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Research paper

Accumulation of sunscreens and other compounds in keratinous substrates

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Abstract

Several cosmetic ingredients, especially sunscreens, should be substantive, which means they are to be adsorbed to specific binding sites within the upper skin layers, particularly keratinized structures of the stratum corneum, and thus show resistance to washing off. We investigated the affinity of 10 non-ionic compounds, among these UV-absorbing chemicals, antioxidants, antimicrobial compounds and a repellent to animal keratin and human callus. In each case a linear relationship between the drug amount, which has accumulated in the respective keratin, and the remaining free concentration of the applied solution could be established. Moreover, drug affinities to keratinous substrates are in direct proportion to the octanol/vehicle partition coefficients, pointing to the fact, that drug enrichment in keratinic substrates is clearly governed by lipophilicity, while specific adsorption, i.e. genuine substantivity, does not seem to occur. After application of a saturated solution, non-ionic compounds with a pronounced keratin/vehicle partition coefficient will build up the highest concentration within the stratum corneum. If these compounds show, at the same time, a high solubility in the vehicle, they will penetrate the skin most easily. The used callous tissue seems to be a suitable substrate to simulate and quantify solute uptake into human skin.

Keywords: Binding; Differential scanning calorimetry; Keratin; Partition coefficient; Percutaneous absorption; Stratum corneum; Substantivity; Sunscreens

1. Introduction

Many substances are commonly applied to the surface of human skin thus getting into intimate contact with components of the uppermost skin layers. However, only little information is available on the possible sorption of these compounds by keratin, the main constituent of the stratum corneum and the intercellular lipid bilayers. Nevertheless, keratin is believed to be of special importance for sorption processes within the skin [1]. This investigation was therefore initiated in an attempt to examine the uptake of cosmetic ingredients, especially sunscreens, by keratinous substrates and to study the influence of the skin lipids on this uptake.

Searching for a suitable substrate as a source of keratin to investigate the sorption behaviour of the 10 test compounds led to excised human callous tissue. Although certain differences in composition may exist between the normal keratin layer of the skin and callus [2], the latter was the only type that could be obtained in adequate amounts for this study. Nevertheless, differential scanning calorimetry (DSC) measurements were carried out in order to characterize some of the above mentioned structural differences. Afterwards a decision should be possible whether or not callus could serve as substitute for stratum corneum.

The adherent properties of materials depositing onto keratin are usually referred to as ‘substantivity’. This term is quite frequently used to describe the ability of a drug or an excipient to be ad- or absorbed by keratinous substrates, thus resulting in retention on subsequent exposure to

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water [3]. Naturally, such properties are of special importance for water-proof sunscreens [4] or insect repellents. Usually, substantivity is expressed as ratio of retained and totally applied substance in terms of percentages [5]:

$$S\% = \frac{\text{amount of substance retained}}{\text{total amount of applied substance}} \times 100$$

Three main driving forces, which may lead to drug accumulation in keratin, have to be considered [3]. Firstly, regular distribution processes have to be mentioned, which are strictly governed by the lipophilicity of the compounds in question. Secondly and thirdly, adsorption can occur. Here, distinction is possible between specific adsorption, caused by strong physico-chemical attraction, e.g. ion-ion interactions, and, on the other hand, unspecific adsorption on the basis of van der Waals forces or hydrophobic interactions. Naturally, the latter is dependent on lipophilicity, too, so that differentiation between distribution and unspecific adsorption may be difficult. As most of the drugs, which are applied to the skin for cosmetical reasons, are to persist on or within the skin in order to delay permeation into deeper tissues or washing off, respectively, the definition of substantivity requires, that only genuine binding processes should be involved. In summary, substantivity implies skin retention on the basis of specific or unspecific adsorption.

Chandrasekaran et al. introduced the so-called 'dual sorption model' to the migration of penetrants through the microhomogeneous media of the skin [6]. The dual sorption theory postulates, that sorption occurs by two mechanisms, the first one being a simple dissolution or partitioning producing mobile and freely diffusible molecules, and the second one being an adsorption process leading to non-mobile molecules, which do not participate in the diffusion process. Accordingly, a sorption isotherm of bound versus free amounts of a penetrant will be the result of two overlapping processes: the linear dissolution or distribution and the saturation of binding sites represented by an adsorption isotherm of the Langmuir type. Thus, a distinct curvature of the graph has to become noticeable [7–9].

