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Research paper

Potential pitfalls in skin permeation experiments: Influence of experimental factors and subsequent data evaluation

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ABSTRACT

There is a growing demand for skin penetration and permeation data considering toxicological and potential drug delivery aspects for an increasing number of substances. Although there are official guidelines available, results from different skin diffusion studies are often inconsistent and sometimes even controversial. The aim of our study is to address and to investigate the influence of experimental parameters as well as mathematical problems for subsequent evaluation of the permeation raw data. To create a reliable database diffusion experiments across human stratum corneum were performed under highly standardized conditions. The experimental data were evaluated using linear and non-linear regression analysis to determine the influence on the permeability coefficient and the lag-time. Additionally, the influence of two critical experimental parameters, temperature and unstirred water layers, on the permeability was investigated in silico. Based on our results we suggest that the influence of temperature on the permeability coefficient is small compared to the effect of other experimental parameters. Thickness of unstirred water layers has a tremendous effect on the permeation and may lead to underestimation of the permeability by more than 90%. Non-linear regression analysis seems to be superior compared to linear algorithms hence is advisable for evaluation of the experimental data. Our findings may help to optimize the experimental set-up and to reduce total costs for future skin diffusion testing. With regards to EU's REACH-Initiative this will also help to create more and reliable data on safety issues of industrial materials.

1. Introduction

There is a constantly growing demand for risk assessment data for old as well as new chemicals in nearly all fields of industry. This demand became even more substantial since the REACH initiative came into force in July 2007. Moreover, there are fundamental changes to be expected in the regulatory field of cosmetics and animal testing by the end of 2009.

Due to these facts it is easily comprehensible that the need for alternative testing methods, especially *in vitro* skin penetration methodology, is constantly growing thereby influencing industrial as well as academic research interests. Hitherto, *in vivo* and *in vitro* skin penetration experiments mainly have been employed as a first-line tool to develop and optimize topically applied pharmaceutics. In this context an extensive database has been generated over the past years by performing innumerable skin diffusion experiments [1]. Although there are official guidelines [2–5] for *in vitro* as well as *in vivo* skin diffusion experiments, results from different studies are often inconsistent and sometimes even controversial. On the one hand this may be explained by the huge variety of methods and test systems available for skin diffusion experiments. On the other hand there are still open questions on how to calculate and interpret results from the complex experimental set-up.

The aim of our study is to address and to discuss potential pitfalls in *in vitro* skin permeability testing from both the experimental side as well as the following aspect on evaluation and interpretation of the experimental outcome.

In vitro skin diffusion experiments across human isolated stratum corneum sheets were performed using static Franz-type diffusion cells and a hydrophilic donor medium containing flufenamic acid as a model drug. By realizing these studies under highly standardized conditions, we generated reliable data for assessing the quality of the ensuing evaluation procedures.

Abbreviations: OECD, Organisation for Economic Co-operation and Development; D, diffusion coefficient; D_{app}, apparent diffusion coefficient, calculated from, e.g. macroscopic dimensions; t_{lag}, lag-time, extrapolated from the linear portion of the permeation curve; BSA, bovine serum albumin; HSA, human serum albumin; *MW*, molecular weight; PSA, porcine serum albumin; UWL, unstirred water layer.

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Next, we evaluated our experimental data using two different methods (linear vs. non-linear) to determine the influence on the calculated values for the permeability coefficient and the lag-time.

Finally, using a computational approach, the impact of two additional factors, temperature and thickness of unstirred water layers (UWL), on the estimated permeability was investigated systematically. Especially these parameters are likely to differ in inter-laboratory comparisons, since, e.g. the thickness of the unstirred water layers is correlated to the diffusion cell geometry and stirring speed. Moreover, diffusion cells in series may establish a temperature gradient not only between donor and receptor compartment but also between the first and the last diffusion cell.

In addition to the official guidelines, the present study provides valuable details on the practical implementation of skin diffusion testing including the repercussion of data evaluation on an intelligent experimental set-up.

Being aware and considering the potential pitfalls outlined in here, by e.g. adequate sampling schemes, suitable arrangement and control of the permeation experiments (taking also cell dimensions and stirring speed into account), may allow to generate reliable and reproducible data. This will help for reducing interlaboratory variance in multicenter studies. Moreover, applying appropriate data evaluation can help to optimize the total number of samples and duration of each experiment. Thus, time and costs in skin diffusion studies can be reduced effectively in the future.

2. Materials and methods

2.1. Franz-diffusion cell experiments

In vitro skin diffusion experiments, intended to provide the raw data for the following linear and non-linear regression studies (see Section 3.1), were performed as described recently [6]. In brief, we employed static Franz-type diffusion cells (Type 4G-01-00-15-12; Perme Gear, Riegelsville, Pa., USA), a widely used and well-established *in vitro* test system, in which the skin preparation separates the donor (max. 5.0 ml) and the receptor (12.1 ml) compartment.

