SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Pharmaceutical characterisation of surfactants applied in self emulsifying drug delivery systems

by Zoltán Ujhelyi PharmD

Supervisor:

Ildikó Bácskay, PhD

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Supervisor: Ildikó Bácskay PhD

Doctoral school of Pharmaceutical Sciences, University of Debrecen

Head of the Examination Committee: Lajos Gergely, DSc

Members of the Examination Committee: Romána Zelkó, DSc
Róbert Gáspár, PhD

The Examination takes place at the Library of Department of Pharmacology, Faculty of Pharmacy, University of Debrecen, 13th of January 2015. at 11:00

Head of the **Defense Committee**: Lajos Gergely, DSc
Reviewers: István Antal, PhD
György Deák, PhD

Members of the Defense Committee: Romána Zelkó, DSc
Róbert Gáspár, PhD

The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 13th of January 2015. at 13:00

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1. Introduction

Surface-active agents are widely used in new pharmaceutical dosage form development to improve the bioavailability of drugs which have low solubility in water. They can influence drug permeability by modifying barriers, by micellar solubilization, membrane fluidization, ion-pair formation, and the inhibition of efflux transporters such as P-glycoprotein. On the other hand, surfactants can cause local irritations, membrane damage and cell death and, therefore, during formulation processes in vitro tests are required for the investigation of cytotoxicity and irritative effects. Tensides are characterized by their critical micelle concentration (CMC) and hydrophilic–lipophilic balance (HLB) values. Primarily, CMC refers to arrangement of surfactants into micelles and indirectly to the solubilizing ability of lipophilic molecules such as membrane lipids. Micellar solubilization on the intestinal membrane permeability above the CMC can predict some lipophilic drug absorption The other important property of tensides which influences membrane integrity is the structures of tensides. The relationship between tenside structures and their cytotoxic effects or modification of membrane integrity has been already described in somecases. The cytotoxicity of a series of homologous polyethylene oxide (PEO) surfactants with a wide span in hydrophilic head-group size and hydrophobic chain lengths was measured for concentration- dependent effects on the transepithelial electrical resistance(TEER) and mannitol permeability across Caco-2 cell monolayers.Correlation of surfactant molecular structure with cell toxicity showed the size of the PEO group to be a more critical parameter than the size of the hydrocarbon chain Nevertheless, a variety of tensides and co-tensides with different structures have been commonly used in combinations in formulations.Microemulsions and SMEDDS often require a high content of surfactants, which can lead to skin or mucosal irritation. Amphiphilic molecules can be ideal surfactants and co-surfactants in SMEDDS , but cytotoxic effects must be screened to prove their harmlessness. It is also very important to asses the integrity of barriers in the presence of tensides and co-tensides Surfactants have been extensively studied to enhance transepithelial permeability for different marker molecules, but their effect on tight junction is less characterized. In a negatively or positively charged SMEDDS preparation, Labrasol is able to increase the paracellular transport of mannitol. It is capable of opening tight junction by modifying F-actin and ZO-1 proteins. Polysorbate 20 showed a concentration dependent paracellular increase in metformin transport as a result of solubilizing

the membrane, in paralell with TEER decrease, however increased permeability correlated with decreased cell viability of Caco-2 cells. The modulation of tight junction by Polysorbates has been investigated in human nasal epithelial cell monolayer, and the result suggested that Polysorbate 80 had no altering effect on tight junction integrity. In another study, cromolyn transport was evaluated using Caco-2 model with surfactant solution and proliposomal beads which contained Polysorbate 80, and no evidence of tight junction modulation was observed.

