Short communication

Chemical ultraviolet absorbers topically applied in a skin barrier mimetic formulation remain in the outer stratum corneum of porcine skin

Haque, T.,[1] Crowther, J.M.,[2] Lane, M.E.,[1*] Moore, D.J.[3]

[1]UCL School of Pharmacy, 29-39 Brunswick Square, London, WC1N 1AX, UK;

[2]GSK Consumer Healthcare UK Ltd, 980 Great West Road, Brentford, Middlesex, TW8 9GS, UK;

[3]GSK Consumer Healthcare, 184 Liberty Corner Road, Suite 200, Warren, New Jersey 07059, United States

*Corresponding author

Email: majella.lane@btinternet.com

Tel: +44 207 7535821

Fax: +44 870 1659275

Abstract

The objective of the present study was to evaluate the fate of three chemical sunscreens, isoamyl p-methoxycinnamate (IPMC), diethylamino hydroxybenzoyl hexyl benzoate (DHHB), and bis-ethylhexylphenol methoxyphenyl triazine (BEMT), topically applied to mammalian skin from a skin barrier mimetic oil-in-water formulation. High Performance Liquid Chromatography (HPLC) methods were developed for the analysis of each molecule and validated. Franz cell permeation studies were conducted following application of finite doses of the formulations to excised porcine skin. A vehicle formulation containing no sunscreens was evaluated as a control. Permeation studies were conducted for 12 h after which full mass balance studies were carried out. Analysis of individual UV sunscreens was achieved with HPLC following application of the formulation to the skin with no interference from the vehicle components. No skin permeation of any of the chemical sunscreens was evident after 12 h. While sunscreens were detected in up to 12 tape strips taken from the SC, 87% or more of the applied doses recovered in the first 5 tape strips. When corrected for the amount of protein removed per tape strip this corresponded to a penetration depth in porcine stratum corneum of ~ 1.7 μm. Mass balance studies indicated total recovery values were within accepted guidelines for cosmetic formulations. Overall, only superficial penetration into the SC was observed for each compound. These findings are consistent with the physicochemical properties of the selected UV absorbing molecules and their formulation into a ordered biomimetic barrier formulation thus support their intended use in topical consumer formulations designed to protect from UV exposure. To our knowledge this is the first report of depth profiling of chemical sunscreens in the SC that combines tape stripping and protein determination following *in vitro* Franz cell studies.

Keywords: Sunscreens, mass balance, permeation studies, porcine skin, tape stripping, topical.
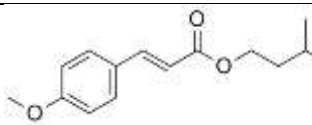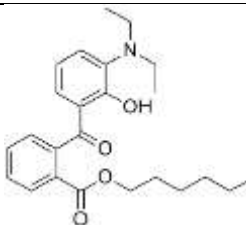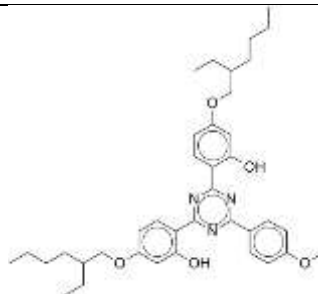
Introduction

Ultraviolet (UV) light has both positive and negative effects on human skin depending on the nature of the radiation and duration of exposure. Exposure of humans to UV radiation is essential for normal production of vitamin D and it is also used in the management of rickets, eczema, psoriasis and jaundice. However, UV may also cause degenerative changes in skin cells, ultimately resulting in skin aging, erythema, photodermatoses, actinic keratosis and skin cancer (Leigh, 2014). UV radiation is further classified based on specific wavelength ranges of the electromagnetic spectrum, namely UVA (320-400 nm), UVB (280-320 nm) and UVC (100-280 nm). UVA radiation may be subdivided further as UVA1 (340-400 nm) and UVA2 (320-340 nm). UVC or short wave radiation is absorbed by ozone and attenuated by the atmosphere and does not normally cause significant irradiation of humans. UVB radiation can only penetrate the superficial layers of the skin and may cause tanning, burning, photo-aging and cancer of the skin. UVA is the most available radiation (95%) on earth's surface and penetrates the deep layers of the skin. It may also induce tanning effects, skin aging, wrinkle formation and skin cancers (Marionnet et al., 2014; D'Orazio et al., 2013).

