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Binding of primaquine to epidermal membranes and keratin

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Abstract

The localisation of primaquine was studied within epidermal membranes following the application of a topical dose. A depth profile was constructed by tape-stripping human epidermis following permeation of a 70 mg ml^{-1} solution of primaquine in Miglyol 840. Comparative binding studies of primaquine were carried out on isolated human stratum corneum and whole epidermis, using normal and delipidised tissue. An additional study was undertaken using bovine keratin powder as a model of human keratin. The depth profile showed that primaquine decreased with depth and decreasing keratin content, and the total primaquine recovered (15.5 mg cm^{-2}) was 300× the amount of extractable lipid. Binding to delipidised skin was saturable, whereas binding to normal skin was unsaturable, reflecting the high miscibility of drug in the lipid domains as opposed to a finite, but large number of binding sites on the corneocytes. Binding was greater for stratum corneum than stratum corneum plus viable epidermis, probably due to greater accessibility of corneocytes keratin. Binding was dose dependent, although binding to delipidised skin was far greater than to normal skin, demonstrating that primaquine had an affinity for lipoidal regions and an even higher affinity for the proteinaceous domains of the stratum corneum. This was supported by high saturable levels of primaquine binding to bovine horn keratin. The results indicated extensive binding to corneocyte keratin has a significant effect on reservoir formation and the permeability of primaquine across human skin. It is speculated that the large amount of keratin presented at the skin surface may be an evolutionary protective process for the sequestration of ingressing molecules.

Keywords: Keratin; Skin; Stratum corneum; Primaquine

1. Introduction

Primaquine is an anti-malarial drug that has previously been observed to permeate skin with relatively high fluxes in vitro from a Miglyol 840 (M840) vehicle across both hairless rat skin (Mayorga et al., 1996) and human skin (Morris et al., 1998). In addition, high flux across human skin was observed in a subsequent evaluation involving primaquine applied as drug-in-adhesive patches (Jeans and Heard, 1999). During the course of our investigations it was noted, upon dismantling the diffusion cells after termination of the permeation experiments, that the diffused areas were deeply stained red—the colour of primaquine—indicating significant drug retention (Walter and Kurz, 1988). The issue of penetration mechanism is recurrent within the field of transdermal drug delivery and current opinion dictates that the major factor involves the diffusion of permeant molecules through the narrow channels between adjacent corneoctyes of the stratum corneum. Interactions with protein, predominantly keratin, which accounts for 95% of the stratum corneum would not be expected to be a significant factor, especially for a lipophilic drug. However,

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apparent saturation of the skin tissue with primaquine suggested otherwise, prompting us to investigate further the localisation of the drug within skin and probe the extent and significance of keratin binding.

Consequently, a series of in vitro experiments were performed aimed at investigating the interaction between primaquine and different domains of human epidermis. Firstly, a depth profile was constructed of primaquine within epidermal tissue following the administration of a topical dose and attainment of steady state flux. This was followed by a comparative study of the binding of primaquine to excised human epidermis and stratum corneum, in both the normal and delipidised states, to determine relative binding to these tissues (Banning and Heard, 2002). Finally, binding of primaquine to bovine horn keratin was examined to model the direct binding of the drug to the keratin present in the epidermis.

2. Materials and methods

2.1. Materials

Primaquine diphosphate was purchased from Sigma (Poole, UK) and converted to the viscous dark red liquid free base (Jeans and Heard, 1999). Acetonitrile, methanol, perchloric acid 60% (all HPLC grade), chloroform (general laboratory reagent) and Ultrafree—MC Millipore centrifuge tubes, containing 0.45 μm internal filter inserts were purchased from Fisher Scientific, Loughborough, UK. M840 was a gift from Condea Chemie, GmbH, Germany. Beef pancreatic trypsin, was obtained from (Merck, Poole, UK). Keratin powder, prepared from processed and purified bovine horn, was obtained from ICN Biomedicals, Poole, UK. Ultrapure water was drawn from a Barnstead E-pure system (Fisher Scientific, Loughborough, UK). Phosphate buffer saline (pH 7.4) was prepared by standard methods. Abdominal female cadaver skin (age range 55–85) was obtained from a local mortuary and stored at −20 °C until required.

