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Design, development, and characterization of an idebenone-loaded poly-ε-caprolactone intravitreal implant as a new therapeutic approach for LHON treatment

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ABSTRACT

Leber's Hereditary Optic Neuropathy (LHON) is a hereditary mitochondrial neurodegenerative disease of unclear etiology and lack of available therapeutic alternatives. The main goal of the current pilot study was based on the evaluation of the feasibility and characteristics of prolonged and controlled idebenone release from a PCL intravitreal implant. The design, development, and characterization of idebenone-loaded PCL implants prepared by an homogenization/extrusion/solvent evaporation method allowed the obtention of high PY, EE and LC values. *In vitro* characterization was completed by the assessment of mechanical and instrumental properties. The *in vitro* release of idebenone from the PCL implants was assessed and the implant erosion was monitored by the mass loss and surface morphology changes. DSC was used to estimate stability and interaction among implant's components. The present work demonstrated the controlled and prolonged idebenone delivery from the PCL implants in an *in vitro* model. A consistent preclinical base was established, supporting the idea of idebenone-loaded PCL implants as a new strategy of long-term sustained intraocular delivery for the LHON treatment.

1. Introduction

Chronic retinal disorders account for 84% of visual damage worldwide [1]. Leber's Hereditary Optic Neuropathy (LHON) is a hereditary mitochondrial neurodegenerative disease of the posterior segment of the eye, characterized by a progressive degeneration of the optic nerve as a result of a functional anergy of the retinal ganglion cells (RGCs) [2]. Its etiology remains unclear, although high oxidative stress levels due to different DNA point mutations in the coding process of the mitochondrial NADH dehydrogenase complex appear to be the main trigger of the dysfunction and subsequent apoptosis of the RGCs cells.

The interval between RGCs dysfunction and cell apoptosis may be contemplated as a chance to restore vision by normalizing mitochondrial respiratory chain functionality. Presently, there are no effective LHON treatment options. Idebenone, a new generation CoQ10 analogue [3], is a short-chain benzoquinone with a potent antioxidant activity, being able to protect cell membranes and mitochondria from the action of the reactive oxygen species (ROS), as well as retard lipid peroxidation, thus minimizing oxidative stress processes [4,5]. Idebenone has a molecular weigh of 338.4 g/mol, an aqueous solubility of 12.5 mg/L and log P of 4,5 (Drugbank®). After oral administration Idebenone is quickly absorbed but intensively metabolized in the liver (99%) suffering an intense first pass effect [6]. Consequently, it poor aqueous solubility and the intense first pass effect produce a very low bioavailability (<1%) limiting the amount of pharmacologically active parent idebenone in the plasma and tissues [6]. In 2015, Raxone®, an idebenone film-coated tablet formulation (900 mg/day for 24 weeks), was commercialized as a new LHON treatment [7]. Nevertheless, low bioavailability and effectiveness, high interindividual variability and high occurrence of systemic adverse effects seemed to be the main drawbacks of this

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treatment, due to the difficulty in obtaining effective concentrations in the target tissue [3].

The therapeutic options remain limited to the use of topical ophthalmic or systemic drug delivery [8]. Topical ophthalmic administration is a commonly used and non-invasive route, although a high dosage frequency is required, leading to a patient's adherence-to-treatment reduction [9,10]. Likewise, systemic administration of drugs intended for the treatment of ocular posterior segment diseases is associated with high incidence of side effects [11,12].

Extended levels of the drug at the action site may improve treatment's efficacy, minimizing associated side effects. Long-acting injections and implants present several benefits in comparison to conventional administration routes [13]. Certainly, long-acting DDS may lead to drug stable levels that are preserved within the therapeutic interval for a long time [14,15], improving the drug pharmacokinetic and pharmacodynamic profiles and, subsequently, the therapeutic response [16,17]. Furthermore, the versatility of long-acting injections and implants may show these systems as appropriate DDS both for systemic and local drug delivery [18]. Attached to this, less frequent administration may result in a patient's compliance enhancement and significant economic savings for the healthcare systems. Nevertheless, maintaining drug's constant concentration levels is a tough challenge [17,19,20]. Ideally, the drug's concentration at the target site should remain constant within the boundaries of the therapeutic interval. Long-acting injections and implants show a combination of prolonged and controlled drug release and elimination profiles, resulting in a drug constant concentration at the site of interest [21]. Even so, pharmacodynamic response may be affected by several parameters, changing drug's maximum concentration and the overall treatment duration [22,23].

An extensive selection of long-acting DDS was designed and developed in the past few decades. Nevertheless, the design of these systems requires an extensive knowledge of the system and drug pharmacokinetic and pharmacodynamic properties in order to guarantee effective and predictable drug behavior, as well as the foreign body response understanding [24,25]. Injection-site biological characteristics that are involved in drug transport and uptake must also be considered.

Implant's local injection or implantation was reported to overcome several drawbacks of repeated topical ophthalmic or systemic administration, leading to prolonged drug levels into specific target sites within the therapeutic interval [26,27]. Nevertheless, invasive administration procedures and fibrous encapsulation created by the foreign body are the major drawback of its administration. The implant administration for long-term drug delivery may give rise to the formation of fibrous capsules because of a large foreign body reaction, causing undesirable effects in terms of drug release [28].

A major improvement has been seen in the past few years due to the polymer technology development, especially in the field of high-biocompatibility polymers. Nowadays, biodegradable polymers are characteristically attractive for drug delivery applications due to two possible key features: (I) sustained release profile, which may be reached when erosion is the main polymer degradation process, and (II) biodegradability, which leads to a complete polymer erosion, preventing the need for an ulterior removal procedure of the long-acting DDS at the end of the delivery lifetime.

Poly-ε-caprolactone (PCL) is an extensively studied synthetic polymer approved by the Food and Drug Administration (FDA) for ocular controlled drug delivery [29,30], due to its customizable physical and mechanical properties, as well as biocompatibility, biodegradability and non-toxicity [31,32].

The purpose of the present work was based on the design, development, and physicochemical characterization of a biodegradable drug-loaded poly-ε-caprolactone implant that may provide flexibility in the choice of the active substance and controlled release for a long period; for this purpose, idebenone was employed as a model drug. A preclinical consistent base was obtained by means of the determination and assessment of the physicochemical, mechanical, and thermal properties, as well as the stability, cytotoxicity, and *in vitro* release. Additional studies were carried out to complete this preclinical background.

2. Materials

Poly-ε-caprolactone (Mw: 45 KDa) was bought from Polusciences (Warrington, UK). Idebenone was provided by Acofarma® (Terrasa, Barcelona). Dichloromethane was purchased from Labkem Labware SL (Vilassar de Dalt, Barcelona). Dialysis cellulose membranes were bought from Sigma Aldrich (St. Louis, Missouri). Additional substances and reagents might be used throughout the present work, being of the highest analytical grade.

3. Methods

3.1. Preparation of idebenone-loaded PCL implants

Idebenone-loaded long-term implants were prepared by homogenization/extrusion/solvent evaporation method. All components (poly-ε-caprolactone and idebenone) were physically premixed in three different proportions (1:1, 1:5 and 1:10 drug:polymer ratio) using a mechanic homogenizer (Ultra-Turrax T25, IKA® Laboratories, Staufen, Germany), until complete homogenization of the resulting powder. Organic solvent (dichloromethane) was then added into the mixture until the dissolution process was completed, under magnetic stirring (28 g) and at room temperature (25 ± 2 °C). The resulting mixture was included into a 5 mL syringe, loaded with a 22G needle, and subsequently manually extruded into defined-size capillary tubes (0.8 mm diameter). Mixture-loaded tubes were then included into a vacuum-drying oven at room temperature for 24 h to promote organic solvent evaporation, with a predefined staggered pressure decrease. Idebenone-loaded implants were finally obtained and stored into a desiccator for further analysis.

3.2. Physicochemical characterization of implants

3.2.1. Size, size distribution and weight uniformity

Size and distribution size of idebenone-loaded PCL implants are critical parameters to consider for intravitreal delivery to the eye by conditioning the type and method of administration. Implant diameter measurements were carried out by using a digital caliper (RS Pro Electronic Digital Caliper, Madrid, Spain), while the weight data of the implants were obtained by means of the use of an analytical scale (Denver Instrument APX-200, USA), to obtain the final physical dimensions of the implants, as well as assess the preparation procedure's reproducibility.

3.2.2. Morphological evaluation

The implant's internal and external morphology as well as its microstructure was assessed by macroscopic observation, prior to the scanning electron microscopy (SEM) analysis, after drying process. The macroscopic analysis of the implants was carried out by depositing the sample on a slide and subsequent observation by an electronic magnifying glass/camera system (Olympus® SZ-CTV/Olympus® SC100) in order to assess the external and internal structure of the resulting implants and verify the shape uniformity during the manufacturing process.

