h \quad (3)$$

and L is the lag time (the intercept of the straight line on the time axis):

$$L = \frac{h^2}{6D}. \quad (4)$$

In practice, J_s and L were calculated for each compound using a linear regression obtained with the aid of a computer (Vax 11/750, Digital Corporation, Bedford, Ma., USA) and standard software (RS/Explore BBN Software Product Corporation, Bedford, Ma., USA).

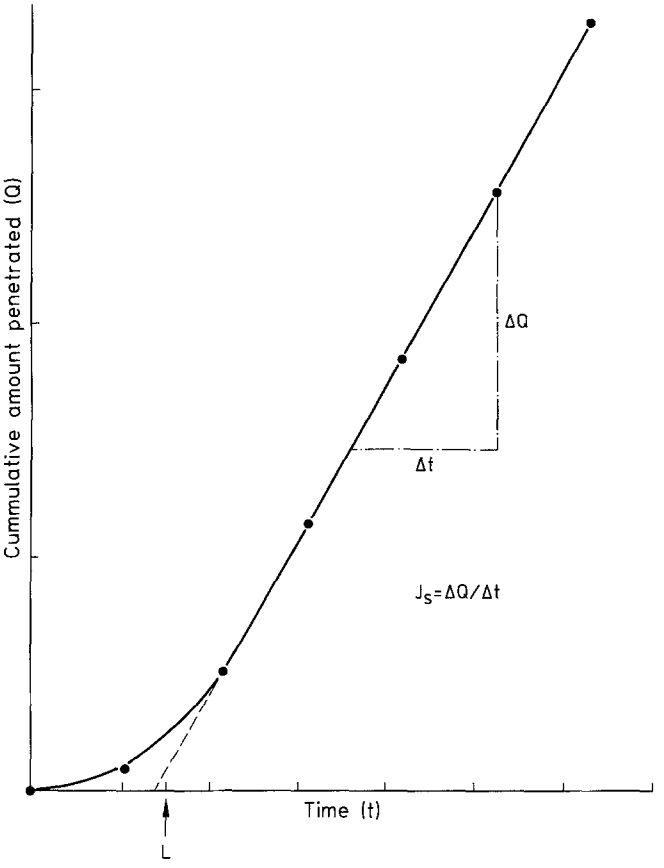

Fig. 2. Typical profile of concentration versus time for diffusion through the stratum corneum

Results

Figure 3 shows that, irrespective of the nature of the compound tested, the plot of the cumulative amount of solute which passes through unit surface area of the SC as a function of the application time appears to be linear ($r = 0.99, p < 0.001$). As shown in Fig. 3 and Table 2, the steady state values (J_s) are strongly molecule-dependent. Thus, the values for inulin and benzoic acid differ by a factor of 40. The rank order of the J_s values is:

inulin < hydrocortisone < thiourea < caffeine < benzoic acid.

In contrast, although the physicochemical properties of the molecules under study vary widely, their lag times are very close. The mean value can be estimated graphically to be about 11 min. This value is in agreement with the establishment of a constant flux during the first hour of administration since, in theory, the steady-state is attained after about 2.7 times the lag time [3].

In the present case, a constant flux of penetration ought to be attained within a contact time of about 30 min (11×2.7). According to Zatz [34] the attainment of a constant flux would be expected to coincide with the delivery of a constant amount at the SC. In order to test this hypothesis we measured the amount present in the horny layer after an application time of 30 min (it should be recalled that the time of 30 min corresponds to that used in the stripping method). The results obtained for the five molecules are shown in Table 3. The total amounts of solute accumulated in the first six strippings rank as follows:

inulin < hydrocortisone < thiourea < caffeine < benzoic acid.

