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A Collaborative Evaluation of the Cytotoxicity of Two Surfactants by Using the Human Corneal Epithelial Cell Line and the WST-1 Test

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ABSTRACT

This study was undertaken to investigate the use of the *in vitro* test WST-1, an assay of cell proliferation and viability, for a preliminary safety evaluation of topical ophthalmic preparations. The cytotoxicity of two surfactants, benzalkonium chloride (BAC) and polyoxyethylene-20-stearyl ether (Brij®78, PSE) was independently investigated in four laboratories in the EU by using an immortalized human corneal epithelial (HCE) cell line. The HCE cells were exposed to BAC and PSE for 5 min, 15 min, and 1 hour, and the results of the HCE-WST-1 tests were collected and compared. After one-hour exposure, the EC_{50} values in BAC-treated cells in the presence of serum ranged between 0.0650 ± 0.0284 (mean ± SD) mM, and those in the absence of serum 0.0296 ± 0.0081 mM. The corresponding values for PSE were 0.0581 ± .0300 mM and 0.0228 ± .0063 mM. There were variations in the results between different laboratories, with coefficients of variation ranging from 31 to 121%, mean 58%. The use of one-hour exposure time is to be preferred, and the elimination of serum in the culture medium is recommended to avoid both underestimation of toxic effects and variability of the test results.

INTRODUCTION

In recent years ethical considerations have emphasized the necessity of reducing the use of laboratory animals for the safety testing of various chemicals and formulations, and considerable efforts have been directed towards the development of *in vitro* alternatives to *in vivo* animal tests (1–9). In particular, the time-honored Draize eye irritation test (10) has been extensively criticized for its subjectivity, lack of discrimination of fine response differences, and overestimation of the human re-

sponse (2,11–13). Many *in vitro* methods have been proposed as alternatives (14–21). To date, however, no test or testing strategy has been universally accepted as a complete replacement for the Draize test.

The purpose of this study was to assess and investigate the reproducibility and the test conditions of an alternative *in vitro* cytotoxicity test, the WST-1 {4[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} assay, for a preliminary safety evaluation of topical ophthalmic preparations. Immortalized human corneal epithelial (HCE) cells were used as a model culture, since they cover the outer surface of the eye, which is primarily exposed to the outside environment and to topical ophthalmic treatments. WST-1, a colorimetric assay for non-radioactive quantification of cell proliferation and viability, is based on the cleavage of the tetrazolium salt WST-1 (slightly red) to formazan (dark red), occurring only in the active mitochondria of living cells. Two surfactants were used as test chemicals: benzalkonium chloride (BAC), a common preservative of ophthalmic preparations, and polyoxyethylene-20-stearyl ether (Brij®78, PSE), used in ophthalmic preparations chiefly as a solubilizing agent. The effects of exposure time and serum content in the culture medium were evaluated. The cytotoxicity tests were independently carried out in four laboratories in the EU, and the results were collected and compared.

MATERIALS AND METHODS

The methods used in this study, i.e., cell culture techniques, exposure of cells to test substances, and the WST-1 test procedure were mutually agreed to by all the participating laboratories. The tested substances, BAC and PSE, were simultaneously distributed to the participating laboratories by Santen Oy (Tampere, Finland).

Immortalized Human Corneal Epithelial Cells

An immortalized human corneal epithelial cell line (HCE) was established by Araki-Sasaki and colleagues (22) by infecting primary human corneal epithelial cells with a recombinant SV40-adenovirus vector and by cloning the cells three times to obtain a continuously growing cell line. The SV40-cell line used in this study was a generous gift of the Araki-Sasaki research group. Initially, HCE cells were grown in a culture medium containing 1 vol of Dulbecco's modified Eagle's medium and 1 vol of Ham's nutrient mixture F-12 supplemented with 15% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) antibiotic-antimycotic solution (penicillin 10,000 U/ml, streptomycin 10,000 g/ml and amphotericin B 25 μg/ml), 2 mM L-glutamine (Gibco, UK), 5 μg/ml insulin, 10 ng/ml human epithelial growth factor, 0.5% (vol/vol) dimethylsulfoxide (Sigma Chemical Co., St. Louis, MO), and 0.1 μg/ml cholera toxin (Calbiochem, San Diego, CA). Subsequently, dimethylsulfoxide and cholera toxin were eliminated from the growth medium to better simulate the natural physiological conditions. The elimination of these substances did not affect the cell morphology, although a slight decrease in growth rate was observed.

