Formulation and Intestinal Absorption Enhancement Evaluation of Water-in-Oil Microemulsions Incorporating Medium-Chain Glycerides

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We developed self-emulsifying water-in-oil (w/o) microemulsions incorporating medium-chain glycerides and measured their conductance, viscosity, refractive index and particle size. Formulation of Calcein (a water-soluble marker molecule, MW = 623), or SK&F 106760 (a water-soluble RGD peptide, MW = 634) in a w/o microemulsion having a composition of Captex 355/Capmul MCM/Tween 80/Aqueous (65/22/10/3, % w/w), resulted in significant bioavailability enhancement in rats relative to their aqueous formulations. Upon intraduodenal administration the bioavailability was enhanced from 2% for Calcein in isotonic Tris, pH 7.4 to 45% in the microemulsion and from 0.5% for SK&F 106760 in physiological saline to 27% in the microemulsion formulation. The microemulsion did not induce gross changes in GI mucosa at a dosing volume of 3.3 ml/kg. These results suggest that water-in-oil microemulsion systems may be utilized for enhancement of intestinal drug absorption.

KEY WORDS: water-in-oil microemulsions; medium-chain glycerides; enhancer; intestinal absorption; fibrinogen receptor antagonist.

INTRODUCTION

In recent years, much attention has been given to the design of new oral dosage forms, with particular emphasis on the development of lipid-based systems. Much of the activity in this area has been focused on the development of microemulsions as drug solubilisation and absorption enhancement systems (1,2).

Microemulsions can be defined in general as thermodynamically stable, isotropically clear dispersions of two immiscible liquids stabilized by interfacial films of surface-active molecules (3). The formation of microemulsions usually involves a combination of three to five components, namely, oil, water, surfactant, cosurfactant and electrolyte. The tendency toward a water-in-oil (w/o) or an oil-in-water (o/w) microemulsion is dependent on the properties of the oil and the surfactant. The role of the cosurfactant, usually a short-chain alcohol, is to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film due to the void space among surfactant molecules (3). However, the use of cosurfactant in microemulsions is not mandatory and alcohol-free self-emulsifying microemulsion systems have been described in the literature (4).

Lipid-based microemulsions (o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (1). Drug delivery advantages offered by microemulsions include: improved drug solubilisation and protection against enzymatic hydrolysis, as well as the potential for enhanced absorption afforded by surfactant-induced membrane fluidity and thus permeability changes (5).

In the present work, w/o microemulsion systems have been developed (6) using commercially available and pharmaceutically acceptable components. They consist of an oil, a blend of a low and high hydrophilic–lipophilic balance (HLB) surfactant and an aqueous phase. We have focused on formulations containing medium-chain glycerides (MCG), based on several reports in the literature that indicate absorption enhancement of different compounds/drugs by these neutral lipids (7–16). Thus medium-chain (C_6–C_{12}) fatty acids, mono-, di-, and tri-glycerides, particularly C_8/C_{10} mono-/di-glycerides, have independently been used in mixed micelle and emulsion formulations as absorption enhancers of a number of different drugs (7). Although bioavailability enhancement was primarily observed after rectal instillation particularly with medium chain fatty acids (8–10) or mono-/diglycerides (11–14), medium chain triglycerides (15) and/or mono-/diglycerides (14,16) have been reported to promote the oral absorption of ceftriaxone (14), cefoxitin (15) and Cyclosporine A (16). We have combined the low HLB surfactant (Capmul MCM) with a high HLB surfactant, such as Polysorbate 80 in order to form stable w/o microemulsion.

The water-soluble RGD peptide SK&F 106760 [cyclo(S,S)-(2-mercapto)benzoyl-(Nα-methyl)-Arg-Gly-Asp-(2-mercapto)-phenylamide] (Fig. 1) is a potent fibrinogen receptor antagonist (17). This cyclic tetrapeptide is enzymatically stable towards hydrolysis by intestinal enzymes and has low membrane permeability (unpublished data). As a result of its low membrane permeability, the oral bioavailability of this peptide is low, making it a good candidate for absorption enhancement evaluation from w/o microemulsion formulations.