Experiments utilizing isolated stratum corneum sheets (n = 3–4) were performed using flufenamic acid (FFA), which is a lipophilic (log $K_{octanol/water}$ = 4.88), ionizable (pK_a = 3.9) derivative of anthranilic acid, as a model drug. Stratum corneum (SC) sheets were prepared by trypsinisation of full-thickness skin (FTS) punches according to the protocol by Kligman [7]. FTS was incubated in phosphate buffer, containing 0.15% trypsin at 32 °C. After incubation for 24 h, stratum corneum had separated from deeper skin layers and was isolated from the trypsin solution. Adherent trypsin was removed by washing the stratum corneum four times with both phosphate buffer and purified water. Following the washing step, the floating stratum corneum sheets were carefully loaded on teflon foil and transferred to the diffusion cells. Positioning of the diffusion barrier on top of the receptor compartment was realized by gently rinsing the sheets off of the teflon supply. Infinite dose conditions were ensured by applying 500 µl donor solution containing 1000 µg/ml FFA in hydrophilic Soerensen phosphate buffer, pH 7.4, ($Na_2HPO_4{\cdot}2H_2O$ = 9.20 g; H_2PO_4 = 2.00 g; purified water ad 1000 ml). FFA in the samples was quantified by HPLC according to the method described by Wagner et al. [8]. Prior to the experiments, the diffusion cells were incubated for 1 h in a water bath (32 ± 1 °C) to adjust equal temperature in all cells. Samples (0.4 ml) were drawn via a syringe from the center of the acceptor compartment, before each experiment and after different predetermined time intervals. The removed sample volume was immediately replaced with fresh acceptor medium (Phosphate-buffered saline, pH 7.4; consisting of NaCl = 8.00 g; KCl = 0.20 g; $Na_2HPO_4{\cdot}2H_2O$ = 1.75 g; KH_2PO_4 = 0.20 g; purified water ad 1000 ml). Sink conditions were ensured in all experiments after initial testing of FFA saturation concentration in the acceptor medium.

2.2. Evaluation of in vitro skin diffusion experiments

Linear regression: To determine the permeability coefficients and the lag-times we employed the most simple approach by fitting the linear portion of the experimental curves and calculated the slope and the intercept with the time-axis.

Non-linear regression: In addition, the parameters of an analytical solution for Fick's 2nd law of diffusion [9] were fitted to our experimental permeation data (Eq. (1)). This analytical solution was previously used to fit skin permeation data [10–12].

$$Q(t) = AK_{lip/aq}lc_0\left[\frac{D}{l^2} - \frac{1}{6} - \frac{2}{\pi}\sum_{1}^{\infty}\left(\frac{-1^n}{n^2}\right)\exp(-Dn^2\pi^2 t/l^2)\right] \quad (1)$$

Here, $Q(t)$ is the total mass of drug in the acceptor compartment at time t, A accounts for the area available for diffusion, $K_{lip/aq}$ describes the partitioning between the stratum corneum lipids and the donor, l is the length of the diffusion pathway, and c_0 is the constant concentration of the drug in the donor fluid. For both the linear and the non-linear fit we studied the influence of the evaluation procedure on the fitted parameters by (a) fitting the single curves, (b) fitting the pooled data, (c) using the squared standard deviations of the experimental values for the weighting of the averaged data during the fit in (b). When performing linear regression we iteratively removed data points at earlier times to study the influence of the data sampling both on the values and on the stability of the computed results. For the non-linear regression, we employed a similar approach by successively removing data points at later times to seek for an eligible number of data points necessary to give reliable results. All regressions were performed using Origin 7 (OriginLab Corporation, Northampton, MA, USA).

2.3. In silico diffusion modeling through stratum corneum

All simulations were run using amylobarbitone (MW = 226.0, XlogP = 2.1) as a model drug. We chose this compound since it features molecular properties very similar to the average properties (MW = 224.23, XlogP = 2.07) of two recently published compilations of permeability coefficients [13,14]. The total amount of diffusant in the acceptor compartment may be calculated by an appropriate solution to the diffusion equation [9]. In simple cases, e.g. infinite donor conditions and perfect sink, it is possible to derive an analytical solution which allows for computing the concentrations due to diffusion at any given point in space and time. For other, more complex, conditions, it is difficult or even impossible to find an analytical solution. Here, finite-difference schemes or similar approaches may be used to approximate the mathematical solutions.