The cells were incubated in a humidified atmosphere at 37°C in 5% CO_2/95% air in 75 cm^2 T-flasks and serially passaged at a split ratio of 1:5 twice a week. For cytotoxicity testing, HCE cells were plated at the density of 30,000 cells/well in a 96-well microtiter plate. The cells were exposed to the test solutions 24 hours after plating, at 70% confluence, and before the cultures started to grow in multi-layered fashion.

Treatment of Cells for Cytotoxicity Assays

The test substances were benzalkonium chloride (BAC, average MW 360, FeF Chemicals A/S, Køke, Denmark) and polyoxyethylene-20-stearyl ether (PSE, Brij®78, average MW 1152, Fluka,

Buchs, Switzerland). For cytotoxicity testing, the compounds were dissolved in serum-free medium or in medium containing 15% (vol/vol) fetal bovine serum (FBS). All dilutions were made using the appropriate culture medium.

24 hours after plating, the medium was discarded and 100 μl of the test solutions were added to the culture wells for treatment periods of 5, 15, or 60 min. After removal of the test medium, the cells were rinsed once with serum-free basal medium (Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12, 1:1), and then 100 μl of fresh growth medium containing 15% (vol/vol) FBS was added. The cells were returned to the incubator for a one-hour recovery period, and then the WST-1 test was performed.

WST-1 Cytotoxicity Test

The cytotoxicity test was based on the ready-to-use cell proliferation reagent WST-1 (cat no. 1644807, Roche Diagnostics GmbH, Germany). After one-hour recovery, 10 μl of WST-1 reagent was added to the medium in each well. The cells were incubated in a humidified atmosphere at 37°C in 5% CO_2/95% air for two hours, then the multi-titer plate was thoroughly shaken for one minute and absorbances were read at 450 nm. The use of a two-hour incubation period was based on a series of preliminary experiments. The background absorbance was measured on wells containing only the dye solution and the culture medium. Cytotoxicity data in each participating laboratory were obtained from at least three experiments with at least six wells for each concentration in separate 96-well plates. The mean optical density values corresponding to the non-treated controls were taken as 100%. The results were expressed as percentages of the optical density of treated vs. untreated controls.

Statistical Methods

EC_{50} values (mM) were determined by GraphPad Prism software (GraphPad™, San Diego, US) using non-linear regression analysis, as a concentration of the test agent that decreased the WST-1 formazan reduction values to 50% of the control values. The sigmoidal dose-response was defined by the following equation:

$$Y = BOTTOM + (TOP\text{-}BOTTOM)/[1 + 10^{(LogEC50\text{-}X)} * R]$$

where X is the logarithm of the concentration, Y the response, and R is the hillslope. Y starts at bottom (0) and goes to top (100) with a sigmoid shape.

The coefficient of variation (CV%), an overall estimate of the inter-laboratory variability, was calculated for both test substances and for all times of exposure. The statistical significance of the differences of the EC_{50} values between cultures exposed to test substances without FBS and with 15% (vol/vol) FBS were determined with Student's two-tailed t-test (GraphPad™).

