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ORIGINAL ARTICLE

Characterization of new eye drops with choline salicylate and assessment of their irritancy by *in vitro* short time exposure tests



Katarzyna Wroblewska ^a, Małgorzata Kucinska ^b, Marek Murias ^b,
Janina Lulek ^{a,*}

^a Department of Pharmaceutical Technology, Poznan University of Medical Sciences, 6 Grunwaldzka Street, 60-780 Poznan, Poland

^b Department of Toxicology, Poznan University of Medical Sciences, 30 Dojazd Street, 60-631 Poznan, Poland

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Abstract The aim of our study was to examine the irritation potential of new eye drops containing 2% choline salicylate (CS) as an active pharmaceutical ingredient (API) and various polymers increasing eye drop viscosity (hydroxyethylcellulose, hydroxypropyl methylcellulose, methylcellulose, polyvinyl alcohol, polyvinylpyrrolidone). The standard method for assessing the potential of irritating substances has been the Draize rabbit eye test. However the European Centre for Validation of Alternative Methods and the Coordinating Committee for Validation of Alternative Methods recommend, short time exposure (STE) *in vitro* tests as an alternative method for assessing eye irritation. The eye irritation potential was determined using cytotoxicity test methods for rabbit corneal cell line (SIRC) after 5 min exposure. The viability of cells was determined using two cytotoxicity assays: MTT and Neutral Red Uptake. According to the irritation rankings for the short time exposure test, all tested eye drops are classified as non-irritating (cell viability > 70%).

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1. Introduction

From a number of pharmaceutical dosage forms applied topically to the eye, the most common and widely used remain

continuously eye drops (Lu, 2010). Their preparation requires special consideration with regard to sterility, preservation, isotonicity, buffering, viscosity and ocular bioavailability. In addition to physiological factors affecting ocular bioavailability, other factors such as physicochemical characteristics of the active pharmaceutical ingredient (API) and excipients are important. The evaluation of irritation potential of API as well as of the excipients is essential to secure the patient safety. For several years the method of choice to determine eye irritation potential was the Draize rabbit eye test (Wilhelmus, 2001). However, ethical considerations and the limited value of animal models including lack of reproducibility and overestimation of

* Corresponding author.

E-mail address: jlulek@ump.edu.pl (J. Lulek).

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human responses became the impetus for the development of alternative *in vitro* tests (Vinardell and Mitjans, 2008). As the ocular surface is a very complex system (corneal and conjunctival epithelial cells, the underlying stroma, and associated cells), it is difficult to develop the tests with a physiological and mechanistic base that are capable of eliminating the need for animals. Generally the *in vitro* model has been designed for only one tissue but in the eye there are more tissues. This is useful in obtaining more detailed data on the mechanical irritation of the eye. However, experiments on animals have to be replaced with several *in vitro* studies, such as: red blood cell test, isolated cornea and eye tests, as well as culture cell tests, which have different targets (da Nobrega et al., 2012; Donahue et al., 2011; Gerner et al., 2005; McNamee et al., 2009).

The European Centre for Validation of Alternative Methods (ECVAM) and the Interagency Coordinating Committee for Validation of Alternative Methods (ICCVAM) supervise the development of alternative methods. Both organizations recommend short time exposure (STE) *in vitro* test using a rabbit corneal cell line (SIRC) as an alternative method for assessing eye irritation (EVCAM, 2007; Li et al., 2009; Prinsen, 2006; Sakaguchi et al., 2011; Takahashi et al., 2008).

The aim of our study was to apply two *in vitro* tests (MTT and NRU) for assessing the irritation potential of new eye drops containing choline salicylate as API and various polymers improving eye drops viscosity (Repetto et al., 2008).

2. Materials and methods

2.1. Chemicals

Choline salicylate (CS) was kindly obtained from ICN Polfa Rzeszów S.A. The following polymers: hydroxyethylcellulose (HEC), hydroxypropyl methylcellulose (HPMC), methylcellulose (MC) and polyvinylpyrrolidone (PVP) were supplied from Sigma–Aldrich. Polyvinyl alcohol (PVA) and disodium ethylenediaminetetraacetate (Na₂EDTA) were purchased from POCH SA. Sodium chloride from Pharma Cosmetics, sodium bicarbonate from PPH Galfarm, and sodium metabisulfite from Fluka were used. Ultrapure water was produced by Millipore Simplicity UV system.

