Albumin Effects on Drug Absorption and Metabolism in Reconstructed Epidermis and Excised Pig Skin

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Summary

To replace animal experiments for the risk assessment in cutaneous absorption European and Non-European regulatory authorities urge to develop in vitro test systems. A variety of methods have been established which, however, vary in several respects, such as the nature of the barrier and used media. Since both affect drug uptake we have compared the influence of albumin (BSA) in the acceptor medium on the permeation and metabolism of highly lipophilic steroidal drugs, testosterone and prednicarbate (PC). Surprisingly, the addition of BSA to the acceptor medium slightly reduced the steroid permeation, especially when formulations of poor PC uptake were tested. Moreover, with slow drug permeation the metabolite pattern changed as compared to PC metabolites to be found in albumin-free acceptor medium. This was clearly less the case with PC incorporated into newly developed solid lipid nanoparticles accelerating PC uptake about fourfold. The penetration of testosterone was not influenced by BSA in the acceptor medium.

Summarising, these results contribute essentially to the development of appropriate in vitro methods for testing of the cutaneous absorption of drugs, ingredients of cosmetics and for the risk assessment of xenobiotics, pesticides and biozides.

Zusammenfassung: Zum Einfluss von Albumin auf Arzneistoffresorption und -metabolismus bei rekonstruierter Epidermis und Schweinehaut

Europäische und außereuropäische Behörden stimulieren die Entwicklung geeigneter in vitro Testverfahren, um die derzeit üblichen Tierexperimente zur Risikoabschätzung der kutanen Resorption von chemischen Substanzen zu ersetzen. Mehrere Methoden sind bereits etabliert worden, die jedoch vielfach, wie z.B. in der verwendeten Barriere oder den Medien, variieren. Da diese Faktoren die Resorption wesentlich beeinflussen, wurde der Einfluss von Albumin im Akzeptormedium auf die kutane Permeation und den Metabolismus von lipophilen steroidalen Arzneistoffen, Prednicarbat (PC) und Testosteron, getestet. Überraschenderweise reduzierte der Zusatz von BSA zum Akzeptormedium die PC Aufnahme geringfügig, insbesondere bei der Testung von Zubereitungen, die zu einer geringen Wirkstoffaufnahme führten. Dann trat zudem eine Veränderung im Metabolitenspektrum auf. Die Kinetik von PC inkorporiert in feste Lipidnanopartikel, die die Penetration deutlich erhöhen, unterlag keinerlei Veränderungen durch den Zusatz von BSA im Akzeptormedium. Auch die Testosteronaufnahme wurde durch BSA nicht beeinflusst.

Zusammenfassend kann festgestellt werden, dass diese Ergebnisse essentiell zur Entwicklung geeigneter Testverfahren zur Risikobewertung von Substanzen im Hinblick auf deren kutane Resorption beitragen. Dies gilt gleichermaßen für Arzneistoffe und Bestandteile von Kosmetika wie für eine Risikobewertung von Xenobiotika, Pestiziden und Bioziden.

Keywords: human skin models, experimental conditions, cutaneous metabolism, albumin effects, glucocorticoids, prednicarbate, testosterone

1 Introduction

Investigative new drugs require intensive preclinical testing before introduction into human studies. With respect to innovative topical dermatics this includes the investigation of cutaneous uptake. This is also indispensable for risk assessment of chemicals, pesticides and ingredients of cosmetics. Excised skin of animal or human origin, reconstructed human epidermis and sometimes simply synthetic membranes serve for *in vitro* testing to evaluate penetration and permeation, thereby in part replacing animal experiments (Zghoul et al., 2001; Barber et al., 1992; Kitagawa and Li, 1999; Gysler et al., 1999b; Wagner et al., 2001). Moreover, more complex perfusion models have been described (Bäumer and Kietzmann, 2000). While viable skin and reconstructed epidermis are able to metabolise drugs (Gysler et al., 1999a), non-viable models such as heat-separated epidermis prepared from cryoconserved skin or synthetic membranes are

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not. Whether drug metabolism effects cutaneous penetration and permeation and thus is of relevance for *in vitro* testing is a matter of current debate. While many scientists consider cutaneous metabolism to be of minor importance if at all (Diembeck et al., 1999) others report on drug metabolism to enhance uptake (Howes et al., 1996). The recent OECD draft guideline for *in vitro* testing of dermal uptake (OECD, 2000) favours the use of living skin because of the still active drug metabolising enzymes.