Skin protection against harmful UV radiation may be achieved by topical application of (i) physical barriers which deflect and scatter radiation or (ii) actives which absorb UV radiation. Titanium dioxide (TiO_2) and zinc oxide (ZnO) are the most commonly used materials in physical barriers whereas a much larger range of compounds are available for use as chemical UV absorbers (Skotarczak et al., 2015). Although a number of *in vitro* studies have examined the interaction of TiO_2 and ZnO particles with skin, comparatively fewer studies have been reported for chemical sunscreens (Gamer et al., 2006; Cross et al., 2007; Senzui et al., 2010). Knowledge of the fate of these materials following application to the skin is important in order to ensure that they are effective and that their residence time is adequate to assure UV protection. The aim of the present work was, therefore, to evaluate the skin disposition of three UV absorbers *in vitro* following application in a barrier mimetic oil-in-water topical formulation. The specific UV absorbers selected for study were isoamyl p-methoxycinnamate (IPMC), diethylamino hydroxybenzoyl hexyl benzoate (DHHB) and bis-ethylhexyloxyphenol methoxyphenol triazine (BEMT). These three molecules were evaluated because they span a range of molecular weights and physical states (Table 1) and when

formulated in combination provide protection from UVB and UVA radiation. IPMC (also known as amiloxate) is an efficient UVB absorber and is a liquid at room temperature. It is a lipophilic molecule and the maximum amount used in topical formulations is 10% (Couteau et al., 2007; Environment Agency, 2008). DHHB is a white to light salmon coloured powder and is an oil soluble UVA filter. It may be used alone or in combination with other UV filters to a maximum amount of 10% (European Commission, 2008). BEMT (also known as bemotrizinol) is a broad spectrum UV absorber which is effective against both UVA and UVB radiation. In appearance, it is a light yellow powder and is typically incorporated in formulations at a level of 5%, although it is approved for use up to 10% (Ruiz, 2000). We also report a new HPLC method for analysis of each molecule. Finally, the overall distribution of each of the UV absorbers, in and on the skin, is accounted for, via a mass balance approach.

Table I Experimental and predicted physicochemical parameters of isoamyl p-methoxycinnamate (IPMC), diethylamino hydroxybenzoyl hexyl benzoate (DHHB) and bis-ethylhexyloxyphenol methoxyphenol triazine (BEMT).

	Isoamyl P-methoxycinnamate	Diethylamino hydroxybenzoyl hexyl benzoate	Bis-ethylhexyloxyphenol methoxyphenol triazine
Chemical structure			
Brand name	Neo Heliopan® E1000	Uvinul® A Plus	Tinosorb® S
Molecular weight (Da)	248.3	397.5	627.8
Log P	3.6*	5.7*	12.6*
		6.2†	>5.7‡
Water solubility (mg/L)	4.9 (25°C)$\overline{\text{T}}$	<0.01 (20°C)†	$<10^{-4}$‡
Melting point (°C)	N/A	54; 314 (decomposition temperature)†	80.40±0.10‡

*Calculated with ChemBioDraw®
†Reference 10
‡Reference 11
$\overline{\text{T}}$Reference 9

Materials and methods

Materials

IPMC (NeoHeliopan® E1000), DHHB (Uvinul® A Plus) and BEMT (Tinosorb® S) were provided by GSK. The three sunscreens were incorporated in a biomimetic lamellar oil-in-water (o/w) formulation at amounts (w/w) typically used in personal care products and the same formulation without sunscreens was used as a control. The formulation also contained the following materials: hydrogenated lecithin, capric caprylic triglyceride, shea butter, glycerine, olus oil, isostearyl isostearate, dicapryl carbonate, xylitol, panthenol, niacinamide, pentylene glycol and 1,2 hexanediol. HPLC grade unstabilised tetrahydrofuran (THF), acetonitrile (ACN), and trifluoroacetic acid (TFA) were obtained from Fisher Scientific. Water and Brij^{TM} 98 were purchased from (Sigma Aldrich, Dorset, UK). Phosphate buffered saline (PBS) was prepared by dissolving PBS tablets (Dulbecco A, Oxoid Limited, UK) in deionised water (pH 7.4±0.2).