The most simple finite-difference methods divide space and time into discrete intervals and calculate the concentrations at each point in time and space. These techniques have been applied repeatedly to solve the equations for diffusion or heat conduction [15,16]. In our study we employed a finite-difference forward scheme to solve the diffusion equation in one dimension. In brief, for each step forward in time we calculated the new concentration $c_{i,j+1}$ which results from the difference in the current concentrations between this central point $c_{i,j}$ and the two neighbouring points in space, $c_{i-1,j}$ and $c_{i+1,j}$ (Eq. (2)).

$$c_{i,j+1} = \left(1 - \frac{2Dk}{h^2}\right)c_{i,j} + D\frac{k}{h^2}(c_{i+1,j} + c_{i-1,j}) \quad (2)$$

The rate of transport between any two neighbouring points in space is proportional to the diffusion coefficient D and the length of a time-step k and is inversely proportional to the squared space-interval h. The diffusion coefficient in the aqueous media was calculated by non-linear fitting published data on diffusion coefficients of weak and non-electrolytes at 25 °C [17] to Eq. (3) and correcting the fitted value to account for the change in solvent viscosity and kinetic energy of the diffusant due to the increase in temperature to 32 °C. The diffusion coefficient in the stratum corneum lipids was obtained in a similar manner by fitting diffusion coefficients in the extracted stratum corneum lipids to Eq. (3) [18].

$$D = aMW^b \tag{3}$$

Here, a accounts for the influence of the temperature and the viscosity of the medium, while b describes the dependency of the diffusion coefficient on the diffusant's molecular weight MW. At the interface between donor and barrier as well as between barrier and acceptor we assumed immediate partitioning. The donor concentration was kept at a constant concentration throughout the simulation (infinite dose conditions), while for the acceptor the concentration was set to zero for all time points (perfect sink conditions). This is in agreement with the established Potts–Guy equation [19] and with the boundary conditions used when deriving Eq. (1).

Diffusion through the stratum corneum was assumed to occur only through the inter-cellular lipids. The pathlength l and surface fraction f were 180 μm and 0.2%, respectively, which is in accordance with experimental observations [20] and previous calculations [21]. Unless otherwise noted, a temperature of 32 °C was assumed in all compartments and the simulations were stopped when the flux into the acceptor reached a constant value.

2.4. Influence of unstirred water layers

To study the influence of unstirred water layers (UWL) adjacent to the stratum corneum, we performed simulations using several combinations of UWL thicknesses. Different values for UWL thickness were based on previously reported data [22–24]. In detail we employed the following conditions (see also Tables 3–5): no UWL in donor (don) and acceptor (acc); UWL_{don} = 50 μm, UWL_{acc} = 0 μm; UWL_{don} = UWL_{acc} = 50 μm; UWL_{don} = 1000 μm, UWL_{acc} = 50 μm; UWL_{don} = UWL_{acc} = 1000 μm.

From the calculated permeability coefficients k_p and lag-times t_{lag}, we computed the apparent diffusion coefficient D_{app} using only the values for the lipid channel and neglecting the unstirred water layers using Eqs. (4) and (5), respectively. This corresponds probably best to the approach most investigators employ to determine the diffusion coefficient from *in vitro* permeation experiments.

$$D_{app} = k_p l/(fK_{lip/aq}) \tag{4}$$

$$D_{app} = l^2/(6t_{lag}) \tag{5}$$

2.5. Influence of temperature

To investigate the influence of temperature, we varied the parameter a (Eq. (3)) to account for both the change in viscosity and in kinetic energy of the diffusant at 25 °C. To correct the diffusion coefficient in the donor and acceptor phases, we utilized known values for the solvent's viscosity [17], whereas for the stratum corneum lipids, we used a linear interpolation of the temperature-dependent permeability of water vapor to estimate the change in diffusivity [25].

3. Results

3.1. Evaluation of the in vitro skin diffusion experiments

To determine the lag-time and permeability coefficient from experimental curves we applied different mathematical approaches (see Section 2.2).

For the stratum corneum, a plot of cumulative permeated mass versus time (Fig. 1) shows that the difference between the curves for different diffusion cells grows over time. To a certain extent this is due to natural variations in barrier permeability between skin specimens leading to slight differences in the slopes of the experimental curves. Hence, averaged data points at the end of the experiment possess a higher standard deviation (SD) than data points at earlier times. After two hours we observed a standard deviation of 0.41 μg/cm^2, whereas after 30 h the SD was 5.07 μg/cm^2. On the other hand, the relative standard deviation was highest after two hours (33%) and dropped to only 12% after 30 h.

3.1.1. Linear regression

To determine the permeability coefficient and the lag-time, we performed a linear fit of the single curves (see Section 2.2a) and the pooled data (see Section 2.2b), while iteratively removing data points at earlier times. Both approaches led to nearly identical results, the only difference being the slightly higher standard deviations of the mean values obtained using the first approach. Thus, we only present the results for the averaged curve (Table 1).

For all linear analyses, the regression coefficients r were invariably high and usually greater than 0.99 (data not shown). Hence, we found r unsuited for assessment of the influence of the fitting procedure on the results and do not report those values.

The number of data points taken into account during the regression analysis had a high impact on the lag-time. For all linear regressions, we observed a shorter lag-time when using the last eight data points starting at two hours. Using only the last three points resulted in negative lag-times for all curves.