In a recently conducted study the transdermal in vivo fluxes of 10 compounds were investigated [10], because those fractions of the respective chemicals should be quantified, which really reach the organism. The respective compounds, their function and the used abbreviations are listed in Table 1. Fig. 1 shows typical courses of maximum fluxes J_{max}, which were measured during a time interval of 6 h. In this case, the maximum flux was determined in terms of mass disappearance from an applied saturated solution. In general, the flux is high in the beginning and then decreases to a constant level. Presumably, these pronounced initial effects are caused by those amounts of penetrants, which are required to saturate the stratum corneum. Steady state permeation rates were only achieved after a certain amount of permeant had accumulated in the skin. These findings led to the question, if the observed drug deposition in the stratum corneum can be referred to as genuine substantivity. Moreover, the underlying accumulation mechanisms should be clarified.

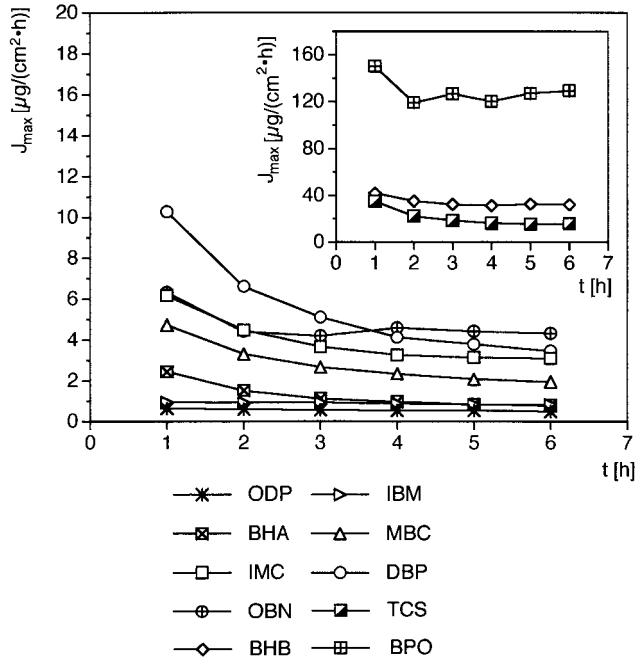

Fig. 1. Time courses of in vivo maximum fluxes J_{max} over 6 h. The inserted plot shows the profiles of the highest fluxes separately. Each point represents the geometric mean of 12 volunteers. SDs are about 30% and not included for clarity reasons.

2. Materials and methods

2.1. Chemicals

All substances (see Table 1) were obtained with a purity > 99% and were used as received. Octyldimethyl p-aminobenzoic acid, oxybenzone, 4-isopropyl-dibenzoylmethane, 3-(4-methylbenzylidene)-camphor, 3,5-di-t-butyl-4-hydroxyanisol and dibutyl phthalate, were kindly donated by E. Merck (Darmstadt, Germany), isoamyl-4-methoxycinnamate by Haarmann und Reimer (Holzminden, Germany), butyl-4-hydroxybenzoate by NIPA-Laboratories GmbH (Norderstedt, Germany), triclosane (2,4,4′-trichlor-2-hydroxydiphenylether) by Ciba Geigy GmbH (Basel, Switzerland), biphenyl-2-ol by Schülke and Mayr (Norderstedt, Germany). 1,2-Propandiol was a gift of BASF AG (Ludwigshafen, Germany). HPLC grade methanol, n-octanol and animal keratin were purchased from E. Merck (Darmstadt, Germany). Water was used freshly distilled.