The Dulbecco's Modified Eagle's Medium (DMEM) without phenol red, fetal bovine serum (FBS), 0.25% trypsin EDTA solution, phosphate buffered saline (PBS),

methylthiazolyldiphenyl-tetrazolium bromide (MTT), neutral red were obtained from Sigma Aldrich (St. Louis, Mi, USA). Penicillin–streptomycin and L-glutamine solution were obtained from Gibco Invitrogen Corp. (Grand Island, NY, USA). Dimethyl sulfoxide was obtained from POCh, S.A. (Gliwice, Poland).

2.2. Eye drops

Three series of eye drops without polymers, containing increasing quantities of choline salicylate (1.0% CS, 2.0% CS, and 3.0% CS) were prepared. Appropriate amounts of choline salicylate were dissolved in water containing suitable quantities of tonicity adjusting agent (sodium chloride). After mixing, the drops were filtered through filter Schott G3, poured into infusion bottles of 100 ml and sterilized in the Exacta M.O.COM autoclave at the temperature of 122 ± 2 °C for 20 min. under steam pressure 101.4 kPa. After 12 h of sterilization physicochemical parameters of drops were tested.

In the next stage six types of eye drops with increased viscosity (A-F) containing 2.0% choline salicylate were prepared. Received eye drops differed in type and concentration of the polymer used, and comprised respectively: 0.25% hydroxyethylcellulose (A), 0.5% hydroxyethylcellulose (B), 0.5% hydroxypropyl methylcellulose (C), 0.25% methylcellulose (D), 2.0% polyvinyl alcohol (E) or 5% polyvinylpyrrolidone (F). Their compositions are presented in Table 1. As in the case of eye drops mentioned above, eye drops of increased viscosity (A-F) were poured into infusion bottles and sterilized in the Exacta M.O.COM autoclave at the temperature of 122 ± 2 °C for 20 min. under steam pressure 101.4 kPa. After 12 h of sterilization physicochemical parameters of eye drops were tested.

2.3. Cell culture

The cytotoxicity study was determined for rabbit corneal cell line (SIRC) obtained from European Collection of Cell Cultures (Salisbury, UK). The cell line was cultured in DMEM medium, without phenol red supplemented with 10% (v/v) FBS, 1% penicillin–streptomycin and 1% L-glutamine. The cells were cultured at temperature 37 °C, in a humidified atmosphere containing 5% CO₂. The cytotoxicity assays for three eye drops formulations (1% CS, 2% CS and 3% CS) and new eye drops (A-F), with 2% choline salicylate and different

Table 1 Composition of examined eye drops with choline salicylate.

Active ingredient/excipient	Content in eye drops (g)					
	A	B	C	D	E	F
Choline salicylate (CS)				2.00		
Hydroxyethylcellulose (HEC)	0.25	0.50				
Hydroxypropyl methylcellulose (HPMC)			0.50			
Methylcellulose (MC)				0.25		
Polyvinyl alcohol (PVA)					2.00	
Polyvinylpyrrolidone (PVP)						5.00
Sodium chloride	+	+	+	+	+	+
Sodium bicarbonate	+	+	+	+	+	+
Disodium ethylenediaminetetraacetate (Na ₂ EDTA)						+
Sodium metabisulfite						+
Water ultrapure				ad 100		

thickening agents were performed using SIRC cell line. Control cells were exposed to solution containing all ingredients without the active compound. The 0.9% solution of sodium chloride was used as a control for cells treated only with drops containing choline salicylate. The relative viability after adding the test sample and exposing for 5 min was the targets of the STE test.

2.4. The short time exposure (STE) tests

2.4.1. MTT assay

The MTT assay was employed to assess rabbit corneal cell viability after treatment with examined eye drops. Cells were seeded in 96-well plates at a density 2×10^4 cells per well and incubated overnight at 37 °C before experiment. Subsequently, tested solutions were given to each well except the well with control solution, for 5 min. After incubation the cells were rinsed twice with PBS. Next 170 µl of reaction solution containing methylthiazolyldiphenyl-tetrazolium bromide solution (0.5 mg/mL) in culture medium was added to each well. The plates were incubated for 3 h at 37 °C, and then centrifuged at 1300 rpm for 3 min. The formazan crystals were extracted with 200 µl DMSO and plates were shaken for 10 min. The absorbance was measured at 570 nm with plate reader (Biotek Instruments, Elx-800). Cell viability was calculated as a percentage of the control.

