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ARTICLE *in* MOLECULAR PHARMACEUTICS · AUGUST 2010

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Uptake of Microemulsion Components into the Stratum Corneum and Their Molecular Effects on Skin Barrier Function

Rania M. Hathout,[†,‡] Samar Mansour,[†] Nahed D. Mortada,[†] Ahmed S. Geneidi,[†] and Richard H. Guy*[,‡]

Faculty of Pharmacy, Ain Shams University, Cairo, Egypt, and Department of Pharmacy & Pharmacology, University of Bath, Bath, U.K.

Received March 15, 2010; Revised Manuscript Received May 17, 2010; Accepted June 14, 2010

Abstract: This research determined the uptake of individual components of topically applied microemulsions into the stratum corneum (SC) and assessed their molecular effects on skin barrier function. The microemulsions comprised oleic acid, Tween20, Transcutol and water. The effects of selected formulations, and of the individual components, on the conformational order of the SC intercellular lipids, and on SC hydration, were assessed by infrared spectroscopy. Measurements were made as a function of SC depth by progressively tape-stripping the membrane in the normal way. SC uptake of microemulsion components was quantified via extraction and analysis of the collected tape strips. SC hydration increased in proportion to the water content of the microemulsion. Each of the microemulsion components penetrated into the SC, but to different extents. Oleic acid decreased the conformational order of the SC lipids, and induced some phase separation, as revealed by the frequency shifts and peak areas of the absorbances associated with $-CH_2$ symmetric and asymmetric stretching vibrations. Tween20 extracted some of the SC intercellular lipids. In summary, SC structure was perturbed by all components of the microemulsions, and the degree of the effects detected was proportional to the level of the respective component present in the skin.

Keywords: Microemulsion; skin; stratum corneum; tape stripping; infrared spectroscopy

Introduction

Success of the transdermal route depends on the ability of drugs to permeate the skin at a rate and in amounts sufficient to attain effective plasma concentrations. The stratum corneum (SC) is the outermost skin layer and represents the principal rate limiting barrier to percutaneous delivery. The SC is composed of keratin-rich cells embedded in multiple lipid bilayers, composed mainly of ceramides, cholesterol and free fatty acids. It is widely accepted that the intercellular lipid domain is the main pathway for the permeation of most drugs through the SC.[1] This conclusion is based upon visualization studies and from the reduced barrier function of the SC induced by lipid extraction.[2,3] While there have been many investigations examining how the composition of a formulation may influence the delivery of a drug across the skin, and the mechanisms by which this effect is achieved (e.g., lipid extraction or increased lipid disorder),[4,5] the correlation of the latter with the SC uptake of specific vehicle constituents has rarely been determined. Attenuated total reflectance Fourier transform infrared spec-

* To whom correspondence should be addressed at Department of Pharmacy & Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, U.K. Tel: +44 (0) 1225 384901. Fax: +44 (0) 1225 386114. E-mail: r.h.guy@bath.ac.uk.

† Ain Shams University.

‡ University of Bath.

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10.1021/mp100068s
Published on Web 06/14/2010

troscopy (ATR-FTIR) has been used, in particular, to reveal specific penetration enhancer effects on the SC,[6] and to track the permeation of model compounds with distinct IR absorbance profiles (such as cyanophenol).[7,8] However, unless the formulation excipients are available in perdeuterated form, it has proved difficult to quantify their uptake into the SC and to relate this level to the IR-detected molecular effects on the barrier.[9,10]

Recently, microemulsions have been shown to have potential as efficient formulations for transdermal drug delivery.[11] Components of microemulsions can clearly interact with SC intercellular lipids, facilitating drug transport across the barrier. The objective of the present study was to combine ATR-FTIR measurements with SC tape-stripping and sensitive quantification of microemulsion constituents using liquid chromatography−mass spectroscopy (LC−MS), to correlate the molecular-level effects of the formulation on the barrier with the uptake and spatial distribution of specific excipients in the SC. The key components of the microemulsions considered (in addition to water) were oleic acid, Tween20 and Transcutol. Oleic acid is an unsaturated fatty acid, and a known skin penetration enhancer.[12] Tween20 is a stable, nontoxic and biodegradable nonionic surfactant of low irritation potential.[13−15] Transcutol is a cosolvent, effective in solubilizing a wide range of drugs, and having excellent compatibility both with skin and with an array of typical topical excipients.[16] Ultimately, this work strives to demonstrate a causal link between the physical presence of an excipient, as a function of position within the stratum corneum (SC), and its impact upon intercellular lipid biophysical parameters related to skin barrier function. While the results are able to confirm (at least, in part) mechanistic insight already deduced from earlier investigations, the practical value of the approach described, and of the data obtained, is significant, providing a tool with which the performance (or not) of a formulation can be assessed and ultimately optimized.