2.2. Stability studies

0.5 mg ml^{-1} solutions of primaquine in M840, ethanol and mobile phase (see HPLC analysis) were maintained at 37 °C for 7 days in clear glass and amber glass vials. Periodically, 100 μl aliquots were sampled and the primaquine concentration determined.

2.3. Preparation of human epidermal tissue

After thawing, subcutaneous fat was removed from the skin by blunt dissection. The skin specimens were then immersed in water at 60 °C for 55 s enabling the epidermal membranes to be carefully peeled away from the underlying tissue using forceps. These were then floated onto aluminium foil, dried gently using tissue and finally cut into either 1 or 2 cm^2 sections using a surgical blade.

2.4. Depth profiling

Heat separated epidermal membranes (2 cm^2) were placed upon filter paper supports, mounted in six Franz-type diffusion cells, and receptor phase added (pH 7.4 PBS). Cells were dosed with 1 ml aliquots of 70 mg ml^{-1} primaquine in M840 and at steady state flux (Morris et al., 1998) the cells were dismantled, the membranes recovered, wiped then immobilised on PVC board using cyanoacrylate adhesive. They were then subjected to tape-stripping using regular adhesive tape (Perkins and Heard, 1999). The first four strips were discarded, but each subsequent strip was placed into 15 ml screw-capped vials. To each vial 3 ml of methanol was then added such that the tape was completely immersed in the liquid and the vials shaken for 24 h on a shaking plate. Solutions were decanted into glass tubes and the methanol evaporated in a vacuum oven overnight. The residues were redissolved in 500 μl of methanol and mixed on a vortex mixer for 3 × 10 s per tube. These solutions were then decanted into Eppendorf vials and centrifuged at 14,000 rpm for 5 min. Finally, 200 μl aliquots of the supernatant were transferred to HPLC autosampler vials. The mean primaquine extracted from each individual tape strip was plotted against the strip number in order to construct a depth profile. It was not possible to carry out a similar study using delipised skin as the tissue was not sufficiently robust.

2.5. Isolation of stratum corneum

Epidermal specimens (2 cm^2) were immersed in 1 × 10^{-4}% trypsin in phosphate buffer saline solution (pH

7.4) for 24 h at 37 °C. This method was similar to a previously reported method (Pellett et al., 1997), except that upon removal from the trypsin solution, de-ionised water was used to was the tissue, rather than hexane in order to avoid potential delipidisation.

2.6. Delipidisation of epidermis/stratum corneum

Delipidisation of viable epidermis and stratum corneum samples was achieved using a previously published method (Wertz and Downing, 1987). Briefly, the tissue was extracted by successively shaking in three solvent mixtures (2:1, 1:1, and 1:2 chloroform:methanol) each for 2 h. The skin specimens were finally shaken overnight in methanol then dried under vacuum.

2.7. Skin binding studies

The skin samples were placed into Eppendorf vials, each containing 1 ml of primaquine in M840 solution (0.1, 0.5, 1, 2, and 5 mg ml^{-1}). Four replicates of each concentration were performed. The samples were mixed for 18 h using a blood tube rotator (Bibby Stuart Scientific, UK) in an incubator at 32 °C. At the end of this period the skin samples were separated from unbound primaquine and M840 by ultracentrifugation: 14,000 rpm for 30 min in a Centurion 8000 centrifuge. Bound primaquine was extracted from epidermal or stratum corneum specimens with a three-stage extraction process, involving 3 × 3 ml of chloroform:methanol solution (2:1). Extracts were pooled and evaporated to dryness using a rotary evaporator (40 °C). The residue was reconstituted with 2 ml of HPLC mobile phase. To validate the primaquine extraction method, four epidermal specimens (normal and delipidised) were treated as described above, using primaquine concentrations of 0.1 and 5 mg ml^{-1}. After 18 h three successive extractions were performed (as above) and the extracts evaporated to dryness, reconstituted with 2 ml of mobile phase and analysed by HPLC.