The objectives of the present work were: a) to design and develop stable w/o microemulsions containing medium-chain glycerides based on the corresponding phase diagrams, b) to identify stable model formulations incorporating Calcein, a water-soluble marker or SK&F 106760 (Fig. 1) for subsequent *in vivo* testing in rats for bioavailability assessment. The results indicate significant absorption enhancement of both molecules from a microemulsion formulation incorporating Capmul MCM, without gross tissue damage of the gastrointestinal mucosa. The pharmacological activity of SK&F 106760 is not compromised by formulation in a microemulsion.

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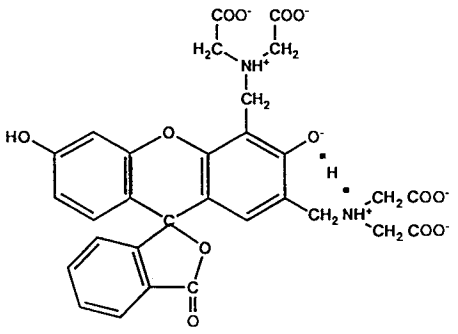
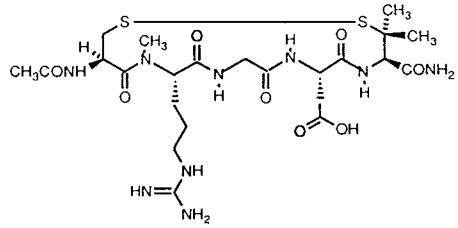


Calcein, pH 7



SKF 106760

Ac-Cys-(NMe)Arg-Gly-Asp-Pen-NH_2

Figure 1: Structures of Calcein and SK&F 106760. At physiological pH Calcein is negatively charged whereas, SK&F 106760 is zwitterionic. The molecular weight of Calcein and SK&F 106760 (acetate salt) is 623 and 634, respectively.

MATERIALS AND METHODS

Materials

Captex 355 (C_8/C_{10} triglycerides) and Capmul MCM (C_8/C_{10} mono-/di-glycerides) were supplied by Karlshamns Lipid Specialties (Columbus, OH). The fatty acid distribution in Captex 355 according to the manufacturer is: caprylic (C_8): 55%, capric (C_{10}): 42%, and caproic (C_6): 2%. Capmul MCM is approximately a 1:1 mixture of C_8/C_{10} mono-/di-glycerides with 2% free glycerol and it has the following fatty acid distribution: caprylic (C_8): 55%, capric (C_{10}): 30%, caproic (C_6): 3.2% and palmitic (C_{16}): < 1%. Tween 80 [polyoxyethylene (20) sorbitan monooleate] was purchased from Sigma Chemical Co. (St. Louis, MO). Physiological saline (0.9% sodium chloride, USP) with measured pH of 6.0 and osmolarity of 300 mOsm/liter was obtained from Baxter (Deerfield, IL). The universal Ringer salt solution was prepared containing the following salts (mM): Na^+, 141; K^+, 5; Ca^{2+}, 1.2; Mg^{2+}, 1.2; Cl^-, 122; HCO_3^-, 25; $H_2PO_4^-$, 0.4 and HPO_4^{2-}, 1.6. High purity Calcein [5(6)-carboxyfluorescein, MW = 623] was obtained from Molecular Probes, Inc. (Eugene, OR). The acetate salt of SK&F 106760 (MW 634) was provided by the Peptidomimetic Chemistry Department, SmithKline Beecham Pharmaceuticals (King of Prussia, PA). All drug supplies were stored at −20°C in a dessicator. The structures of Calcein and SK&F 106760 are shown in Figure 1.

Oil/Water Partitioning

The oil/water partitioning of Calcein or SK&F 106760 was determined by mixing and equilibrating each compound with a 1:1 mixture of Capmul MCM and Ringer's buffer, pH 7.4 (13). After a 2-hr equilibration at 37°C, the mixtures were centrifuged at 30,000 × g and the concentration of Calcein or SK&F 106760 in the two phases were determined by fluorescence or HPLC, respectively. A Perkin-Elmer LS 50 luminescence spectrometer (Perkin-Elmer Instruments, Exton PA) at excitation and emission wavelengths of 490 and 515 nm, respectively, was employed to measure Calcein concentration. An HPLC post-column fluorescence derivatization assay was used to determine SK&F 106760 (18).