Experimental Section

Materials. Oleic acid and polyethylene 20 sorbitan monolaurate (Tween20) (Sigma-Aldrich, Gillingham, U.K.), Transcutol (Gattefosse, Lyon, France), acetonitrile, ethanol (HPLC grade) (Fisher Scientific, Loughbrough, U.K.), sodium chloride, potassium chloride, sodium phosphate (monobasic) and potassium phosphate (dibasic) (Acros Organics, Geel, Belgium) (Sigma-Aldrich, St. Louis, MO). All aqueous solutions were prepared using high-purity deionized water (18.2 MΩ·cm) (Barnstead Nanopure Diamond, Dubuque, IA).

Methods. *Construction of Pseudoternary Phase Diagrams.* To determine the concentration range of components necessary for the formation of microemulsions, a pseudoternary phase diagram was constructed using the water titration method at ambient temperature (25 °C).[17] The phase diagram was prepared with a 1:1 weight ratio of Tween20 to Transcutol. At this specific surfactant/cosurfactant weight ratio, the ratios of oleic acid (oil) to the mixture of surfactant and cosurfactant were varied as follows: 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1. The oil, surfactant and cosurfactant mixtures were diluted dropwise with water under moderate magnetic stirring. After equilibration, the mixtures were assessed visually and categorized as single-phase transparent microemulsions or two-phase mixtures. Turbidity was considered as an indication of phase separation. Dynamic light scattering measurements indicated that the average colloid diameters in the microemulsions were in the range of 10 to 13 nm (Malvern Zetasizer, Malvern, Worcestershire, U.K.). To obtain these data, microemulsions were prefiltered (0.45 μm) and loaded into 1 cm^2 cuvettes in a thermostatted chamber. Triplicate measurements were made. Every

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Table 1. Composition (% w/w) of Selected Microemulsion Formulations[a]

	A	B	C	D	E	F
oleic acid	19	18	17	16.2	16	15.4
Tween20	38	36	34	32.9	32	30.8
Transcutol	38	36	34	32.9	32	30.8
water	5	10	15	18	20	23

[a] The pH of the microemulsions ranged from 4.45 to 5.24.

sample that remained transparent and homogeneous after vigorous vortexing was assigned to the monophasic area in the phase diagram.[18] Selected microemulsions (Table 1) were chosen, starting at a composition (%w/w) of oleic acid:Tween20:Transcutol of 20:40:40, for ATR-FTIR studies. Full details of the physicochemical characterization of the microemulsions have been published.[18]

ATR-FTIR Studies. Porcine skin was used for all the experiments as it is considered to be a relevant animal model for the human counterpart.[19] Tissue was obtained post-sacrifice of the animal, dermatomed to a thickness of 750 μm and stored frozen for no longer than one month before use. Once defrosted, a 2 cm^2 piece of skin was clamped between the two halves of a conventional Franz diffusion cell (Permeagear, Hellertown, PA). The lower, subdermal compartment contained phosphate-buffered saline (PBS) at pH 7.4. The skin surface was treated with 200 μL of a microemulsion formulation which remained in contact with the tissue for 2 h; all experiments were performed in triplicate. At the end of the treatment period, the microemulsion was removed and the skin patted dry with an absorbent tissue. The skin was removed from the cell and then placed SC surface down on the internal reflectance element (ZnSe with a trapezoidal cut at 45°) of an ATR-FTIR spectrophotometer (Nicolet 520P, Thermo Electron Corp., Madison, WI) equipped with a liquid nitrogen cooled mercury−cadmium−telluride detector. Spectra were recorded as the average of 100 scans in the frequency range 4000−500 cm^{-1} at a resolution of 2 cm^{-1}. Peak positions were determined with the aid of OMNIC software (Thermo Fisher Scientific, Waltham, MA). To minimize intersample variability associated with inconsistencies in the degree of contact between the skin and the ATR crystal, spectra of the skin were taken before treatment and a normalization procedure previously described was employed when analyzing the results.[20] The IR features examined to assess the effect of the microemulsion components on the SC were: (1) the intensity and frequency shift of the CH_2 symmetric and asymmetric stretching absorbances, at ~2920 cm^{-1} and ~2850 cm^{-1}, respectively, which are sensitive to perturbations in the amount and the conformational order of the SC intercellular lipids; (2) the amide I (C═O stretching) to amide II (N−H bending) absorbance intensity ratio which reflects SC hydration; and (3) the intensities of the C═O and C−O stretching vibrations at ~1710 cm^{-1} and ~1100 cm^{-1}, respectively, which report of the presence of oleic acid, Tween20 and Transcutol in the SC.

