J. Pharm. Pharmacol. 2000, 52: 157–162
Received May 19, 1999
Accepted September 28, 1999

Effects of Some Non-ionic Surfactants on Transepithelial Permeability in Caco-2 Cells

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Abstract

The effects of the non-ionic surfactants polysorbate 20, polysorbate 60, polysorbate 85, cholesteryl poly (24) oxyethylene ether (Solulan C24) and the lanolin-based poly (16) oxyethylene ether (Solulan 16) on the epithelial integrity of monolayers of human intestinal epithelial (Caco-2) cells has been studied using metformin as a model drug. The aim was to identify the surfactants and their optimal concentrations capable of enhancing drug transport while causing no, or only minor, cellular damage. Effects on cell permeability were assessed by measurements of the transport of metformin, a hydrophilic drug, by monitoring transepithelial electrical resistance. Cell viability was determined by the diphenyltetrazolium bromide test (the MTT test).

All the surfactants studied demonstrated concentration-dependent effects on cell permeability and cell viability. The effects on transepithelial electrical resistance correlated with cell viability, i.e. increased transepithelial electrical resistance and increased cell-monolayer permeability for metformin corresponded to decreased cell viability. The results indicate that the Solulan and polysorbate surfactants were active as absorption enhancers, Solulan C24 and 16 being more effective than polysorbates 20, 60 or 85, causing an increase in metformin transport at lower concentrations than the polysorbates. Polysorbate 20 exerted its greatest effect at a concentration of 5%—increasing the flux of metformin after 3 h by a factor of around 20 over the control. Large increases in the transport of metformin, especially at surfactant levels of 0·05%, 0·1% and 0·5%, were related to the effect of Solulan C24 and Solulan 16 on the cell permeability.

The Caco-2 cell monolayer experiments confirmed the ability, especially of polysorbate 20, Solulan C24 and Solulan 16, to increase the absorption of metformin. The polysorbates increased permeability as a result of solubilisation of membrane components, while Solulans did so by penetrating and solubilising the membrane. Correlation between increase in membrane permeability and the toxicity of the surfactants towards the cell membrane has been established.

A wide variety of surfactants enhance drug absorption across epithelial barriers (Attwood & Florence 1983; Anderberg et al 1992, 1993; Anderberg & Artursson 1993). Surface-active absorption enhancers increase the permeability of cell membranes in a concentration-dependent manner. It has generally been accepted that surfactants increase drug permeability by the transcellular pathway although some recent studies using Caco-2 cell monolayers show that several absorption enhancers such as sodium dodecyl sulphate, sodium caprate and long-chain acylcarnitines increase permeability through paracellular pathways (Anderberg et al 1993; Hochman & Artursson 1994).

This paper describes some studies aimed at increasing the absorption of the hydrophilic drug metformin (dimethylbiguanide), an antihyperglycaemic agent used widely for the treatment of non-insulin-dependent diabetes mellitus. In man it has an oral bioavailability of 50–60% (Noel 1979; Pentikainen et al 1979; Tucker et al 1981). We

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have used Caco-2 cells, which are widely accepted in studies of epithelial integrity, drug transport and absorption-enhancing effects on cell membrane (Wilson et al 1990; Artursson 1991; Artursson et al 1996).

The effect of polysorbates (Tween) 20, 60 and 85 and the cholesteryl poly (24) oxyethylene ether (Solulan C24) and the mixture of poly (16) oxyethylene ethers of lanolin alcohol (Solulan 16) on [^{14}C]metformin transport across Caco-2 cell monolayers was determined, and membrane monolayer integrity was assessed by measuring transepithelial electrical resistance. We analysed the relationship between the structural and physical characteristics of the surfactants and their activity. Cell viability measured by the diphenyltetrazolium bromide test (MTT test) was also used to establish the cytotoxicity of the surfactants. The relationship between increase in metformin transport and the effects of the surfactants on the Caco-2 cell membrane was explored.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), gentamicin (10 mg mL^{-1}), non-essential amino acids (NEAA), foetal calf serum (FCS), phosphate-buffered saline (PBS) and trypsin/EDTA were obtained from Gibco Life Technologies, Paisley, Scotland. Polysorbate (Tween) 20 (polyoxyethylene sorbitan monolaurate), polysorbate (Tween) 60 (polyoxyethylene sorbitan monostearate) and polysorbate (Tween) 85 (polyoxyethylene sorbitan trioleate) were from Fluka Chemica, UK. Solulan C24 (a cholesterol poly (24) oxyethylene ether) and Solulan 16 (mixture of poly (16) oxyethylene ethers of lanolin alcohol) were obtained from Anstead, UK. Trypan blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethylsulphoxide (DMSO) were obtained from Sigma, UK. Hionic-Fluor liquid scintillation fluid was obtained from Packard Instrument company. Transwell polycarbonate cell culture inserts (diameter 12 mm, pore diameter 0·4 μm) were from the Costar Corporation, Cambridge, UK. [^{14}C]metformin hydrochloride (MW 163; 66 mCi mmol^{-1}) was a gift from Lipha Pharmaceuticals, UK.