Besides the variety of test models, *in vitro* studies make use of other different experimental conditions including the media. Physiologic media, especially the addition of bovine serum albumin (BSA) or fetal calf serum (FCS) increase the integrity and viability of the skin and skin equivalents (Kratz, 1998). Moreover, BSA has been reported to increase cutaneous permeation of lipophilic drugs (Howes et al., 1996). Increased costs, however, make the use of these ingredients less attractive for routine testing. To evaluate a possible influence of the acceptor medium and of metabolism in viable skin (reconstructed epidermis) on the permeation/penetration, we have studied the effects of BSA addition. Since most pronounced effects are to be expected with lipophilic agents we have focused on two lipophilic model drugs, PC and testosterone, both known to be metabolised in skin (Gysler et al., 1997; Gysler et al., 1999a; Fritsch et al., 2001). The glucocorticoid double ester PC has been tested in different formulations known to influence drug uptake considerably (Maia et al., 2000; Santos Maia et al., 2002). The influence of BSA addition on PC metabolism and on penetration is compared to the effects on testosterone uptake by split pig skin.

2 Materials and methods

2.1 Materials

Prednicarbate (PC, log P = 3.83), its metabolites prednisolone 17-ethylcarbonate (P17EC), prednisolone 21-ethylcarbonate (P21EC) and prednisolone (PD, log P = 1.69) as well as prednicarbate 0.25% cream (Dermatop® Creme) were donated by Aventis (Frankfurt/M., Germany). ^{3}H-Testosterone (100 Ci/mmol) was supplied by Amersham Biosciences (Freiburg, Germany), non labelled testosterone (log P = 3.47) was from Sigma (Deisenhofen, Germany).

2.2 Preparation and characterisation of solid lipid nanoparticles (SLN)

PC-SLN were prepared according to Jenning et al. (1999) and zur Mühlen et al. (1998). Particles made from 5% Compritol, 2.5% Poloxamer 188 and 0.125% PC are very well tolerated by reconstructed epidermis (Santos Maia et al., 2002). Particle size analysis was performed by dynamic light scattering using a Zetasizer (Malvern Zetasizer IV, Malvern Instruments, Malvern, UK).

2.3 Preparation of ^{3}H-testosterone solutions

Ethanolic solutions of 1% testosterone containing 120,000 counts of 1,2,6,7-^{3}H-testosterone were prepared by the respective volume of a radioactive labelled testosterone solution. After evaporation of the solvent the residue was dissolved in ethanol containing the appropriate amount of non labelled drug.

2.4 Reconstructed epidermis

Reconstructed epidermis was purchased from SkinEthic™ (Laboratoire SkinEthic, Nice, France). The quality of the horny layer barrier was regularly checked by measuring the transepidermal water loss (TEWL; Evaporimeter EP1, Servomed, Kinna, Sweden). To be included into the experiments, TEWL had to be < 15 g/m^2 h.

Cellular viability of the reconstructed epidermis during the experiment was proven by monitoring the LDH activity. Results from permeation studies with skin showing LDH activities above 10 U/l were excluded from the evaluation. LDH was measured using routine tests of clinical chemistry.

2.5 Excised pig skin

Pig skin was excised from the leg of female pigs of the breed *Deutsche Landrasse Hybride* at a local slaughter house being able to avoid the procedure of soaking the cadaver in boiling water. The pigs were slaughtered at an age of 5-8 month, corresponding to a weight of 60-100 kg. The skin was transported in cell cultivating medium (DMEM) containing additions of 1 µg/ml gentamicin and 0.8 µg/ml vancomycine. Split skin (1 mm) was prepared and used at the same day (Dermatome GA 630, Aesculap AG, Tuttlingen, Germany). Quality and viability (TEWL, LDH) were tested before and during the experiment as described for reconstructed epidermis.

