



## **CLINICAL TRIAL PROTOCOL**

**Aqueous humour concentrations after topical application of combined levofloxacin-dexamethasone eye drops and of its single components: a randomized, assessor-blinded, parallel-group study in patients undergoing cataract surgery - iPERME**

**PROTOCOL NUMBER: LevoDesa\_05-2017**

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OF NTC S.r.l.**

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## STATEMENT OF COMPLIANCE

The trial will be conducted in accordance with International Conference on Harmonisation Good Clinical Practice (ICH GCP) and all applicable laws and regulations. The Principal Investigator will assure that no deviation from, or changes to the protocol will take place without prior agreement from the sponsor and documented approval from the Independent Ethics Committee (IEC) and/or Competent Authority, except where necessary to eliminate an immediate hazard(s) to the trial participants. All personnel involved in the conduct of this study have completed ICH GCP Training.

The protocol, informed consent form(s), and all participant materials will be submitted to the IEC for review and approval. Approval of both the protocol and the consent form must be obtained before any participant is enrolled. Any substantial amendment to the protocol will require review and approval by the IEC before the changes are implemented. All changes to the consent form will be IEC approved; a determination will be made regarding whether a new consent needs to be obtained from participants who provided consent, using a previously approved consent form.



## PROTOCOL APPROVERS

### CHAIRMAN:

Prof. Marco Nardi

U.O. Oculistica Universitaria

Azienda Ospedaliera Universitaria Pisana

Presidio Ospedaliero di Cisanello

Pisa

\_\_\_\_\_  
*signature*

\_\_\_\_\_  
*date*

### SPONSOR:

Dr. Federico Bertocchi

*Head of R&D*

NTC – Novelty Technology Care

\_\_\_\_\_  
*signature*

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*date*

### CONTRACT RESEARCH ORGANIZATION:

Dr. Chiara Costantini

*Scientific Director*

OPIS s.r.l.

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*date*

Dr. Laura Ambrosoli

*Medical Director*

OPIS s.r.l.

\_\_\_\_\_  
*signature*

\_\_\_\_\_  
*date*

## INVESTIGATOR APPROVAL SIGNATURES:

### Clinical Study Protocol: LevoDesa\_05-2017

#### Investigator signature

I have read the protocol and agree to conduct this trial in accordance with all stipulations of the protocol, with applicable laws and regulations and in accordance with the ethical principles outlined in the Declaration of Helsinki.

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Investigator

---

Signature

---

Date

Center name and address:

# 1 PROTOCOL SUMMARY

## 1.1 SYNOPSIS

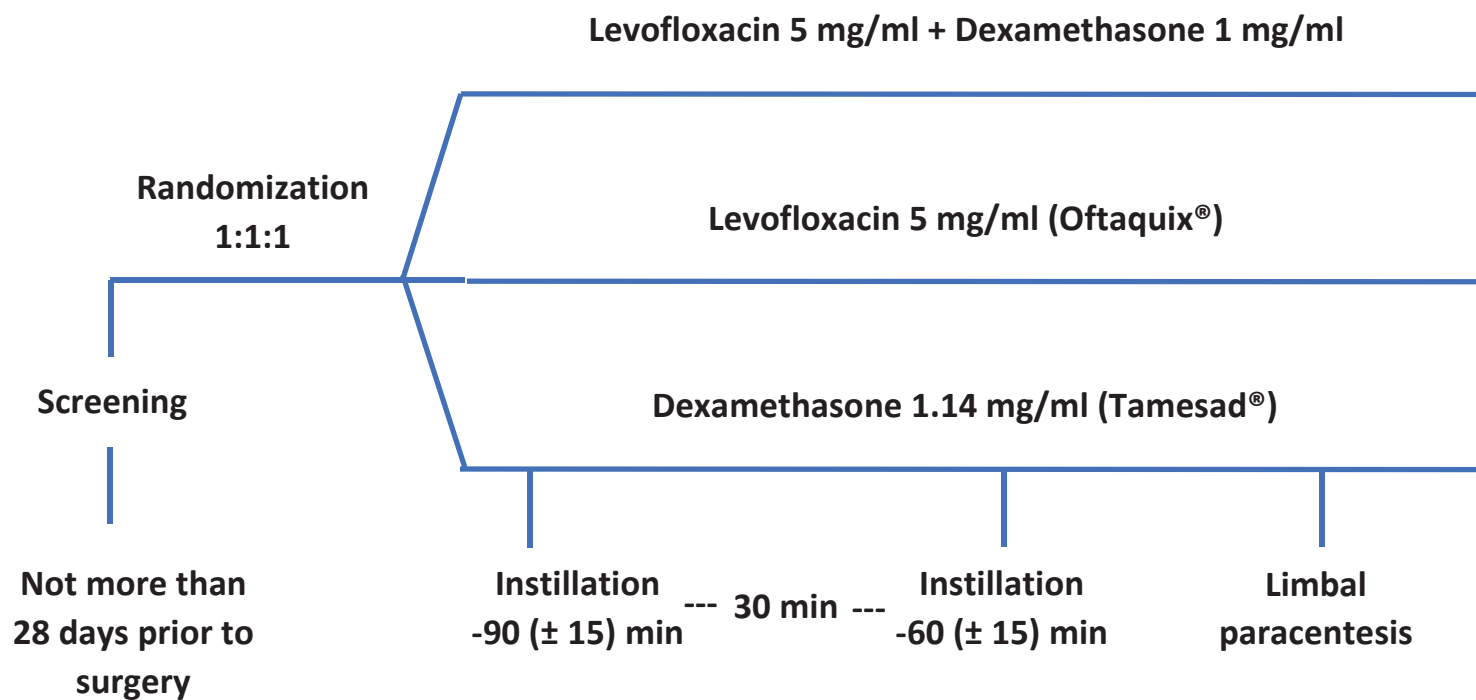
<b>Protocol number</b>	LevoDesa_05-2017
<b>EudraCT number</b>	2018-001149-15
<b>Title of the Study</b>	Aqueous humour concentrations after topical application of combined levofloxacin-dexamethasone eye drops and of its single components: a randomized, assessor-blinded, parallel-group study in patients undergoing cataract surgery - iPERME
<b>Phase</b>	II
<b>Sponsor</b>	NTC S.r.l.
<b>Investigational Sites</b>	<ul style="list-style-type: none"> <li>• Prof. Marco Nardi: U.O. Oculistica Universitaria – Azienda Ospedaliera Universitaria Pisana – Presidio Ospedaliero di Cisanello – Pisa</li> <li>• Prof. Luca Rossetti: Clinica Oculistica – Presidio Ospedale San Paolo – Milan</li> </ul>
<b>Study Objective</b>	To evaluate the penetration of levofloxacin and dexamethasone 21-phosphate into the aqueous humour after ocular administration in combination or as single active ingredients.
<b>Study Endpoint</b>	Aqueous humour concentration of levofloxacin, dexamethasone 21-phosphate and dexamethasone.
<b>Type of Study</b>	This is a randomized, assessor-blinded, parallel-group study.
<b>Number of Patients</b>	The study will enrol 120 patients scheduled for cataract surgery.
<b>Inclusion Criteria</b>	<ol style="list-style-type: none"> <li>1. Written informed consent must be obtained before any assessment is performed</li> <li>2. Male or female patients, aged <math>\geq 40</math> years</li> <li>3. Patient undergoing phacoemulsification</li> <li>4. Corneal thickness between 450 <math>\mu\text{m}</math> and 600 <math>\mu\text{m}</math> as measured by means of pachymetry</li> </ol>

	<ol style="list-style-type: none"> <li>5. Corneal integrity confirmed by means of fluorescein test (Oxford scheme grade 0)</li> <li>6. Adequate pupil dilation assessed at screening</li> <li>7. Female patients of childbearing potential must have a negative pregnancy test</li> <li>8. Ability to fully understand all study procedures</li> </ol>
<b>Exclusion Criteria</b>	<ol style="list-style-type: none"> <li>1. Corneal epithelium integrity not confirmed by fluorescein test</li> <li>2. History of corneal disease or dystrophy (e.g. ocular herpes, conjunctivitis, keratitis)</li> <li>3. History of ocular trauma with corneal damage</li> <li>4. History of acute ocular inflammation (including uveitis) in the 6 months prior to screening</li> <li>5. Previous ocular surgery (including laser treatment)</li> <li>6. Glaucoma</li> <li>7. Treatment with an ophthalmic investigational drug in the 3 months prior to screening</li> <li>8. Treatment with any topical ocular drug within 12 hours before start of cataract surgery other than study drugs and instillation of topical anaesthetic (oxybuprocaine hydrochloride - Novesina®) followed by povidone-iodine (Oftasteril®) within 10 minutes before start of surgery</li> <li>9. Treatment with any topical steroid or antibiotic drug in the 7 days prior to cataract surgery (artificial tears without BAK are allowed)</li> <li>10. Treatment with any systemic steroid or antibiotic drug in the 7 days prior to cataract surgery</li> <li>11. Known hypersensitivity to levofloxacin or other fluoroquinolones and/or dexamethasone or other steroids</li> <li>12. Pregnant or lactating women</li> <li>13. Patients who have received any investigational drug during the preceding 30 days or 5 times the plasma half-life, whichever is longer, or who have previously participated in this trial</li> </ol>
<b>Study Drugs</b>	<ul style="list-style-type: none"> <li>• Levofloxacin hemihydrate 5.12 mg/ml, corresponding to 5 mg/ml levofloxacin + dexamethasone 21-phosphate 1.32 mg/ml, corresponding to dexamethasone 1 mg/ml: two 30 µl doses 30 minutes apart.</li> <li>• Levofloxacin hemihydrate 5.12 mg/ml (Oftaquix®), corresponding to 5 mg/ml levofloxacin: two 30 µl doses 30 minutes apart.</li> <li>• Dexamethasone 21-phosphate 1,50 mg/ml (Tamesad®), corresponding to dexamethasone 1.14 mg/ml: two 26 µl doses 30 minutes apart.</li> </ul>

<p><b>Study Procedures</b></p>	<p>After providing informed consent, patients scheduled for cataract surgery will undergo screening assessments to verify eligibility for the study. Screening must not take place more than 28 days prior to surgery.</p> <p>Eligible patients will be randomly assigned to levofloxacin 5 mg/ml + dexamethasone 1 mg/ml (eye drops, test drug), or to levofloxacin (Oftraquix®) or to dexamethasone (Tamesad®) in a 1:1:1 ratio.</p> <p>Administration of study drugs will be performed by qualified health care personnel using a micropipette and dispenser. Two doses of one of the study treatments will be administered, the first <math>90 \pm 15</math> minutes prior to surgery, and the second <math>60 \pm 15</math> minutes before surgery. The two doses must be administered 30 minutes apart. Doses are to be administered at the lateral canthus while applying pressure at the medial canthus to prevent drainage of the drug.</p> <p>Before cataract surgery, a limbal paracentesis will be performed and an aqueous humour sample of approximately 0.05 ml will be drawn from the anterior chamber with a 30-gauge needle insulin syringe. The aqueous humour will be stored immediately in vials at <math>-80^{\circ}\text{C}</math> and subsequently analysed at a central laboratory. Vial labels will contain the kit number but no information regarding the drug received. Laboratory analysts will thus be blinded to the drug administered and samples will be analysed for the concentration of all three molecules (levofloxacin, dexamethasone 21-phosphate and dexamethasone) by means of LC-MS-MS.</p> <p>Following surgery, patients will be treated with antibiotics and/or steroids according to local clinical practice.</p>
<p><b>Sample size calculation</b></p>	<p>The study plans to enrol 120 patients: 40 will be assigned to the dexamethasone group, 40 to the levofloxacin group and 40 to the dexamethasone-levofloxacin combination.</p> <p>The sample size has been estimated based on the expected precision (i.e. width of the 95% confidence interval) of the estimates of the study drug concentrations.</p> <p>Assessment of dexamethasone concentration: When the sample size is 40, a two-sided 95% confidence interval for a single mean will extend 4.648 ng/ml from the observed mean, assuming that the standard deviation is known to be 15 ng/ml and the confidence interval is based on the large sample z statistic.</p>

	Assessment of levofloxacin concentration: When the sample size is 40, a two-sided 95% confidence interval for a single mean will extend 61.676 ng/ml from the observed mean, assuming that the standard deviation is known to be 199.022 ng/ml and the confidence interval is based on the large sample z statistic.
<b>Drug analysis</b>	All biological samples will be sent to the central laboratory, Ticinumlab, Novara (Italy), which will perform in one run the LC-MS-MS (triple quadrupole) analyses for the simultaneous determination of levofloxacin, dexamethasone and dexamethasone 21-phosphate.
<b>Statistical considerations</b>	<p>Continuous data will be summarized with standard descriptive statistics (i.e. the mean, standard deviation, minimum, median and maximum, 95% confidence limits). Categorical data will be summarized by frequencies and percentages.</p> <p>All analyses will be performed using SAS version 9.4 or later.</p>
<b>Duration of the study</b>	<p>Estimated date of the first visit of the first patient (FPFV): Q2, 2018</p> <p>Estimated date of the first visit of the last patient (LPFV): Q1, 2019</p> <p>Estimated date of the last visit of the last patient (LPLV): Q1, 2019</p>
<b>Version and date</b>	V 1.0 – 21 March 2018

## 1.2 STUDY DESIGN



### 1.3 SCHEDULE OF ACTIVITIES (SOA)

Procedures	Screening <sup>a</sup>	Day of surgery			
		Pre-dose	First dose -90 (±15) min	Second dose -60 (±15) min	Limbal Paracentesis
Informed consent	X				
Demographics	X				
Medical history	X				
Concomitant medications	X	X			
Pachymetry	X				
Fluorescein test	X				
Inclusion/Exclusion criteria	X	X			
Pregnancy test <sup>b</sup>		X			
Randomization		X			
Study drug administration <sup>c</sup>			X	X	
Limbal paracentesis					X
Administration of Mydrane <sup>®d</sup>					X
Adverse event review and evaluation <sup>e</sup>	X	X	X	X	X

<sup>a</sup> Screening must take place not more than 28 days prior to surgery.

<sup>b</sup> Urine pregnancy test (women of childbearing potential).

<sup>c</sup> Two doses 30 minutes apart, the first 90 (± 15) minutes prior to surgery, and the second 60 (± 15) minutes prior to surgery.

<sup>d</sup> Mydrane<sup>®</sup> is to be administered following withdrawal of aqueous humour.

<sup>e</sup> Adverse events are to be reported until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation.



