Effects of sterilization on drug loaded ophthalmic lenses materials

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ABSTRACT

The use of ophthalmic lenses as drug carriers seems a promising option for the sustained ocular drug delivery. However, to solve the problem of incorporating sufficient amounts of drug and achieving a sustained release while ensuring sterility is still a challenge. Sterilization is mandatory to reduce the risk of infection but it is imperative to preserve the drugs and materials properties. This work presents an investigation of the effects of two methods of sterilization, steam heat (SH) and gamma radiation, on several ophthalmic drugs (diclofenac, ketorolac, moxifloxacin and a combination of the last two), on two polymeric materials used for the production of ophthalmic lenses (a contact lens and an intraocular lens material), and on the drug loaded materials. The role of different conditions of SH sterilization, loading (in particular, the loading temperature), release and storage, was evaluated using the intraocular lens material loaded with moxifloxacin. The impact of the sterilization procedures and of the loading, release and storage conditions on the stability and antimicrobial activity of the drugs as well as on the properties of lenses materials (before and after drug-loading) was assessed. For all studied systems, SH sterilization stood out as an excellent method: it did not affect the drugs/materials and even improved the release profiles of the intraocular lens material loaded with all the studied drugs. For the intraocular lens material loaded with moxifloxacin, higher loading temperatures and longer storage times also significantly improved the release profiles.

Keywords: ocular drug delivery, ophthalmic drugs, ophthalmic lenses materials, gamma radiation sterilization, steam heat sterilization, loading conditions.

1. INTRODUCTION

Over the last years, several strategies for ocular drug delivery have been considered towards the development of novel and safe drug delivery systems capable of surpass the ocular barriers and maintain adequate drug levels in the ocular tissues. Though, until today none of the new methods became as important as eye drops. In fact, they are the most common form of ocular treatment, representing 90% of the marketed ophthalmic formulations. However, they have a low ocular bioavailability: less than 5% of the applied dose reaches the deeper ocular tissues. Thus, more investigation is needed in order to provide an improved system capable of deliver adequate amounts of drug to the target tissues during an appropriate time, avoiding the side effects associated with the current existing drug delivery methods.¹,²

Contact lenses (CL) and intraocular lenses (IOL) have arisen as promising options for the sustained release of drug into the ocular tissues from both segments of the eye. To turn these new drug carriers in safe and efficient options, besides the incorporation of sufficient amounts of drug into the lens matrix and the achievement of a sustaining drug release for a desired time, some issues concerning sterilization and storage, need also to be explored to ensure that all the restrict criteria are fulfilled, such as, therapeutic effectiveness, comfort, safety, biocompatibility and sterility. Sterilization is mandatory for the ophthalmic products/lenses due to the potential risk of infection. Moreover, every method of sterilization needs to be validated with respect to the assurance of sterility and should warrant that no adverse effects occur within the product/material. For drug loaded lenses, this issue becomes even more complex, since despite the maintenance of the lenses proprieties it is necessary to ensure that loaded drugs remain stable and active after the sterilization.³,⁴

The main objective of this research is to determine the effect of two different methods of sterilization, SH and gamma radiation (GR), on several ophthalmic drugs, on two polymeric materials currently used for the production of ophthalmic lenses and on the drug loaded lenses materials. Focusing in one drug loaded IOL material, it will be investigated the role of different conditions of SH sterilization, loading and release. Special attention will be given to the effect of loading temperature. Furthermore, the effect of storage of the drug loaded IOL in the drug solution will also be investigated.
Thus, to assess the impact of the different sterilization procedures, methodologies of loading, release and storage conditions, the drugs, lenses materials and drug loaded materials were subjected to many analytical assays to characterize the materials properties, to study the stability and antimicrobial activity of the drugs and to evaluate eventual changes in the drug release behaviour of the loaded materials.

2. EXPERIMENTAL

2.1. Materials

The drugs used in this work were Moxifloxacin (MXF) hydrochloride $\geq 98\%$ from Carbasynth Ltd., VIGAMOX® (GVMX) $5\, \text{mg/mL}$ ophthalmic solution, from Alcon, Diclofenac (DFN) sodium salt $\geq 99\%$ and Ketorolac (KTL) tris salt $\geq 99\%$ both obtained from Sigma-Aldrich.

The ophthalmic lenses materials, kindly provided by Physiol SA (Liège - Belgium), were Contaflex 58 (CFL58), and BenzFlex 26% Natural Yellow™ (B26Y). Before the utilization of the materials, they were submitted to impurity extraction by soxhlet extraction.

The excipient used for radio-protection of the drugs was D-Mannitol $\geq 98.0\%$ (Sigma-Aldrich).

For the antimicrobial assays and sterility tests the following products/materials were used: Staphylococcus aureus ATCC 25923, Pseudomonas aeruginosa ATCC 15442, Escherichia coli ATTC 10536, Candida albicans ATCC 10231 and Aspergillus niger ATCC 16404, all obtained from American Type Culture Collection (ATCC); Staphylococcus epidermidis CECT 231 from Colección Española de Cultivos Tipo (CECT); blank antimicrobial susceptibility disks (Oxoid), tryptone soya agar (TSA) (Oxoid) and Mueller-Hinton Agar (MH) (Oxoid), all purchased in Thermo Fisher Scientific.

For the production of the mobile phases of High Performance Liquid Chromatography (HPLC), for each drug, were used: ortho-phosphoric acid (technical grade) 85% (Panreac), methanol (HPLC grade) $\geq 99.8\%$ (Fisher Scientific), acetonitrile (HPLC Plus gradient) $\geq 99.95\%$ (Carlo Erba Reagents), triethylamine $\geq 99\%$ (Sigma-Aldrich), potassium phosphate monobasic $\geq 99\%$ (Sigma-Aldrich) and potassium chloride $\geq 99\%$, (Sigma-Aldrich).

All aqueous solutions were prepared with distilled and deionized (DD) water, obtained using a Millipore Milli-Q water purification system.