SEM analysis were further executed by implant deposition on a metal plate, attached to an appropriate adhesive tape, prior to morphology examination, under different magnifications. Samples were incorporated as longitudinal and transversal cuts to observe both the external and internal morphology of the implants. Each batch was assessed in triplicate.

3.2.3. Production yield (PY, %)

The production yield (PY) (%) of each formulation was obtained by using a mathematical equation, dividing implant's weight after drying by the total amount of the components used in the formulation process, as follows:

$$PY\ (\%) = \frac{Implant's\ weight}{Total\ amount\ of\ components\ in\ the\ formulation} \cdot 100$$

Likewise, a standardization value was established after taking 10 randomized samples from different batches for each formulation to guarantee the elaboration process.

3.2.4. Encapsulation Efficiency (EE, %)

Encapsulation Efficiency (EE) of implants was determined by a centrifugation method, followed by the free-drug quantification by UV–Vis spectrophotometry, after polymer precipitation [33,34]. Briefly, implants were dissolved into 2.5 mL of methylene chloride, followed by a mechanic stirring process (Vortex®, VWR International, Germany) to promote polymer dissolution. 6 mL methanol were then added and subsequently vortexed to foster polymer precipitation. Samples were later centrifuged (2800 g, 5 min), supernatant was subsequently collected and idebenone amount was determined at a 279 nm wavelength. This procedure was repeated using 10 randomized batches for each formulation. EE was finally estimated by the following mathematical equation:

$$EE\ (\%) = \frac{Amount\ of\ released\ drug\ from\ the\ implant}{Total\ amount\ of\ drug} \cdot 100$$

3.2.5. Loading Capacity (LC, %)

Loading Capacity (LC) of implants was determined by a centrifugation method followed by the free-drug granulometric analysis (Denver Instrument APX-200, USA), after polymer precipitation [33,34]. Briefly, implants were dissolved into 2.5 mL of methylene chloride, followed by a mechanic stirring process (Vortex®, VWR International, Germany) to promote polymer dissolution. 6 mL methanol were then added and subsequently vortexed to foster polymer precipitation. Samples were then centrifuged (2800 g, 5 min) and supernatant was subsequently collected, conducted to evaporation to obtain the resulting powder, which would correspond to the total amount of free drug released from the implant. This procedure was repeated using 10 randomized batches for each formulation. Loading capacity was then estimated by the following mathematical equation:

$$LC\ (\%) = \frac{Weight\ of\ released\ drug\ from\ the\ implant}{Implant\ weight} \cdot 100$$

3.3. Determination of the mechanical properties: Tensile and elongation at break studies

Tensile strength and elongation at break are commonly used studies to obtain mechanical properties of solid materials [35]. These parameters were determined by using an industrial hydraulic press (Shimadzu® AGS-X Series, Kyoto, Japan). The length (L_0) and the diameter of each implant were gauged, and surface area was estimated prior to the assay. Each implant was subjected to a controlled tension increase (0.5 mm/min speed) until mechanical failure, where tension force and distance changes (ΔL) were recorded during the entire process. Samples were gripped by the hydraulic press at the two ends. It is imperative to validate that the sample is tightly grasped without slippage or breakdown phenomena from the holding segments. Tensile strength and elongation at break were subsequently calculated as presented:

$$Tensile\ strength\ (MPa) = \frac{Force\ at\ break}{Area\ of\ cross\ section}$$

$$Elongation\ at\ break\ (\%) = \frac{\Delta L}{L_0} \cdot 100$$

At predetermined times (0, 1, 7, 15, 30, 60, 120, 240, and 360 days), three implants from each batch were extracted from their respective media, dried to remove the excess of water, and mechanical properties were then assessed. Each measurement was performed in triplicate.

3.4. Friability test

The abrasion resistance of the idebenone-loaded PCL implants was evaluated through a friability test. Ten implants from each batch were randomly selected, weighed, and then included in a friabilator (Campbell Electronics Friabilator FTA-20, Mumbai, India). Each batch was test in triplicate. A predefined test sequence was established, based on a 1-g constant speed for 5 min at room temperature (25 ± 2 °C). After this time, implants were weighed again, and the friability was calculated as presented (see equation below). A friability percentage of ≤1% was established as the optimal value.

$$F\ (\%) = \frac{Initial\ weight - Final\ weight}{Initial\ weight} \cdot 100$$

3.5. Differential scanning Calorimetry (DSC)

Possible interactions between idebenone and PCL were studied by DSC, which was performed using a TA Instruments® Q1000 DSC/TGA/IR analyzer, standardized with indium. Samples (10 mg) were weighed and placed in sealed aluminum capsules. DSC curves were obtained by a 10 °C/min scanning rate, conducted through a 0–150 °C temperature range, in a nitrogen environment, using a 50 mL/min flow rate. Each formulation was tested in triplicate.

3.6. Thermogravimetry analysis (TGA)

Thermal stability of the implant components as well as the determination of the presence/absence of residual solvents in the formulations were assessed by thermogravimetry analysis [36]. The procedure was similar to that described for the DSC analysis. Briefly, each implant loaded into an hermetic aluminum plate and heated from 0 to 150 °C at a heating rate of 10 °C/min by using a TA Instruments® Q1000 DSC/TGA/IR analyzer (Madrid, Spain). Each batch was tested in triplicate to confirm the presence or absence of dichloromethane in the final formulations, having been used as a solvent to solubilize the components prior the implant extrusion.

3.7. Fourier-Transformed infrared spectroscopy (FTIR)

FTIR assay was performed to determine implant's structural characterization. Certainly, samples were scanned through the IR interval, ranging from 400 to 4000 cm^{-1}, using a 4 cm^{-1} resolution and dry air as a background blank to stabilize the FTIR system (GladiATR™, Varian Pike Technologies). 64 scans/min were applied to each sample, obtaining 4084 different points, and resulting data were collected in transmittance values (%) and processed through the Resolutions Pro® software.

3.8. X-Ray diffractometry analysis

X-ray diffraction is a widely used technique in the study of solid materials, although it has also demonstrated an important application in the analysis of disordered states of matter. At present, this technique is moving towards the analysis of structured samples in submicron dimensions and towards the structural analysis of non-solid systems, by conducting a wide variety of studies including, not only qualitative and quantitative analysis of materials, but also microtextural, crystallinity,

non-homogeneous deformation analysis and/or phase change analysis, among others.

The X-ray diffractometry study was carried out following the ISO 9001: 2015 standard normative in terms of reception, sample management and analysis using monocrystalline, crystalline powder and X-ray fluorescence techniques. All samples were kept at room temperature, in vials, waiting to be measured. The material's handling was carried out with an agate mortar for spraying/homogenizing the physical samples.

Implant's crystalline structure was examined by wide-angle X ray powder diffraction in an X-ray diffractometer (Philips X'Pert, USA), operated with a PW170 control unit, a PW1820/00 vertical goniometer and a Enraf Nonius FR590 generator, locating and assigning the most significant mathematical values of the peaks of the diffractograms present. Samples were scanned from a Cu Kα source, monochromated with a graphite monochromator (λ = 1.5406 Å), at 40 kV and 30 mA, using 2θ from 2° to 50° at a scan rate of 0.04°·min^{-1}. Samples were rotated during the analysis to obtain the most optimal peak profiles for the diffractograms, as well as to minimize the effect of the preferred orientation. It also must be taken into account that samples were deposited in oriented glass bases (Si-511 plate) to avoid background noise caused by a vitreous support. The mathematical analysis of the diffractograms was carried out by using the HighScore Plus® 3.0d software.

3.9. In vitro release study

The dialysis method is frequently employed to quantity drug release from DDS. In this process, two spaces are physically divided by a dialysis membrane (diffusion surface 0.784 cm^2), as previously explained [37], making an proper system to evaluate the drug delivery pattern from a variety of DDS [38-40].