2.6 Cutaneous uptake

Permeation studies were performed using Franz flow-through diffusion cells of 9 mm diameter (Crown Scientific, Somerville, NJ). Reconstructed epidermis (plus the supporting membrane) or porcine skin was placed between donor and acceptor compartment the lower site of the skin equivalent, the supporting membrane, or of the dermis making contact with acceptor medium consisting of MEME (with or without 5% BSA) supplemented with 20 µg/ml gentamicin, 50 ng/ml amphotericin B, 2 mM glutamine and 0.1% glucose. The tissue was equilibrated with acceptor medium (flow 6 ml/h, 37°C) for 30 min. During the equilibration period and once more after the end of the penetration period of 6 h medium was removed for LDH measurement. Following drug application acceptor medium was collected in fractions of 1 h for drug analysis. To avoid hydrolysis of prednisolone esters in the acceptor medium the fluid was collected in tubes containing 20 mg NaF for enzyme inhibition (Gysler et al., 1999a). After 6 h the skin was removed and wiped twice with cotton balls soaked in 70% ethanol. The reconstructed skin was striped twice with Tesa™ tape, while excised pig skin was striped four times. From 9 mm punches of pig skin two 100 µm horizontal slices were cut by a freeze-microtome (Frigocut 2800 MN, Leica, Bensheim, Germany).

2.7 Drug analysis

Native PC and its metabolites were quantified in the acceptor fluid by ethyl acetate extraction and HPLC; betamethasone served as internal standard (Gysler et al., 1999a; Gysler et al., 1997). The ethyl acetate extract was centrifuged and exsiccated by vacuum rotation. The

residue was dissolved in methanol, centrifugation and exsiccation were repeated. Following dissolution in 100 µl methanol, 50 µl were injected to RP HPLC (LaChrom™, Merck-Hitachi, Darmstadt, Germany). The recovery for PC and its metabolites was estimated for the different acceptor media. The following data were obtained for media without and with BSA addition, respectively: for PC 83.7% and 79.8%, for P17EC 101.8% and 102.6%, for P21EC 98.8% and 101.4%, and for PD 84.9% and 85.9%.

9 mm punches of the epidermis corresponding to the diffusional area were cut in small pieces and subjected to ultra turrax extraction (3 times 10 sec each) in ethyl acetate. Centrifugation and exsiccation followed as described above.

^{3}H-testosterone was quantified in skin slices of a depth as indicated in the table legend, cut in small pieces by extraction into methanol/PBS (70/30 v/v) for 1 h. Then testosterone was transferred from methanol/PBS into ethylacetate. The solvent was removed and evaporated. The residues were dissolved in PBS, szintillation cocktail (Optiphase SuperMix, Wallac, Turku, Finland) was added and testosterone was measured using a 1450 Microbeta Plus counter (Wallac, Turku, Finland).

2.8 Data analysis and statistics

All experiments were run threefold, using three different skin batches. All data are presented as arithmetic mean values ± standard deviations ($\bar{x}$ ± S.D.). For individual experiments cumulative drug, metabolite, and total PC (sum of native drug plus metabolites) amounts in the acceptor medium were calculated. Then the values (3) at each time point served to determine $\bar{x}$ ± S.D. for PC and metabolites as well as total PC. Tissue levels give $\bar{x}$ ± S.D. of data from three different batches, too.

3 Results

3.1 Influence of BSA on PC permeation

First, the influence of BSA in the acceptor medium was tested with respect to the total amount of permeated PC (plus metabolites) comparing PC-cream and the SLN preparation. With the latter, there is a fourfold increase in PC uptake (Santos Maia et al., 2002). As depicted in Figure 1, BSA addition to the acceptor medium did not favour total drug permeation.

3.2 Epidermal PC metabolism

Investigating PC metabolism in human keratinocyte cultures and human skin, we described the pathway as follows: The prednisolone 21-ester is rapidly cleaved by epidermal enzymes present in high amounts. The resulting P17EC rapidly rearranges by acyl migration forming thermodynamic more stable P21EC which once more is cleaved by esterases to the final metabolite prednisolone (Gysler et al., 1999a; Gysler et al., 1997).

Looking at the metabolite pattern, BSA effects on epidermal PC metabolism become obvious (Fig. 2). Following PC-cream cumulative amounts of PC and its metabolites in the protein-free acceptor medium (Fig. 2A) demonstrated an essentially complete metabolism since prednisolone (51% of the total permeated drug) dominated in the acceptor medium.