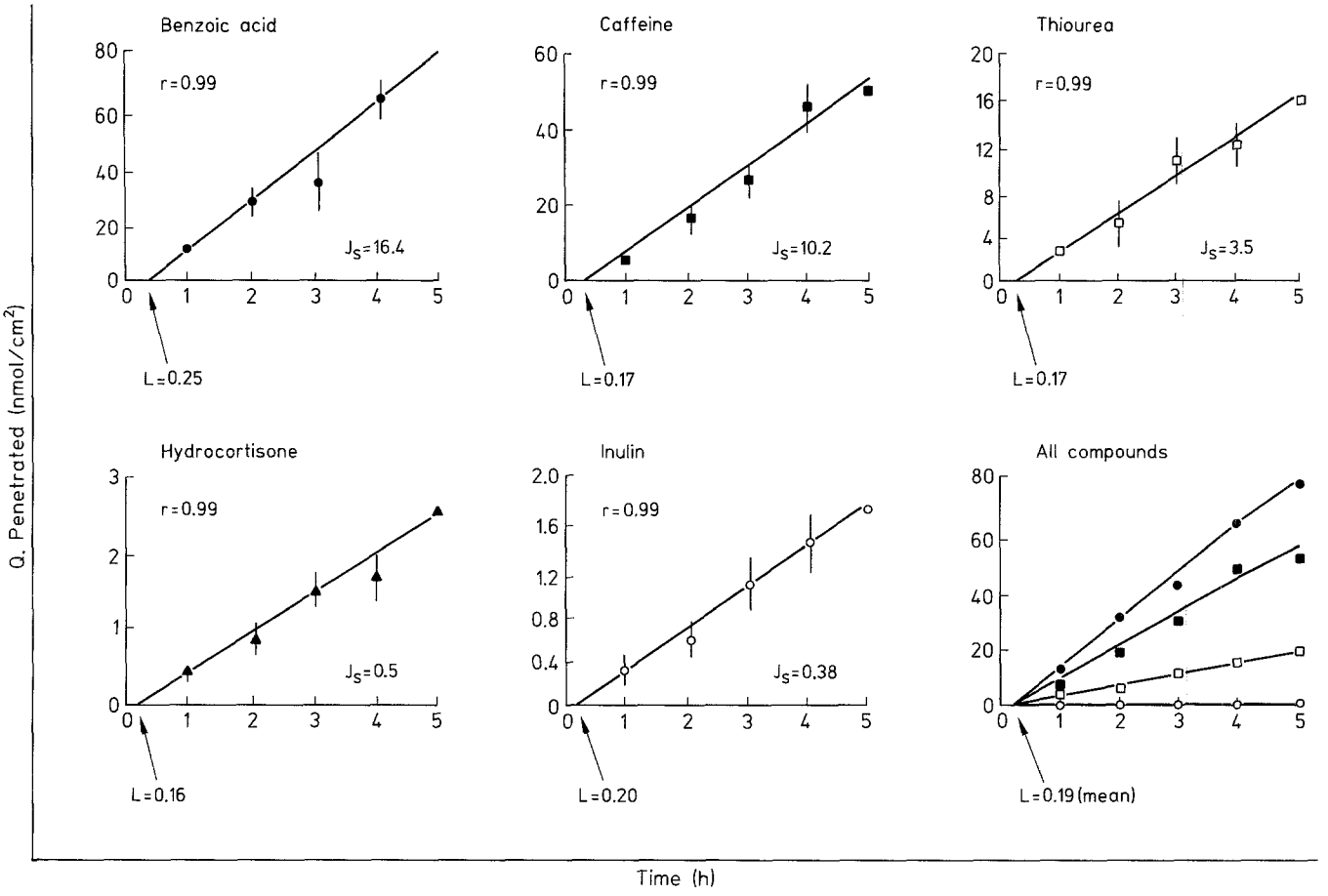

Fig. 3. Cumulative amount of solute penetrating through the stratum corneum as a function of application time. (J_S Steady-state flux (nmol/cm^2 per h); L = Lag time (h)

