Development of a new contact lens multipurpose solution: Comparative analysis of microbiological, biological and clinical performance

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Received 26 March 2010; accepted 9 June 2010

Abstract

Purpose: Antimicrobial, cytotoxicity and clinical performance of a new soft contact lens multi-purpose solution (COMPLETE® RevitaLens) based on polyquaternium-1 and alexidine dihydrochloride (NuMPS) was evaluated.

Methods: Antimicrobial efficacy was assessed according to ISO 14729 for both biocidal and regimen performance against bacteria and fungi. *Acanthamoeba* efficacy was tested along with ability to retain antimicrobial activity on partial evaporation. *In vitro* cytotoxicity of NuMPS and OPTI-FREE® Replenish® MPS (MPS-3) was assessed based on ISO 10993-5 and United States Pharmacopeia (USP) methods. In addition, a 3 month, double-masked, parallel group clinical trial comparing safety and acceptability with respect to MPS-3 was conducted with 4 silicone hydrogel (SHy) and FDA Group IV lens types.

Results: NuMPS showed broad antimicrobial efficacy, including *Acanthamoeba*, giving a 3-4 log₁₀ reduction in viability after 6 hours contact time. NuMPS also passed ISO 14729 regimens with SHy and etafilcon lenses for bacteria, fungi and also *Acanthamoeba*. The cytotoxicity of NuMPS was equivalent or better compared to MPS-3. In the clinical trial, there was no statistically significant between-group difference in corneal staining (p > 0.05). Patients using MPS-3 had more adverse events than patients using NuMPS: 11.8% (11/93) versus 2.8% (5/177), respectively, (p < 0.05).

Conclusion: Taken together, the results of these studies indicate that the NuMPS is a novel and effective soft contact lens care solution.

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Desarrollo de una nueva solución multiusos para lentes de contacto: análisis comparativo de su rendimiento microbiológico, biológico y clínico

Resumen
Objetivos: Se evaluó el rendimiento clínico, antimicrobiano y citotóxico de una nueva solución multiusos para lentes de contacto blandas (COMPLETE® RevitaLens) basada en poliquaternio-1 y diclorhidrato de alexidina (NuMPS).

Métodos: Se evaluó la eficacia antimicrobiana y la citotoxicidad de una nueva solución multiusos con 20 y 40 pacientes que utilizaron MPS (MPS-2 y MPS-3). Además, se llevó a cabo un ensayo clínico simultáneo, a doble ciego, y de 3 meses de duración que comparó la seguridad y la aceptabilidad de MPS-3 para 4 lentes de hidrogel de silicona (SHy) y lentes del grupo IV de la FDA.

Resultados: La solución NuMPS mostró una eficacia antimicrobiana amplia, incluso contra Acant hamoeba, y produjo una reducción logarítmica de 3-4 (log10) en la viabilidad tras 6 horas de contacto. Dicha solución también superó ISO 14729 para regímenes con SHy y lentes etafilcon para bacterias, hongos y Acant hamoeba. La citotoxicidad de NuMPS fue equivalente o mejor que la de MPS-3. Se evaluó el rendimiento microbiológico, biocida como en regímenes de higiene contra bacterias y hongos. Se comprobó la eficacia antimicrobiana y la citotoxicidad de una nueva solución multiusos y de OPTI-FREE® RepleniSH® MPS (MPS-3). Además, se llevó a cabo un ensayo clínico simultáneo, a doble ciego y de 3 meses de duración que comparó la seguridad y la aceptabilidad de MPS-3 para 4 lentes de hidrogel de silicona (SHy) y lentes del grupo IV de la FDA.

Conclusiones: En su conjunto, los resultados de estos estudios indican que la NuMPS es una nueva solución eficaz para el cuidado de lentes de contacto blandas.

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Introduction

Multi-purpose solutions (MPS) represent the majority of systems used for the care of soft contact lenses. They comprise a single solution for the rinsing, disinfection and storage of lenses and are typically composed of a preservative, buffer system and other agents to aid lens comfort and cleaning. The ability of these components to achieve sufficient antimicrobial efficacy is fundamental to safe contact lens use.

