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Biocompatibility investigation of different pharmaceutical excipients used in liquid dosage forms

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Aqueous pharmaceutical solutions provide prosperous living conditions for microbiological agents. In order to eliminate these microbes, we use preservatives which can harm human cells as well. Their cytotoxicity is concentration-dependent and the aim of our study was to find how other pharmaceutical excipients modify the cytotoxic attributes of preservatives. We tested the following compounds: methylparaben, benzalkonium chloride, polysorbate 20, Labrasol® and hydroxyethyl cellulose. The MTT tests indicated that surfactants increase the cytotoxicity while polymers may decrease it in some cases.

1. Introduction

Nowadays, biocompatibility studies are becoming more important in the field of pharmaceutical sciences, because concerns of the consumers are intensively growing, regarding the safety of pharmaceutical excipients. Liquid dosage forms containing the widest selection of excipients for professional formulations, have a large share on the drug market. Orally administered solutions, emulsions and suspensions contain high amount of purified water as a solvent, which is an excellent environment for the spread of microbes like bacteria, fungi and eukaryotic unicellulars. Their metabolism can lead to the inefficiency of the product (change of pH, degradation of important excipients or the active substance, etc.) and result in serious stability problems as well. Preservatives are widely used to prevent the microbial contamination of pharmaceutical products, particularly, because aseptic techniques might not be enough to prevent the contamination (Gargiulo et al. 2016). Preservatives have a wide and unclear mechanism of action, but mostly they distort the structure of the phospholipid bilayer by acting as a detergent (Flasiński et al. 2016).

Benzalkonium chloride is a cheap and still popular preservative in eye drops and other ophthalmological preparations despite its well-known toxic effects in animals (Kwon et al. 2015) and on human cells (Ammar et al. 2010). Apart from the external use, benzalkonium chloride can be a part of nebulizer solutions, but recent studies showed the concern of toxic effects after local application (Kim and Ahn 2004). Parabens are also widely used antimicrobial agents in both internal and external pharmaceutical dosage forms, but they may cause allergic contact dermatitis (Cheng et al. 2014) or increase the risk of oestrogen dependent tumours (Lillo et al. 2016). Thus, the safety of these preservatives is disputable, although they are widely spread in foods, pharmaceuticals and cosmetics.

Liquid dosage forms contain other excipients apart from preservatives. Surfactants are commonly used to increase the solubility of lipophilic drugs and to stabilize emulsions and suspensions. Due to their amphiphilic nature, they can disturb the integrity of the cell membranes (Ménard et al. 2012). Also, they can modify the transport of active pharmaceutical ingredients but only at the cost of the lower biocompatibility (Dimitrijevic et al. 2000). Labrasol® is known to activate death receptors as well (Sigward et al. 2013). Consequently, preservatives and surfactants both have different cytotoxic effects, but the interference of their respective toxicity is not particularly studied. It is not clarified, what are the exact sites of actions, whether the targets can be shared or separated between these excipient groups or not. Thickening agents are used for spherical stabilization in disperse systems and they also increase the viscosity of the system. Hydroxyethyl cellulose is known to be harmless for human cells, proved by MTT and LDH cell viability assays even, if it is chemically modified (Leonaviciute et al. 2016). Previous studies showed that the cell damage caused by surfactants was lowered in their presence (Calejo et al. 2012).

Nowadays, every new active substance must undergo years of testing before entering the market. The same strict industrial qualifications are needed for the approval of new excipients. The implementations of different animal tests are limited because of ethical and financial concerns (Festing and Wilkinson 2007). Also in the EU, these tests are controlled by the 440/2008 EC regulation, which prefers cell lines over animal tests. This led to a highly increasing significance of human cell culture models in biocompatibility studies. These cells can represent human cells, having the same expression patterns, membrane proteins, enzymes, etc. However, they cannot represent a real human tissue, because the lack of extracellular elements and structure.

The objective of our research was to investigate the cytotoxic properties of methylparaben and benzalkonium chloride in a Caco-2 cell culture model. We also tested their synergetic cytotoxic effect with detergents (polysorbate 20 and Labrasol®) and polymer (hydroxyethyl cellulose). Previous studies summarized the cytotoxic effect of these excipient groups, but they were focused on separate cell damage mechanisms.