Table 3. Percutaneous absorptions parameters of the tested compounds

Compound	Log P octanol/water[a]	K_b	Q_{SC}[c] calculated (nmol/cm^2)	Q_{SC} measured (nmol/cm^2)	Steady-state flux (J_s) (nmol/cm^2/h)	
					Predicted from Eq. (8)	Measured
Benzoic acid	1.87	0.30	8.77	9.07 (SE 0.66[d])	15.87 (SE 1.15)	16.4 (SE 0.80)
Caffeine	−0.07	0.14	6.46	5.92 (SE 0.46)	10.36 (SE 0.80)	10.2 (SE 0.60)
Thiourea	−1.02	0.066	3.86	3.34 (SE 0.2)	5.85 (SE 0.35)	3.5 (SE 0.20)
Hydrocortisone	1.61	0.077	0.52	2.36 (SE 0.09)	4.1 (SE 0.16)	0.5 (SE 0.04)
Inulin	−3.58	0.078	0.46	0.85 (SE 0.12)	1.49 (SE 0.20)	0.38 (SE 0.02)

[a] From reference [12]
[b] Partition coefficient calculated from Eq. (5) (h = 13 μm, L = 11 min, C_0 see below)
[c] Q_{SC} calculated from Eq. (6) (h = 13 μm, K = calculated from Eq. (5), C_0 = see below)
[d] Standard error
C_0 = Solute concentration within the vehicles; (taking into account vehicle evaporation (Fig. 4): benzoic acid, thiourea, inulin = 4.5×10^4 nmol/cm^3; Hydrocortisone = 5.2×10^4 nmol/cm^3; caffeine = 7.1×10^4 nmol/cm^3

Discussion

Our results in vivo, like those in vitro, show that the phenomenon of transport across the SC can be considered as a process obeying the general laws governing passive membrane diffusion. Thus, after a time to attain equilibrium, a constant flux of penetration is established. From a theoretical point of view this can occur only if the solute distribution within the membrane remains constant. This implies that the solute concentration in the outermost layer of the membrane has to remain constant throughout the entire experiment (infinite dose condition), and that the solute concentration in the innermost layer of the membrane has to remain constant and be negligible (sink condition).

As shown in Fig. 4, the amount of the vehicle applied (20 μl/cm^2) changes only during the first hour of

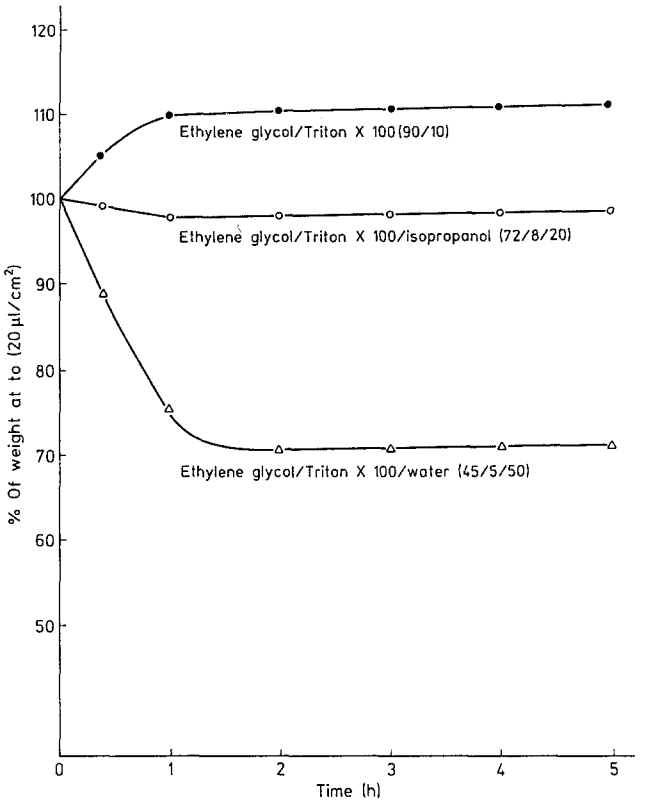

Fig. 4. Modification of the vehicles during their administration (20 µl/cm², room temperature 27° C)

Table 4. Effect of the applied vehicles on the barrier function of the stratum corneum (transepidermal water loss)

Ethylene glycol/ Triton X100 (90/10) (g/m²/h)	Ethylene glycol/ Triton X100/ water (45/5/50) (g/m²/h)	Ethylene glycol/ Triton X100/ isopropanol (72/8/20) (g/m²/h)	Stratum corneum removed (10 strippings) (g/m²/h)
5.8 (SE 0.3)	5.7 (0.4)	5.6 (0.3)	91 (3.0)
Controls			
5.1 (SE 0.3)	5.3 (0.4)	5.1 (0.3)	5.1 (0.3)

administration. Then remains constant throughout the time of percutaneous absorption measurements (1 – 5 h). In the case of the most penetrating compound (benzoic acid), the amount which penetrated after 5 h of application (78 nmol) was far below the amount applied (1,000 nmol). It can therefore be assumed that the solute concentration in the vehicle remained relatively constant between 1 and 5 h. Experimentally, we can consider that the first condition is met.