2.8. Binding to bovine horn keratin

Bovine keratin powder (0.04 g) was weighed into 6 × 15 ml glass vials and 1 ml samples of primaquine free base in M840 (2, 4, 6, 8, and 10 mg ml^{-1}) were added to each (Banning and Heard, 2002). The resulting suspensions were stirred on an autostirrer to maintain homogeneity. The experiment was carried out at room temperature, with a 21 h contact time (an equilibrium study using a primaquine concentration of 2 mg ml^{-1} indicated that a contact time of 21 h was required before the binding process reached completion). The keratin powder (together with bound primaquine) was separated from remaining unbound primaquine free base by ultracentrifugation (13,000 rpm, 2 h). Bound primaquine was extracted from recovered samples of keratin (0.015 g) by sonication (5 min) with chloroform:methanol 2:1 (1.5 ml). The liquid filtrates obtained were collected, and the keratin was recovered once again. The extraction process was repeated on a further two occasions and the liquid filtrates combined. The product was evaporated to dryness using a rotary evaporator (40 °C, 5 min) and the residue was reconstituted in 2 ml of mobile phase. The binding study was run three times in total.

2.9. HPLC analysis

HPLC analysis was performed using a Hewlett-Packard 1100 automated system. Data was acquired using JCL6000 software (Jones Chromatography, Hengoed, UK). A Phenomenex Kingsorb 5 μm C18 (250 mm × 4.6 mm) column was used along with a mobile phase composed of acetonitrile, methanol, 60% perchloric acid and ultrapure water (25.9:4.91:0.56:68.63) and flow rate of 1.2 ml min^{-1}. The injection volume was 20 μl and detection was by UV at 254 nm. The retention time under these conditions was approximately 8 min. Standard solutions of 0.0125, 0.025, 0.05, and 0.1 mg ml^{-1} receptor phase were used in the construction of a calibration plot, which provided a linear regression of 1 and calibration coefficient of 3.438 e^4 mol^{-1}.

3. Results and discussion

In the clear glass vials decomposition was observed in all three test solutions: M840, the mobile phase and ethanol (k_R: 5.95 e^{-6}, 6.21 e^{-6} and 3.42 e^{-6} mol l^{-1} h^{-1}, respectively). The solutions in the amber glass vials were unaffected, indicating that decomposition was a photochemically induced

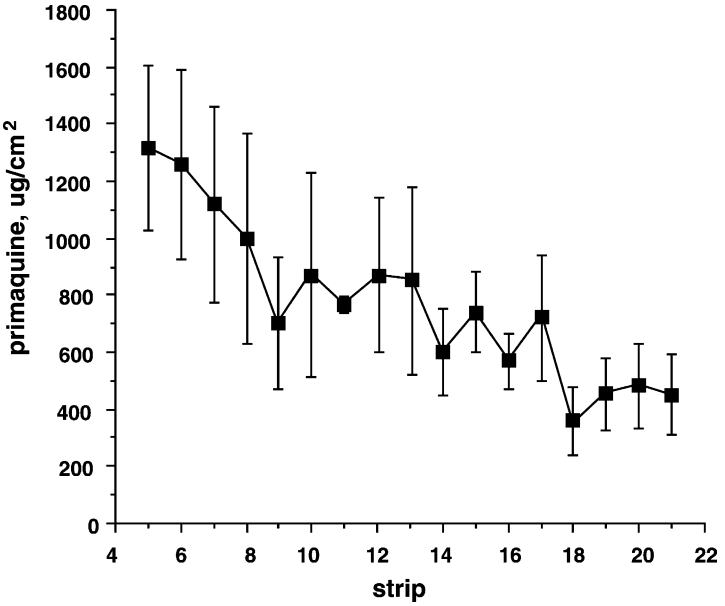

Fig. 1. Depth profile for the localisation of primaquine in human skin ($n = 6$, ±SEM).

process. All experimentation was subsequently performed under light exclusion.

