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# Probing the skin permeation of fish oil/EPA and ketoprofen 1. NMR spectroscopy and molecular modelling

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**Abstract**

The complexation of EPA with ketoprofen was probed in order to rationalise co-operative skin permeation enhancement behaviour observed previously. The modulation of aromatic protons of ketoprofen was determined using $^1$H NMR spectra from different formulations containing varying concentrations of fish oil and a control saturated triglyceride. Molecular modelling of possible complexes of ketoprofen with constituents of fish oil was performed. NMR data revealed a dose-dependent change in chemical shift in the aromatic protons of ketoprofen on addition of fish oil and/or EPA. Similar patterns were observed in both cases, although the free fatty acid induced changes in more protons. Molecular modelling results indicate quite large binding energies of all complexes considered, varying between *ca.* 90 and 160 kJ mol$^{-1}$. The geometries of these complexes shows strong O–H···O hydrogen bonds in all cases, and in the case of the complex of ketoprofen with free EPA there is also some evidence of C–H···π and/or π–π interactions, giving rise to regiospecifically solvated complexes. If strongly bound ketoprofen:EPA complexes can form, then the permeation enhancement of EPA by ketoprofen could be attributed to such a complex. Once the complex is formed, the triglyceride/free fatty acid could aid permeation of associated ketoprofen into the lipophilic stratum corneum via the pull effect. Once permeated, the more hydrophilic ketoprofen could aid the permeation of the triglyceride/free fatty acid through the epidermis, again via the pull effect. This could explain the synergistic permeation enhancement seen with these compounds.


*Keywords:* Skin; Transdermal delivery; NMR; Molecular modelling; Ketoprofen; Eicosapentaenoic acid

## 1. Introduction

The principal polyunsaturated fatty acids present in fish oil, eicosapentaenoic acid (EPA) (approximately 33% of total) and docosahexaenoic acid (DHA) (approximately 21% of total) have been demonstrated to possess numerous properties beneficial to health (Calder, 2006). Topical delivery of the oil offers the ability to avoid wide distribution and other pitfalls associated with oral doses and the transcutaneous delivery of EPA and DHA from a fish oil vehicle has previously been demonstrated in vitro (Heard et al., 2003) with potential value in the treatment of arthritis (Thomas and Heard, 2005); furthermore topically applied fish oil was found to exert an anti-inflammatory activity against UVB-induced erythema in vivo (Puglia et al., 2005). As a novel multi-pronged topical anti-inflammatory, the simultaneous delivery of EPA, DHA and ketoprofen has been demonstrated in vitro, where the data also suggested that an interrelationship existed between the permeation of EPA, DHA and ketoprofen (Heard et al., 2003; Thomas and Heard, 2005). Ketoprofen permeation was enhanced by the presence of EPA, but it was difficult to explain the enhancement of EPA by ketoprofen using regular skin permeation theory.

The classic viewpoint of drugs within a formulation being considered implicitly as free distinct species has been challenged recently by consideration of the existence of drug molecules within solvation cages, comprised of molecules of the fluid phase. Although the phenomenon of a pull effect, whereby transdermal delivery of the vehicle facilitates that of the solute was first suggested some time ago (Kadir et al., 1987; Pardo et al., 1991), it is only recently that evidence for the permeation of intact complexes across skin has been demonstrated (Heard et al., 2003, 2006; Karia et al., 2004). Mechanisms of skin permeation enhancement are often the subject of publications (Hadgraft, 1999; Williams and Barry, 2003; Karande et al., 2006)

