molecules

MDPI

Article

Interaction between Different Pharmaceutical Excipients in Liquid Dosage Forms—Assessment of Cytotoxicity and Antimicrobial Activity

Dániel Nemes [1], Renátó Kovács [2], Fruzsina Nagy [2], Mirtill Mező [1], Nikolett Poczok [1], Zoltán Ujhelyi [1], Ágota Pető [1], Pálma Fehér [1], Ferenc Fenyvesi [1], Judit Váradi [1], Miklós Vecsernyés [1] and Ildikó Bácskay [1,*]

[1] Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Debrecen, Debrecen 4032, Hungary; nemes.daniel@pharm.unideb.hu (D.N.); mmirtill95@gmail.com (M.M.); poczok.niki@freemail.hu (N.P.); ujhelyi.zoltan@pharm.unideb.hu (Z.U.); agota713@gmail.com (Á.P.); feher.palma@pharm.unideb.hu (P.F.); fenyvesi.ferenc@pharm.unideb.hu (F.F.); varadi.judit@pharm.unideb.hu (J.V.); vecsernyes.miklos@pharm.unideb.hu (M.V.)

[2] Department of Medical Microbiology, Faculty of Medicine, University of Debrecen, Debrecen 4032, Hungary; kovacs.renato@med.unideb.hu (R.K.); nagyfru@freemail.hu (F.N.)

* Correspondence: bacskay.ildiko@pharm.unideb.hu

Received: 25 June 2018; Accepted: 19 July 2018; Published: 23 July 2018

Abstract: Nowadays, the safety of parabens as pharmaceutical preservatives is debated. Recent studies investigated their interference with the oestrogen receptors, nevertheless their carcinogenic activity was also proved. That was the reason why the re-evaluation of the biocompatibility and antimicrobial activity of parabens is required using modern investigation methods. We aimed to test the cytotoxic, antifungal and antibacterial effect of parabens on Caco-2 cells, *C. albicans*, *C. parapsilosis*, *C. glabrata*, *E. coli*, *P. aeruginosa* and *S. aureus*. Two complex systems (glycerol—Polysorbate 20; ethanol—Capryol PGMC™) were formulated to study—with the MTT-assay and microdilution method, respectively—how other excipients may modify the biocompatibility and antimicrobial effect of parabens. In the case of cytotoxicity, the toxicity of these two systems was highly influenced by co-solvents and surfactants. The fungi and bacteria had significantly different resistance in the formulations and in some cases the excipients could highly modify the effectiveness of parabens both in an agonistic and in a counteractive way. These results indicate that with appropriate selection, non-preservative excipients can contribute to the antimicrobial safety of the products, thus they may decrease the required preservative concentration.

Keywords: excipient interaction; surfactant; liquid dosage forms; cytotoxicity; preservative; Caco-2 cells

1. Introduction

Although tablets and capsules are the most popular types of pharmaceutical dosage forms, different oral liquid formulations (syrups, herbal extracts, suspensions, emulsions, etc.) still have specific therapeutic indications, mainly in paediatrics. Flavouring is a crucial part of these formulations because patient compliance is highly dependent on the taste of the product. Usually they contain high amount of sweet carbohydrates (glycose, fructose, maltitol, xylitol, sorbitol, etc.), which can be metabolized by different microorganisms, thus the product can be easily contaminated [1]. It must be noted, that these liquid preparations are opened and closed multiple times during their life-time and each application increases the possibility of contamination. In order to avoid it, an appropriate

amount of preservatives must be used, which can kill or inhibit the growth of bacteria, fungi and other unicellular. The exact mechanism of action of preservatives is unclear in some cases, but as the cell membrane is the only common subcellular component in these microbes, they mostly distort the structure of the membrane resulting in several consequences [2]. Their cytotoxicity is mostly based on these effects as well [3].

One of the most widely used group of pharmaceutical preservatives is the parabens. They are derivatives of 4-hydroxybenzoic acid in the form of its carboxylic esters. The most commonly used parabens (Figure 1) are methyl paraben (MP) (E218), ethyl paraben (EP) (E214), propyl paraben (PP) (E216), butyl paraben (BP), heptyl paraben and their respective sodium salts. The longer the alkyl chain, the lower the solubility in water is. Hence, some co-solvent such as ethanol is usually required to increase their solubility and it must also be noted, that the sodium salts are less frequent in different formulations. Generally, they are considered as synthetic compounds, but in the recent years many natural sources were found [4–6]. They are preferred in the pharmaceutical and cosmetic industries, because of their odourless and tasteless characteristics, great chemical stability over a wide range of pH values and a broad spectrum of antimicrobial activity [7].