RESULTS

Four laboratories in the EU (University of Tampere in Finland, University of Bremen in Germany, University of Ioannina in Greece, and University of Pisa in Italy) took part in this multi-laboratory study. Both laboratories with years of experience in cell culture techniques and laboratories just starting with cell culture methods were involved. Figures 1–6, based on data obtained with the WST-1 test, illustrate the effects, as per cent of WST-1 formazan reduction (viability) compared to the controls, produced by the exposure of HCE cells to the test agents under study, BAC and PSE,

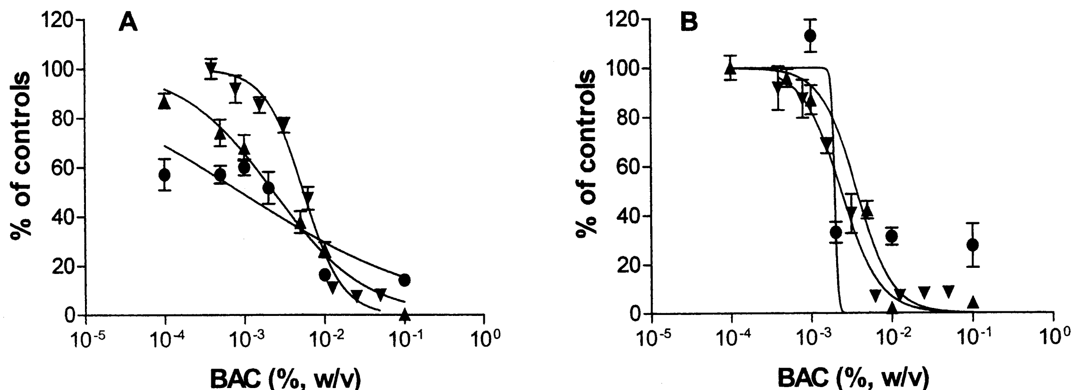

FIGURE 1. Reduction of WST-1 {4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} after 5-Minutes Exposure to Benzalkonium Chloride (BAC) in Serum-Containing (A) and in Serum-Free (B) Medium. In All Figures Each Point Represents the Mean SEM of at Least Three Separate Experiments. Lab 1 (◆), Lab 3 (●), and Lab 4 (◆).

for 5, 15 and 60 min. The cytotoxicity of BAC and PSE was dose-dependent, and it was influenced by the time of exposure and the presence of serum (FBS) in the culture medium. However, the cytotoxicity data were not influenced by the laboratory cell culture experience.

The EC_{50} values (in mM) obtained by the four laboratories for the tested agents at different incubation times in the presence and absence of FBS are reported in Table 1. The values were obtained by non-linear regression analysis from the viability compared to controls vs. concentration graphs such as those shown in Figures 1–6. The EC_{50} mean values with the relevant standard deviations are reported in Table 2, together with the respective coefficients of variation (CV%).

DISCUSSION

In the present study the cytotoxicity of two surfactants was investigated in four laboratories by using an immortalized human corneal epithelial cell line and the WST-1 assay. The tested substances were BAC, a cationic surfactant used as a preservative in most eye-drops, and PSE, a non-ionic sur-

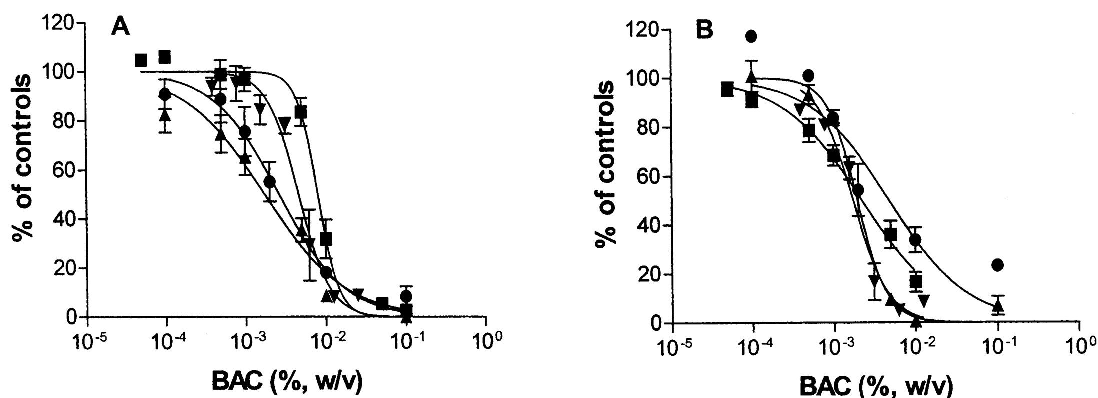

FIGURE 2. Reduction of WST-1 after 15-Minutes Exposure to BAC in Serum-Containing (A) and in Serum-Free (B) Medium. Lab 1 (◆), Lab 2 (■), Lab 3 (●), and Lab 4 (◆).