## 2 INTRODUCTION

### 2.1 STUDY RATIONALE

In most cases, postoperative care after cataract surgery consists of anti-inflammatory and antibacterial drug therapy. The use of an ophthalmic solution containing the combination of a steroid and an antibiotic is routinely used in clinical practice also because it favours the proper administration of the two agents, reduces the possibility of inaccurate dosage and improves patient compliance with medication. To reduce the emergence of antibiotic resistance, treatment with an antibiotic should however not be longer than the time necessary for the healing of surgically induced epithelial lesions, and therefore should not last more than a week.

Based on these considerations, there is clear interest in having a combination of a broad-spectrum antibiotic with a highly effective corticosteroid that can be used for a short period of time (one week). The combination of dexamethasone and levofloxacin tested in this study is the first combination of a steroid and a quinolone under development and represents a major step forward in the prevention and treatment of post-operative inflammation and prevention of post-operative infections.

Levofloxacin 0.5% – dexamethasone 0.1% ophthalmic solution is an anti-inflammatory/antibiotic combination indicated for the prevention and treatment of inflammation and prevention of infection associated with cataract surgery in adults. The active components of this combination are currently available in marketed ophthalmic products. The purpose of this study is to measure the concentrations of levofloxacin and dexamethasone in the aqueous humour (AH) after topical application of the combined ophthalmic solution and its single components.

### 2.2 BACKGROUND

Cataract is an ocular condition that causes vision impairment due to changes in the opacity of the ocular lens. Cataract is an age-related condition, but smoking, ocular trauma, exposure to ultraviolet light, diabetes and drug induced metabolic changes in the lens are risk factors for lens opacification causing cataract.<sup>1</sup>

The only cure for cataract is surgical, with phacoemulsification with lens implantation as the treatment of choice.<sup>2</sup> Recovery after surgery is easier than it was in the past and patients usually no longer require in-patient hospital care, with recovery usually uneventful and pain-free. However, adverse events may occur due to corneal trauma, ocular inflammation and dryness, which can result in ocular irritation during the recovery period.<sup>3-5</sup> These conditions may cause symptoms such as pain, burning, stinging, foreign body sensation, itchiness and glare in the operated eye. The incidence of ocular irritation symptoms experienced during the first postoperative hours may be as high as 98%.<sup>6</sup> Conjunctival hyperaemia, anterior chamber cells and flare and cystoid macular oedema are some possible signs of inflammation after cataract surgery.

In many cases, inflammation is self-limiting, but drug therapy is often used to shorten resolution time and improve ocular comfort.<sup>7</sup>

Steroidal and non-steroidal anti-inflammatory drugs are used to prevent and treat postoperative inflammation. Topical corticosteroids are usually administered for 1 to 6 weeks after cataract surgery. According to the practice pattern studies published by the American Society of Cataract and Refractive Surgery (2012), Canadian Ophthalmological Society members study 2011<sup>8</sup> and European Society of Cataract and Refractive Surgeons (ESCRS), there are variations in postoperative drug therapy provided to individual patients and among different countries. In Europe, dexamethasone was the most commonly used steroidal anti-inflammatory drug and was chosen as the preferred drug by 71% of ophthalmological surgeons.<sup>9</sup>

Endophthalmitis, is a rare yet severe condition that occurs in 0.03–0.3% of patients undergoing cataract surgery.<sup>10,11</sup> This condition usually appears during the first days after surgery, and in 80% of cases, endophthalmitis will have developed during the first 6 weeks after surgery.<sup>11,12</sup> Topically applied antibiotics are routinely used for the prophylaxis of postoperative bacterial ocular infections such as endophthalmitis. Intraocular, usually intracameral or subconjunctival antibiotics are used during surgery, and both pre- and postoperative topical applications are also provided.<sup>10</sup> To increase the efficacy of this approach, a broad-spectrum antibiotic should be chosen.

A prospective observational study was conducted to determine the antibiotic susceptibility patterns of conjunctival bacterial flora isolated preoperatively from patients undergoing anterior segment surgery. Of the 120 eyes studied, 21 (18%) showed no bacterial growth. Of the 143 bacterial strains isolated from the remaining 99 eyes, 112 (78%) were coagulase-negative staphylococci (CNS). Among the CNS, greater than 90% were susceptible to cefotaxime, levofloxacin, imipenem, meropenem, vancomycin, and each of the aminoglycosides except neomycin. Between 70% and 90% of the CNS were susceptible to cefazolin, neomycin, ciprofloxacin, ofloxacin, norfloxacin, and chloramphenicol.<sup>13</sup>

Based on these results, quinolones clearly represent a rational approach to the prevention of ocular infections after cataract surgery. Indeed, among available antibiotics for ophthalmic use, quinolones are characterized by a broad spectrum that includes both the Gram-positive and Gram-negative bacteria that are most frequently responsible for ocular bacterial infections.

In most cases, postoperative care after cataract surgery consists of anti-inflammatory and antibacterial drug therapy. Topical anti-inflammatory agents and antibiotics are both routinely provided after cataract surgery. The combined administration of corticosteroids and antibiotics guarantees a powerful anti-inflammatory effect, and contemporarily a preventive/therapeutic action against infections related either to the intensity of the inflammatory process or to the immunosuppressive effect of the steroid.

However, use of such a combination should also be in line with the modern approach to antimicrobial therapy, which advocates preventing increasing bacterial resistance caused by the

inappropriate use of antibiotics. The ESCRS guidelines do not indicate a specific antibiotic to be used for topical postoperative treatment, nor a precise duration of such treatment, although they emphasize the importance of rational use to limit the development of bacterial resistance, especially when a broad-spectrum antibiotic is used.<sup>14</sup> For this reason, antibiotic treatment should not be prolonged beyond the time necessary for the healing of surgically induced epithelial lesions, and therefore should not last more than a week.<sup>15</sup>

In light of these considerations, having the combination of a broad-spectrum antibiotic and a highly effective corticosteroid to be used for a short period of time would be of great clinical interest. The association of dexamethasone 1 mg/ml and levofloxacin 5 mg/ml tested in this study is the first combination of a steroid and a quinolone under development and represents a major step forward in this direction.

The concentration of dexamethasone (1 mg/ml) is well-established in ophthalmic solutions both as sole active ingredient or in combination with an antibiotic to prevent ocular inflammation following cataract surgery. After topical administration of 0.1% solutions of this agent, the concentration in the aqueous humour is adequate to exert its potent anti-inflammatory activity at the site of action.<sup>16</sup> The dexamethasone 21-phosphate salt is freely soluble in water and thus the solution does not require shaking before application and guarantees consistency of the content of the active ingredient at each application.

The pharmacokinetics of both levofloxacin and dexamethasone administered individually confirm that after application as an eye drop solution, both active drugs reach significant concentrations in the aqueous humour, with peaks of aqueous levels between 90 and 150 minutes, and detectable concentrations after 12 hours from dosing.<sup>16-21</sup> Conversely, the concentrations in plasma are negligible, which guarantees lack of systemic effects during a short-course treatment.

The purpose of this study is to analyse the aqueous humour drug concentrations of levofloxacin and dexamethasone administered topically in combination and as single components to assess the penetration of these agents and provide data for clinical use.

## 2.3 RISK/BENEFIT ASSESSMENT

### 2.3.1 KNOWN POTENTIAL RISKS

In toxicity studies, the only toxicity data reported for dexamethasone were those related to an exaggerated pharmacological activity including a negative feedback on the pituitary-adrenal axis. Systemic effects are however not expected or are negligible when the drug is topically administered as eye drops and for a limited period of time. Nevertheless, its potential effect on foetal development and on newborns suggests it be used very carefully in pregnant women and to discard milk during lactation.<sup>22,23</sup>

Possible adverse effects of topical ocular corticosteroid therapy consist of an increase of intraocular pressure (IOP), inhibition of corneal wound healing and increased likelihood of infection and serious complications, but systemic adverse effects are rare.<sup>7,24</sup>

Levofloxacin is not toxic on reproduction and does not have mutagenic or carcinogenic potential. It is not irritating after eye instillation, but it is phototoxic at very high doses and photosensitization may not be excluded. Thus, it is suggested not to expose patients to direct sunlight during treatment with eye drops containing levofloxacin.

### 2.3.2 KNOWN POTENTIAL BENEFITS

The efficacy of dexamethasone as anti-inflammatory agent and of levofloxacin as antibiotic in ophthalmology is well established. Combining the two agents would favour their proper administration, reduce the possibility of inaccurate dosage and improve patient compliance. The combination would also allow the implementation of a shorter antibiotic treatment period as compared to other marketed products and thus prevent the emergence of antibiotic resistance.

### 2.3.3 ASSESSMENT OF POTENTIAL RISKS AND BENEFITS

All things considered, the potential benefits of this trial surely outweigh their potential risks.

## 3 OBJECTIVES AND ENDPOINTS

OBJECTIVE	ENDPOINT	JUSTIFICATION FOR ENDPOINT
To evaluate the penetration of levofloxacin/dexamethasone 21-phosphate into the aqueous humour after ocular administration in combination or as single active ingredients.	Aqueous humour concentration of levofloxacin and dexamethasone.	The endpoint is standard for penetration studies of ophthalmic preparations into the aqueous humour.

## 4 STUDY DESIGN

### 4.1 OVERALL DESIGN

This is a randomized, assessor-blinded, parallel-group clinical study.

One hundred and twenty (120) patients scheduled for cataract surgery at 2 centres who have provided written informed consent will undergo screening procedures during a preoperative visit. Prior to surgery, and once eligibility criteria have been confirmed, eligible patients will be assigned to one of the three following treatment groups in a 1:1:1 ratio:

- Levofloxacin 5 mg/ml + dexamethasone 1 mg/ml (test drug)
- Levofloxacin (Oftaquix<sup>®</sup>)

- Dexamethasone (Tamesad®)

Administration of study drugs will be performed by qualified health care personnel using a micropipette and dispenser. Two doses of one of the study treatments will be administered, the first  $90 \pm 15$  minutes prior to surgery, and the second  $60 \pm 15$  minutes before surgery. The two doses must be administered 30 minutes apart. Eye drops are to be administered at the lateral canthus while applying pressure at the medial canthus to prevent drainage of the drug.

Phacoemulsification and limbal paracentesis will be performed by an experienced surgeon. Before the main incisions of phacoemulsification, approximately 0.05 ml of aqueous humour will be drawn from the anterior chamber with a 30-gauge needle syringe. Mydrane®, a combined solution (phenylephrine [0.31%], tropicamide [0.02%] and lidocaine [1%]) for intracameral injection indicated for cataract surgery to obtain mydriasis and intraocular anaesthesia during the surgical procedure is to be administered following paracentesis. The patient's participation in the study will end with the administration of Mydrane®. Following surgery, patients will be treated with antibiotics and/or steroids according to local clinical practice.

The aqueous humour will be stored immediately in vials at  $-80^{\circ}\text{C}$  and subsequently analysed at a central laboratory. Vial labels will contain the study kit number but no information regarding the drug received. The laboratory analyst will be blinded to the drug administered and samples will be analysed for the concentration of all three molecules (levofloxacin, dexamethasone 21-phosphate and dexamethasone) by means of an LC tandem mass spectrometry method. Quality control samples blinded to the analyst will be included following advice from BfArM. To that end, the randomization list will include kit numbers corresponding to quality control samples. A different analyst will prepare twelve samples (4 of each active principle) with synthetic aqueous humour and a known concentration of the active ingredients in pre-labelled vials at the central laboratory in the presence of personnel belonging to a Contract Research Organization (CRO) (OPIS s.r.l.) working on behalf of the Sponsor. The twelve quality control samples correspond to 10% of the true biological samples obtained from the patients.

## 4.2 SCIENTIFIC RATIONALE FOR STUDY DESIGN

The randomized design is standard for studies investigating the penetration of ophthalmic preparations into the aqueous humour. The laboratory analyst will be blinded to the drug administered to ensure an unbiased analysis (see [Section 4.1](#)).

#### 4.3 JUSTIFICATION FOR DOSE

The concentration of dexamethasone (1 mg/ml) in the combination treatment is well established in ophthalmic solutions, both as sole active ingredient or in combination with an antibiotic. Levofloxacin is also well established as sole active ingredient of ophthalmic solutions at a concentration of 5 mg/ml.

Two 30 µl doses administered by micropipette and dispenser of combined levofloxacin hemihydrate 5.12 mg/ml, corresponding to 5 mg /ml levofloxacin + dexamethasone 21-phosphate 1.32 mg/ml, corresponding to dexamethasone 1 mg/ml administered 90 minutes and 60 minutes prior to surgery were deemed suitable for investigating the penetration of the active principles into the aqueous humour. AH concentration of levofloxacin and dexamethasone will be analysed also using marketed products containing the single active principles; levofloxacin hemihydrate 5.12 mg/ml, corresponding to 5mg/ml levofloxacin (Oftaquix<sup>®</sup>) will be administered at the same dose (two 30 µl instillations) given the same concentration of the active principle, whereas dexamethasone 21-phosphate 1.50 mg/ml, corresponding to dexamethasone 1.14 mg / ml (Tamesad<sup>®</sup>) will be administered at a dose of two 26 µl instillations due to the higher concentration of the active principle in this product respect to that of the combination product. The dose of 26 µl was calculated as follows:

Tamesad<sup>®</sup> 0.15%:1 ml contains 1.5 mg of dexamethasone sodium phosphate

$1.5 \text{ mg} / \text{molecular weight dexamethasone sodium phosphate} \times \text{molecular weight dexamethasone} = 1.14 \text{ mg dexamethasone.}$

Tamesad<sup>®</sup> therefore contains 1.14 mg dexamethasone / ml.

$30 \text{ µl} \times 0.1\% / 0.114\% = 26.3 \text{ µl}$ , i.e. 26.3 µl of the 1.5 mg/ml solution will have the same content in dexamethasone as 30 µl of the 1 mg/ml solution.

#### 4.4 END OF STUDY DEFINITION

A participant is considered as having completed the study if he or she has completed all phases of the study including the last procedure as indicated in the Schedule of Activities (SoA) (paracentesis and administration of Mydrane<sup>®</sup>), [Section 1.3](#). The patient will subsequently be treated according to local clinical practice.

The end of the study is defined as completion of the last procedure in the trial globally.

### 5 STUDY POPULATION

The study population will be made up of 120 patients scheduled for cataract surgery.