The red-stained diffusional areas were circular corresponding exactly to the diffused areas, indicating that there had been no discernible lateral diffusion of drug within the skin tissue. The depth profile of primaquine in the epidermal membrane is shown in Fig. 1. The plot, which terminated at strip number 22 (epidermal/dermal junction), indicated that primaquine was distributed throughout the stratum corneum and viable epidermis in high concentrations. As is typically observed with such procedures, there was an inverse relationship between the amount of primaquine and depth. The amount of primaquine on each strip was summated to provide an indication of the total primaquine per unit area: in this case the total was approximately $13\,\mathrm{mg\,cm^{-2}}$ (19% of applied dose). This mass is some 300 times greater than the amount of extractable lipid present in the same unit area in the stratum corneum: approximately $52.3\,\mu\mathrm{g\,cm^{-2}}$ (derived from Abrams et al., 1993). In terms of moles, the factor was 600 ($6.0 \times 10^{-5}\,\mathrm{mol\,cm^{-2}}$ primaquine; in the order of $1 \times 10^{-7}\,\mathrm{mol\,cm^{-2}}$ lipids). Although these figures do not account for the covalently bound lipids of the stratum corneum or the extractable lipids in the viable epidermis, it remains highly implausible that so much drug could be accomodated entirely within the intercellular lipid domains. In earlier work involving doxycycline (Banning and Heard, 2002) the skin extraction/extractable lipid concentration factor was lower at approximately 9×. Doxycycline is much more hydrophilic than primaquine, and the lower value may have been a result of greater thermodynamic barriers experienced in crossing corneocyte/lipid interfaces.

Tape-stripping is at best a crude technique for probing skin localisation as, for example, the amount of material removed per strip can vary considerably and is subject to much operator variability. Nevertheless, it is worth considering the significance of the progressively lower amounts of primaquine associated with deeper stratigraphic levels within the epidermal tissue, as flux had been at steady state and the tissue effectively saturated. The stratum corneum is continually being renewed and is the end product of epidermal differentiation, whereby living cells undergo cornification as they progress outwards through the different layers of the underlying viable epidermis. A feature of the epidermal cornification process is the increasing relative proportion of corneocytes and keratin as the epidermis evolves. Significant drug–corneocyte interaction, and in particular keratin which comprises the vast majority of the corneocytes/stratum corneum,

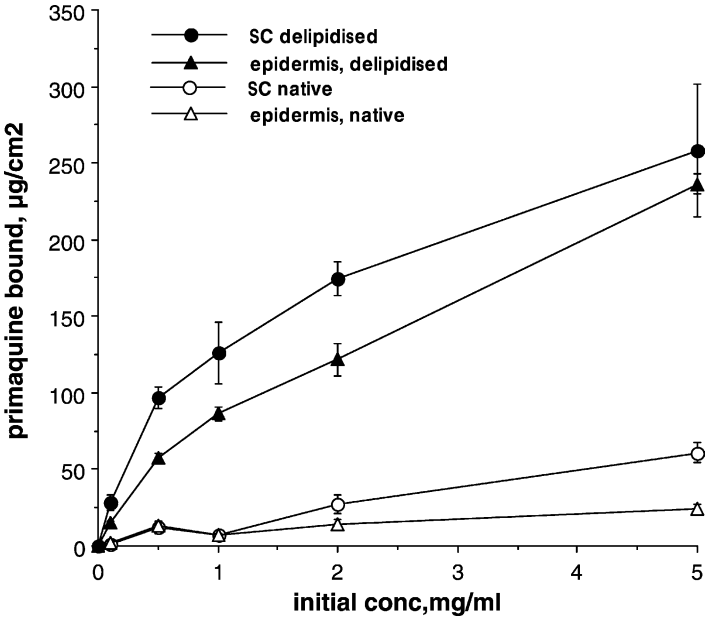

Fig. 2. Binding or primaquine to excised human epidermis and stratum corneum (native and delipidised) ($n = 4$, ±SEM).

offers a plausible explanation of both the high amount of primaquine bound in the epidermal tissue and the increasing amount of drug in the outer reaches of the tissue. Osamura et al. (1984) attributed this effect to the higher water content of the lower layers and poorer adherence of the guinea pig skin cells to the discs. However, visual inspection of each stripping showed this was not the case on this occasion.

Fig. 2 shows the results obtained from the skin equilibrium-binding experiments. It displays binding as a function of primaquine concentration, for epidermal and stratum corneum tissues, in both normal and delipidised states. Binding of primaquine to all skin samples was concentration dependent. Significantly greater amounts of primaquine bound to delipidised tissue than the normal tissue. This was surprising given high lipophilicity of primaquine and skin lipid, yet it supports the hypothesis in the previous section. An explanation may involve increased accessibility of drug molecules to the binding sites following the removal of the non-covalently bound lipid which effectively block access to such sites.