Methods

Analysis of chemical sunscreens

All UV filters were analysed using an Agilent 1200 HPLC system consisting of an Agilent G1322A degasser, Agilent G1311A quaternary pump, Agilent 1329A auto sampler, Agilent G1316A thermostat column compartment and Agilent G1314B UV absorbance detector (Agilent Technologies, Cheadle, United Kingdom). Separation of the molecules was performed using a Capcell Pak® C$_{18}$ column (type MG II, 4.6 mm x 250 mm) purchased from Shiseido Ltd. (Tokyo, Japan). A gradient mobile phase method was developed and the solvent compositions and run times for each gradient stage are shown in Table 2. The column temperature was set at 40°C and the flow rate and injection volume were 1.2 mL/min and 10 μL, respectively. Calibration curves were constructed by dissolving known amounts of the sunscreens in THF from 0.01 - 100 μg/mL.

Table II Mobile phase proportions and flow rate for the different gradient stages.

Time	Water (with 0.1% TFA)	THF	ACN
0	30	10	60
6	15	15	70
20	10	70	20
21	30	10	60
30	30	10	60

In vitro skin permeation and mass balance studies

Full thickness porcine skin (1.00±0.05 mm) was prepared from pig ears, classified as waste tissue, obtained from a local abattoir and stored in a freezer at -20°C prior to use. *In vitro* permeation studies were subsequently conducted as described in detail elsewhere (Oliveira et al., 2012). The receptor phase consisted of 6% Brij in PBS to ensure sink conditions and was constantly stirred with a magnetic stirrer (OECD, 2004a,b). A dose equivalent to 5 $\mu L/cm^2$ of the formulation was applied on the skin surface and the exact weights of applied amounts of the formulation were noted. The clinical efficacy of sun screen formulations is typically evaluated after dosing *in vivo* at 2 mg/cm^2. A higher dose has been employed in this study because, to our knowledge, this is the first time these compounds have been examined in skin *in vitro*. The skin was not occluded throughout the permeation period and at 12 h, the receptor solution (200 μL) was removed and analysed by HPLC.

At the end of the permeation experiment, the skin was removed from the Franz cells and placed on a cork board. Sequential tape stripping was performed using D-Square[TM] Discs (Cuderm, Dallas, TX, USA) with a diameter of 14 mm. Fifteen tape strips were taken from the skin surface, as preliminary experiments indicated no penetration of the sunscreens past tape strip 12. The absorbance values of each tape strip were measured using a SquameScan[TM] 850A (Heiland Electronic, Wetzlar, Germany). From the tape absorbance value the protein content of each tape was calculated in $\mu g/cm^2$ as reported previously (Klang et al., 2011). The thickness of the stratum corneum (SC) removed by the tape-strips was subsequently calculated, considering the amount of protein removed from each tape-strip and assuming that the value for SC density is 1 g/cm^3 (Anderson and Cassidy, 1973). Tape strips were then placed into Eppendorf® tubes and extracted with 0.5 mL of ACN:THF (50:50)

solution. The tubes were placed in a rotor (Stuart, UK) at 40 rpm in a temperature controlled oven (Jouan, Saint Herblain, France) for 16 h at 32.0±0.5°C. After extraction, each tube was centrifuged at 12,000 rpm and at 32°C for 30 min (Centrifuge 5415R, Eppendorf AG, Hamburg, Germany). Supernatant solution was taken, diluted where necessary, and the sunscreens were subsequently analysed by HPLC. The total recovery of the sunscreens was calculated from the percentage of the applied dose recovered from the strips, the receptor solution and following extraction of the tissue.

Statistical analysis

All data were processed with Microsoft Excel® 2007 and results are presented as mean± standard deviation (SD). Statistical analysis was performed using SPSS software (IBM SPSS Statistics, version 22). One way analysis of variance (ANOVA) followed by a post hoc Tukey test was conducted for multiple comparisons between groups. A value of $p<0.05$ was considered as a significant difference.

