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# Preferential π–π complexation between tamoxifen and borage oil/γ linolenic acid: Transcutaneous delivery and NMR spectral modulation

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**Abstract**

The effect of different proportions of borage oil on the in vitro transcutaneous delivery of tamoxifen were studied, with the aim of developing a gel capable of the simultaneous delivery of tamoxifen and γ linolenic acid across (breast) skin. Supplementary work probed $^{1}$H NMR spectral data for tamoxifen in the presence of different proportions of polyunsaturated or unsaturated fatty acids. Typical, non-aqueous gels were modified to contain 1% tamoxifen and three levels of borage oil (∼25% γ linolenic acid) and the transcutaneous delivery of both tamoxifen and GLA across full thickness skin determined in vitro. Both tamoxifen and γ linolenic acid permeated the skin with the ratio of moles being consistent at approximately 4:1. This was irrespective of time, amount of borage oil contained in the formulation (above a minimum) and the presence of other (unsaturated) excipients: mineral oil, Miglyiol 810N, white soft paraffin, PEG400 and Cabosil M5. Dose-dependent downfield shifts of tamoxifen aromatic protons were observed in the presence of borage oil and linolenic acid (γ and α), but not saturated triacyl glycerol. The permeation data suggested vehicular complexation between tamoxifen and polyunsaturated constituents of borage oil and that such complexes permeated the skin intact. The $^{1}$H NMR data supported the hypothesis that such complexation was a consequence of preferential π–π orbital interactions between the phenyl groups of tamoxifen and the multiple double bonds of GLA. The mechanism for the permeation of intact complexes across skin remains to be elucidated.


*Keywords:* Tamoxifen; Borage oil; γ Linolenic acid; GLA; π–π interactions; Vehicle; Transcutaneous delivery; Skin; NMR; Complexation

## 1. Introduction

Tamoxifen is the hormonal treatment of choice for women who have ER+ (oestrogen receptor) breast

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cancer (Mehta, 2004) and also has demonstrated efficacy as a prophylactic (Cuzick et al., 2003). Certain essential polyunsaturated fatty acids (PUFAs) exhibit selective toxicity towards tumour cells and the $n-6$ PUFA γ linolenic acid (GLA) has been used cases of benign cyclical mastalgia where it is thought to act by attenuating the sensitivity of breast hormone receptors to circulating oestrogens (Kenny et al., 2001). More recently, the potential of administering GLA in combination with tamoxifen in the management of breast cancer has been studied using oral dosed regimens (Kenny et al., 2000, 2001). The findings of these two studies indicated that the major actions of GLA in ER+ breast cancer occur via the ER pathway and that GLA in combination with tamoxifen could be a valuable addition to the current treatments for ER+ breast cancer. However, oral administration of both drugs, either in combination or as single doses poses potential problems, as the wide-distribution of tamoxifen in the body can lead to the undesirable effects on the CNS, uterus and liver and such wide-distribution of GLA could potentially decrease its therapeutic efficacy. This work concerns the development of transcutaneous delivery systems for simultaneous delivery of both tamoxifen and GLA directly to the breast, where successful delivery would provide therapeutic levels of tamoxifen and GLA local to the site of action (subcutaneous breast tissue). Several studies have investigated the enhancement of the percutaneous absorption of tamoxifen using oleic acid (Gao and Singh, 1998a) and terpenes (Gao and Singh, 1998b; Zhao and Singh, 1998; Zhao et al., 2001), although no topical products are currently available. The in vitro delivery of substantial amounts of tamoxifen and GLA across full-thickness skin from simple tamoxifen/borage oil (25% GLA) formulations was recently reported (Karia et al., 2004) where a fixed relationship between the amounts of tamoxifen and GLA permeating the skin was noted. This suggested the formation of vehicular complexes that permeated the skin intact. A further report on the simultaneous delivery of GLA and tamoxifen was recently published where the effect of the penetration enhancer 1,8-cineole was examined (Ho et al., 2004).