Overall, the lowest relative error for the lag-time was 12%, whereas the errors for k_p are usually less than 3% of the fitted value.

Employing averaged data and weighting by error (see Section 2.2c) increases the relative error for both k_p and t_{lag}. Using five or fewer data points, the relative error of k_p amounts to more than

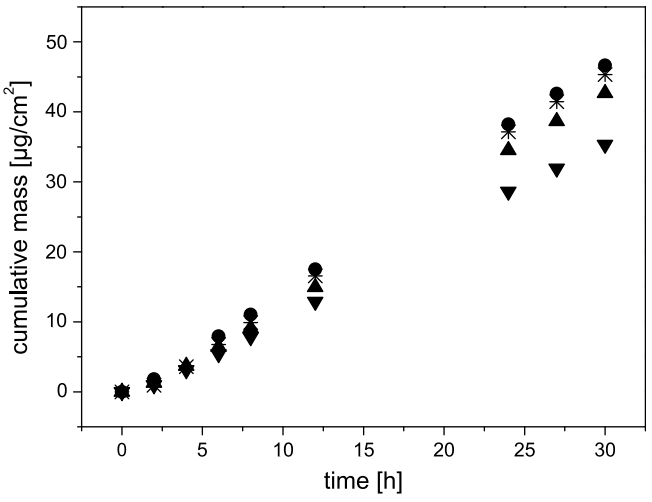

Fig. 1. Cumulative amounts of permeated flufenamic acid across human stratum corneum. The figure shows the results of four different diffusion experiments performed with skin originating from only a single donor.

Table 1
Results of the unweighted and weighted linear fit on the pooled experimental data

#	Lag-time						Permeability coefficient					
	No weighting			Weighting $1/SD^2$			No weighting			Weighting $1/SD^2$		
	t_{lag} [h]	SD	SD [%]	t_{lag} [h]	SD	SD [%]	k_p [cm/h]	SD	SD [%]	k_p [cm/h]	SD	SD [%]
8	1.56	0.19	12.18	1.44	0.31	21.53	1.51E-03	1.60E-05	1.06	1.45E-03	9.02E-05	6.22
7	1.71	0.20	11.70	1.73	0.39	22.54	1.52E-03	1.59E-05	1.05	1.52E-03	1.04E-04	6.84
6	1.70	0.27	15.88	1.70	1.00	58.82	1.52E-03	2.03E-05	1.34	1.52E-03	1.35E-04	8.88
5	1.75	0.41	23.43	1.84	1.54	83.70	1.53E-03	2.81E-05	1.84	1.53E-03	1.60E-04	10.46
4	1.66	0.79	47.59	1.90	3.15	165.79	1.52E-03	4.82E-05	3.17	1.54E-03	2.26E-04	14.68
3	-2.43	0.38	15.64	-2.39	41.31	1728.45	1.31E-03	1.83E-05	1.40	1.31E-03	1.10E-03	83.97

The number of data points (#) was reduced iteratively starting with the first data point while performing linear regression analysis.

10%, while the error for t_{lag} is of the order of the measured value (84%).

3.1.2. Non-linear regression

In addition to the linear regression, we fitted a non-linear solution to Fick's 2nd law of diffusion (Eq. (1)) to our experimental data (Table 2). In comparison to the linear regression analysis, this procedure allows to include the non-linear part of the curve too. Instead of removing the first data points, we removed later data points starting with the last to investigate the influence of stopping the experiment at an earlier point of time.

Non-linear regression produced very stable results if the number of data points was more than five (Table 2). In contrast to the linear fit (Table 1), the relative errors associated with t_{lag} were smaller than those for k_p.

Comparing the results with those of the linear fit, we observed larger errors for k_p in the case of the non-linear fit. In general, errors for t_{lag} were smaller compared to the linear fit.

Using weighted regression analysis, we found higher relative errors again with the errors for k_p being greater than those for t_{lag}.

3.1.3. In silico diffusion modeling

To compare the results calculated using different possible experimental conditions, we performed all simulations for a representative compound. We chose amylobarbitone as our model drug since this molecule features the typical physico-chemical characteristics of the drugs tested for transdermal permeation. For our model drug, using a non-linear fit (Eq. (3)) of previously published diffusion coefficients, we computed a diffusion coefficient in the aqueous donor and receptor phase of $D_{aq} = 2.67 * 10^{-2}$ cm^2/h and a diffusion coefficient in the lipid phase of $D_{lip} = 7.53 * 10^{-5}$ cm^2/h. The partition coefficient between stratum corneum lipids and aqueous phase was $K_{lip/aq} = 2183$. Assuming a pathlength of 180 μm and that only 0.2% of the macroscopic surface are available for diffusion, the permeability coefficient through the stratum corneum was calculated to be $k_p = 1.82 * 10^{-2}$ cm/h.