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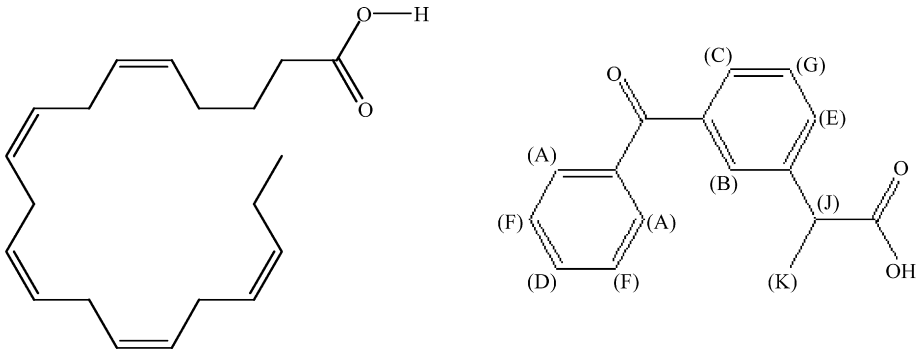


Fig. 1. Structures of EPA and ketoprofen, with proton assignment illustrated for ketoprofen.

although the fate of the enhancer itself is rarely mentioned. However, it has recently been shown that ethanol and 1,8-cineole used as enhancers can permeate the skin in a concentration-dependent manner and enhance permeation of solute accordingly (Heard et al., 2006) *via* a manifestation of the pull effect (Kadir et al., 1987). Such considerations highlight the importance of understanding the fate of enhancers and solvents in addition to the primary active in the formulation.

In an earlier paper (Thomas and Heard, 2005) the permeation of EPA was found to be enhanced by the presence of ketoprofen and similarly, permeation of ketoprofen enhanced by the presence of fish oil. Indeed, only negligible amounts of EPA were found to permeate skin in the absence of ketoprofen. This interrelationship, which cannot be explained by current accepted theory of skin permeation, led to the proposal of a vehicular complexation between EPA and ketoprofen, which was sufficiently stable to permeate through the skin intact. Of particular significance in these structures is the presence of π-electrons on C=C bonds in EPA, and within aromatic rings in ketoprofen.

Attractive interactions between π-systems such as these are possible, arising from electrostatic and dispersion-based forces. Such attractive interactions between π-systems play an important role in biological systems and molecular recognition. To date, most research has focussed on the benzene dimer (Sinnokrot et al., 2002) taken as a small model system of representative interactions in biological molecules, such as proteins or DNA (Hunter and Sanders, 1990). The interaction energies of the face-to-face dimer configuration are comparable to weak hydrogen bonds at approximately 10–15 kJ $mol^{-1}$. In this paper, we consider the interactions of much bigger molecules, namely the unsaturated regions of EPA and the aromatic regions of ketoprofen. The energy of such interactions should be approximately additive, such that larger binding energies than found for the benzene dimer are anticipated. The theory of π–π interactions has been investigated by Hunter and Sanders, who have published much significant work on them, and established several rules when considering such attractions. They also illustrate how π–π interactions are fundamental to DNA helix formation and to the intercalation of drugs into a DNA helix via face-to-face π stacked complexes with the aromatic DNA bases (Hunter and Sanders, 1990).

The structures of EPA (five double bonds) and ketoprofen (two aromatic rings) suggest that complexation of the two compounds could involve π–π interactions. The nature of the molecular interactions involved in two structurally similar compounds to this work has recently been examined: tamoxifen, a hormonal treatment for oestrogen receptor positive (ER+) cancer containing three π-aromatic rings, and γ linolenic acid, an $n-6$ polyunsaturated fatty acid with anti-cancer properties (Heard et al., 2005).

The current paper is part of an ongoing study attempting to elucidate the unusual inter-relationship between permeation of EPA and ketoprofen (Fig. 1). In the current paper the vehicular formation of a fish oil/EPA–ketoprofen complex is investigated using $^1$H NMR spectroscopy to determine the possibility of complexation involving adjacent π–π interactions between unsaturated regions of EPA and the aromatic regions of ketoprofen. Molecular models were then constructed to suggest possible geometrical parameters of EPA or DHA–ketoprofen complexes and their binding energies determined.