The idebenone release rate from implants was obtained by UV–Visible spectrophotometry, by means of Franz diffusion cells. The receiving compartment was 6 mL PBS filled, while donating compartment was filled with 3 mL of synthetic vitreous humor (VH). Two different compositions of synthetic VH were employed in order to simulate the physiological VH composition, these being: VH-A, containing an 0.1% (w/v) agar and 0.5% (w/v) hyaluronic acid, and VH-B, composed of an 0.1% (w/v) agar and 0.4% (w/v) hyaluronic acid. One implant (l = 5 mm and d = 0.8 mm) was then added to the donor chamber and Franz cells were then positioned in an orbital shaker at 1 g and 37 °C for the whole study. At prearranged intervals, 1 mL from the receiving compartment was taken, and an equal volume of fresh PBS solution was then added to it to preserve sink conditions during the entire assay. The assay was run for a one-year period, and each formulation was tested in triplicate. Idebenone concentrations were measured by UV–Vis spectrophotometry (λ = 279.0 nm). Analytic validation was previously made by our group and showed linearity (R = 0.999) over a concentration interval of 1.5625–250 μg/mL, 96.3% accuracy, of a 98.6% relative standard deviation (RSD) intra-day precision, and limit of detection (LOD) and limit of quantification (LOQ) values of 0.7813 μg/mL and 1.5625 mg/mL, respectively (data not published).

3.10. Water uptake, mass loss and mass remaining studies

The erosion study of the implants was carried out in line with the *in vitro* release study, over a one-year period. Water uptake, mass loss and mass remaining were gravimetrically assessed by using a granulometric scale (Denver Instrument APX-200, USA).

Freshly prepared implants were screened, and subsequently placed into amber vials containing 5 mL PBS (pH 7.4). All implants were accurately weighted prior to the immersion in the medium (initial weight). The extraction and renewal of the medium was performed at the same time as for the *in vitro* release study of the implants in order to maintain the sink conditions and allow the drug release from implants. Thirty implants from each batch were studied (l = 5 mm, d = 0.8 mm), using three different batches randomly chosen.

At predetermined times (0, 1, 7, 15, 30, 60, 120, 240, and 360 days), three implants from each batch were extracted from their respective media and weighted (wet weight). A drying process was then applied to remove the excess of water and weighted again until constant weight by using a vacuum oven (Heraeus RVT360, Gemini® BV, Apeldoorn, The Netherlands) (dry weight), at room temperature. The volume of water absorbed, and the quantity of polymer eroded were calculated and expressed as percentage. Each sample was separately immersed into the release medium and subsequently taken out; dried implants were later completely dried (dry weight). Water uptake, mass loss and mass remaining values were then estimated as presented:

$$Water\ \ uptake\ \ (\%) = \frac{Wet\ \ weight - Dry\ \ weight}{Dry\ \ weight} \cdot 100$$

$$Mass\ \ loss\ \ (\%) = \frac{Initial\ \ weight - Dry\ \ weight}{Initial\ \ weight} \cdot 100$$

$$Mass\ \ remaining\ \ (\%) = \frac{Dry\ \ weight}{Initial\ \ weight} \cdot 100$$

The implants morphology was also assessed by scanning electron microscopy (SEM) analysis over time, as previously described (see Section 3.2.2), to determine the existence or absence of morphological changes along the studied period.

3.11. Stability studies

3.11.1. Stability to storage

Implants were placed in screw-capped glass containers and stored at three temperature subsets, these being: (1) 4 ± 2 °C, (2) 25 ± 2 °C, and (3) 37 ± 2 °C for a six-month period. Stability was assessed through drug content uniformity, where zero-time samples were used as controls.

Drug content assay (n = 3 per batch) was carried out by weighting each implant, following the same procedure that has been applied for the LC (%) determination. Drug content was finally determined by UV–Vis spectrophotometry at a 279 nm wavelength. Each formulation was also macroscopically assessed in search of physical state changes (color and consistency).

3.11.2. Stability under physiological conditions

3.11.2.1. Stability to pH. The physical evaluation of the pH-dependent implant stability was carried out on freshly prepared formulations. The methodology of this procedure was based on the suspension of each implant (n = 3 per batch) in 5 mL of Milli-Q® water at different predetermined pH values (from 2 to 12) (Hanna® HI5522, Hanna Instruments®, Spain), previously adjusted by means of HCl or a NaOH aqueous solutions, as necessary. Samples were then kept refrigerated (T = 4 °C ± 2 °C) for 24 h so as to be subsequently centrifuged at 2800 g for 10 min. Finally, the determination of the free idebenone concentration in the solution was carried out at a 279 nm wavelength in order to verify the existence of implant collapse or breakage phenomena with the consequent drug release into the medium. Each formulation was tested in triplicate for all set pH values.

3.11.2.2. Stability to ionic strength. The physical evaluation of the ionic-strength-dependent implant stability was performed on freshly prepared formulations. The methodology of this procedure was based on the suspension of each implant (n = 3 per batch) in 5 mL of NaCl aqueous solutions with different predetermined ionic strength values (from 0.2 M to 2 M). Samples were then kept refrigerated (T = 4 °C ± 2 °C) for 24 h so as to be subsequently centrifuged at 2800*g* for 10 min. Finally, the determination of the free idebenone concentration in the solution was carried out at a 279 nm wavelength in order to verify the existence of implant collapse or breakage phenomena with the consequent drug

release into the medium. Each formulation was tested in triplicate for all set ionic strength values.

3.12. Injectability of implants

Injectability is a key parameter in the preclinical evaluation of implants for intravitreal administration. This factor alludes to the performance of the implant during injection [41]. Suitably injectability properties guarantee implant's intraocular administration.

Injectability studies of implants enable to establish the optimal characteristics of the intravitreal needles (size and length), by calculating the needed force to inject a predetermined formulation. Suitably injectability properties guarantee an accurate procedure for the implant to be intraocularly administered. A 12 N force over 10 s was set as the maximum ejection force, being appropriate for a proper intraocular injection [42].

Implant preparation procedure was based on the preparation of a 5 mm × 0.8 mm; implant dimensions were properly selected in order to accomplish the equipment requirements. Once prepared, implants were included in a syringe system, equipped with different types of needles (15G, 16G and 17G), in order to compare them for an appropriate administration [43] (see details in Table 1).

Ejection force values were respectively obtained by using an Autograph Table-TOP Precision Universal Tester (Shimadzu® AGS-X Series, Kyoto, Japan), managed through a specific software (Trapezium®). Established injectability and syringeability conditions were as presented in Table 2. All the samples were tested in triplicate for all the different syringe/needle systems.

3.13. Cytotoxicity analysis: Hen's egg test on the chorioallantoic membrane (HET-CAM)

The HET-CAM assay, an alternative organotypic model to the standard Draize rabbit eye test, is commonly used for the assessment and quantification (irritation score (IS) determination) of three potential undesired effects that may be caused by irritative compounds, these being hemorrhage, lysis and coagulation [44], taking into account the Kalweit et al. criteria [45]. All actions with eggs accomplished the standard guidelines for animal materials management.

The procedure was adjusted to the instructions formerly established by Spielmann and Liebsch [46], with minor changes. Briefly, fertilized eggs were incubated for nine days at 37 ± 0.5 °C and 65% ± 5% RH, under automatic rotation (every 2 h) for the first eight days. On the last day, rotation was blocked, and the eggs were vertically positioned for the correct chorioallantoic membrane (CAM) location. A cavity was subsequently made on the end of the eggs, through which implant's incubation media (300 μL) were straightforwardly administered onto the CAM. To point out, the intravitreal implants were not directly placed onto the CAM, since an observation period of 5 min would be too short and insufficient to assess the effect that they would have on the membrane vascular system. However, the instillation of the solution in which the implants were immersed during the degradation process over time (at t = 360 days) was carried out in order to evaluate whether the implant degradation products could become irritating to the retina and choroid. The chorioallantoic membrane used in HET CAM assay is a highly vascularized membrane found in the eggs that can be used to mimic highly vascularized membranes or tissues as the conjunctiva, retina, choroid, or even tumor tissues (CAM tumor models).

An Olympus SZ61TR Stereomicroscope and an Olympus CellSens Entry software were used for the aforementioned factors evaluation, along a 5 min period. Images were taken at the beginning and at the end of the test, and each duplicate was recorded along the study to determine potential undesired consequences (if required). These observations were individually taken into account and later merged to obtain the IS (if possible), which was employed to categorize the irritancy potential. A 1.8% (w/v) NaOH solution and a BSS solution were used as positive and negative control solutions respectively, as previously described [37]. Each batch was evaluated in triplicate.

Table 1
Specifications of the different needles used during the syringeability and injectability assays.

Needle gauge	Inner diameter (mm)	Length (mm)
16	1.16	40
17	1.01	38
18	0.838	38

Table 2
Conditions developed in the Shimadzu® AGS-X test machine for injectability and syringeability test of idebenone-loaded PCL intravitreal implants.