Microbial keratitis is a rare but significant risk among soft contact lens wearers with a reported incidence of 4 cases per 10,000 users per year. In keratitis, due to the free-living amoebae Acanthamoeba, almost 90% of cases occur in contact lens wearers and the reported incidence varies from 1-2 cases per million in the USA or 17-20 in the UK. Accordingly, the use of MPS with effective disinfectant properties, in conjunction with good compliance to lens care hygiene, reduces the incidence of microbial keratitis through the prevention or inhibition of potentially pathogenic organism growth on the lens surface and within the lens storage case. The majority of MPS utilize the preservative polyhexamethylene biguanide (PHMB) but others use the quaternary ammonium compound polyquaternium-1 (PQ-1) alone or in combination with the amido amine myristamidopropl dimethylamine (MAPD). According to international standards, including ISO 14729 and FDA 510(k) guidance, contact lens solutions are required to exhibit antimicrobial efficacy against select reference strains of common bacterial and fungal ocular pathogens. However, the rigor of such testing has been questioned following the association of two MPS with a significant rise in cases of keratitis due to the filamentous mould Fusarium and to Acanthamoeba.

The purpose of this review is to evaluate the antimicrobial, cytotoxicity and clinical performance of a new MPS (NuMPS: COMPLETE® RevitaLens) based on the novel dual disinfection combination of PQ-1 and the bis-biguanide alexidine.

Materials and methods

Test organisms and solutions

For the microbiological analysis, the following organisms were studied: Pseudomonas aeruginosa (ATCC 9027), Serratia marcescens (ATCC 13880), Staphylococcus aureus (ATCC 6538), Candida albicans (ATCC 10231), Fusarium solani (ATCC 36031) and Acanthamoeba castellanii (ATCC 50370). Test solutions studied are given in Table 1.

For the bacteria and fungi, stand-alone (biocidal) and regimen assays were performed according to ISO 14729. Briefly, in the stand-alone procedure the test solutions were challenged with 1 × 10^5 ml organism and the number of survivors determined by culture viability at 0 and 6 hr using the WASP Spiral Plater and ProtoCOL colony counter system. At each time point the test samples were first diluted 1:10 into DifcoW Letheen Broth neutralizing broth for the prevention or inhibition of potentially pathogenic organism growth on the lens surface and within the lens storage case. The majority of MPS utilize the preservative polyhexamethylene biguanide (PHMB) but others use the quaternary ammonium compound polyquaternium-1 (PQ-1) alone or in combination with the amido amine myristamidopropl dimethylamine (MAPD). According to international standards, including ISO 14729 and FDA 510(k) guidance, contact lens solutions are required to exhibit antimicrobial efficacy against select reference strains of common bacterial and fungal ocular pathogens. However,
In the regimen assay, four sets of the following lenses were tested per organism: Solfens® 38 (polymacon, Bausch & Lomb), Acuvue® 2 (etafilcon A, Vistakon), Acuvue® Advance® (galafilcon, Vistakon), O₂Optix®/AirOptix® (lotrafilcon B, CIBA Vision), PureVision® (balafilcon A, Bausch & Lomb) and Biofinity® (comfilcon A, CooperVision) lenses. Although not required by the protocol, organic soil was included in the organism challenge inoculum and comprised a final concentration of 300–3000 heat killed Saccharomyces cerevisiae (ATCC 9763) in 0.003 % heat-inactivated fetal bovine serum. Briefly, four lenses of each test type were inoculated with approximately 2 × 10⁶ organisms and left to adhere for 5 minutes. Each lens was then subjected to a “No Rub but Rinse” regimen with the NuMPS® as the positive control solution. Loss of MPS antimicrobial efficacy following partial evaporation was also studied. The solutions were evaporated under a stream of air to 2x and 4x concentration by weight, challenged with F. solani or A. castellanii trophozoites and the level of organism kill determined after 6 hr contact time.

Cytotoxicity studies

In vitro cytotoxicity potential of NuMPS and MPS-3 (OPTI-FREE® Multi-Purpose Solution) was evaluated according to USP < 87 > and ISO 10993-5,11,20,21 Five soft contact lens types were studied: Solfens® 38 (polymacon, Bausch & Lomb), Acuvue Advance® (galafilcon A, Vistakon), O₂Optix® (lotrafilcon B, CIBA Vision), PureVision® (balafilcon A, Bausch & Lomb) and Biofinity® (comfilcon A, CooperVision) lenses. Briefly, the lenses were soaked in 100 ml of the test solutions, in triplicate, for four days. Confluent monolayers of L929 mouse fibroblasts were then exposed to treated contact lenses for 24 hours and scored for reactivity according to USP Direct Contact Test criteria (Table 2).21 Polypropylene pellets and latex rubber served as negative and positive controls, respectively.
Clinical investigation