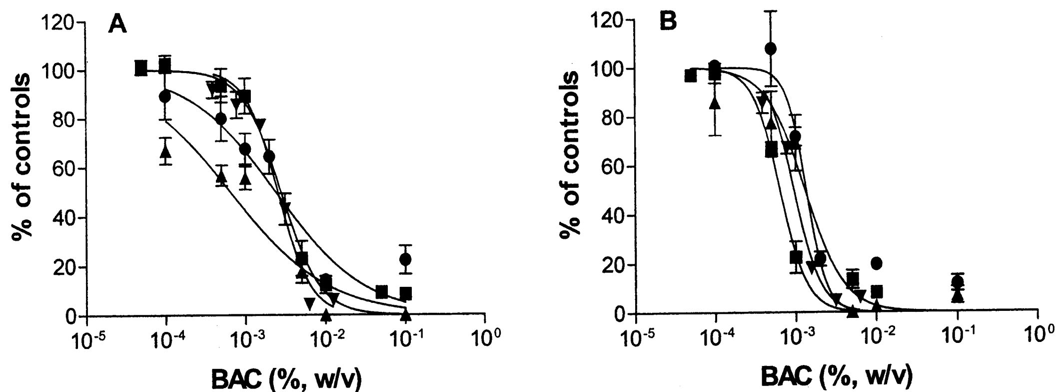

FIGURE 3. Reduction of WST-1 after One-Hour Exposure to BAC in Serum-Containing (A) and in Serum-Free (B) Medium. Lab 1 (◆), Lab 2 (■), Lab 3 (●), and Lab 4 (◆).

factant used in ophthalmic preparations chiefly as a solubilizing agent, and also in many other topical pharmaceutical vehicles as an emulsifying and gelatinous agent. The toxicity of BAC has been investigated in the past by several *in vitro* cytotoxicity tests such as [^{3}H]uridine uptake assay (23), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test, the best-known member of the tetrazolium salt family used for assaying cell proliferation and viability (24–27), and EZ4U assay using modified XTT (sodium 3,3′-{1-(phenylamino)-carbonyl-3,4-tetrazolium}-bis(4-methoxy-6-nitro)-benzenesulfonic acid hydrate), another member of the tetrazolium salt family (28). Furthermore, the cytotoxicity of BAC has been evaluated by the following tests: lactate dehydrogenase release (24, 26), neutral red uptake (23–25), propidium iodide staining (26,27), intracellular calcium (27) and intracellular pH (27).

WST-1 or 4[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate is a relatively recent member of the tetrazolium salt family. The tetrazolium salt MTT is the most widely used among various tetrazolium salts employed for the measurement of cell proliferation and viability, based on the measurement of the function of various mitochondrial dehydrogenases (29). The tetrazolium salts are cleaved to formazan by the cellular enzymes. An increase in the amount of formazan dye correlates directly to the number of metabolically active cells in the culture. Unlike the