Condition	Parameter	Injectability specifications
System	Assay mode	Simple
	Assay type	Compression
	Force polarity	Compression
	Force sense	Downwards
Sensor	Force scale	1000 N
	Displacement	500 mm
Assay	Action	Force
	Control	Displacement
	Sampling	10 seg/sample
	Breakage sensitivity	10%
	Sensor	Displacement
Sample	Material	–
	Shape	Cylinder
	Dimensions	Diameter and height
Processed data	Maximum force	–

3.14. Statistical analysis

Pairs of groups were matched by carrying out one-tailed Student's *t*-test, while multiple group comparison analysis was performed by parametric or non-parametric analysis (if necessary) of variance, with a 95% significance level ($p < 0.05$), using the GraphPad Prism® v.8.00 software. All data were presented as a mean value with its standard deviation. Additional test (Tukey's, Bonferroni's or Dunnet's tests) might be employed for post-hoc contrast, if possible.

4. Results and discussion

4.1. Preparation and optimization of idebenone-loaded PCL implants

The idebenone-loaded PCL implants were successfully obtained by a homogenization/extrusion/solvent evaporation method. The preparation method enabled the drug inclusion inside the polymer matrix, favored by the drug physicochemical properties, mainly by its hydrophobicity. Certainly, the versatility of hydrophobic polymers permits the elaboration of intravitreal administration implants with a variety of shapes or sizes [29,31]. Firstly, premixed implant components were dissolved by an organic solvent, included into a syringe/needle system, and extruded into capillary molds prior to the solvent evaporation. After placing in a vacuum chamber, evaporation led to a polymer precipitation phenomenon and implants solidified, entrapping the drug in its matrix. After the vacuum-drying process, all idebenone-loaded PCL implants showed a cylindrical form and a uniform orange color.

Three different proportions (1:1, 1:5, and 1:10 drug:polymer ratio) were tested and 1:5 drug:polymer ratio was selected as the optimum quotient, discarding the other two tested drug:polymer ratios. Certainly, 1:1 drug:polymer ratio was rejected because the polymer plastic properties were compromised by the large amount of incorporated drug, increasing the breakage and fragility phenomena of the implants. On the

other hand, 1:10 drug:polymer ratio was discarded because the drug: polymer proportion was too high, being associated to high variability in terms of idebenone content uniformity along the polymeric matrix.

4.2. Implant characterization

4.2.1. Mean size, size distribution and weight uniformity

The idebenone-loaded PCL implants were extracted from the capillary molds after the vacuum-drying process and their size was subsequently adjusted to a 5 mm length. Afterwards, the diameter and weight of the resulting implants were determined by measuring 10 different implants randomly selected, and the study was carried out in triplicate. Resulting data showed a diameter interval of 0.0731 ± 0.0162 mm, as well as a weight interval ranging from 6.15 ± 0.41 mg.

4.2.2. Morphological evaluation

The evaluation of the implant morphology was firstly carried out by means of a batch macroscopic examination, prior to the SEM analysis. The macroscopic observation was performed as a preliminary analysis to confirm the existence of a shape uniformity of the resulting pharmaceutical form, in order to verify the adequacy of the preparation method to the desired objective.

Macroscopic analysis of the samples allowed to conclude that the resulting idebenone-loaded PCL implants showed uniformity of shape and color, as well as a cylindrical appearance (see Fig. 1). The implant surface could be assumed to be smooth and clean, with no adhered artifacts. The internal structure of the implants seems to follow the same pattern described.

Morphological evaluation of the implants shape and surface was also carried out by SEM assessment, taking into account that implant morphological characterization may surface characteristics with the pharmacokinetic behavior. All idebenone-loaded PCL implants prepared by a homogenization/extrusion/solvent evaporation method were without pores, and showed a uniform although not completely smooth surface, as it can be seen in SEM images (see Fig. 2). In addition, the implant cross section showed a compact and consistent structure, ensuring the homogeneous drug distribution along its entire length. Images also reveal the methylene chloride complete removal during the elaboration process.

In order to point out, it seems that the implant surface shows a polymer stratification, this being supported by the polymer physicochemical characteristics, which highlights for its semi-crystalline aspect. Nevertheless, this appearance was not observed in the polymer internal structure. The main reason may strive to the fact that the drug is included into the internal polymer matrix, while the implant surface was mainly composed of a higher polymer concentration, acting as a coating film.

4.2.3. Production yield (PY), encapsulation efficiency (EE), and loading capacity (LC)

The homogenization/extrusion/solvent evaporation method is ideal for obtaining high production performance, encapsulation efficiency and load capacity values. This is additionally enhanced by the hydrophobicity of petroleum-derived polymers (e.g., polycaprolactone) and the low water solubility of hydrophobic drugs such as idebenone, making them good candidates for the incorporation into intraocular polymeric implants. Fig. 3 shows the results for production yield, encapsulation efficiency and loading capacity of idebenone-loaded PCL implants. All formulations showed appropriate PY, EE and LC values for the preparation method.

High PY values (almost 90%) were obtained, demonstrating the idea that the chosen preparation method is suitable, effective, and reproducible for the implant elaboration. Furthermore, the EE values were ranging from 90 to 95%, while LC values were around 20% but, considering that a 1:5 drug:polymer ratio was employed, the adjusted LC values varied from 90 to 95%, supporting the idea that the preparation process was reproducible and efficient. Resulting data are reinforced by the polycaprolactone hydrophobicity and the idebenone low aqueous solubility, providing high drug entrapment into polymeric matrix.

4.3. Determination of mechanical properties: Tensile strength and elongation at break

Fig. 4 shows the tensile strength (MPa) and elongation at break (%) values for the idebenone-loaded PCL implants. One way ANOVA of both tensile strength and elongation at break show significant differences from day 60. In this context, it is possible to observe a slight decrease in both parameters over time, which may be associated with a progressive degradation process of the implant structure by the medium action in which implants were immersed. Taking into account the tensile strength data, it can be seen that, as the measurement time advances, the force gradually decreases. This phenomenon may be a result of the progressive degradation of the implant external structure, which ends in greater structural fragility. Nevertheless, the variability of the pressure values

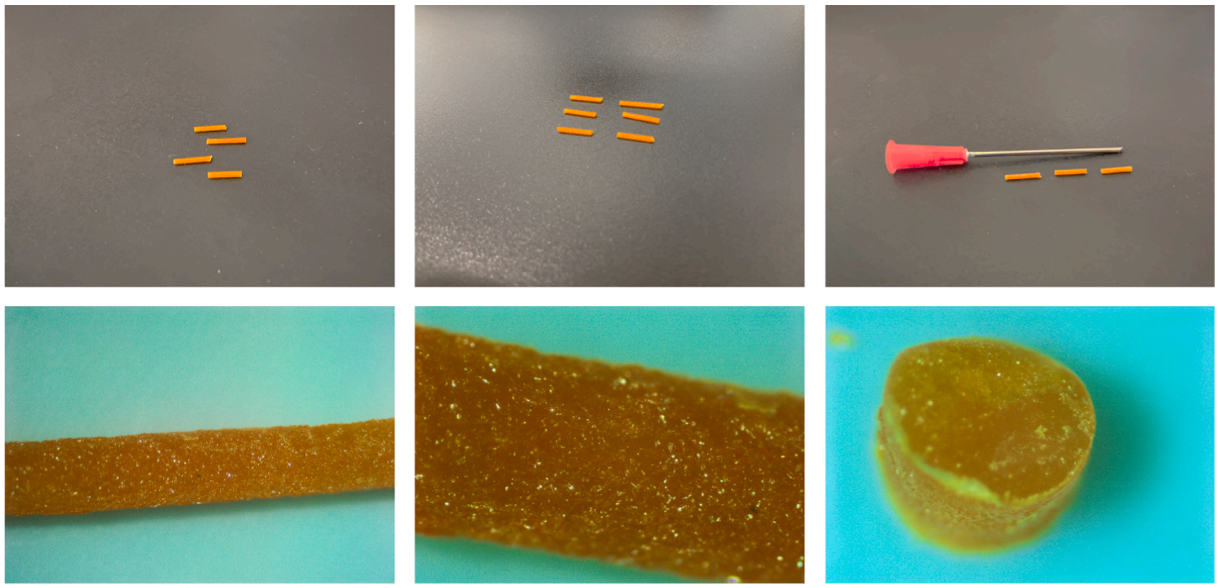

Fig. 1. Macroscopic images of the idebenone-loaded PCL implants.