Results

HPLC analysis of sunscreens

A linear relationship was observed ($r^2>0.99$) from 1 - 100 µg/mL for IPMC and from 0.5 - 100 µg/mL for DHHB and BEMT. Retention times for IPMC, DHHB and BEMT were 6.5, 8.3 and 19.7 min respectively, in line with their calculated Log P values (Table 1). The accuracy values at three concentration levels for all methods were within 100±5% (n=9). The limit of quantitation (LOQ) value was 1, 0.5 and 0.5 µg/mL for IPMC, DHHB and BEMT, respectively, while the corresponding values for limit of detection (LOD) were 0.5, 0.1 and 0.1 µg/mL, respectively.

Permeation and tape stripping studies

At the end of the 12 h permeation studies none of the UV absorbers could be detected in the receptor chambers of the Franz cells. The cumulative amounts of protein and estimated

SC thickness removed by the tape stripping procedure, following application of the formulation containing the sunscreens are shown in Figure 1.
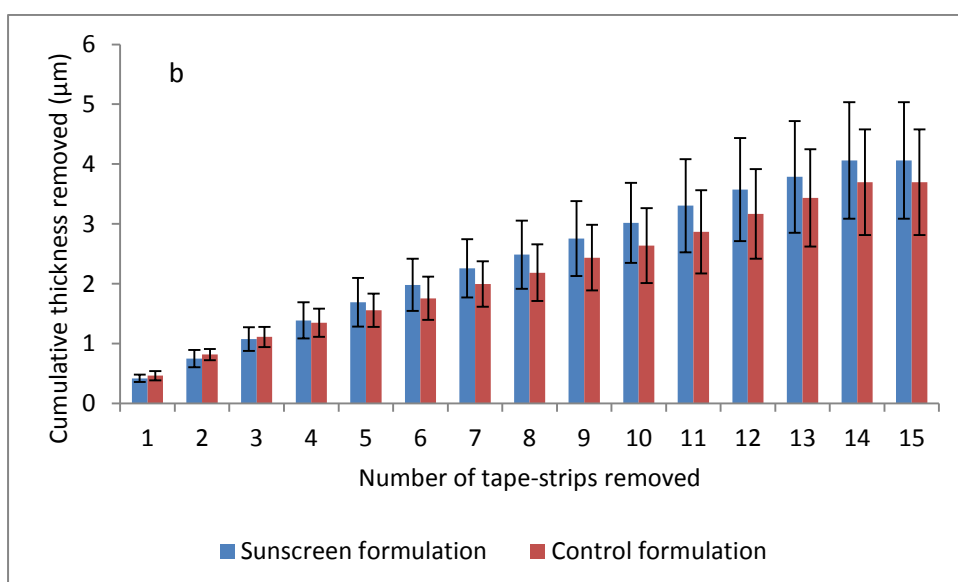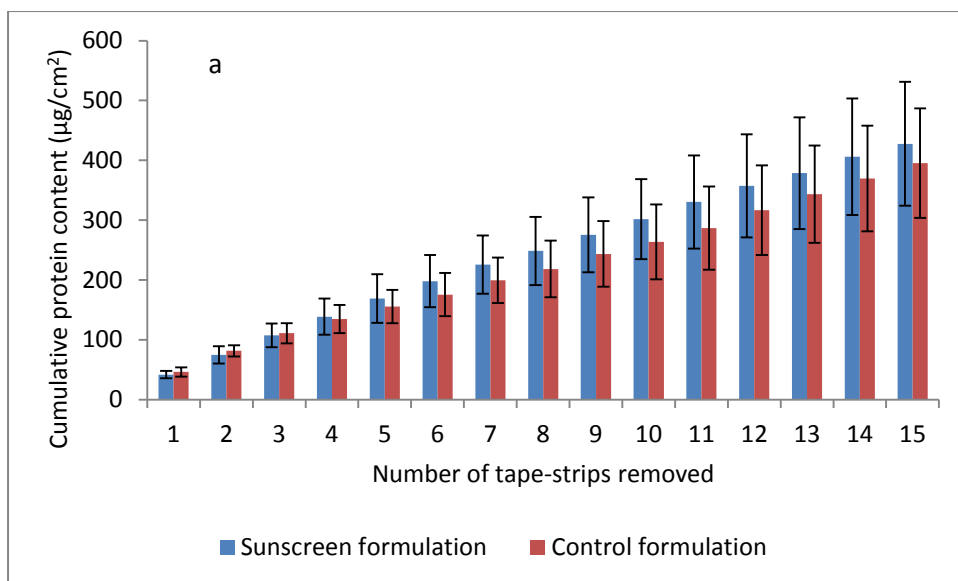
Figure 1 (a) Cumulative amount of protein removed by tape stripping (b) Cumulative SC thickness removed by tape-stripping for control formulation and for formulation containing IPMC, DHHB, BEMT (Mean±SD, n=6).