2.4.2. Neutral Red Uptake (NRU)

The SIRC cells were seeded at a density 2×10^4 cells per well in 96-well plates and incubated overnight under cell culture condition. After incubation, the cells were exposed to tested solutions for 5 min. Subsequently, plates were washed twice with PBS and the 100 µl of neutral red (5 mg/mL in distilled water) in medium was added into each well. The cells were incubated with neutral red for 2 h at 37 °C. The dye was extracted with a mixture of 1% acetic acid : 50% ethanol, and the absorbance was measured using a spectrophotometer at 540 nm with plate reader (Biotek Instruments, Elx-800). The results were calculated as a percentage of the NRU by untreated cells (Repetto et al., 2008).

2.5. Statistical analysis in cytotoxicity tests

The results are presented as the mean \pm SD from three independent experiments repeated six times. The values were calculated using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA. Data were compared for statistical significance by the Tukey's Multiple Comparison Test, a probability value (*P*) of less than 0.05 was considered significant.

3. Results and discussion

The aim of the conducted research was to characterize as well as to check the potential irritant effect of the new eye drops with choline salicylate.

Analyzing the qualitative composition of the developed eye drops (Table 1) it can be concluded that all the excipients used have been monographed in the current pharmacopeias which guarantees their quality required by regulatory agencies

(ORMPMDBP, 2011). However, taking into consideration that there is no data available on the behavior of choline salicylate in contact with the cornea cells, it seemed to be necessary to check its potential irritant effect. Due to the existing guidance on the possible limitations of the studies involving experimental animals, short-term exposure tests were used in the assessment of ocular irritation. Among the several possibilities, in our study two STE tests with different endpoints were chosen. The first was MTT assay which is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), to blue formazan crystals. MTT reduction is associated not only with mitochondria, but also with the cytoplasm and with non-mitochondrial membranes including the endosome/lysosome compartment and the plasma membrane (Berridge et al., 2005). The second test applied was the Neutral Red Uptake assay (NRU) which is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes (Repetto et al., 2008). Therefore, the results of both assays may give a more complete picture of the mechanisms of the corneal cell damage.

Considering the fact that the choline salicylate is currently not used in any ophthalmic drug dosage form it was purposeful to examine its impact on corneal cells. Therefore, eye irritation assessment was carried out for drops containing only choline salicylate, isotonized with sodium chloride solution (without the polymers). For this purpose, three series of drops containing 1%, 2% and 3% of choline salicylate were prepared and characterized. As it is apparent from the data presented in Table 2 the analyzed drops did not differ significantly as regards pH values (7.4–7.6), and osmotic pressure (289–305 mOsm/L). The values of both the aforementioned parameters resembled the physiological values of lacrimal fluid.

Analyzing the results of short time exposure (STE) tests performed for SIRC cell line (Fig. 1), it can be concluded that increase of SC concentration in eye drops does not affect corneal cell viability. The relative cell viability values ranged from 101.77% to 93.71% and showed no significant differences between the control and tested solutions. The CV > 70% indicates, that all tested drops may be considered as non-irritant (NI) according to Sakaguchi et al. classification (Sakaguchi et al., 2011).

The next step of our study referred to six series of developed eye drops containing 2% of CS, and characterized by different viscosity (Table 1). As thickeners were chosen five synthetic and semisynthetic polymers commonly used in pharmaceutical manufacturing and extemporaneous compounding (Rowe et al., 2012).

Table 2 Physicochemical properties of eye drops with various concentrations of choline salicylate without polymers increased viscosity.

Parameter	Eye drops (mean \pm SD, <i>n</i> = 3)		
	1% CS	2% CS	3% CS
pH (mean \pm SD)	7.4 \pm 0.01	7.6 \pm 0.02	7.5 \pm 0.02
Tonicity (mOsm/L) (mean \pm SD)	289 \pm 0.5	295 \pm 0.6	305 \pm 0.6

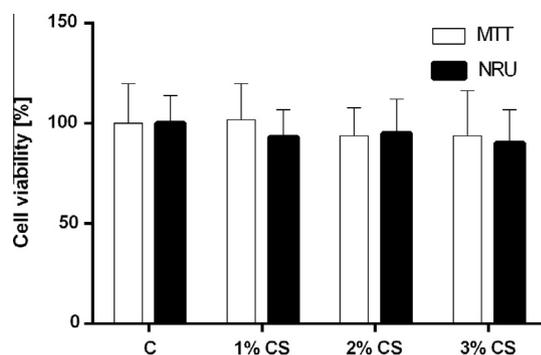


Figure 1 The viability of SIRC cell line after exposition to drops containing increasing concentration of choline salicylate, using MTT and NRU assays. Data represented as mean \pm standard deviation. Statistical significance between groups was assessed by Tukey's Multiple Comparison Test ($p < 0.05$). *Abbreviations:* C – control, CS – choline salicylate.