## 2. Methods

### 2.1. NMR spectrometry

$^1$H NMR spectra were obtained using a Bruker Avance DPX400 spectrometer operating at 400 MHz and 27 °C. Subsaturated solutions of ketoprofen (2.5%, w/w) in fish oil, mixtures of fish oil and Miglyol 812N (a synthetic saturated triglyceride), 1:10, 1:20, 1:1, 10:1 and 20:1 were prepared, including pure Miglyol 812N as a control formulation (Heard et al., 2005). A fixed volume of formulation (25 μl) was added to separate NMR tubes and $CDCl_3$ (475 μl) was added as solvent. These volumes were found to produce suitability strong NMR signals. The shifts of the aromatic protons were determined relative to ketoprofen control and the individual proton shifts were plotted as a function of concentration. These experiments were repeated using the primary constituents of fish oil, namely EPA and DHA free fatty acids. Due to the small amount of material available, serial dilutions of a 25 mg $ml^{-1}$ solution in Miglyol 812N were prepared and saturated with ketoprofen.

Ketoprofen proton assignments were assigned using the Spectral Database for Organic Compounds, as illustrated in Fig. 1. An example of the aromatic region of the ketoprofen spectrum produced from 2.5% ketoprofen in Miglyol 812N with the

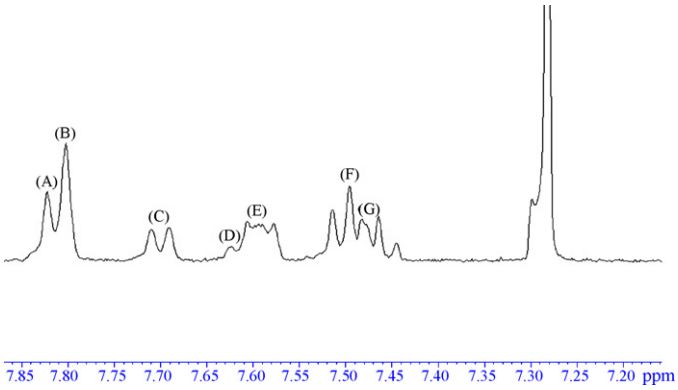


Fig. 2. NMR spectrum of ketoprofen aromatic region showing proton assignments.

assigned protons and their relevant positions within the spectrum is illustrated in Fig. 2. As the region affected by π–π bonding is the aromatic region of ketoprofen, this is the region which has been the focus of these studies. Assignments (J) and (K) represent a single proton and a methyl group, respectively (three equivalent protons). Neither signal is in the aromatic region having shifts of approximately 3.8 and 1.5 ppm, respectively, and has therefore been omitted from analysis.

### 2.2. Molecular modelling

Individual molecules of ketoprofen, EPA and the triglyceride of this fatty acid were built using the Molecular Builder function of the MOE package (http://www.chemcomp.com), and minimised using the MMFF-94 force-field (Halgren, 1996) until no force on any nucleus exceeded 0.05 kcal/(Å mol). Complexes between ketoprofen and EPA or DHA were constructed by bringing together optimised structures and re-optimising using the same method. This resulted in little or no change to the conformation of either drug or fatty acid. The conformational energy landscape of these complexes was explored by randomly altering the mutual orientation of drug and fatty acid as well as the dihedral angles of any rotatable bonds. This was followed by full energy minimisation. Throughout this procedure, the *cis* orientation of the C=C bonds in fatty acids was maintained using harmonic restraints, which were subsequently removed in the final minimisation. These stochastic searches proceeded until either 1000 conformations were found, or no new low-energy conformations were found after 1000 searches.

Binding energies were calculated by subtracting the energies of the individual molecules from the total energy of the complex, all calculated using the MMFF-94 force-field. In all cases the contribution to binding energy from bond length, angle and dihedral terms was effectively zero, such that binding was a combination solely of electrostatic and Van der Waals terms. Of course, static calculations such as these cannot fully represent the dynamic behaviour of flexible molecules at room temperature. However, they can at least probe possible interactions between molecules, yielding information on their strength and any close contacts of specific groups.