For the native samples, binding was approximately linear, indicating that the stratum corneum in normal skin has a net non-saturable primaquine capacity at the concentrations investigated. This observation is consistent with those made in previous studies, where the concentration-binding relationship of drugs to skin and skin layers was linear and non-saturable (Menczel and Maibach, 1972; Artuc et al., 1980; Menczel et al., 1984). This could perhaps be rationalised in terms of regular solution theory, in that primaquine, M840 and the stratum corneum all possess a solubility parameter of approximately 10 (Morris et al., 1998; Fedors, 1974; Liron and Cohen, 1984) and are therefore theoretically extensively miscible. However, as discussed earlier, the differences in the amounts recovered from the skin and the capacity of the extractable lipids, makes this unlikely unless there was considerable expansion of these domains. For the delipidised skin, the relationship was non-linear suggesting that binding to delipidised (essentially proteinaceous) stratum corneum was saturable. This again can be rationalised in that, without the presence of the lipid domains, the drug would be no longer miscible with the tissue and saturability implies that corneocytes (including keratin contained within) possess a finite (yet quite large) number of binding sites.

Raykar et al. (1988) proposed that partitioning into the stratum corneum is the sum of partitioning into

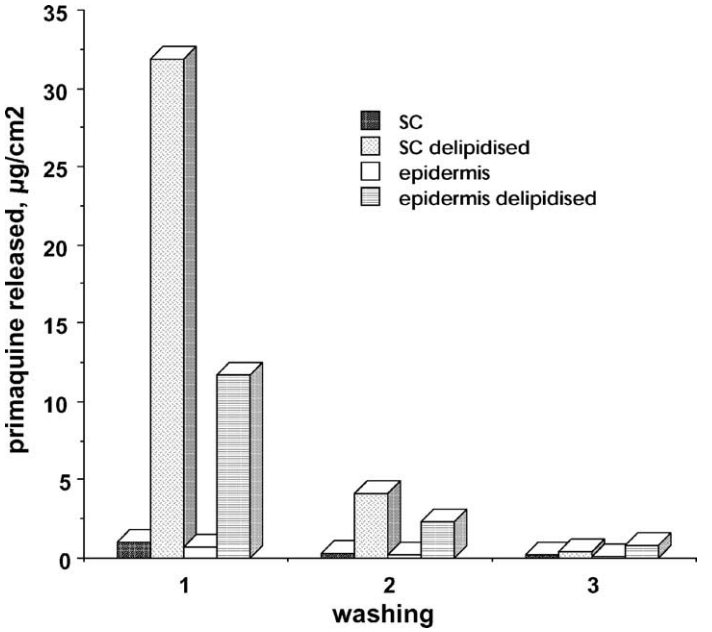

Fig. 3. Validation of the extraction process, following determination of primaquine from three successive extractions of epidermal samples.

the lipids, the corneocytes and a solvent. According to their theory, the stratum corneum lipid removal should have resulted in a decrease of partitioning into the stratum corneum as opposed to the increase observed. However, several other researchers have reported this apparent increase in drug binding, following delipidisation (Walter and Kurz, 1988; Surber et al., 1990; Kurz and Fichtl, 2001). Partitioning of drug into the stratum corneum cannot simply be considered as partitioning of the drug independently into the two separate phases. It follows that the binding of primaquine to corneocytes in delipidised stratum corneum is not indicative of the degree of corneocyte binding in intact stratum corneum. However, it does demonstrate the high affinity of primaquine for corneocytes/keratin.

More primaquine was found bind to the stratum corneum than the stratum corneum plus viable epidermis, both normal and delipidised. This probably reflects greater accessibility of the drug to the stratum corneum keratin, as in the case of stratum corneum plus viable epidermis, only one face of tissue was stratum corneum—the other face was the lower reaches of the viable epidermis with much lower amount of keratin. It should be borne in mind that the covalently bound lipid component of the corneocyte envelope was not removed by the delipidisation process. Some of the primaquine which bound to the delipidised stratum corneum sample, and indeed to the normal stratum corneum sample, may therefore have been localised in this covalently bound lipid monolayer. This is more likely should it exist in the form of discrete domains (Percot and Lafleur, 2001). Validation experiments showed that the recovery procedures used extracted some >95% of total primaquine bound to the tissues (Fig. 3).