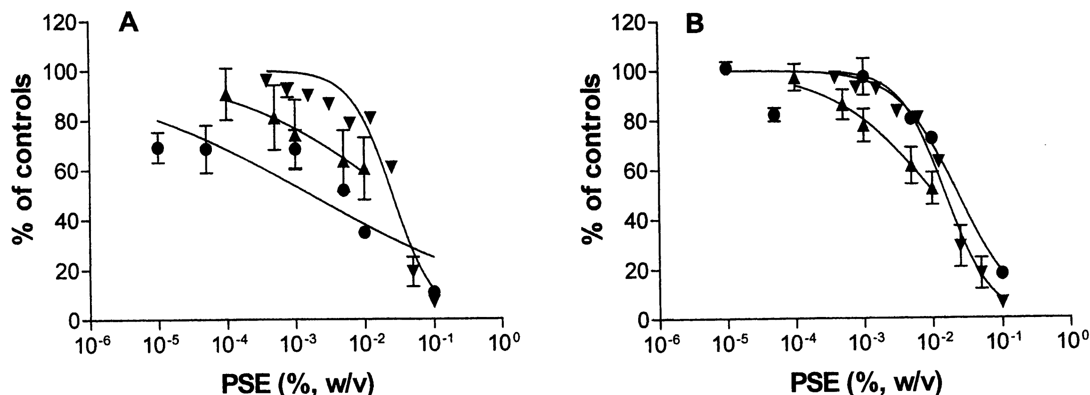

FIGURE 4. Reduction of WST-1 {4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} after 5-Minutes Exposure to Polyoxyethylene-20-Strearyl Ether (PSE) in Serum-Containing (A) and in Serum-Free (B) Medium. Lab 1 (◆), Lab 3 (●), and Lab 4 (◆).

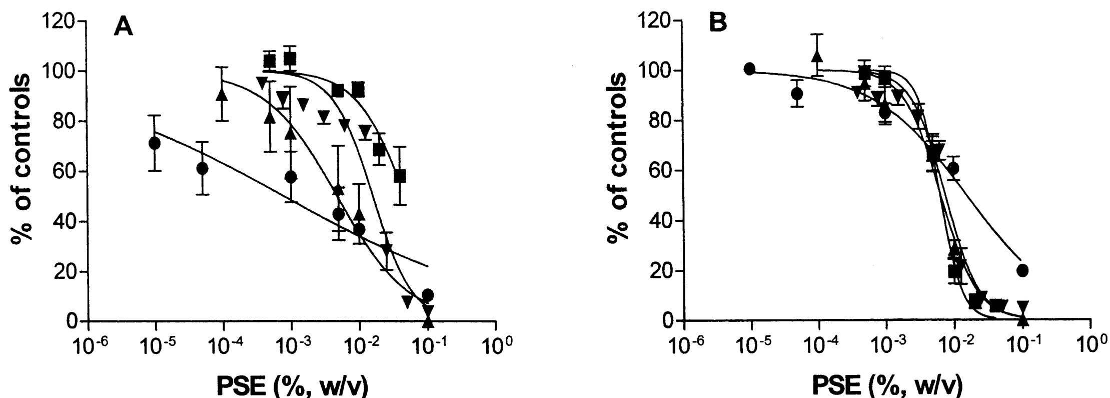

FIGURE 5. Reduction of WST-1 after 15-Minutes Exposure to PSE in Serum-Containing (A) and in Serum-Free (B) Medium. Lab 1 (◆), Lab 2 (■), Lab 3 (●), and Lab 4 (◆).

MTT test, WST-1 yields water-soluble cleavage products and thus does not require any additional solubilization steps before photometric measurements. Because organic solvent extraction is not required, the WST-1 assay is simpler, faster, and environmentally more favorable than the traditional MTT test. In recent studies, WST-1 was used to evaluate cytotoxicity of ophthalmic adjuvants on ocular cell lines (30).

The reported EC_{50} values for BAC for one-hour treatment period in the medium containing 5% serum evaluated by the MTT test on primary cultures of rabbit corneal epithelial cells vary from 5.1 ± 0.4 (mean ± SEM) μg/ml (24) to 7.0 ± 0.1 μg/ml (26) and 8.0 μg/ml (27). In another study, in the presence of 15% FBS serum, for the same HCE cell line as used in the present study the reported EC_{50} value obtained by the MTT test after one-hour exposure was 47 ± 5 (mean ± SEM) μg/ml (18). In an earlier study at the University of Tampere with the same HCE cell line and culture conditions as in the present study (31), the EC_{50} value for one-hour exposure in the absence of serum was 10.8 ± 0.9 (mean ± SEM) μg/ml (0.0300 ± 0.0025 mM), and in the presence of serum 7 ± 2.1 μg/ml (0.0547 ± 0.0058 mM). The EC_{50} values for one-hour exposure to BAC reported in this study are in