The two solutions used throughout this work were: sodium chloride (NaCl) and Hank’s balanced salt solution (HBSS) (pH of 7.3). NaCl was prepared at 130 mM with sodium chloride 99.5% (Panreac) and HBSS was prepared in 1 L of DD water by dissolving 8 g of NaCl 99.5% (Panreac), 0.4 g of potassium chloride (Sigma-Aldrich), 0.0358 g of disodium hydrogen phosphate (Sigma-Aldrich), 0.060 g of potassium phosphate monobasic $\geq 99\%$ (Sigma-Aldrich), 0.144 g of calcium chloride (Panreac), 0.246 g of magnesium sulfate heptahydrate (Sigma-Aldrich) and lastly 0.350 g of sodium bicarbonate (Sigma-AI.), with a gentle stirring.$^5$

2.2. Methods

2.2.1. Sterilization methods

In the present work two methods of sterilization were applied to the drugs, the ophthalmic lenses materials and the drug loaded disks: SH and GR. The SH sterilization was accomplished in a vertical steam sterilizer, an UNICLEAVE 88/75L and the sterilization parameters used were 60 min at 121°C and 1 bar. In some cases, drug loaded materials were also sterilized for 30 or 90 min. GR sterilization was performed in atmospheric air at room temperature, using a $^{60}\text{Co}$ as a source of gamma rays. As the dosing rate was approximately 5 kGy/h, the time used for each dose tested (5, 15 and 25 kGy) was 1, 3 and 5 hours respectively. Depending on the studied propose, sometimes only one or two of the radiation doses were used.

2.2.1.1. Sterilization of drugs

The drugs were sterilized in solution by SH (60 min) and by GR (5, 15 and 25 kGy), which concentrations are different depending on the studied propose. Sterilized drug solutions of DFN, KTL, MXF and a combination of MXF with KTL (MXF+KTL) were prepared in NaCl solution with a [drug] of 2 mg/mL. For GR sterilization, solutions with 5% of mannitol and in powder were also tested. MXF solutions were prepared as well with a [drug] of 2 and 5 mg/mL and sterilized by SH (60 min). Additionally, MXF and VGMX solutions were prepared in HBSS solution with a [drug] of 5 mg/mL and sterilized by SH (30 min).

2.2.1.2. Sterilization of lenses materials

For the materials characterization, CLs and IOLs were sterilized by SH (60 min) and by GR (5, 15 or 25 kGy) in NaCl solution (henceforward, also called just NaCl or aqueous solution). IOLs were also sterilized by SH (60 min) and by GR (5 kGy) in drug solutions with a concentration of 2 mg/mL of DFN (only for SH), KTL, MXF or MXF+KTL.

2.2.2. Drug loading and drug release

For the drug loading/release assays disks were cut in 5 mm of diameter. Before the loading/release experiments all lenses materials were properly dried for 7 days at 36°C and their height was measured for further calculations. In all the experiments, loading was accomplished using the drug soaking method, where disks were immersed in a drug solution for 4 days at a defined temperature. After drug incorporation, loaded disks were removed from the drug solution, rinsed with DD water and blotted with dry absorbent paper to remove the excess of drug on the surfaces. For the drug release experiments, carried out in static sink conditions, lenses materials were immersed in 3 mL of drug solvent solution (NaCl or HBSS) and placed in a shaker (Incubating Mini Shaker from VWR) to mimic the eye conditions, at 36°C and 180 rpm. To determine the amount of drug release (methodology of quantification will be described hereinafter), aliquots of 0.3 mL were collected and replaced with the same volume of fresh solvent solution. The aliquots were taken over time, until the plateau was reach, point at which no more drug was released. All the release experiments were done at least in triplicate.

Regarding the release studies, several conditions of loading, sterilization, storage and release were used.

For the study of the effects of SH and GR sterilization on the release profiles of loaded CLs and IOLs materials, the disks were loaded in 0.5 mL of drug solution (prepared in NaCl) at 4°C with DFN, KTL, MXF and MXF+KTL with a [drug] of 2 mg/mL and sterilized on the 3rd day of loading or before loading by SH (60 min) and GR (5 kGy).

To study the effects of SH sterilization and loading temperature on the release profiles of loaded IOLs materials, disks were loaded in 0.5 mL of MXF solution prepared in NaCl and sterilized, or not, by SH in different conditions. Loading was done at 4, 36, 60 and 80°C with a [drug] of 2 or 5 mg/mL and sterilization was performed during 30, 60 or 90 min on the 1st or 3rd days of loading.

For the study of the effect of the release conditions and reversibility of the drug release profiles of loaded
IOLs materials, the disks were loaded in 0.5 mL of MXF solution at 2 mg/mL, prepared in NaCl, at 4 or 60°C and non-sterilized. The aliquots collection and replacement were change between conditions in terms of rate and volume (0.3 or 3 mL, respectively) with a total n° of aliquots per experimental condition of 30 or 44 correspondingly) for the study of the effect of the release conditions.

Lastly, to study the effect of storage on the release profiles of loaded IOL materials, disks were loaded at 60 or 80°C with MXF or VGMX at 5 mg/mL and sterilized, or not, by SH (30 min) after the loading period.

### 2.2.3. Drugs analysis

#### 2.2.3.1. Quantification of released drugs and determination of drugs degradation

The amount of drug released was determined with both, an UV–Vis spectrophotometer (Multiskan™ GO Microplate Spectrophotometer from Thermo Scientific) and a HPLC (Waters Alliance 2695) incorporated with a Nova-Pak C18 RP column (60 Å, 4 μm, 3.9 mm × 150 mm) and a Photodiode Array (PDA) detector (Waters 396). The data collected from the HPLC were processed with the Empower PDA Software (from Waters).

The drug quantifications were performed by reading the absorbance of each aliquot collected, selecting an appropriated wavelength for each drug (276 nm for DFN, 315 nm for KTL and 290 nm for MXF and VGMX) 6–11. For each aliquot, at least, two measurements were done and for each drug, several calibration curves were produced during all the experiments and all the quantifications were done in the linear range of the calibration curves.