3.1.4. Influence of unstirred water layers on permeability and lag-time

The influence of unstirred water layers on the permeability coefficient was investigated by varying the thickness of the UWL in the donor and acceptor compartments (Table 3). Assuming no UWL, the calculated permeability coefficient of amylobarbitone ($k_p = 1.82 * 10^{-2}$ cm/h) was much higher than the experimental value ($k_p = 0.23 * 10^{-2}$ cm/h) [26]. Using a UWL of 50 μm in both the donor and acceptor compartment decreases the calculated permeability coefficient to $k_p = 0.42 * 10^{-2}$ cm/h and increases the agreement with the experimental value. Increasing the thickness of one UWL from 50 to 1000 μm further decreases the calculated permeability coefficient to $k_p = 0.049 * 10^{-2}$ cm/h, a value much lower than the experimental value.

To study the difference between determining the diffusion coefficient from either permeability (Eq. (4)) or lag-time (Eq. (5)), we used the calculated permeability coefficients and lag-times to compute apparent diffusion coefficients in the lipid channel (Table 4). Both methods give quite similar results. For an UWL of 50 μm in both the donor and acceptor, we observe that the apparent diffusion coefficient is only between 16% and 23% of the true diffusion coefficient in the lipids. Increasing the thickness of the UWL in

Table 3
Calculated permeability coefficients and lag-times for amylobarbitone in dependence on the thickness of unstirred aqueous layers

UWL thickness [μm]		k_p [cm/h]	t_{lag} [h]
Donor	Acceptor		
0	0	$1.82 * 10^{-2}$	0.75
50	0	$0.68 * 10^{-2}$	1.64
50	50	$0.42 * 10^{-2}$	4.53
50	1000	$0.05 * 10^{-2}$	8.56
1000	1000	$0.03 * 10^{-2}$	

The experimentally determined permeability coefficient is $k_p = 0.23 * 10^{-2}$ cm/h [26].

Table 2
Results of the unweighted and weighted non-linear fit on the pooled experimental data

#	Lag-time						Permeability coefficient					
	No weighting			Weighting $1/SD^2$			No weighting			Weighting $1/SD^2$		
	t_{lag} [h]	SD	SD [%]	t_{lag} [h]	SD	SD [%]	k_p [cm/h]	SD	SD [%]	k_p [cm/h]	SD	SD [%]
8	1.69	0.18	10.53	1.67	0.29	17.52	1.52E-03	3.33E-04	21.90	1.51E-03	6.12E-04	40.69
7	1.78	0.13	7.50	1.67	0.32	18.88	1.54E-03	2.43E-04	15.74	1.51E-03	6.72E-04	44.59
6	1.84	0.14	7.60	1.65	0.35	21.38	1.56E-03	2.52E-04	16.18	1.50E-03	7.69E-04	51.38
5	1.56	0.14	8.74	1.55	0.43	27.66	1.48E-03	2.86E-04	19.35	1.45E-03	9.73E-04	67.29
4	1.48	0.22	15.01	1.45	0.54	37.34	1.45E-03	5.00E-04	34.48	1.39E-03	1.28E-03	91.79
3	1.45	0.42	28.70	1.25	0.69	54.70	1.43E-03	9.83E-04	68.57	1.29E-03	1.72E-03	133.07

The number of data points was (#) reduced iteratively starting with the last data point while performing linear regression analysis.

Table 4
Dependence of the apparent diffusion coefficient on the thickness of the unstirred layers

UWL thickness [μm]		D_{app} calculated from k_p [cm²/h]	D_{app} calculated from t_{lag} [cm²/h]
Donor	Acceptor		
0	0	$7.50 * 10^{-05}$	$7.20 * 10^{-05}$
50	0	$2.79 * 10^{-05}$	$3.30 * 10^{-05}$
50	50	$1.72 * 10^{-05}$	$1.20 * 10^{-05}$
50	1000	$0.20 * 10^{-05}$	$0.63 * 10^{-05}$
1000	1000	$0.11 * 10^{-05}$	

The apparent diffusion coefficient was computed from the permeability coefficient or the lag-time using Eq. (3) or Eq. (4), respectively.

Table 5
Permeability coefficients calculated for a temperature of 25 °C

UWL thickness [μm]		k_p [cm/h]
Donor	Acceptor	
0	0	$0.98 * 10^{-2}$
50	0	$0.48 * 10^{-2}$
50	50	$0.32 * 10^{-2}$
50	1000	$0.04 * 10^{-2}$
1000	1000	$0.02 * 10^{-2}$

The differences to the permeability coefficients at 32 °C (Table 3) decrease with increasing thickness of UWLs.

the acceptor to 1000 μm leads to an apparent diffusion coefficient between 2.7% and 8.4% of the true value.

3.1.5. Influence of temperature on permeability

The impact of the temperature on the permeability coefficient was investigated by adjusting the diffusion coefficients in the aqueous and lipid phases. For the water phase, the diffusion coefficient at 25 °C is lowered to 84% due to the increased solvent viscosity, while the diffusion coefficient in the lipids is reduced to 54% compared to the value at 32 °C.