Cells

Caco-2 cells were obtained from the European Collection of Animal Cell Culture (Wiltshire, UK). Cells were maintained in DMEM supplemented with 1% NEAA, 10% FCS and gentamicin (50 μg mL^{-1}) at 37°C, 10% CO_2, in 95% relative humidity. The cells were expanded in tissue culture flasks (75 cm^2 growth area). Caco-2 cells, passage number 70–75, were used. If the cell viability was satisfactory when assessed by the trypan blue exclusion method of Freshney (1991), the cells were cultured on permeable cell-culture inserts as described elsewhere (Artursson 1990). A cell suspension (0·5 mL, 1×10^6 cells mL^{-1}) was added to the apical sides of Costar Transwell cell-culture inserts and the cells were allowed to grow and differentiate for up to 30 days, refeeding every other day.

Osmolality

The osmolality of solutions of the surfactants was determined with an electronic osmometer (Knauer) by the freezing-point method, calibrated with a 400-mOsmol kg^{-1} solution of NaCl.

Absorption studies

Metformin solutions were prepared by mixing a solution of the radiolabelled isotope of [^{14}C]metformin and the corresponding unlabelled compound in DMEM to give 100 μM metformin solution of specific activity of 0·52 mCi mmol^{-1}. All transport experiments were performed at 95% relative humidity and 37°C in DMEM containing 1% NEAA. The transepithelial transport of metformin was studied for a period of 4 h. The radiolabelled drug solutions (control) and mixture of metformin and different concentrations of surfactants were added to the apical chamber. Samples (450 μL each) were taken from each basolateral chamber out of 1500 μL (3 times for each well) and replaced with fresh medium. The samples were measured in a liquid scintillation counter (Beckman). The results were expressed as percentage dose transported from the apical to the basolateral chamber.

Transepithelial electrical resistance (TEER)

The integrity of the monolayers was determined by measuring the potential difference between the apical and the basolateral sides of the monolayer as previously described (Artursson 1990). TEER was measured every hour during the experiments and was expressed as a percentage of TEER at $t = 0$.

Determination of the critical micellar concentration (CMC)

The CAHN DCA-312 System was used with a Wilhelmy plate to determine the surface tension (mN m^{-1}) vs log concentration relationship of Solulan C24 and Solulan 16 in distilled water at