Apart from quantifying the released drugs, HPLC was also used to analyse the eventual degradation of the drugs, after being submitted to the treatments such as sterilization, heating or storage. For HPLC assays, two mobile phases were prepared considering the studied drug. The mobile phase for DFN was produced by using orto-phosphoric acid (at 0.05 M), acetonitrile and methanol in the ratio of 40/48/12 v/v/v, respectively.6 The mobile phase for KTL, MXF, MXF+KTL and VGMX was prepared using phosphate buffer (2.72 g of potassium phosphate monobasic per 1 L of DD water and 1 mL of triethylamine at pH 3.0 – adjusted with orto-phosphoric acid) and methanol in the ratio of 30/3/35/35 v/v/v/v, respectively.6 All mobile phases were introduced into the column at a flow rate of 1 mL/min.

The injection volume was 20 μL, the monitoring by the detector was in the range of 210 – 395 nm and all the quantifications were run at 25 ± 5°C.

To analyse the drugs degradation, DFN and KTL formulations were diluted in NaCl solution until reaching the concentration of 100 μg/mL, MXF, MXF+KTL and VGMX were diluted in NaCl solution or HBSS until achieving the concentration of 35 μg/mL. All samples were prepared, at least in triplicate, and for each sample two measurements were performed. The relative concentration of drug solutions was determined using the following equation:

\[
\text{% Relative concentration} = \frac{[\text{drug after the experiments}]}{[\text{drug before the experiments}]} \times 100
\]

where the experiments refer to the procedures of sterilization, heating or storage.

#### 2.2.3.2. Determination of MICs and drugs activity

The antimicrobial agents used were MXF, MXF+KTL and VGMX against two strains of gram-positive bacteria: Staphylococcus aureus (SA) and Staphylococcus epidermidis (SE). Before starting the microbiological assays, both strains were previously cultured in TSA medium for 24 h at 37°C. The agar diffusion method was used to determine the activity of the studied drugs and the agar dilution method was used to determine the MIC values and the calibration curves necessary to estimate the drug concentration. In the latter case, drug solutions were prepared with the concentrations of 0.25, 0.5, 1, 2, 4, 8, 16 and 32 μg/mL. For both proposals of study, all the inoculated mediums were prepared in quadrangular petri plates (120 × 120 mm) by adding 350 μL of the bacterial suspension (with a concentration of 0.5 McFarland in DD water) and 50 mL of Mueller Hinton (MH) agar (38 g of MH agar / 1 L of DD water), previously sterilized by SH at 121°C for 15 min and kept in a water bath at 50°C until stabilize the temperature. Filter paper discs with 6 mm diameter were placed on the agar plates with 15 μL of each sample solution and, at least, one negative control per plate (15 μL of sterilized solvent solution). Then, all petri plates were incubated for 24 h at 37 ± 2°C. After the incubation period, the inhibition halos were measured (in mm) using a digital calliper (0 – 150 mm/0.01 mm ± 0.02 mm).

The antimicrobial activity (%) was determined in two different ways. To evaluate the activity of drugs after the sterilization procedures, MXF and MXF+KTL solutions were prepared with a concentration of 10 μg/mL for all the experimental conditions. Therefore, the activity, before and after sterilization, was determined by the following expression:

\[
\text{% Antimicrobial activity} = \frac{\text{[drug determined by microbiological assay]}}{\text{[drug determined by HPLC]}} \times 100
\]

These microbiological assays were done at least three times in duplicate.

To determine the activity of the released drugs (MXF and VGMX from day 3 and 7 of release), the total concentration of the release solution was previously quantified by HPLC. Then, the activity was determined with the following expression:

\[
\text{% Antimicrobial activity} = \frac{\text{[drug determined by HPLC]}}{\text{[drug determined by microbiological assay]}} \times 100
\]

These microbiological assays were accomplished only once in duplicate.

#### 2.2.3.3. Sterility tests

The membrane filtration technique was chosen to verify the sterility of drug solutions. Sterilized drug solutions by SH (15 mL of DFN, KTL, MXF and MXF+KTL) and by GR (5kGy) (15 mL of KTL, MXF and MXF+KTL) were totally filtered through a sterile membrane (0.45 – micron). The membrane was aseptically divided and placed in two culture media, fluid thioglycolate medium (FTM) for aerobic and anaerobic bacteria and tryptone soya broth (TSB) for aerobic bacteria, fungi and yeasts. Both media were incubated for 30°C and 25°C, respectively, for 14 days. Positive controls and negative controls were also prepared and incubated together with the testing samples. The positive controls consisted in 4 infected membranes (2 per medium) by the passage of 15 mL of suspensions (1 per membrane) previously prepared with sterilized DD water and 4 different microorganisms at 10³ (qualitatively). The microorganisms used as positive controls for FTM were Pseudomonas aeruginosa and Escherichia coli, and for TSB were Candida albicans and Aspergillus niger. The negative controls consist in 2 membranes (1 per medium).
used to filter 15 mL of sterilized DD water. After the incubation period, cultures were analysed in terms of turbidity.10