Combined ATR-FTIR and Tape-Stripping Experiments. In a second set of experiments, skin was treated either with microemulsion F or with each of its components separately in the same quantity as present in the complete formulation: i.e., 30.8 mg of oleic acid, 61.6 mg of Tween20, and 61.6 mg of Transcutol (see Table 1). Subsequently, postcleaning of the skin surface and removal from the diffusion cell, the SC was subjected to repeated tape-stripping (Scotch Book tape 845, 3M, St. Paul, MN) using a procedure identical to that already described in the literature.[21] Precise weight measurements of the tapes pre- and post-stripping allowed the mass of SC removed per cm^2 of skin to be determined, and hence the depth to which the barrier had been stripped could be estimated. On a piece of skin from the same porcine donor, the identical stripping procedure was performed and transepidermal water loss was measured as the SC was progressively removed. It was then possible to deduce (together with the information from the weight of SC removed) the total thickness of the SC,[22] and hence all measurements, for each individual piece of skin, could then be presented as a function of "relative position" within the barrier (with 0 corresponding to the SC surface, and 1 to the SC-stratum granulosum interface).[23,24] The latter measurements were undertaken on a separate skin sample so that the principal experiment designed to determine the quantities of the microemulsion constituents in the SC was not unnecessarily prolonged and such that their concentration profiles were not altered as a result. All experiments were performed in triplicate.

Extraction and Quantification of the Microemulsion Components in the Tape Strips. After reweighing, each tape strip was rolled and placed in a 1.5 mL HPLC vial. The microemulsion components were quantitatively extracted

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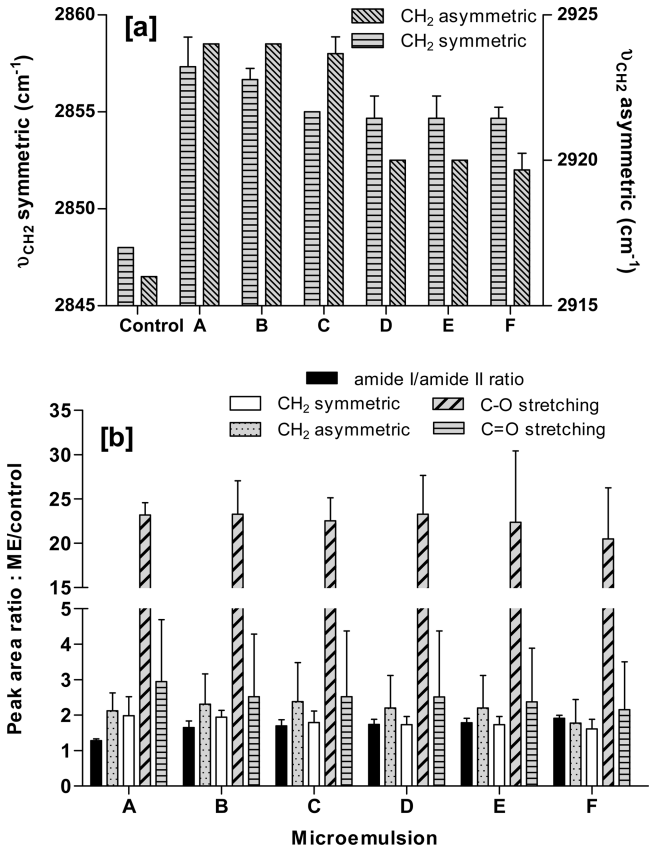

Figure 1. Changes in SC (a) CH_2 symmetric and asymmetric peak positions and (b) fundamental peak areas, after application of the selected microemulsion formulations (mean ± SD; $n = 3$; absence of an SD indicates a value too small to be seen on the scale of the figure). An analysis of variance on each of the spectral features showed only a significant difference for the amide I to amide II ratio with microemulsion A registering a smaller value compared to the other formulations.

