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Assessment of the hemolytic activity and cytotoxicity of different PEG-based solubilizing agents

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Received October 30, 2012, accepted December 5, 2012

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Pharmazie 68: 383–384 (2013)
doi: 10.1691/ph.2013.2207

The hemolytic activity and the cytotoxicity of PEG-based solubilizing agents on Caco-2 monolayer were investigated. *In vitro* tests can predict the irritancy potential and the delayed toxicity of the surfactants. There were significant concentration dependent differences between the result of the MTT (3-(4,5-dimethylthiazol-2-yl))-2,5-diphenyltetrazolium bromide) test and the data of the hemolytic activity test. Our investigations show that safer and more applicable tensides can be selected in order to form a more biocompatible medicament.

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 (Flaten et al. 2008). 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 (Hamid et al. 2009).

Polyethylene glycol (PEG) based solubiliziers (Kolliphors formerly known as Cremophors) as tensides or as co-tensides can be used to design these peroral formulations (Wang et al. 2011). The self-nanoemulsifying capacity of Cremophor RH 40 and Cremophor EL was tested in SMEDDS containing atorvastatin calcium. The optimal concentration of Cremophors without any cytotoxic effect was determined on a Caco-2 cell monolayer (Bandivadeker et al. 2012). 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 (Gursoy et al. 2003). Cremophor EL was reported to be responsible for hypersensitivity reactions in patients who were treated with parenteral formulations (Rowinsky et al. 1993). 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 (Ujhelyi et al. 2012). In spite of the above mentioned extensive studies, there is limited information about the toxic effect of Cremophor solubilizing agents.

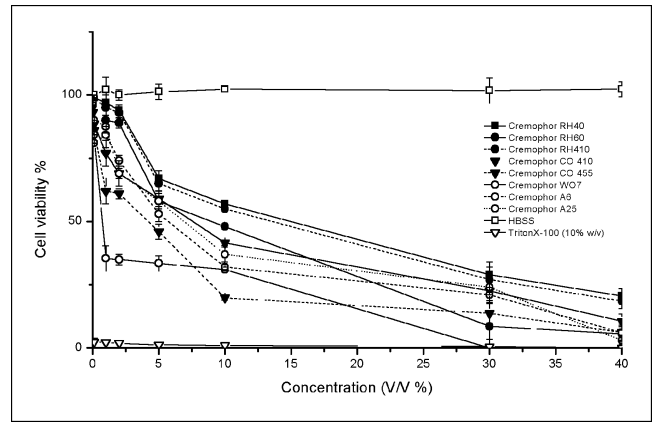

Fig.: Cytotoxic effects of Kolliphor formerly known as Cremophor surfactants on Caco-2 cells determined by MTT-test. Cell viability was expressed as the percentage of untreated control in the function of surfactant concentration. Positive control: Triton X 100 (10% w/v), negative control: HBSS (Hanks Balanced Salt Solution). Values presented are means ± SD, n = 5

In the present study, our aim was to assess the cytotoxicity and the hemolytic activity of PEG based surfactants. The correlation between IC_{50} and HC_{50} values (the ratio at which 50% hemolysis occurred) was also determined. Cytotoxicity was tested using the MTT (3-(4,5-dimethylthiazol-2-yl))-2,5-diphenyltetrazolium bromide) cell viability test on Caco-2 cells (Palamakula et al. 2004). Caco-2 cells were originally derived from a human colon adenocarcinoma. This *in vitro* model is used for the rapid screening of cytotoxicity of orally administered drugs. The hemolytic potential of the tensides was evaluated using a modified method of Nornoo et al. (2008).

There are significant concentration-dependent differences in the cytotoxic properties of PEG based surfactants (Cremophors) (Fig.). 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 (Table): 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 (Table). 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. MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria. The result of this assay refers to the damage of the mitochondrial function (Mosmann et al. 1983). Tensides, in a concentration dependent manner, were capable of interfering with the mitochondrial enzymes (Korzeniewski et al. 1983). Single surfactant micelles can directly interact with the cell membrane and mitochondrial enzymes and cause cellular toxicity (Gursoy et al. 2003). The cytoxicity might be decreased by the application of special pharmaceutical dosage forms (i.e. SMEDDS) or the blends of surface active agents (Buyukozturk et al. 2010).