2.2.4. Materials characterization

2.2.4.1. Transmittance

Optical clarity studies were carried out by measuring the absorbance of visible light through samples, i.e., disks, cut in a half. This characterization technique was performed for both types of materials that were sterilized in NaCl solution and also for loaded IOLs materials sterilized in drug solution with a concentration of 2 mg/mL. Samples were previously hydrated in DD water for 24 hours and placed in a lateral surface of a quartz cuvette. The absorbance was measured at room temperature in the wavelength range of 200 ≤ λ (nm) ≤ 760 (for non-loaded lenses) and 360 ≤ λ (nm) < 760 (for loaded lenses). The procedure was held, at least once, using both sides of each sample and in three different regions, with a total of 6 measurements per sample. The percentage of transmittance was calculated using the follow relation:

\[ \%T = 10^{-\text{Abs} \times 100} \quad \text{Eq. 4} \]

where T is the transmittance and \text{Abs} is the absorbance.13

2.2.4.2. Swelling kinetics

Both materials were cut in small lenses with 5 mm of diameter to perform the swelling assays. The swelling kinetics was determined in DD water and, in some cases, in MFX solution (2 mg/mL), at 4, 36, 60°C. After dried the materials their dry weight was measured and then, each disk was immersed in 1 mL of the solution and incubated at the respective temperature. During the assays sample disks were taken out of the solution, gently blotted with absorbent paper and weighed, until achieving the equilibrium. The procedure was done at 2, 4, 6, 9, 24 and 48 hours. The swelling ratio (%SR), and the equilibrium water content (%EWC) were estimated, respectively, using the following expressions:

\[ \%SR = \frac{W_e-W_w}{W_o} \times 100 \quad \text{Eq. 5} \]
\[ \%EWC = \frac{W_e-W_w}{W_{sw}} \times 100 \quad \text{Eq. 6} \]

where \( W_w \) is the weight of the swollen hydrogel at time \( t \), \( W_e \) is the weight of the swollen hydrogel at the equilibrium and \( W_o \) is the weight of the dry hydrogel.14,15

2.2.4.3. Wettability

For the wettability studies, the water contact angle was measured using the captive bubble method at room temperature in disks cut in a half. CLs and IOLs materials, non-sterilized and sterilized in NaCl solution, were previously hydrated in DD water for 24 h and then fixed to a porcelain support and placed downwards inside a liquid cell (with quartz windows), which was full of DD water. Then, with an inverted needle from a micrometric syringe, an air bubble of approximately 3 – 6 µL was placed on the lens surface. For each type of lens, at least 13 bubbles in both sides of a lens were monitored for 1 minute, being recorded 14 images along the time, for each bubble. A video camera (JAI CV-AS0) connected to a microscope (Wild M3Z) was used to acquire the images and the video signal was transmitted to a frame grabber (Data Translation DT3135). The image analysis was performed with ADSA-P software (Axisymmetric Drop Shape Analysis Profile).16

2.2.4.4. Ion permeability

The ion permeability of the lenses materials was measured using a poly(methyl methacrylate) (PMMA) horizontal diffusion cell with two compartments, the donor and receiver chambers. The samples (entire disks) fully hydrated in DD water were mounted between the two compartments. The donor chamber was filled with 24 mL of NaCl solution and the receiver chamber with 32 mL of DD water. After a suitable calibration, the conductivity was measured at each hour for at least sixteen hours using a conductivity measuring cell (TetraCon 325 from WTW) inserted in the receiver chamber and connected to a conductivity meter (Cond 340i from WTW) to set the measurements and record the values. Calibration curves were prepared to convert conductivity data (in µS/cm) into NaCl concentrations (in µg/mL). The NaCl concentrations were plotted as a function of time and the rate of ion transport (\( F \)) was given from the slope of the linear regression. The ion permeability was then calculated applying the Fick’s law:

\[ \frac{dC}{dx} = \frac{D \times C_0}{A} \quad \text{Eq. 7} \]

where \( F \) is the rate of ion transport, \( V_r \) is the volume of the solution in the receiving chamber, \( A \) is the area of the lens and \( \frac{dC}{dx} \) is the initial concentration gradient of NaCl that across the hydrogel with a defined thickness, \( dx \).17 The experiments were done at 36°C and in triplicate.

2.2.4.5. Morphology

To access the surface morphology, disks of CLs and IOLs materials (5 mm of diameter) were previously hydrated in DD water for 24 h, carefully cleaned with an absorbent paper and then placed in a –80°C freezer for 3 h and lyophilized overnight. Then, the samples were coated with a 15 nm layer of Cr by sputtering, using a turbo-pumped sputter coater (Q150T ES, from Quorum Technologies) and analysed in vacuum conditions with a Field Emission Gun - Scanning Electron Microscope (FEG-SEM) (JEOL JSM-7001F model, from JEOL).

2.2.4.6. Thermotropic behaviour

The thermotropic behaviour study was performed with a differential scanning calorimeter (DSC) (200 F3 Maia model, from NETZSCH). After a suitable calibration, the measurements were run under a nitrogen atmosphere. The Proteus software was used to acquire data and evaluate the results. IOLs materials (3 mm of diameter) were characterized both unloaded and loaded with [MXF] 2 mg/mL at 4°C and 60°C. For each experimental condition, three DSC runs were taken to determine the average \( T_g \) value. Before the experiments, the samples were taken out of DD water or the loading solution, properly cleaned and dried in a vacuum oven at 36°C for 7 days to remove as much as possible the free and loosely bound water. Then, samples were weighted before and after being placed inside a concavus pan (Al crucible with a lid (Ø: 5 mm, C = 30/40 µl – from NETZSCH), sealed with a sealing press (from NETZSCH) and placed together with an empty sealed reference pan in the heating block of the equipment. The DSC thermograms were recorded during two successive heating cycles using the following experimental conditions: (i) isothermal scan for 10 min at 20°C; (ii) heating scan from 20 to 140°C (10°C/min); (iii) isothermal scan for 10 min at 140°C; (iv) cooling scan
from 140 to 20°C (10°C/min); (v) isothermal scan for 10 min at 20°C; (vi) and, a heating scan from 20 to 140°C (10°C/min). After processing the data, the glass transition temperatures were considered as the midpoints of the steps in the baseline.