2.2. Keratinous substrates

2.2.1. Human callus

The shavings, which were obtained from chiropodists,

Table 1
Comparison of keratin/vehicle partition coefficients, n-octanol/vehicle partition coefficients and solubilities in the vehicle

Compound	Abbreviation	$PC^{a}_{C/V}$	$PC^{b}_{DC/V}$	$PC^{c}_{AK/V}$	$PC^{d}_{Oct/V}$	c^{e}_{sV}
Sunscreens (INCI)						
Octyl dimethyl 4-aminobenzoic acid	ODP	805.8	351.4	2891.7	46773.5	2.1
4-Isopropyl dibenzoylmethane	IBM	933.3	293.3	2386.8	184419.4	3.1
3-(4-Methylbenzylidene)-camphor	MBC	137.7	59.7	520.2	13808.7	23.0
Isoamyl 4-methoxycinnamate	IMC	153.0	72.4	683.4	6760.8	30.1
Oxybenzone	OBN	35.7	20.9	137.7	796.9	83.7
Antioxidant						
3,5-Di-t-butyl-4-hydroxyanisol	BHA	86.7	41.3	234.6	6133.3	12.0
Repellent						
Dibutyl phthalate	DBP	66.3	34.2	571.2	2691.5	73.2
Antimicrobial compounds						
Butyl 4-hydroxybenzoate	BHB	20.4	18.4	51.0	272.6	1957.0
Triclosane	TCS	198.9	160.6	948.6	4070.7	209.4
Biphenyl-2-ol	BPO	15.3	14.3	56.1	166.4	4432.1

[a]Partition coefficient between callus and vehicle (30% propylene glycol/water mixture) at 32°C, drug concentration in callus calculated as mass ratio (m/m).
[b]Partition coefficient between delipidized callus and vehicle at 32°C, drug concentration in delipidized callus was calculated as mass ratio (m/m).
[c]Partition coefficient between animal keratin and vehicle at 32°C, drug concentration in animal keratin was calculated as mass ratio (m/m).
[d]Partition coefficient between n-octanol and vehicle at 32°C.
[e]Solubility in the vehicle at 32°C [mg/l].

were allowed to dry sufficiently by storing them in a desiccator with silica gel. Afterwards they were milled by means of a water-cooled mill (IKA Analysenmühle A 10, IKA-Werk Janke and Kunkel, Staufen i. Breisgau, Germany). The powder was classified. All sorption experiments were carried out using a fraction with a particle size < 160 μm to make sure, that equilibration is complete within 24 h.

2.2.2. Lipid-depleted human callus

In order to make differentiation possible between the contribution of both keratin and tissue lipids to the sorption processes in question, human callus was delipidized. For this 30 g of the powdered tissue were treated with 1000 ml of a chloroform/methanol mixture (2:1 v/v). This suspension was stirred for 6 h and filtered afterwards. This procedure was repeated twice with fresh solvent. The third repetition finally yielded no residue. In order to obtain the extracted tissue lipids, the solvent phases were evaporated to dryness at 40°C. The residue amounted to 8.6% of the initial tissue mass. Keratin powders and extracted lipids were stored in a desiccator with silica gel until required.

2.2.3. Animal keratin

To complete the keratin spectrum, an easily obtainable animal keratin extracted from bovine hoof and horn material was included in the experiments, too (Tierkeratin, E. Merck, Darmstadt, Germany, fraction with a particle size < 160 μm).

2.3. Hydration of keratin

In order to investigate the influence of hydration on the thermal behaviour of the used keratin species, the latter were stored at elevated relative humidity (72.5%) for 7 days. The water content of all samples was determined by Karl Fischer titration (Karl-Fischer-Titrator DL 18, Mettler AG, Greifensee, Switzerland).

2.4. Differential scanning calorimetry measurements

All keratinized substrates were characterized by DSC to track down some of the differences in composition that may exist between the selected keratins and the normal stratum corneum of human skin. About 50 mg of the keratin samples were transferred to DSC steel pans and analyzed at high sensitivity over a heating range of 0–150°C at 2 K/min (DSC 30 with TA 3000 Processor, Mettler AG, Greifensee, Switzerland).

2.5. Sorption studies

For the conducted sorption studies very simple test conditions were chosen. Increasing amounts (100, 200, 400, 600, 800 mg) of the respective keratin were slurried in 50 ml of the solutions of the test compounds in a 30% propylene glycol/water mixture, which were previously saturated at 25°C. Only the saturated solutions of BPO were used in a 1:50 dilution. The propylene glycol admixture was selected in order to increase the poor solubility of the compounds. It has to be taken into account, that in this study an increase of the keratin amount replaces the necessary concentration changes of the applied solution. The resulting suspensions of the keratins were gently agitated in a water bath at 32 ± 1°C. After 24 h, that is after equilibration, they were filtered through paper filter. Loss of compounds caused by adsorption to filter material was minimized by choosing a sufficiently high forerun. The solute concentration was measured in the filtrate either spectrophotometrically in consideration of a blank value (BHB, BPO, IMC, MBC, OBN, TCS) (Lambda 2, Perkin Elmer, Überlingen, Germany) or