It can be assumed that the epidermis and the uppermost part of the papillary layer of the dermis constitutes a negligible barrier in comparison with the SC [24], and the microvascularization of the dermal papillae prevents solute accumulation in the region of the capillaries. Thus, the solute concentration in the innermost layer of the SC can be considered to be negligible in comparison with the concentration in the outermost layer. Hence, the sink condition is apparently fulfilled.

Although the existence of a steady-state flux of penetration in vivo was predicted about 20 years ago by Tregear [31] and subsequently by others [2, 21, 33], the problem is still the subject of debate [11, 30]. Our results clearly demonstrate (Fig. 3) that a constant flux can be achieved in vivo just as in in vitro experiments. Although this seems to be quite logical in our view, this is the first time that it has been demonstrated experimentally. Our results thus fill a gap in the understanding of the mechanisms governing in vivo percutaneous absorption. It should, however, be emphasized that the existence of such a gap is in no way due to negligence on the part of investigators in the field, but rather to the technical difficulties of measuring a steady-state flux of penetration in vivo.

Our results show (Fig. 3, Table 2) that lag times for the different molecules tested are very close and extremely short. One explanation is that the vehicles used alter the SC and therefore modify the barrier to penetration. However, Table 4 clearly shows that TEWL is not affected by vehicle treatment, whereas removing the SC by ten successive strippings increases TEWL by a factor of 18.

It is therefore possible to consider that, until the contrary is demonstrated experimentally, such a situation may exist in vivo, even if it upsets some theories which have been built upon in vitro studies. It is important to emphasize that such observations have rarely been made in vitro, perhaps because sink conditions are not necessarily met in vitro. In a dynamic in vitro system, a concentration close to zero in the medium bathing the tissue does not necessarily imply that the concentration in the innermost layer of the membrane is constant with time [24]. It is not unusual to observe an increase in the concentration of solute with time in the innermost layers of the epidermis or dermis, depending on the in vitro model adopted [2, 11, 13, 24, 30, 31, 33, 35]. This increase may be linked either to incomplete resorption by the bathing fluid [6 – 8, 28] or to a possible affinity of the molecule for these structures [18].

On the basis of knowledge concerning the thickness of the SC ($h = 13 \pm 2$ µm) and the value for the mean lag time ($L = 11$ min), it is possible to deduce a mean value for the apparent diffusion coefficient (D_m) by using Eq. (4): $D_m = 4.3 \times 10^{-10}$ cm² s^{-1}. This does not mean that the values of the diffusion coefficients for molecules having physicochemical properties as different as those used in this study are identical. It means that it is impossible to control, with the required degree of precision, all the physical, physicochemical and biological parameters likely to affect diffusion through a membrane as complex as the SC. From a purely practical point of view, it is thus possible, as a first approximation, to consider different molecules as having the same apparent diffusion coefficient in the case of percutaneous absorption in vivo. On the other hand, it is reasonable to ask whether this