2.2.4.7. Structural properties

Powdered samples of MXF (~200 mg) or swollen IOLs materials (8 discs of 5 mm diameter per experiment) were packed into a cylindrical zirconia rotor (Ø: 7 mm; L: 18 mm) and analysed by solid state Nuclear Magnetic Resonance (ssNMR). Before the experiments, the IOLs materials were hydrated in saline solution or loaded with MFX in different experimental conditions (different times, temperatures and solution concentrations), then the materials were rinsed and cleaned properly with absorbent paper to remove the excess of solution from their surfaces. $^{13}$C CP/MAS – TOSS spectra without and with 30 μs of Dipolar dephasing (DT) were obtained at 75.49 MHz on a Tecmag (Redstone)/Bruker 300 WB spectrometer, at a MAS rate of 3.3 – 3.7 kHz with 90° RF pulses of about 4.5 μs, contact time of 2 ms and a relaxation delay of 10 s. $^{13}$C CP/MAS spectra with suppression of $^{13}$C non-quaternary signals were achieved by interrupting proton decoupling during 30 μs before the acquisition period. $^{13}$C chemical shifts were referenced with respect to an external glyicine sample ($^{13}$CO observed at 176.03 ppm). The number of scans accumulated for each spectrum was: 1000 and 8480 for MXF without and with DT respectively; more than 7000 for the IOLs materials loaded with MFX solution; and 1256 for swollen IOLs materials in NaCl solution.

2.2.4.8. Sterility tests

The direct inoculation method was chosen to verify the sterility of lenses materials. Both types of lenses materials (with 5 mm of diameter) were sterilized by SH and by GR (5 kGy) in NaCl solution, and then placed directly in two culture media (FTM and TSB) where they were incubated, respectively, at 30°C and 25°C for 14 days. Positive controls and negative controls were also prepared and incubated together with the testing samples. The positive controls consisted in four inoculated culture mediums (1 per microorganism) with microorganism’s suspensions of $10^3$ (qualitatively); two FTM inoculated with Pseudomonas aeruginosa and Escherichia coli; and two TSB inoculated with Candida albicans and Aspergillus niger. The negative controls consisted in two non-inoculated culture media, one of FTM and another of TSB. After the incubation period, cultures were analysed in terms of turbidity.

3. RESULTS AND DISCUSSION

3.1. Effects of sterilization

3.1.1. Effects of sterilization on drugs

To evaluate the degradation of the studied drugs, as a consequence of having undergone sterilization, HPLC was used to analyse the samples before and after the sterilization. The quantification of drugs degradation is presented in Figure 1.

Nonetheless, when sterilized with 5 kGy of GR in solution, there was a minimal degradation of MXF and MXF+KTL but some degradation of KTL and DFN, being DFN the most affected. Generally, anti-inflammatories were more degraded by GR than antibiotics, when in solution. Moreover, the results suggest that KTL and MXF are better resistant to GR when combined, especially for higher doses of GR. For all drugs, it seems also mannitol had no visible effect on the drug degradation prevention as was previously reported by literature.18

The antimicrobial activity assays, performed against SA and SE presented almost exactly the same results as HPLC for the tested antibiotics, but in terms of loss of activity. The MIC values obtained experimentally for both MXF and MXF+KTL were in the range of 0.5 – 1 μg/mL for both strains of bacteria.

The sterility tests, done by membrane filtration technique, indicated that only SH was able to ensure the sterility of all four drugs. Concerning GR, only KTL was sterile. MXF and MXF+KTL present some bacterial growth, meaning that 5 kGy was not sufficient to sterilize the 15 mL of drug solutions. However, the tested volume was 15 to 30 times more than the volume planned to be used during loadings.

3.1.2. Effects of sterilization on lenses materials

The studied properties were transmittance, swelling, wettability, ion permeability and morphology, where in all studies, the non-sterilized materials (IOLs and CLs) were always compared to the sterilized ones, in aqueous solution.

For transmittance, swelling and wettability assays, the non-sterilized materials were compared to the sterilized ones, by SH or GR (5, 15 and 25 kGy). For the ion permeability and sterility tests, only sterilized samples by SH or with 5 kGy of GR were studied. The morphological characterization was done for the materials sterilized by SH and GR (5 and 25 kGy).

In the transmittance experiments the control lens material was hydrated in DD water and was not sterilized. The spectra obtained are illustrated in Figure 2. The results obtained for B26Y did not present differences in the visible range, however between 400 – 500 nm, the increase of the radiation dose leads to an increase in %T due to the partial degradation of the violet light filtering chromophore,
that is present in the material. Concerning CFL58, the increase in the radiation dose leads to a decrease in %T, placing the material sterilized with 15 and 25 kGy of GR below 90%, the threshold established for an appropriate optical transmissibility of visible light in a CL.

The swelling profiles and EWC were determined in DD water at two temperatures (4 and 36°C). The sterilization did not affect the swelling behaviour of B26Y but for CFL58, GR sterilization affects the swelling behaviour decreasing the EWC with the increase of the radiation dose. In both materials the swelling profiles shown that the water uptake was faster at 36°C and the EWC increased at 4°C.

Contact angles were measured by the captive bubble method in the wettability studies. The results are presented for both materials in Figure 3.

No significant differences were seen among the contact angles for both materials after sterilization. The largest variation was approximately ±8° between a non-sterilized lens and a lens sterilized with 25 kGy of GR.

The values of the ionoflux diffusion coefficients (D_{ion}) were calculated from the measured conductivities. Sterilization only slightly affected the ion permeability and all the obtained D_{ion} coefficients, before and after sterilization, are above of the minimum required value, at least by one order of magnitude (1.5 x 10^{-7} mm²/min).

The surface analysis of the materials was performed by SEM. After sterilization the images did not reveal any alteration, and, for this reason, only non-sterilized materials are shown in Figure 4.

The sterility tests for both materials submitted to SH and GR (5 kGy) in aq. solution reveal the materials were sterile.

### 3.1.3. Effects of sterilization on drug loaded materials

Drug release experiments were carried out in both materials to assess the effect of the sterilization methods on the drug release behaviour. Regarding the previous results obtained for the sterilization of drugs and materials, the chosen sterilization methods to study the release were SH and 5 kGy of GR, except for DFN-loaded materials which were not submitted to GR. The materials were sterilized in drug solutions and in NaCl solution. All the drug quantifications were done by HPLC. The release profiles from the materials sterilized in the drug solutions are presented in Figure 5 (B26Y) and Figure 6 (CFL58). In all cases the profiles obtained with the correspondent non-sterilised samples are included.