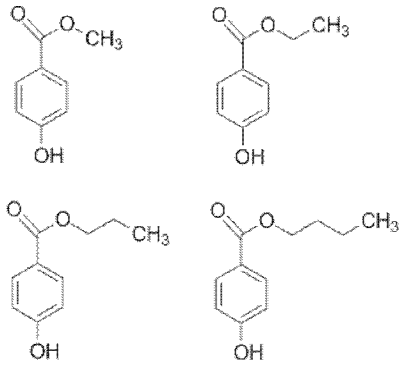

Figure 1. Chemical structure of the most commonly used parabens in growing alkyl chain length: methyl paraben, ethyl paraben, propyl paraben, butyl paraben.

The esters of 4-hydroxybenzoic acid also have certain well-known risks. In the case of topical application, contact dermatitis is a well-known problem [8,9] however, the latest results are controversial, describing a low occurrence of allergic reactions caused by parabens [10,11] or a severe influence on sensitization [12]. Recent studies have indicated the carcinogenic effect of parabens, as they interfere with oestrogen receptors [13,14]. Furthermore, in vivo evidence suggests that urine paraben levels can be associated with menstrual cycle problems [15]. They are able to penetrate through the skin from cosmetic products [16,17]. Their direct cytotoxic behaviour has been reported on corneal epithelial cells [18], on dermal fibroblasts [19] and on liver cells [20]. Paraben exposure is not only restricted to the users of cosmetics [21], as they can pass through the placenta [22] and can be measured in the milk of lactating mothers [23]. These results suggest a decline in the use of these 4-hydroxybenzoic acid derivatives in oral and topical formulations during the next few years.

An oral, liquid pharmaceutical preparation contains many excipients, which is the reason why cytotoxicity tests of each chemical by itself is not enough to gain a comprehensive view of the biocompatibility profile of the product. There are only few studies on how the biocompatibility of an excipient is influenced if other components are present in the test systems. However, in order to get authorized by governmental authorities, the whole product cannot be toxic, but positive interactions might decrease the appropriate concentration of additives i.e., the quantity of preservatives may also be reduced. However, serious cytotoxicity values may be measured, if the excipients can potentiate their harmful effects [24]. As the cytotoxic effects of surface-active agents are well-known [25], they might have synergetic antimicrobial activity with preservatives. Different co-solvent mixtures can

have different biocompatibility profiles and might modify the toxicity of preservatives, increasing their effect on the cell membranes by creating a better chemical environment at the site of action [24].

In this study, our objective was to investigate the cytotoxicity and antimicrobial properties on Caco-2 cells and on various pathogenic microorganisms of different 4-hydroxybenzoic acid derivatives alone and in two complex co-solvent systems to explore interferences between the preservatives and the component of the co-solvent systems. Caco-2 cells are widely applied as an in vitro model of human gastrointestinal transport and mainly used as a monolayer rather than individual cells, however several assays are performed prior to reach complete integrity, such as end point or non-invasive cell viability assays (MTT assay, LDH test, RT-CES, etc.) [26]. In our antimicrobial experiments, our test solutions were tested on clinically relevant pathogens: *S. aureus* as a Gram-positive facultative anaerobe, *E. coli* and *P. aeruginosa* as a Gram-negative aerobes and *C. albicans* as the most common fungal pathogen and C. *parapsilosis* and C. *glabrata* as the top *Candida* species opportunistic pathogens different from *C. albicans* [27].

The formulations of the investigated systems contain a co-solvent and a surface-active agent. The first formulation (S1) consisted of 30% (*v*/*v*) glycerol and 0.002% (*v*/*v*) Polysorbate 20. The surfactant of the second formulation (S2) was 0.5% (*v*/*v*) Capryol PGMC™ and the parabens in the form of their 10 (*w*/*w*)% solutions, dissolved in 70% (*v*/*v*) ethanol. Tables 1 and 2 summarize the composition of every solution used in our experiments. The experimental design is presented in Figure 2.

