

FORMULATION BASED ON POLYHEXAMETHYLENE BIGUANIDE FOR USE IN THE TREATMENT OF ACANTHAMOEBA KERATITIS AND/OR FUNGAL INFECTIONS

The present invention relates to a stable formulation based on polyhexamethylene biguanide (PHMB) suitable for administration at an ophthalmic level, a process for its preparation and
5 relative dosage regimen effective for the treatment of *Acanthamoeba* keratitis, and in particular for the eradication of cysts.

Acanthamoeba keratitis is an infection of the cornea, the transparent tissue that lines the front part of the eye. The infection is caused by *Acanthamoeba*, a microorganism generally present both in streams and water basins (lakes, oceans and rivers), and in home tap water, swimming
10 pools, hydromassage tubs, in the soil and in the air.

Acanthamoeba keratitis is more common in contact-lens wearers [1], but anyone with a corneal lesion can become susceptible to developing this serious eye infection.

In general, *Acanthamoeba* has a life cycle in two phases: an active form (when the microorganism feeds and replicates) and a dormant form (when the microorganism protects itself
15 from possible attacks by turning into a cyst). Cysts are the actual target for the development of effective antiamebic drugs.

Co-infection with bacterial species is common in patients with *Acanthamoeba* keratitis. Specifically, *Staphylococcus* spp and *Pseudomonas aeruginosa* are the most common co-isolated bacterial pathogens [2,3]. In particular, for the latter microorganism an *in vivo* study suggests
20 that the presence of bacteria is an essential factor for the development and severity of *Acanthamoeba* keratitis [4].

Acanthamoeba keratitis can be extremely painful as the cornea is one of the most sensitive organs in the human body, with the highest density of pain receptors and the products used for treating *Acanthamoeba* keratitis can be quite difficult to bear and, in some cases, they can
25 also irritate the surface of the cornea. About 25% of cases of *Acanthamoeba* keratitis treated even end up with a corneal transplantation. Without treatment, the disease progresses to blindness as a result of corneal vascularization and corneal scarring or perforation.

To date, there are no drugs approved for the treatment of *Acanthamoeba* keratitis. Biguanides and diamidines have shown efficacy for *Acanthamoeba* keratitis in a number of clinical cases,
30 although the treatment regimens and concentration of these drugs have been developed empirically.

Among the different options available, treatment with polyhexamethylene biguanide (PHMB) in an ophthalmic formulation at a concentration of 0.02% alone [5] or in combination with chlorhexidine 0.02%, propamidine 0.1% or desomidine 0.1% is the most promising choice in the treatment of patients suffering from *Acanthamoeba* keratitis.

- 5 Current therapies for *Acanthamoeba* keratitis are lengthy and challenging. They start with hourly doses during the day, avoiding the night, for about 1-2 weeks, then reducing according to the response. Although every patient is different, those who have had an early diagnosis and have started receiving adequate treatment immediately, can generally expect from 3 to 6 months of therapeutic treatment.
- 10 Furthermore, the repeated and prolonged use of antibiotics and disinfectants, such as those mentioned above, risks significantly altering the composition of the conjunctival flora by increasing the percentage of pathogens such as *Staphylococcus epidermidis* to the detriment of the normal commensal flora and this can have significant clinical implications as *Staphylococcus epidermidis* is one of the main causes of eye diseases such as conjunctivitis, keratitis
- 15 and endophthalmitis [6].

PHMB is composed of repeating (n) times biguanide units connected by hexamethylene chains, which create a cationic and amphipathic structure (Figure 1).

- PHMB-based preparations are mixtures of biguanide-based polymers with a molecular weight that can vary from 400 to 8,000 amu and a variable polymerization degree wherein n
- 20 can range from 2 to 40.

- The action mechanism of PHMB has been the subject of various studies in *in vitro* experimental models. The SUV membrane is a system that mimics the chemistry of the cell membrane of the human corneal epithelium. When PHMB interacts with the double layer of the SUV membrane, the cooperation between the phospholipids increases, the water is excluded
- 25 from the double layer of acyl chains and polar heads, the region with the acyl chains becomes less dynamic and selective, and the PHMB is absorbed by the surface of the double layer [7].
- The cationic nature of PHMB allows interaction with anionic polymers, such as DNA. In literature, it has been indicated that PHMB interacts with DNA in aqueous solution forming a complex between the two species. The reaction involves the electrostatic interaction be-
- 30 tween the cationic biguanide groups and the anionic phosphate groups of the DNA deoxyribonucleotides in a molar ratio of approximately 1:1 [8].

The structure of *Acanthamoeba*, characterized by the exposure of binding sites to the DNA,

allows an easier access of the PHMB to the DNA of the protozoan, facilitating the chromatin condensation process and the destruction of the same microorganism, as already documented for other bacteria such as *Escherichia Coli*, *Salmonella enteritidis* and *Staphylococcus aureus* [9]. Although these studies are not specific for *Acanthamoeba*, they have been useful to inventors in identifying the optimal conditions in which PHMB binds to the DNA of the protozoan and exerts its best e Phase efficacy in the treatment of infectious keratitis.

In a previous clinical study of Papa V. et al. [10], it has been shown that even higher PHMB concentrations of 0.04%, 0.06%, 0.08% are safe and well-tolerated in healthy volunteers [8]. The pH and osmolarity of the formulation are not described, much less the molecular weight and the polydispersion index of the PHMB polymer used. The concentration of PHMB alone, however, is not sufficient for maximizing the interaction of PHMB with the DNA of *Acanthamoeba* and the effectiveness of the treatment as will be demonstrated hereunder.

Patent application US2007/0140897 A1 [12] describes ophthalmic formulations based on active ingredients containing biguanide (including PHMB) at a pH of 4-6 for the treatment of acanthamoeba keratitis. The only formulations exemplified however are based on alexidine and not on PHMB. In any case, the relevance of the MW and PDI of biguanide-based polymers for interaction with the DNA of the pathogens, or the specific concentrations of use of the biguanide-based polymers in combination with the above-mentioned parameters, are not described.