A three-month, double-masked, parallel group clinical trial comparing the safety and acceptability (comfort and lens cleanliness) of the NuMPS (test) to MPS-3 (control) was conducted with four types of silicone hydrogel contact lenses: Acuvue® Advance® (galyficon A, Vistakon), PureVision® (balaficon A, Bausch & Lomb), O2Optix® (lotralficon B, CIBA Vision), Biofinity® (comfilcon A, CooperVision) and a FDA Group IV material (e.g., Acuvue® 2, [etafilcon A Vistakon]). Subjects were enrolled in a 2:1 ratio (test vs. control) for each lens material. Of the 270 subjects enrolled, 177 were assigned to the test regimen and 93 to the control. In the test group the subjects used a “Rub and Rinse” regimen in their lens care whereas for the control a “No Rub but Rinse” regimen was used. Safety was primarily assessed through slit-lamp observations and the occurrence of adverse events (e.g. keratitis, hypersensitivity or visual acuity loss). All slit lamp evaluation findings were graded on a 0 to 4 scale, where 0 = None, 1 = Trace, 2 = Mild, 3 = Moderate, and 4 = Severe. Findings were categorized as: corneal edema, corneal neovascularization, corneal staining, bulbar hyperemia, palpebral conjunctiva and other findings. Acceptability was assessed via lens cleanliness (lens deposition as described below) and lens wearing comfort rated on a scale from 0-10 (0 = intolerable and 10 = cannot feel lens) compared to baseline assessments.

Protein and lipid deposit assessment of worn lenses

Lenses worn in a daily mode using either the test (NuMPS) or control multipurpose solution (MPS-3; see above section, Clinical investigation) were assessed at the end of a wear period (2 wks or 30 days, depending on lens type): n > 30/15 for test/control lenses for protein (left lens) or lipid (right lens) analysis for each lens type tested in the clinical investigation (see above section). All involved parties (including the analyst) were masked regarding control and test lenses. Protein analysis was by a modified Lowry technique utilizing acetonitrile:water (1:1) containing 0.1% trifluoroacetic acid to extract protein from lenses. Lipid analysis involved extracting lipids from lenses with toluene:isopropanol (4:1), followed by deposition of the extracted lipids on a Teflon circular membrane (6 mm diameter) in a well of a 96-well plate (glass) by air evaporation.

Mid-infrared transmission analysis of the Teflon membrane with deposited lipids, based upon functional groups specific to lipid types, was done using a Nicolet Avatar-370 Fourier Transform Infrared Spectrometer. Glyceryl tristearate (99% Aldrich, USA) was used to prepare standards for the analysis.

Statistical analysis

Cochran-Mantel-Haenszel test was used to compare difference in overall corneal staining between the two regimens and Fisher’s exact test for adverse events. General Linear Model analysis was used to analyze acceptability measures for overall cleanliness and lens wearing comfort. Analysis of protein and lipid deposition on lenses was performed using Wilcoxon rank-sum test.

Results

Microbiology

The log10 reduction in bacterial and fungal viability 6 hours exposure time to the test solutions is shown in Table 3. With the exception of MPS-3, which gave a 2.3 log10 kill for S. aureus, all commercial MPS and Perox gave > 4.0 log10 reduction in bacterial viability. A greater range in efficacy was observed with the fungi. For C. albicans, MPS-1 and MPS-4 gave 1.0–1.1 log10 kill respectively and > 3.0 log10 for the other solutions, including Perox. For F. solani, a 1.7–2.1 log10 reduction was obtained for MPS-4 and MPS-3, respectively, and > 3.0 log10 for all other commercial solutions. NuMPS showed > 4.0 and > 3.0 log10 reduction for all bacteria and fungi at 6 hours, respectively.

The results for the Acanthamoeba biocidal studies are shown in Table 4. All the solutions showed activity against trophozoites, ranging from 2.2–3.6 log10 kill after 6 hours. The results for the cysts were more variable and ranged from 0.1–2.4 log10 for MPS-3 and Perox, respectively, and > 3.0 log10 kill for the others.