Due to a great diversity of eye structures and the requirements concerning the properties of eye medicines, the critical attributes affecting patient comfort as well as stability and safety of use have to be considered in the preparation step.

Besides sterility, pH, viscosity, and isotonicity are of great importance.

Having analyzed the data presented in Table 3 it might be noted that pH (5.65–7.79) of all formulations is in a good agreement with the pH range of most commercial products (5–7), and except of drops E with 2% PVA, pH of remaining drops was adjusted to within the normal tear fluid pH of 7.14–7.80 (Al-Achi et al., 2013).

The values of drops osmolality did not differ more than by 10%, and fell in the range from 288.7 mOsm/L (drops E) to 324.7 mOsm/L (drops B). Taking into account, that normal osmolality of tears ranges from 290 to 310 mOsm/L, it can be assumed that the all developed eye drops should be well tolerated.

A possible approach to extend API residence time in the eye, thereby prolonging drug absorption, is to incorporate the viscosity increasing agents into the vehicle. Analyzing viscosity of particular eye drops series containing polymers it is possible to observe that its value does not exceed the admissible value of 20 mPa s (ORMPMDBP, 2011) and remains within the range of 2.44 mPa s (drops F) to 11.42 mPa s (drops B).

It can be concluded that the viscosity of the tested eye drops, as well as their pH and osmotic pressure values, meets

Table 3 Physicochemical properties of examined eye drops with choline salicylate.

Parameter	Eye drops (mean \pm SD, $n = 3$)					
	A	B	C	D	E	F
pH	7.79 \pm 0.011	7.50 \pm 0.073	7.45 \pm 0.010	7.68 \pm 0.066	5.65 \pm 0.028	7.21 \pm 0.108
Viscosity (mPa s)	5.08 \pm 0.055	11.42 \pm 0.095	4.21 \pm 0.066	5.90 \pm 0.079	4.14 \pm 0.111	2.44 \pm 0.049
Tonicity (mOsm/L)	299.3 \pm 11.6	324.7 \pm 5.7	292.7 \pm 17.5	292.0 \pm 11.5	288.7 \pm 9.5	319.7 \pm 6.7
Polymer	HEC		HPMC	MC	PVA	PVP

Abbreviations: PVA – polyvinyl alcohol, PVP – polyvinylpyrrolidone, HPMC – hydroxypropyl methylcellulose, HEC – hydroxyethylcellulose, MC – methylcellulose

Table 4 The results of short time exposure (STE) tests performed for SIRC cell line.

Sample	Cell viability (%)				STE classification ^a
	MTT		NRU		
	Mean	SD	Mean	SD	
Control	100.00	19.71	100.00	13.63	
1% CS	101.77	17.90	93.72	13.63	NI
2% CS	93.79	13.97	95.44	17.06	NI
3% CS	93.71	22.66	90.97	16.66	NI
Control	100.00	7.21	100.00	9.43	
Drops A _{0.25%} HEC	95.93	11.14	103.81	14.00	NI
Control	100.00	9.85	100.00	15.14	
Drops B _{0.5%} HEC	103.75	8.02	97.50	11.91	NI
Control	100.00	8.46	100.00	10.16	
Drops C _{0.5%} HPMC	93.94	8.13	91.88	11.27	NI
Control	100.00	14.03	100.00	15.88	
Drops D _{0.25%} MC	97.20	12.97	101.57	9.15	NI
Control	100.00	9.14	100.00	13.73	
Drops E _{2%} PVA	97.29	13.90	76.43	13.47	NI
Control	100.00	9.98	100.00	13.42	
Drops F _{5%} PVP	81.82	12.25	102.61	9.28	NI

Abbreviations: CS – choline salicylate, PVA – polyvinyl alcohol, PVP – polyvinylpyrrolidone, HPMC – hydroxypropyl methylcellulose, HEC – hydroxyethylcellulose, MC – methylcellulose, NI – non-irritant, CV – cell viability

^a Eye irritation potential classification STE. The result CV > 70% is classified as non-irritant (Sakaguchi et al., 2011).

the criteria required for this type of pharmaceutical dosage form.

In the last step of our study the irritancy potential of developed formulations was assessed by *in vitro* STE assays. The cell viability (CV) as a critical attribute, was determined using the MTT assay and additionally was extended with NRU assay.