A stability study of the formulations based on PHMB 0.02% with pH = 4 by Bouattour et al. 2018 [13] reveals the formation of degradation products (BP) already after 90 days. The authors conclude that PHMB-based formulations cannot be stored for more than 60 days in EOS-LDPE bottles at 25°C. The authors of the present invention have now identified a polyhexamethylene biguanide-based formulation for ophthalmic administration that maintains stability and efficacy, at the highest concentrations of active ingredient ranging from 0.04% (w/v) to 0.08% (w/v) thanks to the control of the molecular weight (MW) and the polydispersion index (PDI) of the PHMB polymer, together with the pH and osmolarity.

Studies have indicated that the use of PHMB with a high molecular weight and polydispersion index together with a specific buffer system that maintains the pH of the PHMB formulation 0.04% -0.08% (w/v) within the range of 5 - 6.5 synergistically creates the optimal conditions for stability at least up to 24 months and for the activity of the active ingredient. For the reasons indicated above, in fact, the PHMB molecule must be in its protonated form

in order to exert its biocidal action mechanism at its best. In the protonated ionized form, PHMB crosses the epithelium of the cornea infected by *Acanthamoeba* and accumulates in the stroma, thanks to the electrostatic interaction with the negative charges of the proteoglycans. It is here that PHMB exerts its biocidal and cysticidal activity with an action mechanism which, according to the most recent scientific research, is expressed through a bond with the DNA through extensive interactions with the phosphate groups of the latter. This bond blocks the replication process of the DNA of the amoeba causing the death of the pathogen [11]. In this sense, the effectiveness of the above-mentioned interaction between PHMB and DNA, and therefore the activity of PHMB itself, are strongly influenced by the stability of the formulation containing the active ingredient. Furthermore, the administration of the formulation according to the invention in a precise graduated dosage regimen in patients affected by *Acanthamoeba* keratitis has proved to be particularly effective in terms of activity against the complete eradication of *Acanthamoeba* cysts.

In this sense, as the formulation object of the invention is a monotherapy, it favours the patient's compliance and adherence to the therapeutic regimen specifically because it avoids the repeated instillation of various products during the day. Furthermore, considering the selectivity and specificity of the product towards the target microorganism, a greater rapidity of action is ensured with a consequent faster resolution of the pathology, avoiding lengthy periods of treatment of 3-6 months.

Again, compared to the multi-drug therapeutic approach composed of powerful antiseptics and broad-spectrum antibiotics currently used, monotherapy (highly selective and specific) with 0.08% PHMB is certainly a safer option for preserving the diversity of ocular microbiota during treatment and therefore for avoiding the appearance of multi-resistant *Staphylococcus epidermidis* strains.

The present invention therefore relates to a liquid formulation based on polyhexamethylene biguanide or its salts at a concentration ranging from 0.04% to 0.08% comprising a buffer system for keeping the pH within the range of 5-6.5 and an isotonicizing agent for maintaining an osmolarity within the range of 270-330 mOsm/Kg, wherein the molecular weight of the polyhexamethylene biguanide ranges from 2,300 to 6,000 amu and the polydispersion index of the polymer ranges from 1.5 to 1.9, preferably from 1.7 to 1.8, suitable for ophthalmic administration for use in the treatment of *Acanthamoeba* keratitis or fungal infections.

Said fungal infections are preferably keratitis or keratomycosis mediated by a pathogen

selected from the group consisting of *Candida albicans*, *Fusarium solani*, *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus flavus*, and *Cladosporium*.

The above-mentioned fungal infections can also be present in conjunction with *Acanthamoeba* infection, in particular *Fusarium solani* and *Cladosporium* infections.

- 5 In an alternative embodiment of the invention, said treatment of infectious *Acanthamoeba* keratitis is particularly effective in the case of co-infection with *Pseudomonas aeruginosa* or *Staphylococcus epidermis*.

The therapeutic use of the formulation of 0.08% PHMB which is active against *Pseudomonas aeruginosa* (data not shown, internal APE test study: ORPHAN DRUG - *in vitro* assay -
10 1305) proves in fact to be highly performing for a rapid and effective resolution of the pathology in the cases of co-infection indicated above. The polyhexamethylene biguanide can be in the form of an inorganic or organic salt, selected from the group consisting of chloride, bromide, sulfate, phosphate, mesylate, formate, citrate or maleate.

In addition to the need for maintaining a slightly acid pH in order to ensure the maximum
15 protonation state of the PHMB, a further objective of the present invention is to maintain the osmolarity of the formulation at a value comparable to the physiological tear fluid value (which ranges from 290 to 320 mOsm/Kg), preferably by the addition of an ionic isotonizing agent, selected from the group consisting of sodium chloride, potassium chloride, or non-ionic, such as glycerol, mannitol, sorbitol; ionic buffer systems such as phosphate, citrate,
20 bicarbonate or borate buffer or non-ionic buffers such as trometamol, histidine, glycine, HEPES which also provide an osmotic contribution.

The osmotic pressure in fact mainly depends on the ions present in the aqueous layer (Na^+ , K^+ , Cl^- and HCO_3^-).

According to preferred embodiments of the present invention, the isotonizing agent is used
25 in the following concentrations: NaCl (0.01% -0.9%), KCl (0.01% -1.19%), glycerol (0.01% -2.6%), mannitol (0.01% -5.1%) and sorbitol (0.01% -5.5%).

In a particular embodiment of the liquid formulation based on polyhexamethylene biguanide, the buffer system is an ionic buffer such as a phosphate, citrate, bicarbonate or borate buffer or a non-ionic buffer such as trometamol, histidine, glycine, HEPES, or a mixed system.

- 30 The buffer system preferably comprises disodium phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$). The disodium phosphate dodecahydrate is preferably used at a concentration within the range of 0.01%-4.5% and the

monobasic sodium phosphate is used at a concentration within the range of 0.01% -3.5%.

Alternatively, a citrated phosphate buffer comprising disodium phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$) and citric acid ($\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$) or Tris/HCl or boric acid/borate, can be used.

- 5 If the formulation according to the invention is in the form of an ophthalmic gel or a viscous solution, it is preferable to use a phosphate-free buffer, such as, for example, a Tris buffer and/or boric acid or borates.