The novelty of our work was to investigate the cytotoxic relationship between the excipients, how they can modify their biocompatibilities.

2. Investigations, results and discussion

In our study, those substances were investigated, which are officially used in the pharmaceutical and food industries. The stock solutions represented the applied concentrations of each tested chemical in pharmaceuticals. Caco-2 cell lines is an approved model of the absorption, transport of active pharmaceutical ingredients and their respective cytotoxicity in the GIT tract. The concentration of surfactants was set according to the previously measured IC_{50} values (Ujhelyi et al. 2012).

Our results (Fig. 1) showed, that the cytotoxicity of methylparaben was severely increased by the presence of polysorbate 20. The IC_{50} value of methylparaben decreased from 0.47 $^{m/m}\%$ to 0.33 $^{m/m}\%$. In the presence of the hydroxyethyl cellulose, the cytotoxicity decreased and in spite of the increasing concentration of the preser-

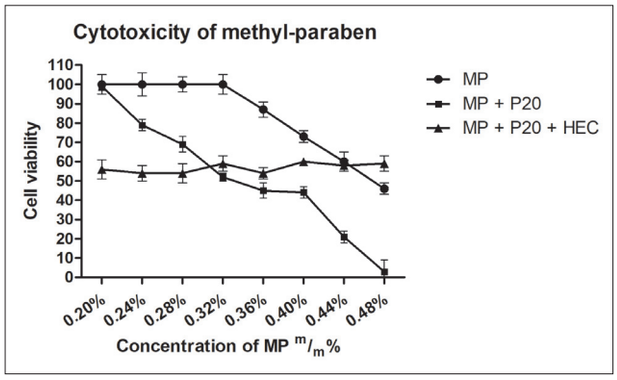

Fig. 1: Cytotoxic effects of methylparaben on Caco-2 cells determined by MTT-assay.
Cell viability was expressed as the percentage of untreated control in the function of surfactant concentration. MP: methylparaben
MP + P20: methylparaben + 0.004 v/v% polysorbate 20
MP + P20 + HEC: methylparaben + 0.004 v/v% polysorbate 20 + 30 v/v% hydroxyethyl cellulose
Values presented are means ± SD (n=6), are compared to the untreated control cell viability and are significantly changed ($p<0.05$).
We compared cell viability values and found: 0.004 v/v% polysorbate 20 greatly increased cytotoxicity, however hydroxyethyl cellulose showed significant cytoprotective effect.

vative, the cell viability was not below 54%. The cytotoxicity of benzalkonium chloride was highly increased in the presence of the Labrasol® (Fig. 2) which meant that the cell viabilities diminished from 83% to 9.6%. However, the thickener had no significant influence on the viability of the cells.

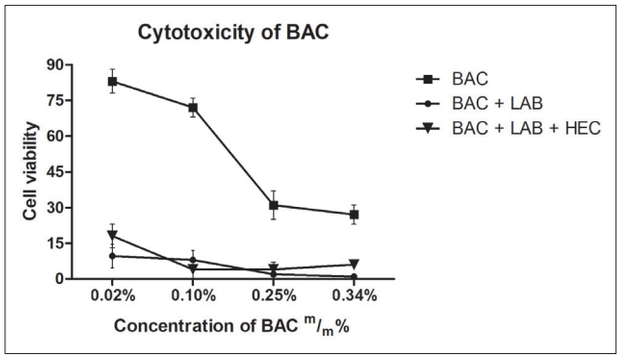

Fig. 2: Cytotoxic effects of benzalkonium-chloride on Caco-2 cells determined by MTT-assay.
Cell viability was expressed as the percentage of untreated control in the function of surfactant concentration.
BAC: benzalkonium chloride
BAC + LAB: benzalkonium chloride + 0.11 v/v% Labrasol®
BAC + LAB + HEC: benzalkonium chloride + 0.11 v/v% Labrasol® + 30 v/v% hydroxyethyl cellulose
Values presented are means ± SD (n=6), are compared to the untreated control cell viability and are significantly changed ($p<0.05$).
We compared cell viability values and found: 0.11 v/v% Labrasol® severely increased cytotoxicity and hydroxyethyl cellulose had no cytoprotective effect.