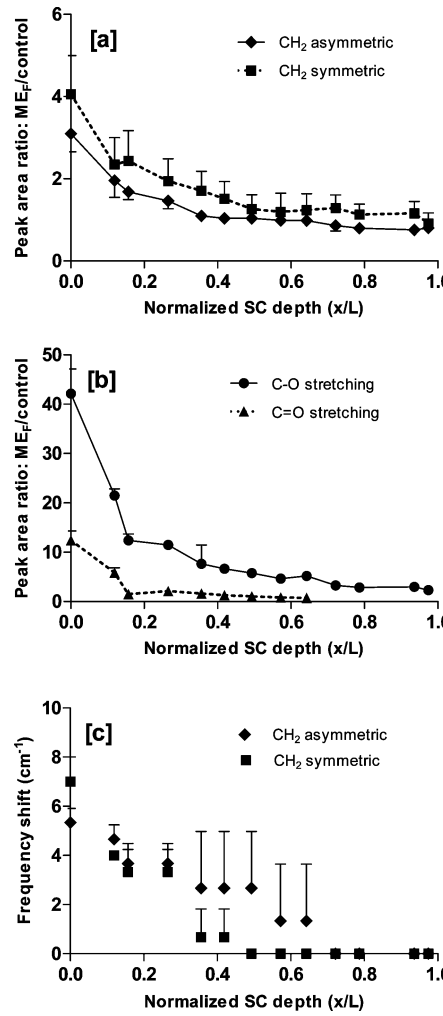

Figure 2. IR changes as a function of SC depth following application of microemulsion F: (a) CH_2 asymmetric stretching and CH_2 symmetric stretching; (b) C−O and C═O stretching; (c) frequency shift of CH_2 asymmetric and symmetric absorbances (mean ± SD; $n = 3$).

with 95% ethanol over 12 h. The procedure was validated by spiking tape-stripped samples of untreated SC with known amounts of excipient chosen to bracket the expected range of concentrations to be found in the samples. The tape-strip extracts were filtered using 0.45 μm Nalgene Millipore syringe filters (Thermo Fisher Scientific, Waltham, MA), and oleic acid, Tween20 and Transcutol in the SC were quantified by liquid chromatography mass spectroscopy (LCMS-2010 EV liquid chromatograph mass spectrometer, Shimadzu, Kyoto, Japan). All components were resolved on a 5 μm, 2 mm, 50 mm GEMINI NX C18 column (Phenomenex, Torrance, CA).

For oleic acid, 7 μL of the extracted sample was injected. The flow rate was 0.3 mL/min with an isocratic mixture of 90% acetonitrile and 10% water. The mass spectra were collected in negative ion mode at 281 *m/z*. The detector voltage was 1500 V, the detector wavelength was 210 nm and the temperature was 25 °C. The retention time (t_R) was 1.6 min with a limit of quantitation of 300 ng/mL.

To quantify Tween20, 20 μL of the sample was injected. The flow rate was again 0.3 mL/min with an isocratic mixture of 30% acetonitrile and 70% water. The mass spectra were collected in positive ion mode at 1151 *m/z*. The detector voltage was 2000 V, the detector wavelength was 254 nm and the temperature was 25 °C. t_R was 1.4 min, and the limit of quantitation equaled 0.5 μg/mL.

In the case of Transcutol, 5 μL samples were required. The flow rate was 0.2 mL/min with the same mobile phase as Tween20. Mass spectra were again collected in positive ion mode, this time at 135 *m/z*. The detector voltage was 1500 V, and the detector wavelength and temperature were the same as those for Tween20. t_R was 1.7 min, and the limit of quantitation was 1 μg/mL.

For all these microemulsion components, concentrations were calculated from relative peak areas. In the case of oleic acid, repeating the analysis of the same samples on different days resulted in essentially identical results, with a coefficient of variation of 0.25%; for Tween20 and Transcutol, the CVs were 0.74% and 0.56%, respectively.