In the current paper, we examined the in vitro transcutaneous delivery of tamoxifen and GLA from a series of non-aqueous gels containing a fixed 1% tamoxifen and various concentrations of borage oil. Furthermore, we sought further insights into the nature of the molecular interactions involved in tamoxifen/GLA complexes that could give rise to apparent preferential complexation. Within the formulation employed, only borage oil possessed double bonds within the acyl chains of the constituent fatty acids, thus we were particularly interested in the potential significance of π–π orbital attraction between the aromatic moieties of tamoxifen and regions of unsaturation that exist in polyunsaturated fatty acids. To this end, $^1$H NMR spectroscopy was used to probe modulation of tamoxifen aromatic proton signals in the presence and absence of borage oil, in addition to GLA and α linolenic acid (ALA) as free acids.

## 2. Materials and methods

### 2.1. Materials

Tamoxifen, borage oil, mineral oil, polyethylene glycol 400, butylated hydroxyanisole (BHA), ALA and GLA were obtained from Sigma Chemical Co., Poole, UK. Trifluoroacetic acid and dioxane were obtained from Aldrich Chemical Company Ltd., Gillingham, UK. HPLC grade chloroform, methanol, white soft paraffin, analytical grade diethylether and petroleum ether (60–80 °C bp) were obtained from Fisher Scientific UK, Loughborough, UK. Miglyols 812N and 810N were gifts from Contensio Chemicals, Witten, Germany and Black (Import and Export Ltd.), Hertford, UK, respectively. Cetrimide was obtained from Thornton & Ross, Huddersfield, UK, and Cabosil® M-5 was a gift from Cabot Carbon Ltd., Barry, UK. Deuterated chloroform ($CDCl_3$) was obtained from Cambridge Isotope Laboratories, Andover, MA, USA. Freshly excised porcine ears were obtained from a local abattoir prior to steam cleaning.

### 2.2. Preparation of gel formulations

Tamoxifen is highly lipophilic ($\log P$ 7.9). To aid solubilisation of tamoxifen, a standard non-aqueous gel formulation was chosen (Lund, 1994) modified to contain 1% tamoxifen with three different concentrations of borage oil (Table 1). Borage oil, tamoxifen, mineral oil, Miglyol 810N, white soft paraffin and PEG 400 were individually weighed and combined into a glass beaker. The beaker was covered using parafilm, placed into a thermostatically controlled

Table 1
Composition of gels (w/w)

| Gel | Tamoxifen | Borage oil | Mineral oil | Miglyiol 810N | White soft paraffin | PEG 400 | Cabosil |
|---|---|---|---|---|---|---|---|
| Control | 1 | 0 | 30 | 40 | 13 | 10 | 6 |
| I | 1 | 5 | 25 | 40 | 13 | 10 | 6 |
| II | 1 | 10 | 20 | 40 | 13 | 10 | 6 |
| III | 1 | 15 | 15 | 40 | 13 | 10 | 6 |

water bath (Fisher Scientific, Loughborough, UK), at a temperature of 75 °C and periodically stirred until a melt was formed. Cab-O-Sil® M5, an approved thickening agent based upon fumed silica, was weighed and gradually added to the mixture until a gel was formed. The gels were transferred to glass screw-topped jars and stored at 2–4 °C until required.

### 2.3. Skin permeation experiments

Using a scalpel, skin was removed from the dorsal side of freshly excised pig ears (Simon and Maibach, 2000). Hair was removed using electric clippers and the full-thickness membranes cut into 2 cm diameter discs using a borer. These were then placed onto pre-greased glass Franz-type diffusion cells, and the donor compartments clamped into position. The receptor chambers were filled with de-gassed receptor phase, comprised of 30 mg ml$^{-1}$ cetrimide + 0.05% BHA. This particular receptor phase has been previously demonstrated to have no deleterious effects on skin (Maguire et al., 2002). To each cell a magnetic stirrer bars was added and a visual inspection conducted to ensure no air bubbles were present. The cells were then dosed with 0.5 ml of gel and the receptor chambers sealed with minimally greased microscope slides. At appropriate time-points the entire contents of each receptor chamber was removed using a dedicated tube-tipped syringe, and transferred to a glass vial. From each receptor sample, a 400 μl aliquot was transferred to an autosampler vial for UV analysis of tamoxifen and a 1 ml aliquot was transferred to a second vial for analysis by GC. Samples were stored at −20 °C until required for analysis. Six replicates were performed for each treatment.