1.1 Surfactants

Tensides are widely used auxiliary materials usually applied in great quantities for the formulation of different pharmaceutical dosage forms. Surfactants are able to increase the solubility of lipophilic drugs and enhance the permeability of active agents The objective of this study was to examine the cellular effects of the members of two non-ionic amphiphilic tenside groups and their mixtures on human Caco-2 cell monolayers as dependent upon their chemical structures and physicochemical properties. The first group of polyethylene glycol esters is represented by Polysorbates and Labrasol alone and in blends, while the members of the second group. Capryol 90, Capryol PGMC, Lauroglycol 90 and Lauroglycol FCC were used as propylene glycol esters. They are increasingly used in SMEDDS as recent tensides or co-tensides to increase hydrophobic bioavailability of a drug. Microemulsions and SMEDDS (Self-Micro Emulsifying Drug Delivery Systems) often require a high content of surfactants, which can lead to the alteration of intestinal membrane barrier functions and can cause damage to the intestinal epithelium. Polyethylene glycol (PEG) based solubiliziers (Kolliphors formerly known as Cremophors) as tensides or as co-tensides can be used to design these peroral formulations PEG based surfactants can be also chosen to prepare parenteral medicaments, such as intravenous (i.v.) injections. In a small amount, these surface active agents can cause hemolysis of erythrocytes (Li et al. 2011). That was the reason for developing a novel Cremophor-free self-emulsifying drug delivery system for the i.v. or peroral delivery of paclitaxel. Cremophor EL was reported to be responsible for the hypersensitivity reactions in patients who were treated with parenteral formulations. Tensides with high IC_{50} values (the concentration of test substances reducing cell viability by 50 % compared to the untreated control) may ensure a secure application. In spite of the above mentioned extensive studies, there is limited information about the toxic effect of Cremophor solubilizing agents.

1.2.Caco-2 cell line

The Caco-2 cell line is an immortalized culture of heterogeneous human epithelial colorectal adenocarcinoma cells. Caco-2 cells are used extensively as an in vitro model for the rapid screening of intestinal absorption and cytotoxicity. However these cells were originated from a colon carcinoma, under specific conditions the cells become differentiated and polarized and their morphology and function, compares the enterocytes lining of the small intestine. Caco-2 culture cells form a highly functionalized epithelial barrier. Caco-2 monolayers express a number of enzymes, tight junctions, and transporters. The Caco-2 monolayer is widely used to investigate the biological effects of various surfactants since the viability and the paracellular permeability of the monolayer can be influenced by pharmaceutical auxiliary compounds.

2. Objectives

The aim of this study was to examine the cellular effects of non-ionic amphiphilic tensides and their mixtures on human erythrocytes and Caco-2 cell monolayers as dependent upon their chemical structures and physicochemical properties. To evaluate the accurate physical and biological properties, the following experiments have been performed:

1. Assesment of solubilishing ability of non-ionic, amphiphilic surfactants by CMC determination.

2. Investigation of cytotoxicity of the compounds on Caco-2 cell monolayers.

3. Determination of haemolitic activity of surfactants used in parenteral drug delivery systems.

4. Evaluation of Caco-2 membrane alterations, influenced by the investigated surfactants

5. Classification of non-ionic, amphiphilic surfactants applicability according to their physical and biological properties

3. Materials and methods

3.1. Surfactants

Polysorbates (20, 60, 80,) were obtained from Sigma-Aldrich. PEG-based surfactants (traditional names are Cremophors, new trade names are Kolliphors) were obtained from BASF, Germany and evaluated at a concentration between 0.02-40 v/v %. Capryol 90®, Capryol PGMC®, Labrasol®, Lauroglycol 90®, and Lauroglycol FCC® (Table 1.) were kind gifts from Gattefossé, France. We investigated three compositions: 0.05 % Labrasol – 0.001 % Polysorbate 20; 0.05 % Labrasol – 0.001 % Polysorbate 60; 0.05 % Labrasol – 0.001 % Polysorbate 80 (Table 1.). An LDH Cytotoxicity Detection Kit was purchased from Roche Diagnostics (Mannheim, Germany). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), Hank's Balanced Salt Solution (HBSS), phosphate buffered saline (PBS), Trypsin-EDTA, Heat-inactivated fetal bovine serum (FBS), L-glutamine, non-essential amino acids solution, penicillin-streptomycin, and Lucifer yellow were purchased from Sigma-Aldrich. Transwell® inserts were obtained from Corning, New York (USA).