The monoesters make up another 40.5% (P17EC 34%, P21EC 6.5%) while only 8.5% of glucocorticoids present was the native PC. The results clearly demonstrate the high hydrolytic activity of the reconstructed epidermis. BSA addition to the acceptor medium improving the solubility of highly lipophilic drugs appeared to interfere with PC metabolism. As to be seen from Figure 2B, the formerly high amount of PD decreased dramatically to only 1.8% of the total recovery. In parallel P21EC and native PC increased to 43% and 39%, respectively. Therefore BSA appears to influence PC permeation/metabolism by an increased washing out of the lipophilic monoesters and PC (98% with vs. 49% without BSA) from the tissue.

With PC formulations improving drug release (PC-SLN; Santos Maia et al., 2002) drug concentrations in the acceptor medium were clearly less influenced by BSA addition (Fig. 2C, D). The relation of PC and metabolites was close to the one seen with the PC-cream following BSA addition to the acceptor medium. Therefore, rapidly increasing PC concentrations in the skin may overwhelm epidermal hydrolytic enzymes. Results are summarised in Table 1.

3.3 Influence of BSA on PC penetration

HPLC analysis of epidermal extracts revealed high concentrations of native PC and lower ones of the monoesters (Tab. 2).

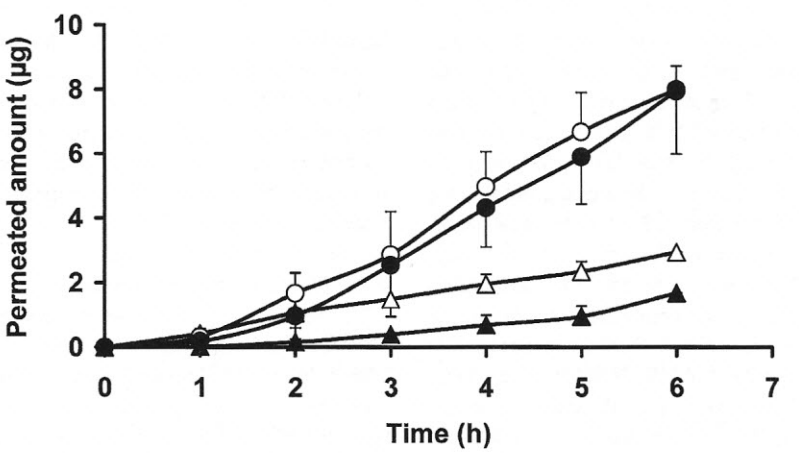

Fig. 1: Cumulative permeation of total PC (native drug plus metabolites) through reconstructed epidermis following the topical application of 125 µg PC incorporated into o/w cream (triangles) and SLN (circles). Permeation was determined by Franz flow-through cells. Open symbols represent acceptor medium without BSA, closed symbols represent acceptor medium containing 5% BSA (n=3, mean ± S.D.).