With the regimen testing, the NuMPS gave < 10 cfu surviving bacteria and fungi on the lenses or in the soaking solution under No Rub but Rinse conditions and < 10 viable...
Acanthamoeba trophozoites or cysts when Rub and Rinse was used.

No evidence for trophozoite encystment (0%–5%) was observed during 24 hr incubation in any of the test or NuMPS solutions. The control encystment solution of Complete® MoisturePlus® MPS gave 30% encystment.

The efficacy of test solutions against F. solani and A. castellanii trophozoites after evaporation to 2× and 4× concentration and a 6 hr disinfection time is shown in Table 5. For F. solani, MPS5 and MPS3 lost 85%–98% and 70%–85% of activity at 2× and 4× concentration, respectively. No loss in trophozoite efficacy on concentration was found with MPS2 or NuMPS. For A. castellanii trophozoites, MPS5 showed a 13%–51% reduction in efficacy and MPS3 a 38%–9% loss at 2× concentration and 4×, respectively. MPS2 gave a 14% loss in activity at 2× concentration and 0% at 4×. No loss in trophozoite efficacy on concentration was found with NuMPS.

Cytotoxicity

In vitro cytotoxicity results are described in Table 6. Here NuMPS showed grade 1 (slight) with PureVision® and grade 2 (mild) with Acuvue® Advance® and O₂Optix® lenses, compared to MPS3 which exhibited a grade 3 (moderate) for these Shy lenses. Both solutions scored a grade 2 for Soflens® 38 lenses and grade 3 for Biofinity® lenses.
acceptability measures, there was no significant difference in corneal staining between the two regimens. Corneal staining incidence at Day 90 was 18% trace and 2.9% mild with NuMPS; and 11.9% trace, 3.6% mild, and 1.2% moderate with MPS-3 (p > 0.05). However, the incidence of adverse events in control subjects was significantly greater than the test subjects with 11.8% (11/93) versus 2.8% (5/177), respectively (p < 0.05). These adverse events were not specific to any one lens type, but were more often inflammatory (keratitis, conjunctivitis) or hypersensitivity responses (solution sensitivity, redness, tearing, etc.) with no cases of confirmed microbial infection. With regard to acceptability measures, there was no significant difference between the two regimens among all lenses tested in terms of overall cleanliness and lens wearing comfort, with lens wearing comfort mean ratings in the test and control groups of 8.7 and 8.6, respectively (p > 0.05).

Protein and lipid deposit assessment of worn lenses

The conventional etafilcon A (Acuvue 2®) worn lenses were found to contain much higher levels of protein than any of the four silicone hydrogel types, with average protein levels of 50-60 μg/ mg of dry lens material (Table 7). Although the average protein for this lens type (FDA Group IV) slightly favored the control solution (MPS-3), it was not statistically significant (p = 0.10). All four silicone hydrogel types showed an average of less than 1 μg protein per mg of dry lens material for lenses used with either the control (MPS-3) or test solution (NuMPS). No statistically significant difference was found between the solutions regarding protein deposition on three of the silicone hydrogel lens types (p = 0.70-0.97). However, lotrafilcon B lenses (O₂ Optix®) exhibited a significantly lower level of protein deposition with NuMPS (0.04 μg/ mg of lens) compared to the control MPS-3 (0.12 μg/ mg, p = 0.01).

As shown in Tables 8-9, lipid levels were significantly higher for all worn silicone hydrogel lenses (1-5 μg/ mg of dry lens material, depending on lens type) than for the conventional etafilcon A (Acuvue 2®) lens type (0.4 μg/ mg of lens). Of the silicone hydrogel lenses, galyfilcon A (Acuvue Advance®) showed the highest total lipid deposition (~4.0 μg/ mg) and lotrafilcon B (O₂ Optix®) exhibited the lowest (~1.0 μg/ mg). There were no statistically significant differences in the amount of lipid deposition between the test and control solutions, although balafilcon A lenses (PureVision®) deposited less ester-type lipids (such as aliphatic glyceride esters) with NuMPS than MPS-3 with data tending toward significance (p = 0.06). Overall, the average amount of total lipid deposition for all lenses was identical for the two products and averaged 2.2 μg of lipid per mg of lens.