PEG-based surfactants are also known to cause red blood cell hemolysis. There might be a connection between erythtrocytes and surfactants. Erythrocytes and emulsions stabilized with lecithins showed interaction in a former study (Nornoo et al.

Table: Trade names and chemical definitions of Kolliphors formerly known as Cremophors (data supported by manufacturers)

Types of Kolliphors formerly known as Cremophors	Chemical structure	IC_{50} Values (V/V%)	HC_{50} Values (V/V%)
RH 40	PEG-40 Hydrogenated castor oil	14.34 ± 0.35	5.12 ± 0.08
RH 60	PEG-60 Hydrogenated castor oil	9.01 ± 0.38	2.78 ± 0.02
RH 410	90% PEG-40 Hydrogenated castor oil and 10% water	12.88 ± 0.21	3.98 ± 0.08
CO 410	90% PEG-40 Hydrogenated castor oil and 10% water	6.71 ± 0.10	1.54 ± 0.01
CO 455	PEG-40 Hydrogenated castor oil and propylene glycol	4.12 ± 0.11	0.98 ± 0.01
WO 7	PEG-7 Hydrogenated Castor Oil	0.67 ± 0.02	0.09 ± 0.001
A 6	Ceteareth-6 (and) Stearyl Alcohol	7.33 ± 0.15	1.58 ± 0.07
A 25	Ceteareth-25 (and) Stearyl Alcohol	6.45 ± 0.19	1.53 ± 0.08

IC_{50} (The concentration of test substances reducing the cell viability by 50% compared to the untreated control), HC_{50} (The ratio at which 50% hemolysis occurred) values of the applied surfactants (mean ± SD; n = 5).

2008). Hemolytic potential may be favorably influenced by the application of more biocompatible tensides and advantageous emulsion systems (Huo et al. 2010).

It can be concluded that assessment of the *in vitro* toxic properties of single non-ionic surfactants is required, although they are used in blends or in several pharmaceutical dosage forms (Bandivaker et al. 2012). In some cases, the results of *in vitro* tests correlate with those of the *in vivo* experiments (Utreja et al. 2011) and with the help of these data, safer pharmaceutical dosage forms with reduced toxicity can be developed.

Experimental

1. Cells

Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC) and maintained in Dulbecco's Modified Eagel's medium (DMEM) in plastic cell culture flasks, supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and 100 mg/L gentamycin at 37 °C in a 5% CO_2 atmosphere.

2. Chemicals

PEG-based surfactants (traditional names are Cremophors, new trade names are Kolliphors)(BASF, Germany) were dissolved in phosphate buffered saline (PBS) at a concentration between 0.02–40 v/v %.

3. Cytotoxicity assay

The cytotoxic effect of Cremophors was evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl))-2,5-diphenyltetrazolium bromide) cytotoxicity method. Caco-2 cells in complete medium were seeded on 24-well plate at a final density of 8×10^4 cells/well. After 2–3 days the medium was removed, the cells were washed with PBS and the surfactant test solution was added. The cells were then incubated for 30 min, then the samples were removed, and the cells were washed twice with 1 ml PBS. In the end, 0.9 ml medium and 100 µl MTT solution (5 mg/ml) was added to each well. The plates were incubated for 4 h, the MTT solution was removed and 2 ml of isopropanol:hydrochloride acid,HCl(25:1) was added to dissolve the formed formazan crystals. The absorbance was measured at 570 nm against a 690 nm reference with FLUOstar OPTIMA Microplate Reader. Cell viability was expressed as the percentage of untreated control.

4. Hemolytic activity

Erythrocytes were separated from citrated blood by centrifugation at $2500 \times g$ 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 $5000 \times g$. 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.

Acknowledgement: This publication is supported by the TÁMOP-4.2.2.A-11/1/KONV-2012-0025 project. The project is co-financed by the European Union and the European Social Fund.

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