![Figure 2. Transmittance (%) of ophthalmic lenses materials, non-sterilized and sterilized in aqueous solution: (A) – B26Y; (B) – CFL58.](image)

Figure 2. Transmittance (%) of ophthalmic lenses materials, non-sterilized and sterilized in aqueous solution: (A) – B26Y; (B) – CFL58.

![Figure 3. Water contact angles of ophthalmic lenses materials, non-sterilized and sterilized in aqueous solution: (A) – B26Y; (B) – CFL58.](image)

Figure 3. Water contact angles of ophthalmic lenses materials, non-sterilized and sterilized in aqueous solution: (A) – B26Y; (B) – CFL58.

![Figure 4. SEM images of the surface of a non-sterilized B26Y (A and B) and CFL58 (C and D). (A and C) – Magnification of 1000x. (B and D) – Magnification of 3000x.](image)

Figure 4. SEM images of the surface of a non-sterilized B26Y (A and B) and CFL58 (C and D). (A and C) – Magnification of 1000x. (B and D) – Magnification of 3000x.

![Figure 5. Cumulative release profiles of DFN (A), KTL (B), MXF (C) and MXF+KTL (D) – MXF and (E) – KTL) from B26Y, determined by HPLC. Samples were sterilized on the 3rd day of loading.](image)

Figure 5. Cumulative release profiles of DFN (A), KTL (B), MXF (C) and MXF+KTL (D) – MXF and (E) – KTL) from B26Y, determined by HPLC. Samples were sterilized on the 3rd day of loading.

![Figure 6. Cumulative release profiles of DFN (A), KTL (B), MXF (C) and MXF+KTL (D) – MXF and (E) – KTL) from CFL58, determined by HPLC. Samples were sterilized on the 3rd day of loading.](image)

Figure 6. Cumulative release profiles of DFN (A), KTL (B), MXF (C) and MXF+KTL (D) – MXF and (E) – KTL) from CFL58, determined by HPLC. Samples were sterilized on the 3rd day of loading.

Overall for B26Y, GR slightly decreases the released amount, while SH improves all the release profiles, not
only in the amount but also in the kinetics of release. In this material, DFN loaded more followed by KTL and then MXF. Between MXF and KTL and the combination of MXF+KTL, the release was slightly increased for both drugs in the mixture.

For CFL58, in all cases, there was an initial burst in the released drug and almost all drug was released in the first 24 h. In this material, as in B26Y, DFN loaded more followed by KTL and then MXF. Between MXF and KTL and the combination of MXF+KTL the uptake/release was practically the same. GR leads to a lower release of the drug and SH did not affect the drug release. When compared with B26Y, the release from CFL58 was faster and yield higher amounts of drugs.

The release profiles were practically not altered when soaking was done after sterilization, which means the proprieties of both lenses materials, seems to have been preserved. In both materials, when sterilization was performed on the 3rd day of loading, the HPLC chromatograms (of the aliquots collected at the 48 h of the release) reveal degradation peaks, which justify the decrease in the amount of released drug.

The studied properties of loaded materials after sterilization were transmittance and morphology. The results obtained for the %T of loaded IOLs materials only presents differences in the transmissibility of irradiated lenses loaded with KTL. The other systems weren’t affected by the presence of the drug neither due sterilization during loading. The surface analysis of the loaded materials did not reveal any alteration.

3.2. Effect of different experimental SH sterilization and loading/release conditions on the properties and release profiles of MXF loaded IOLs materials

In face of the promising results presented for the release of drugs from the loaded IOL material sterilized by SH and in order to clarify which factors, during the SH sterilization process, could have contribute for such results, several loading/release and sterilization conditions were varied with the objective of understanding their effect on the drug release behaviour and on the intrinsic properties of the material. For these studies only one drug was used - MXF.

3.2.1. Effect of SH sterilization conditions

All samples were sterilized in MXF solution by SH in different conditions of loading or sterilization. The release curves are presented in Figure 7.

In what concerns the effect of the moment of autoclaving (Figure 7 (A)) the results revealed that sterilizing in the 1st day or in the 3rd day of loading period does not affect the release profiles. In turn, with respect to the effect of the duration, sterilizing during 30, 60 or 90 min (Figure 7 (B)) also did not change the release profiles, which was probably due to the fact that the total time that each sample remained inside the autoclave was almost the same. In other hand, the increase in the concentration of the loading/sterilization solution (Figure 7 (C)) did not affect the release when the materials were non-sterilized, but after sterilization the amount released clearly increased with the concentration. After normalizing the release curves (Figure 7 (D)), it was possible to see that the increase in the concentration only affect the amount of drug released, the release rate was exactly the same after the SH sterilization. The comparison of the release curves obtained in all cases with the one obtained with non-sterilized samples shows that SH sterilization induces significant changes in the release of the drug. SH sterilized samples present a sustained release over almost 60 days.

The release profiles confirm that the higher the loading temperature, the greater the amount released and the longer the release time.

HPLC assays revealed that no degradation occurs after 4 days of exposure to 60°C and 80°C.

The diffusional mechanism determined using the Korsmeyer–Peppas model denoted a non-Fickian drug diffusion or anomalous drug transport when temperatures up from 36°C were used during loadings.

With the intend of explaining why the hydrogels when subjected to high temperatures display a significant improvement in the drug release behaviour, several techniques were used to verify if the material suffers significant changes when exposed to high temperature.

The swelling profiles (in Figure 9) demonstrate that with the increase in temperature, the solution uptake becomes faster and the EWC slightly decreases. Moreover, when compared with DD water, the EWC performed in MXF-2 mg/mL slightly decreases.