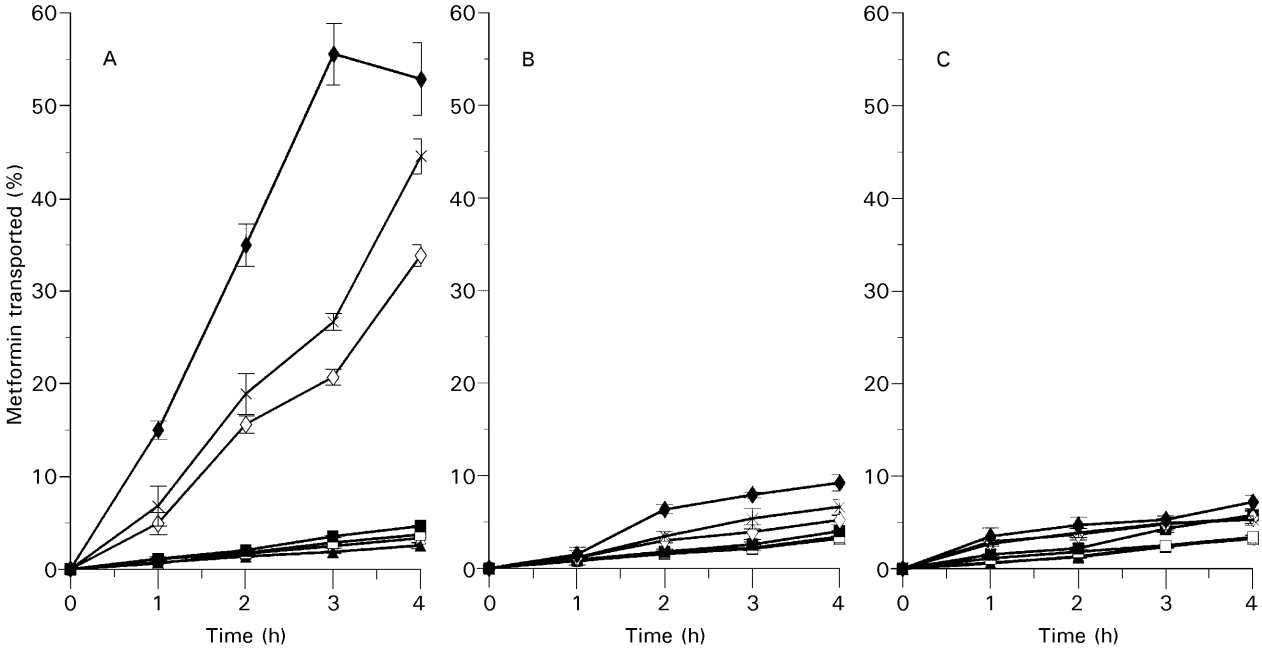

Figure 1. Transport studies of [^{14}C]metformin across Caco-2 cell monolayers as a function of the concentration of polysorbate 20 (A), polysorbate 60 (B) and polysorbate 85 (C). control (△, drug only), 0·005% (▲), 0·1% (□), 0·5% (■), 1% (◇), 2% (×), 5% (◆).

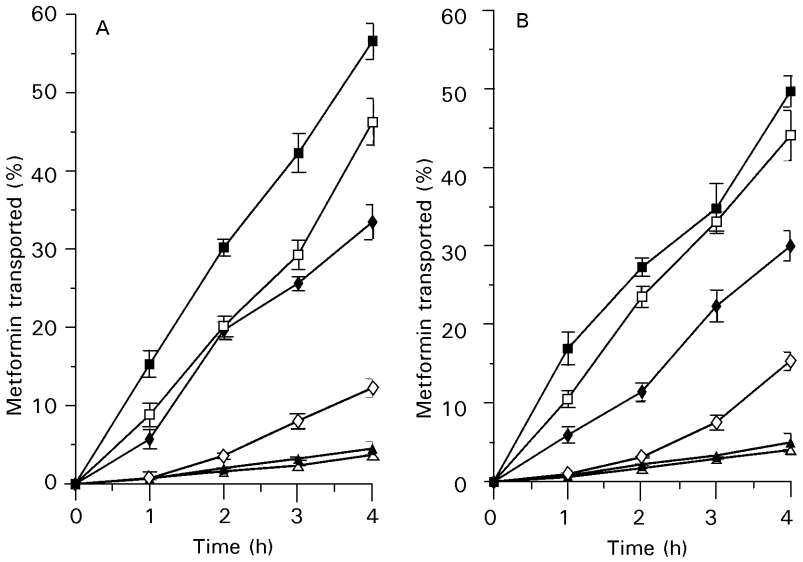

Figure 2. Transport studies of [^{14}C]metformin across Caco-2 cell monolayers as a function of the concentration of Solulan C24 (A) and Solulan 16 (B): control (△, drug only), 0·005% (▲), 0·01% (◇), 0·05% (◆), 0·1% (□), 0·5% (■).

23 ± 1°C. The CMC values obtained from these data were for Solulan C24 (0·009% w/v) and for Solulan 16 (0·01% w/v) to provide a comparison with data on the polysorbates.

Cell viability (MTT test)

The effect of the surfactants on intracellular dehydrogenase activity was determined by the MTT test described by Mosmann (1983). MTT is a tetrazolium salt that is reduced by mitochondrial dehydrogenase in living, but not dead, cells to give a dark-blue product, formazan, in amounts proportional to the number of the viable cells present. Immediately after incubation with surfactants, 100 μL of MTT (5 mg mL^{-1} in PBS) was added on to the cells and the Costar plates were incubated for another 5 h (37°C, 10% CO_2). The solutions were removed and 500 μL of DMSO added to dissolve the formazan. The optical density was measured on a microtitre-plate reader at 550 nm.