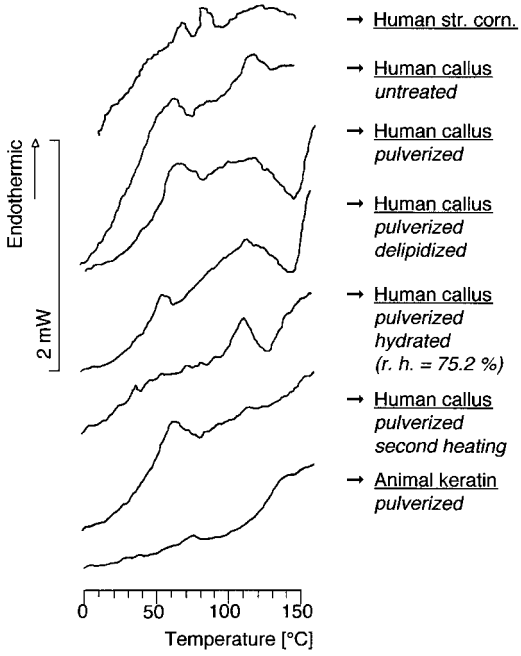

Fig. 2. DSC thermograms for different keratinous substrates and stratum corneum (if not specified otherwise, unhydrated samples).

by HPLC (BHA, DBP, IBM, ODP) (LC-6A, Shimadzu, Duisburg, Germany).

The modular HPLC unit was equipped with an automatic sample injection module (SIL-6B), system controller (SCL-6B), UV-VIS-spectrophotometer (SPD-6AV), adjustable to variable wavelengths, and an integrator (CR 4AX Chromatopac). The analyses were carried out at ambient temperature with a 123 × 4 mm column packed with LiChrospher® 100 RP-18, 5 μm-particle diameter (E. Merck, Darmstadt, Germany). The mobile phase, consisting of a 85% methanol/water mixture was pumped at flow rates ranging from 1.5 to 2.0 ml/min. Injection volumes varied between 20 and 50 μl. The concentration of the samples was calculated from peak areas by the external standard method.

The amount of substance, which accumulated in the respective keratin quantity, was calculated from the difference between initial and final solute concentration and was expressed as drug amount per 200 mg keratin.

Measuring of sorption in dependence on time revealed, that for all test substances, the drug uptake is complete within only a few hours [11].

2.6. Partition coefficients between n-octanol and 30% propylene glycol/water $PC_{Oct/V}$

An experimental determination is not possible due to the partial miscibility of the two solvents. Partition coefficients were calculated either as the ratios of the respective solubilities in n-octanol and in the 30% propylene glycol mixture. In the case of those substances, which show complete miscibility with n-octanol (ODP, IMC, DBP), they were derived by regression calculation from the n-octanol/water partition coefficients, which could be experimentally determined [10].

3. Results and discussion

3.1. Human callus and animal keratin as substitutes for stratum corneum in the sorption experiments

Fig. 2 shows a comparison of the respective DSC thermograms. Stratum corneum exhibits at least two endothermic lipid transitions during heating, the first one at about 75°C, the second at about 85°C. Both represent phase transitions of the intercellular lipid bilayers from the lamellar gel state to the liquid crystalline state [12–15]. A third endothermic peak observed at 105°C can be attributed to the denaturation of α-keratin [13,16].

Human callus, no matter whether pulverized or as shavings, shows only one lipid transition at about 60°C and a quite pronounced keratin peak at 105°C. In spite of thorough lipid extraction with a potent solvent, the lipid peak diminishes perceptibly but does not disappear completely. This phenomenon has already been observed by other authors and usually is attributed to lipids covalently bound to the cell envelope proteins [12,13].

As expected, hydration results in a more pronounced protein denaturation peak and, in addition, disappearance of the lipid peak because of lipid fluidization. Table 2 shows the water contents of the keratin samples after storage over silica gel and at 72.5% r.h., respectively. Lipid depletion decreases the water uptake of callus. Obviously, the lipid lamellae contribute considerably to water absorption. Animal keratin shows the lowest water content after hydration.