3.2. Measurement of surface tension

Surface tension was measured using a computer-controlled tensiometer (Attension/Sigma 700 Surface tensiometer). Prior to measurements, an aqueous stock solution of 0.10% (v/v) concentration was prepared for each tenside. From each stock solution, appropriate dilutions were made to produce solutions with concentrations ranging from 0.0001 to 1% (v/v). All solutions were allowed to stand overnight in their containers, and then their surface tension (y) values were measured. Surface tension data, as a function of concentration (c), was determined employing the ring-detachment method. An accuracy check on the tensiometer was conducted by measuring the y of pure water: y water = 72 dynes/cm ± 0.1 at 25 ±0.5°C, which is in agreement with literature data.The following standardized procedures were adopted: 1. All measurements were made at 25±0.5°C. 2. All reported values were averaged. 3. All glass apparatus, as well as the platinum ring, were cleaned with distilled water and then thoroughly rinsed with 96% alcohol. 4. Each

solution stored in the dish was set aside for 10 minutes prior to the actual measurement of y. This procedure was to compensate for any adsorption by the glass apparatus. The dish was then thoroughly drained, a fresh volume of the same solution was added, the ring was submerged just below the liquid surface, and the whole was allowed to equilibrate undisturbed for 15 minutes before taking the measurement of y. This procedure was done to make allowance for aging effects, as mentioned previously.In our investigation, critical micelle concentration (CMC) was measured by the determination of surface tension. CMC assessment with du Nouy tensiometer is a well-known method. This method has been widely used to characterize different surfactants in aqueous solution at different temperatures.

3.3. Determination of haemolitic activity

Erythrocytes were separated from citrated blood by centrifugation at 2500 × *g* for 10 min., washed three times with PBS and resuspended in the same solution. Aliquots of the cell suspension with the respective red blood cell number of 5×10^7 were added to the buffer solution (PBS pH 7.2) containing increasing concentrations of the samples investigated in the study. After mixing them gently, each solution was incubated at 37 °C for 10 min and then centrifuged at 5000 × *g*. Finally, the absorbance of the hemoglobin released into the supernatant was measured at 540 nm with FLUOstar OPTIMA Microplate Reader. The percentage of hemolysis was expressed as the ratio of hemoglobin in the supernatant of the sample solutions related to the hemoglobin concentration after the complete hemolysis of erythrocytes in water. The dose–response curve was determined, and the concentration inducing hemolysis in 50% of the erythrocyte population (HC_{50}) was subsequently calculated.

3.4. Cell culture

Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were grown in plastic cell culture flasks in Dulbecco's Modified Eagle's Medium, supplemented with 3,7 g/l $NaHCO_3$, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) non-essential amino acids solution, 1% (v/v) L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin at 37ºC in an atmosphere of 5% CO_2. The cells were routinely maintained by regular passaging. For cytotoxic and transport experiments, cells were used between passage numbers 20 and 40. The culture media was replaced with fresh media in every 72 hours (Bigansoli et al., 1999).

3.5. Cell viability measurements, MTT, LDH assays

MTT method was used to determine the viability of Caco-2 cells . Cells were seeded on flat bottom 96-well tissue culture plates at a density of 10^4 cells/well and allowed to grow in a CO_2 incubator at 37 ºC for 7 days. For these studies, the culture medium was removed, surfactant solutions were added, and the cells were incubated for a further 30 minutes. After removing the samples, another 3-hour-incubation in a medium containing MTT at the concentration of 0.5 mg/ml followed. The dark blue formazan crystals were dissolved in acidic isopropanol (isopropanol: 1.0 N hydrochloric acid = 25:1). The absorbance was measured at 570 nm against a 690 nm reference with FLUOstar OPTIMA Microplate Reader (BMG LABTECH, Offenburg, Germany). Cell viability was expressed as the percentage of the untreated control . A lactate dehydrogenase (LDH) cell viability test was also used to detect membrane damages caused by surfactants (Sha et al., 2004). For this test, $2x10^3$ cells/well were seeded in 96-well plates incubated in a CO_2 incubator at 37 ºC for 2 days. The culture medium was replaced by the test solutions and the LDH release from damaged cells into the extracellular fluid was determined after 30 minutes of treatment with the test agents in different concentrations. The measurement was performed according to the manufacturer's instructions using the LDH Cytotoxicity Detection Kit (Roche Diagnostics, Mannheim, Germany). All tests were carried out in triplicate and the results of the cytotoxicity assays were expressed as IC_{50}, which showed the concentration of test substances reducing the cell viability by 50% compared to the untreated control