Table 1. Properties of the surfactant used.

Chemical name	Other names	MW	HLB*	CMC (g/100 mL)
Polyoxyethylene sorbitan monolaurate	Polysorbate 20	1227	16·7	0·0060[a]
Polyoxyethylene sorbitan monostearate	Polysorbate 60	1312	14·9	0·0028[a]
Polyoxyethylene sorbitan trioleate	Polysorbate 85	1839	11·0	0·0023[a]
Cholesteryl poly (24) oxyethylene ether	Solulan C24	1660[b]	14·0	0·0090
Lanolin-based poly (16) oxyethylene ether	Solulan 16	1000[b]	15·0	0·0100

*Hydrophile-lipophile balance. [a]Data from Wan & Lee (1974). [b]MW was determined using matrix-assisted laser desorption mass spectroscopy.

Results and Discussion

Before each experiment the osmolality of the samples was determined to establish that hypo- or hyperosmolality was not the reason for any observed change in permeability of Caco-2 cell monolayers. Since the osmolality of human plasma is about 290 mOsm kg^{-1}, it is reasonable to assume that this is the optimum for human cells in-vitro. The osmolality of control solution (metformin only) was $295 \pm 3{\cdot}5$ mOsm kg^{-1}. Osmolalities of surfactant samples were in the range 290–310 mOsm kg^{-1}. Freshney (1991) states that conditions between 260 and 320 mOsm kg^{-1} are accepted by most cells.

The effects of the surfactants on cell permeability were assessed by measurements of the transport of metformin, transepithelial electrical resistance and cell viability. Metformin transport under the influence of the polysorbates is shown in Figure 1. Polysorbate 20 exerts its greatest effect at a concentration of 5%, increasing the flux of metformin after 3 h by a factor of some 20 times over the control. Polysorbate 60 appears to have little effect and polysorbate 85 has no effect on metformin transport. Large increases in the transport of metformin, especially at concentrations of 0·05, 0·1 or 0.5% can be related to the effect of Solulans C24 and 16 on epithelial cell monolayers as shown in Figure 2.

Table 1 shows that the effective concentrations are all above the CMCs. In the case of polysorbate 20, its CMC is 0·006%, at which concentration it has no effect on permeability. However the CMC of both Solulans is $\sim$0·01% w/v (Table 1) and there is evidence of biological activity at this value (Figure 2).

If the data on metformin transport and transepithelial electrical resistance measurements are combined for all the surfactants at all concentrations, as in Figure 3, surfactant structural influences are not evident in the relationship between the reduction in TEER and metformin transport enhancement, indicating at least a common mechanism of action. The cell viability (MTT) test is used as a measure of toxicity to the cellular system. Figure 4 shows data for the polysorbates and for the Solulans. It is interesting that there are few observed effects until the threshold concentration of 0·1% w/v is reached with the polysorbates. Polysorbate 85 has little interaction with cell membranes, while the most active penetration enhancer (polysorbate 20) shows increased toxicity. At the 5% level of polysorbates 20 and 60, at which metformin transport is increased to the greatest extent, cell damage is severe. There is little difference between the two Solulans and they are both more toxic than the polysorbates by the test as