Figure 7. Cumulative or fractional release profiles of MXF from B26Y determined by UV–Vis spectrophotometry. (A) – Study of the effect of the moment of autoclaving. (B) – Study of the effect of the duration of sterilization. (C and D) – Study of the effect of the drug concentration in the loading/sterilization solution.

From the fit of the first 60% drug release data in the kinetic model described by Korsmeyer–Peppas21 the release of the experimental curves was mainly controlled by Fickian or quasi-Fickian diffusion.

3.2.2. Effect of loading temperature

Following the previous study and considering that SH sterilization was performed at 121°C, several temperatures were chosen to perform the loadings without autoclaving. The release curves are presented in Figure 8.

The release profiles confirm that the higher the loading temperature, the greater the amount released and the longer the release time.

HPLC assays revealed that no degradation occurs after 4 days of exposure to 60°C and 80°C.

The diffusional mechanism determined using the Korsmeyer–Peppas model denoted a non-Fickian drug diffusion or anomalous drug transport when temperatures up from 36°C were used during loadings.

With the intend of explaining why the hydrogels when subjected to high temperatures display a significant improvement in the drug release behaviour, several techniques were used to verify if the material suffers significant changes when exposed to high temperature.

The swelling profiles (in Figure 9) demonstrate that with the increase in temperature, the solution uptake becomes faster and the EWC slightly decreases. Moreover, when compared with DD water, the EWC performed in MXF-2 mg/mL slightly decreases.
With the acquisition of the DSC thermograms, $T_g$ values were determined considering the midpoint of the step in the baseline of the first heating cycle. The results revealed that, when the polymeric material is placed in a drug solution at 4 or 60°C, its transition temperature is not affected either by the presence of the drug or by the temperature at which it was exposed.

ssNMR spectroscopy was performed to powdered samples of MXF, IOLs materials hydrated in DD water (for 24 h at 4°C) and IOLs materials loaded with MXF (during 4 days with 2 mg/mL at 4 or 60°C and during 10 days with 10 mg/mL at 36 or 60°C). The structural formulas of the main components of the polymeric material are presented in Figure 10 and Figure 11 shows the acquired spectra for all the B26Y tested samples.

The MXF spectra are not presented, because the signs of MXF did not appear in the same regions of the loaded hydrogels. The numbers indicate the carbons on the PMMA and PHEMA molecules and (A) and (B) represent different areas of chemical shift from the same spectra.

The release curves obtained with the two types of samples were named: Release A and Release B. To evaluate the reversibility of the loading/release behaviour, samples were loaded and submitted to release assays under identical conditions (loading: MXF 2 mg/mL at 4°C), but one set of samples was new, and the other one reused (samples used in Release B), i.e., the first set of materials was not previously submitted to any procedure but the second set was previously soaked at 60°C for 4 days and used after finishing the release study. The release curves obtained with the two types of samples were named 1st Loading (new) and 2nd Loading (reused).

The results are presented in Figure 13, for both studies.

The drug release results, from Release A and B, appear to have been affected by the change in the rates of collection of aliquots during the release assay. However, it seems that only the amount released was influenced, while the kinetic, in terms of percentage released, was maintained.
For the 1st and 2nd Loading curves, identical release profiles were observed which demonstrates that the loading/release behaviour was reversible, and hence, the interactions between the polymer and the drug, when loaded loaded at 60°C may also be reversible.

In addition, the drug uptake (determined from depletion measurements) and the drug release from the samples used in Release B and 2nd Loading were compared and the results shown that in both, all the drug (or the majority of the drug) entered in the disk was released.

3.3. Effect of storage of IOLs materials loaded with MXF

To explore the effect of storage on the release profiles, the materials were previously soaked for 4 days at 60 and 80°C in 1 mL of MXF solution (commercial or prepared in the lab), and sterilized by SH in drug solution. Then, the disks were stored for periods of 1, 2 and 3 months, in their loading solutions, protected from light and at room temperature. UV–Vis spectrophotometry was used to determine the amount of MXF released. The release profiles are presented in Figure 14.

The results show that for longer storage times, IOLs materials release more and the release kinetic was lightly improved. When soaked at 60°C, both formulations reached the equilibrium after 2 months. VGXM seems to be also a viable option for loading and storage the IOLs, if proved that it does not affect the materials properties. Moreover, for loadings performed at high temperatures, sterilization had a minor or neglectable effect on the profiles.

To evaluate the stability of the drug released after 3 months of storage of the disks loaded at 60°C and 80°C with MXF and VGXM solutions and SH sterilized, microbiological assays were performed with the objective of determining the drugs activity against SA and SE. For this purpose, the solutions collected at day 3 and 7 were analysed and no degradation of the released drug was observed, being active in their maximum performance after 3 months of storage.

![Figure 14. Cumulative release profiles of MXF from B26Y samples loaded at 60°C (green) or 80°C (rouge) and stored in MXF (A and C) or VGXM (B and D) solutions. The quantifications were determined by UV–Vis spectrophotometry.](image)

A mathematical model that considers the physiological conditions of the anterior chamber of the eye was applied to all the data obtained in the study of the effect of storage, to estimate the MXF concentration in the aqueous humor (AH) over time, after implantation of a drug loaded IOL. The model was adapted to the proposed by R. Galante et al. and the parameters used were: a renewal rate of the AH of 2.4 ± 0.6 µL/min and a total volume of the AH of 240 ± 12 µL.

The results show that the loaded lenses shall be efficient at least 20 days. In some cases, the concentrations remain above the MIC up to 80 – 90 days. This means that the loading conditions may need to be adjusted, so that the release only occurs during the therapeutic period set (1 – 2 weeks) and does not bring the risk of inducing bacteria resistance to the antibiotic.