3.6. Caco-2 Permeability Experiments

Caco-2 cells were seeded on 12-well Transwell inserts (Corning Transwell Clear, diameter: 6, 5 mm, pore size: 3, 0 μm) at a density of 2×10^5 cells/cm^2 and grown for 21–28 days. The inserts were used for experiments when transepithelial electrical resistance (TEER) values reached 1000 Ωcm^2. The culture medium was removed and the apical and basolateral compartments of the Caco-2 cell monolayers were pre-incubated in HBSS at 37°C for 30 minutes. After that, the transport solution was added which contained surfactants and Lucifer yellow (40 μg/ml) was added to the apical chambers. In transport experiments, two concentrations of the surfactants were considered: 0.001 v/v%, and 0.05 v/v%. The negative control was HBBS. Samples were collected from the basolateral compartment every 15 minutes for up to 60 minutes and replaced with fresh HBSS. Fluorescence of the samples was measured at an excitation wavelength of 450 nm and an emission wavelength of 520 nm with FLUOstar OPTIMA Microplate Reader. We depicted the permeated quantities of Lucifer yellow as a function of time for all experiments.

3.7. Transepithelial Electrical Resistance Measurements

To investigate the Caco-2 membrane integrity alteration transepithelial electrical resistance measurements have been performed. In TEER values were measured in every 15 minutes and monitored during the following 24 hours with MILLIPORE MILLICELL-ERS 00001.

3.8. Immunohistochemistry

The treated and control Caco-2 cells cultured on glass cover slips were washed in phosphate buffered saline (PBS, pH 7.3) and fixed with methanol - acetone (1:1) solution for 10 minutes. Cells were blocked with 3 % BSA and incubated with 1:200 dilutions of primary antibodies against ZO-1 (Invitrogen, USA), claudin-1 (Invitrogen, USA), β-catenin (Sigma-Aldrich, Hungary) in blocking buffer for overnight. Incubation with secondary antibody Cy3-labelled anti-rabbit IgG (1:400, Sigma-Aldrich, Hungary) for ZO-1 and β-catenin, Alexa 488 anti-rabbit IgG (1:400, Life Technologies) for claudin-1and bisbenzimide (10 μM) to stain cell nuclei lasted for 1 hour. Between incubations cells were washed three times with PBS. Cover slips with the stained cells were mounted in Gel Mount (Biomeda, USA) to glass slides and examined by a NikonEclipse TE2000 fluorescent microscope (Nikon, Japan) and photographed by a Spot RT digital camera (Diagnostic Instruments, USA).

3.9. Statistical Analysis

Data were analyzed using SigmaStat (version 3.1; SPSS, Inc.) and presented as means ± S.D. Comparison of the groups was performed by one-way ANOVA. This ANOVA was used to compare the differences of each values belong to certain concentrations in MTT, LDH, TEER. We signed the significant differences with asterices in these figures. After that, the results among the groups were presented by Tukey's test. Differences were regarded as significant, with $p < 0.05$. All experiments were carried out in triplicates and repeated at least three times.