## 3. Results and discussion

NMR spectra have been shown to be highly sensitive to local chemical environment and the technique has been used previously to probe π–π interactions where such processes are manifested as up or downfield shifts depending on the magnitude of shielding/deshielding modulation (Kelly et al., 2001). Fig. 3 shows the modulation of the ketoprofen aromatic protons signals in the different formulations. It is clear that addition of fish oil to ketoprofen in Miglyol 812N resulted in dose-dependent downfield chemical shifts of signals from aromatic protons on the ketoprofen structure. From examination of the NMR spectra it would appear that the protons most susceptible to shift when in the presence of fish oil or Miglyol are those labelled protons (F), which are *meta* to the carbonyl group on the unsubstituted phenyl ring. This pattern seems logical, since the ring on which the carboxylic acid group is located is more sterically hindered to potential π–π interactions. A single EPA molecule or EPA-triacylglyceride would therefore experience difficulty in approaching this ring, whereas the free phenyl ring, which is also likely to be in a different plane, apparently represents a more attractive option for any possible interaction.

Fig. 4(a) shows the lowest energy geometry located for the complex of an EPA triglyceride with ketoprofen, which would have a molecular weight of approximately 1163. The highlighted protons (F) are the most noticeably affected in the NMR spectra. Somewhat unexpectedly, these are amongst the furthest away from the bulk of the triglyceride, and would therefore be expected to be the least affected of all the ketoprofen protons. This complex contains a hydrogen bond between the carboxylic acid group of ketoprofen and a carbonyl of the triglyceride, contributing to the calculated binding energy of 95.4 kJ mol$^{-1}$. This large binding energy and resulting stability makes it understandable how this complex could permeate the skin as a whole, as there is no obvious mechanism in the passage through the skin that could supply sufficient energy to cause dissociation. These results strengthen the hypothesis of permeation enhancement of EPA by ketoprofen discussed above.

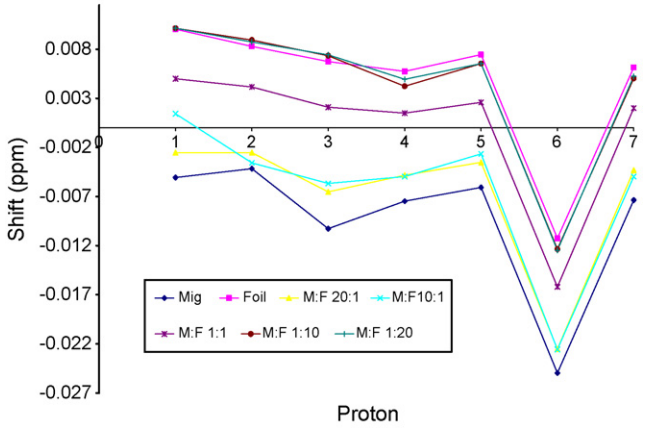


Fig. 3. Chemical shifts of ketoprofen aromatic protons in fish oil/Miglyol formulations.