Fig. 4 indicates dose dependent binding of primaquine to the bovine horn keratin. The keratin binding–concentration relationship was generally sigmoidal, indicating that a co-operative binding process was occurring and again suggests that, at sufficiently high primaquine concentrations, keratin binding sites present in corneocytes are saturable. Although not particularly representative of human epidermal keratin, bovine horn keratin does provide an indicative model (Wertz and Downing, 1987; Idei et al., 2001).

Overall, the data indicated that the binding of primaquine to the epidermal tissue gave rise to the deposition of a considerable concentration of drug within the skin—the so-called ‘reservoir’. The data suggests that the major location for the binding was probably

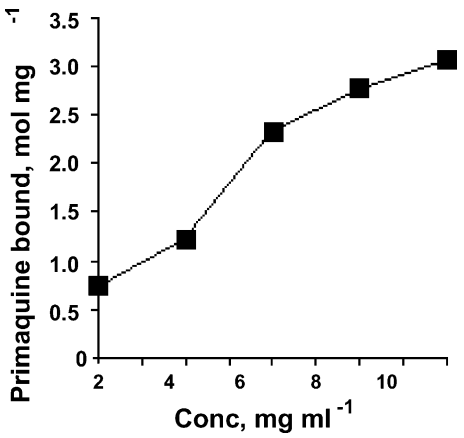

Fig. 4. Binding of primaquine to bovine horn keratin as a function of initial concentration of primaquine in M840 solution ($n = 3$, ±SEM).

the keratin which forms the bulk of corneocytes (Downing, 1992), although other proteins (e.g. corneocyte envelope) might also have been involved (Idei et al., 2001). Binding to proteins present in corneocytes can therefore greatly influence the distribution and pharmacokinetics of drugs, administered either dermally (Hashiguchi et al., 1998); or transdermally (Walter and Kurz, 1988; Banning and Heard, 2002). On the other hand, low protein binding was found to produce a tendency for greater subcutaneous targeting (Yanagimoto et al., 1999). Implicit in this is that the permeation process involved sequential passage through relatively hydrophilic corneocytes and hydrophobic lipid bilayers. This is contrary to regular skin permeation theory as it is widely believed that the multiple partitioning processes required to traverse the stratum corneum in such a manner make it an unlikely route, although it is consistent with Chandrasekaran et al. (1980) who proposed the existence of bound and freely diffusible drug molecules within the stratum corneum. We would further postulate that upon commencement of the permeation process, permeant molecules are adsorbed onto keratin throughout the epidermis of the diffused skin until a situation is reached where all the binding sites become fully occupied or saturated. At this stage further permeant molecules would then be able to bypass corneocyte binding sites relatively unhindered and, with the permeation of additional molecules altogether bypassing corneocytes via the intercellular lipids (Roberts and Pugh, 1996), steady state is attained.

Keratin binding would therefore appear to dictate the retentive capacity of the stratum corneum/epidermis, depending on the affinity of the permeant. Moreover, the common view of the skin as a ‘barrier’ may be too simplistic as such sequestration has more in common with a cattle grid. It is speculated that keratin binding may in fact be a deliberate evolutionary process designed to deactivate potentially harmful permeant chemicals before they are able to reach the system.

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References

Abrams, K., Harvell, J., Shriner, D., Wertz, P., Maibach, H.I., Rehfeld, S., 1993. Effect of organic solvents on in vitro human skin water barrier function. Soc. Investig. Dermatol. 101, 609–613.

Artuc, M., Reinhold, C., Stuttgen, G., Gazith, J., 1980. A rapid method for measuring drug enrichment in epidermis. Arch. Dermatol. Res. 268, 129–140.

Banning, T.P., Heard, C.M., 2002. Binding of doxycycline to keratin, melanin and human epidermal tissue. Int. J. Pharm. 235, 219–227.

Chandrasekaran, S.K., Campbell, P.S., Watanabe, T., 1980. Application of the ‘dual sorption’ model to drug transport through skin. Polym. Eng. Science 20, 36–39.