Discussion

Microbial keratitis is a rare but significant risk among contact lens wearers and studies have indicated that the risk of infection is around 2 cases per 10,000 soft contact lens wearers per year, rising to 19.5-25.4 cases per 10,000 for conventional hydrogel and silicone hydrogel lenses.
Table 9: Total lipid (ester and non-ester types) deposition levels (μg/mg of dry lens material) after continuous daily use of particular lens types (for the maximum recommended time) of NuMPS relative to MPS-3 as assessed by ex-vivo analysis of human worn lenses

<table>
<thead>
<tr>
<th>Contact Lens Type</th>
<th>Galilficon A (Acuvue® Advance®)</th>
<th>Balafilcon A (PureVision®)</th>
<th>Lotrafilcon B (O2Optix®)</th>
<th>Etafilcon A (Acuvue® 2)</th>
<th>Comofilcon A (Biofinity®)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NuMPS</td>
<td>MPS-3</td>
<td>NuMPS</td>
<td>MPS-3</td>
<td>NuMPS</td>
</tr>
<tr>
<td>N</td>
<td>32</td>
<td>17</td>
<td>31</td>
<td>15</td>
<td>31</td>
</tr>
<tr>
<td>Mean</td>
<td>4.2</td>
<td>3.5</td>
<td>2.4</td>
<td>3.0</td>
<td>0.88</td>
</tr>
<tr>
<td>SD</td>
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<td>2.5</td>
<td>1.6</td>
<td>3.9</td>
<td>0.70</td>
</tr>
<tr>
<td>P-value</td>
<td>0.34</td>
<td>0.42</td>
<td>0.13</td>
<td>0.63</td>
<td>0.50</td>
</tr>
</tbody>
</table>

respectively, if worn overnight. In Acanthamoeba keratitis almost 90% of cases occur in contact lens wearers and can result in permanent blindness. The reported incidence of Acanthamoeba keratitis varies from 1-2 cases per million in the USA or 17-20 in the UK.4-6 According to the manufacturer’s instructions for use (e.g. rubbing, rinsing, soaking). Contact lenses are inoculated with at least 5 log10 reduction in the three bacteria, with at least 1 log10 occurring for each bacterium.10 If this condition is met the solution is eligible for the Secondary Regimen assessment. Here, the test evaluated the antimicrobial efficacy of the entire lens care regimen according to the manufacturer’s instructions for use (e.g. rubbing, rinsing, soaking). Contact lenses are inoculated with at least 5 log10 of organism and following the Regimen an average of not more than 10 viable organisms should remain on the lens or the soaking solution.10 To date, no requirement has been established for testing against Acanthamoeba.10

With the exception of MPS-3, which produced only a 2.3 log10 with S. aureus (ATCC 6538), all the commercial solutions and NuMPS studied here met the ISO 14729 Primary Stand Alone (biocidal) criteria for bacterial and fungal efficacy at 6 hr contact time. Failure to pass biocidal criteria does not preclude a MPS from the market place providing it passes the secondary regimen requirement.10 In addition, all of the solutions tested including the NuMPS showed biocidal efficacy against A. castellanii trophozoites and, with the exception of MPS-3, the cysts. The NuMPS also met the ISO 14729 Secondary Regimen test for bacteria and fungi using a No Rub and Rinse procedure. For the Acanthamoeba only a Rub and Rinse regimen was used as previous studies have shown that the organism adheres with great affinity to silicone hydrogel lenses.5,27 As such, only the inclusion of a rub step can ensure satisfactory removal of trophozoites or cysts, as was demonstrated here.5 Therefore, it is recommended that a rubbing step always be included in contact lens care regimen procedures to ensure effective removal of microorganisms and to aid lens cleaning.

MPS-5 (ReNu with MoistureLoc®) was withdrawn from the market in 2006 due to its association with a significant rise in Fusarium keratitis cases.12,13 The formulation, containing alexidine (0.00045 %), showed good activity against F. solani in biocidal assays. However, it was subsequently shown to have lost efficacy on film formation through desiccation in the lens case, most probably exacerbated by the presence of polymer PO10 in the formulation.14,15 The findings presented here extended this observation, showing that even on partial evaporation the solution lost 85-98% of Fusarium activity. Loss in Acanthamoeba trophozoite efficacy was also observed, with a 51% reduction in activity at 4x concentration upon evaporation. This finding is similar to that described previously with ReNu with MoistureLoc® in which a 70% loss in Fusarium efficacy was obtained with solutions formulated (rather than evaporated, as was done here) to 2x and 4x concentration. MPS-3 containing PO-1 (0.001 %) and myristamidopropyl dimethylamine (0.0005%) also showed a significant loss in Fusarium activity ranging from 70% at 2x and 4x concentration. However, there are no reports associating MPS-3 with an increased risk of Fusarium keratitis, suggesting additional factors besides loss in biocidal efficacy through evaporation may have been involved in the outbreak. In contrast, the NuMPS formulation, containing PO-1 and alexidine showed no loss in Fusarium or Acanthamoeba efficacy on evaporation to 2 times and 4 times the concentration.