4.Results and Discussion

4.1. Measurements of surface tension and determination of critical micelle concentration

For the determination of surface tension of each surfactant, a computer-controlled ring-detachment method was used. WE determined the surface tension (y) values versus concentration (v/v %). All plots show that y varies linearly with the concentration up to a certain concentration characteristic of each compound, where y remains essentially constant, with further increases in concentration. Our measurements demonstrate that CMC values decrease in the following order: Capryol 90 > Capryol PGMC > Lauroglycol 90 > Lauroglycol FCC > Polysorbate 20 > Polysorbate 60 > Polysorbate 80 > Labrasol

4.2. Cell viability measurements, MTT and LDH tests

There are significant differences in the cytotoxic properties of surfactants in a concentration-dependent manner. Polyoxyethylene glycol based tensides are more toxic surfactants than propylene glycol esters. Polysorbates showed higher cytotoxicity than polyethylene glycol based Labrasol with different degree of esterification. In the propylenglycol esters group (Capryol 90, Capryol PGMC, Lauroglycol 90 and Lauroglycol FCC) Capryol 90 proved to be the most toxic in Caco-2 cells. Capryol PGMC, Lauroglycol 90, and Lauroglycol FCC showed no difference in effects on cell viability compared to the untreated control measured by the MTT. In the case of the LDH assay, the trend was similar; both tests were in agreement with the rankings of the cytotoxicity of the investigated surfactants. 0.001 % Polysorbate 20, 60, 80 and 0.05 % Labrasol in blend did not show any cytotoxicity by MTT and LDH as well. There are noteworthy differences between the IC_{50} values revealed by the MTT and LDH assays, respectively.

4.3. Evaluation of Caco-2 cell membrane alteration

Polysorbates and Labrasol as polyethylene glycol esters were able to increase Lucifer yellow permeability significantly ($p<0.05$) in non-toxic concentrations, while propylene glycol ester surfactants did not alter the integrity of monolayers. Increased Lucifer Yellow permeability was measured in the case of polyethylene glycol esters in blends. To determine whether the

pronounced effect of surfactants (Polysorbates, Labrasol and blends) on TEER of Caco-2 monolayers is reversible, the apical buffers containing the surfactants were replaced with a fresh cell culture medium after treatments. TEER values significantly decreased after 1 hour of incubation with surfactants. The 24 hours TEER measurement revealed that the transepithelial electric resistance started to increase in the fresh culture medium. TEER values of Labrasol and Polysorbates-treated monolayers increased from 35 % to 60 % of baseline after 3 hours. Moreover, transepithelial electric resistance values increased even further. 75% of baseline TEER values were observed after 6 hours and TEER values reached the baseline at the end of experiment. These results indicate that the monolayers treated with 0.05% Labrasol, 0.001 % Polysorbates 20, 60, 80 and their mixtures, fully recovered their barrier properties within 24 hours, as assessed by the TEER, and did not lead to the irreversible disruption of the Caco-2 monolayers.

4.4. MTT cell viability tests and haemolytic activity measurements

The correlation between IC_{50} and HC_{50} values of PEG based surfactants was determined. Our results have shown that, there are significant concentration-dependent differences in the cytotoxic properties of PEG based surfactants (Cremophors). Cremophor WO7 proved to be the most toxic on Caco-2 monolayers. The IC_{50} values increase and in vitro cytotoxicities decrease in the following order: Cremophor WO7>CO455>CO410>A6> A25> RH60 and RH410 > RH40. PEG 40 based Cremophors showed less toxic effect than PEG 7, PEG 35, PEG 60 based materials, Ceteareth and Stearylalcohol derivatives. Higher IC_{50} values were measured in the case of Cremophor CO 410 and 455 surfactants with PEG 40 based structures. This result is in agreement with that offered by the manufacturer because they are only suggested for external use.

There were significant differences in the hemolytic potentials of the investigated surfactants as well. HC_{50} and IC_{50} values showed significant correlation because the order is the same in both cases. In the case of the evaluated PEG-based surfactants, the impairment of the mitochondrial function is less expressed than the hemolytic potential. This is confirmed by our experiments, because HC_{50} values are higher than IC_{50} values.