The change in k_p due to the lower temperature is greatest assuming no unstirred water layers (Table 5). Here, the permeability coefficient is approximately 54% of the value at 32 °C. With increasing thickness of the unstirred water layers, the influence of the decreased permeability of the stratum corneum itself becomes negligible, and the ratio between the permeability at 25 °C and 32 °C approaches a value of 84%. This value corresponds well to the expected effect due to the lower diffusivity in the aqueous phase.

4. Discussion

4.1. Experimental set-up

The experimentalist will easily find information about different types and basic handling of the set-up [27–31]. In general all systems consist of a donor and a receptor compartment in-between which the skin or an other membrane may be placed as a diffusion barrier. Sampling after predetermined time intervals is normally done from the receptor compartment. In addition to determination of drug concentration in the acceptor compartment, total recovery of the analyte (including donor, receptor and residual analyte within the diffusion barrier) is strongly recommended to avoid an experimental bias due to e.g. drug degradation processes.

A next step for the experimentalist is to choose which diffusion barrier to use for the experiments. As most studies are intended to predict skin absorption in man, human skin should be used in first instance whenever available. Viable skin should be preferred to mimic *in vivo* conditions best possible [32]. Normally skin originating from medical or plastic surgery of human breast or abdomen is employed. For mechanistic studies, skin from a single donor should be used to minimize the variability, whereas skin from different donors should be employed when developing formulations or conducting bioavailability studies. Despite their advantages, human skin samples are rarely used due to limited resources, high logistic effort and regulatory issues. Therefore porcine (ear) skin, bovine (udder) skin, or rat skin is usually employed as an alternative. It is commonly known that animal skin is often more permeable than human skin, hence overestimation for human skin absorption has to be taken into account for toxicological interpretation of the results [33–35].

For the future and with respect to the above-mentioned regulatory changes in the field of cosmetics and animal testing by the end of 2009, artificial skin models might become a powerful tool in skin irritation, penetration and permeation testing. Several *in vitro* models representing the human epidermis (Episkin®, EpiDerm®, Skinethic®) or full-thickness skin (Phenion®) are already available and may also help to standardize future experimental approaches [36–39]. However, these artificial skin models frequently exhibit much higher permeabilities than human or animal skin.

Besides skin source, handling aspects and storage of the skin probes prior to the experimental use have to be realized in best controlled manner. Pretreatment of the skin with disinfecting solution is an inevitable surgical standard procedure and cannot be avoided. Hitherto there was no systematical investigation on influences and possible effects of surgical or other pretreatment on skin permeability reported. However, (poly-)alcohols and other disinfecting agents are predestined thus likely to alter skin barrier function, as is already known for ethyleneglycol and ethanol [40].

Upon organizing a skin source and appropriate transportation, storage and handling, the experimentalist must select one out of different diffusion barriers and hence skin preparation techniques: trypsin-isolated stratum corneum, heat-separated epidermis or dermis, dermatomed skin, or full-thickness skin. This decision is important in terms of thickness and structure of the membrane. Increased barrier thickness is primarily correlated with a longer lag-time (t_{lag}), but also with changes in barrier structure due to hydration and dimensional problems like the edge effect [41].

For experiments lasting longer than 24 h the addition of preservatives (e.g. sodium azide, ethanol) is recommended to prevent enzymatic and microbiological degradation of the commonly organic diffusion barrier. However, this is likely to influence barrier properties [42], and may change the UWL thickness (see also Section 4.3) thus affecting the lag-time and overall duration of the experiment.

So far it is suggested that human trypsin-isolated stratum corneum and heat-separated epidermis are equivalent concerning permeation rates. Employing heat-separated epidermis for a hydrophilic drug may result in reduced permeation and longer lag-times due to the additional partitioning between stratum corneum and viable epidermis. Nonetheless heat-separated epidermis probably reflects the in vivo situation better. Together with the simpler handling and the shorter preparation time, heat-separated epidermis appears to be preferable over the rather fragile stratum corneum which may be easily damaged and thus may lose its barrier integrity.

Full-thickness skin should be avoided in human skin *in vitro* diffusion experiments due to a prohibitively long lag-time. Degradation of the barrier or drug molecules cannot be excluded for full-thickness skin experiments and should be investigated in more detail as well as a possible influence of the edge effect [6]. A possible alternative might be dermatomed skin which retains most of the advantages of heat-separated epidermis.

4.2. Influence of temperature

Although *static* as well as *flow-through* cells are available, most of the experiments are conducted using *static* (Franz-type) diffusion cells. This is primarily due to easy handling and tempering, thus minimizing time required per experiment and as a consequence also reducing costs and manpower needed. However, insufficient temperature control within the set-up is likely to act as a first source for interlaboratory variations. If tempering of the diffusion cell series is realized via a single flow cycle, a constantly increasing difference in temperature between first and last cell will be established with an increasing number of diffusion cells. As a consequence temperature control via several flow cycles or tempering units can help to reduce temperature gradients between the cells. Besides the inter-cell temperature gradient, there also might occur differences in temperature (depending on the ambient temperature) between the donor and the acceptor compartment. Thus, tempering of all cells within a tempering oven seems to be favorable over the series tempering using one or more fluid cycles.