## 5.1 INCLUSION CRITERIA

To be eligible to participate in this study, a subject must meet all the following criteria:

1. Written informed consent must be obtained before any assessment is performed
2. Male or female patients, aged  $\geq 40$  years
3. Patient undergoing phacoemulsification
4. Corneal thickness between 450  $\mu\text{m}$  and 600  $\mu\text{m}$  as measured by means of pachymetry
5. Corneal integrity confirmed by means of fluorescein test (Oxford scheme grade 0)
6. Adequate pupil dilation assessed at screening
7. Female patients of childbearing potential must have a negative pregnancy test
8. Ability to fully understand all study procedures

## 5.2 EXCLUSION CRITERIA

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Corneal epithelium integrity not confirmed by fluorescein test
2. History of corneal disease or dystrophy (e.g. ocular herpes, conjunctivitis, keratitis)
3. History of ocular trauma with corneal damage
4. History of acute ocular inflammation (including uveitis) in the 6 months prior to screening
5. Previous ocular surgery (including laser treatment)
6. Glaucoma
7. Treatment with an ophthalmic investigational drug in the 3 months prior to screening
8. Treatment with any topical ocular drug within 12 hours before start of cataract surgery other than study drugs and instillation of topical anaesthetic (oxybuprocaine hydrochloride - Novesina<sup>®</sup>) followed by povidone-iodine (Oftasteril<sup>®</sup>) within 10 minutes before start of surgery
9. Treatment with any topical steroid or antibiotic drug in the 7 days prior to cataract surgery (artificial tears without BAK are allowed)
10. Treatment with any systemic steroid or antibiotic drug in the 7 days prior to cataract surgery
11. Known hypersensitivity to levofloxacin, other fluoroquinolones or dexamethasone
12. Pregnant or lactating women
13. Patients who have received any investigational drug during the preceding 30 days or 5 times the plasma half-life, whichever is longer, or who have previously participated in this trial



### 5.3 SCREEN FAILURES

Screen failures are defined as participants who consent to participate in the clinical trial but are not subsequently randomly assigned to the study intervention. A minimum set of screen failure information is required to ensure transparent reporting of screen failure participants, to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements and to respond to queries from regulatory authorities. Minimal information includes demography, screen failure details, eligibility criteria, and any serious adverse event (SAE).

### 5.4 STRATEGIES FOR RECRUITMENT AND RETENTION

This study will enrol subjects already scheduled for cataract surgery and no other recruitment strategies are required. No strategy for retaining subjects is necessary given the duration of the study.

## 6 STUDY INTERVENTION

### 6.1 STUDY INTERVENTION(S) ADMINISTRATION

#### 6.1.1 STUDY INTERVENTION DESCRIPTION

The study drugs are as follows:

- Levofloxacin hemihydrate 5.12 mg/ml + dexamethasone 21-phosphate 1.32 mg/ml (eye drops solution)
- Levofloxacin hemihydrate 5.12 mg/ml (Oftaquix<sup>®</sup> eye drops)
- Dexamethasone 21-phosphate 1,50 mg/ml (Tamesad<sup>®</sup> eye drops)

#### 6.1.2 DOSING AND ADMINISTRATION

Eligible patients will be randomly assigned to one of the three following treatment groups in a 1:1:1 ratio:

- Levofloxacin 5 mg/ml + dexamethasone 1 mg/ml (test drug): two 30 µl doses, 30 minutes apart, one 90 ± 15 minutes prior to surgery and the other 60 ± 15 minutes prior to surgery.
- Levofloxacin (Oftaquix<sup>®</sup>): two 30 µl doses, 30 minutes apart, one 90 ± 15 minutes prior to surgery and the other 60 ± 15 minutes prior to surgery.
- Dexamethasone (Tamesad<sup>®</sup>): two 26 µl doses, 30 minutes apart, one 90 ± 15 minutes prior to surgery and the other 60 ± 15 minutes prior to surgery



Administration of study drugs will be performed by qualified health care personnel using a micropipette and dispenser. Doses are to be administered at the lateral canthus while applying pressure at the medial canthus to prevent drainage of the study drug.

Should the treatment schedule not be respected, subjects will not undergo aqueous humour extraction and will be followed for safety purposes only.

## 6.2 PREPARATION/HANDLING/STORAGE/ACCOUNTABILITY

### 6.2.1 ACQUISITION AND ACCOUNTABILITY

Study treatments will be provided by the Sponsor. The investigator, or a pharmacist or other appropriate individual who is designated by the investigator, will maintain accurate records of the product's delivery to the study site, the inventory at the site, the use for each subject, and the return to the sponsor or alternative disposition of unused products. Drug-related documentation will be reviewed by the field monitor during the study conduct.

### 6.2.2 FORMULATION, APPEARANCE, PACKAGING, AND LABELING

- Test drug: levofloxacin 5 mg/ml – dexamethasone 1 mg/ml eye drops

Composition in active ingredients: levofloxacin hemihydrate 5.12 mg/ml, corresponding to 5mg/ml, and dexamethasone 21-phosphate 1.32 mg/ml, corresponding to dexamethasone 1 mg/ml.

The Investigational Medicinal Product (IMP) is a sterile, clear, greenish-yellow isotonic ophthalmic solution with a pH of 7.2. The finished product is packaged into a light-resistant LDPE bottle with ophthalmic dropper and PP screw cap.

- Levofloxacin 5.0 mg/ml (Oftaquix<sup>®</sup>) 0.5% eye drops

Transparent, light yellow or light yellow-green solution in a multi-dose 5 ml vial. 1 ml contains: 5,12 mg di levofloxacin hemihydrate equal to 5 mg of levofloxacin.

- Dexamethasone 1.5 mg/ml (Tamesad<sup>®</sup>) 0,15% eye drops

Solution in multi-dose vial. One ml of solution contains: dexamethasone sodium phosphate 1.5 mg.

### 6.2.3 PRODUCT STORAGE AND STABILITY

All drugs to be administered to patients enrolled in this trial as per study protocol are to be stored at room temperature not higher than 25°C, not frozen and not refrigerated.

### 6.2.4 PREPARATION

Study drugs are ready for use and do not require preparation.

### 6.3 MEASURES TO MINIMIZE BIAS: RANDOMIZATION AND BLINDING

Patients will be randomized to one of the three treatment groups described in the sections above in a 1:1:1 ratio. Randomization is stratified by centre.

The randomization numbers will be generated using procedures that ensure that treatment assignment is unbiased. A patient randomization list will be produced using a validated system that automates the random assignment of patient numbers to randomization numbers.

Prior to surgery, patients who fulfil all inclusion criteria and none of the exclusion criteria will be randomized via an Interactive Web Response System (IWRS) that will assign the patient a randomization number linked to a treatment arm.

Vials containing frozen humour aqueous samples will be labelled and shipped to a centralized laboratory for analysis. Vial labels will contain the kit numbers but no information regarding the drug received. Laboratory analysts will thus be blinded to the drug administered and samples will be analysed for the concentration of all three molecules (levofloxacin, dexamethasone 21-phosphate and dexamethasone. Quality control samples blinded to the analyst will be included. To that end, the randomization list will include kit numbers corresponding to quality control samples. Twelve samples (4 of each active principle) will be prepared with synthetic aqueous humour and a known concentration of the active ingredients in pre-labelled vials at the central laboratory by a different analyst in presence of OPIS personnel.

### 6.4 STUDY INTERVENTION COMPLIANCE

Study personnel will be responsible for respecting administration times and recording them in a Dose and Sampling Log. Compliance will not be evaluated as patients will receive two doses of treatment within the first two hours prior to cataract surgery.

### 6.5 CONCOMITANT MEDICATIONS

Medications to be reported in the electronic Case Report Form (eCRF) are concomitant prescription medications, over-the-counter medications and supplements.

Treatment with any topical ocular drug other than study drugs before start of cataract surgery the day of surgery is prohibited [a topical anaesthetic (oxybuprocaine hydrochloride - Novesina®) followed by povidone-iodine (Oftasteril®) instillation is allowed within 10 minutes before start of surgery). Treatment with any topical or systemic steroid or antibiotic drug in the 7 days prior to cataract surgery is also not permitted (artificial tears without BAK are allowed).

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#### 6.5.1 RESCUE THERAPY

Not applicable.

## **7 STUDY INTERVENTION DISCONTINUATION AND PARTICIPANT DISCONTINUATION/WITHDRAWAL**

### **7.1 DISCONTINUATION OF STUDY INTERVENTION**

If a patient receives only one dose of the study drug (but not the second), this does not mean discontinuation from the study, however aqueous humour will not be extracted from patients if the treatment schedule is not respected. These patients will be followed for safety purposes only. If a clinically significant finding is identified after enrolment, the investigator or qualified designee will determine if any change in participant management is needed. Any new clinically relevant finding leading to discontinuation from study treatment will be reported as an adverse event (AE).

### **7.2 PARTICIPANT DISCONTINUATION/WITHDRAWAL FROM THE STUDY**

Participants are free to withdraw from participation in the study at any time upon request. An investigator may discontinue or withdraw a participant from the study for the following reasons:

- Pregnancy.
- If any clinical adverse event (AE) or other medical condition or situation occurs such that continued participation in the study would not be in the best interest of the participant.
- If the participant meets an exclusion criterion (either newly developed or not previously recognized) that precludes further study participation.

The reason for participant discontinuation or withdrawal from the study will be recorded on the eCRF. Subjects who sign the informed consent form and are randomized but do not complete the study will not be replaced.

### **7.3 LOST TO FOLLOW-UP**

A participant will be considered lost to follow-up if he or she does not complete study procedures.

## **8 STUDY ASSESSMENTS AND PROCEDURES**

### **8.1 ASSESSMENTS**

At screening, which must take place not more than 28 days before surgery, information will be collected concerning demographics, medical history (a diagnosis should be provided and not symptoms) and the use of concomitant medications. Pachymetry will be performed to measure corneal thickness and a fluorescein test will be performed to confirm corneal epithelium integrity, defined as Oxford scheme grade 0.

Prior to surgery, a pre-dose visit will be performed to confirm eligibility criteria are met. Women of childbearing potential will be required to undergo a urine pregnancy test. Study drugs will be

administered prior to surgery by qualified health care personnel using a micropipette and dispenser. Two doses of one of the study treatments will be administered, the first  $90 \pm 15$  minutes prior to surgery, and the second  $60 \pm 15$  minutes before surgery. The two doses must be administered 30 minutes apart. Eye drops are to be administered at the lateral canthus while applying pressure at the medial canthus to prevent drainage of the drug.

Immediately before cataract surgery, a limbal paracentesis will be performed and an aqueous humour sample of approximately 0.05 ml will be drawn from the anterior chamber with a 30-gauge needle insulin syringe. The aqueous humour will be stored immediately in vials at  $-80^{\circ}\text{C}$ .

Treatment with any topical drug (including mydriatic eye drops) other than study drugs within 12 hours before the start of cataract surgery is prohibited to prevent variations of the penetration of the study drugs. Oxybuprocaine hydrochloride (Novesina<sup>®</sup>) followed by povidone-iodine (Oftasteril<sup>®</sup>) instillation are allowed within 10 minutes before the start of surgery. Mydrane<sup>®</sup> is a combined solution (phenylephrine [0.31%], tropicamide [0.02%] and lidocaine [1%]) for intracameral injection indicated for cataract surgery to obtain mydriasis and intraocular anaesthesia during the surgical procedure. Mydrane<sup>®</sup> is to be administered following paracentesis to avoid potential interference with the penetration of study drugs. Mydrane<sup>®</sup> will be provided free of charge by the Sponsor.

## 8.2 LABORATORY ANALYSES

The concentration of levofloxacin, dexamethasone 21-phosphate and dexamethasone will be measured by LC tandem mass spectrometry at the centralised laboratory Ticinumlab (Novara, Italy). Ticinumlab is a laboratory working according to Good Laboratory Practice (GLP) and qualified by the Health Authority for the analysis of biological samples during human pharmacokinetic studies. The laboratory will develop and validate a specific analytical procedure via LC tandem mass spectrometry by triple quadrupole. The method is able to detect the three analytes at once, namely levofloxacin, dexamethasone and dexamethasone 21-phosphate. This method will allow a minimum quantity of aqueous humour (i.e. 50  $\mu\text{l}$ ) to be withdrawn from each patient. The vials containing frozen aqueous humour will contain no information regarding the drug received. Quality control samples blinded to the analyst will be included following advice from BfArM. To that end, the randomization list will include kit numbers corresponding to quality control samples. Twelve samples will be prepared with synthetic aqueous humour and a known concentration of the active ingredients in pre-labelled vials at the central laboratory by a different analyst in presence of OPIS personnel. The twelve quality control samples correspond to 10% of the true biological samples obtained from the patients.

The laboratory analyst will thus be blinded to the drug administered and samples will be analysed for the concentration of all three molecules (levofloxacin, dexamethasone 21-phosphate and dexamethasone). All the analyses will be done in a single run and will not be duplicated.

Further details are provided in the laboratory protocol (*Appendix I*).

### 8.3 SAFETY

The assessment of safety consists in evaluating all adverse events.

### 8.4 ADVERSE EVENTS AND SERIOUS ADVERSE EVENTS

#### 8.4.1 DEFINITION OF ADVERSE EVENTS (AE)

An adverse event can be defined as any untoward medical occurrence associated with the use of an intervention in humans after providing written informed consent for participation in the study until the end of study visit, whether considered intervention-related or not.

#### 8.4.2 DEFINITION OF SERIOUS ADVERSE EVENTS (SAE)

An adverse event (AE) or suspected adverse reaction is considered "serious" if, in the view of either the investigator or sponsor, it results in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions, or a congenital anomaly/birth defect. Important medical events that may not result in death, be life-threatening, or require hospitalization may be considered serious when, based upon appropriate medical judgment, they may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed in this definition.

#### 8.4.3 CLASSIFICATION OF AN ADVERSE EVENT

##### 8.4.3.1 SEVERITY OF EVENT

The following guidelines will be used to describe severity.

- Mild – Events require minimal or no treatment and do not interfere with the participant's daily activities.
- Moderate – Events result in a low level of inconvenience or concern with the therapeutic measures. Moderate events may cause some interference with functioning.
- Severe – Events interrupt a participant's usual daily activity and may require systemic drug therapy or other treatment. Severe events are usually potentially life-threatening or incapacitating. Of note, the term "severe" does not necessarily equate to "serious".

##### 8.4.3.2 RELATIONSHIP TO STUDY INTERVENTION

All adverse events (AEs) must have their relationship to study intervention assessed by the clinician who examines and evaluates the participant based on temporal relationship and his/her

clinical judgment. Investigators are to judge the causal relationship of the event with the study intervention as “suspected”, “unsuspected” or “unknown”.