According to a preferred embodiment of the liquid formulation based on polyhexamethylene biguanide, the pH is 5.8.

- 10 In a preferred embodiment of the liquid formulation based on polyhexamethylene biguanide of the invention, the concentration of PHMB is selected from the group consisting of 0.04% (w/v), 0.05% (w/v), 0.06% (w/v), 0.07% (w/v) and 0.08% (w/v), preferably 0.08% (w/v).

The liquid formulation based on polyhexamethylene biguanide according to the invention suitable for ophthalmic administration is a sterile solution which can be in the form of eye
15 drops.

Alternatively, in the presence of a viscosifying agent (e.g. xanthan gum, gellan gum, polyvinyl alcohol, hyaluronic acid, sodium hyaluronate, cellulose derivatives, such as carboxymethyl cellulose or hydroxypropyl cellulose) the polyhexamethylene biguanide-based formulation according to the invention can be in the form of an ophthalmic gel or viscous solution.

- 20 According to a further preferred embodiment of the invention, the liquid formulation based on polyhexamethylene biguanide according to the invention further comprises a penetration promoter which can be selected from Tween 80 and benzalkonium chloride.

Again according to a preferred embodiment, the liquid formulation based on polyhexamethylene biguanide for use according to the invention can further comprise 0.02% of chlorhexidine, 0.1% of propamidine, 0.1% of desomidine in combination with PHMB. When the
25 PHMB is administered in combination with a second active agent, the administration can be effected sequentially, simultaneously or separately, i.e. in the same eye drop formulation or in separate formulations.

- The present invention further relates to the liquid formulation based on polyhexamethylene biguanide according to the invention for the treatment of *Acanthamoeba* keratitis by administering a graduated dosage of 16 drops per day for 5 days, 8 drops per day for 7 days, 6 drops
30 per day for 7 days and 4 drops per day until clinical resolution. Clinical resolution was 88%

in an average time of 4 months. This graduated dosage differs from the intensive dosage previously described in the Phase I study conducted by Papa et al. [10] as it intervenes more effectively in the eradication of cysts and therefore in the clinical resolution of *Acanthamoeba* keratitis.

5 The present invention further relates to a process for the preparation of the liquid formulation based on polyhexamethylene biguanide according to the invention which comprises the following steps:

(i) addition of the buffer system at a concentration ranging from 0.01 to 4.5% (w/v), and the isotonicizing agent at a concentration ranging from 0.01% to 5.5% (w/v), in purified water
10 under gentle and continuous stirring; optional addition of a viscosifying agent selected from sodium hyaluronate, xanthan gum, polyvinyl alcohol, carboxymethyl cellulose and hydroxypropyl cellulose;

(ii) addition of polyhexamethylene biguanide having a molecular weight within the range of 2,300-6,000 amu and a polydispersion index within the range of 1.5-1.9, preferably 1.7-
15 1.8, at a concentration ranging from 0.04% to 0.08% (w/v), under mild stirring;

(iii) bringing to volume by the addition of purified water;

(iv) sterilization by filtration or heat.

In the case of a viscous solution, the addition of a viscosifying agent selected from sodium hyaluronate, xanthan gum, polyvinyl alcohol, carboxymethyl cellulose and hydroxypropyl
20 cellulose should also be added after step (i). In this case, in the presence of non-filterable polymers, heat sterilization is used instead of sterilization by filtration.

The solution is then divided into single-dose containers or vials which are then sealed. The single-dose containers can be made of low-density polyethylene (LDPE), every package can contain 5 units of single dose.

25 In a particular embodiment of the process for preparing the liquid formulation based on polyhexamethylene biguanide, the buffer system for maintaining the pH within the range of 5-6.5 is an ionic buffer such as a phosphate, citrate, bicarbonate, Tris, borate or non-ionic buffer such as trometamol, histidine, glycine, HEPES, or a mixed system. The buffer system is preferably selected from the group consisting of disodium phosphate dodecahydrate/monobasic
30 sodium phosphate, disodium phosphate dodecahydrate/citric acid, Tris/HCl or boric acid/borate.

According to a preferred embodiment, the buffer system is based on disodium phosphate

dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$) and monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$). The disodium phosphate dodecahydrate is preferably used at a concentration within the range of 0.01% -4.5% (w/v), and monobasic sodium phosphate is used at a concentration within the range of 0.01% -3.5% (w/v).

- 5 The isotonicizing agent for keeping an osmolarity within the range of 270-330 mOsm/Kg is preferably sodium chloride.

The preparation process of the formulation was developed for also ensuring that both the initial molecular weight and the polydispersion index of the active ingredient PHMB are kept in the finished product within the range of 2,300-6,000 amu and 1.5-1.9, preferably 1.7-1.8,
10 respectively. These parameters have proved to be responsible for the effectiveness of the finished product.

Finally, the present invention relates to therapeutic eye lenses loaded or derivatized with the liquid formulation based on polyhexamethylene biguanide according to the invention for the controlled release of the active ingredient for the treatment of *Acanthamoeba* keratitis or co-
15 infections by *Pseudomonas aeruginosa*. This embodiment allows for the continuous release of the formulation for prolonged periods and with a further gain in terms of compliance and adherence to therapy on the part of the patient.

The present invention will now be described for illustrative but non-limiting purposes, according to a preferred embodiment with particular reference to the attached figures, in which:

- 20 - Figure 1 shows the structure of the repeating unit of hexamethylene biguanide in PHMB;
- Figure 2 shows a flow chart of the preparation process of the PHMB-based formulations;
- Figure 3 illustrates the chromatogram obtained by gel-permeation chromatography of the PHMB in API.
- Figure 4 illustrates the chromatogram obtained by gel-permeation chromatography of the
25 PHMB in the finished product; from a comparison with the chromatogram of Figure 2 referring to the raw material, it is evident how the production process is capable of keeping the molecular-weight distribution of the raw material unchanged, up to the finished product.
- Figure 5 shows the comparison between the stability data of the 0.08% PHMB-based formulation at pH 7.4 and pH 5.8.
30 - Figure 6 shows a comparison between the PHMB-DNA bond in relation to the molecular weight. From the trend of the graph it can be appreciated that using PHMB with a high molecular weight the polymer-DNA bond is more effective as one or more nucleic acid

molecules are involved. The high-molecular-weight polymer is capable of forming cross-bridge links between different nucleic acid molecules forming a network of bonds (line with circles). Vice versa, the line with squares reflects the establishment of a linear type bond between PHMB and DNA, in which the polymer characterized by a lower molecular weight cannot show an aptitude for forming cross-bridge bonds but establishes a bond in which only one nucleic acid molecule is involved.