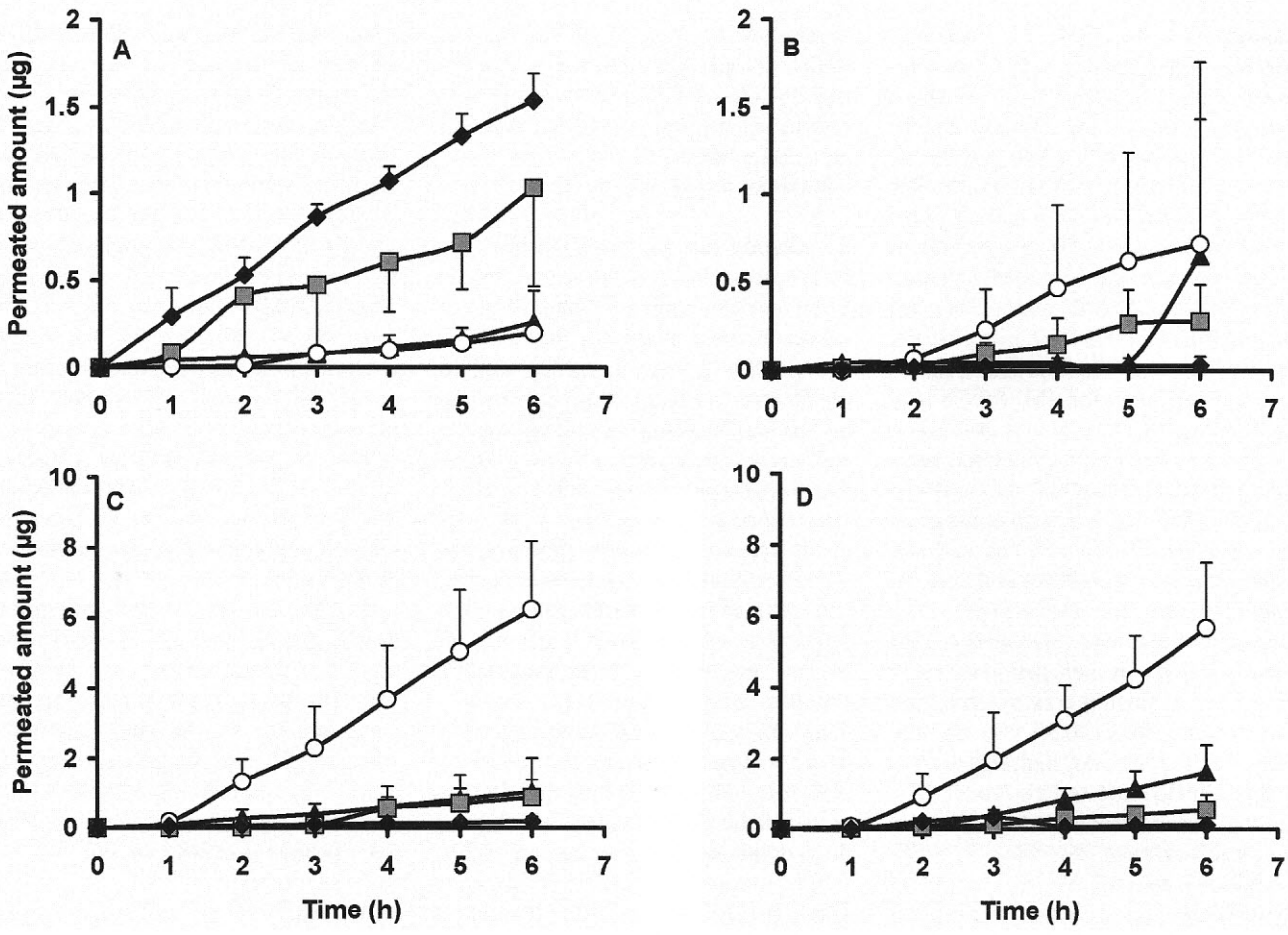

Fig. 2: Cumulative permeation of PC and its metabolites through reconstructed epidermis. A cream (A and B) or a SLN dispersion (C and D) containing 125 µg PC was applied to the top of the skin. PC (▲) and its metabolites P17EC (■), P21EC (○) and PD (■) were determined in A, C: BSA-free acceptor medium. B, D: acceptor medium containing 5% BSA (n=3, mean ± S.D.).

As to be expected, using BSA-free acceptor medium glucocorticoid uptake was less with PC-cream (0.44 µg in total) compared to PC-SLN (1.56 µg). Following the cream only native PC was detectable. PC-SLN improving total drug uptake 3.5 fold, 33% of the total penetrated drug was recovered as P17EC suggesting a non saturated metabolisation capacity of the keratinocytes. Due to the low levels of the P21EC precursor PD was not detected at all. Moreover, viable epidermis directly contacting the acceptor medium facilitated PD to enter the acceptor medium.

Irrespective of the formulation, addition of BSA to the medium appeared to enhance epidermal concentration although the increase failed to be significant (Tab. 2). Increased tissue levels, however, were not reflected by glucocorticoid concentrations in the acceptor medium (Tab. 1). Total PC recovery from the acceptor medium containing BSA was reduced even by 1.34 µg and 0.35 µg following the cream and SLN preparation, respectively.

3.4 Influence of BSA on the penetration of testosterone into excised pig skin

The influence of BSA on the penetration and permeation of split pig skin (1 mm) was tested using an ethanolic testosterone solution which was dotted with ^{3}H-testosterone. Following the application of 0.1 mg testosterone and using the most sensitive assay procedure, no permeation has been observed neither with nor without the addition of 5% BSA to the acceptor medium.