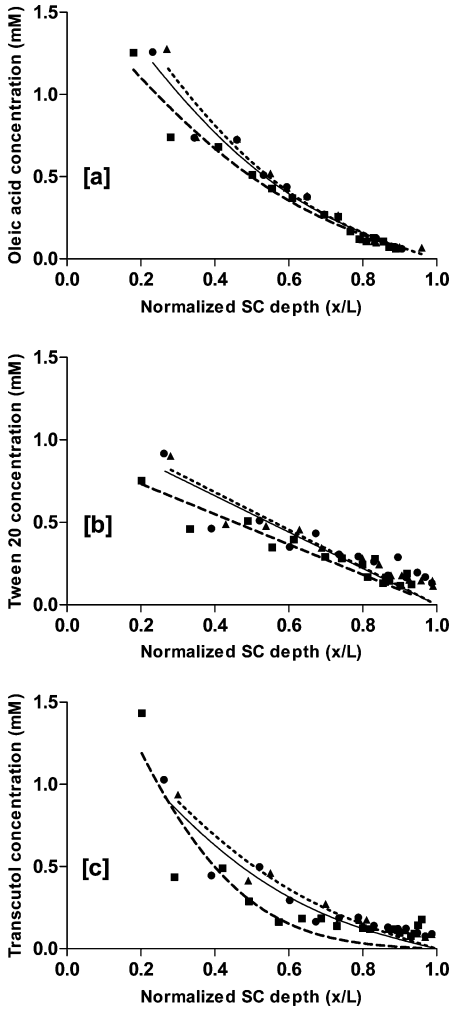

Figure 3. SC concentrations of the different components of microemulsion F: (a) oleic acid, (b) Tween20, (c) Transcutol. The results from three replicate experiments are shown.

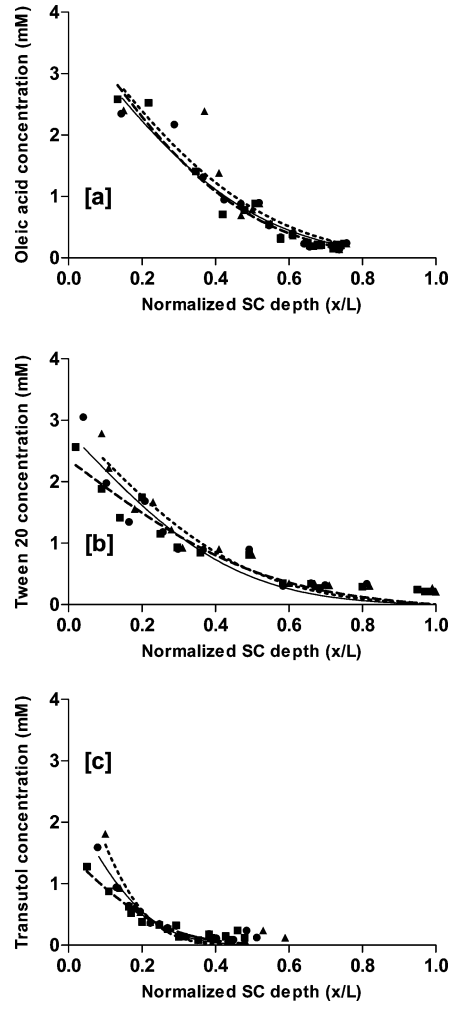

Figure 4. SC concentrations of (a) oleic acid, (b) Tween20, and (c) Transcutol, following their separate application to the skin. The results from three replicate experiments are shown.

Results and Discussion

Figure 1 summarizes the results from the first series of IR experiments. All the microemulsions tested provoked clear changes in the reflectance spectrum from the SC. Figure 1a focuses on the CH_2 symmetric and asymmetric stretching vibrations, an increase in the frequency of which indicates a decrease in the conformational order of the lipids under observation.[25,26] As all the microemulsions contain oleic acid, the enhanced conformational disorder observed is attributable simply to the presence of the liquid fatty acid within the stiffer, intercellular lipids of the SC.[9,10] Extensive previous work has indicated that oleic acid mostly phase separates producing fluid domains within the more solid SC lipids;[10] the interfaces between these phase boundaries are believed to offer more permeable pathways across the barrier, and this phenomenon has been proposed for oleic acid's action as a skin penetration enhancer.[9,10]