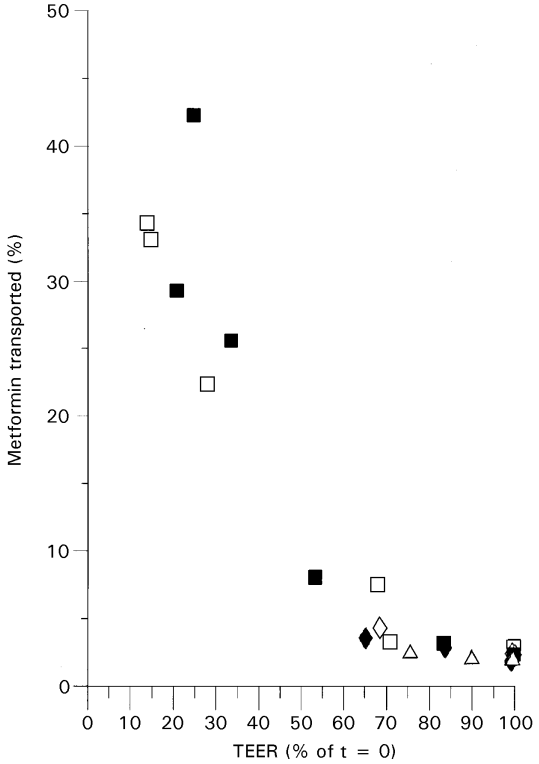

Figure 3. Correlation between transepithelial electrical resistance and percentage of [^{14}C]metformin transported across Caco-2 cell monolayers at t = 3h: Solulan 16 (□), polysorbate 85 (◇), Solulan C24 (■), polysorbate 20 (◆), polysorbate 60 (△) (the concentration of the surfactants: 0·005%, 0·01%, 0·05%, 0·1%, 0·5% for Solulan C24 and Solulan 16, and 0·005%, 0·1%, 0·5%, 1%, 2%, 5% for polysorbate 20, 60 and 85).

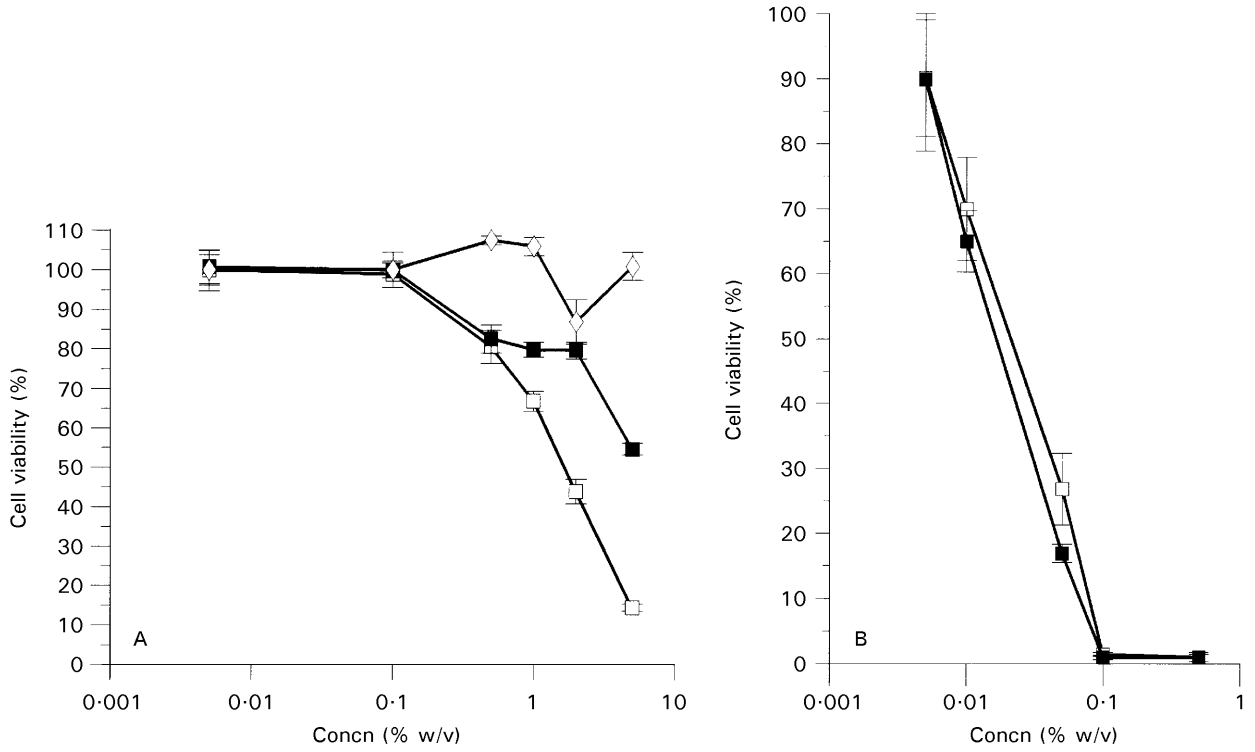

Figure 4. Cytotoxicity of polysorbate 20 (□), 60 (■) and 85 (◇) (A) and Solulan C24 (□) and Solulan 16 (■) (B) on Caco-2 cells, as measured by MTT test. Incubation period was 4 h.

well as having greater absorption enhancing properties. The effects on TEER correlated with cell viability, i.e. increased TEER and increased cell-monolayer permeability for metformin corresponded to decreased cell viability.