Quantifying the penetrated amount of testosterone by measurement of ^{3}H-testosterone in the skin a difference between the experiments run with or without addition of 5% BSA was not detected either (Tab. 2). In total 2.08% and 3.37% (without strips; including strips 4.54% and 4.9%) of the applied drug was recovered from the tissue when the experiment was run in the absence and presence of BSA, respectively.

4 Discussion

Facing the still increasing use of new drugs and cosmetics applied to the skin, but also the extensive production and use of chemicals and pesticides which may

induce systemic toxicity following contact to the skin, there is a strong need for alternative testing methods. Only then will it be possible to obtain sufficient information on possible hazards while saving animal experiments during development. Since there is a lack of knowledge on the pros and cons of the various techniques described, there is a strong need to compare the results of the different experimental approaches. Most often cutaneous absorption is studied in excised pig skin, excised human skin or reconstructed human epidermis. Non physiologic buffer systems frequently serve as acceptor medium, and drug metabolism in the skin is often ignored. Xenobiotics, however, can undergo metabolic transformation in human skin (Gysler et al., 1999a; Gysler et al., 1997; Baron and Merk, 2001). *In vitro* cutaneous metabolism is due to tissue viability (OECD, 2000) which is influenced by the acceptor medium ingredients. Pro-

Tab. 1: PC permeation and metabolism in reconstructed epidermis determined by Franz flow-through cells (n=3, mean ± S.D.).

	Glucocorticoid	permeated amount (µg)	% recovery of applied drug	% of total recovery	monoester/PD ratio
PC-cream					
BSA-free	PC	0.258 ± 0.182	0.21 ± 0.15	8.5 ± 6.0	
	P17EC	1.028 ± 0.563	0.82 ± 0.45	34.0 ± 18.6	
	P21EC	0.198 ± 0.017	0.16 ± 0.01	6.5 ± 0.6	
	PD	1.537 ± 0.155	1.23 ± 0.12	50.8 ± 5.1	0.79
	Total drug	**3.021 ± 0.005**			
5% BSA	PC	0.651 ± 1.107	0.52 ± 0.08	38.7 ± 65.8	
	P17EC	0.281 ± 0.206	0.22 ± 0.16	16.7 ± 12.2	
	P21EC	0.718 ± 0.240	0.57 ± 0.19	42.7 ± 14.3	
	PD	0.031 ± 0.049	0.02 ± 0.04	1.8 ± 2.9	32.5
	Total drug	**1.681 ± 0.727**			
PC-SLN					
BSA-free	PC	1.020 ± 0.363	0.82 ± 0.29	12.3 ± 4.4	
	P17EC	0.867 ± 1.138	0.69 ± 0.91	10.4 ± 13.7	
	P21EC	6.243 ± 1.944	4.99 ± 1.55	75.1 ± 23.4	
	PD	0.183 ± 0.139	0.15 ± 0.11	2.2 ± 1.7	38.8
	Total drug	**8.313 ± 0.735**			
5% BSA	PC	1.623 ± 0.763	1.29 ± 0.61	20.4 ± 9.6	
	P17EC	0.546 ± 0.418	0.44 ± 0.33	6.8 ± 5.2	
	P21EC	5.674 ± 1.843	4.54 ± 1.47	71.2 ± 23.1	
	PD	0.120 ± 0.056	0.09 ± 0.04	1.5 ± 0.7	51.8
	Total drug	**7.963 ± 1.963**			

Tab. 2: Penetration into split pig skin and reconstructed epidermis following the application of 0.1 mg testosterone for 6 h and the PC-formulations. Acceptor medium without BSA or with addition of 5% BSA (n=3, mean ± S.D., except PC-cream, tissue, BSA-free n=1).

drug	tissue		drug penetration (µg) BSA-free medium	drug penetration (µg) 5% BSA containing medium
testosterone	**porcine skin**	**(mm)**		
		0.0 - 0.1	0.484 ± 0.113	0.609 ± 0.078
		0.1 - 0.2	0.384 ± 0.109	0.446 ± 0.131
		0.2 - 1.0	2.510 ± 1.688	1.020 ± 0.164
PC-cream	**reconstructed epidermis**	**PC**	0.444	0.829 ± 0.747
		P17EC	0	0.250 ± 0.253
		P21EC	0	0.007 ± 0.008
PC-SLN		**PC**	0.877 ± 0.082	2.130 ± 2.450
		P17EC	0.515 ± 0.006	0.725 ± 0.694
		P21EC	0.165 ± 0.208	0.027 ± 0.029

teins present in the acceptor medium may also interfere with the solubility of lipophilic drugs, yet currently to an unknown extent. Favouring drug transport from the skin to the acceptor medium, protein binding may also influence cutaneous metabolism.