Microemulsion Formulation/Phase Diagrams

Pseudo-ternary phase diagrams were constructed (6) with systems comprising four components: a medium-chain fatty acid triglyceride (Captex 355), a low HLB surfactant (Capmul MCM, HLB = 5.0–6.0), a high HLB surfactant (Tween 80, HLB = 15.0) and an aqueous phase (deionized water or saline). As this system comprises four components, the ratio of the oil (Captex 355) to low HLB surfactant (Capmul MCM) is kept constant so that there are only three variables, each of which can be represented by one side of the triangle. The regions of the phase diagram in which w/o microemulsions exist were determined by titrating a mixture of the oil and low HLB surfactant (in a fixed ratio) against the high HLB surfactant and the hydrophilic phase noting points of phase separation, turbidity and transparency. Clear, transparent formulations were indicative of a stable microemulsion. Once stable transparent formulations were obtained, simple tests, such as solubilisation of a water-soluble dye (Calcein), non-dispersability in water and extremely low conductance were employed to verify that the microemulsions formed were of the w/o type. In addition, microemulsions were examined under polarizing light and found to be non-birefringent as expected from their isotropic nature.

Microemulsion Preparation and Drug Incorporation

Once the microemulsion existence field on the phase diagram was identified, w/o microemulsions were readily prepared by admixing appropriate quantities of the various components with gentle hand-mixing or stirring to ensure thorough mixing. Calcein or peptide was first dissolved in the hydrophilic phase, either directly or by dilution of a stock solution, then the high HLB surfactant (Tween 80) was added followed by a pre-mix combination of the oil (Captex 355) and the low HLB surfactant (Capmul MCM). Upon complete mixing, either by hand or stirring via a magnetic bar, a clear and transparent formulation incorporating the water-soluble molecule was formed. Microemulsions incorporating Calcein or SK&F 106760 were equilibrated at ambient temperature overnight before dosing to the rats.

Physical Characterization of Microemulsions

Photon Correlation Spectroscopy. A Malvern Photon Correlation Spectrometer model 4700 (Southborough, MA) equipped with an argon laser model 2000 from Spectra-Physics (Spectra-Physics, Inc. Mount View, CA) was employed to monitor the particle size of microemulsions. Light scattering was monitored at 90° angle and 25°C with polystyrene beads used as a standard.

Viscosity and Refractive Index Measurements. The kinematic viscosity of microemulsions was monitored by a Cannon-Manning Semi-micro viscometer (Baxter/Scientific Products, McGaw Park, IL) with a constant of 0.0984 mm^2/S^2 in cSt/s (centistokes/sec). Multiplying this value by the density of the sample gives the viscosity in centipoise (cP). For measuring refractive index, a Milton Roy refractometer (Thomas Scientific, Swedesboro, NJ) was used. Both instruments were calibrated with oleic acid.

Conductance Determination. The conductance of microemulsions was determined using a YSI model 32 (Yellow Spring Instruments Co. Inc., Yellow Springs, OH) conductivity meter coupled to a YSI B3403 cell having a constant of 1.0/cm. Deionized water and saline were used to calibrate the instrument.

Polarized Light Microscopy. An Optiphot-Pol NIKON 144850 microscope (Nikon Inc. Garden City, NY) equipped with a camera was employed to examine the various fields (phases) of the phase diagram and to verify the isotropic behavior of microemulsions. A drop of sample was placed between a coverslip and a glass slide and then examined under polarized light. Pictures were taken at 10X and 20X magnification.

Absorption Studies

Sprague-Dawley, male rats that had been fasted overnight were employed for the absorption studies. Intravenous (i.v.) or intraduodenal (i.d.) administration of Calcein or SK&F 106760 either from a solution or a microemulsion was carried out using conventional methods (19,20).