- Figure 7 (A) shows the results of the stability study of 0.08% PHMB formulations using PHMB batches with different molecular weights (MW) and polydispersion indexes (PDI), expressed as % recovery (w/v) of PHMB at T0 and after 12 months (T12).

- Figure 7 (B) shows the results of the stability study of a 0.08% PHMB formulation, using a PHMB batch characterized by a MW = 2,641 amu and a PDI = 1.6. The study was carried out under three different temperature conditions: long term stability 25°C, RH 40%; intermediate stability 30°C, RH 65%; accelerated stability 40°C RH 25%. Stability results expressed as % recovery (w/v) of PHMB at T0 and after 3 months (T3) are indicated.

The following non-limiting examples are now provided for a better illustration of the invention, wherein various PHMB-based formulations were tested and compared, evaluating the impact on the stability of some formulation parameters such as pH, osmolarity, molecular weight and polydispersion index of the active ingredient PHMB.

EXAMPLE 1: Preparation process of PHMB-based formulations

The process for preparing the formulation according to the invention is illustrated in the diagram of Figure 2.

Disodium phosphate dodecahydrate ($\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$), monobasic sodium phosphate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) and sodium chloride are added in succession and dissolved in a volume of purified water under continuous gentle stirring.

The stirring step is critical for maintaining the initial molecular weight and the polydispersion index of PHMB and must be effected avoiding the formation of foam or vortexes in the solution. A stir bar or wheel to be immersed in the solution for stirring is preferably used. Finally, PHMB (0.08%) is added.

The solution is brought to volume (100 ml) by adding a sufficient quantity of purified water and under continuous stirring. The pH and appearance of the solution are then controlled.

Sterilization is effected by filtration through a 0.2 μM filter. The nature of the filter membrane and the dynamics (pressure, flow) are selected so as to prevent the product from being forced

through the membrane to avoid depolymerization or breakage of the polymer chain.

After filtration, the integrity of the 0.2 µm filter is controlled by means of an electronic control system.

Finally, the sterile solution is used for filling single-dose containers using the Blow/Fill/Seal

5 (B/F/S) technology under aseptic conditions.

This automated technology allows a vial to be filled and sealed, under aseptic conditions with solution volumes ranging from 0.1 to 1,000 cm³.

The PHMB solution appears as a clear colourless or pale yellow liquid.

At the end of the production process, the characterization of the finished product in its main

10 components is effected, including any impurities with suitable chromatographic methods.

The comparison between the PHMB chromatogram in the API (Figure 3) and the PHMB chromatogram following the preparation process of the formulation (Figure 4), shows that the molecular-weight distribution of the PHMB is unaltered.

EXAMPLE 2: *Comparison studies on the stability of PHMB-based formulations*

15 The PHMB-based formulations produced are sterile solutions whose relevant physicochemical parameters are pH and osmolarity.

As detailed, the choice of a slightly acidic pH is governed by the necessity of obtaining a stable formulation that cannot be obtained at a pH around neutrality.

For this purpose, various formulations based on PHMB 0.08% at different pH values (5.8,

20 6.2, 7.4) were developed, subsequently verifying their stability at 25°C, 40°C and 60°C.

For the preparation of the formulation based on PHMB 0.08%, pH 5.8 the following ingredients were weighed:

25

Table 1

Ingredient	Function	% (w/v)
NaH ₂ PO ₄ · H ₂ O	buffer	0.827 g
Na ₂ HPO ₄ · 12 H ₂ O	buffer	0.240 g
NaCl	isotonizing agent	0.520 g
PHMB	active ingredient	0.08 g

Sterile water	solvent	complement to 100 ml
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Table 2 hereunder indicates the physico-chemical characteristics of the final formulation at pH 5.8 and during the phases of the preparation process of the same.

Table 2

	pH	Osmolarity (mOsm/Kg)	Active UV spectrum	Recovery (UV quantitative)
Buffer solution (Before adding PHMB)	-	289	-	-
Buffer with PHMB (before sterilization)	5.8	290	corresponds	-
Formulation sterilized by filtration	5.8	297	corresponds	100.4

5

For the preparation of the formulation based on PHMB 0.08%, pH 6.2 the following ingredients were weighed following the same process described above:

Table 3

Ingredient	Function	% (w/v)
NaH ₂ PO ₄ · H ₂ O	buffer	0.735 g
Na ₂ HPO ₄ · 12 H ₂ O	buffer	0.480 g
NaCl	isotonizing agent	0.510 g
PHMB	active ingredient	0.08 g
Sterile water	solvent	complement to 100 ml

10 Table 4 hereunder indicates the physico-chemical characteristics of the final formulation at pH 6.2 and during the phases of the preparation process of the same.

Table 4

	pH	Osmolarity (mOsm/Kg)	Active UV spectrum	Recovery (UV quanti- tative)
Buffer solution (Before adding PHMB)	6.05	291	-	-
Buffer with PHMB (before sterilization)	6.07	295	corresponds	-
Sterilized formulation by filtration	6.20	287	corresponds	100.9

For the preparation of the formulation based on 0.08% PHMB, pH 7.4, the following ingredients were weighed:

5

Table 5

Ingredient	Function	% (w/v)
NaH ₂ PO ₄ · H ₂ O	buffer	1.91 g
Na ₂ HPO ₄ · 12 H ₂ O	buffer	0.19 g
NaCl	isotonizing agent	0.44 g
PHMB	active ingredient	0.08 g
Sterile water	solvent	comple- ment to 100 ml

Table 6 hereunder indicates the physico-chemical characteristics of the final formulation at pH 7.4 and during the phases of the preparation process of the same.