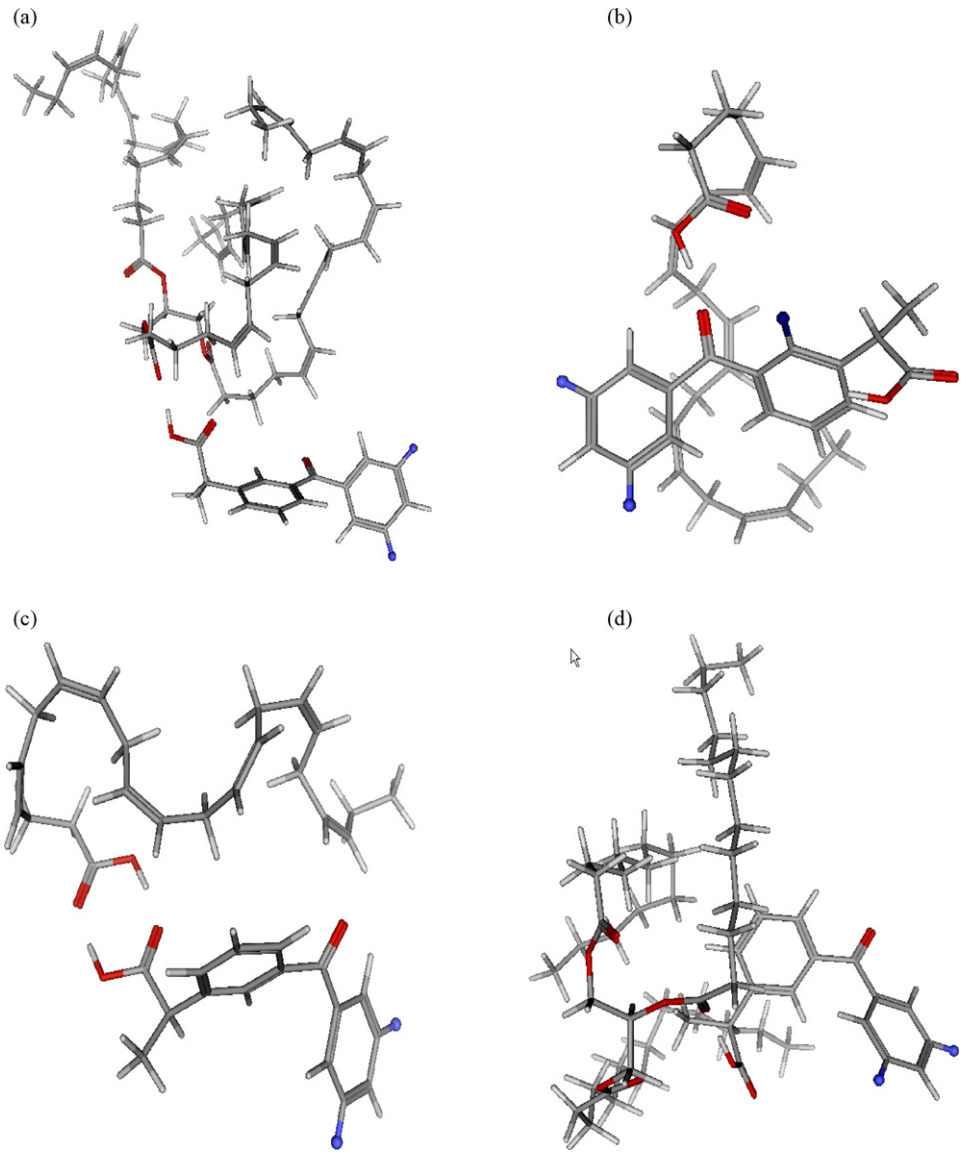


Fig. 4. Molecular model graphics: (a) EPA triglyceride/ketoprofen complex; (b) EPA free fatty acid/ketoprofen complex; (c) DHA free fatty acid/ketoprofen complex; (d) Miglyol/ketoprofen complex.

$^{1}$H NMR was carried out using differing concentrations of pure EPA free fatty acid and dose-dependent downfield shifts were again observed (Fig. 5). As with the entire fish oil, proton (F) appeared to be most susceptible to such a shift, suggesting that this effect may be due to individual fatty acids within the fish oil. The main difference between the free fatty acid and the triacylglycerol appears to be the effect upon the (B) proton of ketoprofen. This proton lies on the opposite ring to proton (F), *ortho*—to both carbonyl and carboxylic acid groups. This difference in the shifts may be due to the lesser steric hindrance of the free fatty acid, allowing it to approach closer to ketoprofen and hence exert a greater effect on other protons, specifically (B). Alternatively, free EPA may be susceptible to dimerisation between pairs of carboxylate groups unlike in the triglyceride, which may be partly responsible for the differences in the spectra.