For the i.v. administration, fasted rats were anesthetized with an intraperitoneal injection of a mixture of Rompun (5 mg/kg) and Ketaset (35 mg/kg) and a jugular catheter was implanted (21). Rats were allowed to recover from surgery for 1 day. Catheterized rats were fasted for 18 hr prior the administration of Calcein or peptide. Each compound was administered by lateral tail-vein administration. Blood samples of 0.5 ml aliquots were collected at 0, 1, 3, 5, 15, 30, 45, 60, 90, 120, 150, and 180 min. The 0 min sample was taken 15 min prior to administration of the dose. Plasma was removed from whole blood by centrifugation at 1600 × g for 5 min, and then stored at −20°C in 250 μl aliquots per sample. The blood pellet was reconstituted with 12.5 units heparinized saline and returned to the appropriate rat via the jugular catheter. After the experiment, rats were euthanized with i.v. administration of pentobarbital.

For the i.d. administration, in addition to jugular catheters, duodenal catheters were surgically implanted in anesthetized rats and the animals allowed to recover from surgery for 4–5 days. Calcein or SK&F 106760 was administered either from a solution or microemulsion via the duodenal catheter. Blood samples of 0.5 ml aliquots were collected via jugular catheter in heparinized eppendorf tubes at 0, 10, 30, 60, 120, 180, 240, and 1440 min. The 0 min sample was taken 15 min prior to administration of the dose. Plasma was collected for analysis and the blood returned to rats as described in the i.v. administration protocol. The stool of each rat over time was evaluated for consistency by a rank of soft, soft/watery, or mucoid.

Upon termination of the absorption study (4–6, or 24 hrs post-dosing) the animals were euthanized with asphyxiation using carbon dioxide and exsanguinated. An abdominal incision was then made, the entire GI tract removed and observed under a microscope (Nikon model SMZ-10 binocular microscope) at 50X magnification.

Plasma levels of Calcein were determined by fluorescence spectroscopy and those of SK&F 106760 using an HPLC post-column fluorescence derivatization assay (18), as described in the oil/water partitioning study. The bioavailability (% F) was calculated from the AUC (area under the plasma concentration-time curve) following i.d. or i.v. dosing using the following equation (Eq. 1):

$$\% F = (AUC_{id}/AUC_{iv}) \times (Dose_{iv}/Dose_{id}) \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Microemulsion Formulation/Phase Diagrams

A representative pseudo-ternary phase diagram of a system containing: C_8/C_{10} triglycerides (oil), low HLB surfactant (C_8/C_{10} mono-/diglycerides), high HLB surfactant (Tween 80) and water is shown as Figure 2. The mixture of oil plus low HLB surfactant, at a fixed ratio of 3/1, is indi-

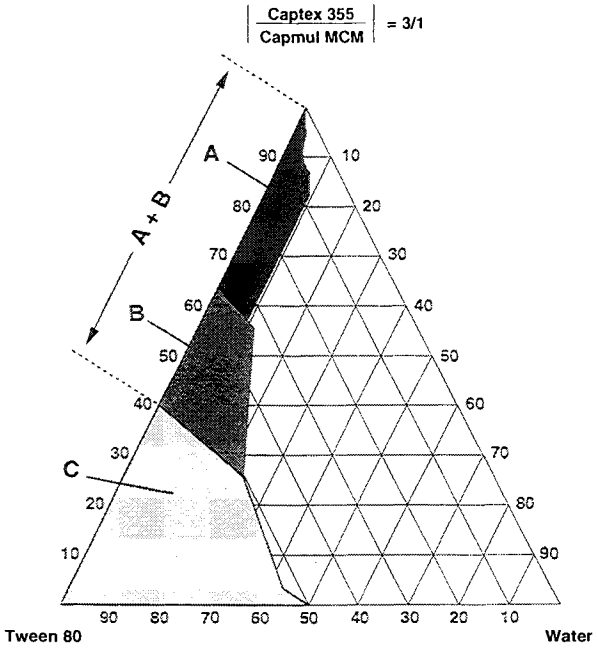


Figure 2: Water-in-Oil Microemulsion Existence Field in the Pseudo-Ternary Phase Diagram of the System Captex 355/Capmul MCM/Tween 80/Water at Ambient Temperature. The w/o microemulsion field extends through regions A, B and C of the phase diagram. The w/o microemulsion evaluated for absorption enhancement with Calcein or SK&F 106760 was from the upper part of field A. Other phases produced by this system are not shown.