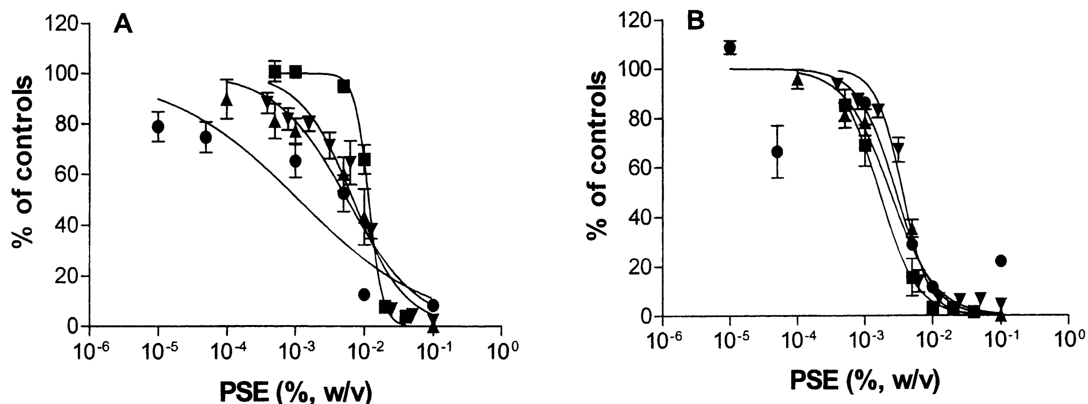

FIGURE 6. Reduction of WST-1 after One-Hour Exposure to PSE in Serum-Containing (A) and in Serum-Free (B) Medium. Lab 1 (◆), Lab 2 (■), Lab 3 (●), and Lab 4 (◆).

Table 1. EC_{50} values (mM) of Benzalkonium Chloride (BAC) and Polyxyethylene-20-Stearyl Ether (PSE) in the presence of fetal bovine serum (+FBS) or in the absence of serum (−FBS) evaluated by using immortalized human corneal epithelial cells and the WST-1 {4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate} test. The R^2 values describing the goodness of the curve fitting used to determine the EC_{50} values are in brackets below the corresponding EC_{50} values.

Substance (+FBS/−FBS) and exposure time	*Laboratory*			
	1	*2*	*3*	*4*
BAC +FBS, 5 min	0.1526 (0.9681)	*	0.0251 (0.6775)	0.0674 (0.8667)
BAC −FBS, 5 min	0.0651 (0.9383)	*	0.0538 (0.9390)	0.1034 (0.9207)
BAC +FBS, 15 min	0.1277 (0.9156)	0.2225 (0.9495)	0.0716 (0.8254)	0.0484 (0.7676)
BAC −FBS, 15 min	0.0481 (0.9442)	0.0616 (0.9155)	0.1327 (0.7960)	0.0542 (0.9577)
BAC +FBS, 1 h	0.0711 (0.9537)	0.0776 (0.9371)	0.0936 (0.4100)	0.0180 (0.7506)
BAC −FBS, 1 h	0.0265 (0.9738)	0.0178 (0.9321)	0.0378 (0.8595)	0.0363 (0.7294)
PSE +FBS, 5 min	0.2224 (0.9225)	*	0.0146 (0.6365)	0.2298 (0.2032)
PSE −FBS, 5 min	0.1343 (0.9609)	*	0.2035 (0.8857)	0.0960 (0.3978)
PSE +FBS, 15 min	0.1388 (0.9149)	0.4155 (0.6791)	0.0064 (0.4379)	0.0484 (0.5742)
PSE −FBS, 15 min	0.0654 (0.9528)	0.0549 (0.9429)	0.1311 (0.9451)	0.0556 (0.7910)
PSE +FBS, 1 h	0.0596 (0.9223)	0.1006 (0.9802)	0.0158 (0.6560)	0.0563 (0.6218)
PSE −FBS, 1 h	0.0313 (0.9594)	0.0142 (0.9089)	0.0250 (0.7437)	0.0205 (0.8601)

*No Data.

the range 6.4–13.6 μg/ml (0.0178–0.0378 mM) in the absence of FBS, and 6.5–33.7 μg/ml (0.0180–0.0936 mM) in the presence of 15% serum.