Molecular models of ketoprofen with free EPA. Fig. 4(b) indicates significantly stronger binding (157.0 kJ mol$^{-1}$) than

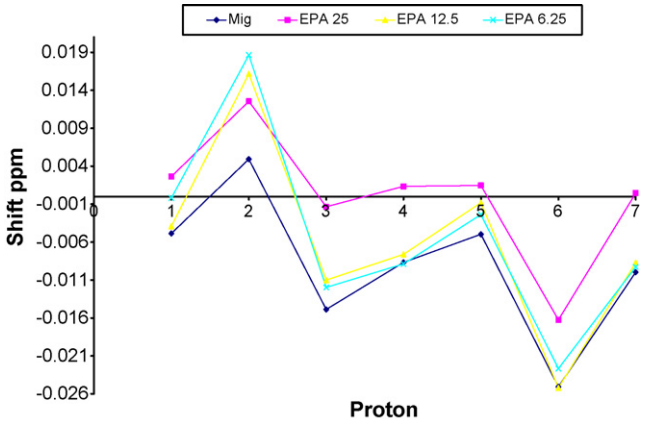


Fig. 5. Chemical shifts of ketoprofen aromatic protons in EPA free fatty acid/Miglyol formulations.

was found for the triglyceride of EPA discussed above. Once again, a hydrogen bond is formed between the molecules: in this complex, this is from the O–H of EPA to the central carbonyl of ketoprofen, which presumably accounts for much of the binding energy. Unlike in the triglyceride, however, there are reasonably close contacts between the aromatic rings of ketoprofen and C=C double bonds in the EPA chain, a possible source of the increased stability of this complex. In particular, protons (F) are found close to the penultimate C=C bond in EPA, with H···H distances of 3.5–3.7 Å. The orientation of the groups involved in this interaction is such that π···π stacking seems unlikely, and this might be better described as C—H···π contact. Proton (B) is also highlighted in Fig. 4, and is located close to the carboxylic acid group of EPA (3.0 Å), which could explain the shift seen for this proton that was not present with the triglyceride. Overall, the proximity of these groups is striking when compared with NMR spectra.

The formulations containing DHA and Miglyol follow a similar trend, with a large shift difference seen on protons (B) and (F) (Fig. 6). DHA at a level of 6.25 mg ml$^{-1}$ also effects proton (E). As DHA has an additional two carbon atoms within the alkene chain it is possible that they are responsible for the effect on this proton. At higher concentrations DHA is not able to fit as easily around the ketoprofen molecule due to competition for space and so the effect is not as marked. The molecular modelling of this compound with ketoprofen (approximate molecular weight of complex, 580) is shown in Fig. 4(c). Once again a strong binding energy (132.1 kJ mol$^{-1}$) was found but unlike free EPA this high binding energy is most likely to be due to the O–H attractions between the carboxylate groups of the DHA and ketoprofen, with a probable contribution from π orbital overlap. However, no clear link between this complex and the difference in proton (F) shifts can be determined.

It is clear that the ketoprofen $^1$H NMR shifts are distinctly different in the presence of polyunsaturated-containing fish oil and fully saturated Miglyol. Thus even though π-bonding between adjacent molecules of ketoprofen is possible, this arrangement was apparently disrupted by the presence of the fish oil/EPA. Furthermore, the same general pattern of shifts is observed when pure EPA and DHA were used in place of fish oil.