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#### 8.4.3.3 EXPECTEDNESS

The Sponsor will be responsible for determining whether an adverse event (AE) is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention.

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### 8.4.4 TIME PERIOD AND FREQUENCY FOR EVENT ASSESSMENT AND FOLLOW-UP

The occurrence of an adverse event (AE) or serious adverse event (SAE) may come to the attention of study personnel during study visits and interviews of a study participant presenting for medical care, or upon review by a study monitor.

All AEs including local and systemic reactions will be captured on the appropriate case report form (eCRF). Information to be collected includes event description (a diagnosis and not symptoms should be provided, if possible), time of onset, clinician’s assessment of severity, relationship to study product (assessed only by those with the training and authority to make a diagnosis), and time of resolution/stabilization of the event. All AEs occurring while on study must be documented appropriately regardless of relationship. All AEs will be followed to adequate resolution.

Any medical condition that is present at the time that the participant is screened will be considered as baseline and not reported as an AE. However, if the study participant’s condition deteriorates at any time during the study, it will be recorded as an AE.

Changes in the severity of an AE will be documented to allow an assessment of the duration of the event at each level of severity to be performed. AEs characterized as intermittent require documentation of onset and duration of each episode.

The investigator will record all reportable events with onset dates occurring any time after informed consent is obtained until 7 (for non-serious AEs) or 30 days (for SAEs) after the last day of study participation. Events will be followed for outcome information until resolution or stabilization.

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### 8.4.5 NON-SERIOUS ADVERSE EVENT REPORTING

All identified non-serious AEs (related and unrelated) must be recorded and described on the eCRF.

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#### 8.4.6 SERIOUS ADVERSE EVENT REPORTING

Every SAE, regardless of suspected causality, occurring after the subject has provided informed consent and until 30 days after the last day of study participation must be reported to the sponsor within 24 hours of site awareness.

Any SAE experienced after this 30-day period should only be reported to the sponsor if the investigator suspects a causal relationship to the study treatment. Recurrent episodes, complications, or progression of the initial SAE must be reported as follow-up to the original episode within 24 hours of the investigator receiving the follow-up information.

Information about all SAEs will be recorded on the eCRF. In case of technical difficulties, SAE notification can be carried out by contacting the CRO (OPIS) in charge of Pharmacovigilance, via email at [all\\_phv@opis.it](mailto:all_phv@opis.it) or by fax using the following number: +39 0362 633622.

All SAEs will be followed until satisfactory resolution or until the site investigator deems the event to be chronic or the adherence to be stable. Other supporting documentation of the event may be requested by the study sponsor and should be provided as soon as possible. The study sponsor will be responsible for notifying Health Authorities of any unexpected fatal or life-threatening suspected adverse reaction as soon as possible, but in no case later than 7 calendar days after the sponsor's initial receipt of the information.

Suspected Unexpected Serious Adverse Reactions (SUSARs) will be collected and reported to the competent authorities and relevant Ethics Committees in accordance with Directive 2001/20/EC or as per national regulatory requirements.

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#### 8.4.7 REPORTING EVENTS TO PARTICIPANTS

Should an event occur that changes the overall benefit/risk ratio of the study, the Sponsor shall evaluate if a risk minimization measure is needed. Should this measure require a substantial amendment to the protocol, the informed consent and patient information will be revised and submitted to the patient for written consent.

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#### 8.4.8 EVENTS OF SPECIAL INTEREST

Not applicable.



#### 8.4.9 REPORTING OF PREGNANCY

Pregnant women will not be permitted to participate in this study. A negative pregnancy test will be required to enter the study and, given the very short duration of the study, the occurrence of a pregnancy is highly unlikely. Nevertheless, the investigator shall report all pregnancy exposure occurring in a female patient within 24 hours to the sponsor using the Pregnancy Reporting Form of the eCRF. In case of technical difficulties, pregnancy notification can be carried out by contacting the Pharmacovigilance Officer via email at [all\\_phv@opis.it](mailto:all_phv@opis.it) or by fax using the following number: +39 0362 633622. The investigator should counsel the subject; discuss the risks of continuing with the pregnancy and the possible effects on the foetus potentially induced by participation in the study. Monitoring of the subject should continue until conclusion of the pregnancy.

### 9 STATISTICAL CONSIDERATIONS

#### 9.1 STATISTICAL HYPOTHESES

No formal statistical hypothesis has been formulated. All analyses will be descriptive in nature and no inferential statistical analyses are planned.

#### 9.2 SAMPLE SIZE DETERMINATION

The study plans to enrol 120 patients: 40 will be assigned to the dexamethasone group, 40 to the levofloxacin group and 40 to the dexamethasone-levofloxacin combination.

The sample size has been estimated based on the expected precision (i.e. width of the 95% confidence interval) of the estimates of the study drug concentrations.

For assessment of dexamethasone concentration:

When the sample size is 40, a two-sided 95% confidence interval for a single mean will extend 4.648 ng/ml from the observed mean, assuming that the standard deviation is known to be 15 ng/ml<sup>25</sup> and the confidence interval is based on the large sample z statistic.

For assessment of levofloxacin concentration:

When the sample size is 40, a two-sided 95% confidence interval for a single mean will extend 61.676 ng/ml from the observed mean, assuming that the standard deviation is known to be 199.022 ng/ml<sup>26</sup> and the confidence interval is based on the large sample z statistic.



### 9.3 POPULATIONS FOR ANALYSES

The descriptive analysis will be carried out on the following populations:

- Per Protocol (PP): all patients who have completed the study without any major protocol deviations.
- Full analysis Set (FAS): all randomized patients. According to Intention To Treat (ITT) principles all patients will be analyzed considering the randomized study treatment assigned by IWRS.

The safety analysis will be performed on the Safety Population, made up of all patients who have received at least one dose of study treatment.

### 9.4 STATISTICAL ANALYSES

#### 9.4.1 GENERAL APPROACH

Continuous data will be summarized with standard descriptive statistics (i.e. the mean, standard deviation, minimum, median and maximum, 95% confidence limits). Categorical data will be summarized by frequencies and percentages.

Missing values will not be replaced.

All statistical tables, listings and analyses will be produced using SAS<sup>®</sup> release 9.4 or later (SAS Institute, Inc., Cary, NC, USA).

#### 9.4.2 ANALYSIS OF THE ENDPOINT

Aqueous humour concentration of levofloxacin and dexamethasone, measured by LC-MS-MS, will be descriptively summarized by treatment group and 95% confidence limits will be provided.

#### 9.4.3 SAFETY ANALYSES

The incidence of Adverse Events (AEs) and Serious Adverse Events (SAEs) recorded throughout the study will be presented overall and by treatment group respectively.

Depending on the onset date of the event, AEs will be defined as follows:

- Treatment-emergent AEs, those events with an onset date after any treatment initiation.
- Non-treatment-emergent AEs, those events with an onset date between informed consent and any treatment initiation.

Non-treatment-emergent AEs will be listed only.

Treatment-emergent AEs will be summarized using the Medical Dictionary for Regulatory Activities (MedDRA) System Organ Class (SOC) and Preferred Term (PT). A summary of treatment-emergent AEs by SOC, PT and severity will be also provided. All related treatment-emergent AEs, treatment-emergent SAEs, treatment-emergent AEs with an outcome of death, treatment-emergent AEs leading to discontinuation of treatment will be summarized by MedDRA SOC and PT and will also be listed.

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#### 9.4.4 BASELINE DESCRIPTIVE STATISTICS

Medical history will be described according to SOC and PT using MedDRA and will be presented by treatment group.

Previous/concomitant medications will be described according to ATC 2 level and PT using the World Health Organization Drug Dictionary (WHO-DD) and will be presented by treatment group.

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#### 9.4.5 PLANNED INTERIM ANALYSES

Not applicable

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#### 9.4.6 SUB-GROUP ANALYSES

No sub-group analysis is planned.

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#### 9.4.7 TABULATION OF INDIVIDUAL PARTICIPANT DATA

Patient listings will be provided.

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#### 9.4.8 EXPLORATORY ANALYSES

No exploratory analysis is planned.

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### 10 SUPPORTING DOCUMENTATION AND OPERATIONAL CONSIDERATIONS

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#### 10.1 REGULATORY, ETHICAL, AND STUDY OVERSIGHT CONSIDERATIONS

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##### 10.1.1 INFORMED CONSENT PROCESS

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###### 10.1.1.1 CONSENT/ASSENT AND OTHER INFORMATIONAL DOCUMENTS PROVIDED TO PARTICIPANTS

Consent forms describing in detail the study intervention, study procedures, benefits and risks are given to the participant and written documentation of informed consent is required prior to undertaking any study-related procedures.

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#### 10.1.1.2 CONSENT PROCEDURES AND DOCUMENTATION

Informed consent is a process that is initiated prior to the individual's agreeing to participate in the study and continues throughout the individual's study participation. Consent forms will be approved by the IEC and the participant will be asked to read and review the document. The investigator will explain the research study to the participant and answer any questions that may arise. A verbal explanation will be provided in terms suited to the participant's comprehension of the purposes, procedures, and potential risks of the study and of their rights as research participants. Participants will have the opportunity to carefully review the written consent form and ask questions prior to signing. The participants should have the opportunity to discuss the study with their family or surrogates and think about it prior to agreeing to participate. The participant will sign the informed consent document prior to any procedures being done specifically for the study. Participants must be informed that participation is voluntary and that they may withdraw from the study at any time, without prejudice. A copy of the informed consent document will be given to the participants for their records. The informed consent process will be conducted and documented in the source document (including the date), and the form signed, before the participant undergoes any study-specific procedures. The rights and welfare of the participants will be protected by emphasizing to them that the quality of their medical care will not be adversely affected if they decline to participate in this study.

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#### 10.1.2 STUDY DISCONTINUATION AND CLOSURE

This study may be temporarily suspended or prematurely terminated if there is sufficient reasonable cause. Written notification, documenting the reason for study suspension or termination, will be provided by the suspending or terminating party to study participants, investigator, IEC, sponsor and regulatory authorities. If the study is prematurely terminated or suspended, the Principal Investigator will promptly inform study participants, the IEC, and sponsor and will provide the reason(s) for the termination or suspension. Study participants will be contacted, as applicable, and be informed of changes to the study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable

The study may resume once concerns about safety, protocol compliance, and data quality are addressed, and satisfy the sponsor, IEC and/or regulatory authorities.

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### 10.1.3 CONFIDENTIALITY AND PRIVACY

Participant confidentiality and privacy is strictly held in trust by the participating investigators, their staff, and the sponsor(s) and their interventions. This confidentiality is extended to cover testing of biological samples in addition to the clinical information relating to participants. Therefore, the study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the sponsor.

All research activities will be conducted in as private a setting as possible.

The study monitor, other authorized representatives of the sponsor, representatives of the IEC, regulatory agencies or pharmaceutical company supplying study product may inspect all documents and records required to be maintained by the investigator, including but not limited to, medical records (office, clinic, or hospital) and pharmacy records for the participants in this study. The clinical study site will permit access to such records.

The study participant's contact information will be securely stored at each clinical site for internal use during the study. At the end of the study, all records will continue to be kept in a secure location for as long a period as dictated by the reviewing IEC, Institutional policies, or sponsor requirements.

Study participant research data, which is for purposes of statistical analysis and scientific reporting, will be transmitted to and stored at the CRO (OPIS) working on behalf of the Sponsor. This will not include the participant's contact or identifying information. Rather, individual participants and their research data will be identified by a unique study identification number. Only the study centre will be able to link the study ID number to the patient's identity. The study data entry and study management systems used by clinical sites and by OPIS research staff will be secured and password protected.

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### 10.1.4 FUTURE USE OF STORED SPECIMENS AND DATA

Not applicable, samples of aqueous humour will be destroyed after the concentration analyses.

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### 10.1.5 SAFETY OVERSIGHT

Given the nature of the study, oversight of safety by a Data and Safety Monitoring Board was not deemed necessary.

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### 10.1.6 CLINICAL MONITORING

Clinical site monitoring is conducted to ensure that the rights and well-being of trial participants are protected, that the reported trial data are accurate, complete, and verifiable, and that the conduct of the trial is in compliance with the currently approved protocol/amendment(s), with International

Conference on Harmonisation Good Clinical Practice (ICH GCP), and with applicable regulatory requirement(s).

The field monitor will visit the site to check the completeness of patient records, the accuracy of entries on the eCRFs, the adherence to the protocol and to GCP, the progress of enrolment, and to ensure that study treatment is being stored, dispensed, and accounted for according to specifications. Key study personnel must be available to assist the field monitor during these visits.

The investigator must maintain source documents for each patient in the study, consisting of case and visit notes (hospital or clinic medical records) containing demographic and medical information, and Dose and Sampling Logs. All information on eCRFs must be traceable to these source documents in the patient's file. The investigator must also keep the original informed consent form signed by the patient (a signed copy is given to the patient).

Monitoring standards require full verification for the presence of informed consent, adherence to the inclusion/exclusion criteria, documentation of SAEs, and of data that will be used for all primary variables. Additional checks of the consistency of the source data with the eCRFs are performed according to the study-specific Monitoring Plan (MP). No information in source documents about the identity of the patients/subjects will be disclosed.

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#### 10.1.7 QUALITY ASSURANCE AND QUALITY CONTROL

Following written Standard Operating Procedures (SOPs), monitors will verify that the clinical trial is conducted and data are generated, documented (recorded), and reported in compliance with the protocol, International Conference on Harmonisation Good Clinical Practice (ICH GCP), GLP and applicable regulatory requirements.

The investigational site and central laboratory will provide direct access to all trial-related facilities, source data/documents, and reports for the purpose of monitoring and auditing by the sponsor, and inspection by local and regulatory authorities.

Independent audits may be conducted to ensure monitoring practices are performed consistently across all participating sites and that monitors are following the MP. Independent audits may be conducted by the Sponsor to ensure monitoring practices are performed consistently across all participating sites and that monitors are following the MP.

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## 10.1.8 DATA HANDLING AND RECORD KEEPING

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### 10.1.8.1 DATA COLLECTION AND MANAGEMENT RESPONSIBILITIES

#### **Data collection**

Designated investigator staff will enter the data required by the protocol into the eCRF using fully validated software that conforms to 21 CFR Part 11 requirements. Designated investigator site staff will not be given access to the electronic data capture system until they are trained.