Figure 1b presents the post- to pretreatment ratios of different IR absorbance peaks. The symmetric and asymmetric CH_2 and the C═O carbonyl stretching absorbance ratios are all greater than 1, supporting the uptake of oleic acid into the SC from the microemulsions. Equally, substantial increases in the absorbance ratio associated with −C−O stretching (at ∼1100 cm^{-1}) were seen. These signals, which originate from C−O bonds in esters and alcohols,[27] strongly implicate the uptake of Tween20 and Transcutol into the SC. Finally, the ratio of the peak amide I to amide II absorption increased as the percentage of water in the microemulsion increased, suggesting a gradual increase in SC hydration.[28]

IR Spectroscopy and Concentration Profiles of Microemulsion Constituents. From the second series of experiments, in which IR spectra were recorded as the SC was

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Table 2. Stratum Corneum Uptake (Mean ± SD) of Microemulsion Components Following Their Application as Pure Chemicals or as Constituents of Microemulsion F

applied vehicle	SC uptake, mM (% of applied dose)		
	oleic acid	Tween 20	Transcutol
pure	11 ± 0.21	14 ± 0.01	6.0 ± 0.14
	(0.058 ± 0.001)	(0.08 ± 0.001)	(0.002 ± 0.0004)
microemulsion F	5.4 ± 0.04	4.8 ± 0.09	3.7 ± 0.20
	(0.023 ± 0.002)	(0.05 ± 0.001)	(0.004 ± 0.0003)

progressively stripped, it was possible to determine the extent of the spectral changes as a function of depth into the barrier (Figure 2). Given the similarity in the results in Figure 1, this part of the study was performed using just microemulsion F (see Table 1 for composition). This formulation contains the highest fraction of water and was particularly efficient in terms of the delivery of testosterone in a recently published investigation.[18] Not unexpectedly, given the relatively short duration of contact between microemulsion F and the skin, the effects are most noticeable near the surface and then fall off with increasing depth into the barrier.

Further details of the interactions between the microemulsion components and the SC are revealed by the quantification of the concentration profiles of oleic acid, Tween20 and Transcutol (Figure 3). All constituents of the microemulsion are taken up into the SC, and the reproducibility between different skin samples is quite good. Notably, the concentration profile of Tween20 appeared to be linear (certainly in

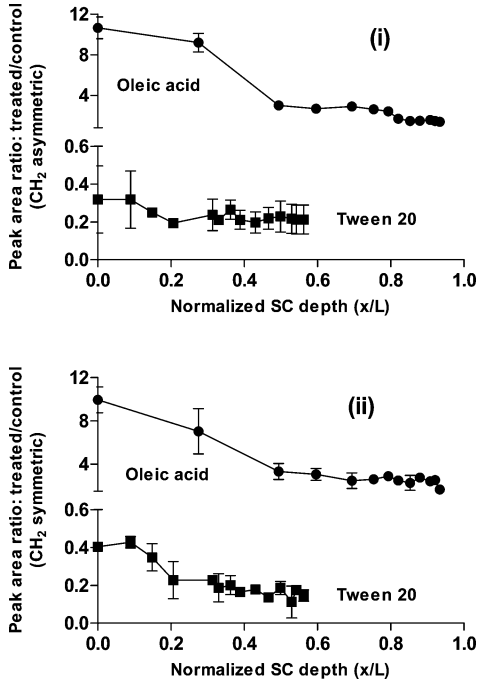

Figure 5. (i) CH_2 asymmetric stretching absorbance area and (ii) CH_2 symmetric stretching absorbance area, as a function of SC depth, following application of oleic acid, or Tween 20, relative to the corresponding peak areas from control, untreated skin samples (mean ± SD; $n = 3$).

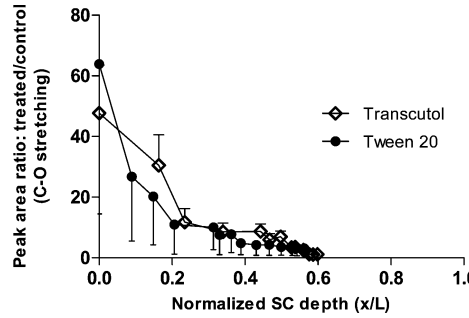

Figure 6. C−O stretching absorbance area as a function of SC depth following application of Transcutol and Tween 20, relative to the corresponding peak areas from control, untreated skin samples (mean ± SD; $n = 3$).