### 2.4. Determination of tamoxifen by UV spectrophotometry

Using matched quartz cells, the concentration of tamoxifen present in the receptor phase samples was determined using a Unicam Heλios UV–vis spectrophotometer (v. 1.30) (Thermo Spectronic, Cambridge, UK) at 258 nm. Where appropriate, dioxane was used as sample diluent and absorbance readings automatically corrected for receptor phase background using the double beam facility. Calibration curves were constructed from standard solutions of tamoxifen (range 0.1–20 μg ml$^{-1}$) that contained the relative same proportions of constituents contained in the receptor phase samples. Subsequent analysis of samples by HPLC confirmed that no other species were present which also absorbed at 258 nm, confirming the sole quantitation of tamoxifen by UV analysis.

### 2.5. Determination of GLA by GC

To determine the total amount of GLA permeating the skin (either as glyceride or as free fatty acid) receptor phase samples were firstly extracted by vortex mixing with 3× 1 ml redistilled diethyl ether and washing with 2× 1 ml water, and removing the diethyl ether layer between washes. Lipid extracts were dried under nitrogen and fatty acid methyl esters (FAMEs) prepared by acid-catalysed methanolysis. FAMEs were analysed using an Autosystem XL gas chromatograph (Perkin-Elmer Instruments, Beaconsfield, UK) and separation in a glass column (1.5 m × 3.0 mm) packed with 10% SP-2330 on 100/120 mesh Supelcoport (Supelco, Sigma–Aldrich Company Ltd., Poole, UK), using a previously reported method (Curtis et al., 2000). A gradient temperature program was used in which the initial oven temperature was 150 °C, following sample loading, the temperature was held for 0.5 min, and was subsequently increased at 2 °C min$^{-1}$ to a final temperature of 240 °C. This temperature was then held for a further 10 min. Identification GLA methyl ester was made by reference to standards (Nu-Check Prep Inc., Elysian, USA) and quantified using the in-built Autosystem Perkin-Elmer software using a pentadecanoate internal standard.

### 2.6. $^1H$ NMR spectroscopy

Saturated solutions of tamoxifen in borage oil, mixtures of borage oil and Miglyol 812N 3:1, 1:1, 1.3 and pure Miglyol 812N were prepared. To achieve a constant amount of tamoxifen, equivalent volumes of saturated solutions were transferred into NMR tubes, according to the solubility of tamoxifen in each solution. NMR spectra were acquired on Bruker Avance DPX300 spectrometer operating at 300 MHz and 27 °C. For miscibility with borage oil, Miglyol 812N and 810N, $CDCl_3$ was used as a solvent. Similar experiments were performed using GLA and ALA. Control experiments were carried out using saturated solutions of tamoxifen in Miglyol 810N (saturated triacylglycerol) and Miglyol 810/Miglyol 812N mixtures.

### 2.7. Data analysis

Tamoxifen and fatty acid concentrations were corrected for sampling effects and cumulative amounts permeated, per unit skin surface area, were plotted against time. Due to early loss of steady state kinetics, cumulative permeation data were determined at 6, 12, 24 and 48 h. As GLA is distributed randomly within borage oil, the concentration in the vehicle ($C_v$), was estimated from the fatty acid composition of borage oil, as previously determined (Redden et al., 1995). $^1H$ NMR shifts for tamoxifen aromatic proton absorptions in samples containing borage oil, either up- or downfield, were accurately measured (δ, ppm) relative to spectra obtained from the control (free of borage oil).

### 2.8. Statistical analysis

Statistical comparisons were made using Mann–Whitney *U*-tests and performed using SPSS for Windows, Version 10.

## 3. Results and discussion

### 3.1. Transcutaneous permeation of tamoxifen

Steady state permeation was short lived, <6 h (not shown). As only 1% (w/w) tamoxifen was contained in the gels, coupled with the entire-receptor phase sampling protocol, it is probable that depletion of tamoxifen occurred, and that this was exacerbated by binding to the silica-based thickening agent (Gallagher et al., 2003). Also, given that tamoxifen is a highly lipophilic molecule, and the vehicle itself is lipophilic, the net thermodynamic activity of tamoxifen in the formulation was relatively low. Despite these issues Table 2 reveals that all the gels containing borage oil resulted in higher tamoxifen permeation in comparison to the control. At 48 h, tamoxifen permeation from the gel containing 15% borage oil was 112.1 μg cm$^{-2}$ versus the control, which was 99.9 μg cm$^{-2}$: a 12.1% increase in tamoxifen permeation ($P = 0.054$, i.e. 94.6% probability of a significant difference). Further inspection of Table 2 reveals that there was no overt relationship between the permeation of tamoxifen and the proportion of borage oil in the gel (the reader may wish to re-plot the data from Table 2 for visual confirmation).