Reheating of the samples does not affect the lipid peak and indeed shows the expected missing of the keratin denaturation. The used animal keratin was obtained by a denaturating manufacturing process. Accordingly, in the respective thermogram a keratin peak cannot be detected.

Via the phase transition enthalpy, i.e. the area below the peak of the extracted callus lipids (Fig. 3), quantification of the lipid contents of human callus is possible. The calculated value of about 10% is in good agreement with values reported on lipid fractions of human stratum corneum, as well as with the gravimetrically determined fraction of the extracted skin lipids (about 8.6%).

Summarizing these findings, it can be concluded, that the lipid phases of normal stratum corneum sheets compared to

Table 2

Water contents of keratin samples after storage at two different humidities

	% H_2O after storage over silica gel	% H_2O after storage at 72.5% r.h.
Callus	3.4	16.0
Delipidized callus	4.1	13.0
Animal keratin	3.3	8.8

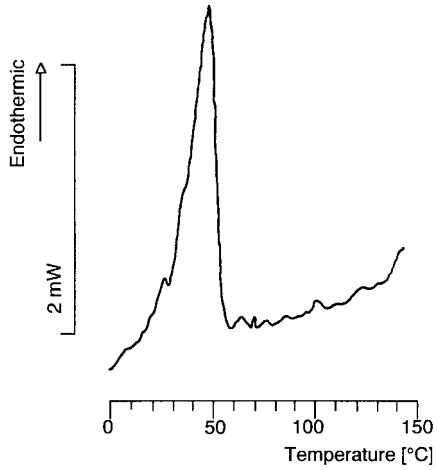

Fig. 3. DSC thermogram for lipids extracted from human callus.

those of callus certainly show some structural differences, while the thermal protein behaviour permits the assumption of a close resemblance between the selected keratin species. Consequently, it seems tolerable for the sorption studies to substitute ordinary stratum corneum by human callus.

3.2. Sorption studies with different keratinous substrates

Fig. 4 shows the calculated drug amounts, which were bound by 200 mg of powdered human callus as a function of those amounts, which remained in 100 ml of the applied solution. In each case a linear relationship between bound and free amounts of the respective agent could be established, the slope of the straight line representing the constant ratio of both variables in terms of a partition coefficient between keratin and vehicle. Similar sorption isotherms were found with animal keratin and delipidized callus, but will not be presented here.

Hyperbolic interrelations, which were postulated by the dual sorption model for processes, in which specific adsorption is involved, cannot be confirmed. These findings correspond to results reported by other authors [1,5,17,18]. Accordingly, the uptake of the 10 test compounds by keratinous substrates has to be considered as partitioning-like and non-saturable, which of course has to be restricted to the investigated concentration range.

From the slopes of the sorption isotherms of the three keratin species (callus (C), delipidized callus (DC), animal keratin (AK)) the respective keratin/vehicle partition coefficients $PC_{K/V}$ ($PC_{C/V}$, $PC_{DC/V}$, $PC_{AK/V}$) could be calculated. These were correlated with the respective octanol/vehicle partition coefficients $PC_{Oct/V}$ in consideration of the regression method postulated by Collander [19]. The $PC_{Oct/V}$ can be regarded as parameters measuring lipophilicity and are widely used to predict the behaviour of drugs towards biological tissues. Especially the polarity of human stratum corneum is often reported to be similar to that of octanol. The determined $PC_{Oct/V}$ cover an interval of four orders of magnitude. Corresponding to the previous results, it does not come as a surprise, that linear relationships for all three substrates were found (Fig. 5). This observation corresponds to that of other authors [1,17,20]. The low regression coefficients ranging from 0.47 to 0.62 indicate the inferior lipophilicity of keratinous substrates in comparison to octanol, which obviously is a less appropriate medium to simulate the polarity of the human horny layer. As expected, delipidized callus (DC) as the most hydrophilic substrate shows the lowest slope. The lipophilicity of animal keratin (AK) exceeds that of human callus.