4. CONCLUSIONS AND FUTURE WORK

4.1. Conclusions

The main conclusion from the results obtained with the stability studies and antimicrobial assays of the studied drugs is that the effects of sterilization depend, not only on the method, but also on the type of drug preparation. For all studied drugs there was no degradation when they were sterilized by SH (in solution) or with all doses of GR (in powder). However, all drugs degraded with higher doses of GR (15 and 25 kGy) when they were sterilized in solution. Generally, anti-inflammatories were more degraded by GR than antibiotics when in solution, being DFN the most affected. Moreover, KTL and MXF were better resistant to GR when combined, especially for higher doses of GR. Also, the addition of mannitol as a radio-protective excipient did not present the expected effect of protection. Lastly, the sterility tests indicated that only SH was able to ensure the sterility state, while the results for GR were not fully conclusive.

The main conclusion about the effects of sterilization on the materials properties is that SH is the more appropriate method for the sterilization of both materials, but also GR (5 kGy) for both materials and all doses for IOLs materials (without the UV-A blocking and the violet light filtering chromophore) may be used. Moreover, the sterility tests made for both materials sterilized with SH and GR (5 kGy) revealed that both lenses were sterile. Other modifications in the lenses materials matrix may also had occurred however they were not detected by the studied properties.

From the drug release experiments for IOLs material, GR slightly decreased the release of the studied drugs, while SH improved the release profile, not only in the amount released but also in the kinetics of release. Referring to CLs material, GR led to a lower release of the drug, while SH did not affect the drug release.

For both materials sterilized in drug solution with 5 kGy of GR, the chromatograms of all the drug release solutions revealed degradation peaks. In regard to the effect of the sterilization on the proprieties of drug loaded IOLs materials only the transmissibility of irradiated lenses loaded with KTL was affected.

Generally, for the effects of sterilization on the drug loaded materials, GR may not be an option for the sterilization if the degradation products prove to be harmful for the patient ocular tissues. However, SH is a great option for both materials, it didn’t cause any harmful effect and improved the quantity of drug released, the release rate and delivered the drugs for a longer period.

It is well known that is not possible to achieve a single sterilization method for all drug delivery systems. Instead, for each system, sterilization studies must be performed.
The MXF loaded IOLs materials were chosen for further investigation of the effects of SH sterilization and of the loading/release conditions on the properties and release profiles, due to the promising results presented above for the release of drugs from this material when sterilized by SH.

The most relevant conclusions obtained from the study of the effects of SH sterilization conditions on the release profiles are: no effect of the moment of sterilization (1st day or 3rd day of the loading period); no effect of the sterilizing period (30, 60 or 90 min); and the increase in drug concentration of the loading/sterilization solution increases the quantity released but does not affect the release rate. For all SH sterilizing conditions, a sustained release was observed for more than 60 days.

Relatively to the loading conditions, we restricted the study to the effect of temperature. The increase in temperature led to the increase on the amount of drug released and highly improved the drug release kinetics. MXF was not degraded as a consequence of having been exposed to 60°C and 80°C during 4 days. Application of the Korsmeyer–Peppas model to the release data showed that when lenses materials are soaked at 4°C, independently of being sterilized, the diffusion mechanism is mostly Fickian. However, for temperature \( \geq 36°C \), the diffusion turns into a non-Fickian process.

Concerning the properties of IOLs materials loaded at high temperatures: the swelling profiles demonstrate that, even in the presence of the drug, the solution uptake is faster and the EWC decreases with the increase of temperature; the \( T_w \) values obtained for loaded IOLs at 4 or 60°C with MXF, were not affected either by the presence of the drug or by the temperature at which the material was exposed; from the NMR studies the main conclusions were that the sustained release of MXF from materials loaded at high temperature results from the plasticizing effect of the water and, mainly, from the interactions between MXF and the polymeric matrix, specially with the PMMA component; the results from the reversibility studies of loading/release behavior suggest that these interactions are reversible.

Finally, the modifications of the release conditions led to conclude that the increase in the frequency of aliquots collection and consequently, in the volume used to perform the release, increased the amount released but did not affect the kinetics in percentage terms.

Analysis of the influence of the time of storage on the drug release profiles was done using the IOLs materials loaded in different conditions. For longer storage times, IOLs materials release more and the release kinetic was lightly improved, reaching the equilibrium after 2 months. For loadings performed at high temperatures, sterilization had a minor or neglectable effect on the profiles. Moreover, VGMX seems to be a viable option for loading and storage the IOLs. No degradation of the released drug was observed being active in their maximum performance after 3 months of storage. From the estimation of the in vivo efficacy of drug loaded IOLs materials, it was concluded that the drugs may be released, in some cases during 80 — 90 days above the MIC. Considering that the MXF therapeutic period after cataract surgery is 1-2 weeks, this means that the loading conditions may need to be adjusted by changing temperature, loading time and concentration of loading solution. Furthermore, we must stress that the model applied to estimate the in vivo efficacy is a simplistic approach which does not take into account other clearance mechanisms of the drug in the eye, and that the predicted values will depend on the fitting to the experimental data.

4.2. Future work

Concerning drug sterilization, possible strategies to reduce the degradation caused by GR should be explored. To better understand what happens to the drug loaded materials upon sterilization, mechanical properties should be investigated. Other methods of sterilization are necessary to be explored for CLs materials. In addition, toxicological assays are required to analyse the degradation products from the studied drugs, if GR was eventually chosen as sterilization method. Finally, other studies, such as electron paramagnetic resonance (EPR) are recommended to assess the stability of the drugs.

Regarding the effects of SH sterilization conditions, only temperature was analysed in this study; it would be interesting to test the effect of different pressures (without temperature) on the drug release behaviour. Following the study of the effects of loading temperature, pH values should be monitored and controlled to keep within the best range to perform the loading experiments. To better approach the release behaviour from the real one, release assays should be performed in dynamic conditions, using a microfluidic cell. Additionally, besides MXF, other drugs, particularly inflammatory drugs should be considered to test with the Bz2Y material. Finally, the drug loaded systems with the best performance shall be submitted to bacterial resistance tests (if loaded with antibiotic) and tested in in vivo experiments with animals to conclude on their safety and efficiency.

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