For tape strip 1, the mean amount of protein removed for the formulation containing the three sunscreens was 41.9 ± 6.3 μg/cm^2, which was not significantly different than the value for the control formulation (46.3 ± 7.7 μg/cm^2). Mean amounts of protein removed per

strip were not significantly different for tape strips 1 to 5 for this formulation ($p>0.05$); however by tape strip 6 a significant reduction ($p<0.05$) in the amounts of protein removed per strip was evident (28.9 ± 9.1 $\mu g/cm^2$). A similar trend was evident for the control formulation (data not shown) and the corresponding amount that could be removed by the sixth tape strip was 20.0 ± 11.0 $\mu g/cm^2$. There are a number of factors which may contribute to lower protein recovery on tapes with sequential tape stripping. The presence of excipients in the skin may affect the adhesiveness of the tape; it has also been suggested that corneocytes are more cohesive in the deeper layers of the SC (Dreher et al., 1998; Bashir et al., 2001; Mohammed et al., 2011a). It is also interesting to note the greater amounts of protein removed here from porcine SC compared with human SC, i.e. for a given number of strips more of the stratum corneum is removed from the pig skin when compared to human skin. Using the same tape strips and method for protein measurement ~50 $\mu g/cm^2$ of protein were collected by three tape strippings of the untreated volar forearm (Mohammed et al., 2011a). When the forearm was treated with an oil-in-water cream, values of protein recovered per individual tape strip did not exceed ~20 $\mu g/cm^2$ (Mohammed et al., 2011b) which is approximately half the amount of protein removed in the first tape strip in Figure 1a. The results are consistent with the lower barrier function of porcine ear skin compared with human skin (Dick and Scott, 1992; Vallet et al., 2007). The actual cumulative amounts of protein removed with tape strip number increased in a linear manner ($r^2>0.99$) for both the sunscreen (Figure 1b) and control formulations. Similarly, the thickness of SC removed with increasing tape strip number also increased in a linear manner ($r^2>0.99$) for both the sunscreen (Figure 1b) and control formulation.

Cumulative SC thickness values removed for 5, 10 and 15 tape strips for the sunscreen formulation were 1.69 ± 0.41, 3.02 ± 0.67 and 4.06 ± 0.97 μm, respectively and corresponding values for the control formulation were 1.55 ± 0.20, 2.64 ± 0.63 and 3.70 ± 0.88 μm, respectively. Again, considering available data for human subjects, following a 28 day treatment with an oil-in-water cream, a total of 20 tape strippings of the forearm removed a SC thickness of 7.6 ± 1.1 μm (Mohammed et al., 2011b). In the present study it was possible to remove further SC from the tissue however the number of tape strips was limited to 15 as the sunscreens could not be detected after the 12th tape strip. To our knowledge this is the first report of this approach to quantify both the active and its depth of penetration in porcine skin following *in vitro* Franz cell studies.