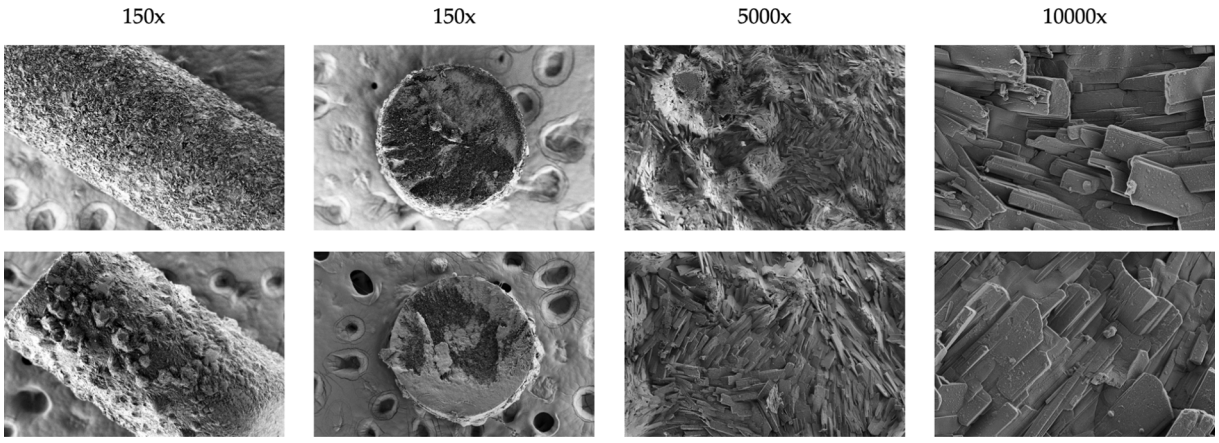

Fig. 2. SEM images of idebenone-loaded PCL implants. The first-column images show the implant's longitudinal morphology. The second-column images show an implant cross-section, observing the implant internal structure. The last two columns show the polymer structure along the implant in more detail, at two different magnifications.

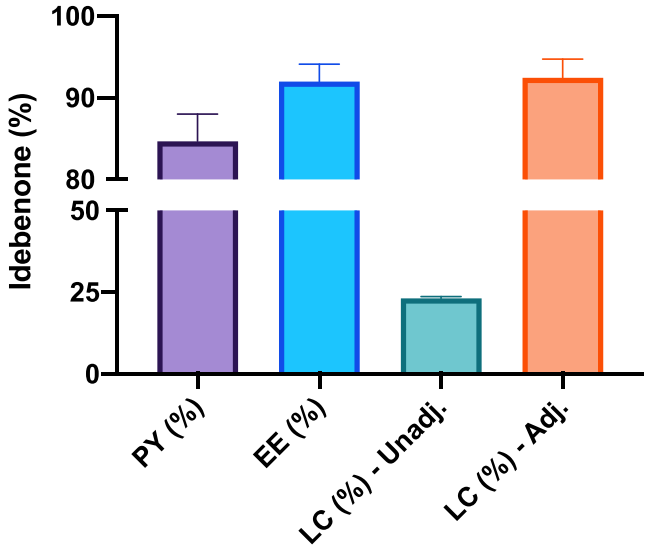

Fig. 3. Production yield (PY), encapsulation efficiency (EE) and loading capacity (LC) values (unadjusted and adjusted, respectively) for the idebenone-loaded PCL implants.

along the progressive determinations was minimal, supporting the fact that the drug was homogeneously dispersed along the polymer matrix.

A gradual reduction in elongation at break values was also observed as the trial time progressed. Resulting data proved that polymer matrix ductility gradually decreased over time. Even so, elongation at break values remained high over almost the entire studied period, typical of a robust polymer structure, evidencing the PCL plastic deformation behavior. This is supported by the low melting temperature (around 60 °C) of this polymer, making implants relatively flexible at room temperature.

4.4. Friability test

Friability measuring of the idebenone-loaded PCL implants may tend to powder or fragment. This test is narrowly related to hardness properties, being designed to assess the implant ability to resist abrasion along the management process. Friability results were in the optimal range (0.90 ± 0.04%), suggesting that the final formulations show adequate resistance to abrasion or attrition processes.

4.5. Differential scanning calorimetry (DSC) and thermogravimetry (TGA) analysis

DSC and TGA curves of idebenone-loaded PCL implants were measured as a way to determine the existence of interactions between the polymer and the drug within the polymer net of this type of intravitreal implants and the determination of residual solvents.

Fig. 5 shows the resulting DSC data for the idebenone-loaded PCL implants. As presented, the DSC trace showed a melting endotherm behavior at a T_{peak} of 65.39 °C (T_{offset} 58.78 °C, 50.85 J/g of melting enthalpy) that correspond to the PCL transition temperature (around 60 °C). Besides, a slightly detectable melting point at T_{peak} of 52.87 °C (T_{offset} 52.40 °C and 3.59 J/g of melting enthalpy) was early found for idebenone, by exhibiting a lower melting temperature (52–55 °C).

TGA/IR analysis showed no change in the implant's weight due to

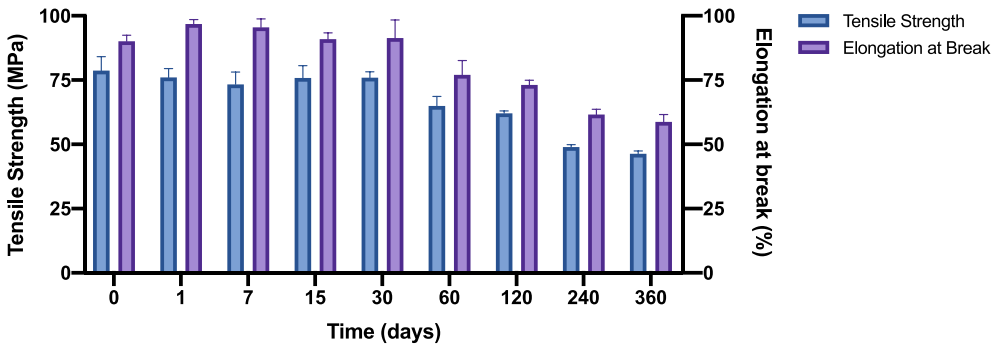

Fig. 4. Tensile strength (MPa) and elongation at break (%) values for the idebenone-loaded PCL implants.

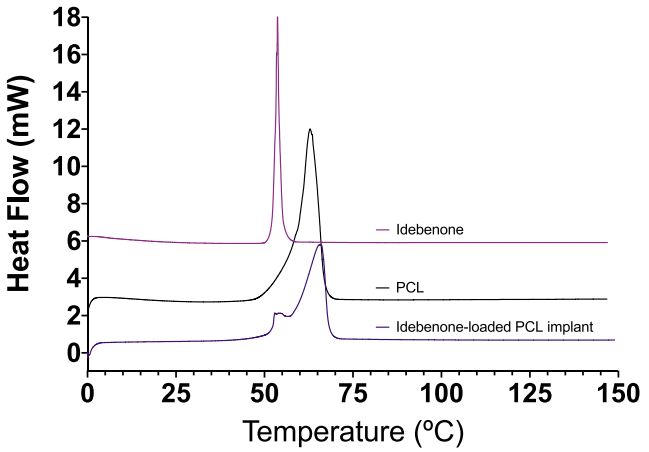

Fig. 5. Differential Scanning Calorimetry (DSC) analysis of idebenone-loaded PCL implants.

the temperature increase, and no residues of the dichloromethane used in the implant fabrication were detected by the IR analyzer associated with the TGA equipment.

4.6. FTIR analysis

FTIR spectroscopy analysis was used to study possible chemical interactions between the drug and polymer. The FTIR spectra of idebenone, PCL, and idebenone-loaded PCL implants are shown in Fig. 6.

The idebenone FTIR spectrum showed several characteristic bands for the O—H, C—H, C═O groups and C═C ring, with stretching vibrations at 3568, 2920, 2850, 1648 and 1609 cm^{-1}, respectively. The PCL spectrum showed a doublet at 2945 and 2872 cm^{-1} due to the C—H stretching vibration of the methylene groups as well as a characteristic single band that corresponded to the carbonyl group. Implants presented a mixture of the bands of both components, and no important changes were observed in the signals position. Thus, no interaction between idebenone and PCL was observed, where drug and polymer chemical structures were not modified during the preparation process.

4.7. X-Ray diffractometry analysis

The physical state of the idebenone-loaded implants and its components were characterized by X-ray diffraction studies. Fig. 7 shows the idebenone-loaded PCL implant diffractograms, as well as the comparison with the drug (idebenone) and the pure polymer (PCL). The pure PCL and drug diffractograms show that both materials are in crystalline state. PCL diffractograms shows three main peaks at 21.4°, 22.0° e 23.7°

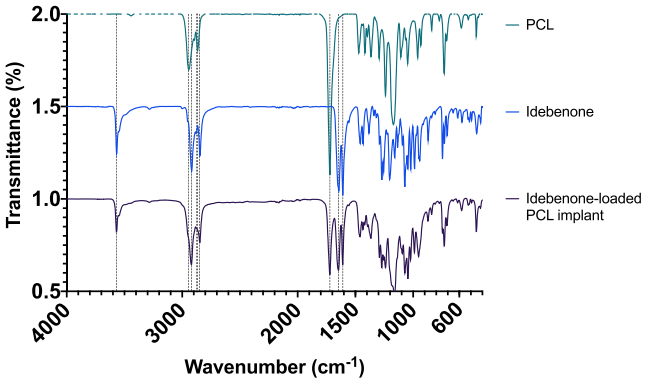

Fig. 6. FTIR results for the components (idebenone and polycaprolactone) and the idebenone-loaded PCL implants.