Downing, D.T., 1992. Lipid and protein structures in the permeability barrier of mammalian epidermis. J. Lipid Res. 33, 301–313.

Fedors, R., 1974. A method for estimating both the solubility parameters and molar volumes of liquids. Polym. Eng. Sci. 14, 147–154.

Hashiguchi, T., Kodama, A., Ryu, A., Otagiri, M., 1998. Retention capacity of topical imidazole antifungal agents in the skin. Int. J. Pharm. 161, 195–204.

Idei, T., Takahashi, K., Nobuhara, S., Miyachi, Y., 2001. Cloning and characterization of a novel keratin binding protein, occulibin, which links keratin filaments to the nuclear envelope. J. Investig. Dermatol. 117, 135.

Jeans, C.W., Heard, C.M., 1999. A therapeutic dose of primaquine can be delivered across excised human skin from simple transdermal patches. Int. J. Pharm. 189, 1–6.

Kurz, H., Fichtl, B., 2001. Binding of drugs to tissues. Drug Metab. Rev. 14, 467–510.

Liron, Z., Cohen, S., 1984. Percutaneous absorption of alkanoic acids 1: a study of operational conditions. J. Pharm. Sci. 73, 534–537.

Mayorga, P., Puisieux, F., Courraze, G., 1996. Formulation study of a transdermal delivery system of primaquine. Int. J. Pharm. 132, 71–79.

Menczel, E., Maibach, H.I., 1972. Chemical binding to human dermis in vitro testosterone and benzyl alcohol. Acta Dermatologica-Venereologica 52, 38–42.

Menczel, E, Bucks, D., Maibach, H.I., Wester, R., 1984. Lindane binding to section of human skin: skin capacity and isotherm determinations. Ibid 276, 326–329.

Morris, A., Kee, W.J., Brain, K.R., Heard, C.M., Watkinson, A.C., 1998. Vehicle effects in the permeation of primaquine across human skin in vitro. In: Brain, K.R., James, V.J., Walters, K. (Eds.), Perspectives in Percutaneous Penetration, vol. 5b. STS Publishing, Cardiff, pp. 180–182.

Osamura, H., Jimbo, Y., Ishihari, M., 1984. Skin penetration of nicotinic acid, methyl nicotinate and butyl nicotinate in guinea pig. J. Dermatol. 11, 471–481.

Pellett, M.A., Watkinson, A.C., Hadgraft, J., Brain, K.R., 1997. Comparison of permeability data from traditional diffusion cells and ATR- FTIR spectroscopy. Part II. Determination of diffusional pathlengths in synthetic membranes and human stratum corneum. Int. J. Pharm. 154, 217–227.

Percot, A., Lafleur, M., 2001. Direct observation of domains in model stratum corneum lipid mixtures by Raman microspectroscopy. Biophys. J. 81, 2144–2153.

Perkins, N.C., Heard, C.M., 1999. In vitro dermal and transdermal delivery of doxycycline from ethanol/Miglyol 840. Int. J. Pharm. 190, 155–164.

Raykar, P.V., Fung, M., Anderson, B.D., 1988. The role of protein and lipid domains in the uptake of solutes by human stratum corneum. Pharm. Res. 5, 140–150.

Roberts, M.S., Pugh, W.J., 1996. Epidermal permeability: penetrant structure relationships. 2. The effect of H-bonding groups in penetrants on their diffusion through the stratum corneaum. Int. J. Pharm. 132, 23–32.

Surber, C., Wilhelm, K.P., Hori, M., Maibach, H.I., Guy, R.H., 1990. Optimisation of topical therapy. Partitioning of drugs into stratum corneum. Pharm. Res. 7, 1320–1324.

Walter, K., Kurz, H., 1988. Binding of drugs to human skin: influencing factors and the role of tissue lipids. J. Pharm. Pharmacol. 40, 689–693.

Wertz, P.W., Downing, D.T., 1987. Covalently bound omega—hydroxyacylsphingosine in the stratum corneum. Biochim. Biophys. Acta 917, 108–111.

Yanagimoto, G., Hayashi, T., Hasegawa, T., Seki, T., Juni, K., Sugibayashi, K., Morimoto, Y., 1999. Skin disposition of drugs after topical application in hairless rats. Chem. Pharm. Bull. 47, 749–754.