Our calculations indicate that the influence of reducing the temperature from 32 °C to 25 °C on the permeability coefficient is highest when the experiments are performed under thorough stirring. Under these circumstances, the rate-determining step in crossing the barrier is the diffusion in the stratum corneum lipids. Since the permeability of the stratum corneum shows a strong temperature-dependency, lowering the temperature by 8 °C has a pronounced effect on the permeability. If the main diffusion resistance is due to thick UWLs, that is when neither donor nor acceptor is stirred, the rate-determining step is the diffusion through the UWLs. Since the diffusion coefficient in the UWLs is less susceptible to temperature changes, the influence of varying the temperature is small compared to the effect of other experimental parameters. In summary, temperature control is more important when performing experiments with thin UWLs.

4.3. Influence of unstirred water layers

Regarding donor and receptor fluids, only limited information is provided by the guidelines [2–5]. Acceptable receptor fluids must meet the solubility criteria and ensure sink condition in the receptor compartment throughout the entire experiment. Sink conditions are guaranteed according to the USP if "*the volume of medium is at least three times that required in order to form a saturated solution of drug substance*".

To meet these requirements, especially for low soluble drugs, it may be helpful to adopt a corresponding sampling frequency and sample volume for the diffusion experiment [43]. The OECD recommended to increase total solubility of a substance by addition of ethanol or polyethyleneglycol to the receptor fluid. It is important to note that such additives are likely to alter the barrier function of a biological membrane as is already known for alcohols, emulsifying agents and various lipophilic compounds [40,44–46]. Nonetheless, these additives may also decrease the thickness of the UWL adjacent to the biological barrier [47].

Our results demonstrate that the thickness of the UWL has a tremendous effect on the calculated permeability coefficient (Table 3). Using a rather conservative thickness of 50 μm for both the donor and acceptor compartment, we observed a reduction of k_p to 23%. No stirring in one of the compartments led to an underestimate of the permeability of our model drug by a factor of 1/37 (2.8%) compared to the perfect stirring where no UWLs are present. When applying ointments or finite doses in small volumes of aqueous solution, it is impossible to stir the donor, while the acceptor may be well stirred. Under these settings, we estimate for aqueous media that the UWLs in the donor and acceptor will be 250–500 μm and 0–150 μm, respectively.

Since the actual thickness of the unstirred layer depends on both stirring speed and geometry of the diffusion cells, it will be difficult to compare data from different laboratories. Especially this finding may also explain the variations reported in the literature for several substances.

To overcome the problem of reduced flux and prolonged lag-time due to the existence of UWLs, *bovine* serum albumin (BSA) may be added to the receptor fluid [48]. *In vivo* many drugs adsorb quite efficiently onto this protein. Thus addition of BSA, or if available *human* alternatively *porcine* serum albumin, seems to be advisable in terms of reducing the risk for altered barrier function. BSA changes the macroscopic solubility of drug molecules by reversibly binding these molecules. By the same mechanism, BSA reduces the local concentration of free drug in the UWL. This in turn decreases the UWL thickness and thereby lowers the resistance of the UWL. However, if there is substantial drug binding to the albumin, this may complicate the analytical procedures to quantify the permeant.

4.4. Sampling and evaluation of in vitro skin diffusion experiments

For the stratum corneum, our experimental curves represent a typical behavior (Fig. 1). Each skin sample behaves slightly different than the other and hence the cumulative permeated masses will differ from one cell to the other. The same is true for skin from two different donors, but the differences will be larger. This effect is well known from pharmacokinetics, where appropriate models have to take into account the intra- and interindividual differences.

In our experience, when evaluating the curves obtained with skin from a single donor, the relative standard deviation is always >5%, which is usually greater than the relative standard deviation resulting from the analytical method employed.

Linear fitting: The most simple approach to assess the lag-time and the permeability coefficient is to fit the linear portion of the curves by linear regression. It is well known that this procedure is prone to errors if gross outliers occur. However, we did not observe such outliers in our studies. For our experiments performed with skin originating from a single donor, the exact fitting procedure showed no marked influence on the values for lag-time and permeability coefficient.

Based on the relative errors, the value for t_{lag} is much more uncertain than the value for k_p and subsequent calculations using t_{lag}, e.g. to estimate the diffusion coefficient, should take this uncertainty into account.

Regarding the number of data points to be used during the fitting procedure, it is not possible to determine an arbitrary number of data points. Obviously, the more data points are used the smaller is the error of the fitted values. A possible way would be to start linear regression analysis including all data points and to successively remove early data points for the following regression analyses. In each step, the root-mean squared error should be calculated to assess the quality of the fit. Initially, this value will decrease, and as more points are removed this value will increase again. The first computed local minimum of the root-mean squared error might serve as a good indicator for the number of points to be included.