Table 6

	pH	Osmolarity (mOsm/Kg)	Active UV spectrum
Buffer solution (Before adding PHMB)	-	-	-
Buffer with PHMB	7.45	286	corresponds

(before sterilization)			
Formulation sterilized by filtration	7.45	286	corresponds
Formulation sterilized in an auto-clave	7.33	295	corresponds
Filtered formulation, after 2 weeks at 25°C	7.36	293	corresponds

Comparisons were then made on the stability of 0.08% PHMB-based formulations in a single dose container at a pH of 5.8 and 7.4 at 25°C (long term), 40°C (accelerated) and 60°C (stress conditions).

5 The results of the stability analyses are indicated hereunder:

• Stability data:

- 0.08% PHMB formulation, pH 5.8 at 25°C $\pm$ 2°C and relative humidity 40% $\pm$ 5 (long term)

Table 7

Analyses	Limits			Weeks						
	Meas. Unit	Min	Max	0	13	26	39	52	65	104
Appearance	Transparent, free of foreign particles			corr.*	corr.	corr.	corr.	corr.	corr.	corr.
Colour	Colourless			corr.	corr.	corr.	corr.	corr.	corr.	corr.
pH		5.3	6.3	5.7	5.6	5.8	5.8	5.8	5.8	5.8
Osmolarity	mOsm/Kg	270	330	290	274	281	276	293	277	276
Biguanide (HPLC)	Corresponds to standard			corr.	corr.	corr.	corr.	corr.	corr.	corr.
Biguanide test (UV)	% L.S.	90	110	97.6	97.8	99.7	96.2	98.8	92.5	94.6
Impurity A	% a.s.		5.0	0.8	1.0	1.0	1.8	0.8	1.0	0.9
Impurities	% a.s.		5.0	0.9	0.9	1.2	2.4	1.1	1.4	1.1

B+ C										
Water loss	%		5.0		0.0	0.1	0.1	0.1	0.1	0.2
Sterility	According to European pharmacopoeia			ster-ile						ster-ile

*corr.: corresponding

- 0.08% PHMB formulation, pH 5.8 at 40°C ± 2° C and relative humidity 25% (accelerated)

Table 8

Analysis	Limits			Weeks		
	Meas. Unit	Min.	Max.	0	13	26
Appearance	Transparent, free of foreign particles			corr.	corr.	corr.
Colour	Colourless			corr.	corr.	corr.
pH		5.3	6.3	5.7	5.6	5.8
Osmolarity	mOsm/Kg	270	330	290	274	276
Biguanide (HPLC)	Corresponding to the standard			corr.		corr.
Biguanide test (UV)	% L.S.	90	110	97.6	96.7	99.2
Impurity A	% a.s.		5.0	0.8	1.0	0.9
Impur. B+ C	% a.s.		5.0	0.9	1.0	1.3
Water loss	%		5.0		0.1	0.2
Sterility	According to European Pharmacopoeia			sterile		

*corr.: corresponding

- 5 0.08% PHMB formulation, pH 7.4 at 60°C ± 2°C and relative humidity 20% (stress conditions)

Table 9

Analysis	Limits			Weeks		
	Meas.units	Min.	Max.	0	1	2
Appearance	Transparent, free of foreign particles			corr.	corr.	corr.
Colour	Colourless			corr.	corr.	corr.
pH		7.0	7.8	7.3	7.2	7.3

Osmolarity	mOsm/Kg	270	330	276	294	299
Biguanide (ABS ratio 235/220nm)				1.5	1.4	1.3
Sterility	According to European Pharmacopoeia			sterile		sterile

*corr.: corresponding

PHMB 0.08% formulation, pH 5.8 at 60°C ± 2°C and relative humidity 20% (stress conditions)

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Table 10

Analysis	Limits			Weeks		
	Meas.units	Min.	Max.	0	1	2
Appearance	Transparent, free of foreign particles			corr.	corr.	corr.
Colour	Colourless			corr.	corr.	corr.
pH		5.3	6.3	5.8	5.6	5.7
Osmolarity	mOsm/Kg	270	330	289	296	302
Biguanide (ABS ratio 235/220nm)				1.4	1.5	1.5
Sterility	According to European Pharmacopoeia			sterile		sterile

*corr.: corresponding

The results shown in Figure 5 show that the stability of the PHMB-based formulation obtained by maintaining a pH within the range of 5-6.5 is not achieved with a pH around neutral (pH 7.4).

10 EXAMPLE 3: Study of the PHMB-DNA bond

A study on the PHMB-DNA bond was carried out in order to evaluate the equivalence between low-molecular-weight PHMB and high-molecular-weight PHMB.

The binding capacity of PHMB to DNA can be considered as being independent of the specific type of DNA studied as indicated in literature [8], whereas the binding capacity and effectiveness to DNA depend on the molecular weight and polydispersion index of the

15

PHMB polymer. When the polymer has a molecular weight ranging from 2,300 to 6,000 amu and a polydispersion index within the range of 1.5-1.9, the binding with DNA is more effective, probably because the length of the polymer is more suitable for the formation of cross-bridge bonds involving multiple nucleic acid molecules according to the mechanism proposed by Allen et al. [8]. Vice versa, when the polymer is characterized by a low molecular weight the bond will be linear and will involve a single nucleic acid molecule.

A comparative study was then conducted with different PHMB polymer samples in order to evaluate the binding capacity to nucleic acid molecules. The PHMB (sample A) was degraded by acid and high-temperature treatment for 5 hours to obtain low-molecular-weight PHMB polymers. The PHMB (sample B) did not receive any type of treatment and is characterized by a molecular weight that falls within the range claimed.

The capacity of both samples of binding nucleic acid molecules was then evaluated. The experimental method proposed herein is based on the interaction between an aqueous solution of PHMB and an aqueous solution of DNA. The quantity of the PHMB-DNA complex was measured as the difference between the initial quantity of both species in solution and the residual quantity after the end of the reaction. The quantity of both species in solution is monitored by spectrophotometric measurements at 2 different wavelengths, 260 nm for DNA and 235 nm for PHMB, respectively.