cated as component 1, water as component 2 and the high HLB surfactant as component 3. This system produced a wide range of clear and transparent microemulsions which are shown in the phase diagram as the microemulsion existence field (shaded area), which field may usefully be subdivided into regions (A), (B) and (C). The sub-division is based primarily on differences in conductance, viscosity and dilutability in the presence of excess water (dispersed or internal phase). Both the viscosity and conductance increase from region (A) to (C), with major changes between (B) and (C). Thus, with an aqueous phase of saline at 3% (w/w), the conductance of microemulsions within regions (A), (B) and (C) varied between 0.5 and 4.0 μmhos/cm and the viscosity from 50–150 cP. In the presence of excess water or saline (100-fold), microemulsions of regions (A) and (B) are inverted to turbid emulsions (o/w) whereas microemulsions of region (C) remained clear indicative of a conversion to an o/w microemulsion. The calculated final HLB values for the blend of low and high HLB surfactants in the regions (A), (B) and (C) are 7 to 11, 11 to 13 and 13 to 15, respectively. The microemulsion formulation used for absorption enhancement evaluation with Calcein or SK&F 106760 is from region A and it has the following composition: Captex 355/Capmul MCM/Tween 80/Aqueous (65/22/10/3, % w/w). Pseudo-ternary phase diagrams similar to the one shown in Fig. 1 have also been constructed at other oil to low HLB ratios (6). This ratio was preferably maintained between 4:1 and 2:1.

The advantages of a microemulsion over conventional emulsions or other lipid carriers are improved stability and solubilisation characteristics (2). The microemulsions of the present study form spontaneously at ambient temperature when their components are brought into contact, that is without the application of high energy or the inclusion of short-chain alcohols that are known to cause tissue irritation (4). Formulation at ambient temperature is particularly advantageous for thermolabile drugs, particularly peptides. Since the components used to formulate the microemulsions of the present study are non-ionic, both the formation and stability of these microemulsions should not be affected by the pH of the aqueous phase in the range between 3 and 10 (6), a property that can be beneficial for drugs exhibiting higher solubility and/or stability at low or high pH.

Physical Characterization of Microemulsions

Table I summarizes some of the physical properties of a w/o microemulsion from region (A) of Figure 1, comprising a 3/1 mixture of Captex 355 and Capmul MCM (87%), Tween 80 (10%) and saline (3%). The extremely low conductance of this microemulsion (0.540 μmhos/cm) is characteristic of a w/o particle. For comparison, the conductance of a saline solution is 13,400 μmhos/cm. The thermodynamic stability is evident from the very small particle size (15.2 ± 4.1 nm). These microemulsions can be stored at 4°, 30° and 40°C for several months, without any phase separation and/or precipitation. Microemulsions were routinely examined under polarized light and found to be non-birefringent as expected from their isotropic behavior.

Table I. Physical Properties of a Captex 355/Capmul MCM/Tween 80/Saline (65/22/10/3, % w/w) Water-in-Oil Microemulsion

Physical property	Determined value[a]
Density	0.9677
Refractive Index	1.449
Viscosity	56.7 cP
Conductance	0.540 μmhos/cm
Particle Size[b] (mean diameter ± sd)	15.2 ± 4.1 nm
Polydispersity[b]	0.153

[a] At ambient temperature.

[b] Both expressed as particle number results; a polystyrene beads standard of 63 nm produced a particle size of 64.2 ± 15.1 nm and a polydispersity of 0.031.

The oil/water partitioning of Calcein or SK&F 106760 in the Capmul MCM/Ringer's buffer system was determined as described under Methods and found to be 7/93 for Calcein and 13/87 for SK&F 106760. That is, as expected from their high aqueous solubility, both compounds were primarily found in the aqueous phase of this two-phase system and this is likely their distribution between the oil and aqueous phase in the microemulsion particle.