Actually, keratin affinities of the investigated substances are dependent on lipophilicity. In addition, a fairly close

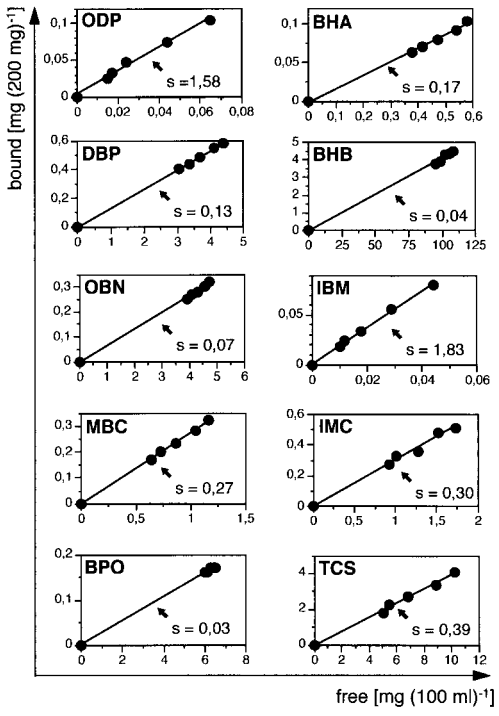

Fig. 4. Sorption isotherms, accumulation of the test compounds in human callus.

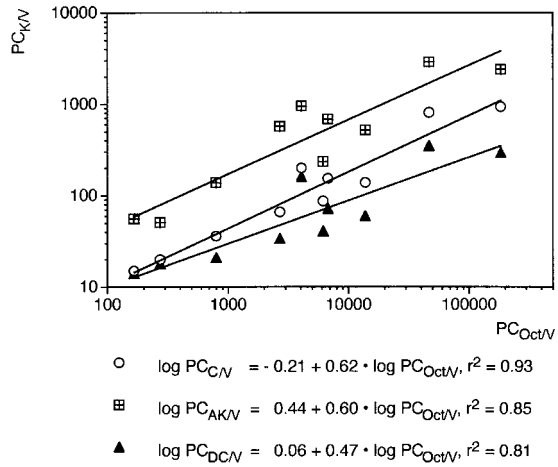

Fig. 5. Relationship between different keratin/vehicle partition coefficients $PC_{K/V}$ and n-octanol/vehicle partition coefficients $PC_{Oct/V}$.

inverse relationship can be observed between the logarithms of the callus/vehicle partition coefficients $PC_{C/V}$ and of the solubilities in the vehicle c_{sV} (Fig. 6). However, the partition parameters do not correspond adequately to the calculated solubility in callus. Accordingly, high keratin affinities seem to be predominantly a consequence of low solubilities in the vehicle. Similar results have already been published [1,21]. In summary, such findings confirm, that specific adsorption caused by strong attraction forces cannot be of major importance for the accumulation processes under investigation.

Table 1 presents a comparison of the different partition coefficients and the solubilities in the vehicle. Differences between the $PC_{Oct/V}$ and $PC_{C/V}$ are at least about two orders of magnitude. Obviously, human keratin is a significantly more polar substrate than n-octanol. Animal keratin as a substrate overestimates the lipophilicity or sorption ability of untreated and delipidized human keratin by one order of magnitude. It does not seem to be appropriate to substitute a respective human tissue. Generally, delipidization changes the keratin affinity only to a moderate extent. Accordingly, reservoir formation in keratinized substrates seems to be mainly governed by proteins, which of course, represent the major weight fraction of about 40%. Interestingly enough, hydrophilic compounds are rather insensitive to delipidization. In the case of the more lipophilic compounds, however, lipid extraction leads to a measurable decrease of keratin affinity. Probably, these substances reside in both the intercellular lipid lamella and the corneocytes, whereas the more hydrophilic agents seem to prefer the protein domains. Besides, it has to be taken into account, that although the protein domains may contribute significantly to solute uptake into the skin, steady state transport may proceed predominantly by a lipid pathway [22].

As shown by Fig. 7, steady state permeabilities (P), which were deduced from results of the previously conducted in vivo penetration experiments [10], correlate excellently with the determined callus/vehicle partition coefficients. As the slope of the straight line is below unity, normal (hydrated) stratum corneum has to be considered as an even slightly more hydrophilic substrate than callus.