### 3.2. Transcutaneous permeation of GLA

Even though relatively small amounts of borage oil were incorporated into the gels (up to 15%, w/w) substantial amounts of GLA was shown to be delivered transcutaneously across full thickness porcine ear skin (at 48 h approximately 300 μg cm$^{-2}$). As seen with tamoxifen, steady-state flux of GLA was short-lived and cumulative permeation of GLA exhibited depletion after 6 h. Unexpectedly, as the amount of borage oil present in the gel formulation increased, no significant differences were observed for the permeation of GLA.

### 3.3. Relationship between permeation of tamoxifen and GLA

Table 3 reveals that the ratios of the moles of GLA to tamoxifen permeated are quite consistent: $x_{12} = 3.75$ (±S.D., 0.61) (Fig. 1). On the grounds of conventional skin permeation theory, one would have anticipated that the ratio would either increase or decrease as a consequence of differences in either thermodynamic activity and/or donor concentration.

However, a fixed relationship between the amount of tamoxifen and GLA from simple solution (Karia et al., 2004) and also NSAID and essential fatty acids from a fish oil vehicle were recently described (Heard et al.,

Table 2
Cumulative masses and moles of tamoxifen (TAM, $m_w$ 371.5) and GLA ($m_w$ 278.4) permeated at 6, 12, 24 and 48 h from three gels containing different amounts of borage oil (BO), $n = 6 \pm$ S.E.M.

| Permeant | $Q_6$ (μg cm$^{-2}$) | $Q_6$ (nmol cm$^{-2}$) | $Q_{12}$ (μg cm$^{-2}$) | $Q_{12}$ (nmol cm$^{-2}$) | $Q_{24}$ (μg cm$^{-2}$) | $Q_{24}$ (nmol cm$^{-2}$) | $Q_{48}$ (μg cm$^{-2}$) | $Q_{48}$ (nmol cm$^{-2}$) |
|---|---|---|---|---|---|---|---|---|
| TAM (0% BO) | 32.0 ± 2.22 | 86.1 ± 6.0 | 48.2 ± 2.12 | 129.7 ± 5.7 | 68.6 ± 4.22 | 184.7 ± 11.4 | 99.9 ± 5.87 | 268.9 ± 15.8 |
| TAM (5% BO) | 42.9 ± 4.01 | 115.5 ± 10.8 | 56.5 ± 6.00 | 152.1 ± 16.2 | 75.4 ± 3.25 | 203.0 ± 8.8 | 108.7 ± 6.61 | 292.6 ± 17.8 |
| TAM (10% BO) | 44.1 ± 3.88 | 118.7 ± 10.4 | 59.2 ± 7.03 | 159.4 ± 18.9 | 79.0 ± 7.33 | 212.7 ± 19.7 | 109.4 ± 8.84 | 294.5 ± 23.8 |
| TAM (15% BO) | 40.5 ± 3.20 | 109.0 ± 8.6 | 58.5 ± 4.33 | 157.5 ± 11.7 | 81.6 ± 7.10 | 220.0 ± 19.1 | 112.1 ± 7.77 | 301.8 ± 20.9 |
| GLA (5% BO) | 125.5 ± 7.02 | 450.8 ± 25.2 | 169.9 ± 11.23 | 610.3 ± 40.3 | 235.0 ± 16.08 | 844.1 ± 57.6 | 282.1 ± 18.02 | 1013.3 ± 64.7 |
| GLA (10% BO) | 114.8 ± 8.88 | 412.4 ± 31.9 | 180.3 ± 12.84 | 647.6 ± 46.1 | 275.0 ± 20.03 | 987.8 ± 72.0 | 325.9 ± 22.59 | 1170.6 ± 81.1 |
| GLA (15% BO) | 101.6 ± 7.02 | 364.9 ± 25.2 | 162.8 ± 13.39 | 584.8 ± 48.1 | 237.1 ± 16.87 | 851.7 ± 60.6 | 305.9 ± 28.89 | 1098.8 ± 103.8 |