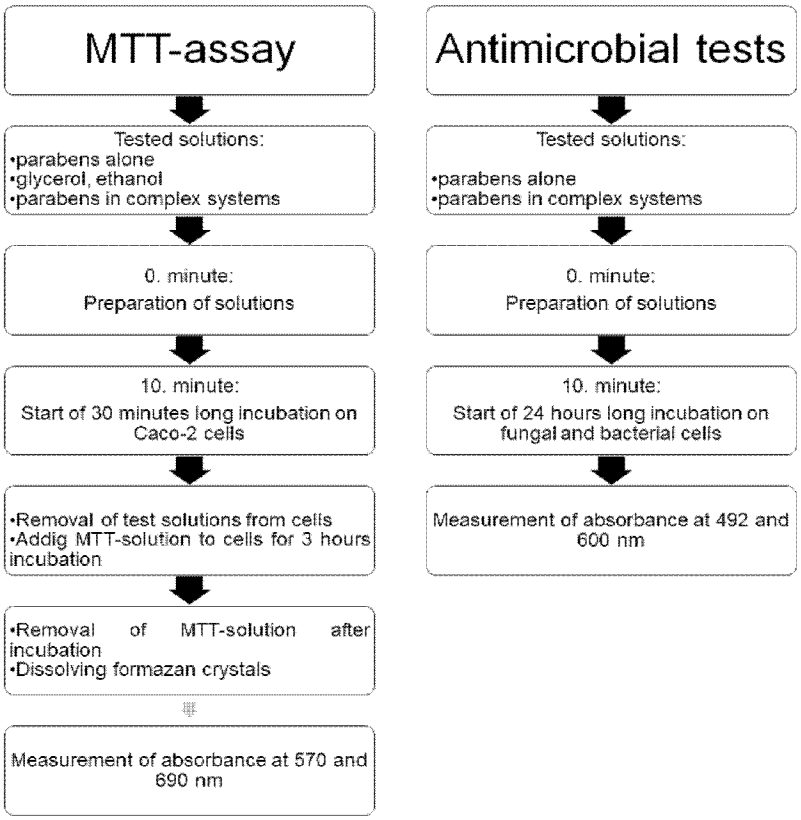

Figure 2. Experimental design.

Table 1. Composition of test solutions for cytotoxicity tests.

Component	S1	S2
Paraben	0.2 *w*/*w*%, 0.02 *w*/*w*%, 0.002 *w*/*w*%, 0.0002 *w*/*w*%	
Glycerol	30 *v*/*v*%, 3 *v*/*v*%, 0.3 *v*/*v*%, 0.03 *v*/*v*%	-
Polysorbate 20	0.002 *v*/*v*%, 0.0002 *v*/*v*%, 0.00002 *v*/*v*%, 0.000002 *v*/*v*%	-
Capryol PGMC™	-	0.5 *v*/*v*%, 0.05 *v*/*v*%, 0.005 *v*/*v*%, 0.0005 *v*/*v*%
Ethanol	-	1.4 *v*/*v*%, 0.14 *v*/*v*%, 0.014 *v*/*v*%, 0.0014 *v*/*v*%
PBS	solvent, used for tenfold, hundredfold, thousand-fold dilution	

Table 2. Composition of test solutions for antimicrobial tests.

Component	S1	S2	Control
Paraben		0.1 *w*/*w*%, 0.15 *w*/*w*%, 0.25 *w*/*w*%	
Glycerol	30 *v*/*v*%	-	-
Polysorbate 20	0.002 *v*/*v*%	-	-
Capryol PGMC™	-	0.5 *v*/*v*%	-
Ethanol	-	0.7 *v*/*v*%, 1.05 *v*/*v*%, 1.75 *v*/*v*%	0.7 *v*/*v*%, 1.05 *v*/*v*%, 1.75 *v*/*v*%
RPMI-1640	solvent for antifungal tests		
Mueller-Hinton broth	solvent for antibacterial tests		

Test solutions were prepared in situ, 10 min before the inoculation for antimicrobial investigations. Caco-2 cells were incubated for 30 min with the test solutions, then these solutions were removed and the MTT-solution was added for a 3 h long reaction. The converted formazan crystals were dissolved in appropriate solvents after the unreacted MTT was removed. Absorbance was measured at two different wavelengths and the cell viability was calculated. After seeding the bacterial and fungal cells in appropriate concentrations into 96-well microplates, a 24 h long incubation was started. Optical density was measured at two wavelengths at the end of the incubation period.