Another contact lens solution recall occurred in 2007 with the withdrawal of Complete® MoisturePlus® after it was linked to a number of Acanthamoeba keratitis cases.13,15 It was subsequently shown that the solution induced significant Acanthamoeba trophozoite encystment due, primarily, to the presence of propylene glycol in the formulation.19,30 In this study, none of the solutions, including the NuMPS, were found to induce trophozoite encystment. The root cause for the association between Complete® MoisturePlus® and Acanthamoeba keratitis remains unclear and, despite the recall, the number of cases of infection related to the use of other contact lens solutions continues to be reported with increased frequency.31
findings of this study indicate that greater stringency is required. Studies have also shown Fusarium strain variation in susceptibility to some MPS and, along with C. albicans, are significantly more resistant to disinfection when present as biofilms compared to the planktonic state. Based on these observations, it is recommended that the standard assessment of antimicrobial efficacy of contact lens care solutions should be extended to include additional clinical strains of the ISO 14729 reference organisms along with Acanthamoeba for biocidal, regimen and encystment testing. Furthermore, the effect of evaporation on the loss of antimicrobial efficacy should also be considered.

Clinical trials with NuMPS are ongoing and will be published in detail elsewhere. In the study described here, corneal staining change from baseline with NuMPS was found to be no greater than that with the control. Moreover, NuMPS was associated with appreciably fewer adverse events, none of which could be attributed to microbial keratitis. The trial also showed that NuMPS was comparable to the control solution with respect to lens cleanliness or wearing comfort.

Results from the present study showed NuMPS to have only slight to moderate cytotoxicity for all lens types studied and these findings were superior or equivalent to the control solution MPS-3. As the use of MPS-3 with some silicone hydrogel lenses has recently been associated with an increased incidence of corneal infiltrative events, the clinical implications of the cytotoxicity results from this study warrant further investigation to correlate in vitro biocompatibility to in vivo performance of the various MPS-CL combinations at the ocular surface. Previous in vitro studies have also reported that certain MPS may exert negative effects on cell viability and possibly other changes in corneal function including permeability, suggesting some cytotoxic influences from these MPS at the ocular surface. Findings from these studies and others have demonstrated good correlation between cytotoxicity observed in human corneal epithelial cells (HCEC) compared to that in L929 cells.

Tear deposit assessment for total protein from worn lenses in the clinical trials showed greater deposition with the Group IV lenses than the silicone hydrogel lenses and that there were no statistically significant difference in the levels between users of NuMPS and the control solution. Lipid levels were significantly higher for all worn silicone hydrogel lenses compared to the group IV lenses. The observation of higher levels of protein deposition on conventional hydrogel compared with silicone hydrogel lenses is consistent with previous reports, as is the converse finding that less lipid binds to silicone hydrogel lenses.

In conclusion, the NuMPS is a novel contact lens care solution based on the dual disinfection system of PQ-1 and alexidine. This solution was shown to provide antimicrobial activity against the pathogenic bacteria and fungi stipulated under ISO 14729 guidance as well as A. castellanii trophozoites and cysts. Unlike ReNu with MoistureLoc® MPS, which also contained alexidine, NuMPS maintained efficacy on partial evaporation against Fusarium and Acanthamoeba. Furthermore, the NuMPS performed very well in both the in vitro cytotoxicity testing and clinical trials.

Conflict of interest
The authors are employed by Abbott Medical Optics Inc. The authors have no proprietary, financial or commercial interest in any material or method mentioned in this study.

Acknowledgements
The authors would like to thank Anthony Lam, Marina Nikolic, Nancy Brady, Simon Cheung, Adriana Park, Lisa Hoong and Lauren Crawford from the Corneal Research & Development Department for their technical assistance in conducting this study.

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