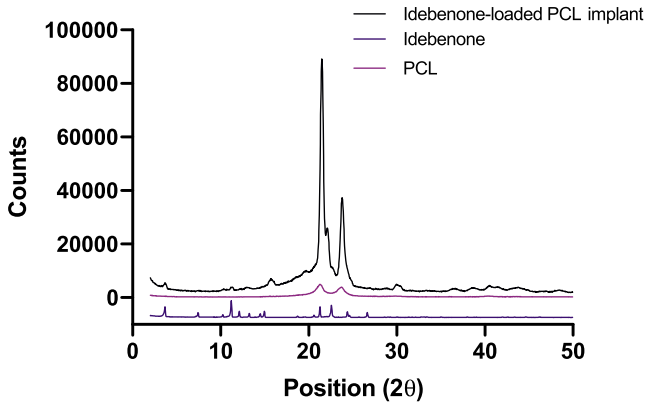

Fig. 7. X-Ray diffractograms of idebenone-loaded PCL implants. Two different transformations ($Y = -5000Y_0 + 1$ and $Y = 5000Y_0 + 1$) were respectively applied on idebenone and polycaprolactone graph representations to promote the wide-ranging observation and comparison of the FTIR data.

at 2θ, being characteristic of the polymeric crystalline structure and observed in the idebenone-loaded PCL implants. Idebenone shows numerous characteristic peaks in its crystalline structure between 22° and 50° at 2θ that remain in the implant diffractograms.

4.8. Stability studies

4.8.1. Stability to storage

The stability-to-storage study of idebenone-loaded PCL implants was carried out by determining the drug content uniformity by maintaining samples at three different temperature sets for a 180-day period. Resulting data showed no major changes during the assay (see Fig. 8). Insignificant changes in the drug content uniformity may be associated with interferences coming from the sensitivity of the quantification method. Thus, the idebenone entrapment into the PCL matrix was appropriate, providing drug stability and protection against variations in ambient temperature.

A two-way ANOVA was also conducted, and no statistically significant differences ($p > 0.05$) were detected among the three different storage conditions for the studied time. Thus, it can be concluded that idebenone-loaded PCL implants were stable over the considered interval.

4.8.2. Stability to pH

Extreme pH conditions of the environment might lead to a faster drug release speed from the implants due to variations in the release mechanism. Stability-to-pH changes were assessed through idebenone uniformity content by UV–Vis spectrophotometry (279.0 nm wavelength), as explained in previous sections. Physical characteristics of the formulations were also evaluated for appearance color variations.

Fig. 9 shows the stability-to-pH study data of idebenone-loaded PCL implants. The hydrophobic nature of the polycaprolactone and idebenone would generate a synergic effect, preventing the drug diffusion to the medium, thus enhancing the controlled delivery of idebenone from the PCL implants, as well as protecting the drug from degradation processes. Furthermore, no alterations in physical appearance or color were observed for the studied pH conditions. A two-way ANOVA was also applied, and no statistically significant differences ($p > 0.05$) were observed along the studied period. Henceforth, idebenone-loaded PCL implants were stable over the studied pH interval.

4.8.3. Stability to ionic strength

The medium's ionic strength value may promote a quicker drug release from the polymeric implants due to changes in the release mechanism. Stability-to-ionic-strength variations were assessed through idebenone uniformity content by UV–Vis spectrophotometry (279.0 nm

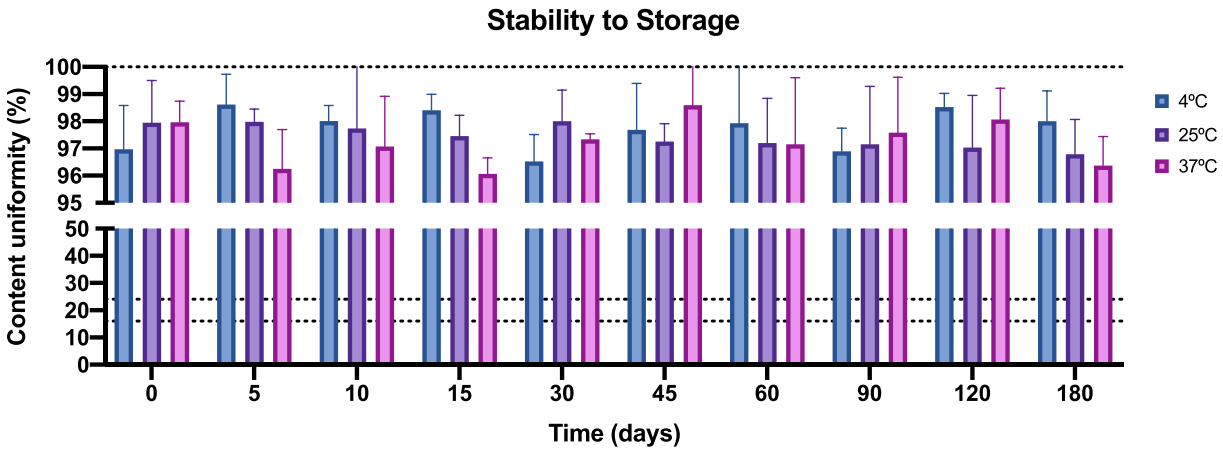

Fig. 8. Idebenone content uniformity values (%) for idebenone-loaded PCL implants during the stability-to-storage study.

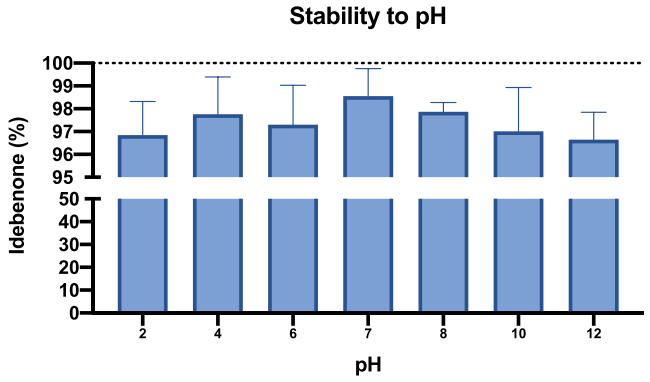

Fig. 9. Idebenone content uniformity values (%) for idebenone-loaded PCL implants during the stability-to-pH study.

wavelength), as described in previous sections. Physical characteristics of the formulations were also inspected for appearance or color changes.

Fig. 10 shows the resulting data for the idebenone-loaded PCL implants. The DDS stability was maintained unchanged regardless of the variations in the medium's ionic strength values. The hydrophobic characteristics of the drug and the polymer would prevent the fast release of idebenone from the PCL implants to the surrounding medium, as well as protecting the drug from degradation processes. A two-way ANOVA was also performed, and no statistically significant differences ($p > 0.05$) were observed along the studied interval, supporting the idea that idebenone-loaded PCL implants would be stable against ionic strength changes of the medium.

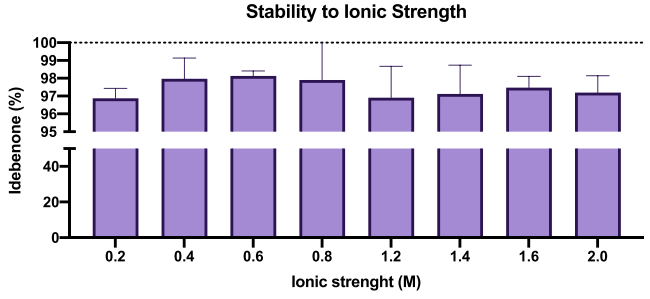

Fig. 10. Idebenone content uniformity values (%) for idebenone-loaded PCL implants during the stability-to-ionic-strength study.