The cytotoxicity of PSE has been less investigated than that of BAC. The EC_{50} value for one-hour exposure time in the presence of 10% FBS evaluated on the promonocytic human cell line U937 by the EZ4U tetrazolium salt reduction assay is 7 μg/ml (28). Our EC_{50} values for one-hour exposure range from 16.3 to 36.1 μg/ml (0.0142–0.0313 mM) in the absence of FBS, and from 18.2 to 115.9 μg/ml (0.0158–0.1006 mM) in serum-containing medium.

The generally high variability (mean 58%) in this study can be attributed to a low number of data (n = 3 to 4), very short exposure times, and the use of serum. Short exposure times were used in this study to simulate an *in vivo* acute exposure response to a test compound in the clinical situations. In the present study, it was apparent that longer exposure times resulted to lower inter-laboratory variability. One-hour exposure time induces more reproducible results and therefore it is recommended rather than shorter exposure times. The serum in the culture medium simulated the possibly neutralizing effect of proteins present in the tear film *in vivo*. In fact, the serum used in the study contains 4.0–4.5% of proteins. After dilution with the growth medium, the protein concentration drops to approximately 0.6%, which is not far from the value indicated in the literature

Table 2. Mean EC_{50} values of Benzalkonium Chloride (BAC) and Polyxyethylene-20-Stearyl Ether (PSE), Standard Deviations (SD), number of tests and Coefficients of Variation (CV%).

Substance (+FBS/−FBS) and exposure time	*Mean EC_{50} value (mM)*	*SD*	*Data number (n)*	*CV%*
BAC +FBS, 5 min	0.0817	0.0530	3	79.5
BAC −FBS, 5 min	0.0741	0.0212	3	35.1
BAC +FBS, 15 min	0.1175	0.0671	4	65.9
BAC −FBS, 15 min	0.0742	0.0342	4	53.2
BAC +FBS, 1 h	0.0650	0.0284	4	50.4
BAC −FBS, 1 h	0.0296	0.0081	4	31.5
PSE +FBS, 5 min	0.1556	0.0998	3	78.5
PSE −FBS, 5 min	0.1446	0.0445	3	37.7
PSE +FBS, 15 min	0.1523	0.1593	4	120.8
PSE −FBS, 15 min	0.0768	0.0316	4	47.6
PSE +FBS, 1 h	0.0581	0.0300	4	59.6
PSE −FBS, 1 h	0.0228	0.0063	4	31.8

*Fetal Bovine Serum.

for the concentration (0.6–0.9%) of tear proteins (32). The protective nature of serum was evident in the present study. The EC_{50} values were twice as high in the presence of serum as in the absence of serum in exposures for 15 min or one hour. In 5-minute exposures the effect of serum was less apparent. The inter-laboratory variability was noticeably higher when serum was used. This variability could be due to the distinct lots of serum used in the study. Since serum contains a mixture of unknown growth factors and its content varies batch by batch, it is easy to understand its influence on the variability of the test results. Consequently, the use of serum is not recommended in the estimation of the acute adverse effects of topical ophthalmic preparations, firstly to avoid underestimation of the acute toxic effects of the test substances, and secondly to avoid unnecessary variability in the test results.

The good inter-laboratory reproducibility of an *in vitro* assay is important when considering its use as a screening tool or as an alternative or replacement test of the test battery for the Draize eye irritation test. Only an assay with excellent inter-laboratory reproducibility can be accepted for validation, which is, in addition, needed for the authoritative acceptance of an *in vitro* test. Immortalized human corneal epithelial cells and the WST-1 test, here designated as the HCE-WST-1 test, is a promising screening tool for evaluating cytotoxicity of ophthalmic preparations. However, more specific mechanism based *in vitro* alternative methods are also needed in the test battery when considering the replacement of the traditional Draize test.

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