Analyzing the results of two *in vitro* STE tests, after 5 min. of exposition to particular series of eyes drops with increased viscosity (Table 4) it can be seen that all series (A-F) of new eye drops both in the MTT and NRU assay exhibited corneal cell viability greater than 70%. It means that all examined formulations could be classified as a non-irritating, according to Sakaguchi et al. classification (Sakaguchi et al., 2011).

Statistically significant differences in cell viability between control and tested solutions were observed for eye drops F of 5% PVP in the MTT assay ($CV = 81.82 \pm 12.25\%$), and for eye drops E of 2% PVA in NRU assay ($CV = 76.43 \pm 13.47\%$) (Figs. 2 and 3).

In case of eye drops of 5% PVP, smaller corneal cell viability may result from their hypertonicity (319.7 mOsm/L), and from the addition of adjuvants used in this formulation

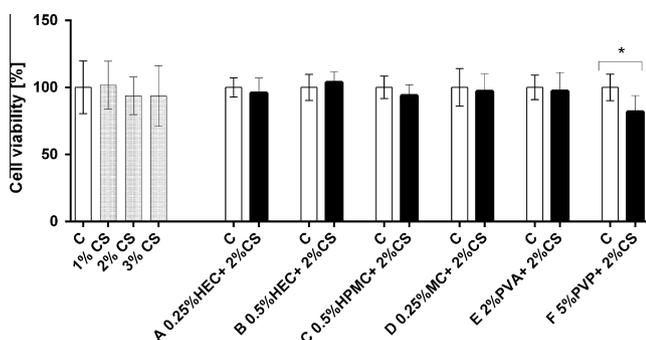


Figure 2 The viability of SIRC cell line using MTT assay. Data represented as mean \pm standard deviation. Statistical significance (*) between groups was assessed by Tukey's Multiple Comparison Test ($p < 0.05$). Abbreviations: C – control, CS – choline salicylate, A-F – examined eye drops, PVA – polyvinyl alcohol, PVP – polyvinylpyrrolidone, HPMC – hydroxypropyl methylcellulose, HEC – hydroxyethylcellulose, MC – methylcellulose.

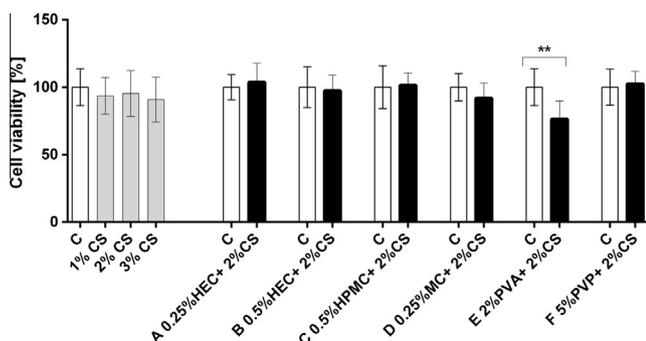


Figure 3 The results from NRU assay. Data represented as mean \pm standard deviation. Statistical significance (**) between groups was assessed by Tukey's Multiple Comparison Test ($p < 0.05$). Abbreviations: C – control, CS – choline salicylate, A-F – examined eye drops, PVA – polyvinyl alcohol, PVP – polyvinylpyrrolidone, HPMC – hydroxypropyl methylcellulose, HEC – hydroxyethylcellulose, MC – methylcellulose.

– sodium metabisulfite and Na_2EDTA , which exhibit antioxidant activity and may themselves act as irritants. For eye drops of 2% PVA CV was 81.82%. This does not indicate a damaging effect on the corneal cells, but may be related to their relatively low pH (5.62) which is in the ranges accepted for eye drops but it is about 25% lower than the physiological pH of tear fluid. Patients applying such eye drops may feel discomfort of burning and tearing.

4. Conclusions

Basing on the obtained results it can be concluded that choline salicylate in tested formulations will not exert irritant effect after administration of eye drops into the conjunctival sac. Higher, 3% choline salicylate concentration in the eye drops does not increase the irritation of the cornea cells, which is confirmed by no statistically significant differences between control and tested eye drop formulation for both the concentration of 1% and 3% CS.

High CV values obtained for eye drop formulations, which includes semisynthetic polymers to increase the viscosity, suggest that cellulose derivatives (HEC, HPMC, MC) will be better tolerated by the patient upon administration to the eye, that those with PVP and PVA.

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