In relating surfactant structure to their interactions with the membrane, Figure 5 may assist. The size and shape of both the alkyl chain (hydrophobic) and the polar (polyoxyethylene) group of the surfactant is important in determining its ability to increase membrane permeability. The polysorbates generally have a complex structure. In particular, polysorbate 85 which is a trio-oleate, should have some difficulties in penetrating cell bilayers to enhance fluidity. It is possible that polysorbate 85, because of its bulk, is prevented from interacting intimately with the lipid membrane. This suggests that, as a class, the effect of polysorbates on membrane permeability results from solubilisation of membrane components rather than penetration hence their lack of effectiveness at low (pre-CMC) concentration. It is known that surfactants increase the permeability of membranes in a concentration-dependent manner. When a small amount of surfactant is present (below the CMC), monomers may be incorporated into the membrane, changing the physical properties of the membrane. At higher concentrations, mixed protein–lipid–surfactant micelles in equilibrium with surfactant micelles and free surfactant molecules occur as the membrane components are fluidized (Gulik-Kraywicki 1975; Helenius & Simons 1975).

Walters et al (1981) demonstrated that the unsaturated oleyl surfactant derivatives are more effective in increasing absorption of paraquat across rabbit isolated gastric mucosa than their

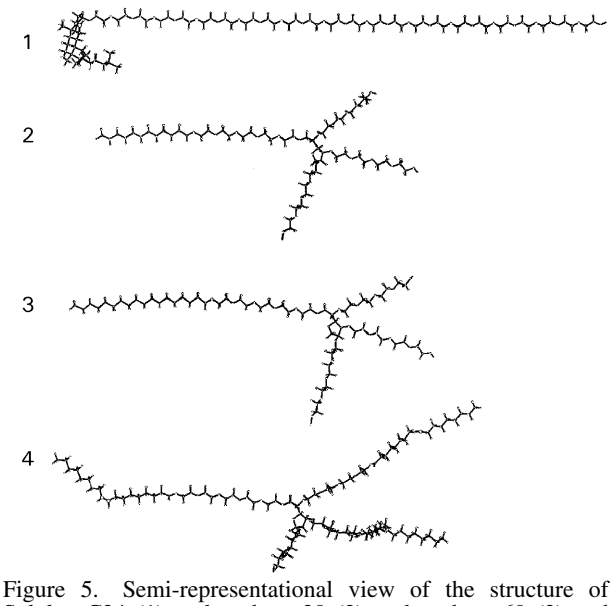

Figure 5. Semi-representational view of the structure of Solulan C24 (1), polysorbate 20 (2), polysorbate 60 (3) and polysorbate 85 (4).

saturated analogues. From these findings, one might expect that polysorbate 85 has a greater effect on absorption compared with polysorbate 20 or 60, but its bulky character may be responsible for the results obtained.

Many reports of intestinal absorption experiments have indicated an optimum in the length of the acyl chain between C8 and C12 (Ishizawa et al 1987; Swenson & Curatolo 1992). One unique aspect of the C12 chain is its intermediate solubility between oil and water (Florence 1981). Optimal effects for surfactants with a C12 hydrocarbon chain (such as polysorbate 20) probably correspond with the optimal size and shape for surfactant penetration. These findings are in agreement with our results, since polysorbate 20 appeared to be the most potent enhancer of the polysorbate series.

Surfactants which are very hydrophilic cannot partition with ease into the hydrophobic environment; surfactants with large ethylene oxide residues (Solulan C24 and Solulan 16) fall into this category (Florence & Gillan 1975). The cholesteryl ethers, although they possess quite bulky hydrophilic groups, have the cholesteryl moiety which has an affinity for membranes (Solulan C24 and Solulan 16). Their higher order of toxicity and interaction with membranes at low concentrations imply that their effects arise from a combination of penetration and solubilisation.

In conclusion, the Caco-2 cell monolayer experiments confirmed the ability, especially of polysorbate 20 and Solulan C24 and 16, to increase the absorption of metformin. The polysorbates increase permeability as a result of solubilisation of membranes, while Solulans do so by penetrating and solubilising the membrane. Correlation between increase in metformin permeability and toxicity of the surfactants towards the cell membrane has been established. The in-vitro results indicate that the Solulans and polysorbates were active as absorption enhancers in different concentration ranges.

Acknowledgements

This work was supported by a grant from Lipha Pharmaceuticals Ltd. We are grateful to Mr Mark Domin for the mass-spectrometry and Dr Colin James for molecular modelling.

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