In conclusion, the data show an increased capacity of high-molecular-weight PHMB in interacting with DNA through the formation of cross-bridge bonds with multiple nucleic acid molecules and therefore a consequent greater efficacy in the treatment of infectious *Acanthamoeba* keratitis or of co-infections from *Pseudomonas aeruginosa*. The type of bond between the PHMB polymer and DNA that is created is much more effective, as shown in the graph in Figure 6.

EXAMPLE 4: Stability study in relation to the molecular weight and the polydispersion index of PHMB

A comparative study was carried out on three different PHMB-based starting batches characterized by different molecular weights and polydispersion indexes (PDI):

- batch L-17GR185627, MW = 2517 amu, PDI = 1.73,
- batch F-693/LU/101, MW = 1170 amu, PDI = 1.93;
- batch F-693/LU/118, MW = 440 amu, PDI = 2.14.

For each lot a 0.08% PHMB formulation was prepared characterized by pH = 5.8, osmolarity

0.280 osmol/Kg.

The stability of the PHMB formulation was then evaluated at T0 and after 12 months (T = 12) at a temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, RH $40 \pm 5\%$. The parameters considered are the following:

- 5 - Appearance
- Recovery% (w/v) of the active ingredient

The results are indicated in the following Table 11 and in the graph of Figure 7A.

Table 11

Batch	MW (Da)	PDI	Test	Months	
				T= 0	T= 12
L-17GR185627	2517	1.73	Appearance	Acceptable	Acceptable
			Recovery % PHMB (UV)	101.5	97.1
F-693/LU/101	1170	1.93	Appearance	Acceptable	Not acceptable
			Recovery % PHMB (UV)	100	35.8
F-693/LU/118	440	2.14	Appearance	Acceptable	Non acceptable
			Recovery % PHMB (UV)	100	12.2

- 10 The stability study confirms that the most stable 0.08% PHMB formulation is that characterized by the PHMB with the highest molecular weight, i.e. batch L-17GR185627, MW = 2517 amu, pH = 5.8 and with a PDI value of 1.73. No significant degradation products (BP) were observed throughout the duration of the stability study.

0.08% PHMB solutions characterized by a low molecular weight (batch F-693/LU/101, MW = 1170 amu - batch F-693/LU/118, MW = 440 amu) indicate a reduced recovery % of PHMB after 12 months and therefore instability of the formulation and reduced effectiveness.

It is therefore evident that the parameters of the molecular weight and polydispersion index of PHMB together with the pH of the solution contribute synergistically to providing a stable and effective formulation.

- 20 Furthermore, a stability study was carried out on 0.08% PHMB solutions, at pH 5.8 and osmolality 0.290 osmol/Kg, characterized by a MW = 2641 amu and a PDI = 1.6. The study was carried out under three different temperatures conditions: Long term stability 25°C , RH 40%; Intermediate stability 30°C , RH 65%; Accelerated stability 40°C RH 25%. The stability

results are indicated hereunder, and in Figure 7 (B):

Table 12

Conditions	Test	Months	
		T= 0	T= 3
25°C, RH 40%	Appearance	Acceptable	Acceptable
	Recovery % PHMB (UV)	103.7	103.1
30°C, RH 65%;	Appearance	Acceptable	Acceptable
	Recovery % PHMB (UV)	103.7	101.7
40°C RH 25%	Appearance	Acceptable	Acceptable
	Recovery % PHMB (UV)	103.7	105.3

- 5 The results of the stability tests show that the product is stable under all the temperature conditions investigated, as the recovery % of PHMB falls within the predetermined specifications limit , 90 - 110% (w/v). The other chemical-physical parameters investigated (data not shown), such as pH, osmolality and recovery assay of the impurities, remain stable and within the specified specification limits, under all of the three climatic conditions tested.
- 10 EXAMPLE 5: Clinical study
- A randomized, multicentre, double-blind, parallel-group Phase 3 study is described in detail hereunder, for evaluating the efficacy, safety and tolerability of the 0.08% PHMB formulation of the invention compared to a conventional combination therapy 0.02% PHMB + 0.1% propamidine in male and female adult subjects suffering from *Acanthamoeba* keratitis.
- 15 The study is conceived as a superiority study in accordance with EMA requirements (CPMP/EWP/482/99). The study comprises a screening visit for inclusion, a treatment period that includes short outpatient visits and follow-up visits.
- A total of 130 subjects with *Acanthamoeba* keratitis were assigned to the following two treatment groups in a 1:1 ratio.
- 20 Group 1: 0.08% PHMB + placebo

Group 2: 0.02% PHMB + 0.1% combination therapy.

Patients:

The study was conducted in male and female subjects suffering from *Acanthamoeba* keratitis, ≥ 12 years of age, inclusive.

5 **Objective:**

The primary objective of the study is the comparison of the Clinical Resolution Rate (CRR) 12 months after randomization (CRR₁₂) between the 0.08% PHMB + placebo formulation and the combination therapy 0.02% PHMB + 0.1% propamidine, evaluating the difference between CRR₁₂ in relation to the degree of uncertainty, and testing the therapeutic superiority or non-inferiority of monotherapy with 0.08% PHMB

10

A further objective of the study is to obtain information on the safety of the ophthalmic formulation based on 0.08% PHMB according to the invention.

Hypothesis:

The primary hypothesis to be tested is that the CRR₁₂ of the subjects treated with monotherapy with the 0.08% PHMB formulation is higher or not inferior, lower by a still acceptable margin (Δ), with respect to the CRR₁₂ of the combination therapy 0.02% PHMB + 0.1% propamidine, administered according to the treatment protocol indicated hereunder.

15

The secondary hypotheses are:

- the undesirable effects relating to toxicity are lower with the 0.08% PHMB monotherapy compared to the combination therapy;
- *time to cure* is lower in subjects receiving the 0.08% PHMB monotherapy compared to the combination therapy.

20

The clinical resolution obtained within 12 months of starting treatment is 67%, as indicated in the sponsor's retrospective study (Study 038/SI).