5. Discussion

In our experiments the cytotoxicity of two tenside groups with different chemical structures and HLB were evaluated. These surfactants have similar amphiphilic properties, esters structures, but the head in the molecules, the carbon chain type and length and the degree of esterification are different. To evaluate the cytotoxicity of surfactants, two markers of toxicity were employed: 1. the plasma membrane integrity as characterized by LDH release; and 2. the mitochondrial dehydrogenase activity by the reduction of MTT by viable cells. We assume that all of these surfactants damage the cell membrane in concentration-dependent manner, but only in higher concentrations did they interfere with the mitochondrial enzymes. Polyethylene glycoles (Polysorbates and Labrasol) are more toxic surfactants than propylene glycols. Polysorbates with the highest HLB are the most toxic compounds. In the case of Labrasol, the degree of esterification (mono-, di-, triester components) and the lack of sorbit component reduced cytotoxicity compared to Polysorbates. A previous study observed that Labrasol induced cytotoxicity dramatically in a concentration-dependent manner .Labrasol 0.05 % and 0.001 % Polysorbates 20, 60, 80 in blends did not affect the cytotoxicity than the surfactants alone, in our experiments. This result is in contrast to Buyukozturk et al. investigations, because they found that Labrasol and Capmul MCM in mixture showed higher cytotoxicity levels compared surfactants alone and in emulsion formulation. Surfactant type, surfactant ratio in mixture and their interaction might affect the cytotoxicity. Nevertheless, surfactants in emulsion and their stable or unstable formulations can also influence the cytotoxicity. Labrasol, above the CMC can increase membrane lipid fluidity in a concentration dependent manner. Labrasol can also interact the membrane lipids. 0.001 % Polysorbates concentrations are below the CMC. Polysorbates are in the monomers. In blends there are interactions between Labrasol micelles and Polysorbates monomers. The complex micelle formation may exist and it depends on the ratio of applied surfactants. These micelles might effect on the membrane components in different manner. The cytotoxicity mechanism may be different.According to our immunohistochemistry studies none of the surfactants disrupted visibly the monolayer in the applied concentration. For this reason the tight junction modulations are not due to the cytotoxic effects of surfactants. Regarding the structures, in the case, when the hydrophilic head was changed from polyethylene glycol to propylene glycol, the main determining factor of cytotoxicity could be the monoester content and the length of the carbon chain. According to previous studies the optimum chain length is

between C_8-C_{12}. However, chain length in itself is not a determining factor for the increase of intestinal absorption, some other structural parameters such as the degree of esterification, the position of unsaturation and the nature of substituents can also influence surfactants to act as penetration enhancers. The differences between MTT and LDH methods are well-characterized. This fact can be explained by the nature of each assay. The comparison of MTT and LDH methods can show not only the differences between cytotoxicity methods but also the sensitivity of cell lines.Lactate dehydrogenase (LDH) is an intracellular enzyme common in most cells, and LDH leakage is an established marker for cell toxicity and cell membrane damage. Changes in membrane integrity are good indicators of severe injury prior to cell death, and can be assayed by measuring lactate dehydrogenase (a stable cytostolic enzyme) activity (LDH) in the extracellular medium. MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria. Some toxic agents i.e. cadmium-chloride can result in the inhibition of mitochondrial respiration. In this case the MTT assay detects early cytotoxicity compared to the LDH. The cytotoxicity mechanism of surfactants are different than cadmium-chloride exposure. The effect of polysorbates on membrane integrity results in solubilisation of membrane components. In this case the first cell damage mechanism is the intracellular membrane solubilisation. For this reason the LDH assay shows lower IC_{50} values than MTT assay in our experiments. The following effect might be the damage of the mitochondrial function so that the IC_{50} values measured by MTT are higher. Same cytotoxicity values of Polysorbate 20 were measured by LDH and MTT in our investigations. It is thought that Polysorbate 20 in low concentration (0.004 %) can cause either intracellular membrane or mitochondrial damage. These in vitro assay systems can predict irritancy, potential and delayed toxicity of surfactants. Nevertheless, cytotoxicity data alone are not necessarily predictive of in vivo issues, but complemented with results of paracellular permeability the in vivo toxicity data may be estimated. Critical micelle concentration refers to the ability of micelle solubilisation of surfactants. Polysorbates and Labrasol are hydrophilic tensides (HLB = 14 – 16.7), hence they have much lower CMC and good solubilisation abilities than propylene glycol esters with their lipophil properties, low HLB (4 - 6)(Capryol 90, Capryol PGMC, Lauroglycol 90 and Lauroglycol FCC).Polysorbates can show expressed cytotoxicity below their CMC's. It can be explained that Polysorbate monomers in small amount may be incorporated into the membrane and can change the physical properties of membrane. At concentrations below the CMC, higher levels of surfactant monomers are available to penetrate