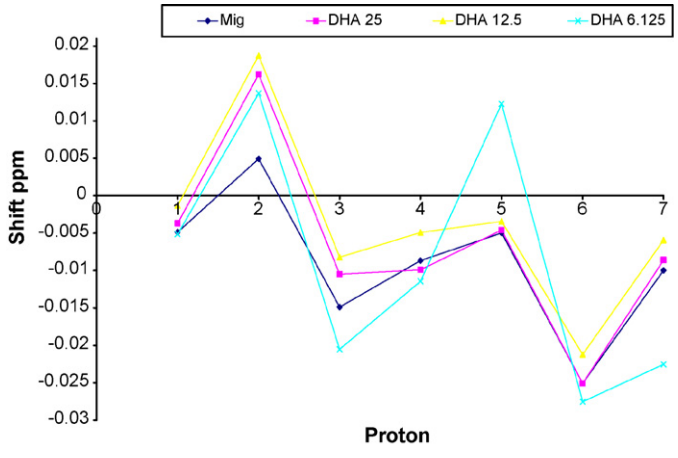


Fig. 6. Chemical shifts of ketoprofen aromatic protons in DHA/Miglyol formulations.

To compare the binding energies and structures of the EPA/ketoprofen complex and the control formulation, a complex of Miglyol with ketoprofen, with approximate molecular weight 764, was also modelled (Fig. 4(d)). The binding energy for this synthetic triglyceride complex is 109.1 kJ mol$^{-1}$, again a rather large value. Indeed, this is slightly larger than that found for the EPA-triglyceride complex, but not as large as that found for free EPA. Taken together, these data suggest that the saturated and unsaturated triglycerides have similar binding energies, but that a very strongly bound complex with EPA as a free fatty acid can be formed, which may displace another compound to form a stronger complex, thus further supporting our hypothesis that a EPA/ketoprofen complex may be able to permeate skin intact. It has been demonstrated (Thomas and Heard, 2005) that very little EPA permeated from a fish oil vehicle alone, underpinning the need for a complexant (ketoprofen) in the formulation. If the complex forms within the formulation then a co-operative enhancing effect could be in operation. The lipophilic EPA triglyceride/fatty acid permeates the lipophilic SC taking the more hydrophilic ketoprofen with it. Then in the more hydrophilic layers of the viable epidermis and dermis the reverse occurs and the ketoprofen permeates transporting the bound EPA with it.

Work carried out by Thomas and Heard (in press) have shown the molecular ratio of EPA:ketoprofen that is found post-transcutaneous delivery is approximately 10:1, with the ratio within the formulation of approximately 6:1. The molecular modelling demonstrated in the current paper has shown that a triglyceride is capable of binding to a single ketoprofen molecule. Further modelling studies could indicate the possibility of further binding with another triglyceride or free fatty acids present within the formulation. Also, it is important to consider the effect of biological processes within skin on these complexes (and vice versa). This is addressed in terms of metabolism of EPA (Thomas and Heard, in press) and activity on epidermal cyclooxygenase and lipoxygenase (Thomas et al., submitted for publication) in further articles.

It appears that the formula weights of the overall complexes have no significant effect upon their binding energies and it is the unsaturation of the EPA chains that has the over-riding effect, again supporting the notion of bonding by a complexation interaction. It is difficult to determine the exact mechanism of interaction between both the fish oil/ketoprofen complex and the free fatty acid/ketoprofen complex, as the triglyceride is present as a random mixture of fatty acids and not all EPA or DHA fatty acids. The trend seen in NMR shifts however are very similar between the triglyceride and free fatty acid indicating the shifts are more likely to be due to EPA and DHA/ketoprofen interactions than other fatty acids.

## 4. Conclusions

This work supports the hypothesis of complexation between EPA and/or DHA present as both free fatty acids and triacylglycerides, and ketoprofen. NMR data shows a clear pattern of

changes in chemical shift of aromatic protons in ketoprofen, the magnitude of which depends on the concentration of fatty acid. Molecular modelling results indicate quite large binding energies of all complexes considered, varying between *ca.* 90 and 160 kJ $mol^{-1}$ and the geometries of these complexes give rise to regiospecifically solvated complexes. Permeation of these intact complexes across skin could, at least in part, account for increased permeation reported for mixtures of ketoprofen with fish oils, EPA or DHA.

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