25 **Concurrent and previous treatments**

The subjects are authorized to take or have taken the following drugs:

Antibiotics: Topical moxifloxacin is allowed for the treatment of concomitant bacterial infections. It is not permitted however as a prophylactic antibiotic for the treatment of patients with corneal ulcers; PHMB is already a broad-spectrum antibacterial and an additional antibacterial is not needed. Topical moxifloxacin is not permitted for use as a prophylactic antibiotic in patients with corneal ulcers; PHMB is a good broad-spectrum anti-bacterial and an additional antibacterial is not needed for this.

30

Antiviral and antifungal drugs: The use of these drugs is not allowed during the study. Any intake of these drugs at the onset of the study must be discontinued.

Anti-inflammatory drugs: For subjects under steroid treatment at the onset of the study (who are already taking local steroids, e.g. for a misdiagnosis of HSV keratitis or as an adjuvant treatment for bacterial keratitis) the following options exist:

- a. Interruption, maintenance or reduction of steroid doses. Dexamethasone (0.1% or 0.15%) is the only local steroid allowed in this trial. Patients using other topical steroids at the time of the start of the study must change the frequency of administration. Diclofenac is the only oral NSAID allowed in the trial and is added in the appropriate doses (75 mg - 150 mg per day, divided into 2-3 doses).
 - b. Subjects using NSAIDs or cyclosporine at the time of the start of the clinical study must stop treatment after randomization.
 - c. Subjects who are not using local steroids at the time of entry into the study can start them together with oral NSAIDs (recommended diclofenac; 75 mg - 150 mg per day, divided into 2-3 doses) during the study as specified in the protocol.
- Other topical therapeutic treatments permitted: lubricants, mydriatics (cyclopentolate, homatropine or atropine) and anti-glaucoma drugs.

Dosage

The dosage applied in the clinical efficacy study is as follows:

- administration of a graduated dosage of 16 drops per day for 5 days, 8 drops per day for 7 days, 6 drops per day for 7 days and 4 drops per day until clinical resolution.

Treatment assignment:

- Protocol for bilateral pathology: if both eyes are affected only one of the two (the right unless there is a difference in severity such as to require treatment of the more compromised eye) will be treated according to the dosage of the treatment and considered for the study. The other eye will be treated according to standard clinical practice.

Table 13 below summarizes the demographics and distribution of patients participating in the study.

Table 13

	PHMB 0.08% + placebo	PHMB 0.02% + 0.1% Pro- pamidine	Total
Screened	69	66	135
Screening failures	0	0	0
Randomized	69	66	135
Did not receive the study drug ¹	0	1 (1.5%)	1 (0.7%)
Safety analysis set ¹	69 (100%)	65 (98.5%)	134 (99.3%)
Study completion ²	58 (84.1%)	57 (87.7%)	115 (85.8%)
Premature withdrawals from the study ²	11 (15.9%)	7 (10.8%)	18 (13.4%)
did not receive the study drug	0	1 (100.0%)	1 (100.0%)
Full analysis set ¹	66 (95.7%)	61 (92.4%)	127 (94.1%)
Study completion ²	56 (84.8%)	53 (86.9%)	109 (85.8%)
Premature withdrawals from the study ²	10 (15.2%)	7 (11.5%)	17 (13.4%)
DX not confirmed	3 (100%)	4 (80%)	7 (87.5%)
Absence of primary efficacy evaluation	0	1 (20%)	1 (12.5%)
Protocol analysis set ¹	62 (89.9%)	57 (86.4%)	119 (88.1%)
Study completion ²	54 (87.1%)	50 (87.7%)	104 (87.4%)
Premature withdrawals from the study ²	8 (12.9%)	6 (10.5%)	14 (11.8%)
Maximum PD	4 (57.1%)	4 (44.4%)	8 (50%)
Not FAS	3 (42.9%)	5 (55.6%)	8 (50%)
¹ Percentages based on the number of randomized patients within each treatment group			
² Percentages based on the number of subjects included in the analysis set			

Table 14 shows the 12-month clinical resolution rate. Patients are divided into patients with and without prior steroid treatment.

Table 14

			PHMB		
			0.08%	PHMB	
			PHMB	+ 0.02% +	0.1%
			placebo	Propamidine	Total
Clinical resolution rate 12 months			(N=66)	(N=61)	(N=127)
5	Prior steroid-treated patients*	Unresolved	7 (22.6%)	2 (10%)	9 (17.6%)
		Resolution	24 (77.4%)	18 (90%)	42 (82.4%)
10	Patients without prior steroid treatment	Unresolved	3 (8.6%)	5 (12.2%)	8 (10.5%)
		Resolution	32 (91.4%)	36 (87.8%)	68 (89.5%)

* Any use of any of these drugs prior to therapy:

Corticosteroids and anti-infective drugs in combination, corticosteroids

The percentages are based on the number of subjects within each subgroup and treatment

15 Results:

Primary efficacy: 12-month clinical resolution rate

The results of the clinical study reveal the non-inferiority of 0.08% PHMB monotherapy compared to the combination therapy 0.02% PHMB + Propamidine 0.1% in terms of clinical resolution rate at 12 months (CRR₁₂)

20 * CRR₁₂ for 0.08% PHMB monotherapy is equal to 87.1%

* CRR₁₂ for 0.02% PHMB + Propamidine 0.1% is equal to 89.5%

Monotherapy is always preferred to combination therapy and the results do not show any statistically significant differences.

25 The percentage of subjects previously treated with corticosteroid-based therapies are indicated hereunder:

- 22.6% for 0.08% PHMB monotherapy

- 10% for 0.02% PHMB + Propamidine 0.1% combination therapy

The CRR₁₂ clinical resolution rate for patients with and without prior steroid treatment are indicated hereunder:

* CRR₁₂ for monotherapy 0.08% PHMB is equal to 91.4%

5 * CRR₁₂ for 0.02% PHMB + Propamidine 0.1% is equal to 87.8%

The CRR₁₂ obtained with monotherapy is in both cases unexpected, as the combination therapy followed in normal clinical practice provides a CRR₁₂ of approximately 63-67%.

The clinical study therefore not only demonstrates that monotherapy under the formulation conditions described (MW, PDI and PHMB polymer concentration 0.04% -0.008%, pH and

10 osmolality) is equivalent to the combination therapy, but in both cases the results obtained are better than those previously described in clinical practice (retrospective study 083/SI).