Web-based software will be used and no installation procedure is needed. Each site will be authorized by the administrator to access the eCRF. Each site-qualified personnel will be allowed to access the eCRF by means of a 'login mask' requiring user ID and password and may read, modify, and update only the information previously reported at his or her site and according to their profile. Each page reports site code and subject code.

On-line validation programs will check for data discrepancies and, by generating appropriate error messages, allow the data to be confirmed or corrected before transfer to the CRO OPIS working on behalf of the sponsor. The investigator will certify that the data entered into the eCRF are complete and accurate.

Data generated by the central laboratory will automatically be transmitted to the study database according to a predefined transmission protocol.

After database lock, the investigator will receive a CD-ROM of subject data for archiving at the investigational site.

#### **Database management and quality control**

The CRO (OPIS) working on behalf of the Sponsor will review the data entered in the eCRF by investigational staff for completeness and accuracy and instruct site personnel to make any necessary corrections or additions. The Data Manager will perform the cleaning session by reviewing the warning messages raised by on-line checks and by running post-entry checks by means of validation programs and data listings specific for the study. If clarifications are needed, the Data Manager will raise queries by means of data query forms through the web application. Designated investigator site staff will be required to respond to queries and the Data Manager will make the correction to the database according to the responses.

Data collection and query flows, as well as the on-line and off-line checks, are detailed in the Data Management Plan and Data Validation documents.

Concomitant medications and prior medications entered into the database will be coded using the WHO Drug Reference List, which employs the Anatomic Therapeutic Chemical (ATC)

classification system. Medical history/current medical conditions and AEs will be coded using the MedDRA.

Randomization codes are tracked using the eCRF. The system is supplied by a CRO, OPIS s.r.l., who also manages the database.

The occurrence of any protocol deviations will be checked and the database will be locked and made available for data analysis after these actions have been completed and the database has been declared complete and accurate.

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#### 10.1.8.2 STUDY RECORDS RETENTION

The investigator/institution/central laboratory should maintain the trial documents as specified in Essential Documents for the Conduct of a Clinical Trial (ICH E6 Section 8) and as required by applicable regulations and/or guidelines. The investigator/institution/central laboratory should take measures to prevent accidental or premature destruction of these documents.

Essential documents (written and electronic) should be retained for a period of not less than twenty-five (25) years from the completion of the study unless the sponsor provides written permission to dispose of them or, requires their retention for an additional period because of applicable laws, regulations and/or guidelines. The subjects' medical files will be archived in accordance with the national laws.

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#### 10.1.9 PROTOCOL DEVIATIONS

A protocol deviation is any noncompliance with the clinical trial protocol, International Conference on Harmonisation Good Clinical Practice (ICH GCP). The noncompliance may be either on the part of the participant, the investigator, or the study site staff. As a result of deviations, corrective actions are to be developed by the site and implemented promptly.

These practices are consistent with ICH GCP:

- 4.5 Compliance with Protocol, sections 4.5.1, 4.5.2, and 4.5.3
- 5.1 Quality Assurance and Quality Control, section 5.1.1
- 5.20 Noncompliance, sections 5.20.1, and 5.20.2.

It is the responsibility of the site to avoid any protocol deviations and of the site staff and monitors to use continuous vigilance to identify and report deviations. All deviations must be addressed in study source documents.

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#### 10.1.10 INSURANCE

The Sponsor certifies that it has taken out a liability insurance policy covering this clinical trial. This insurance policy is in accordance with local laws and requirements. The insurance of the Sponsor does not relieve the Investigator and the collaborators from any obligation to maintain their own liability insurance policy. An insurance certificate will be provided to the IEC and/or regulatory authorities.

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#### 10.1.11 PUBLICATION AND DATA SHARING POLICY

All data and results and all intellectual property rights in the data and results derived from the study will be the property of the Sponsor, who may utilize them in various ways, such as for submission to government regulatory authorities or disclosure to other investigators.

This study will ensure that the public has access to the published results of the research.

As the study involves more than one center, the first publication must be related to data collected from all patients enrolled and analyzed under the Sponsor's responsibility. The investigator shall not publish or communicate data collected in only one center or part of the centers before the publication of the complete results of the study, unless prior written authorization from the Sponsor has been provided.

Any publication and/or communication project regarding the study and/or its results, whether obtained during the study or after the study end, shall be submitted to the Sponsor at least 30 days for a publication and 15 days for an abstract before the planned date of communication and/or submission for a publication. The Sponsor shall make comments on the project within 15 days of receipt of the project for a publication and within 7 days for an abstract. The investigator who submitted the project shall take the Sponsor's comments into due consideration. Nevertheless, should the investigator who submitted the project decide not to modify the project according to the Sponsor's comments, he/she shall provide the Sponsor with the grounds for his/her decision in writing.

The International Committee of Medical Journal Editors (ICMJE) member journals have adopted a clinical trials registration policy as a condition for publication. The ICMJE defines a clinical trial as any research project that prospectively assigns human subjects to intervention or concurrent comparison or control groups to study the cause-and-effect relationship between a medical intervention and a health outcome. Medical interventions include drugs, surgical procedures, devices, behavioural treatments, process-of-care changes, and the like. Health outcomes include any biomedical or health-related measures obtained in patients or subjects, including pharmacokinetic measures and adverse events. The ICMJE policy requires that all clinical trials be registered in a public trials registry such as ClinicalTrials.gov, which is sponsored by the National Library of Medicine. Other biomedical journals are considering adopting similar policies.



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#### 10.1.12 CONFLICT OF INTEREST POLICY

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the design and conduct of this trial. The study leadership has established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

## 10.2 ABBREVIATIONS

AE	Adverse Event
AH	Aqueous Humour
ATC	Anatomic Therapeutic Chemical
CNS	Coagulase-Negative Staphylococci
CONSORT	Consolidated Standards of Reporting Trials
CRF	Case Report Form
CRO	Contract Research Organization
ESCRS	European Society of Cataract and Refractive Surgeons
eCRF	electronic Case Report Forms
GCP	Good Clinical Practice
GLP	Good Laboratory Practice
HPLC -MS	High Performance Liquid Chromatography – Mass Spectrometry
ICH	International Conference on Harmonisation
ICMJE	International Committee of Medical Journal Editors
IEC	Independent Ethics Committee
IOP	Intraocular Pressure
IWRS	Interactive Web Response System
MedDRA	Medical Dictionary for Regulatory Activities
MP	Monitoring Plan
PT	Preferred Term
QC	Quality Control
SAE	Serious Adverse Event
SoA	Schedule of Activities
SOC	System Organ Class
SOP	Standard Operating Procedure
SUSAR	Suspected Unexpected Serious Adverse Reaction
WHO-DD	World Health Organization Drug Dictionary

## 11 REFERENCES


1. West et al., Epidemiology of risk factors for age-related cataracts. *Surv Ophthalmol* 1995;39:323–334.
2. Kelman CD et al., Phaco-emulsification and aspiration. A new technique of cataract removal. A preliminary report. *Am J Ophthalmol* 1967;64:23–35.
3. Li XM et., Investigation of dry eye disease and analysis of the pathogenic factors in patients after cataract surgery. *Cornea* 2007;26:S16–S20.
4. Rosado-Adames et al., The changing fate of the corneal endothelium in cataract surgery. *Curr Opin Ophthalmol* 2012;23:3–6.
5. Kasetsuwan N et al., Incidence and pattern of dry eye after cataract surgery. *PLoS ONE* 2013;8:e78657.
6. Dell SJ et al., Randomized comparison of postoperative use of hydrogel ocular bandage and collagen corneal shield for wound protection and patient tolerability after cataract surgery. *J Cataract Refract Surg* 2011;37:113–121.
7. Gonzales JA, Gritz DC, Channa R, et al. (2013): Nonsteroidal anti-inflammatory drugs versus corticosteroids for controlling inflammation after uncomplicated cataract surgery. *Cochrane Database of Syst Rev* (5): CD010516. doi: 10.1002/14651858.CD010 516.
8. Ong-Tone L, Bell A & Tan YY. Practice patterns of Canadian Ophthalmological Society members in cataract surgery: 2011 survey. *Can J Ophthalmol* 2012;47:124–130.
9. European Society of Cataract Refractive Surgery (2012): ESCRS Survey 2012 Available at: <http://www.analey.com>.
10. Behndig A, Cochener B, Guell JL et al. Endophthalmitis prophylaxis in cataract surgery: overview of current practice patterns in 9 European countries. *J Cataract Refract Surg*. 2013;39:1421–1431.
11. Gower EW, Lindsley K, Nanji AA et al. (2013): Perioperative antibiotics for prevention of acute endophthalmitis after cataract surgery. *Cochrane Database of Syst Rev* (7): CD006364. doi:10.1002/ 14651858.CD006364.pub2.
12. Barry P, Gardner S, Seal D, Gettinby G, Lees F, Peterson M & Revie C; ESCRS Endophthalmitis Study Group (2009): Clinical observations associated with proven and unproven cases in the ESCRS study of prophylaxis of post-operative endophthalmitis after cataract surgery.
13. Ta CN, Chang RT, Singh K et al. Antibiotic resistance patterns of ocular bacterial flora: a prospective study of patients undergoing anterior segment surgery; *Ophthalmology*. 2003;110: 1946-1951.
14. Barry P et Al. ESCRS Guidelines for prevention and treatment of endophthalmitis following cataract surgery: data, dilemmas and conclusions 2013. Available at: [www. es CRS.org](http://www.es CRS.org).
15. Porela-Tiihonen S. Recovery after cataract surgery *Acta Ophthalmol*. 2016; 94, thesis 2: 1–34.
16. Cagini C, Cometa F, Torroni G et al. Dexamethasone Disodium Phosphate Penetration Into the Human Aqueous Humor After Topical Application. *Curr Eye Res*. 2016;41(7):897-9.




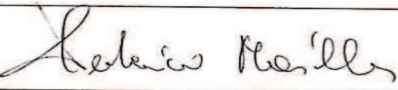
17. Bucci FA. An in vivo study comparing the ocular absorption of levofloxacin and ciprofloxacin prior to phacoemulsification; *Am J Ophthalmol.* 2004;137:308-312.
18. Healy DP, Holland EJ, Nordlund ML et al. Concentrations of levofloxacin, ofloxacin, and ciprofloxacin in human corneal stromal tissue and aqueous humor alter topical administration; *Cornea.* 2004;23:255-263.
19. Puustjarvi T, Terasvirta M, Nurmenniemi P et al. Penetration of topically applied levofloxacin 0.5% and ofloxacin 0.3% into the vitreous of the non-inflamed human eye; *Graefes Arch Clin Exp Ophthalmol* 2006;244:1633-1637.
20. Yamada M, Mochizuki H, Yamada K et al. Aqueous humor levels of topically applied levofloxacin in human eyes; *Curr Eye Res.* 2002;24:403-406.
21. Rosenblum C, Dengler RE Jr, Geoffroy RF. Ocular absorption of dexamethasone phosphate disodium by the rabbit. *Arch Ophthalmol* 1967;77(2):234-7.
22. McDonald TO, Kasten K, Hervey R, Gregg S, Smith D, Robb CA. Comparative toxicity of dexamethasone and its tertiary butyl acetate ester after topical ocular instillation in rabbits. *Am J Ophthalmol.* 1973 Jul;76(1):117-25.
23. Weijtens O, Schoemaker RC, Romijn FP, Cohen AF, Lentjes EG, van Meurs JC. Intraocular penetration and systemic absorption after topical application of dexamethasone disodium phosphate. *Ophthalmology.* 2002;109(10):1887-91.
24. DeCroos FC & Afshari NA: Perioperative antibiotics and anti-inflammatory agents in cataract surgery. *Curr Opin Ophthalmol.* 2008;19:22–26.
25. O. Weijtens, Schoemaker RC, Fred P et al. Intraocular penetration and systemic absorption after topical application of dexamethasone disodium phosphate *Ophthalmology* 2002;109:1887-1891.
26. K. Sundelin Seal D, Gardner S et al. Increased anterior chamber penetration of topical levofloxacin 0.5% after pulse dosing in cataract patients. *Acta Ophthalmol.* 2009;87:160-165.

## 12 APPENDICES

- 12.1 Laboratory Protocol
- 12.2 Oxford Grading Scheme

## 12.1 LABORATORY PROTOCOL

	<p><b>STUDY PROTOCOL</b> Code: GCLP022/01</p>	<p>Page. 1 of 27</p>
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<p>Study title :</p>	<p><b>QUANTITATIVE DETERMINATION OF LEVOFLOXACINE, DEXAMETHASONE 21- PHOSPHATE AND ITS METABOLITE DEXAMETHASONE IN HUMAN AQUEOUS HUMOR SAMPLES BY LC/MS/MS</b></p>	
	<p>Signature PRC TICINUM LAB</p>	<p>Date</p>
<p>Approved by Study Director: Donatella Dellavecchia</p>		<p>13/03/18</p>
<p>Approved by PRC Ticinum Lab Director: Luisa Zangirolami</p>		<p>13.03.18</p>
<p>Audited by QA: Clara Giordanino</p>		<p>13/03/18</p>
	<p>Signature Opis S.r.l.</p>	<p>Date</p>
<p>Authorised by: Federico Mailland</p>		<p>13/03/2018</p>
<p>Study Code</p>	<p>GCLP022/01</p>	
<p>Document history</p>	<p>March 2018: revision 00, first edition revision 01, typing error, addition of long term stability</p>	
<p>Distribution list</p>	<p>PRC Ticinum Lab Opis S.r.l.</p>	



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## **1 INTRODUCTION**

### **1.1 AIM OF THE STUDY**

The aim of this study is to perform a full validation to quantify Levofloxacin, Dexamethasone 21-phosphate and its metabolite Dexamethasone in human aqueous humor to demonstrate that it is suitable for its intended purpose.

Substantially, the method consist of IS addition, precipitation with methanol, then chromatographic separation under gradient conditions and MS/MS detection. The method will be validated to investigate matrix effect, recovery efficiency, within and between-run accuracy, within and between-run precision, linearity, lower limit of quantitation, carry-over effect, dilution integrity, short-term stability in matrix and in frozen matrix, freeze-thaw cycles stability, autosampler stability, to fulfil the requirements of the recently issued EMA's Guideline on bioanalytical method validation [1] and it will be performed in accordance with PRC Ticinum Lab SOPs.

Because the human aqueous humor is a rare matrix and its collection is difficult (since it is only possible by anterior chamber paracentesis), as deviation from EMA's Guideline on bioanalytical method validation [1], method selectivity will be evaluated on a single batch of artificial aqueous humor (AHA) instead on matrix coming from six different sources.