e.g. the skin and increase permeability. At higher concentrations, above CMC there is an equlibrium between mixed protein-lipid surfactant micelles and surfactant saturated analogues and membrane components may be fluidizied. Labrasol resulted in cytotoxicity above its CMC's. The low CMC value refers to good ability of solubilisation and the high IC_{50} values mean better and safer applicability than Polysorbates.The micelle solubilisation ability of propylene glycol esters are worse than hydropilic surfactants. For this reason their CMC's are higher than Polysorbates and Labrasol. Capryol 90 showed cytotoxicity by MTT and LDH below the CMC. Capryol PGMC is cytotoxic by LDH but not by MTT below the CMC. Lauroglycol 90 and Lauroglycol FCC can show cytotoxicity both by MTT and LDH above the CMC. Surfactants below the CMC are in monomers and some surfactant monomers can damage the cell membranes. Tensides above the CMC can cause dose-dependent cytotoxicity because the membrane components are solubilizied.A previous study has shown that surfactants can modify Caco-2 cells membrane integrity, and can cause intestinal membrane damage which results in an alteration of intestinal membrane barrier function. This is in agreement with our study, because our experiments indicate that all of the surfactants interacted with Caco-2 monolayers, decreasing the transepithelial electric resistance (TEER). However, propylene glycol esters surfactants did not modify TEER. TEER values correlate with the IC_{50} values; it is similar to the result of previous study. TEER values were lower in the case of polyethylene glycol esters (Polysorbates and Labrasol) and Lucifer yellow permeability was also altered. The blends (0.001 % Polysorbates 20, 60, 80 – 0.05 % Labrasol) decreased the TEER significantly ($p<0.05$). The degree of TEER decreasing of the blends was the same than Labrasol and Polysorbates alone. Changes in intestinal permeability and epithelial tight junction integrity was assessed by measuring transepithelial electrical resistance (TEER). The decreasing of TEER values was influenced by the surfactant type and the ratio of oil content. This result is consistent with our study because the HLB, chain length of tensides, ratio of mixed surfactants may affect the TEER. Polyethylene glycol esters were able to increase the paracellular transport in non-cytotoxic concentrations. These alterations were reversible, and the monolayers were able to recover after 24 hours. On the other hand, examining the effects of propylene glycol esters on the monolayer integrity, we did not detect alterations in Lucifer yellow permeability. The absorption enhancers increase the permeability of cell membranes in a concentration-dependent manner and it is in agreement with our findings. However, many articles report that surfactants are not applied alone