Skin disposition of UV filters

Figure 2 shows the amounts of each UV filter recovered from each tape strip collected at the end of the permeation study with the sunscreen formulation. It was not possible to detect IPMC after removal of tape strip 10; DHHB could still be detected up to the 11th tape strip and BEMT was not detected after the 12th tape strip. It is interesting to note that although BEMT has the highest molecular weight it is still detected in the deepest tape strip compared with the lower molecular weight sunscreens. The first 5 tape strips account for the majority of the dose applied with values of 238, 151 and 99 µg of IPMC, DHHB and BEMT respectively, recovered, corresponding to 87.1±0.9, 94.2±1.4 and 96.6±2.7% of the respective applied doses. These values also lie within the recovery ranges of 85-115% specified by SCCS guidelines for skin absorption of cosmetic ingredients (SCCS, 2010). For the first ten tape strips total recovery values increase to 90, 98.5 and 101.5% respectively for IPMC, DHHB and BEMT. The lower recovery values for IPMC may reflect the lower LOD and LOQ values for this molecule compared with the other sunscreens. However the possibility of some evaporation of IPMC should also be considered as it is a liquid at room temperature unlike DHHB and BEMT.
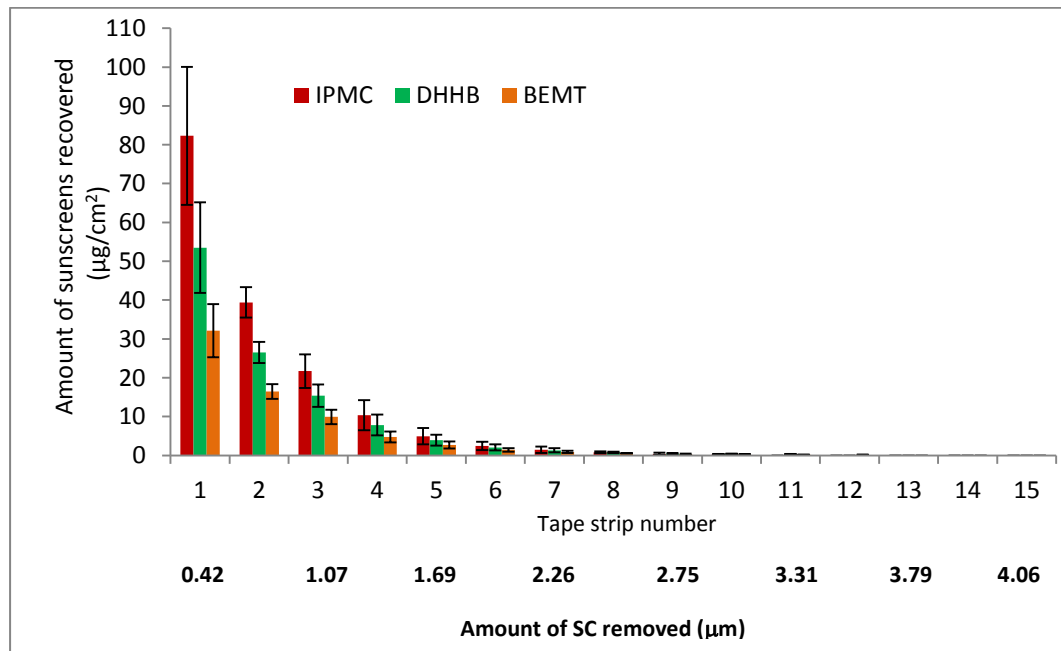

Figure 2 Distribution of IPMC, DHHB and BEMT for each tape strip and corresponding depth of SC removed for tape strip 1, 3, 5, 7, 9, 11, 13 and 15 in bold.