In Vivo Absorption Enhancement Evaluation

Using a conscious rat model (19), the i.d. absorption of Calcein (a water-soluble molecule) and SK&F 106760 (an RGD peptide) either as an aqueous solution or formulated in a Captex 355/Capmul MCM/Tween 80/Saline (65/22/10/3, % w/w) w/o microemulsion (Table I) was determined as described under methods. The plasma level versus time curves for Calcein and SK&F 106760 are shown in Figs 3 and 4, respectively. It is evident from the i.d. absorption profiles shown in Figs. 3 and 4 that much higher plasma levels of both compounds were achieved from the microemulsion formulation than from the aqueous solution. Absorption from microemulsion was rapid with maximum plasma concentra-

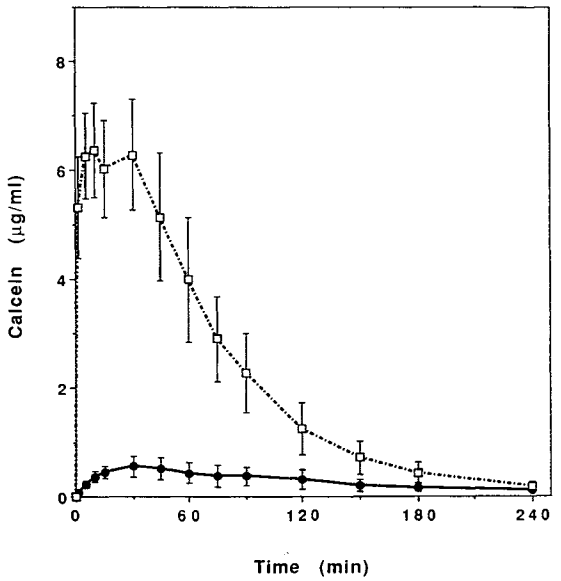


Figure 3: Plasma concentration of Calcein as a function of time following intraduodenal administration from aqueous (●) or a microemulsion (□) formulation. Results are means ± s.d. of four and six animals for the aqueous and microemulsion formulation of Calcein, respectively.

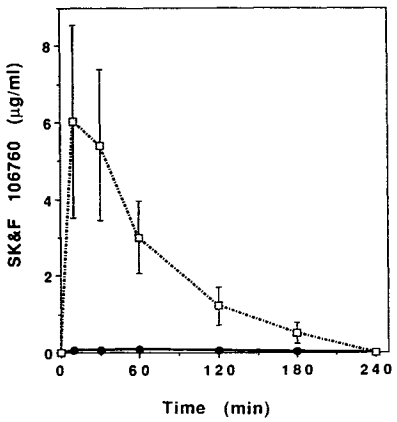


Figure 4: Plasma concentration of SK&F 106760 as a function of time following intraduodenal administration from aqueous (●) or a microemulsion (□) formulation. Results are means ± s.d. of three animals for the aqueous or the microemulsion formulation.

tion (Cmax) being reached at about 10 min for SK&F 106760 and 30 min for Calcein. Table II summarizes the bioavailability data determined from the areas under the plasma concentration-time curve of the i.d. (Figs. 3 and 4) and i.v. dosing. The difference between non-extrapolated and extrapolated AUCs were within 5%. As can be seen from the data in Table II, microemulsion administration of Calcein and SK&F 106760 resulted in significant absorption enhancement. Interestingly, although these molecules have very similar size (MW of about 650), they exhibit quite different physicochemical characteristics. In addition to structural differences (Fig. 1), Calcein is negatively charged at physiological pH while SK&F 106760 is zwitterionic. The aforementioned Capmul MCM/Ringer buffer partitioning data of Calcein and SK&F 106760 suggests that solubilisation enhancement of these compounds by formulation into w/o microemulsions is probably not a consideration factor for the observed absorption enhancement. In animals employed in absorption studies, there were no signs of irritation upon light microscopic examination of the intestinal tract of each rat at 4–6 hrs post dosing. A more definitive histological examination is necessary, however, in order to determine any changes in membrane structure. Microemulsion-formulated SK&F 106760 retained its pharmacologic activity based on platelet aggregation inhibition using the standard assay (17).