Artificial aqueous humor (AHA) will be recreated and utilised as matrix during the validation study. According to literature [2], no differences in the analysis of ophthalmological drugs were found between artificial and human aqueous humor; thus, the artificial one may be used to generate a matrix-based standard curve for method analytes quantification.

Long-term stability in matrix at -80°C will be also evaluated at 1, 3, 6 and 12 months and the obtained results will be reported in interim reports issued at each time points.

Stability of analytes stock solutions has been already validated by Accelera S.r.l. during the validation studies "Eye Drop combi, Levofloxacin/Dexamethasone Eye Drops Solution: Validation of an Analytical Method for the Determination of Levofloxacin in the Rabbit Aqueous Humor by LC-MS/MS" (Report Study 2017-0050) and "Eye Drop combi, Levofloxacin/Dexamethasone Eye Drops Solution: Validation of an Analytical Method for the Determination of Dexamethasone 21-phosphate and Dexamethasone in the Rabbit Aqueous Humor by LC-MS/MS" (Report Study 2017-0051) and do not need to be re-validated.

Stability of Internal Standard stock solutions will be evaluated.



## **1.2 STUDY INFORMATION**

**Study code:** GCLP022/01  
**Study initial date:** Last approval date  
**Study Director:** Donatella Dellavecchia  
(PRC TICINUM LAB, via Bovio 6, 28100 Novara)  
**Study Sponsor:** Opis srl.  
**Head office:** Via Matteotti, 10  
20832 Desio (MB)  
**Contact person:** Dott. Raphaela Schnurbus

## **1.3 REGULATORY COMPLIANCE**

This study will be conducted in accordance with the GCLP regulations of:

- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004;
- ENV/MC/CHEM(98)17 "OECD principles on Good Laboratory Practice – as revised in 1997";
- DM 19/03/1998.

In addition, the study is designed based on the experimental methods indicated in the following guidelines:

- EMEA/CHMP/EWP/192217/2009 "Guideline on bioanalytical method validation", adoption by CHMP date July, 21st 2011.

## **1.4 RESPONSIBILITIES**

The analytical study will be carried out at PRC Ticinum Lab (via Bovio 6, 28100 Novara, Italy) under the responsibility of the Study Director.

Analytical validation study protocol and further amendments or revision will be issued only after proper authorization by the Study Sponsor. In order to achieve this, PRC Ticinum Lab will issue the final Analytical validation study protocol or amendment to the Analytical validation study protocol which will be signed by the Study Director and the Sponsor Representative.

It is responsibility of PRC Ticinum Lab Study Director to carry out the study according to Analytical validation study protocol; to describe, in the final report, the results obtained in relation to the acceptance criteria and to report and justify any deviation related to the Analytical validation study protocol.

It is responsibility of PRC Ticinum Lab to describe results of long-term stability in matrix at -80°C in interim reports issued at each time points.

### **1.5 ARCHIVING**

The original data (protocol and eventual amendments or revision, raw data, final report, records and documentation) generated during the course of the study will be filed in PRC Ticinum Lab archives for a period of ten years since the data of the final report's issue, according to internal procedures.

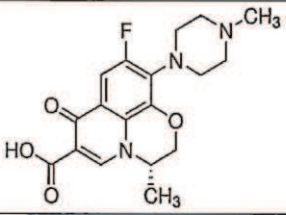
### **1.6 ACRONYMS**

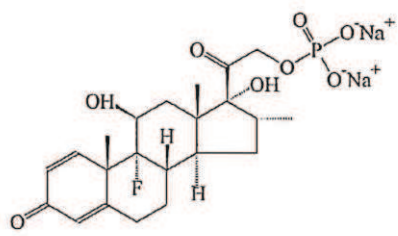
AS	Autosampler
EMA	European Medicines Agency
CHMP	Committee for Medicinal Products for Human Use
MF	Matrix Factor
MS/MS	Tandem Mass Spectrometry
MRM	Multiple Reaction Monitoring
GLP	Good Laboratory Practice
HPLC	High Performance Liquid Chromatography
N/A	Not Applicable
PCF	Purity Correction Factor
IS	Internal Standard
QA	Quality Assurance
SOP	Standard Operation Procedure
S/N	Signal to Noise ratio
CV	Coefficient of Variation
CC	Calibration Curve
QC	Quality control
LLOQ	Lower Limit Of Quantitation
ULOQ	Upper Limit Of Quantitation
LQC	Low Quality Control
MQL	Medium Quality Control
HQL	High Quality Control
WS	Working Solution
rpm	rotations per minute

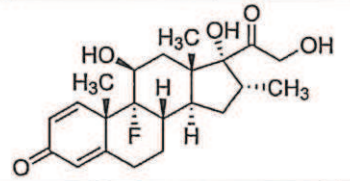


## 1.7 ANALYTICAL STANDARDS

### 1.7.1 Test items

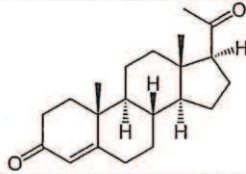
Identification	Levofloxacin	
Molecular Formula	$C_{18}H_{20}FN_3O_4$	
Lot/Batch Number	R07580	
Purity	97.3 %.	
Storage Conditions	Room temperature	
Source and Manufacturer	USP reference standard	
Molecular Weight	361.37 g/mol	
Special Handling Precautions	Usual protection of all personnel conducting the study (mask, gloves and eyeglasses) or According to MSDS (Material Safety Data Sheet)	

Identification	Dexamethasone Sodium Phosphate	
Molecular Formula	$C_{22}H_{30}F Na_2O_8P$	
Lot/Batch Number	R06110	
Purity	99.7%	
Storage Conditions	5°C	
Source and Manufacturer	USP reference standard	
Molecular Weight	516.41 g/mol	
Special Handling Precautions	Usual protection of all personnel conducting the study (mask, gloves and eyeglasses) or According to MSDS (Material Safety Data Sheet)	

Identification	Dexamethasone	
Molecular Formula	$C_{22}H_{29}FO_5$	
Lot/Batch Number	R00520	
Purity	99.2 %	
Storage Conditions	Room temperature	
Source and Manufacturer	USP reference standard	
Molecular Weight	392.46 g/mol	
Special Handling Precautions	Usual protection of all personnel conducting the study (mask, gloves and eyeglasses) or According to MSDS (Material Safety Data Sheet)	

PCF should be applied

### 1.7.2 Internal Standard

Identification	Progesterone	
Molecular Formula	C <sub>21</sub> H <sub>30</sub> O <sub>2</sub>	
Lot/Batch Number	SLBQ9723V	
Purity and Expiry	100% - 12 Jan 2019	
Storage Conditions	Room temperature	
Source and Manufacturer	Sigma-Aldrich	
Molecular Weight	314,47 g/mol	
Special Handling Precautions	Usual protection of all personnel conducting the study (mask, gloves and eyeglasses) or According to MSDS (Material Safety Data Sheet)	

### 1.8 VALIDATION PARAMETERS AND ACCEPTANCE CRITERIA

Data obtained from the entire test will be calculated, in order to verify linearity, precision and accuracy of the method. Table n°1 summarizes the test parameters and acceptance criteria.



**Table 1: Test parameters and acceptance criteria for the method validation study**

PARAMETERS	TEST	ACCEPTANCE CRITERIA
Selectivity	Analyze 6 different blank AHA samples and compare with the 6 different samples at LLOQ level. <sup>1</sup>	Absence of interfering components is accepted where the response is $\leq 20\%$ of the LLOQ for the analytes, and $\leq 5\%$ for the IS.
Matrix effect	Verify, for each level of concentration, the matrix factor (MF) for the analytes and for IS Calculate the CV at LQC and HQC of IS-normalized MF using 6 samples in matrix for each level. Verify IS-normalized MF for AHA, for AHA containing Benzalkonium chloride, for AHA containing oxibuprocaine and for AHA containing iodopovidone	Precision (CV) $\leq 15\%$
Linearity	Calculate correlation coefficient (r) and accuracy (%) of back-calculated concentration obtained from 7 non-zero calibration standard defined by LLOQ and ULOQ. Linearity is evaluated on at least three calibration curves.	$r \geq 0.99$ Accuracy: $\pm 15\%$ of nominal value ( $\pm 20\%$ only at LLOQ) At least 75% of non-zero calibration standard must fulfill this criterion.
Carry-over effect	Analyze one Blank matrix sample injected after ULOQ.	Absence of carry-over effect is accepted where the response is $\leq 20\%$ of the LLOQ for the analytes, and $\leq 5\%$ for the IS.
LLOQ	Calculate S/N ratio, accuracy and precision by analysis of at least 5 independent LLOQ samples in matrix	$S/N \geq 5$ Accuracy: $\pm 20\%$ Precision (CV) $\leq 20\%$
Within-run accuracy and precision	Calculate accuracy and precision by analysis of 6 samples per concentration at 4 different concentration extracted from matrix 6 x LLOQ 6 x LQC 6 x MQC 6 x HQC	Accuracy: $\pm 15\%$ of nominal value ( $\pm 20\%$ only at LLOQ) Precision (CV) $\leq 15\%$ ( $\leq 20\%$ only at LLOQ)  At least 67% of QCs concentration results should be within 15% of their respective nominal values. At least 50% of QCs at each level should be within 15% of their nominal concentration

<sup>1</sup> Because the aqueous humor is a rare matrix and its collection is difficult (since it is only possible by anterior chamber paracentesis), As deviation from EMA's Guideline on bioanalytical method validation [1], method selectivity will be evaluated on a single batch of artificial aqueous humor (AHA) instead on matrix coming from six different sources.

**Table 1 (continue): Test parameters and acceptance criteria for the method validation study**

PARAMETERS	TEST	ACCEPTANCE CRITERIA
Between-run accuracy and precision	Calculate accuracy and precision by analysis of 3 samples per concentration at 4 different concentration extracted from matrix on 5 separated analytical runs, in at least 2 days: 3 x LLOQ 3 x LQC 3 x MQC 3 x HQC	Accuracy: $\pm 15\%$ of nominal value ( $\pm 20\%$ only at LLOQ)  Precision (CV) $\leq 15\%$ ( $\leq 20\%$ only at LLOQ)  At least 67% of QCs concentration results should be within 15% of their respective nominal values. At least 50% of QCs at each level should be within 15% of their nominal concentration
Dilution integrity	Calculate accuracy and precision by analysis of 5 independent samples per concentration at two different concentration higher than ULOQ, 5 x > ULOQ 10 times concentrated 5 x > ULOQ 100 times concentrated after 1:10 and 1:100 dilution with blank matrix Analyze these samples against the calibration curve.	Mean Accuracy: $\pm 15\%$ of nominal value  Precision (CV) $\leq 15\%$
System Suitability Test	Analyze one blank matrix sample.  Analyze 1 LLOQ sample.	Absence of interfering compounds ( $\leq 20\%$ of the LLOQ for the analytes, and $\leq 5\%$ for the IS). Confirm retention time of analytes and IS. Analytes S/N $\geq 5$ .



## **2 MATERIALS, REAGENTS AND METHODS**

### **2.1 MATERIALS AND REAGENTS**

#### **2.1.1 *Materials***

Acetonitrile: LC/MS grade  
Water: LC/MS grade  
Methanol: HPLC grade  
Iso-Propanol: HPLC grade  
Ammonium formiate: for analysis  
98-100% Formic acid: for analysis  
Sodium chloride: for analysis  
Potassium chloride: for analysis  
Calcium chloride: for analysis  
Sodium hydrogen carbonate: for analysis  
glucose: for analysis  
Urea: for analysis  
Albumin: for analysis  
Sodium-lactate: for analysis  
Sodium-ascorbate: for analysis

#### **2.1.2 *Reagents***

*In-vitro* artificial aqueous humor:

AHA is prepared by dissolving NaCl (6.19 g/L), KCl (0.39 g/L), CaCl<sub>2</sub> (0.28 g/L), NaHCO<sub>3</sub> (2.90 g/L), glucose (0.80 g/L), Urea (0.10 g/L), albumin (0.10 g/L), Na-lactate (0.28 g/L) and Na-ascorbate (0.15 g/L) in water. Each substance is added separately in the above mentioned order. The solution is to be stirred constantly and kept at room temperature. Na-lactate and Na -ascorbate are added last and the solution is then adjusted at pH 7.21 with HCl 1M. Aqueous humor is centrifuge at 4000 rpm for 1 minute, supernatant is stored at 4°C until analysis.

1M HCOONH<sub>4</sub>: Weight 6.6 g of HCOONH<sub>4</sub> and transfer in a 100 mL graduated flask, then dilute with H<sub>2</sub>O to volume.



10 mM HCOONH<sub>4</sub>: 10 mL of 1M HCOONH<sub>4</sub> are diluted to 1 L with water

### 2.1.3 Hazard and Precautionary statements

Acetonitrile: H225, H302 + H332 + H312, H319  
P210, P280, P305 + P351 + P338

Methanol: H225, H303 + H311 + H331, H370  
P210, P260, P280, P301 + P310, P311

Ammonium acetate: n/a

Formic acid: H226, H314  
P280, P305 + P351 + P338, P310

## 2.2 DETERMINATION BY LC-MS/MS

### 2.2.1 Instrument

**HPLC:** Agilent 1260 Infinity Series

**Detector:** ABSciexAPI-4500 Triple Quadrupole

**Data system:** Analyst ver. 1.6

### 2.2.2 Chromatographic conditions

**Column:** Thermo Fisher HYPERSYL GOLD C 50x3mm, 3µm

**Mobile phase A:** 1 L 10 mM ammonium formiate + 1 mL formic acid

**Mobile phase B:** 500 mL Acetonitrile + 500 mL Methanol + 1 mL formic acid

**Needle wash:** 50 mL *iso*-Propanol + 450 mL Acetonitrile + 500 mL Methanol + 1 mL formic acid

**Elution mode:** gradient

Time (min)	A %	B%
0	90	10
6	0	100
9	0	100
9.5	90	10
15	90	10

**Flow rate:** 0.7 mL/min

**Column temperature:** Nominally 40°C

**Injection volume:** 2 µL

**Autosampler temperature:** Nominally at 10°C

### 2.2.3 Mass-Spectrometry conditions

**Source:** Turbo Ion Spray (Positive Polarity)

**Source temperature:** 500°C

**Con Voltage:** 5500 eV

**Desolvation / nebulizer gas:** 50/50

**Divert Valve to source:** from 0.5 minutes to 6 minutes

**Ions Monitored:**

**MRM**

ID	Q1 mass amu	Q3 mass amu	CE	Dwell time (msec)
Levofloxacin	362.1	261.0	40	90
Levofloxacin *	362.1	318.0	30	90
Dexamethasone	393.2	373.1	15	90
Dexamethasone*	393.2	355.0	15	90
Dexamethasone 21-phosphate	473.1	435.1	18	90
Dexamethasone 21-phosphate*	473.1	355.0	18	90
Progesterone	315.10	109.0	50	90
Progesterone*	315.10	297.1	35	90

*\* for qualitative use*

Instrument parameters may be adjusted, according to the instrument changes over the time.