Non-linear regression: Although the non-linear regression analysis appears more cumbersome, it has – in contrast to the linear regression analysis – the clear advantage that already the first data points are sufficient to give a good estimate of the permeability and lag-time.

The errors associated with k_p are higher than in the case of linear regression which provides less confidence in the values. On the other hand t_{lag} exhibits smaller errors compared to the linear routine. Overall, we did not find large deviations between the values for k_p or t_{lag} using the two approaches.

Regarding t_{lag} it is important to adapt the sampling scheme to the diffusion barrier used in the experiments. Sampling prior to t_{lag} may help to determine the onset of the steady state flux more precisely, but is of limited use for the final data interpretation when employing linear regression analysis. At the same time this early sampling increases analytical effort and costs.

A major problem of both the linear and non-linear fitting approaches is to advise a priori a suitable time point for stopping the *in vitro* permeation experiment. It seems unfeasible to run linear or non-linear regressions based on the latest analytical results while running the experiment(s) in parallel. However, we recommend to employ non-linear regression if experiments were ended prematurely, that is before the linear portion of the curve was reached. Using the non-linear fit such experiments may still be evaluated, and approximate values for k_p and t_{lag} may be computed. It was pointed out in a previous study [10], that the non-linear regression analysis produced high standard errors of the fitted values [49]. The high standard errors, however, did not result from the non-linear regression, but rather from the high standard errors of the data points for which the fitting was performed [10].

4.4.1. Weighted fitting

The simple weighting we deliberately employed here led to an increase in the relative error because the variance of the averaged data grows with time and hence with cumulative drug amount. Thus, data points determined later during the experiment will have less influence on the fitted parameters than earlier time points. By applying a variance-stabilizing transformation it should be possible to circumvent this issue and to perform a reasonable weighted regression analysis [50]. However, finding a suitable transformation may be difficult due to the contradictory results on the relationship between variance and measured value. While earlier reports hint at a log-normal inter- and intra-individual variability in permeability [51], recent studies found evidence for both normal (Gaussian) and log-normal distributions [52,53].

4.5. Calculation of the diffusion coefficient

The diffusion coefficient in the stratum corneum lipids may be computed either from the permeability coefficient or from the lag-time (Table 5). Both approaches underestimate the true diffusion coefficient depending on the thickness of additional UWLs. Thus, these two methods seem to be on par. Computation of the diffusion coefficient from k_p (Eq. (4)) suffers from the drawback that in addition to the pathlength the surface fraction covered by the lipid channels and the partition coefficient between the lipid matrix and the aqueous phase must be known. While the latter parameter may be accessible by additional experiments, it is rather difficult to determine the surface fraction covered by the lipid slits. On the other hand, any error in the determination of the pathlength will have much more impact on the computed diffusion coefficient when using the lag-time approach (Eq. (5)). Here, D depends on the squared pathlength (Eq. (5)), whereas D is proportional to the pathlength only when using the permeability coefficient (Eq. (4)).

When using, e.g. heat-separated epidermis or full-thickness skin, only the macroscopic thickness of the barrier and its total surface may be easily estimated. Employing a macroscopic stratum corneum thickness of 50 μm [20] instead of the true pathlength and assuming that all the surface area is available for diffusion, we obtain a diffusion coefficient of $D_{app} = 4.17 * 10^{-8}$ cm^2/h for $k_p = 1.82 * 10^{-2}$ cm/h and of $D_{app} = 5.56 * 10^{-6}$ cm^2/h for $t_{lag} = 0.75$ h. Note that in this case the values for D_{app} differ by two orders of magnitude. Although one of the values is closer to the true diffusion coefficient, both values are inherently wrong and there is no reason to prefer one over the other.

5. Conclusions and outlook

There are several factors which may influence the outcome of an *in vitro* permeation experiment. Some of these factors are directly (e.g. temperature) or indirectly (e.g. thickness of the unstirred water layers) under the control of the experimenter. Based on our findings, we conclude that the thickness of the unstirred water layers has an enormous effect on the permeability coefficient and should be reduced by either thorough stirring or, preferably, by using additives such as bovine serum albumin (BSA). The difference in permeability due to the temperature is less pronounced and probably negligible when using skin from different donors or comparing results from different laboratories. As for the evaluation, non-linear regression analysis should be employed as it is fast and tries to balance the errors of lag-time and permeability. This approach can help to design experiments using an optimal number of samples and an adequate duration.

Experiments performed with artificial skin models will be influenced by the same factors as those done using stratum corneum or heat-separated epidermis. Since these skin models usually show higher permeabilities than human or animal skin [36,54], the evaluation of the permeation data will suffer even more severely from the drawbacks and advantages presented and discussed here.

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