Oil-in-water microemulsions incorporating medium-chain glycerides have been reported to enhance peptide absorption (1,16). Very little is known, however, on drug/peptide absorption enhancement from water-in-oil microemulsions (1). Reports appeared in the literature that show enhanced intestinal absorption of water-soluble drugs in the presence of medium-chain glycerides primarily from mixed micelle or emulsion formulations (8–16). For lipophilic drugs/peptides it has been demonstrated that the particle size of the emulsion can affect drug dissolution and thus absorption (19,20). In the case of hydrophilic drugs, however, it is not clear what effect the particle size of the water-in-oil micro-emulsion may have on the rate and extent of their absorption. The mechanism(s) by which w/o microemulsions promote the absorption of a water-soluble drug/peptide are not well understood. *In vivo,* w/o microemulsions undergo phase inversion releasing the encapsulated drug (1,3). The exact nature of the *in vivo* particle, however, as well as, the site of drug release are largely unknown. One of the proposed mechanisms is based on enhancer-induced membrane structure and fluidity changes thus resulting in significant permeability changes. Supporting this mechanism is the fact that several *in vitro* studies have shown that medium-chain

Table II. Intraduodenal Bioavailabilities of Calcein and SK&F 106760 in the Rat

Compound	Administration route[a]	Dose[b]	$AUC_{0\rightarrow\infty}$ mean ± sd	%F[c] mean ± sd
Calcein (Aqueous[d])	Intravenous (n = 3)	0.6	0.114 ± 0.009	—
Calcein (Aqueous[d])	Intraduodenal (n = 4)	18.7	0.081 ± 0.029	2.4 ± 0.9
Calcein (Microemulsion)	Intraduodenal (n = 6)	6.2	0.505 ± 0.107	44.9 ± 9.6
SK&F 106760 (Aqueous[e])	Intravenous (n = 3)	3.0	0.642 ± 0.178	—
SK&F 106760 (Aqueous[e])	Intraduodenal (n = 3)	10.0	0.011 ± 0.005[f]	0.5 ± 0.3
SK&F 106760 (Microemulsion)	Intraduodenal (n = 3)	8.4	0.493 ± 0.160[f]	27.4 ± 8.9

[a] Number of animals per group are indicated in parentheses.
[b] mg/kg; the administered microemulsion volume was 3.3 ml/kg for either Calcein- or SK&F 106760-incorporating microemulsion.
[c] % F = $(AUC_{id}/AUC_{iv}) \times (DOSE_{iv}/DOSE_{id}) \times 100$ where, % F is the percent absolute bioavailability and AUC (mg × min/ml) is the area under the plasma concentration-time curve following intraduodenal (i.d.) or intravenous (i.v.) administration.
[d] Isotonic 10 mM Tris, pH 7.4.
[e] Physiological saline, USP.
[f] $AUC_{0\rightarrow240}$.

glycerides markedly affect the permeability of paracellular markers (7,22,23). Clearly a better understanding of the biophysical characteristics of the epithelial membranes and enhancer-membrane interactions, is necessary in order to elucidate the mechanism by which certain drugs/peptides cross mucosal membranes from microemulsion and other absorption enhancer formulations.

CONCLUSIONS

Stable, alcohol-free, self-emulsifying w/o microemulsions consisting of medium-chain glycerides have been developed. Several water-soluble compounds/peptides, having different physicochemical characteristics can be incorporated into these microemulsions. Significant intraduodenal absorption enhancement of a model compound (Calcein) and a peptide-drug (SK&F 106760) was observed in the rat from a microemulsion incorporating C_8/C_{10} mono-/di-glycerides (Capmul MCM). There was no macroscopic evidence for tissue damage 4–6 hrs after dosing at microemulsion levels up to 3.3 ml/kg. The pharmacological activity of SK&F 106760 is not compromised by microemulsion formulation. These results suggest that microemulsion delivery systems upon proper optimization and safety assessment may be utilized for oral administration of peptidergic drugs. The potential, however, for water-in-oil microemulsions to enhance the absorption of a wide range of peptidergic molecules remains to be established.

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