4.9. *In vitro* release study

The idebenone *in vitro* release profile from the PCL implants was reported to be conditioned by the polymer physicochemical characteristics, as well as by the release mechanisms behavior (i.e., drug desorption, polymer matrix erosion, and drug diffusion, which might even simultaneously happen) [47,48]. The *in vitro* release profile for the idebenone-loaded PCL implants is shown in Fig. 11. Idebenone release from PCL implants seems to follow a biphasic pattern, described by a faster preliminary release phase that lasts up to 120 days and a slower second phase that lasts beyond one year. As observed, the drug release from the implants shows a controlled release profile for at least one year, during which time only 65% of the drug was released, with approximately 35% remaining inside the polymer matrix. Such idebenone controlled release profile might be associated with a diffusion and degradation phenomena; the burst release might be related to the idebenone diffusion process from the implant surface [49], while the controlled and sustained release phase might be regulated by polymer degradation and drug diffusion through the implant matrix or even both concurrently [50,51].

The initial release pattern of idebenone from the PCL implants (75-day period), the delivery followed a linear regression trend, while after 100 days, a greater stabilization was observed in terms of drug release, better adjusting to a non-linear kinetics. In order to point out, it is known

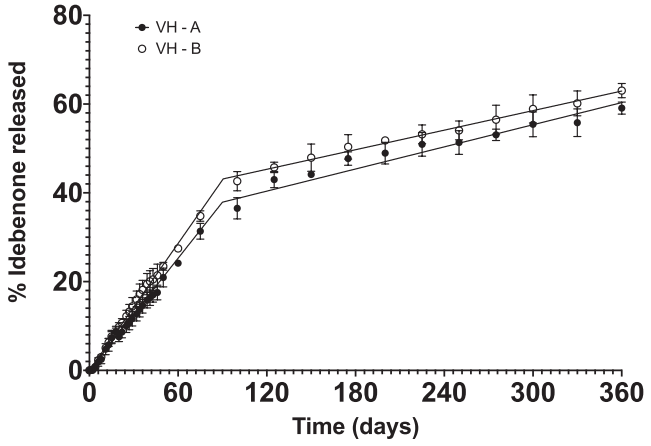

Fig. 11. *In vitro* release study of idebenone-loaded PCL implants. The pharmacokinetic profile of the idebenone-loaded PCL was tested under to different synthetic vitreous humor (VH-A, containing an 0.1% (w/v) agar and 0.5% (w/v) hyaluronic acid, and VH-B, composed of an 0.1% (w/v) agar and 0.4% (w/v) hyaluronic acid).

that the vitreous cavity environment and the nature of the vitreous humor may condition the idebenone release from the PCL implants, generating a concentration gradient within the vitreous cavity [52,53]. Based on these precedents, two different types of vitreous humor were used to perform the release study.

The resulting data of the idebenone cumulative delivery from the PCL implants were fitted to several kinetic models to establish the release behavior. Nevertheless, the greatest correlation data was obtained with a segmental linear regression model, proposing the polymer degradation as the leading release mechanism, combined with the drug diffusion from the implant itself (see details in Table 3).

In order to point out, Heitz et al [54] determined the idebenone pharmacokinetic behavior in plasma, aqueous and vitreous humor after oral administration to a rat model. An estimation of the $t_{1/2}$ values from the results previously obtained by these authors was made, and resulting values of 0.035, 0.026 and 0.153 min^{-1} for plasm, aqueous and vitreous humor were found, respectively. Considering a mean value of 20 μL for vitreous humor in the rat model, it was possible to calculate the mean equilibrium concentration of idebenone into the vitreous humor using a linear monocompartimental model with a zero order (constant) drug incorporation rate. An equilibrium concentration of 1,2 μg/mL in the first initial 90 days and 0.2 μg/mL from the inflexion point (at 90 days) was obtained, with short times to reach the equilibrium concentration (<1h). These predicted values are higher than the idebenone concentrations observed by these authors following once daily administration of idebenone in the diet for 21 days with doses of 2000 mg/kg/day (approx. 14 ng/mL).

4.10. Implant's erosion study: Water uptake, mass loss, and mass remaining

There are numerous key parameters that condition the implant's degradation rate, such as the molecular weight, the crystallinity or the glass transition temperature, among others. Water uptake was proposed as a critical assay to modify the polymer degradation rate, by relating water absorption to the mass decrease of the implant matrix (polymer chain progressive breakage) [55].

Fig. 12 shows the *in vitro* water uptake, mass loss and mass remaining of the idebenone-loaded PCL implants. The water uptake and mass loss profiles revealed that the developed PCL implant exhibits a low degradation rate, related to the high percent of mass remaining in the implants at the 360-day endpoint. Resulting data showed almost no water uptake and weight loss over the first two months, possibly due to a decrease in the polymer molecular weight but without an implant significant mass loss, associated to a slow water penetration into the hydrophobic matrix. However, from the third month onwards, a greater water uptake and mass loss were observed, which might be attributed to a diffusion phenomenon of small polymer fragments that are detached from the implant matrix [56,57]. The overall water uptake and weight loss of the idebenone-loaded PCL implants were around 5% and 10% per year, respectively, suggesting that the drug release mechanism was mostly influenced by a combination of polymer erosion and drug diffusion from the implant matrix, as well as the hydrophobic nature of both components. Thus, a more robust correlation may be established between the drug release mechanism from the polymer matrix and changes in the structure/mass of the polymer over time. These results are in good arrangement with the resulting data obtained in the *in vitro* release assay.

The erosion study was also assessed by the examination of the surface erosion process of the implants. All the tested PCL implants showed comparable structural advancement over the studied period. Both the implant surface and the cross-section were dense and non-porous before the *in vitro* release test (see Section 4.2.2). Moreover, PCL implants showed a homogeneously distributed crystalline structure along the surface at the beginning of the study. Nevertheless, changes in the external surface of the implants were observed throughout the study period, especially after the 240-day determination, going from a defined crystalline structure to a more amorphous one (see Fig. 13). The appearance of pores on the implant surface was also observed as the erosion process progressed, associated to the organic solvent evaporation process during the implant elaboration. However, the internal structure of the implants remained practically unchanged. These results suggest that idebenone was released by polymer erosion and gradual diffusion from the exterior to the interior of the PCL implant.

Table 3
Release data of idebenone-loaded PCL implants into a segmental linear regression.

Vitreous humor	Intercept X = 0	First slope	T inflexion	Second slope	R^2
VH-A	0.28 ± 0.30	0.39 ± 0.01	108.3 ± 2.7	0.07 ± 0.01	0.9980
VH-B	0.26 ± 0.24	0.48 ± 0.01	90.5 ± 1.6	0.07 ± 0.01	0.9990

4.11. Injectability of implants

The injectability assessment constitutes a key-product routine factor of intraocular dosage forms, where the formulation should be delivered by passing through a needle-based system. The injectability of the resulting idebenone-loaded PCL implants was mechanically measured by the panel trial built for the hydraulic press. Furthermore, as the needle gauge might have a critical effect on the patient's comfort and compliance, different needles were tested (see Table 1 in methods section for needle specifications).

All injectability values were appropriate in terms of injection into air, independently of needle diameter or length (see Fig. 14). Nevertheless, the ease of injection into air was more acceptable for wider needle systems. The results of injectability also revealed neither partial nor complete blockage of the polymeric implants flow. Thus, idebenone-loaded PCL implants are suitable for intraocular injection through a 18G needle.

4.12. Cytotoxicity analysis: Hen's egg test on the chorioallantoic membrane (HET-CAM)

The HET-CAM test is commonly used to evaluate the short time cytotoxicity and biocompatibility of ocular formulations. Idebenone-loaded PCL implants were evaluated, and results were compared with those obtained for the negative and positive control formulations. These polymeric implants showed no cytotoxicity properties (Irritation Score = 0), in comparison with the control solutions (see Fig. 15). These results are in good arrangement with formerly published works [58,59], validating that both components and implants are non-toxic and biocompatible. It will be necessary to develop future studies in animal models to evaluate the long-term toxicity of the implants, since implants will be in contact with ocular tissues during long periods of time.