## 2.3 STANDARD SOLUTIONS

### 2.3.1 Stock and working standard solutions for calibration sample.

#### Levofloxacin Stock Standard Solution-1

Weigh accurately about 10 mg of Levofloxacin into a 10 mL volumetric flask, dissolve with methanol then dilute to volume with the same solvent and mix. The concentration of this solution is 2.767 µmol/mL.



Dexamethasone 21-phosphate Stock Standard Solution-1

Weigh accurately about 10 mg of Dexamethasone sodium 21-phosphate into a 10 mL volumetric flask, dissolve with water/methanol 50/50 (v/v) then dilute to volume with the same solvent and mix. The concentration of this solution is 1.936  $\mu\text{mol/mL}$  of Dexamethasone 21-phosphate.

Dexamethasone Stock Standard Solution-1

Weigh accurately about 10 mg of Dexamethasone into a 10 mL volumetric flask, dissolve with water/methanol 50/50 (v/v) then dilute to volume with the same solvent and mix. The concentration of his solution is 2.548  $\mu\text{mol/mL}$ .

Intermediate Standard Solution-1a

Transfer 1 mL of Levofloxacin and Dexamethasone Stock Standard Solution-1 into a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix. The concentration of this solution is 0.277  $\mu\text{mol/mL}$  of Levofloxacin and 0.255  $\mu\text{mol/mL}$  dexamethasone.

Intermediate Standard Solution-1b

Transfer 1 mL of Dexamethasone 21-phosphate Stock Standard Solution-1 into a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix. The concentration of this solution is 0.194  $\mu\text{mol/mL}$  dexamethasone 21-phosphate.

Working Standard Solution A

Transfer 1 mL of Intermediate Standard Solution-1a and -1b in a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

Working Standard Solution B

Transfer 0.9 mL of Intermediate Standard Solution-1a and -1b in a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

Working Standard Solution C

Transfer 0.5 mL of Intermediate Standard Solution-1a and -1b in a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

Working Standard Solution D

Transfer 0.1 mL of Intermediate Standard Solution-1a and -1b in a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

Working Standard Solution E

Transfer 0.5 mL of Intermediate Standard Solution-1a and -1b in a 100 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

#### Working Standard Solution F

Transfer 0.1 mL of Intermediate Standard Solution-1a and 0.4 mL of Intermediate Standard Solution -1b in a 100 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

#### Working Standard Solution G

Transfer 0.05 mL of Intermediate Standard Solution-1a and 0.3 mL of Intermediate Standard Solution -1b in a 100 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

Store all the solutions at nominally 4°C.

### *2.3.2 Stock and working standard solutions for QC sample.*

#### Levofloxacin Stock Standard Solution-2

Weigh accurately about 10 mg of Levofloxacin into a 10 mL volumetric flask, dissolve with methanol then dilute to volume with the same solvent and mix. The concentration of this solution is 2.767  $\mu\text{mol/mL}$ .

#### Dexamethasone 21-phosphate Stock Standard Solution-2

Weigh accurately about 10 mg of Dexamethasone into a 10 mL volumetric flask, dissolve with water/methanol 50/50 (v/v) then dilute to volume with the same solvent and mix. The concentration of his solution is 2.548  $\mu\text{mol/mL}$ .

#### Dexamethasone Stock Standard Solution-2

Weigh accurately about 10 mg of Dexamethasone into a 10 mL volumetric flask, dissolve with water/methanol 50/50 (v/v) then dilute to volume with the same solvent and mix. The concentration of his solution is 2.548  $\mu\text{mol/mL}$ .

#### Intermediate Standard Solution-2a

Transfer 1 mL of Levofloxacin and Dexamethasone Stock Standard Solution-2 into a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix. The concentration of this solution is 0.277  $\mu\text{mol/mL}$  of Levofloxacin and 0.255  $\mu\text{mol/mL}$  dexamethasone.

#### Intermediate Standard Solution-2b

Transfer 1 mL of Dexamethasone 21-phosphate Stock Standard Solution-2 into a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix. The concentration of this solution is 0.194  $\mu\text{mol/mL}$  dexamethasone 21-phosphate.



#### Working Standard Solution H

Transfer 0.8 mL of Intermediate Standard Solution-2a and -2b in a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

#### Working Standard Solution I

Transfer 1 mL of Working Standard Solution H in a 10 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

#### Working Standard Solution L

Transfer 0.15 mL of Intermediate Standard Solution-2a and 0.45 mL of Intermediate Standard Solution -1b in a 100 mL volumetric flask, then dilute to volume with water/methanol 50/50 (v/v) and mix.

Store all the solutions at nominally 4°C.

### *2.3.3 Internal Standard solutions*

#### IS Stock Standard Solution

Weigh accurately about 10 mg of Progesterone into a 10 mL volumetric flask, dissolve with methanol, then dilute to volume with the same solvent and mix.

#### IS Intermediate Standard Solution

Transfer 1 mL of IS Stock Standard Solution (1000 µg/mL) in a 100 mL volumetric flask, then dilute to volume with methanol and mix.

#### IS Working Standard Solution (1.590 nmol/mL)

Transfer 2.5 mL of IS Intermediate Standard Solution (10 µg/mL) in a 50 mL volumetric flask, then dilute to volume with methanol and mix.

Store the solution at nominally 4°C.

## *2.4 BIOANALYTICALPROCEDURE*

### *2.4.1 Preparation of calibration standard (CC) and quality control (QC) samples in artificial aqueous humor (AHA)*

Use an seven-points calibration curve and three-level of QCs.

Prepare each calibration and QC sample by adding 1 mL of each Working Standard Solution from A to L to 9 mL of artificial aqueous humor (AHA), according to the following scheme:

WS concentration (nmol/mL)			AHA concentration (nmol/mL)			ID	SAMPLE
	Levo	DEX	DEX 21	Levo	DEX	DEX 21	
A	27.672	25.480	19.364	2.767	2.548	1.936	CC A
B	24.905	22.932	17.428	2.491	2.293	1.743	CC B
C	13.836	12.740	9.682	1.384	1.274	0.968	CC C
D	2.767	2.548	1.936	0.277	0.255	0.194	CC D
E	1.384	1.274	0.968	0.138	0.127	0.097	CC E
F	0.277	0.255	0.775	0.028	0.025	0.078	CC F
G	0.138	0.127	0.581	0.014	0.013	0.058	CC G
H	22.138	20.384	15.492	2.214	2.038	1.549	HQC
I	2.214	2.038	1.549	0.221	0.204	0.155	MQC
L	0.415	0.382	0.871	0.042	0.038	0.087	LQC

where *Levo* is levofloxacin, *DEX* is dexamethasone and *DEX 21-P* is dexamethasone 21-phosphate

Divide each standard sample in AHA into 20  $\mu$ L aliquots, and store at nominally -80°C until analysis. Process calibration curve and QCs samples exactly as described in §2.4.2. Each calibration curve will include a blank sample (AHA sample processed without internal standard) and a zero blank sample (AHA sample processed with internal standard).

#### 2.4.2 Sample preparation to analyse in AHA

Transfer 20  $\mu$ L of AHA sample to a 1.5mL Eppendorf polypropylene tube; add 20  $\mu$ L of the IS working standard solution and mix well by vortex for about 10sec. Centrifuge at 13000 rpm for 1 min at nominally 4°C, then transfer the supernatant in a autosampler plate and inject.

### 3 DATA PROCESSING

All the data are processed using Analyst software version 1.6.2 associated with the mass spectrometer. Calibration curves are constructed by plotting the peak area ratio of Levofloxacin, Dexamethasone 21-phosphate and Dexamethasone to IS against the theoretical concentration of the analytes.

The concentration values in the study samples are directly calculated by the software using an optimal calibration curve defined during the method validation. Integration will be performed by the software or manually by the operator if the peak shape is anomalous or if the baseline is noisy. The manual integration is reported on the summary table elaborated by the software in the column “record modified”, according to the TicinumLab SOP GCP004.



## **4 METHOD VALIDATION**

### **4.1 SELECTIVITY**

Selectivity is the ability to differentiate the analyte of interest and the IS from the endogenous components in the matrix or other component in the sample.

#### **4.1.1 Procedure**

Analyse 6 different samples in matrix at LLOQ level and compare with of blank artificial aqueous humor (AHA) to verify the interferences with analytes and IS.

#### **4.1.2 Calculation**

Absence of interfering components is accepted if the mean response in blank is  $\leq 20\%$  of the LLOQ for the analytes and  $\leq 5\%$  for the IS.

### **4.2 LINEARITY**

The linearity of an analytical procedure is its ability (within a given range) to obtain results which are directly proportional to the concentration (amount) of analyte in the sample.

#### **4.2.1 Procedure**

Prepare working standard solutions and IS working standard solutions as described in §2.3.1, 2.3.3 and the AHA samples as described in §2.4.1, process the samples as described in §2.4.2

Prepare a blank sample (only blank AHA sample processed) and a zero blank (blank AHA sample processed with internal standard). Inject the samples as described in the following sequence:

N.	SAMPLE
1	Blank
2	Zero blank
4	G
5	F
6	E
7	D
8	C
9	B
10	A

#### 4.2.2 Calculation

Determine the calibration curve that better link nominal analytes AHA concentration to the peak area ratio analyte / internal standard.

Linear or quadratic regression may be used and appropriate weighting factors may be explored and justified, however this weighting factor must be consistent throughout the study.

Evaluate the goodness of the fitting by linear correlation coefficient (r), this should be greater than 0.99.

The back calculated concentrations of CC standards should be within  $\pm 15\%$  of the nominal value, except for LLOQ for which it should be  $\pm 20\%$ . At least 75% of CC must fulfill this criterion.

Linearity is evaluated on at least three calibration curves.

#### 4.3 CARRY-OVER EFFECT

Carry-over effect is assessed by injecting blank AHA sample after CC sample at the ULOQ.

##### 4.3.1 Procedure

Prepare and inject blank AHA sample after CC sample at the ULOQ.

##### 4.3.2 Calculation

Absence of carry-over effect is accepted where the response is  $\leq 20\%$  of the LLOQ for the analytes, and  $\leq 5\%$  for the internal standard.

#### 4.4 LOWER LIMIT OF QUANTITATION (LLOQ)

The lower limit of quantitation is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

The analytical signal of LLOQ should be at least 5 times the signal of the blank sample and it should have accuracy and precision within  $\pm 20\%$  in within-run assay and between-run assay as described in §4.5.

#### 4.5 WITHIN AND BETWEEN-RUN ACCURACY AND PRECISION

The accuracy of an analytical procedure expresses the closeness of the determined value obtained by the method to the nominal concentration of the analyte (expressed in percentage).

The precision of the analytical method describes the closeness of repeated individual measures of analyte and it is expressed as the coefficient of variation (CV).



- Within-run accuracy and precision will be determined by analyzing in a single run 6 samples per level at 4 level of concentration (LLOQ, LQC, MQC, HQC)
- Between-run accuracy and precision: determined by analyzing in five runs, performed in different days, 3 samples per level at 4 level of concentration (LLOQ, LQC, MQC, HQC).

#### 4.5.1 Procedure

Accuracy and precision are assessed on AHA sample spiked with known amount of analyte, quality control (QC). QC samples are prepared independently from the calibration standards (CC) and are analyzed against the calibration curve.

Prepare working standard solutions and IS working standard solutions as described in § 2.3.1, 2.3.2 and 2.3.3 and the AHA samples as described in § 2.4.1; process AHA samples as described in § 2.4.2. For within-run assay during a single analytical run, inject the samples according to the following sequence:

N.	SAMPLE	N.	SAMPLE
1	Blank	18	LQC 3
2	Zero-blank	19	LQC 4
3	LLOQ 1 (G)	20	LQC 5
4	LLOQ 2	21	LQC 6
5	LLOQ 3	22	MQC 1
6	LLOQ 4	23	MQC 2
7	LLOQ 5	24	MQC 3
8	LLOQ 6	25	MQC 4
9	F	26	MQC 5
10	E	27	MQC 6
11	D	28	HQC 1
12	C	29	HQC 2
13	B	30	HQC 3
14	A	31	HQC 4
15	Blank	32	HQC 5
16	LQC 1	33	HQC 6
17	LQC 2		

For between-run assay, for 5 different analytical runs over at least two different days, inject the samples according to the following sequence:

N.	SAMPLE	N.	SAMPLE
1	Blank	12	Blank
2	Zero-blank	13	LQC 1
3	LLOQ 1 (G)	14	LQC 2
4	LLOQ 2	15	LQC 3
5	LLOQ 3	16	MQC 1
6	F	17	MQC 2
7	E	18	MQC 3
8	D	19	HQC 1
9	C	20	HQC2
10	B	21	HQC3
11	A		

#### 4.5.2 Calculations

Accuracy: evaluate each point accuracy according to the following formula:

$$\% Acc = \frac{x_j - x_i}{x_i} * 100$$

where:

$x_j$  = found concentration of j-eme analyte

$x_i$  = actual concentration of j-eme analyte

Accuracy (%) in within-run assay (mean value of 6) and Accuracy (%) in between-run assay (mean value of 18) should be  $\pm 15\%$ , except for LLOQ level which should be  $\pm 20\%$ .

Precision: calculate precision coefficient of variation CV(%), equivalent to relative standard deviation according to USP XXIII, page 1982.

CV% in within-run assay (mean value of 6) and CV% in between-run assay (mean value of 18) should be  $\leq 15\%$ , except for LLOQ level which should be  $\leq 20\%$ .

At least 67% of QCs concentration results should be within 15% of their respective nominal values.  
At least 50% of QCs at each level should be within 15% of their nominal concentration

#### 4.6 MATRIX EFFECT

Matrix effect is evaluated in order to check if the matrix may affect the accuracy of the determination. This determination is performed at a low and at a high level of concentration (LQC and HQC) using matrixes.