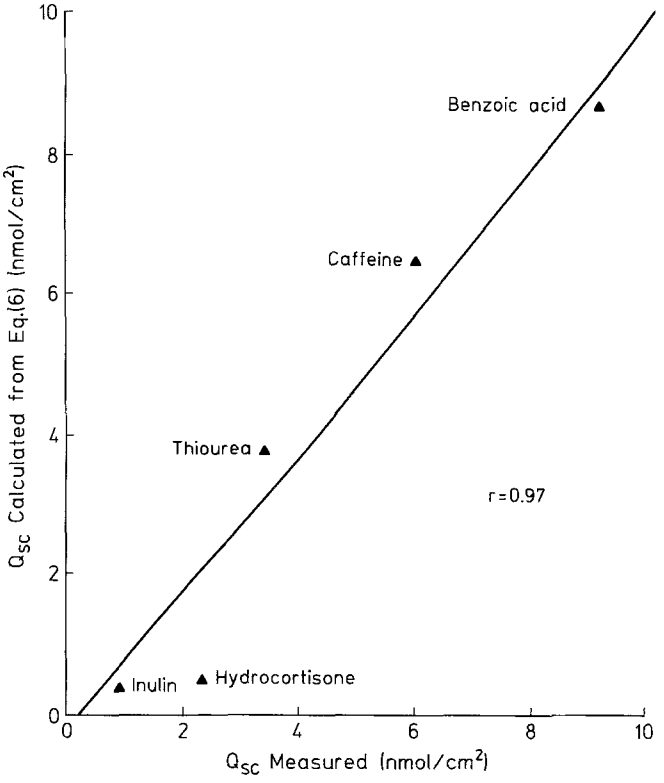

Fig. 5. Relationship between the quantity of chemical within the stratum corneum measured after 30 min of contact and predicted using Eq. (6)

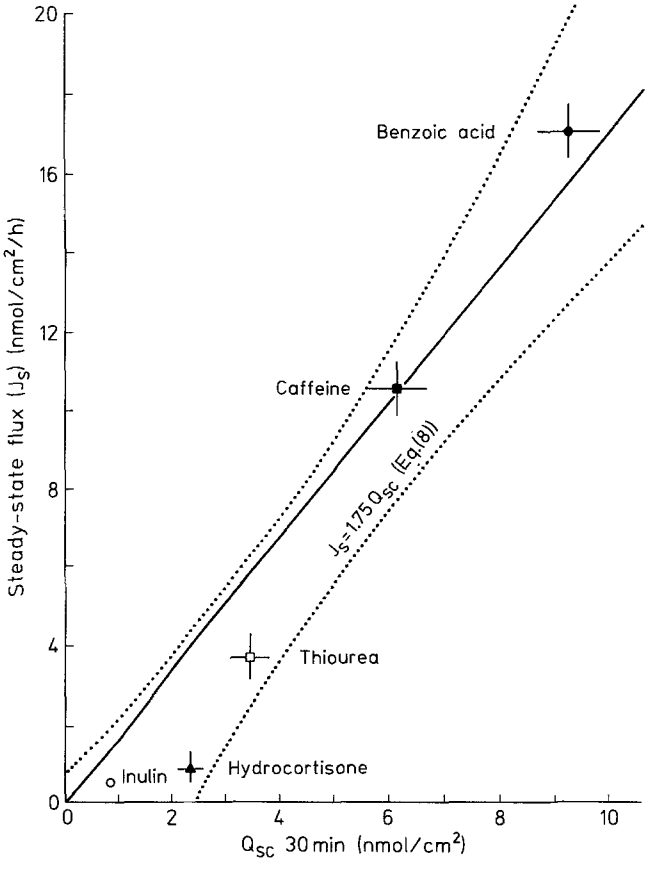

Fig. 6. In vivo relationship between steady-state flux of penetration (J_S) and quantity of solute within the stratum corneum after 30 min of contact

coefficient may vary as a function of parameters such as animal species, anatomical site, age, etc.

It follows from Eqs. (3) and (4) that the flux at equilibrium can be written in the form:

$$J_s = \frac{1}{6} KC_0 \left(\frac{h}{L}\right). \tag{5}$$

As we have shown earlier, the values of the lag times for the five molecules are similar. This results in the apparent 'velocity of diffusion', defined by the ratio h/L, being independent of the nature of the diffusing substance for a given thickness of the horny layer and a given anatomical site. Only the number of molecules in transit (KC_0) would be characteristic for a given substance, and would determine the value of its flux at equilibrium. Since, for a given compound, the value of C_0 may be considered to be constant within the time of percutaneous absorption measurements (1 – 5 h), the value of this flux would depend only on the SC/vehicle partition coefficient (K).