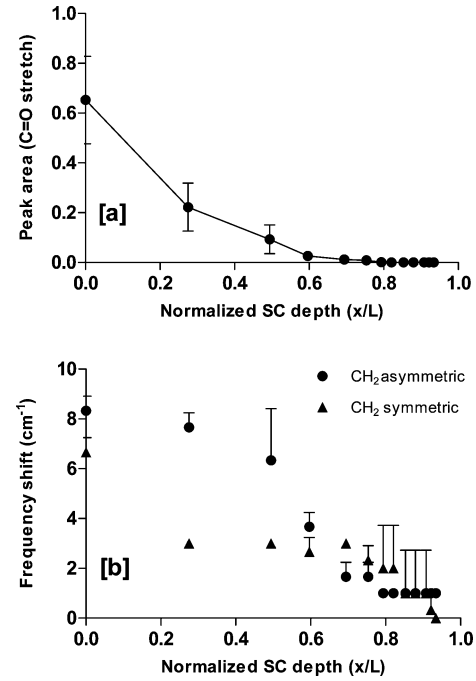

Figure 7. (a) C═O stretching absorbance area at 1710 cm^{-1}, and (b) frequency shift of CH_2 asymmetric and symmetric absorbances, following application of oleic acid (mean ± SD; $n = 3$).

comparison with those of oleic acid and Transcutol), suggesting that this component had diffused from the microemulsion into the SC more quickly than the other constituents.

To discriminate between the actions of the different microemulsion components, porcine skin was subsequently treated individually (adopting the identical experimental approach) with oleic acid, Tween20 and Transcutol alone using amounts equivalent to those in the complete formulation. The individual concentration profiles, which again demonstrate excellent reproducibility, are in Figure 4. Interestingly, when applied alone Tween20 did not achieve a steady-state profile, suggesting (as one might anticipate) a synergistic mechanism at play from the complete microemulsion. SC uptake was calculated by multiplying the area under the concentration profile over the entire thickness of the SC (for microemulsion F and for each individual component when applied separately)

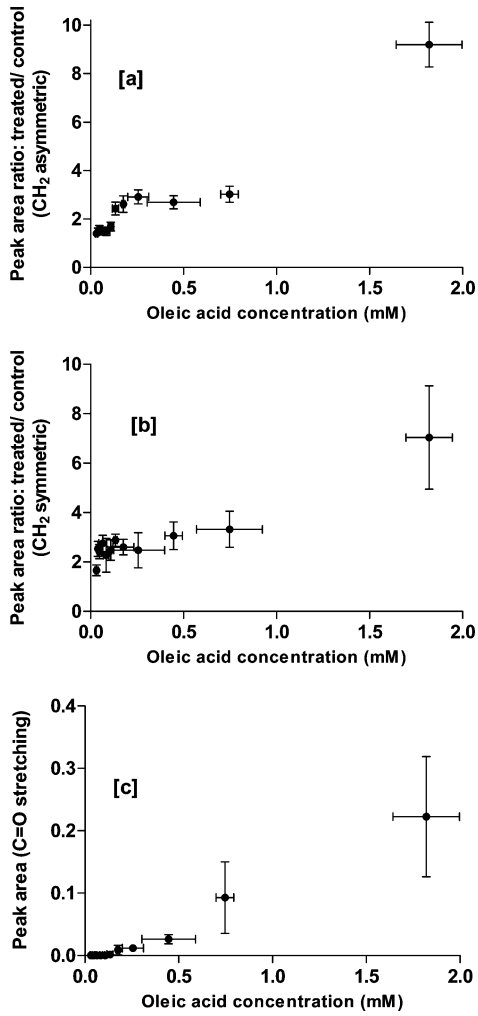

Figure 8. Correlation between oleic acid concentration in the SC and changes in the areas of IR absorbances from the SC, specifically: (a) CH_2 asymmetric stretching, (b) CH_2 symmetric stretching, (c) C=O stretching (mean ± SD; $n = 3$).

by the total volume of the SC (= total mass of SC removed by tape strips × SC density). The percentage of applied chemical taken up into the SC was then simply determined by multiplying the ratio of the mass that penetrated into the barrier to that which was applied by 100. The quantities taken up from the pure components are higher, but the percentage of the applied "dose" absorbed into the SC for each component was, in fact, similar to those from the microemulsion (Table 2). In other words, formulating the components in a microemulsion increased their relative uptake into the SC (compared to the pure components) despite their lower thermodynamic activity. The more efficient uptake from the microemulsion is perhaps not surprising and implies, as articulated above, the possibility of synergy between the different components (that is, a degree of mutual enhancement perhaps).