#### 4.6.1 Procedure

Analyze LQC level (blank matrix spiked with analytes after extraction) in matrix and at the same level (LQC) in absence of matrix (water/methanol 50/50 (v/v)). Repeat the analysis at HQC level. Using the same approach, Matrix effect is also evaluated in order to verify if oxibuprocaine<sup>2</sup> (use as anesthetic before the surgical procedure), iodopovidone (use as antimicrobial) and benzalkonium chloride (present in the drug product formulation) affect the determination. Oxibuprocaine, iodopovidone and benzalkonium chloride levels that should be included in aqueous humor in order to define the matrix effect is defined according to literature. Mean values plus 2 times the standard deviation (SD) is tested.

Benzalkonium chloride (BAK) level found in rabbit aqueous humor after 15 drops of 0.05% BAC dosage [3] is  $0.52 \pm 0.13$  µg/mL: Considering this value as mean  $\pm$  Standard Error (worst case) and being the study performed on 24 eyes, SD is  $0.637^3$ . According to this approach, BAK is tested at 1.8 µg/mL in AHA. This is a worst case if compared to drug product posology in clinical study (2 drops of drug product containing 0.1 mg/mL of BAK).

According to literature [4], lidocaine levels in human aqueous humor after administration of 3 and 6 drops of anesthetic at 4 mg/mL are  $1.4 \pm 0.5$  µg/mL and  $4.2 \pm 1.5$  µg/mL (Mean  $\pm$  SD) respectively. Assuming that the same absorption for oxibuprocaine, it is tested at 7.2 µg/mL in AHA, worst case if compared to anesthetic posology (3 drops).

Iodopovidone (0.5% iodine) is used as antimicrobial and is not instilled inside the eye. It is tested at 7.2 µg/mL in AHA, assuming an absorption similar to oxibuprocaine (worst case).

Prepare WS 1 containing 2.214 nmol/mL of levofloxacin, 2.038 nmol/mL dexamethasone, 1.549 nmol/mL of dexamethasone 21-phosphate and 1.59 nmol/mL of IS in methanol.

Prepare WS 2 containing 0.042 nmol/mL of levofloxacin, 0.038 nmol/mL dexamethasone, 0.087 nmol/mL of dexamethasone 21-phosphate and 1.59 nmol/mL of IS in methanol.

Prepare LQC and HQC in solvent diluting 20 µL of WS with 20 µL of water (use WS1 for HQC and WS2 for LQC)

Prepare LQC and HQC in matrix (use WS1 for HQC and WS2 for LQC):

Matrix 1: 20 µL of WS with 20 µL of AHA

Matrix 2: 20 µL of WS with 20 µL of AHA containing BAK 0.8 µg/mL

<sup>2</sup> Lidocaine may be considered as backup, in case of oxibuprocaine matrix effect.

<sup>3</sup>  $SD = SE \times \sqrt{n}$ , where  $n = 24$

Matrix 3: 20 µL of WS with 20 µL of AHA containing oxibuprocaine 7.2 µg/mL

Matrix 4: 20 µL of WS with 20 µL of AHA containing iodiopovidone (0.5% iodine) 7.2 µg/mL

Inject the samples as described in the following sequence:

N.	SAMPLE	N.	SAMPLE
1	LQC solvent	13	HQC solvent
2	LQC matrix	14	HQC matrix
3	LQC solvent	15	HQC solvent
4	LQC matrix	16	HQC matrix
5	LQC solvent	17	HQC solvent
6	LQC matrix	18	HQC matrix
7	LQC solvent	19	HQC solvent
8	LQC matrix	20	HQC matrix
9	LQC solvent	21	HQC solvent
10	LQC matrix	22	HQC matrix
11	LQC solvent	23	HQC solvent
12	LQC matrix	24	HQC matrix

### Calculation

Verify, for each level of concentration, the matrix factor (MF) for the analytes and for IS by calculating the ratio of peak area in presence of matrix to the peak area in absence of matrix according to the following formulas:

$MF_{Analyte} = \text{Analyte peak area in presence of matrix} / \text{Analyte peak area in absence of matrix}$

$MF_{IS} = \text{IS peak area in presence of matrix} / \text{IS peak area in absence of matrix}$

IS normalized MF is also calculated by dividing the MF of analyte by the MF of IS according to the following formula:

$$IS \text{ normalised } MF = \frac{MF_{Analyte}}{MF_{IS}}$$

The CV% of the IS normalized MF at both concentration levels should be lower than 15%.



#### **4.7 RECOVERY EFFICIENCY**

Recovery efficiency should be demonstrated by comparison of 6 samples in matrix at LQC and HQC levels with samples prepared in blank matrix spiked with analytes after extraction at the same concentrations.

Recovery of the analytes need not be 100%, but should be consistent, precise and reproducible.

#### **4.8 DILUTION INTEGRITY**

Dilution integrity should be demonstrated by spiking the matrix with an analyte concentration above the ULOQ and diluting this sample with blank matrix to obtain a concentration value in the range of calibration curve.

##### **4.8.1 *Procedure***

1. Dilute 800  $\mu\text{L}$  of Intermediate Solution-2a and -2b (see § 2.3.2) to 10 mL of AHA.

Divide AHA samples into 10  $\mu\text{L}$  aliquots and use it to validate the dilution factor 1:10 (5 replicates each).

**1:10** To 10  $\mu\text{L}$  aliquots add 90  $\mu\text{L}$  of blank AHA. The final concentration of analytes in in AHA is 2.214 nmol/mL of levofloxacin, 2.038 nmol mL of dexamethasone and 1.936 nmol/mL of dexamethasone 21-phosphate.

2. Dilute 800  $\mu\text{L}$  of each Stock Standard Solution-2 (see § 2.3.2) to 10 mL of AHA.

Divide HA samples into 10 $\mu\text{L}$  aliquots and use it to validate the dilution factor 1:100 (5 replicates each).

**1:100** → To 10  $\mu\text{L}$  aliquots add 90  $\mu\text{L}$  of blank AHA. Subsequently dilute 10  $\mu\text{L}$  of this diluted AHA sample with 90  $\mu\text{L}$  of blank AHA. The final concentration of analytes in in AHA 2.214 nmol/mL of levofloxacin, 2.038 nmol mL of dexamethasone and 1.936 nmol/mL of dexamethasone 21-phosphate.

##### **4.8.2 *Calculation***

Recalculate the concentration of AHA samples having a concentration higher than the ULOQ taking into account the dilution factor. Mean accuracy should be  $\pm 15\%$  and precision (CV) should be  $\leq 15\%$ .

#### **4.9 STABILITY**

The stability of analytes samples in different conditions are checked

#### *4.9.1 Bench top stability in matrix at room temperature - procedure*

Twelve AHA samples spiked at LQC and HQC levels (six per QC level) and two AHA blank samples are prepared and analysed after 6 hours at room temperature. Six spiked samples for each concentration and one blank sample analysed immediately after the preparation. Samples are analysed against a freshly prepared calibration curve.

#### *4.9.2 Freeze/thaw (F/T) stability - procedure*

Thirty-six AHA samples spiked at LQC and HQC levels (six per QC level) and three AHA blank samples are prepared, frozen in the freezer at -80°C and thereafter thawed at room temperature. After complete thawing, samples are refrozen again applying the same conditions. At each cycle, sample should be frozen for at least 12 hours before they are thawed. One, two and three cycles are tested. Six spiked samples for each concentration and one blank are analysed immediately after the preparation, six spiked samples for each concentration and for each F/T cycle are analysed against a freshly prepared calibration curve.

#### *4.9.3 Short-term stability in frozen matrix- procedure*

Twelve AHA samples spiked at LQC and HQC levels (six per QC level) and two AHA blank samples are prepared and analysed at the end of the method validation at nominal -80°C. Six spiked samples for each concentration and one blank sample analysed immediately after the preparation. Samples are analysed against a freshly prepared calibration curve.

#### *4.9.4 Autosampler stability - procedure*

Twelve AHA samples spiked at LQC and HQC levels and two AHA blank samples are prepared. Six samples and a blank are injected immediately after the preparation and the remaining samples are analysed after about 72 hours at nominal 10°C in autosampler. The samples are analysed against a freshly prepared calibration curve.

#### *4.9.5 Long-term stability in frozen matrix- procedure*

Long term stability is evaluated at 1, 3, 6 and 12 months at -80°C.



AHA samples spiked at LQC and HQC levels (six per QC level) and AHA blank samples are prepared for each time points. Six spiked samples for each concentration and one blank sample are analysed immediately after the preparation and six spiked samples and a blank sample are analysed at each time point at nominal -80°C. The samples are analysed against a freshly prepared calibration curve.

The analysis should be performed within the range showed in the following table:

TIME POINT (months)	RANGE (days)
1	± 5
3	± 10
6	± 15
12	± 30

#### 4.9.6 Calculation

The stability samples (at LQC and HQC levels) are analysed together with a freshly prepared calibration curve and QC. The obtained concentrations are compared to the nominal concentration.

The analytical batch is accepted if at least 75% of CC showed standards is within  $\pm 15\%$  of the nominal value, except for LLOQ for which it should be  $\pm 20\%$  and at least 67% of QCs concentration results is within 15% of their respective nominal values (at least 50% of QCs at each level should be within 15% of their nominal concentration).

Stability of the samples is accepted if mean accuracy of stability samples at each level is within  $\pm 15\%$  of the nominal concentration and precision of at each level is  $\leq 15\%$ .

#### 4.9.7 IS Stock Solution Stability

IS Stock Solution stability at 4°C is evaluated diluting the solution a 0.795 nmol/mL in water / methanol 50/50 (V/V). The obtained area is compared with the area of a freshly prepared solution. Three injections for each sample is performed. Stability is considered acceptable if mean recovery is within  $\pm 15\%$  and precision is  $\leq 15\%$ .

#### 4.10 SYSTEM SUITABILITY TEST

A system suitability test (SST) will be performed before sample processing to verify the performance of the analytical system by injecting a blank AHA sample and a AHA sample with a concentration corresponding to the LLOQ for each analyte.

SST is considered positive if:

- interfering compounds in blank matrix sample have peak area not higher than 20% of peak areas of the analytes at the LLOQ level and not higher than 5% of the IS.
- The retention times of analytes and IS are confirmed and the S/N of analytes is  $\geq 5$ .

SST results will not be part of the validation batch, however the documentation (blank AHA and LLOQ chromatograms) are to be archived together with the validation batch raw-data.






## 5 REFERENCES

1. EMEA/CHMP/EWP/192217/2009: Guideline on bioanalytical method validation
2. Angelo Macri, Valeria Marini, Giorgio Sangalli, Carmen Fucile, Michele Iester, Francesca Mattioli, *An Artificial Aqueous Humor as a Standard Matrix to Assess Drug Concentration in the Anterior Chamber by High Performance Liquid Chromatography Method*, Clin.Lin. Lab. 2015, 61:47-52.
3. Jin KH, Kim DJ, Lee SJ, *The Concentration of Benzalkonium Chloride in Aqueous Humor According to Size of Deepthelized Area of Rabbit Cornea*, J Korean Ophthalmol Soc. 2002. Jan; 43 (1): 189-196.
4. Andes Behndig MD, Christina Linden MD, *Aqueous humor lidocaine concentration in topical and intracameral anesthesia*, Journal of Cataract & Refractive Surgery, Vol. 24, Issue 12, December 1998, Pages 1598-1601.



## 12.2 OXFORD GRADING SCHEME

Staining is represented by punctate dots on a series of panels (A-E). Staining ranges from 0-5 for each panel and 0-15 for the total exposed inter-palpebral conjunctiva and cornea. The dots are ordered on a log scale.

Panel	Grade	Verbal descriptor
A 	0	Absent
B 	I	Minimal
C 	II	Mild
D 	III	Moderate
E 	IV	Marked
>E	V	Severe

### Conduct of Test:

- Dye is instilled.
- Slit-lamp is set (e.g. 16 magnification with x10 oculars with Haag-Streit).
- Cornea: The upper eyelid is lifted slightly to grade the whole corneal surface.
- Conjunctiva: To grade the temporal zone, the subject looks nasally; to grade the nasal zone the subject looks temporally.
- (The upper and lower conjunctiva can also be graded).

## **Fluorescein sodium**

With fluorescein, staining must be graded as quickly as possible after instillation, since the dye then diffuses rapidly into the tissue and its high luminosity blurring the stain margin.

### **1. Quantified drop instillation**

E.g. 2 ml of 2 % sterile fluorescein instilled into each conjunctival sac with a micro-pipette (using a sterile tip). In very dry eye, larger volumes risk the possibility of inadequate dilution into the fluorescent range.

### **2. Unquantified instillation – impregnated paper strips**

This is a convenient approach in the clinic using the following method of application:

- A single drop of unit dose saline is instilled onto a fluorescein-impregnated strip.
- When the drop has saturated the impregnated tip, the excess is shaken into a waste bin with a sharp flick.
- The right lower lid is then pulled down and the strip is tapped onto the lower tarsal conjunctiva. A similar procedure is carried out on the left.

If too large a volume is delivered then the concentration in the tear film will be too high, and the tear film and staining pattern will be non-fluorescent.

### **3. Timing**

The fluorescein break-up time (FBUT) is usually performed prior to grading. Since fluorescein diffuses rapidly into tissues, punctate staining blurs after a short period. It is therefore essential to assess staining rapidly, in sequence, in the right and then the left eye, so that the staining patterns observed are equally crisp.

If it is intended to photograph the staining pattern for grading, then photography should follow immediately after each instillation.

## **Exciter and Barrier Filters**

The absorption peak of fluorescein sodium occurs between 465 - 490 nm and the emission peak between 520 - 530 nm. A suggested filter pair for detection of fluorescein staining is a yellow, Kodak Wratten 12 barrier filter (transmitting above 495 nm) or an orange Wratten 15 filter (transmitting above 510 nm) in combination with a blue Wratten 47 or 47A exciter filter. The 47A shows greater transmittance than the Wratten 47 over the absorption range. The 'cobalt' filter of many slit-lamps is suitable to use with a Wratten 12 or 15 barrier.

Where more light is required for photographic purposes, narrow band-pass, interference filters can be used.

The use of both exciter and barrier filters allows both the cornea and conjunctiva to be assessed using a single stain. This is a major advantage in clinical trials where it is otherwise customary to employ fluorescein to grade corneal staining and rose bengal or lissamine green to grade conjunctival staining.

**References:** Bron A, Evans VE, Smith JA. (2003). Grading of corneal and conjunctival staining in the context of other dry eye tests. *Cornea* 22(7): 640-50.