Secondary efficacy: cure time

The cure times associated with the treatment are:

* 0.08% PHMB group: 138.3 days* (range 32-365 days)

15 * 0.02% PHMB group + Propamidine 0.1%: 117.1* days
(range 54-214 days)

*average value

0.08% PHMB shows a cure time within a wider range. A longer cure time is generally associated with a monotherapy treatment with respect to a combination therapy, but unexpectedly

20 the lower limit of the cure time range for monotherapy shows a resolution at 32 days. The results do not show any statistical difference.

Table 15 shows the results for the time-to-care of patients who have been treated

25

30

Table 15

Treatment time (days)	PHMB 0.08%+ (N=56)	PHMB 0.02% + 0,1% Propami- dine (N=54)
Visit at the end of the study		
n/nmiss	56/0	54/0
Average (DS)	138.3 (67,3)	117.1 (47)
Mean	125	112
Q1, Q3	93. 152	85. 151
Min, Max	32. 365	54. 214
p-value for the difference between treatment groups ¹	0.0934	
n/nmiss = number of subjects with evaluated/missing data		
Q1 = first quartile, Q3 = third quartile, SD = standard deviation		
¹ Mann Whitney U test.		

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CLAIMS

1. A liquid formulation based on polyhexamethylene biguanide comprising polyhexamethylene biguanide at a concentration within the range of 0,04%-0,08% (w/v), a buffer system for keeping the pH within the range of 5-6,5, an isotonizing agent for keeping the osmolarity within the range of 270-330 mOsm/Kg, characterized by a molecular weight of polyhexamethylene biguanide within the range of 2,300-6,000 amu and a polydispersion index of the polymer within the range of 1.5-1.9, suitable for ophthalmic administration for use in the treatment of *Acanthamoeba* keratitis and/or fungal infections.
2. The liquid formulation based on polyhexamethylene biguanide for use according to claim 1, for the treatment of co-infections by *Acanthamoeba* and *Pseudomonas aeruginos* or *Staphylococcus epidermis*.
3. The liquid formulation based on polyhexamethylene biguanide for use according to claims 1-2, wherein the buffer system is selected from the group comprising phosphate, citrate, bicarbonate, borate, Tris, glycerol, mannitol, sorbitol, trometamol, histidine, glycine, HEPES buffer, or a mixed system.
4. The liquid formulation based on polyhexamethylene biguanide for use according to claim 3, wherein said buffer system is selected from the group consisting of disodium phosphate dodecahydrate/monobasic sodium phosphate, disodium phosphate dodecahydrate/citric acid, Tris/HCl or boric acid/borate.
5. The liquid formulation based on polyhexamethylene biguanide for use according to any of claims 1-4, wherein the isotonizing agent is sodium chloride or potassium chloride.
6. The liquid formulation based on polyhexamethylene biguanide for use according to any of claims 1-5, characterized by a polydispersity index of the polymer within the range of 1.7-1.8.
7. The liquid formulation based on polyhexamethylene biguanide for use according to any of claims 1-6, having a pH of 5.8.
8. The liquid formulation based on polyhexamethylene biguanide for use according to any of claims 1-7, in the form of a collyrium, eye drops, ophthalmic gel or viscose solutions.
9. The liquid formulation based on polyhexamethylene biguanide for use according to claim 8, in the form of an ophthalmic gel or a viscose solution, further comprising a viscosifying agent selected from the group consisting of Xanthan gum, gellan gum, polyvinyl alcohol, hyaluronic acid, sodium hyaluronate, carboxymethylcellulose and

hydroxypropylcellulose.

10. The liquid formulation based on polyhexamethylene biguanide for use according to any of claims 1-9, further comprising a penetration enhancer selected from Tween 80 and benzalkonium chloride.

5 11. The liquid formulation based on polyhexamethylene biguanide for use according to any of claims 1-10, further comprising 0.02% chlorhexidine, 0.1% propamidine or 0.1% desomidine.

12. The liquid formulation based on polyhexamethylene biguanide for use according to any of claims 1-11, by ophthalmic administration of a graduated dosage of 16 drops/day for
10 5 days, 8 drops/day for 7 days, 6 drops/day for 7 days and 4 drops/day until clinical resolution.

13. A process for the preparation of a liquid formulation based on polyhexamethylene biguanide according to any of claims 1-12, comprising the following steps:

(i) addition of the buffer system at a concentration ranging from 0,01% to 4,5% (w/v) and
15 of the isotonicizing agent at a concentration ranging from 0,01% and 5,5% (w/v) in purified water under gentle and continuous stirring; optional addition of a viscosifying agent selected from sodium hyaluronate, xanthan gum, polyvinyl alcohol, carboxymethylcellulose and hydroxypropylcellulose;

(ii) addition of polyhexamethylene biguanide having a molecular weight within the range of
20 2,300-6,000 amu and a polydispersion index within the range of 1.5-1.9, preferably 1.7-1.8, at a concentration within the range of 0.04%-0.08% (w/v) under gentle stirring;

(iii) bringing to volume by the addition of purified water;

(iv) sterilization by filtration or heat.

14. The process for the preparation of a liquid formulation based on polyhexamethylene
25 biguanide according to claim 13, wherein said buffer system is selected from the group comprising phosphate, citrate, bicarbonate, borate, Tris, glycerol, mannitol, sorbitol, trometamol, histidine, glycine, HEPES buffer, or a mixed system.

15. The process for the preparation of a liquid formulation based on polyhexamethylene biguanide according to claim 14, wherein said buffer system is selected from the group consisting of disodium phosphate dodecahydrate/monobasic sodium phosphate, disodium phosphate dodecahydrate/citric acid, Tris/HCl or boric acid/borate.
30

16. The process for the preparation of a liquid formulation based on polyhexamethylene

biguanide according to any of claims 13-15, wherein the isotonizing agent is sodium chloride or potassium chloride.

17. An eye lens loaded with the liquid formulation based on polyhexamethylene biguanide according to any of claims 1-11, for the controlled release of the active ingredient for
5 use in the treatment of *Acanthamoeba* keratitis, co-infections by *Acanthamoeba* and *Pseudomonas aeruginosa* or fungal infections.