but in combination with other penetration enhancers as co-surfactants. In some cases, the surfactants in mixture showed higher cytotoxicity and different permeability on Caco-2 monolayers. In our experiments we combined Polysorbates (20, 60, 80,) 0.001 % and Labrasol 0.05 % in non-toxic concentration. These combinations were not cytotoxic, but the effect of Lucifer Yellow permeability was different than when we applied them alone. In our paracellular permeability study of these blends we depicted the permeated quantity as a function of time. The kinetics of the permeated quantities of Lucifer Yellow was not linear. Our investigation presented that the blends of Polysorbates and Labrasol increase Lucifer Yellow permeability on Caco-2 monolayer significantly ($p < 0.05$) but not linearly. The effect of Polysorbate 20 and Labrasol was more expressed than Polysorbate 60, 80 combined with Labrasol. A formulation consisting Maisine 35-1, Cremophor EL and Labrasol resulted in increased paracellular permeability with no toxicity. The effect was mainly attributed to the Labrasol. It is thought that Labrasol has expressed effect on membrane permeability and Polysorbate 20 may enhance this mechanism. Polysorbate 60 and 80 in blends can also improve the effect of Labrasol. The chain length of surfactants and the interaction of tensides might influence the Lucifer Yellow permeability. We wanted to certify whether the higher permeated quantity of Lucifer Yellow attributes to the increased paracellular permeability of Polysorbate 20, and Labrasol or the disruption of cell membrane. Labrasol resulted in stronger realignment of tight junction proteins (claudin-1, β-catenin and ZO-1) than Polysorbate 20. The effect of blend was similar to Polysorbate-20 treated samples. None of the surfactants disrupted visibly the monolayer in the applied concentration. For this reason the tight junction modulations are not due to the cytotoxic effects of surfactants. Our immunohistochemistry experiments certify that Polysorbates and Labrasol, alone and in blends increase the permeability of Caco-2 monolayers toward hydrophilic compounds predominantly by the modulation of the tight junction and not by the disruption of the cell membrane. Sha et al. reported that Labrasol in negatively and positively charged SMEDDS preparation opens the tight junction by involving F-actin related changes and redistribution of ZO-1. It is similar to our findings, because in our experiment Labrasol in itself results in the disappearance of claudin-1 and an increase in the cytoplasmic staining intensity for β-catenin. The effect of Polysorbate 20 and its blend with Labrasol was moderate, because a decrease in claudin-1 and an increase in the cytoplasmic staining intensity for β-catenin were also observable. Permeability experiments, TEER measurement and immunohistochemistry staining clearly show

that Labrasol and Polysorbate 20 treatments increase Lucifer Yellow flux, decrease monolayer integrity and cause redistribution of junction of proteins which lead to the increment of paracellular permeability. These mechanisms do not exclude the possibility of the transcellular pathway. Although it is well-known that Lucifer Yellow is permeated mainly through paracellular pathway, it can also be the marker of fluid-phase endocitosis. In our experimental setup the results show that the effect of the applied surfactants (Labrasol and Polysorbate 20) on paracellular pathway is more pronounced than the transcellular route. Polyamines and bile salts enhance drug transport via both transcellular and paracellular pathways, but several excipients increase drug permeability on Caco-2 monolayers only via paracellular pathways.

6.List of publications

UNIVERSITY OF DEBRECEN
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PUBLICATIONS

Register number: DEENKÉTK/342/2014.
Item number:
Subject: Ph.D. List of Publications

Candidate: Zoltán Ujhelyi
Neptun ID: JLC63G
Doctoral School: Doctoral School of Pharmaceutical Sciences
Mtmt ID: 10036509

List of publications related to the dissertation

1. **Ujhelyi, Z.**, Róka, E., Fenyvesi, F., Fehér, P., Váradi, J., Réti-Nagy, K., Vecsernyés, M., Bácskay, I.: Assessment of the hemolytic activity and cytotoxicity of different PEG-based solubilizing agents.
   Pharmazie. 68, 383-384, 2013.
   DOI: http://dx.doi.org/10.1691/ph.2013.2207
   IF:1.003

2. **Ujhelyi, Z.**, Fenyvesi, F., Váradi, J., Fehér, P., Kiss, T., Veszelka, S., Deli, M., Vecsernyés, M., Bácskay, I.: Evaluation of cytotoxicity of surfactants used in self-micro emulsifying drug delivery systems and their effects on paracellular transport in Caco-2 cell monolayer.
   Eur. J. Pharm. Sci. 47 (3), 564-573, 2012.
   DOI: http://dx.doi.org/10.1016/j.ejps.2012.07.005
   IF:2.987

List of other publications

3. Ujhelyi, J., **Ujhelyi, Z.**, Szalai, A., László, F.J., Cayasso, M., Vecsernyés, M., Pórszász, R.: Analgesic and anti-inflammatory effectiveness of sitagliptin and vildagliptin in mice.
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