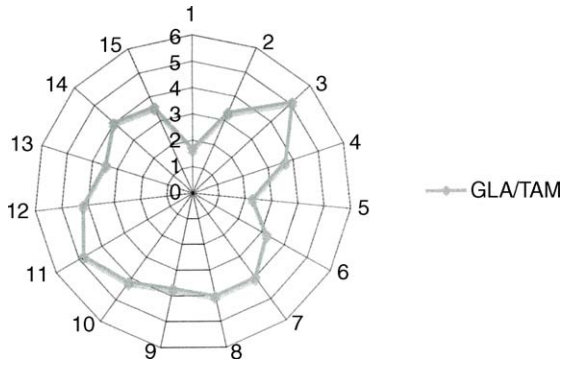


Fig. 1. Radar chart showing ratio of molar masses of GLA:tamoxifen—all observations, $x_{12} = 3.75 \pm 0.61$.

2003), although such processes were not observed in the work of Ho et al., where the presence of the co-solvent/enhancer (1,8-cineole) was believed responsible for disrupting complexation (Ho et al., 2004). The current data suggest that approximately 4 mol of tamoxifen were associated with every 1 mol of GLA, irrespective (above a minimum), of the concentration of borage oil present in the gel. There are some discrepancies, most notably with the 15% gel, which may be attributed to experimental error. Nonetheless, across all other time-points, a consistent 4:1 tamoxifen:GLA molar ratio was observed, again suggesting permeation of intact solvated complexes across skin. Furthermore, even with the higher amounts of borage oil/GLA, no further GLA permeated indicating an inter-dependence between tamoxifen and GLA in the permeation process. Such observations are counter-intuitive in terms of regular skin permeation theory, although a similar phenomenon was recently observed in the skin permeation of ketoprofen and eicosapentaenoic acid (Thomas and Heard, 2005).

The data also suggests preferential complexation between tamoxifen and polyunsaturated fatty acid moieties within the borage oil even in the presence of substantial proportions of the other oily components of the gel, see Table 3. The solubility of tamoxifen in borage oil at 32 °C (as determined by standard methods) was found to be 4.27 mg ml$^{-1}$. However, tamoxifen is also highly soluble in the other components used in the formulation.

In seeking to rationalise these data our attention was turned to the chemical nature of each component of the formulation. Ion-paired transport between

Table 3
Ratios of molar masses of GLA:tamoxifen permeated from the three gels at 6, 12, 24 and 48 h

| Gel | Molar ratio GLA:TAM (in gel) | Molar ratio GLA:TAM (6 h) | Molar ratio GLA:TAM (12 h) | Molar ratio GLA:TAM (24 h) | Molar ratio GLA:TAM (48 h) |
|---|---|---|---|---|---|
| I (5% BO) | 1.67:1 | 3.7:1 | 4:1 | 4.17:1 | 3.46:1 |
| II (10% BO) | 3.33:1 | 2.27:1 | 4:1 | 4.76:1 | 4:1 |
| III (15% BO) | 5:1 | 3.23:1 | 3.71:1 | 4.17:1 | 3.57 |

the amine tamoxifen and free GLA is unlikely as the major components of borage oil are triacylglycerols, of which GLA are a constituent (Redden et al., 1995), and edge-to-face interactions between aromatic moieties are unfavoured in solution (Jennings et al., 2001).

Thus, the key to understanding the processes that gave rise to the observed skin permeation data appears to lie in the fact that borage oil was the only component in the formulation to contain polyunsaturated species, principally GLA, ALA plus other PUFAs. Such molecules possess multiple areas of unsaturation and these electron-rich π orbitals which would provide an attractive force for complexing with the electron rich π orbitals in the aromatic moieties of tamoxifen. The self-assembly of π regions is well documented and, although individually weak, multiple π–π orbital overlapping can yield a substantial net attractive force (Kawahara et al., 2003). For example, π–π interactions play a significant role in molecular recognition and self-assembly processes (McGaughey et al., 1998; Gazit, 2002; Wijnja et al., 2004). Such interactions may be accentuated, although weakly, by the attraction between π-basic regions of the oil and π-acidic regions of tamoxifen (Lipkowitz and Baker, 1990; Horak et al., 2004).