To improve the knowledge on the influences of these experimental conditions we have investigated absorption and metabolism of a glucocorticoid double ester (PC) and testosterone using albumin free and enriched acceptor media. Besides this, we have studied PC drug formulations known to vary with respect to cutaneous absorption (Santos Maia et al., 2002). Despite of PC (log P = 3.83) and testosterone (log P = 3.47) lipophilicity BSA did not increase the total amount of permeated drug. This result was expected with split pig skin which in previous experiments turned out to be almost impermeable in the short time range. Yet the reduced glucocorticoid permeation of reconstructed epidermis by BSA addition to the acceptor medium was surprising (Fig. 1). Moreover, the total PC permeation was clearly less reduced at the BSA containing medium with the SLN preparation which favours cutaneous uptake and permeation. PC concentrations in the acceptor medium (≤ 0.5 µM) were clearly below PC solubility in water (≤ 80 µM, Data sheet Prednicarbate, Aventis) excluding solubility as a limiting parameter. Since solvent extraction of steroids was not influenced by BSA, artefacts due to analytical errors were excluded, too.

Albumin also influenced the glucocorticoid pattern recovered from the acceptor medium. With PC-cream we observed an increased washing out of the lipophilic monoesters and PC by BSA addition, whereas the less lipophilic PD (log P = 1.69) dominated in the BSA-free acceptor medium. Due to the NaF addition to the acceptor fluid, hydrolytic enzyme activity was confined to the tissue excluding the cleavage of permeated PC and P21EC while the non-enzymatic ethylcarbonate migration of the P17EC monoester to form the P21EC monoester was still possible. Consequently, PD esters released from the tissue by BSA accumulated and P17EC rearranged further increasing the P21EC concentration. Moreover, we know from independent experiments that BSA increases the solubility of PC about 5 fold, of P21EC 1.5 fold, P17EC 2.3 fold and of PD about 1.16 fold. With PC-cream PC and P21EC increased about 1.56 fold and 3.6 fold, respectively, while the concentration of P17EC and PD decreased in the BSA-containing acceptor fluid. Therefore, increased solubility due to BSA binding in acceptor medium is obviously not the cause for the changes in the metabolite pattern. The changes of the metabolite pattern clearly exceed the standard deviations of the experiments and therefore should be system immanent.

Albumin effects on PC permeation and metabolism are reflected by the PC-monoester (P17EC plus P21EC)/PD-ratio (Tab. 1). The low ratio of PC-cream using BSA-free acceptor medium of 0.8 increased about fortyfold when 5% BSA is added. The improved PC penetration from the SLN preparation with a high ratio of 38.8% (BSA-free) increased only to 51.8% (5% BSA), demonstrating the influence of BSA on the permeation and ester hydrolysis to be more pronounced with a low PC uptake.

As described, the permeated amount declined with both PC-formulations (cream and SLN) due to BSA addition. This suggests an experimental parameter other than drug binding to BSA in the acceptor fluid to be of relevance. Since the SkinEthic® skin model is grown on a polycarbonate membrane with a pore diameter of 0.2 µm, BSA may penetrate this membrane. It may be speculated that glucocorticoid permeating the epidermis may become bound to BSA and therefore confined to the membrane. This restrains other drugs from leaving the tissue, thereby increasing "tissue" levels and decreasing the concentration in the fluid. The close absolute reduction with both PC-formulations (cream: 1.34 µg; SLN: 0.35 µg) is explainable by the limited number of BSA binding sides within the polycarbonate membrane which is saturable at higher drug levels. Further experiments comparing skin models without and with supporting membranes of differing nature are needed to test this hypothesis.

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Abbreviations

BSA: bovine serum albumine, LDH: lactate dehydrogenase, log P: logarithm of the octanol/water distribution coefficient, PC: prednicarbate, PD: prednisolone, P17EC: prednisolone17-ethylcarbonate, P21EC: prednisolone 21-ethylcarbonate, SLN: solid lipid nanoparticles, TEWL: transepidermal water loss