Using Eq. (5) and the values of flux (J_s) determined experimentally (Table 3) we have calculated the values of K for each of the five molecules (Table 3) taking into account in the C_0 values the evaporation of the vehicles (Fig. 4). The values for the octanol/water partition coefficients (log P) reported in the literature for these five molecules [12] are also shown in the Table 3. It appears that no relationship exists between these values and the values for flux at equilibrium. Although many examples appear to support the use of log P for predicting the degree of penetration of a molecule [4, 26], there are many others which show the limitations of such a procedure [1, 5, 19, 27]. The partition coefficient of a given compound between two solvents can be considered as a constant physical property of that compound. It is now generally accepted that the percutaneous absorption of a compound can vary considerably as a function of the conditions of administration (vehicle, dose, anatomical site, animal species, etc.). This raises the question: how is it possible to predict the value of a variable parameter only from a constant? Thus, in agreement with Scheuplein [25], we consider that, at present, no solvent system is capable of simulating the extreme complexity of the SC. Only the measurement of the partition coefficient between the SC and the vehicle can be representative of reality.

The amount of substance present in the SC at equilibrium (Q_{sc}) can be measured. According to the model adopted, this quantity is related to the partition coefficient by the equation:

$$Q_{sc} = \frac{1}{2} KC_0. \tag{6}$$

As shown in Table 3 and Fig. 5, there exists a very good agreement between the values of Q_{sc} measured by stripping the treated area after 30 min and the values of Q_{sc} calculated from Eq. (6) (C_0 values take into account vehicle evaporation).

In the light of Eqs. (5) and (6), the flux at equilibrium can be written:

$$J_s = \frac{Q_{sc}}{3L}. \quad (7)$$

Since the lag times of the molecules under study are similar, the fluxes at equilibrium would be expected to depend only on the amount present in the SC.

According to the theoretical model adopted, using Eq. (7) and a mean lag time of 0.19 h, the theoretical relationship between J_s and Q_{sc} should be:

$$J_s = 1.75\, Q_{sc} \quad (8)$$

(J_s being expressed in nmol/cm^2 per h). As shown in Fig. 6, the curve derived from Eq. (8) is contained within the 5% confidence limits of the experimental values.

In view of the approximations made in the theoretical model and the inevitable errors arising from the inacurracies of the measurements and biological variation, we can consider that there exists a very satisfactory agreement between experimental values and theory; Eq. (8). Only hydrocortisone does not appear to fit well with the theoretical linear relationship linking steady-state flux of penetration (J_s) and amount in the SC (Q_{sc}). This is not really surprising since steroids are known to form a depot or reservoir within the SC [3, 15]. A fraction of the available molecules may bind to the keratin or other tissue components, while the remainder diffuses slowly downward.

Six years after the development of the stripping method [9, 10, 20–23], the results obtained provide a better understanding of why it is possible to predict the total penetration during 4 days of a substance administered for 30 min with satisfactory precision. As shown in Table 3, from a purely practical point of view, the flux of penetration at equilibrium of a substance administered in vivo in a given vehicle can be predicted using Eq. (8) from the simple measurement of the amount present in the SC (Q_{sc}) after a contact time of 30 min. Since the validity of the stripping method has been verified for many molecules administered under different conditions in different species, it is reasonable to think that it would also hold for the predictive assessment of the in vivo steady state flux of penetration.

Using an original experimental approach we have obtained data leading to a better understanding of the mechanisms implicated in molecular transport across the SC in vivo. Thus, it appears that the SC/vehicle partitioning plays a determining role in the percutaneous absorption of chemicals in vivo.

We can easily conceive that our results, especially those related to lag times and diffusion coefficients, may not be readily accepted. The strength of the data presented lies in the fact that they are experimental. To reason only in terms of in vitro data would be to admit from the outset that there are no differences between the in vitro and in vivo processes of percutaneous absorption. However, considering the theoretical importance of these results, it would be important to see them verified using other chemicals of widely different physicochemical properties. It would also be interesting to ascertain that the theory we have developed concerning the in vivo mechanism of percutaneous absorption is verified when the same chemical is dissolved in different vehicles.

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