5. Conclusion

The treatment of posterior segment ocular diseases remains limited due to the drawbacks in achieving drug effective concentrations into the vitreous chamber through conventional administration routes, both local and systemic, considered as unproductive pathways [60]. The development of biodegradable intravitreal-targeted DDS to the posterior segment of the eye may overcome most of these shortcomings of conventional pharmaceutical forms, as well as providing numerous advantages, including: (I) sustained and controlled drug release into the target tissue, (II) long-term vitreous concentration of the active substance, (III) frequency of administration reduction, (IV) minimization of side effects appearance and, (V) improvement in the patient's adherence

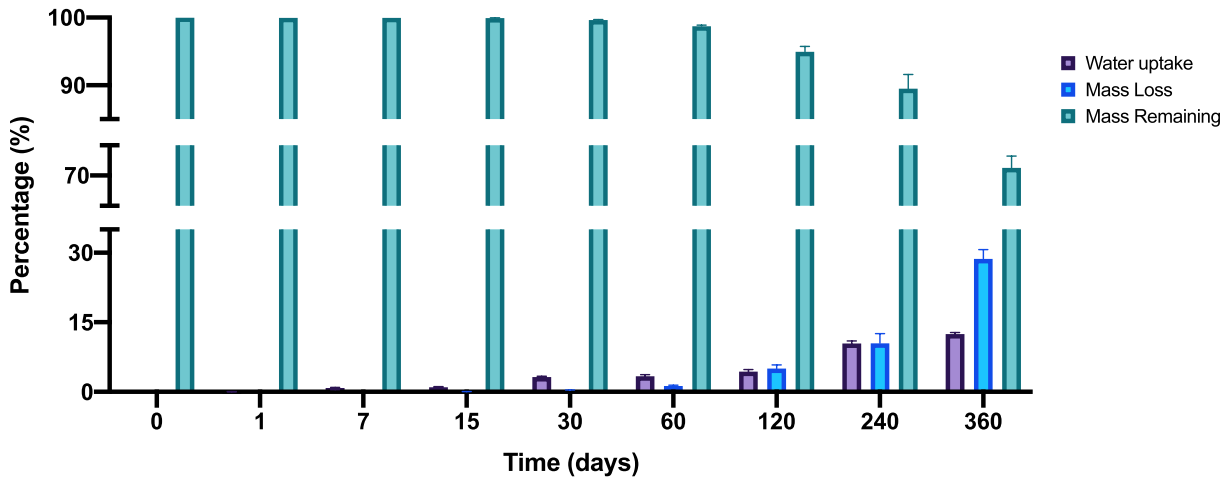

Fig. 12. Erosion study of idebenone-loaded PCL implants. Water uptake, Mass Loss and Mass Remaining data are presented as percentage.

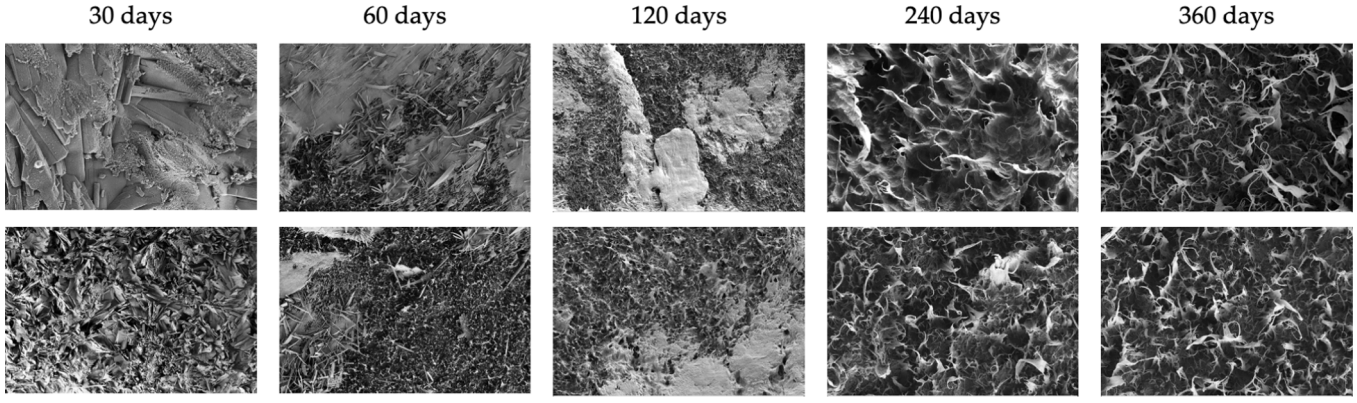

Fig. 13. SEM images of idebenone-loaded PCL implants surface along the erosion study. PCL implant surface was assessed at predetermined time points under the same magnifications (5000x magnification), during the predefined study interval.

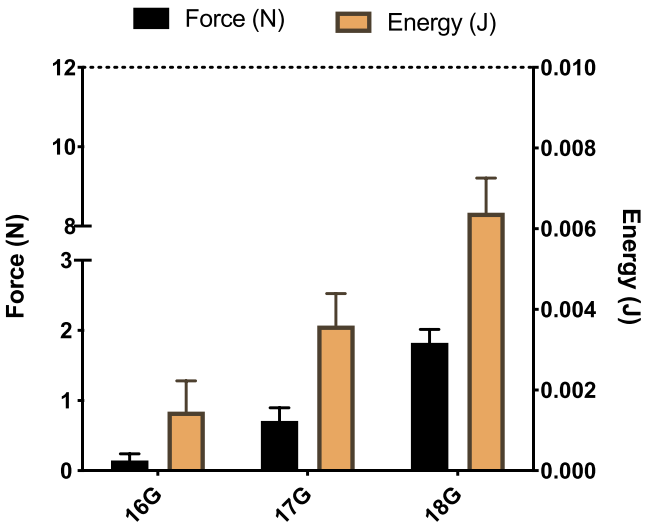

Fig. 14. Injectability results for the idebenone-loaded PCL implants.

to treatment and comfort, among others [29]. Previous research on biodegradable polymers for implant development have been made, although a controlled and sustained drug release for more than six months has not been successfully achieved using biodegradable polymers [61-63].

In the present work, it is proposed, for the first time, a biodegradable long-acting intravitreal implant containing idebenone as a pharmacological alternative for the LHON treatment. PCL was selected as the matrix core compound due to its physicochemical properties, highlighting its extremely low hydrolysis rate of ester bonds and a type III erosion profile, leading to the progressive cleavage of the polymeric chains [57]. A high tolerance by the retinal tissue, without inflammatory response or complications was also observed.

Idebenone-loaded PCL implants were successfully prepared by an homogenization/extrusion/solvent evaporation method. PCL implants showed uniformity of shape and color, as well as a cylindrical appearance, with no evidence of pores or channels, suggesting that drug diffusion is independent of the polymer degradation process. Idebenone release from PCL implants seemed to follow a biphasic pattern, described by a faster preliminary release phase and a slower second phase that lasts beyond one year. Furthermore, high production yield, encapsulation efficiency and loading capacity values were obtained, as well as good mechanical properties. Appropriate physicochemical properties were achieved in terms of DSC/TGA, X-Ray, and FTIR, as well as adequate stability properties with regard to storage, pH and ionic strength. Thus, its design and development were reinforced by an *in vitro* consistent preclinical base. Analogously, idebenone-loaded PCL implants showed appropriate physicochemical properties for intravitreal administration, controlling the idebenone release and guaranteeing the patient's adherence-to-treatment.

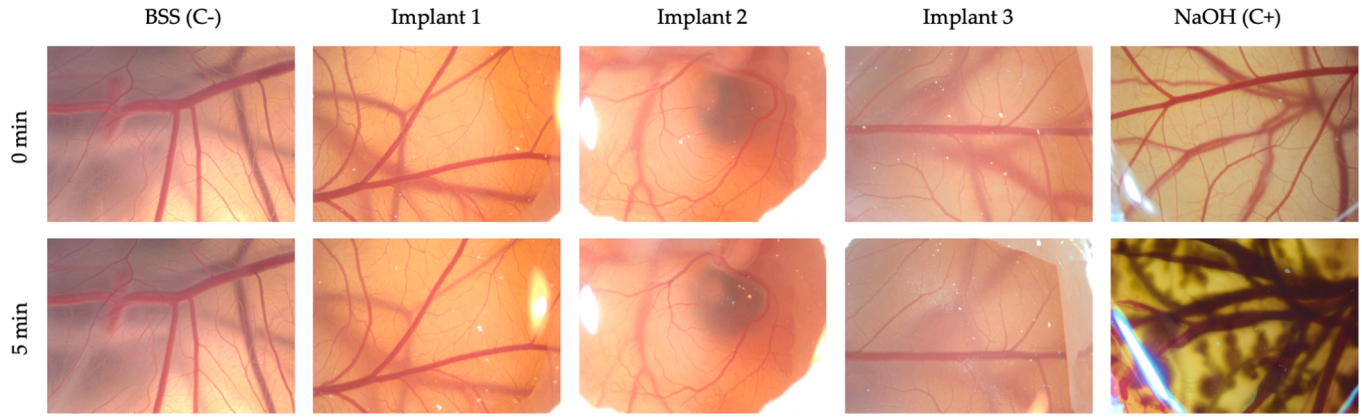

Fig. 15. Images of CAM membranes after the administration of idebenone-loaded PCL intravitreal implant degradation products during the HET-CAM test, compared to the control solutions. (A) idebenone-loaded PCL intravitreal implant degradation products, (B) BSS (negative control), and (C) NaOH solution (positive control).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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