### 3.4. $^1H$ NMR spectroscopy

As NMR spectra are highly sensitive to local chemical environment the technique has been used previously to probe π–π interactions (Hynninen and Lotjonen, 1993) where such processes are manifested as up- or downfield shifts depending on the magnitude of shielding/deshielding modulation (Kelly et al., 2001). The aromatic proton peaks from the $^1$H NMR spectrum for tamoxifen in Mygliol 812N and $CDCl_3$ were assigned as illustrated in Fig. 2. Fig. 3 shows that addition

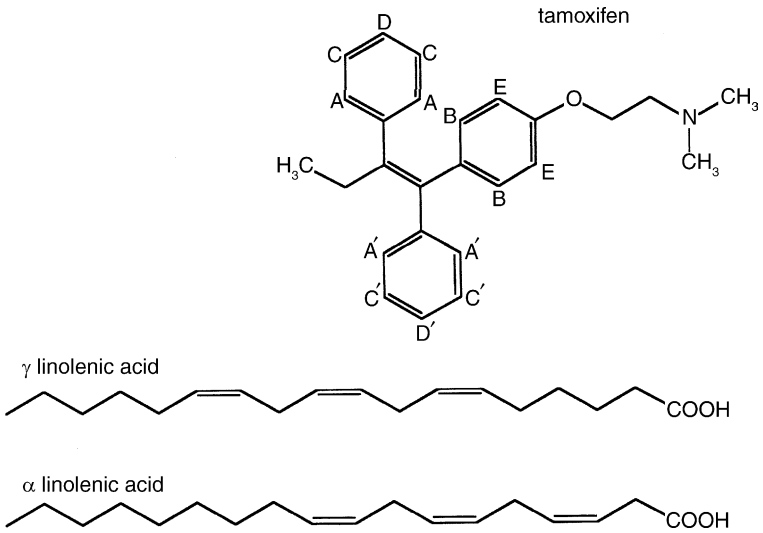


Fig. 2. Structures of tamoxifen, γ linolenic acid (GLA) and α linolenic acid (ALA) showing the assignment of tamoxifen aromatic protons to NMR spectra and potential alignment for ion-pairing/π–π complexation of tamoxifen with free fatty acids.

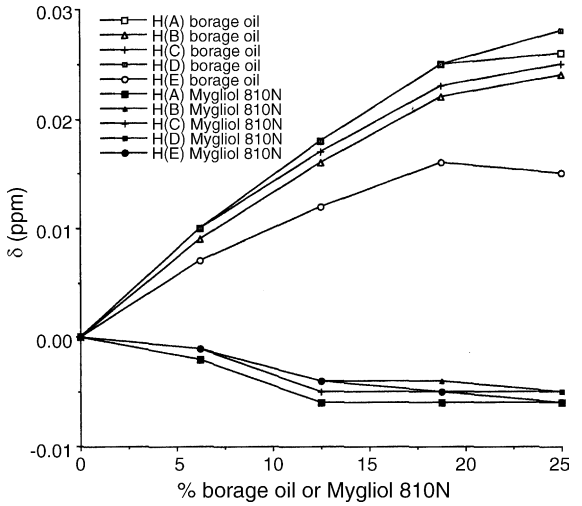


Fig. 3. Shift of tamoxifen aromatic proton signal (see Fig. 1) in $CDCl_3$ in response to different proportions of borage oil (25% GLA and other polyunsaturates) or Mygliol 810N (fully saturated) in Mygliol 812N.

of borage oil to tamoxifen in Miglyol 812 resulted in dose-dependent downfield chemical shifts of signals from aromatic protons on the tamoxifen structure. Slight upfield shifts were observed using solutions of tamoxifen in Miglyol 810—another fully saturated triacylglycerol.

When the same experiment was carried out using GLA and its bioactive isomer ALA in place of borage oil, dose-dependent downfield shifts were again observed (Fig. 4). As with borage oil, proton H(E) appeared the least susceptible to shifting, indicating that the high proportion of GLA in the borage oil (∼24%) was primarily responsible for the pattern of shifts observed with borage oil. GLA and ALA differ only in the location of the three double bonds (GLA: C6, C9, C12; ALA: C9, C12, C15; Fig. 1). Although GLA and ALA performed in a similar manner there appeared to be a difference in that tamoxifen aromatic protons were slightly more sensitive to shifting in the presence of lower concentrations of ALA than GLA, indicating that the position of the carboxylic acid headgroup was of significance. Indeed, cursory examination of the structures of tamoxifen and GLA and ALA free fatty acids reveals the potential for ion-pairing in addition to π–π interaction (Fig. 2), although it is unlikely that the two species would spontaneously protonate