2. Results

2.1. Cytotoxicity Tests

2.1.1. Cytotoxicity of Parabens

In order to mimic the dilution of samples in the gastrointestinal tract, the cytotoxicity of parabens was measured in tenfold, hundredfold and thousand-fold dilutions (Figure 3). The samples were diluted by PBS. At 0.2 (*w*/*w*)% butyl and ethyl paraben had significantly higher cytotoxicity than methyl and ethyl paraben, which had similar toxicity patterns. There was a linear relationship between the cytotoxicity and the dilution ratio of different paraben derivatives. The more concentrated samples decreased the cell viability and resulted in significant cytotoxicity. The higher the ratio of dilution of parabens, the better the cell viability of the Caco-2 cell line was.

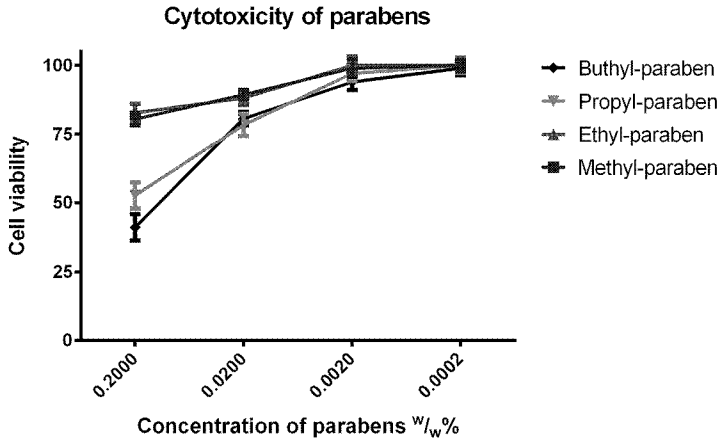

Figure 3. Cytotoxicity of parabens on Caco-2 cells measured by MTT-assay. Cell viability was expressed as the percentage of the absorbance of the untreated control cells. Data expressed as mean ± SEM, n = 12. Cell viability of the samples at 0.2000; 0.0200; 0.0020; 0.0002 (w/w)% concentrations: MP: 81% ± 2.4%; 89% ± 1.6%; 99% ± 3.1%; 100% ± 2%; EP: 83% ± 3.3%; 88% ± 2.6%; 100% ± 3.1%; 100% ± 2.5%; PP: 53% ± 4.7%; 78% ± 4%; 97% ± 2.7%; 100% ± 2.7%; BP: 41% ± 4.6%; 81% ± 2.4%; 94% ± 2.9%; 99% ± 2.5%.

2.1.2. Cytotoxicity of Solvents

Ethanol and glycerol were tested in different concentrations diluted with phosphate buffered saline (PBS) for cytotoxicity experiments. As it can be seen on Figures 4 and 5, the cell viability decreased in a concentration dependent manner in the case of these solvents. The IC_{50} (the inhibitory concentration value, where the 50% cell viability was measured by an MTT test) of glycerol was 45 (v/v)%. In our complex systems, the concentrations of glycerol were 30 (v/v)%, 3 (v/v)%, 0.3 (v/v)%, 0.03 (v/v)% which were lower than this inhibitory concentration.

The concentrations of ethanol (1.75 (v/v)%, 1.4 (v/v)%, 0.14 (v/v)%, 0.014 (v/v)%) in complex systems were applied for cytotoxicity and antimicrobial tests. Based on this cytotoxicity test, the IC_{50} value cannot be determined in these concentration ranges. The cell viability slightly decreased according to the concentration, but the highest concentration (1.75 (v/v)%) decreased the cell viability significantly (80 ± 1.7%).

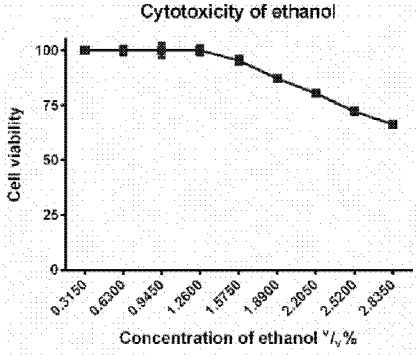

Figure 4. Cytotoxicity of ethanol measured by MTT-assay. Cell viability expressed as the percentage of the absorbance of the untreated control cells. Data expressed as mean ± SEM, n = 12. Cell viability of the samples at the different concentrations: 100% ± 0.2%; 100% ± 1.8%; 100% ± 3.1%; 100% ± 2%; 95% ± 1.7%; 87% ± 0.5%; 81% ± 1.1%; 72% ± 0.9%; 66% ± 1%.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.