Figure 5 shows the post- to pretreatment ratios of the CH_2 asymmetric and symmetric stretching absorbances as a function of depth into the SC after separate and individual application of oleic acid and Tween20. While the absorbances

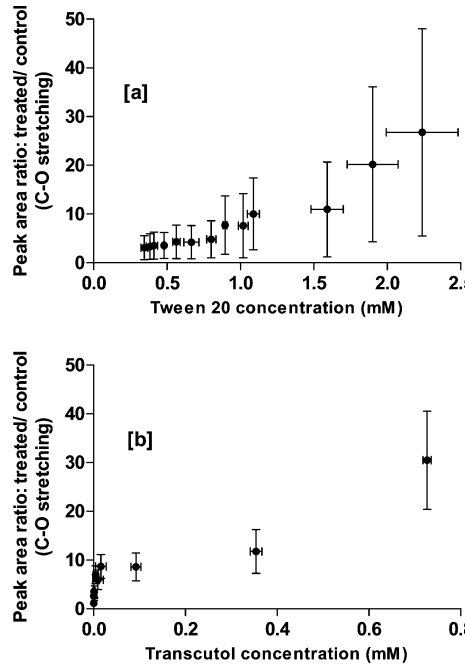

Figure 9. Correlations between (a) Tween20 and (b) Transcutol concentrations in the SC and the C−O single bond stretching absorbance (mean ± SD; $n = 3$).

are augmented by the uptake of oleic acid, treatment with Tween20 actually reduces the CH_2 signals and suggests that this substrate is extracting lipids from the SC,[25] perhaps via the formation of large micelles.[29]

The impact of Tween20 and Transcutol on the SC is illustrated in Figure 6, which shows the post- to pretreatment ratios of the C−O stretching absorbance which originates predominantly from these two excipients. The corresponding results for the C=O stretching absorbance are in Figure 7a and demonstrate a profile consistent with that which has previously been observed for oleic acid when applied in a perdeuterated form.[9] The frequency shifts in the CH_2 symmetric and asymmetric stretching absorbances (Figure 7b) are again consistent with oleic acid penetrating into and through the SC to an appreciable depth.

The strong correlations ($r^2 \geq 0.9$) between the IR spectroscopic changes observed in the SC and the levels of the individual microemulsion components, as a function of SC position, are plotted in Figures 8 and 9 for oleic

(27) Dreher, F.; Walde, P.; Walther, P.; Wehrli, E. Interaction of a lecithin microemulsion gel with human stratum corneum and its effect on transdermal transport. *J. Controlled Release* **1997**, *45*, 131–140.

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(29) Lopez, A.; Llinares, F.; Cortell, C.; Herraez, M. Comparative enhancer effects of Span20 with Tween20 and Azone on the in vitro percutaneous penetration of compounds with different lipophilicities. *Int. J. Pharm.* **2000**, *202*, 133–140.

acid, and for Tween20 and Transcutol, respectively. Taken together with the spectroscopic results described above (which are quite consistent, mechanistically speaking, with findings that have been reported in the literature over several years), these results reveal a causal link between the physical presence of an excipient, as a function of position within the stratum corneum (SC), and its impact upon intercellular lipid biophysical parameters related to skin barrier function. Thus, while the research discussed in this paper may not reveal novel mechanistic information, the practical value of the findings is significant, and provides a tool with which the performance (or not) of a formulation may be objectively assessed and ultimately optimized.

Conclusion

The combination of IR spectroscopy, SC tape-stripping and sensitive analytical chemistry has permitted the quantitative uptake and mechanism of action of microemulsion constituents to be determined. Such "cause-and-effect" relationships are clearly important to the rational and efficient development of topical and transdermal drug delivery systems.

MP100068S