Figure 3 shows the amounts of protein and sunscreens recovered for tape strips 1-5, 6-10 and 11-15. Similar amounts of protein were removed for each set of tape strips (p>0.05). Clearly, most of the sunscreen UV absorbing molecules are collected from tape strips 1-5 with significantly lower amounts being collected for tape strips 6-10 and little or none collected for tape strips 11-15. Considering the thickness of SC removed (Figure 2) this indicates that the UV absorbing molecules are largely distributed in the outer 1.7 μm of the SC, with smaller amounts distributed up to ~3.8 μm. The specific depths of penetration calculated for IPMC, DHHB and BEMT respectively are 3.02±0.67, 3.31±0.78 and 3.57±0.86 μm, again confirming only superficial penetration of these materials and consistent with their intended use. Similar analysis of the receptor phase and tissue was conducted for the control formulation following the 12 h permeation study. No peaks corresponding to the UV absorbing molecules were detected in the receptor solution or tape-strips.
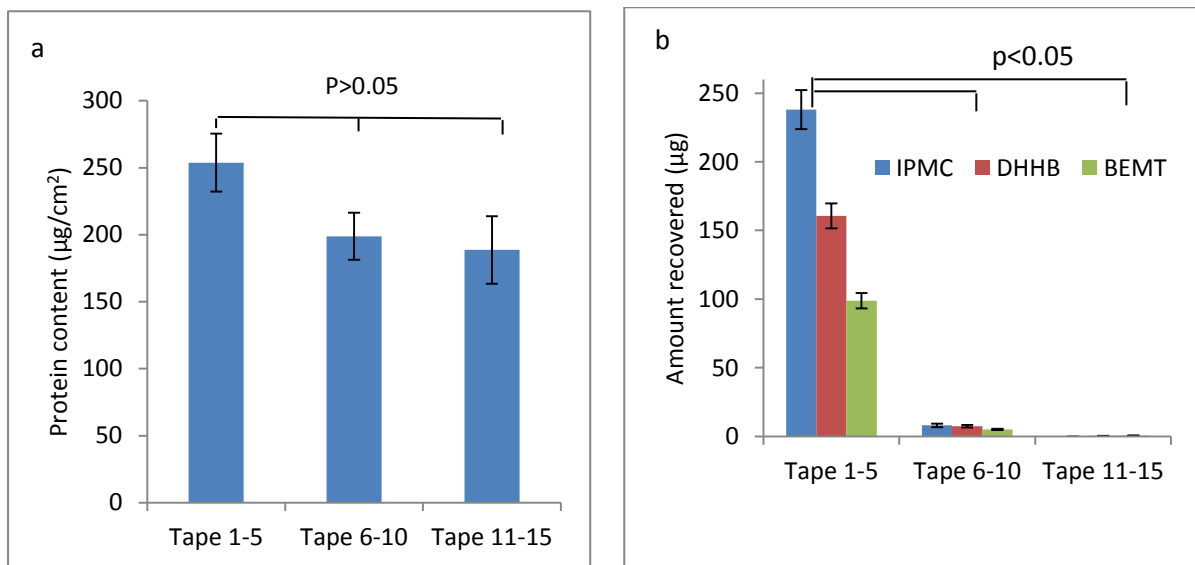

Figure 3 (a) Amounts of protein removed and (b) Amounts of IPMC, DHHB and BEMT removed for tape strips 1-5, 6-10 and 11-15 after 12 h permeation study of sunscreen formulation in porcine skin.

Conclusions

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A sensitive and specific HPLC method was developed which is capable of detecting three different sunscreens spanning a range of physicochemical properties. The sunscreens were successfully detected in tape strips collected from the outer layers of porcine skin following application in a structured biomimetic o/w cream formulation. The HPLC method was validated by conducting similar permeation studies with the same formulation containing no UV absorbers.

Following finite dose Franz cell studies with porcine skin over 12 h none of the molecules could be detected in the receptor phase. Analysis of tape strips collected from the skin at the end of the study confirmed that the sunscreens were largely in the first five tape strips. The maximum depth of penetration of each sunscreen was determined based on protein absorption measurements and confirmed that between $87 - 94\%$ of the applied dose does not penetrate beyond 1.7 μm in the skin. Considering the higher permeability of porcine skin compared with human skin, and the higher finite dose used in this work, this also suggests no effective transdermal permeation of the compounds will occur in normal human skin. As noted, we believe that this is the first report which combines measurement of the disposition of the active as well as its depth profile following *in vitro* permeation studies. This approach could be used to profile the skin disposition of other topical formulation components particularly if their efficacy depends on targeting specific strata of the skin.

References

Anderson, R.L. and Cassidy, J.M. 1973. Variations in physical dimensions and chemical composition of human SC. J. Invest. Dermatol. **61,** 30-32.

Bashir, S.J., Chew, A.L., Anigbogu, A., Dreher, F., Maibach, H.I. 2001. Physical and physiological effects of SC tape stripping. Skin Res. Technol. **7**, 40–48.

Couteau, C., Pommier, M., Paparis, E.*,* Coiffard, L.J. 2007. Study of the efficacy of 18 sun filters authorized in European Union tested in vitro. Pharmazie. **62,** 449-452 (2007).