Preservatives, surfactants and thickening agents are integral parts of disperse systems (real solutions, emulsions, suspensions) for the optimal stabilization. Methylparaben, polysorbate 20 and hydroxyethyl cellulose are official in different Pharmacopoeias (Ph. Eur. 9, Ph. Hg.VIII.) Our results indicated that methylparaben had serious cytotoxicity. Recent studies proved that methylparaben had oestrogen agonistic effects and also many studies reported the limited applicability of this preservative. Nevertheless, polysorbate 20 greatly lowered the biocompatibility of methylparaben because this surfactant may solubilize the membrane proteins, hence disintegrated the structure of the cell membrane (Dimitrijevic et al. 2000) on Caco-2 cells. Hydroxyethyl cellulose may simulate the natural mucus layer of GIT cells, somehow increasing the cell protective signals and reducing cytotoxicity (Calejo et al. 2012).
Bronchoconstrictor effects of benzalkonium chloride (Lee and Kim 2007) in pulmonary drug delivery system have been reported. Our results and this study confirms the theory, that the cytotoxicity of benzalkonium chloride determined on pulmonary or Caco-2 cells could be very intensive and may result in severe side effects in the case of internal applications. New surfactants were developed for the optimal capacity of solubilisation, but the concern of the investigation of additive cytotoxicity is increasing. The concomitant application of benzalkonium chloride and Labrasol® resulted in extremely low cell viability. The polymer solution did not influence the cell viability, so there was no cell protective effect in this case. The reason of severe cell damage was the irreversibly damaged membrane integrity due to the highly increased solubilisation capacity of these surfactants. There was a strong relationship between the cationic surfactant (benzalkonium chloride) and the amphiphilic surfactant (Labrasol®).
The reliable biocompatibility of pharmaceutical products can only be based on the evaluation of concomitant cytotoxicity profiles of different excipients and their combination.

3. Experimental

3.1. 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 foetal bovine serum, 2 mM L-glutamine and 100 mg/L gentamycin at 37 °C in a 5% CO_2 atmosphere.

3.2. Chemicals

Methyl 4-hydroxybenzoate, benzalkonium chloride (N-benzyl-N,N-dimethyl-1-dodecanaminium chloride) and 2-hydroxyethyl cellulose were obtained from Hungaropharma. polysorbate 20 was obtained from Sigma-Aldrich. Labrasol® was a kind gift from Polysorbate. Both the formulations and the other substances were dissolved in phosphate buffered saline (PBS). The tested concentrations of methylparaben were 0.2 %(m/m); 0.24 %(m/m); 0.28 %(m/m); 0.32 %(m/m); 0.36 %(m/m); 0.4 %(m/m); 0.44 %(m/m); 0.48 %(m/m). The tested concentrations of benzalkonium chloride were 0.02 %(m/m); 0.1 %(m/m); 0.25 %(m/m); 0.34 %(m/m). In every experiment, the concentration of polysorbate 20, the Labrasol® and the hydroxyethyl cellulose were 0.004, 0.11 and 30 % (v/v).

3.3. Cytotoxicity assay

The cytotoxic effects of the various substances were 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 96-well plate at a final density of 10.000 cells/well. After 7 days, the medium was removed and the cells were incubated for 30 min with the test solutions. The samples were removed, and a 5 mg/ml MTT solution was added to each well. The plates were incubated for 3 h, then the MTT solution was removed and 0.2 ml of a solution of isopropanol:hydrochloride acid (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.

3.4. Statistical analysis

Data were analyzed using GraphPad Prism (version 5; GrapPad Software, Inc.) and presented as means±SD. 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. After that, the results among the groups were presented by Dunnett's test. All results were regarded as significant, with $p < 0.05$. All experiments were carried out in six, independent and parallel series.

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Conflicts of interest: None declared

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