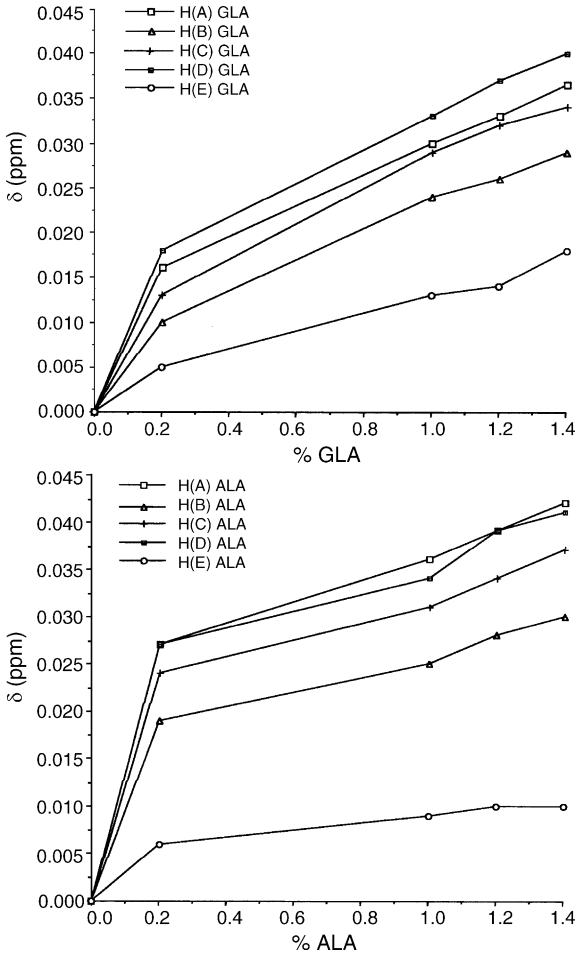


Fig. 4. Shift of tamoxifen aromatic proton signals (Fig. 2) in $CDCl_3$, in response to different proportions of GLA and ALA in Mygliol 812K.

and deprotonate, respectively, under the conditions used.

In the absence of additional factors, the greater the deshielding between two molecules the greater the downfield shift, arising as a consequence of closer proximity between adjacent π orbitals. On this basis it can be proposed that of the aromatic tamoxifen protons, the (A) and (A′) protons of tamoxifen were closest to one of the π orbitals of the double bonds in the GLA structure and the (D), (D′) and (E) were most remote. Whereas an in depth molecular modelling study might cast further light on such processes, it is very difficult to draw many further conclusions as borage oil is a

complex and random mixture and tamoxifen possesses three aromatic moieties each of which may rotate freely relative to the remaining moiety. This is further complicated by the fact that four protons are assigned as equivalent (A) and two as equivalent (E). However, it is clear that tamoxifen in the presence of borage oil and GLA is substantially different to that in the control medium which contains no unsaturated moieties. Furthermore, the same general trend is observed when pure GLA was used in place of the borage oil (Fig. 4). This supports the assertion of preferential vehicular π–π interactions between tamoxifen and regions of unsaturation of the borage oil, probably prior to the addition of the thickening agent to the formulations, giving rise to regiospecifically solvated complexes that could not have applied to other oily components of the gel as they are fully saturated. In a process that remains to be elucidated, the data suggest that these complexes then permeated the skin intact.

## 4. Conclusions

This study has found that using borage oil as a gel formulation vehicle/excipient, flux of tamoxifen and GLA was rapidly achieved with permeation continuing over 24 h. There was evidence that the transcutaneous permeation of tamoxifen and GLA occurred as a solvated complex with a molar ratio of 4:1 tamoxifen:GLA over the duration of the experiment. $^{1}$H NMR spectroscopic data supported the hypothesis that such preferential complexation was a consequence of π–π attraction. However, why such a complex should readily permeate skin is unclear, although oils are generally well absorbed into the stratum corneum and a modest enhancement effect was observed using borage oil. Furthermore, the transport of vehicle across skin simultaneously along with active – the so-called drag effect (Bendas et al., 1995) – is a known phenomenon that is seldom mentioned within the literature, and has recently been linked with vehicular solvation effects (Bowen and Heard, submitted for publication). On a broader scale, it has been demonstrated that selective complexation within a formulation can modulate not only the permeation of the active across skin, but also the complexant.

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