Cross, S.E., Innes, B., Roberts, M.S., Tsuzuki, T., Robertson, T.A., McCormick, P. 2007. Human skin penetration of sunscreen nanoparticles: in-vitro assessment of a novel micronized zinc oxide formulation. Skin Pharmacol. Physiol. *20,* 148-154.

Dick, I.P., Scott, R.C. 1992. Pig ear skin as an in-vitro model for human skin permeability. J. Pharm. Pharmacol. **44,** 640-645.

D'Orazio, J., Jarrett, S., Amaro-Ortiz, A., Scott, T. 2013. UV radiation and the skin. Int. J. Mol. Sci. **14,** 12222-12248.

Dreher, F., Arens, A., Hostynek, J.J.*,* Mudumba, S., Ademola, J., Maibach, H.I. 1998. Colorimetric method for quantifying human SC removed by adhesive-tape stripping. Acta Derm. Venereol. **78**, 186–189.

Gamer, A.O., Leibold, E., van Ravenzwaay, B. 2006. The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin. Toxicol. In Vitro. **20,** 301-307.

Klang, V., Schwarz, J.C., Hartl, A., Valenta, C. 2011. Facilitating in vitro tape stripping: application of infrared densitometry for quantification of porcine SC proteins. Skin Pharmacol. Physiol. **24,** 256-268.

Leigh, I. M. 2014. Progress in skin cancer: the U.K. experience. Br. J. Dermatol. **171,** 443-445.

Marionnet, C., Tricaud, C., Bernerd, F. 2014. Exposure to non-extreme solar UV daylight: spectral characterization, effects on skin and photoprotection. Int. J. Mol. Sci. **16,** 68-90.

Mohammed, D., Matts, P.J., Hadgraft, J., Lane, M.E. 2011a. Depth profiling of SC biophysical and molecular properties. Br. J. Dermatol. **164**, 957-965.

Mohammed, D., Matts, P.J., Hadgraft, J., Lane, M.E. 2011b. Influence of Aqueous Cream BP on corneocyte size, maturity, skin protease activity, protein content and transepidermal water loss. Br. J. Dermatol. **164**, 1304-1310.

Oliveira, G., Hadgraft, J., Lane, M.E. 2012. The influence of volatile solvents on transport across model membranes and human skin. Int. J. Pharm. **435,** 38-49.

Organisation for Economic Co-operation and Development. 2004a. Guidance Document for the Conduct of the Skin Absorption Studies. OECD Environmental Health and Safety Publication Series on Testing and Assessment No. 28; ENV/ JM/MONO. 2004b.

Organisation for Economic Co-operation and Development. 2004b. OECD Guideline for the Testing of Chemicals. Skin Absorption: in vitro Method. Guideline 428.

Ruiz, C.D. 2000. Tinosorb® S. Bis-Ethylhexyloxyphenol Methoxyphenol Triazine (BEMT). Next generation UV filter for sun protection. Ciba Specialty Chemicals Corporation. Citizen's Petition – 78N-0038 -9/5/2000. 22 pages.

SCCS. 2010. Basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients. SCCS/1358/10. European Union.
Scientific Committee on Consumer Products. 2008. Opinion on diethylamino hydroxybenzoyl hexyl benzoate. SCCP/1166/08. European Commission. Brussels, Belgium. 25 pages.

Senzui, M., Tamura, T., Miura, K., Ikarashi, Y., Watanabe, Y., Fujii, M. 2010. Study on penetration of titanium dioxide (TiO(2)) nanoparticles into intact and damaged skin in vitro. J. Toxicol. Sci. **35,** 107-113.

Skotarczak, K., Osmola-Mańkowska, A., Lodyga, M., Polańska, A., Mazur, M., Adamski, Z. 2015. Photoprotection: facts and controversies. Eur. Rev Med. Pharmacol. Sci. **19,** 98-112.

UV filters in cosmetics – prioritisation for environmental assessment. 2008. The Environment Agency. Bristol, United Kingdom. 112 pages.

Vallet, V., Cruz, C., Josse, D., Bazire, A., Lallement, G., Boudry, I. 2007. In vitro percutaneous penetration of organophosphorus compounds using full-thickness and split-thickness pig and human skin.. Toxicol. In Vitro. **21,** 1182-1190.