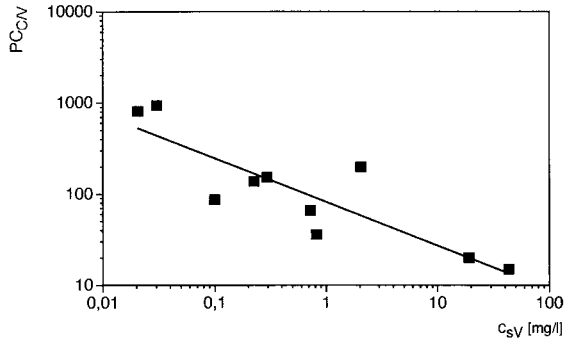

Fig. 6. Dependence of the partition coefficient between human callus and the vehicle $PC_{C/V}$ on the solubility in the vehicle c_{sV}. Regression equation: $\log PC_{C/V} = 2.40 - 0.58 \times \log c_{sV}$, $r^2 = 0.75$.

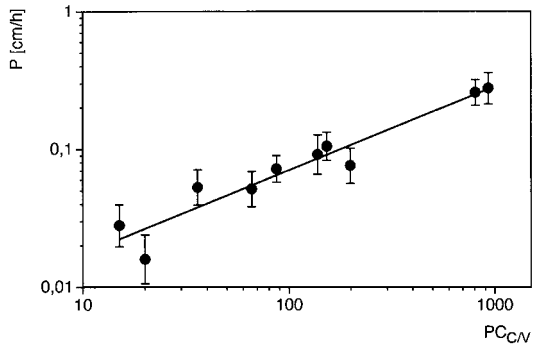

Fig. 7. Relationship between in vivo permeabilities (P) and callus/vehicle partition coefficients $PC_{C/V}$. Geometric means ± SD ($n = 12$). Regression equation: $\log P = -2.36 + 0.61 \times \log PC_{C/V}$, $r^2 = 0.92$.

In each case, however, callus is a suitable substrate to simulate and quantify solute uptake into human skin.

4. Conclusions

In the concentration range investigated the affinity towards the respective keratin is independent on the applied drug concentration. Moreover, the accumulation is predominantly governed by the lipophilicity of applied agents. Therefore, either distribution and/or unspecific adsorption have to be considered as underlying uptake mechanisms.

Tissue lipids only contribute significantly to the uptake of very lipophilic solutes (with an octanol/vehicle partition coefficient exceeding 1000). More hydrophilic substances reside preferably in the protein domains.

As the model lipid octanol as well as animal keratin are distinctly less polar in nature than human keratin, they tend to overestimate the reservoir function of human stratum corneum. Actually, human callous tissue is a more appropriate substrate for binding experiments.

Deduced from Fick's first law, maximum fluxes through the skin, which can be achieved after application of a saturated vehicle, are basically determined by four factors: the diffusivity in the stratum corneum, the thickness of this horny layer, the lipophilicity of the respective drug in terms of a partition coefficient and the solubility in the vehicle. As the first two parameters generally vary only within rather moderate limits, especially the partition behaviour between skin and vehicle as well as the solubility in the applied vehicle determine the extent of skin penetration. If both factors show high values, a considerable quantity of the respective agent will enter the systemic circulation. However, pronounced skin penetration may cause uptake of an alarming drug quantity by the organism, especially, if large areas of the skin are treated with the respective preparation. On the other hand, in the case of UV-absorbing agents, a certain amount of substance is to accumulate within the skin after all, because a dense light-absorbing drug layer shall be built up within the upper skin layers, which, at the same time, will guarantee water resistance.

After the initial accumulation process is complete, diffusion of the permeant into the stratum corneum balances the respective transport out of it into the viable tissue for a certain period of time. Subsequently, the stratum corneum may be very rapidly depleted. As in this case only very low amounts of the applied agents can be re-extracted by water exposure, a high degree of substantivity and retention on exposure to water may be only pretended.

The only answer to solve the problem of poor water resistance seems to be the development of sunscreens, which exhibit specific adsorption during diffusion through the skin. First attempts have been made to introduce groups with positive electric charges into UV-absorbing molecules [4,23–26]. These functional groups are able to interact strongly with negative charges of the protein chains of the keratin. Consequently, a certain fraction of such molecules will be retained by the upper skin layers at least for a certain period of time.

At the moment, however, none of the investigated sunscreens meets the requirement of genuine skin retention and water resistance.

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